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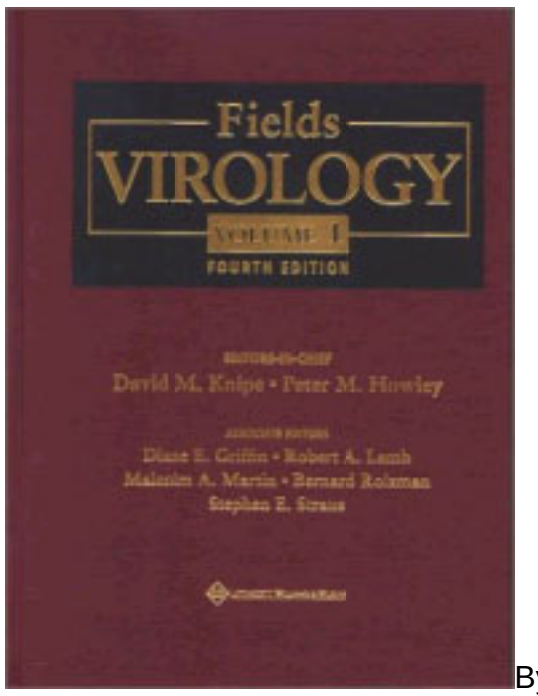

By OkDoKeY

Fields Virology

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Preface

In the early 1980s, Bernie Fields originated the idea of a virology reference textbook that combined the molecular aspects of viral replication with the medical features of viral infections. This broad view of virology reflected Bernie's own research, which applied molecular and genetic analyses to the study of viral pathogenesis, providing an important part of the foundation for the field of molecular pathogenesis. Bernie led the publication of the first three editions of *Virology*.

Unfortunately, we lost Bernie to pancreatic cancer soon after the third edition went into production. The third edition became *Fields Virology* in his memory, and it is entirely fitting that the fourth edition continues to carry his name.

We have retained the general organization of the first three editions for the fourth edition. Part I contains chapters on general aspects of virology, and Part II contains chapters on replication and medical aspects of specific virus families and specific viruses of medical importance. In Part I, we have added new chapters on principles of virology, virus entry and uncoating, replication strategies of RNA viruses and DNA viruses, virus assembly, and virus vectors to enhance the utility of this book as well as that of *Fundamental Virology* as textbooks. In Part II we have added new chapters on the arteriviruses, the *Bornaviridae*, and Kaposi's sarcoma-associated herpes virus. We have also expanded the retrovirus section to include chapters on the nonhuman lentiviruses as well as human and primate viruses other than the lentiviruses and the lymphotropic retroviruses. The main emphasis continues to be on viruses of medical importance and interest, but other viruses are described in specific cases where more is known about their mechanisms of replication or pathogenesis. Although not formally a virus, prions are still included in this edition for historical reasons and because of the intense interest in the infectious spongiform encephalopathies. All of the chapters have been updated to reflect the rapid advances in virology during the last five years.

We wish to thank Anne Snyder, Robin Cook, and Jonathan Pine at Lippincott Williams & Wilkins and Lisa Holik at Harvard Medical School for all of their many important contributions to the preparation of this book.

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CHAPTER 1 The Origins of Virology

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Virology, as a subject matter, has had a remarkable history. Viruses, because of their predatory nature, have shaped the history and evolution of their hosts. Virtually all living organisms, when studied carefully, have viral parasites, and so these smallest of living entities exert significant forces on all life forms, including themselves. The medical consequences of viral infections of humans have altered our history and have resulted in extraordinary efforts on the part of virologists to study, understand, and eradicate these agents. These virologists have elucidated new principles of life processes and taken major new directions in science. Many of the concepts and tools of molecular biology have been derived from the study of viruses and their host cells. This chapter is an attempt to review selected portions of this history as it relates to the development of new concepts in virology (50,51).

THE DEVELOPMENT OF THE CONCEPT OF VIRUSES

The Early Period: The 19th Century

By the last half of the 19th century, the existence of a diverse microbial world of bacteria, fungi, and protozoa was well established. As early as 1840, the noted German anatomist Jacob Henle of Gottingen (the discoverer of Henle's loop and the grandfather of 20th-century virologist Werner Henle) hypothesized the existence of infectious agents that were too small to be observed with the light microscope and that were able to cause specific diseases. In the absence of any direct evidence for such entities, however, his ideas failed to be accepted. It was at this time that three major advances in microbiology came together to set the stage for the development of the concept of a submicroscopic agent that would come to be called a virus.

The first of these ideas was the demonstration that the spontaneous generation of organisms did not occur. This notion had a long history, with experiments both supporting and refuting it. The credit, however, for finally disproving this hypothesis is commonly given to Louis Pasteur (1822–1895), who employed his swan-neck flasks to strike a mortal blow to the concept of spontaneous generation. Pasteur went on to study fermentation by different microbial agents. During these studies, he made it clear that “different kinds of microbes are associated with different kinds of fermentations” and he extended this concept to disease processes. Building on this, Robert Koch (1843–1910), a student of Jacob Henle and a country doctor in a small German village, demonstrated that the anthrax bacillus was the cause of this disease (1876) and that the tubercle bacillus was the cause of tuberculosis in humans (1882). Little of this would have been possible without the third major contribution by Joseph Lister (1827–1912). Once it was clear that organisms reproduce new organisms, the importance of a sterile field, whether in surgery or for the isolation of new organisms, became clear. Lister contributed the technique of limiting dilution to obtain pure cultures of organisms, and Koch developed solid media, the isolation of separate individual colonies of bacteria to obtain pure cultures, and the use of stains to visualize these microorganisms. Although many scientists of that day contributed to these tools and concepts, it was principally Pasteur, Lister, and Koch who put together a new experimental approach for medical science.

These studies formalized some of Jacob Henle's original ideas in what are now termed Koch's postulates for defining whether an organism was indeed the causative agent of a disease. These postulates state that (a) the organism must be regularly found in the lesions of the disease, (b) the organism must be isolated in pure culture, (c) inoculation of such a pure culture of organisms into a host should initiate the disease, and (d) the organism must be recovered once again from the lesions of the host. By the end of the 19th century, these concepts became the dominant paradigm of medical microbiology. They outlined an experimental method to be used in all situations. It was only when these rules broke down and failed to yield a causative agent that the concept of a virus was born.

The Discovery Period: 1886–1903

Adolf Mayer (1843–1942), a German scientist trained in the field of chemical technology (who had studied fermentation and plant nutrition), became the director of the Agricultural Experiment Station at Wageningen, Holland, in 1876. A few years later (1879), he began his research on diseases of tobacco and, although he was not the first to describe such diseases, he named the disease tobacco mosaic disease after the dark and light spots on infected leaves. In one of Mayer's experiments, he inoculated healthy plants with the juice extracted from diseased plants by grinding up the infected leaves in water. This was the first experimental transmission of a viral disease of plants, and Mayer reported that, “in nine cases out of ten (of inoculated plants), one will be successful in making the healthy plant ... heavily diseased” (109). Although these studies established the infectious nature of the disease, neither a bacterial nor a fungal agent could be consistently cultured or detected in these extracts, so Koch's postulates could not be satisfied. In a preliminary communication in 1882 (108), he speculated that the cause could be a “soluble, possibly enzyme-like

contagium, although almost any analogy for such a supposition is failing in science.” However, 4 years later, in his definitive paper on this subject, Mayer concluded that the mosaic disease “is bacterial, but that the infectious forms have not yet been isolated, nor are their forms and mode of life known” (109).

The next step was taken by Dimitri Ivanofsky (1864–1920), a Russian scientist working in St. Petersburg. In 1887 and again in 1890, Ivanofsky was commissioned by the Russian Department of Agriculture to investigate the cause of a tobacco disease on plantations in Bassarabia, Ukraine, and the Crimea. Ivanofsky rapidly repeated Mayer's observations, demonstrating that the sap of infected plants contained an agent able to transmit the disease to healthy plants, and he added one additional step. He passed the infected sap through a filter that blocked the passage of bacteria—the Chamberland filter, made of unglazed porcelain. The Chamberland filter, perfected to purify water by Charles Chamberland, one of Pasteur's collaborators, contained pores small enough to retard most bacteria. On February 12, 1892, Ivanofsky reported to the Academy of Sciences of St. Petersburg that “the sap of leaves infected with tobacco mosaic disease retains its infectious properties even after filtration through Chamberland filter candles” (79). The importance of this experiment is that it provided an operational definition of viruses, an experimental technique by which an agent could qualify as a virus.

Ivanofsky, like Mayer before him, failed to culture an organism from the filtered sap and failed to satisfy Koch's postulates. He, too, was bound by the paradigm of the times suggesting that the filter might be defective or something might be wrong with his methods. He even suggested the possibility that a toxin (not a living/reproducing substance) might pass through the filter and cause the disease. As late as 1903, when Ivanofsky published his thesis (80), he could not depart from the possibility that bacteria caused this disease and that he and others had somehow failed to culture them. The dogma of the times and the obvious success of Koch's postulates kept most scientists from interpreting their data in a different way. It is equally curious that, at this time (1885), Pasteur was working with viruses and developing the rabies vaccine (117) but never investigated the unique nature of that infectious agent.

The third scientist to play a key role in the development of the concept of viruses was Martinus Beijerinck (1851–1931), a Dutch soil microbiologist who collaborated with Adolf Mayer at Wageningen. Beijerinck also showed that the sap of infected tobacco plants could retain its infectivity after filtration through a Chamberland candle filter. [He was unaware of Ivanofsky's work at the time (1898).] He then extended these studies by showing that the filtered sap could be diluted and then regain its “strength” after replication in living, growing tissue of the plant. The agent could reproduce itself (which meant that it was not a toxin) but only in living tissue, not in the cell-free sap of the plant. This explained the failure to culture the pathogen outside its host, and it set the stage for discovery of an organism smaller than bacteria (a filterable agent), not observable in the light microscope, and able to reproduce itself only in living cells or tissue. Beijerinck called this agent a “*contagium vivum fluidum*” (9), or a contagious living liquid. This concept began a 25-year debate about the nature of viruses; were they liquids or particles? This conflict was laid to rest when d'Herelle developed the plaque assay in 1917 (33) and when the first electron micrographs were taken of tobacco mosaic virus (TMV) in 1939 (88).

Thus, Mayer, Ivanofsky, and Beijerinck each contributed to the development of a new concept: a filterable agent too small to observe in the light microscope but able to cause disease by multiplying in living cells. Loeffler and Frosch (101) rapidly described and isolated the first filterable agent from animals, the foot-and-mouth disease virus, and Walter Reed and his team in Cuba (1901) recognized the first human filterable virus, yellow fever virus (122). The term *virus* [from the Latin for slimy liquid or poison (76)] was at that time used interchangeably for any infectious agent and so was applied to TMV. The literature of the first decades of the 20th century most often referred to these infectious entities as filterable agents, and this was indeed the operational definition of viruses. Sometime later, the term *virus* became restricted in use to those agents that fulfilled the criteria developed by Mayer, Ivanofsky, and Beijerinck and that were the first agents to cause a disease that could not be proven by using Koch's postulates.

The Plant Viruses and the Chemical Period: 1929–1956

Tobacco mosaic virus (TMV) continued to play a central role in exploring the nature and properties of viruses. The early decades of the 20th century saw the development of techniques to purify enzymes (proteins). The notion that viruses were proteins and so could be purified in the same way was first appreciated and applied by Vinson and Petre (1927–1931) at the Boyce Thompson Institute in Philadelphia. They precipitated the infectious TMV agent [using an infectivity assay developed by Holmes (75)] from the crude sap of infected plants using selected salts, acetone, or ethyl alcohol (144). They showed that the infectious virus could move in an electric field, just as proteins did. At the same time, H. A. Purdy-Beale, also at the Boyce Thompson Institute, produced in rabbits antibodies directed against TMV that could neutralize the infectivity of this agent (121). At the time, this was taken as further proof of the protein nature of viruses. With the advent of purification procedures for viruses, both physical and chemical measurements of the virus became possible. The strong flow birefringence of purified preparations of TMV was interpreted (correctly) to show an asymmetric particle or rod-shaped particle (136). Max Schlesinger (129), working on purified preparations of bacteriophages in Frankfurt, Germany, showed that they were composed of proteins and contained phosphorus and deoxyribonucleic acid. This led to the first suggestion that viruses were composed of nucleoproteins. The crystallization of TMV in 1935 by Wendell Stanley (132), working at the Rockefeller Institute branch in Princeton, New Jersey, brought this infectious agent into the world of the chemists. Within a year, Bawden and Pirie (7,8) had demonstrated that crystals of TMV contained 0.5% phosphorus and 5% RNA. The first “view” of a virus came from x-ray crystallography using these crystals to show rods of a constant diameter aligned in hexagonal arrays containing RNA and protein (14). The first electron micrographs of any virus, those of TMV, were taken with a microscope built in Germany, and they confirmed the rod shape of the virus particle (88).

The x-ray diffraction patterns (14) suggested that TMV was built up from repeating subunits. These data and other considerations led Crick and Watson (29) to realize that most simple viruses had to consist of one or a few species of identical protein subunits. By 1954–1955, several techniques had been developed to dissociate TMV protein subunits, and TMV could be reconstituted from its RNA and protein subunits (57) to produce infectious virus. The principles of virus self-assembly (20) were appreciated using TMV, and in 1962 Caspar and Klug (23) elegantly described the geometric principles of icosahedral virus structure for many of the isometric viruses. Thus, from 1929 to 1962, both the structures

and the chemical compositions of viruses were elucidated.

As early as 1926, H. H. McKinney (110) reported the isolation of “variants” of TMV with a different plaque morphology that bred true and could be isolated from several geographic locations (111). In 1933, Jensen (85) confirmed McKinney's observations and demonstrated reversion mutations for this phenotype. Thus, viruses, like all living and replicating entities, could mutate and therefore had genetic information. That the genetic information resides in the RNA of TMV was shown 30 years later by an infectious RNA assay (56,65). This was the first demonstration that RNA could be a genetic material. This followed Avery's DNA transformation experiments with pneumococcus (4) and the Hershey-Chase experiment with bacteriophages (73), both of which demonstrated that DNA was the more common genetic material. TMV RNA and its nucleotide sequence helped to confirm codon assignments for the genetic code, added clear evidence for the universality of the genetic code, and helped to elucidate the mechanisms of mutation by several diverse mutagens (55). TMV and its related plant viruses have contributed significantly to both the origins and the development of virology.

THE BACTERIOPHAGES

The Early Period: 1915–1940

In 1915, Frederick W. Twort was the superintendent of the Brown Institution in London. In his research, Twort was looking for variants of vaccinia virus, used in the smallpox vaccine, that would replicate in simple defined media outside living cells. In one of his experiments, he inoculated a culture dish of nutrient agar with an aliquot of the smallpox vaccine and, although the virus failed to replicate, bacterial contaminants grew in the agar dish very readily. As Twort continued to incubate his cultures, he noticed that some bacterial colonies underwent a visible change and became “watery looking” (i.e., more transparent). Such colonies were no longer able to replicate when subcultured (i.e., the bacteria had been killed). Twort called this phenomenon glassy transformation, and he went on to show that infecting a normal colony of bacteria with the glassy transforming principle would kill the bacteria. The glassy entity readily passed through a porcelain filter, could be diluted a million-fold, and when placed upon fresh bacteria would regain its strength, or titer (140,141 and 142).

Twort (140) published a short note describing all this and suggested that one explanation of his observation was a virus of bacteria. Twort's research was interrupted by World War I, in which he served. When he returned to London, he did not continue this line of research and made no further contributions in this area.

At the same time, Felix d'Herelle, a Canadian medical bacteriologist, was working at the Pasteur Institute in Paris. In August 1915, a cavalry squadron of French soldiers were quartered in Maisons-Lafitte, just outside Paris, and a rampant *Shigella* dysentery infection was devastating the entire outfit. D'Herelle readily isolated the dysentery bacillus from filtered emulsions of the feces of sick men and cultured them. As the bacteria grew and covered the surface of the Petri dish, d'Herelle occasionally observed clear circular spots where no bacteria grew, and he called these *taches vierges*, or plaques. D'Herelle (33,34) was able to follow the course of an infection in a single patient, noting when the bacteria were most plentiful and when the plaques appeared. He was able to demonstrate that the plaques appeared on the fourth day after infection and killed the bacteria in the culture dish; interestingly, the patient began to improve on the fourth day after infection.

D'Herelle named these viruses bacteriophages, and he went on to develop techniques utilized to this day in virology. He developed the use of limiting dilutions with the plaque assay to titer the virus preparation. He reasoned that the appearance of plaques showed that the virus was particulate, or “corpuscular.” D'Herelle also demonstrated that the first step of a virus infection was for the agent to attach (adsorb) to the host cell, which he showed by the co-sedimentation of virus and host after mixing the two. (He showed that the virus was lost from the supernatant fluid.) The attachment of a virus occurred only when bacteria sensitive to the virus were mixed with it, demonstrating the host range specificity of a virus at the adsorption step. He described cell lysis and the release of infectious virus in clear and modern terms. D'Herelle (34,35) was in many ways one of the founders of the principles of modern virology.

By 1921, an increasing number of lysogenic bacterial strains had been isolated, and it became impossible to separate the virus from its host in some experiments. This led Jules Bordet (18) of the Pasteur Institute in Brussels to suggest that the transmissible agent described by d'Herelle was nothing more than a bacterial enzyme that stimulates its own production. Although that was an incorrect conclusion, it is remarkably close to the present ideas of prion structure and replication (see Chapter 90).

Throughout the decades of the 1920s and 1930s, d'Herelle focused his efforts on the potential medical applications of his research, but it never bore fruit. What basic research was being carried out at that time was often confused by the interpretations that resulted from the strong personalities of individual scientists in the field. Although it was clear that there were many diverse bacteriophages and that some were lytic while some were lysogenic, their interrelationships remained ill-defined. The highlight of this period was the demonstration by Max Schlesinger (128,130) that purified phages had a maximum linear dimension of 0.1 micron and a mass of about 4×10^{-16} grams and that they were composed of protein and DNA in roughly equal proportions. In 1936, no one quite knew what to make of that observation, but it would begin to make a great deal of sense over the next 20 years.

The Modern Period: 1938–1970

Max Delbrück was trained as a physicist at the University of Gïttingen, and his first position was at the Kaiser Wilhelm Institute for Chemistry in Berlin. There he joined a diverse group of individuals actively discussing how quantum physics related to an understanding of heredity. Delbrück's interest in this area led him to develop a quantum mechanical model of the gene, and in 1937 he applied for and obtained a fellowship to study at the California Institute of Technology. Once at Caltech, he teamed up with another research fellow, Emory Ellis (44), who was working with a group of bacteriophages, T2, T4, T6 (the T-even phages). Delbrück soon appreciated that these viruses were ideal for the study of virus replication. These phages represented a way to probe how genetic information could determine the structure and

function of an organism. From the beginning, these viruses were viewed as model systems for understanding cancer viruses or even for understanding how a sperm fertilizes an egg and a new organism develops. Ellis and Delbrück (45) designed the one-step growth curve experiment, in which an infected bacterium liberates hundreds of phages synchronously after a one-half hour latent or eclipse period. This experiment defined the latent period, when viral infectivity was lost. This became the experimental paradigm of the phage group.

At the outbreak of World War II, Delbrück remained in the United States (at Vanderbilt University) and met an Italian refugee, Salvador E. Luria, who had fled to America and was working at Columbia University in New York (with phages T1 and T2). They met at a meeting in Philadelphia on December 28, 1940, and spent the next 2 days planning experiments at Columbia. These two scientists were to recruit and lead a growing group of researchers focused on using bacterial viruses as a model for understanding life processes. Central to their success was an invitation to spend the summer of 1941 at Cold Spring Harbor Laboratory doing experiments. The result was that a German physicist and an Italian geneticist joined forces throughout the years of World War II to travel throughout the United States and recruit a new generation of biologists who came to be known as the phage group.

Shortly thereafter, Tom Anderson, an electron microscopist at the RCA Laboratories in Princeton, New Jersey, met Delbrück, and by March 1942 the first clear pictures of bacteriophages had been obtained (104). About the same time, the first mutants of these phages were isolated and characterized (102). By 1946, the first phage course was being taught at Cold Spring Harbor, and in March 1947, the first phage meeting attracted eight people. From these humble beginnings grew the field of molecular biology, which focused on the bacterial host and its viruses.

The next 25 years (1950–1975) was an intensely productive period of virus research with bacteriophages. Hundreds of virologists produced thousands of publications that covered three major areas: (a) lytic infection of *E. coli* with the T-even phages; (b) the nature of lysogeny, using lambda phage; and (c) the replication and properties of several unique phages such as fX174 (single-stranded circular DNA), the RNA phages, T7, and so forth. It is simply not possible here to review all this literature, which laid the foundations for modern molecular virology and biology, so only selected highlights of this era will be mentioned.

By 1947–1948, the idea of examining, at the biochemical level, the events occurring in phage-infected cells during the latent period had come into its own. Seymour Cohen [who had trained first with Erwin Chargaff at Columbia University, studying lipids and nucleic acids, and then with Wendell Stanley working on TMV RNA, and who had taken Delbrück's phage course (1946) at Cold Spring Harbor] examined the effects of phage infection on DNA and RNA levels in infected cells using a colorimetric analysis (26,27). These studies showed a dramatic alteration of macromolecular synthesis in phage-infected cells: (a) The net accumulation of RNA stopped in these cells. [Later this would be the basis for detecting a rapidly turning over species of RNA and the first demonstration of messenger RNA (3)]. (b) There was a cessation of DNA synthesis for 7 minutes, followed by a resumption of DNA synthesis at a 5- to 10-fold increased rate. (c) At the same time, Monod and Wollman (112) showed that the synthesis of a cellular enzyme, the inducible b-galactosidase, was inhibited after phage infection. These experiments divided the viral latent period into early (prior to DNA synthesis) and late times. These results, more importantly, made the clear point that a virus could redirect cellular macromolecular synthetic processes in infected cells (28).

By the end of 1952, two experiments had a critical effect on this field. First, Hershey and Chase (73) utilized differentially labeled viral proteins ($^{35}SO_4$) and nucleic acids ($^{32}PO_4$) to follow phage attachment to bacteria. They were able to shear the viral protein coats from the bacteria using a Waring blender and thus leave only the DNA associated with the infected cells. This enabled them to prove that the DNA had all the information needed to reproduce 100 new viruses. The Hershey-Chase experiment came at the right time to be appreciated in light of the novel structure of DNA elucidated by Watson and Crick 1 year later (145). Together, these experiments formed a cornerstone of the molecular biology revolution (21).

The second experiment in virology carried out in 1953, which was to have an important influence, was done by G. R. Wyatt and S. S. Cohen (155). They identified a new base, hydroxymethylcytosine, in T-even phage DNA; this new base took the place of cytosine, which was present in bacterial DNA. This began a 10-year study of how deoxyribonucleotides were synthesized in bacteria and phage-infected cells, and it led to the critical observation that the virus introduces genetic information for a new enzyme into the infected cell (53). By 1964, Mathews et al. (28) had proved that hydroxymethylase does not exist in uninfected cells and must be encoded for by the virus. These experiments introduced the concept of early enzymes, utilized in deoxypyrimidine biosynthesis and DNA replication (90), and provided clear biochemical proof that the virus encoded new information expressed as proteins in an infected cell. A detailed genetic analysis of these phages identified and mapped the genes encoding these phage proteins and added to this concept. Indeed, the genetic analysis of the rII A and B cistrons of T-even phages became one of the best-studied examples of “genetic fine structure” (10,11). The replication of viral DNA using phage mutants and extracts to complement and purify enzyme activities *in vitro* contributed a great deal to our modern understanding of how DNA duplicates itself (1). Finally, a detailed genetic analysis of phage assembly, utilizing the complementation of phage assembly mutants *in vitro*, is a lucid example of how complex structures are built by living organisms using the principles of self-assembly (41). The genetic and biochemical analysis of phage lysozyme helped to elucidate the molecular nature of mutations (135), and phage (amber) mutations provided a clear way to study second-site suppressor mutations at the molecular level (12). The circular genetic map of the T-even phages (134) was explained by the circularly permuted, terminally redundant (giving rise to phage heterozygotes) conformation of these DNAs (139).

The remarkable reprogramming of viral and cellular protein synthesis in phage-infected cells was dramatically revealed by an early use of sodium dodecyl sulfate (SDS)-polyacrylamide gels (93), showing that viral proteins are made in a specific sequence of events ever subdividing the early and late proteins. The underlying mechanism of this temporal regulation led to the discovery of sigma factors modifying RNA polymerase and conferring gene specificity (67). The study of gene regulation at almost every level (transcription, RNA stability, protein synthesis, protein processing) was revealed from a set of original contributions derived from an analysis of phage infections.

Although this remarkable progress had begun with the lytic phages, no one knew quite what to make of the lysogenic phages. This changed in 1949 when André Lwoff at the Pasteur Institute began his studies with *Bacillus megaterium* and its lysogenic phages. By use of a micromanipulator, single bacteria were shown to divide up to 19 times, never liberating a virus. When lysogenic bacteria were lysed from without, no virus was detected. But from time to time a bacterium spontaneously lysed and produced many viruses (106). The influence of ultraviolet light in inducing the release of these viruses was a key observation that began to outline this curious relationship between a virus and its host (107). By 1954, Jacob and Wollman (82,83) at the Pasteur Institute had made the important observation that a genetic cross between a lysogenic bacterial strain (Hfr, lambda) and a nonlysogenic recipient resulted in the induction of the virus after conjugation, a process they called zygotic induction. In fact, the position of the lysogenic phage or prophage in the chromosome of its host *E. coli* could be mapped by the standard interrupted mating experiment after a genetic cross (83). This was one of the most critical experiments in the conceptual understanding of lysogenic viruses for several reasons: (a) It showed that a virus behaved like a bacterial gene on a chromosome in a bacteria; (b) it was one of the first experimental results to suggest that the viral genetic material was kept quiescent in bacteria by negative regulation, which was lost as the chromosome passed from the lysogenic donor bacteria to the nonlysogenic recipient host; and (c) this helped Jacob and Monod to realize as early as 1954 that the “induction of enzyme synthesis and of phage development are the expression of one and the same phenomenon” (106). These experiments laid the foundation for the operon model and the nature of coordinate gene regulation.

Although the structure of DNA was elucidated in 1953 (145) and zygotic induction was described in 1954, the relationship between the bacterial chromosome and the viral chromosome in lysogeny was still referred to as the attachment site and literally thought of in those terms. It was not until Campbell (22) proposed the model for lambda integration of DNA into the bacterial chromosome, based on the fact that the sequence of phage markers was different in the integrated state than in the replicative or vegetative state, that the truly close relationship between a virus and its host was appreciated. This led to the isolation of the negative regulator or repressor of lambda, a clear understanding of immunity in lysogens, and one of the early examples of how genes are regulated coordinately (120). The genetic analysis of the lambda bacteriophage life cycle is one of the great intellectual adventures in microbial genetics (72). It deserves to be reviewed in detail by all students of molecular virology and biology.

The lysogenic phages such as P22 of *Salmonella typhimurium* provided the first example of generalized transduction (157), whereas lambda provided the first example of specialized transduction (114). That viruses could carry within them cellular genes and transfer such genes from one cell to another provided not only a method for fine genetic mapping but also a new concept in virology. As the genetic elements of bacteria were studied in more detail, it became clear that there was a remarkable continuum from lysogenic phages to episomes, transposons and retrotransposons, insertion elements, retroviruses, hepadnoviruses, viroids, virosoids (in plants), and prions. Genetic information moves between viruses and their hosts to the point where definitions and classifications begin to blur.

The genetic and biochemical concepts that derive from the study of bacteriophages made the next phase of virology possible. The lessons of the lytic and lysogenic phages were often relearned and modified as the animal viruses were studied.

ANIMAL VIRUSES

The Early Period (1898–1965): Discovery and Cell Culture

Some of the highlights of the early period of animal virology are summarized in Table 1. Once the concept of a filterable virus took hold, this experimental procedure was applied to many diseased tissues. Filterable agents, unable to be seen in a light microscope, that replicate only in living animal tissue were found. There were truly some surprises, such as a virus—yellow fever virus—transmitted by a mosquito vector (122), specific visible pathologic inclusion bodies (viruses) in infected tissue (80,116), and even viral agents that can “cause cancer” (43,123). Throughout this early time period (1900–1930), a wide variety of viruses were found (see Table 1) and characterized with regard to their size (using the different pore sizes of filters), resistance to chemical or physical agents (e.g., alcohol, ether), and pathogenic effects. Just based on these properties, it became clear that viruses were a very diverse group of agents. Some were even observable in the light microscope (vaccinia in dark-field optics). Some were inactivated by ether, whereas others were not. The range of viral diseases affected every tissue type. Viruses gave rise to chronic or acute disease; they were persistent agents or recurred in a periodic fashion. Viruses might cause cellular destruction or induce cellular proliferation. For the early virologists, unable to see their agents in a light microscope and often confused by this great diversity, there had to be an element of faith in their studies. In 1912, S. B. Wolbach, an American pathologist, remarked, “It is quite possible that when our knowledge of filterable viruses is more complete, our conception of living matter will change considerably, and that we shall cease to attempt to classify the filterable viruses as animal or plant” (151).

Date	Virologist	Discovery
1898	F. Loeffler, P. Frosch	First demonstration of a filterable animal virus (foot-and-mouth disease virus)
1898	G. Sanarelli	Myxomatous virus
1901	W. Reed et al.	First human virus (yellow fever virus)
1901	A. Lode, J. Gruber	Fowl plague virus
1903	P. Remlinger, Riffat Bey	Rabbit virus
1903	A. Negri	The inclusion bodies of rabies virus
1908	V. Ellerman, O. Bang	First demonstration of a leukemia-causing virus
1909	K. Landsteiner, E. Popper	Poliovirus
1911	P. Rous	First demonstration of a solid tumor virus, Rous sarcoma virus
1912	A. Carrel	Tissue culture of chick embryo explant
1913	E. Steinhardt et al.	An early example of virus propagation in tissue culture
1931	J. Furth	Use of mice as a host for viruses
1931	A. Woodruff, E. Goodpasture	Use of the embryonated chicken egg as a host for viruses
1931	R. Shope	Swine influenza virus
1933	W. Smith et al.	Human influenza virus
1933	R. Shope	Rabbit papilloma virus (first DNA tumor virus)
1933	Staff of the Jackson Memorial Laboratory	Mouse mammary tumors
1936	P. Rous, J. Beard	Rabbit papilloma virus induces carcinomas in a different species
1941–50	G. Hirst	Influenza virus hemagglutination (HA), HA inhibition by antibody, receptor-destroying enzyme (neuraminidase—the first virus-associated enzyme)
1948–50	Sanford et al.	Culture of single animal cells
	J. Enders et al.	Nonneural tissue (human) supports poliovirus replication in culture
	G. Gey et al.	Single cell cultures, HeLa cervical carcinoma
	H. Eagle	The optimal medium for growing cells
1952–65	Dulbecco, Dulbecco, Vogt, Temin, Rubin, Baltimore, et al.	Plaque assay and origins of the molecular biology of animal viruses using polioviruses

TABLE 1. *Landmarks in animal virus research: The early period (1898–1965)*

The way out of this early confusion was led by the plant virologists and the development of techniques to purify viruses and characterize both the chemical and physical properties of these agents (see previous section, The Plant Viruses and the Chemical Period: 1929–1956). The second path out of this problem came from the studies with bacteriophages, where single cells infected with viruses in culture were much more amenable to experimental manipulation and clear answers than were virus infections of whole animals. Whereas the plant virologists of that day were tethered to their greenhouses and the animal virologists were bound to their animal facilities, the viruses of bacteria were studied in Petri dishes and test tubes. Progress in simplifying the experimental system under study came one step at a time; from studying animals in the wild, to laboratory animals, such as the mouse (59) or the embryonated chicken eggs (152), to the culture of tissue, and then to single cells in culture. Between 1948 and 1955, a critical transition converting animal virology into a laboratory science came in four important steps: (a) Sanford et al. (126) at the National Institutes of Health (NIH) overcame the difficulty of culturing single cells, (b) George Gey (64) and his colleagues at Johns Hopkins Medical School cultured and passaged human cells for the first time and developed a line of cells (HeLa) from a cervical carcinoma, (c) Harry Eagle (40) at the NIH developed an optimal medium for the culture of single cells, and (d) in a demonstration of the utility of all this, Enders and his colleagues (46) showed that poliovirus could replicate in a nonneuronal human explant of embryonic tissues.

These ideas, technical achievements, and experimental materials had two immediate effects in virology. First, they led to the development of the polio vaccine as the first vaccine produced in cell culture. From 1798 to 1949, all the vaccines in use (smallpox, rabies, yellow fever, influenza) had been grown in animals or embryonated chicken eggs. Poliovirus was grow in monkey kidney cells (74,98) incubated in flasks. Second, the exploitation of cell culture for the study of viruses began the modern era of molecular virology. The first plaque assay for an animal virus in culture was with poliovirus (36), and it led to an analysis of poliovirus every bit as detailed and important as the contemporary work with bacteriophages. The simplest way to document this statement is for the reader to compare the first edition of General Virology by S. E. Luria in 1953 (103) to the second edition by Luria and J. E. Darnell in 1967 (105) and to examine the experimental descriptions of poliovirus infection of cells. The present era of virology had arrived, and it would continue to be full of surprises.

The Modern Period: 1960 to the Present

In this chapter, information has been presented chronologically or in separate virus groups (plant viruses, bacteriophages, and animal viruses), which reflects the historical separation of these fields. In this section, the format changes because the motivation for studying viruses began to change during this period. Virologists began to use viruses to probe questions central to understanding all life processes. Because viruses replicate in and are dependent on their host cells, they must use the rules, signals, and regulatory pathways of the host. Virologists began to make contributions to all facets of biology. These ideas began with the phage group and were continued by the animal virologists. Second, during this period (1970 to the present), the recombinant DNA revolution began, and both bacteriophages and animal viruses played a critical and central role in this revolution. Viruses were used to probe many diverse questions in biology. For these reasons, the organization of this section focuses on the accomplishments of cellular and molecular biology, where viruses were used. Some of the landmarks in virology since 1970 are listed in Table 2.

TABLE 2. *Landmarks in virology: 1970 to present*

The Role of Animal Viruses in Understanding the Basic Outlines of Eukaryotic Gene Regulation

The closed circular and superhelical nature of polyoma virus DNA was first elucidated by Dulbecco and Vogt (37) and Weil and Vinograd (147). The underlying reason for this structure of DNA was first shown to be the packaging of the DNA of simian vacuolating virus 40 (SV40), wound around nucleosomes (63), which produced a superhelix when the histones were removed. These observations proved to be excellent models for the *E. coli* genome (153) and the mammalian chromosome (94). The unique genomes of viruses, with single-stranded DNA (131), plus or minus strand RNA, or double-stranded RNA, are the only life forms that have adopted these modes of information storage.

Important elements of the eukaryotic transcription machinery have been elucidated with viruses. The first transcriptional enhancer element (acts in an orientation- and distance-independent fashion) was described in the SV40 genome (68), as was a distance- and orientation-dependent promoter element observed with the same virus. The transcription factors that bind to the promoter, SP-1 (38), or to the enhancer element, such as AP-1, AP-2 (96), and which are essential to promote transcription along with the basal factors, were first described with SV40. AP-1 is composed of fos and jun family member

proteins, demonstrating the role of transcription factors as oncogenes (17). Indeed, the great majority of experimental data obtained for basal and accessory transcription factors come from *in vitro* transcription systems using the adenovirus major late promoter or the SV40 early enhancer–promoter (146). Our present-day understanding of RNA polymerase III promoter recognition comes, in part, from an analysis of the adenovirus VA gene transcribed by this polymerase (54).

Almost everything we know about the steps of messenger RNA (mRNA) processing began with observations made with viruses. RNA splicing of new transcripts was first described with the adenoviruses (13,25). Polyadenylation of mRNA was first observed with poxviruses (86) using a system where the first DNA-dependent RNA polymerase in a virion was discovered (87). The signal for polyadenylation in the mRNA was first found using SV40 (52). The cap and methylation of bases at the 5' end of mRNA was first detected using reoviruses (60). What little is known about the process of RNA transport out of the nucleus has shown a remarkable discrimination of viral and cellular mRNAs by the adenovirus E1B-55Kd protein (118).

Translational regulation has been profitably studied using poliovirus RNA (or TMV RNA), where internally regulated initiation entry sites (IRES) have been described (84). The discovery of the role of interferon in inducing a set of gene products that act on translational regulatory events owes its origins to virology (77,78). Similarly, the viral defenses against interferon by the adenovirus VA RNA has provided unique insight to the role of eIF-2 phosphorylation events (89). Posttranslational processing of proteins by proteases, carbohydrate addition to proteins in the Golgi apparatus, phosphorylation by a wide variety of important cellular protein kinases, or the addition of fatty acids to membrane-associated proteins have all been profitably studied using viruses. Indeed, a good deal of our present-day knowledge in cell biology of how protein trafficking occurs and is regulated in cells comes from the use of virus-infected cell systems. Clearly, the field of gene regulation has relied on virology for many of its central tenets.

The Role of Animal Viruses in the Recombinant DNA Revolution

The discovery of the enzyme reverse transcriptase in retroviruses (5,138) not only helped to prove how retroviruses replicate but also provided an essential tool to produce complementary DNAs (cDNAs). The first restriction enzyme map of a chromosome, *Hin*dII plus III, was with SV40 DNA (30,31), and the first DNA to show the specificity of a restriction enzyme was SV40 DNA with *Eco*RI (113,115). Some of the earliest DNA cloning experiments used SV40 DNA into lambda, or human b-hemoglobin genes into SV40 DNA, to construct the first mammalian expression vectors (81). Indeed, a debate about these very experiments led to a temporary moratorium on all such recombinant experiments. From the beginning, several animal viruses had been developed into expression vectors for foreign genes, including SV40 (66), the retroviruses (148), the adenoviruses (62,70), and adeno-associated virus (125), which has the remarkable property of site preferential integration (91). Modern day strategies of gene therapy will surely rely on some of these recombinant viruses. The first cDNA cloning of hemoglobin sequences utilized lambda vectors for the cloning and replication of these mRNA copies. In a nice twist of events, the long-elusive hepatitis virus C (non-A, non-B) was cloned from serum using recombinant DNA techniques, reverse transcriptase, and lambda phage vectors (24).

The Role of Animal Virology in Oncology

It is not too strong a statement to say that we owe a great proportion of our present understanding of the origins of human cancers to two major groups of animal viruses, the retroviruses and DNA tumor viruses. The oncogenes were first discovered and proven to exist in a virus and then in the host cell genome using Rous sarcoma virus (133). A wide variety of retroviruses have captured, altered, and delivered oncogenes to the virologists (see Chapter 10). The insertion of retroviruses into the genomes of cancerous cells also helped to locate additional oncogenes (69). The second group of genes that contribute to the origins of human cancers, the tumor suppressor genes (97), has been shown to be intimately associated with the DNA tumor viruses. Genetic alterations at the p53 locus are the single most common mutations known to occur in human cancers (60% to 65% of the time) (99). The p53 protein was first discovered in association with the SV40 large T-antigen (95,100). SV40, the human adenoviruses, and the human papillomaviruses all encode oncogenes that produce proteins that interact with and inactivate the functions of two tumor suppressor gene products, the retinoblastoma susceptibility gene product (Rb) and p53 (32,39,95,100,127,149,150). The cellular oncogenes and the tumor suppressor genes in human cancers have been studied and understood most profitably using these viruses.

The viruses that cause cancers have provided some of the most extraordinary episodes in modern animal virology. The recognition of a new disease and the unique geographic distribution of Burkitt's lymphomas in Africa (19) set off a search for viral agents that cause cancers in humans. From D. Burkitt (19) to Epstein, Achong, and Barr (47) to W. Henle and G. Henle (71), the story of the Epstein-Barr virus and its role in several cancers, as well as in infectious mononucleosis, provides us with the best in detective story science. The story is not yet complete and many mysteries remain. Similarly, the identification of a new pathologic disease, adult T-cell leukemia, in Japan by K. Takatsuki (137,143) led to the isolation of a virus that causes the disease by I. Miyoshi and Y. Hinuma (156) and the realization that this virus [human T-cell leukemia virus (HTLV-1)] had been found previously (119) by Gallo and his colleagues. Although this discovery provided the virus, there is yet to be a satisfactory explanation of how this virus contributes to adult T-cell leukemia.

Equally interesting is the road to the hepatitis B virus and hepatocellular carcinomas. By 1967, S. Krugman and his colleagues (92) had good evidence distinguishing between hepatitis A and B viruses, and in the same year B. Blumberg et al. (16) detected the Australia antigen. Through a tortuous path, it eventually became clear that the Australia antigen was a diagnostic marker (a coat protein) for hepatitis B. Although this freed the blood supply of this dangerous virus, Hilleman at Merck, Sharp and Dohme and the Chiron Corporation (which later isolated the hepatitis C virus) went on to produce the first human vaccine that prevents hepatitis B infections and very likely hepatocellular carcinomas associated with chronic virus infections (see Chapter 87). The idea of a vaccine that can prevent cancer [first proven with the Mareck's disease virus and T-cell lymphomas in chickens (15,42)] comes some 82 to 85 years after the first discoveries of tumor viruses by Ellerman, Bang, and Rous. At present, an experiment is under way in Taiwan, where 63,500 newborn infants have been inoculated to prevent hepatitis B infections. Based on the epidemiologic predictions, this vaccination program should result in 8,300 fewer cases of liver cancer in that population some 40 to 50 years from now.

Vaccines

The Salk and then Sabin poliovirus vaccines were the first beneficial products of the cell culture revolution. In the early 1950s in the United States, just before the introduction of the Salk vaccine, about 21,000 cases of poliomyelitis were reported annually. Today, the number is fewer than 10 (see Chapter 21) (98).

Among the most remarkable achievements of our century is the complete eradication of smallpox, a disease with a history of over 2,000 years (154). In 1966, the World Health Organization began a program to immunize all individuals who had come into contact with an infected person. This strategy, as opposed to trying to immunize an entire population (which simply was not possible), worked and, in October 1977, Ali Maolin of Somalia was the last person in the world to have a naturally occurring case of smallpox (barring laboratory accidents). Because smallpox has no animal reservoir and requires person-to-person contact for its spread, most scientists agree that we are free of this disease (154). What most scientists do not agree on is whether we should store smallpox virus samples as a reference for the future (2).

The viral vaccines used in the past have included live attenuated vaccines, killed virus vaccines, and subunit vaccines. Both the killed virus vaccine (Salk) and the recombinant subunit vaccine (hepatitis B, S antigen) were new to the modern era of virology. In the future, we will see one virus (e.g., vaccinia virus) presenting the antigens of a different virus, the injection of DNA-encoding viral antigens, and the use of specific interleukins or hormones with vaccines to stimulate immunity at specific locations in the host and to elucidate specific immunoglobulin classes. Considering that the first vaccines (for smallpox) were reported in the Chinese literature of the 10th century (48), these ideas can be traced back in time to the origins of virology.

Although vaccines have been extraordinarily successful in preventing specific diseases, there had been very few natural products or chemotherapeutic agents that cured or reduced the symptoms of virus infections. That changed dramatically with the development of acyclovir by Burrows-Wellcome, which requires both a viral enzyme (thymidine kinase) to activate it (by phosphorylation) and a second enzyme to incorporate it into viral DNA, employing its specificity for the viral encoded DNA polymerase. This drug blocks HSV-2 reactivation from latency and stopped a growing epidemic in the 1970s and 1980s (Chapter 15). Similarly, the interferons (Chapter 12) have come into use clinically for hepatitis B and C infections, cancer therapy, and multiple sclerosis. The interferons, found in the course of studying virus interference (77,78), will modulate the immune response and have an increasing role to play in treatments of many clinical syndromes.

An overview of the modern period of animal virology would not be complete without mentioning the appearance of an apparently new virus during the decades of the 1970s and 1980s, the human immunodeficiency virus. It was first recognized as a new disease entity by clinicians and epidemiologists, and they rapidly tracked down the venereal mode of virus transmission. Blood products and transplants provided the fluid and tissue samples for virologists to detect this virus. The first published report of acquired immunodeficiency syndrome (AIDS) was in June 1981. The first publications describing a candidate virus for this disease were in 1983 (6) and then 1984 (61). Had this virus and disease begun in 1961 instead of 1981, neither the nature of retroviruses nor the existence of its host cell (CD-4 helper T-cell) would have been understood. The rapid development of a diagnostic test has helped to remove the virus from the blood supply and to test individuals for the virus.

The development of the first drugs to treat human immunodeficiency virus (HIV) infections (e.g., zidovudine) proved disappointing because of the rapid development of resistance to the drug via mutations in the reverse transcriptase gene. The mutation rates by retroviruses (1 in 10^4 to 10^5 nucleotides polymerized) are very high and the solution to this problem is to give multiple drugs simultaneously, requiring mutations at different loci to develop resistance. The use of two different drugs directed against reverse transcriptase and a third drug that is an HIV protease inhibitor provides a cocktail that can reduce the viral load to a nondetectable level in a patient. Eliminating the integrated and silent copies of the viral DNA from the host cell does not look promising. The highest priority will be the development of an HIV vaccine. This is clearly a major challenge for the future.

Epidemiologic Adventures in Virology

The field of the epidemiology of viruses (see Chapter 14) got a terrific boost in the last half of the 20th century with the advent of specific molecular tools [antibodies, polymerase chain reaction (PCR), rapid diagnostic tests] to detect viruses in body fluids or tissue samples, to compare and classify these agents rapidly, and to determine the relationships between virus strains. The marriage of behavioral, geographic, and molecular epidemiology made this a most powerful science. The previous section reviewing advances in oncology pays tribute to this strategy by D. Burkitt and K. Takatsuki, leading to the identification of Epstein-Barr virus (EBV) and HTLV-1. Similarly, the recombinant DNA revolution overcame the problems of propagating the human papillomaviruses, permitting the isolation of new virus serotypes and setting off epidemiologic correlations for high- or low-risk cancer viruses (158). The human papillomaviruses (see Chapter 65 and Chapter 66) differ in transmission, location on the body, their nature of pathogenesis, persistence, and so forth. Similarly, the first recognition of the so-called slow viruses, which cause spongiform encephalopathies (Chapter 90), came from epidemiologic studies with humans and animals.

With the evolutionary development of a sophisticated immune system in vertebrates, the viruses of these hosts faced a new challenge in that they could no longer productively infect their hosts more than once. For their part, these viruses responded with the establishment of latency (herpesviruses), attacking directly the cells involved in the immune response (HIV), setting up persistent or chronic infections, or devising rapid methods for antigenic changes (influenza A virus). This ability of influenza A to undergo antigenic drift and shift resulted in the development of a lifestyle that uses Darwinian principles in a time frame shorter than that of any other organism. G. Hirst and his colleagues (1941–1950) developed the diagnostic tools to follow this. This permitted both the typing of the hemagglutinin of influenza A strains and the monitoring of the antibody response to it in patients (see Chapter 46 and Chapter 47). This has been used, with more and more sophisticated molecular approaches, to prove the presence of animal reservoirs for this virus, the reassortment of chromosomes between human and animal virus strains (antigenic shift), and a high rate of mutation

(antigenic drift) caused by RNA-dependent RNA synthesis with no known RNA editing or corrective mechanisms. How these molecular events led to episodic local epidemics and worldwide pandemics has now been explained in broad outline. Although most viruses tend to come to equilibrium with their hosts and become endemic [see, for example, the introduction of the North American myxoma–fibroma virus into the wild European rabbit population in Australia (49)], influenza A virus remains epidemic, changing from local to pandemic via an antigenic shift of its HA subunit gene. These studies (Chapter 46 and Chapter 47) have revealed an extraordinary lifestyle. Similarly, the study of the mechanisms of viral pathogenesis and the role of the immune system in this process have led to new insights in the virus–host relationship.

The field of viral epidemiology is changing rapidly with the advent of a new branch termed molecular epidemiology. The PCR technologies permit rapid sampling of viruses without growth in culture or plaque purification. Rapid genome sequencing has revealed sequence relationships between viruses, and sequence heterogeneity within a virus population. Mutation rates are documented and localized in the genome, and their biologic consequences are tested. Just two mutations altered the feline panleukopenia virus into a canine parvovirus, for the first time highly pathogenic and transmissible in dogs. Similarly, the earliest isolates of HIV (ZR 1959) suggest an HIV origin in the 1940s to 1950s derived from a chimpanzee lentivirus in Africa. Rapid mutation forming a population of viruses or clades followed.

Molecular epidemiology will now be required to detect and follow emerging viruses. Whether the next emerging epidemic will result from a novel variant of Ebola virus, Hantan virus, or Norwalk virus, or the more common possibility of a new pandemic variant of influenza virus remains to be seen. What is much clearer, however, is that the demographics of the human population on earth have begun to change. Even as birth rates slow, earth will house 8 to 10 billion people by 2050 to 2100. For the first time, there will be three to four times more people above the age of 60 than below 3 to 4 years of age. We will become an increasingly urban population, with more than 20 to 30 cities containing more than 10 million people. Clearly, human behavioral patterns (increased population density, increased travel, increased ages of the population) will provide the environment for the selection of emerging viruses and the challenges to the new field of molecular epidemiology.

Host–Virus Interactions and Viral Pathogenesis

The modern era of virology (1960 to present), which was developed largely with studies in cell culture, described in great detail the replicative cycles of viruses. Virologists demonstrated the elaborate interactions between viral genomes or viral proteins and host cell proteins. As indicated previously, this resulted in an extraordinary inquiry into the functions of infected or uninfected host cells using the tools of both molecular and cell biology. As this approach matured, the questions became more detailed and of less general interest, and so virologists turned back to the natural host animal, or a related animal model, to address new sets of questions. Chief among these questions was, how does a virus cause disease processes in the animal? How do we quantitate viral virulence and what is the genetic basis of an attenuated virus? These studies have identified, in selected viruses, a set of genes and functions that broadly impact on viral virulence.

Six categories of such factors have been explored to date. First, mutations in genes that impair virus replication in the host reduce the number of viruses produced below a level required to produce disease. These are essential genes (essential for life) *in vivo*. A second class of mutations has been identified in viral genes: These mutations impair virulence but do not alter normal virus replication at least in some cell or tissue types. Here, host or tissue range mutations are most common. Mutations can change the pattern of virus adsorption to a particular cell type and so prevent viral entry into a cell. Mutations in viral enhancer elements can alter the ability of a virus to transcribe its genes in selected cell types. All three strains of the Sabin poliovirus attenuated vaccine contain mutations in the viral RNA 5' untranslated region, which reduce translation of these RNAs in selected cells (i.e., neurons) but still allow replication in other cells and immunization of the host. A third class of genes critical to regulating viral virulence produce gene products that modify the host defenses. HIV infection kills CD-4 T-cells and eliminates an immune response. Some viruses encode genes that produce virokines that are secreted from infected cells and modify the immune response to infection. Other viruses produce proteins that alter the human leukocyte antigen (HLA) presentation of viral antigens in infected cells, whereas some viruses produce superantigens that stimulate or eliminate lymphoid cells of a selected specificity or with a class of receptors. Many viruses produce proteins that block infected cells from undergoing apoptosis in response to a virus infection. Some viruses, such as African swine fever virus, secrete a pro-apoptotic factor that kills lymphocytes and enhances its virulence. A fourth class of genes and products produced by viruses that impacts on virulence enhances the spread of a virus in the host organism. Some viruses contain signals that permit the virus to bud from an infected cell specifically at the apical or basolateral surfaces of a tissue, permitting selected spread *in vivo*. Some of the RNA viruses acquire infectivity by budding out of a cell using specific proteolytic cleavage of their glycoproteins. This is done in some cases by a viral protease and in others by a cellular protease, each requiring a specific amino acid sequence in the glycoprotein for proper cleavage and spread of the virus. Altering this sequence alters the host cell, which can then give rise to infectious virus, and it can also impact on virulence.

A wide variety of polymorphisms or mutations in the host animal can modulate resistance or virulence of a virus. These mutations can even be selected for during viral epidemics, thus changing the hosts' gene pool. In humans, polymorphisms in a cytokine or cytokine receptor gene imparts resistance to HIV infection at the level of viral absorption. Variations in the immune responses of diverse hosts in a population will result in large variations in the virulence of a virus. The mechanisms used by the host to minimize viral diseases after infection will be a major subject for study in the future.

Finally, changes in population density, life styles, cultural traditions, and economic factors have all played a major role in virus virulence. Poliovirus was a minor endemic virus infection for 3,000 years prior to the introduction of improved sanitation in the last century. This created a population that was infected with this virus for the first time at a later age and resulted in poliovirus epidemics that included tens of thousands of cases. It may not have been a coincidence that the worst influenza epidemic in the century, killing 40 million people, started about 1918 toward the end of World War I, with so many people dislocated and moving about the world in very poor conditions. Cultural and environmental changes will

surely play a role in the virulence of viruses.

AFTERWORD: D'HERELLE'S DREAM AND KOCH'S POSTULATES

It was d'Herelle's dream to use bacteriophages as a “magic bullet” to kill bacteria and cure diseases caused by these agents. The ultimate historical irony of that dream was played out by some of the participants described in this chapter over the same time frame.

The story begins in 1884, when Friedrich Johannes Loeffler in Germany used Koch's postulates to isolate and identify the bacterium that causes diphtheria. [Fourteen years later, he and Paul Frosch (both had been trained by R. Koch) would isolate the foot-and-mouth disease virus, the first animal virus to be isolated.] Loeffler was surprised to note, however, that when he inoculated the bacteria into an animal, the bacilli were detected only at the site of the local injection, whereas the abnormalities responsible for the disease were visible in the heart, liver, kidneys, and so forth. Loeffler hypothesized that the bacilli produced a toxin that caused the disease at remote sites in the body with no detectable bacteria. In the next step (in 1888), Emile Roux and A. Yersin (at the Pasteur Institute) demonstrated a heat-labile soluble toxin in the fluid phase above the diphtheria bacillus cultures. An injection of the toxin (which could be filtered through a Chamberland filter) into animals reproduced the symptoms. Emile Roux was a student of Pasteur's who worked with Chamberland on his filters. When Ivanofsky suggested that the filterable agent in his TMV preparations was a toxin, he credited Roux for this idea and his work on diphtheria toxin as precedence (76). In 1898, E. I. E. Nocard and Roux isolated from cows a pleuropneumonia organism that could pass through a Chamberland filter, could be seen as microscopic dots in the light microscope, and could be replicated in collodion sacs in a cell-free meat infusion in the peritoneal cavity of a rabbit. In this way they discovered mycoplasma, a bacteria that violated the “filterable agent” concept of a virus or a liquid that could replicate only in an intracellular environment. In fact, in 1903, Roux challenged Beijerinck's idea of a fluid contagium by calling it “very original” but not distinguishable from his tiny mycoplasma spores, which were microorganisms. In 1903, Roux (124) wrote the first review article on viruses, entitled “Sur les microbes dits 'invisible.'”

By 1890 in Germany, von Behring and Kitasato treated the diphtheria toxin with chemical agents to inactivate it and then immunized animals with it. They demonstrated that serum from immunized animals protected other animals from the toxin and, on Christmas night in 1891 in Berlin, this antitoxin was first given to a child with diphtheria. Shibasaburo Kitasato had also developed a microorganism filter with somewhat finer pores than the Chamberland filter, and he even sold it commercially. Loeffler, in 1898, used both the Chamberland and the Kitasato filters to test whether foot-and-mouth disease virus was retained by or passed through these two filters. This virus readily passed through the Chamberland filter but lost virulence by repeated filtration through a Kitasato filter. Loeffler used these data to claim that the virus was particulate, or corpuscular, partly stopped by the Kitasato filter, and he thus challenged Beijerinck's concept of a fluid contagium (a liquid virus). Beijerinck responded that he felt that the foot-and-mouth disease virus was adsorbed to the Kitasato filter and was not stopped by its smaller pore size. The scientific arguments concerning the nature of viruses continued.

In 1923, G. Ramon introduced a formalin-treated diphtheria toxin as an immunizing agent, and an effective vaccine was in hand that all but eliminated this disease from countries that had an active vaccination program. Although that should have been the end of a good story, it was not. In 1951, V. J. Freeman (58) made the unexpected observation that all virulent strains of *Corynebacterium diphtheriae* are lysogenic with a phage called beta. If the bacteria are cured of this prophage, they fail to produce toxin and are avirulent. Indeed, the gene for this toxin is encoded by the phage genome and is regulated by the metabolic state of the bacteria. The lysogenic virus causes this disease. Koch's postulates had been circumvented by the intimate association of a virus and its host as first described by Lwoff and his colleagues. D'Herelle's dream of a phage curing a disease was given a cruel twist of fate in this case. These filterable agents, be they toxins, viruses, or even viruses that produce toxins when lysogenic in their host and so cannot pass a filter, all have the power to present a most confusing or at the very least a complex picture to the observer.

It is a tribute to the scientists of those times that they came so close to describing reality, while inferring the existence of organisms that could not be seen except by their effect on a host. These 100 years of virology have forged new concepts and provided novel insights into the processes of life.

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CHAPTER 2 Principles of Virology

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Viruses are unique in nature. They are the smallest of all self-replicating organisms, historically characterized by their ability to pass through filters that retain even the smallest bacteria. In their most basic form, viruses consist solely of a small segment of nucleic acid encased in a simple protein shell. They have no metabolism of their own but rather are obliged to invade cells and parasitize subcellular machinery, subverting it to their own purposes. Many have argued that viruses are not even living, although to a seasoned virologist they exhibit a life as robust as any other creature.

The apparent simplicity of viruses is deceptive. As a group, viruses infect virtually every organism in nature, they display a dizzying diversity of structures and lifestyles, and they embody a profound complexity of function.

The study of viruses, virology, must accommodate both the uniqueness and the complexity of these organisms. The singular nature of viruses has spawned novel methods of classification and experimentation entirely peculiar to the discipline of virology. The complexity of viruses is constantly challenging scientists to adjust their thinking and their research to describe and understand some new twist in the central dogma revealed in a “simple” virus infection.

This chapter explores several concepts fundamental to virology as a whole, including virus taxonomy, virus cultivation and assay, and virus genetics. The chapter is not intended as a comprehensive or encyclopedic treatment of these topics but rather as a relatively concise overview with sufficient documentation for more in-depth study. In addition to primary resources and practical experience, the presentation draws heavily on previous editions of *Fields Virology* (29,30 and 31) for both the taxonomy and the genetics material, plus several excellent texts for material on virus cultivation and assay (16,28,35,50,57,60,65). It is hoped that this chapter will be of value to anyone learning virology at any stage: a novice trying to understand basic principles for the first time, an intermediate student of virology trying to understand the technical subtleties of virologic protocols in the literature, or a bewildered scientist in the laboratory wondering why the temperature-sensitive mutant sent by a colleague does not seem to be temperature sensitive.

VIRUS TAXONOMY

A coherent and workable system of classification, a taxonomy, is a critical component of the discipline of virology. However, the unique nature of viruses has defied the strict application of many of the traditional tools of taxonomy used in other disciplines of biology. Thus, scientists who concern themselves with global taxonomy of organisms have traditionally left the viruses scattered throughout the major kingdoms, reasoning that viruses have more in common with their individual hosts than they do with each other (66). By contrast, for practical reasons at least, virologists agree that viruses should be considered together as a separate group of organisms regardless of host, be it plant, animal, fungus, protist, or bacterium, a philosophy borne out by the observation that in several cases viruses now classified in the same family, for example family *Rhabdoviridae*, infect hosts from different kingdoms. Interestingly, the discipline of virus taxonomy brings out the most erudite and thought-provoking, virtually philosophical discussions about the nature of viruses, probably because the decisions that must be made to distinguish one virus from another require the deepest thought about the nature of viruses and virus evolution. In the end, all of nature is a continuum, and the business of taxonomy has the unfortunate obligation of drawing boundaries within this continuum, an artificial and illogical task but necessary nevertheless. The execution of this obligation results today in a free-standing virus taxonomy, overseen by the International Committee on Taxonomy of Viruses (ICTV), with rules and tools unique to the discipline of virology. The process of virus taxonomy that has evolved uses some of the hierarchical nomenclature of traditional taxonomy, identifying virus species and grouping them into genera, genera into families, and families into orders, but at the same time, to cope with both the uniqueness and diversity of viruses as a group, the classification process has been deliberately nonsystematic and thus is “based upon the opinionated usage of data” (73).

Most important, the virus taxonomy that has been developed works well. For the trained virologist, the mention of a virus family or genus name, such as family *Herpesviridae* or genus *Rotavirus*, immediately conjures forth a set of characteristics that form the basis for further discussion or description. Virus taxonomy serves an important practical purpose as well, in that the identification of a limited number of biologic characteristics, such as virion morphology, genome structure, or antigenic properties, quickly provides a focus for identification of an unknown agent for the clinician or epidemiologist and can significantly impact further investigation into treatment or prevention of a virus disease. Virus

taxonomy is an evolving field, and what follows is a summary of the state of the art, including important historical landmarks that influenced the present system of virus taxonomy, a description of the system used for virus taxonomy and the means for implementation of that system, and a very brief overview of the taxonomy of viruses that infect humans and animals.

History and Rationale

Virology as a discipline is scarcely 100 years old, and thus the discipline of virus taxonomy is relatively young. In the early 1900s, viruses were initially classified as distinct from other organisms simply by virtue of their ability to pass through unglazed porcelain filters known to retain the smallest of bacteria. As increasing numbers of filterable agents became recognized, they were distinguished from each other by the only measurable properties available, namely the disease or symptoms caused in an infected organism. Thus animal viruses that caused liver pathology were grouped together as hepatitis viruses, and viruses that caused mottling in plants were grouped together as mosaic viruses. In the 1930s, an explosion of technology spawned a description of the physical properties of many viruses, providing numerous new characteristics for distinguishing viruses one from another. The technologies included procedures for purification of viruses, biochemical characterization of purified virions, serology, and, perhaps most important, electron microscopy, in particular negative staining, which permitted detailed descriptions of virion morphology, even in relatively crude preparations of infected tissue. In the 1950s, these characterizations led to the distinction of three major animal virus groups, the myxoviruses, the herpesviruses, and the poxviruses. By the 1960s, because of the profusion of data describing numerous different viruses, it became clear that an organized effort was required to classify and name viruses, and thus the ICTV (originally the International Committee on the Nomenclature of Viruses, ICNV) was established in 1966. The ICTV functions today as a large, international group of virologists organized into appropriate study groups, whose charge it is to develop rules for the classification and naming of viruses, and to coordinate the activities of study groups in the implementation of these rules.

Early in its history, the ICTV wrestled with the fundamental problem of developing a taxonomic system for classification and naming of viruses that would accommodate the unique properties of viruses as a group, and that could also anticipate advancements in the identification and characterization of viruses. Perhaps the most critical issue was whether the classification of viruses should consider virus properties in a monothetical, hierarchical fashion, or in a polythetical, hierarchical fashion (definition to follow). A monothetical, hierarchical classification, modeled after the Linnean system used for classification of plants and animals, would effectively rank individual virus properties, such as genome structure or virion symmetry, as being more or less important relative to each other, and use these individual characteristics to sort viruses into subphyla, classes, orders, suborders, and families (63). Whereas the hierarchical ordering of viruses into groups and subgroups is desirable, a strictly monothetical approach to using virus properties in making assignments to groups was problematic because both the identification of individual properties to be used in the hierarchy, and the assignment of a hierarchy to individual properties seemed too arbitrary.

A polythetic approach to classification would group viruses by comparing simultaneously numerous properties of individual viruses, without assigning a universal priority to any one property. Thus, using the polythetic approach, a given virus grouping is defined by a collection of properties rather than a single property, and virus groups in different branches of the taxonomy may be characterized by different collections of properties. One argument against the polythetic approach is that a truly systematic and comprehensive comparison of dozens of individual properties would be at least forbidding if not impossible. However, this problem could be avoided by the adoption of a nonsystematic approach, namely, using study groups of virologists within the ICTV to consider together numerous characteristics of a virus and make as rational an assignment to a group as possible. Therefore, the system that is currently being used is a nonsystematic, polythetical, hierarchical system. This system differs from any other taxonomic system in use for bacteria or other organisms, but it is effective and useful, and it has withstood the test of time (72). As our understanding of viruses increases, and as new techniques for characterization are developed, notably comparison of gene and genome sequences, the methods used for taxonomy will undoubtedly continue to evolve.

As a consequence of the polythetic approach to classification, the virus taxonomy that exists today has been filled initially from the middle of the hierarchy by assigning viruses to genera, and then elaborating the taxonomy upward by grouping genera into families and to a limited extent families into orders. By 1970, the ICTV had established two virus families, each containing two genera, 24 floating genera, and 16 plant “groups” (106). A rigorous species definition (100), discussed later, was not approved by the ICTV until 1991, but it has now been applied to the entire taxonomy and has become the primary level of classification for viruses. As of this writing, the complete virus taxonomy comprises 3 orders, 69 families, 9 subfamilies, 243 genera, 1550 species, and 2404 tentative species (101). The complete virus taxonomy is far too extensive to relate here, but examples of the results of the taxonomy are offered in Table 1 and Table 2. Table 1 lists the distinguishing characteristics of the major animal virus families detailed in this edition of *Fields Virology,* and Table 2 provides an example of the entire taxonomic classification of one virus order, the order *Mononegavirales.*

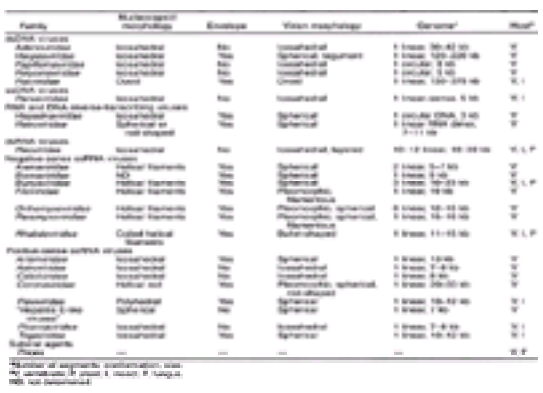

TABLE 1. *Summary of characteristics of major animal virus families*

Order Family	Subfamily	Genus	Type species	Host
Mononegavirales				
Bornaviridae		*Bornavirus*	*Borna disease virus*	Vertebrates
Filoviridae		Marburg-like viruses	*Marburg virus*	Vertebrates
		Ebola-like viruses	*Zaire Ebola virus*	Vertebrates
Paramyxoviridae	Paramyxovirinae	*Respirovirus*	*Sendai virus*	Vertebrates
		Rubulavirus	*Mumps virus*	Vertebrates
		Morbillivirus	*Measles virus*	Vertebrates
	Pneumovirinae	*Pneumovirus*	*Human respiratory syncytial virus*	Vertebrates
		Metapneumovirus	*Turkey rhinotracheitis virus*	Vertebrates
Rhabdoviridae		*Vesiculovirus*	*Vesicular stomatitis Indiana virus*	Vertebrates
		Lyssavirus	*Rabies virus*	Vertebrates
		Ephemerovirus	*Bovine ephemeral fever virus*	Vertebrates
		Novirhabdovirus	*Infectious hematopoietic necrosis virus*	Vertebrates
		Cytorhabdovirus	*Lettuce necrotic yellow virus*	Plants
		Nucleorhabdovirus	*Potato yellow dwarf virus*	Plants

Source: From ref. 101, with permission.

TABLE 2. *Taxonomy of the order* Mononegavirales

The ICTV Universal System of Virus Taxonomy

Structure and Function of the ICTV

The ICTV is a committee of the Virology Division of the International Union of Microbiological Societies. The objectives of the ICTV are to develop an internationally agreed-upon taxonomy and nomenclature for viruses, to maintain an index of virus names, and to communicate the proceedings of the committee to the international community of virologists. The ICTV publishes an update of the taxonomy at approximately 3-year intervals (27,33,68,69,73,106). At the time of this writing, the most recent report is the seventh (101). The ICTV also supports a web site (http://www.ncbi.nlm.nih.gov/ICTV/), which contains all its published information in a conveniently interactive format, plus links to additional sites of interest, including the universal virus database of the ICTV (ICTVdB), described later.

Virus Properties and Their Use in Taxonomy

As introduced previously, the taxonomic method adopted for use in virology is polythetic, meaning that any given virus group is described using a collection of individual properties. The description of a virus group is nonsystematic in that there exists no fixed list of properties that must be considered for all viruses, and no strict formula for the ordered consideration of properties. Instead, a set of properties describing a given virus is simply compared with other viruses described in a similar fashion to formulate rational groupings. Dozens of properties can be listed for description of a virus, but they break down generally into virion morphology, including size, shape, capsid symmetry, and presence or absence of an envelope, virion physical properties, including genome structure, sensitivity to physical or chemical insults; specific features of viral lipids, carbohydrates, and structural and nonstructural proteins; antigenic properties; and biologic properties including replication strategy, host range, mode of transmission, and pathogenicity (72,73).

The Hierarchy

The ICTV has adopted a universal classification scheme that employs the hierarchical levels of order, family, subfamily, genus, and species. Because the polythetic approach to classification introduces viruses into the middle of the hierarchy, and because the ICTV has taken a relatively conservative approach to grouping taxa, levels higher than order are not currently used. Levels lower than species, such as strains and variants, are not officially considered by the ICTV but are left to specialty groups.

A virus species is defined as “a polythetic class of viruses that constitutes a replicating lineage and occupies a particular ecological niche” (100). The formal definition of a polythetic class is “a class whose members always have several properties in common although no single common attribute is present in all of its members” (101). Thus, no single property can be used to define a given species, and application of this formal definition of a polythetic class to species accounts nicely for the inherent variability found among members of a species. The qualification of a replicating lineage implies that members of a species experience evolution over time with consequent variation, but that members share a common ancestor. The qualification of occupation of an ecologic niche acknowledges that the biology of a virus, including such properties as host range, pathogenesis, transmission, and habitat, are fundamental components of the characterization of a virus. In the most recent report of the ICTV, study group members have listed the criteria that identify each species, then listed species according to the criteria. In addition, some viruses are listed as tentative species because their taxonomic status cannot currently be unambiguously determined. Last, a type species, the species used to define the taxon, has been identified for each genus.

Taxonomic levels higher than species are formally defined by the ICTV only in a relative sense—namely, a genus is a group of species sharing certain common characters, a subfamily is a group of genera sharing certain common characters, a family is a group of genera or subfamilies sharing certain common characters, and an order is a group of families sharing certain common characters. As the virus taxonomy has evolved, these higher taxa have acquired some monothetic character. They remain polythetic in that they may be characterized by more than one virus property, but they violate the formal definition of a polythetic class in that one or more defining properties may be required of all candidate viruses for membership in the taxon. Not all taxonomic levels need be used for a given grouping of viruses, thus whereas most species are grouped into genera and genera into families, not all families contain subfamilies, and only a few families have been grouped into orders. Consequently, the family is the highest consistently used taxonomic grouping, it therefore carries the most generalized description of a given virus group, and as a result it has become the benchmark of the taxonomic system. Most families have distinct virion morphology, genome structure, and/or replication strategy (Table

1).

Nomenclature

The ICTV has adopted a formal nomenclature for viruses, specifying suffixes for the various taxa, and rules for written descriptions of viruses. Names for genera, subfamilies, families, and orders must all be single words, ending with the suffixes -virus, -virinae, -viridae, and -virales, respectively. Species names may contain more than one word and have no specific ending. In written usage, the formal virus taxonomic names are capitalized and written in italics, and they are preceded by the name of the taxon, which is neither capitalized nor italicized. For species names that contain more than one word, the first word plus any proper nouns are capitalized. As an example, the full formal written description of human respiratory syncytial virus is order *Mononegavirales*, family *Paramyxoviridae*, subfamily *Pneumovirinae*, genus *Pneumovirus*, species *Human respiratory syncytial virus.*

Order of Presentation of the Viruses

For convenience in presenting or tabulating the virus taxonomy, informal categorical groupings of taxa have been created and together are called an order of presentation. Although superficially the order of presentation appears to function as a higher taxonomic level, it is important to understand that no hierarchy or official taxonomic classification is intended. Currently, the criteria used to specify the order of presentation are nature of the viral genome, strandedness of the viral genome, polarity of the genome, and reverse transcription. In addition, two categories have been created for subviral agents, one for viroids and another for satellites and prions. Finally, a category exists for unassigned viruses. These criteria give rise to nine groupings in the order of presentation, specifically dsDNA viruses, ssDNA viruses, dsRNA viruses, negative-sense ssRNA viruses, positive-sense ssRNA viruses, DNA and RNA reverse transcribing viruses, subviral agents (viroids), subviral agents (satellites and prions), and unassigned viruses. Table 1 provides an example of the usage of the order of presentation.

Universal Virus Database of the ICTV

To facilitate the management and distribution of virologic data, the ICTV has established the universal virus database of the ICTV (ICTVdB). The ICTVdB is accessible on the worldwide web at http://life.anu.edu.au/viruses/welcome.htm, with mirror sites in Europe (http://www.res.bbsrc.ac.uk/mirror/auz/welcome.htm) and North America (http://www.ncbi.nlm.nih.gov/ICTVdB/welcome.htm). The ICTVdB, constructed from virus descriptions in the published reports of the ICTV, comprises searchable descriptions of all virus families, genera, and type species, including microscopic images of many viruses. The ICTVdB is a powerful resource for management of and access to virologic data, and it promises to considerably extend the reach and capability of the ICTV.

VIRUS CULTIVATION AND ASSAY

Different branches of science are defined in large part by their techniques, and virology is no exception. Although the study of viruses uses some general methods that are common to other disciplines, the unique nature of viruses and virus infections requires a unique set of technical tools designed specifically for their investigation. Conversely, what we know and can know about viruses is delimited by the techniques used, and therefore a genuine understanding of virology requires a clear understanding of virologic methods. What follows therefore is a summary of the major techniques essential and unique to all of virology, presented as fundamental background for understanding the discipline.

Initial Detection and Isolation

The presence of a virus is evidenced initially by effects on a host organism, or, in the case of a few animal viruses, by effects on cultured cells. Effects on animal hosts include a broad spectrum of symptoms, including skin and mucous membrane lesions; digestive, respiratory, or neurologic disorders; immune dysfunction; or specific organ failure such as hepatitis or myocarditis. Effects on cultured cells include a variety of morphologic changes in infected cells, termed cytopathic effects and described in detail later in this chapter and in Chapter 18. Both adenovirus (87) and the polyomavirus SV40 (98) were discovered as cell culture contaminants before they were detected in their natural hosts.

Viruses can be isolated from an affected host by harvesting excreted or secreted material, blood, or tissue and testing for induction of the original symptoms in the identical host, or for induction of some abnormal pathology in a substitute host or in cell culture. Historically, dogs, cats, rabbits, rats, guinea pigs, hamsters, mice, and chickens have all been found to be useful in laboratory investigations (57), although most animal methods have now been replaced by cell culture methods (65). Once the presence of a virus has been established, it is often desirable to prepare a genetically pure clone, either by limiting serial dilution or by plaque purification.

Viruses that are cultivated in anything other than the natural host may adapt to the novel situation through acquisition of genetic alterations that provide a replication advantage in the new host. Such adaptive changes may be accompanied by a loss of fitness in the original host, most notably by a loss of virulence or pathogenicity. Although this adaptation and attenuation may present problems to the basic scientist interested in understanding the replication of the virus in its natural state, it also forms the basis of construction of attenuated viral vaccines.

Hosts for Virus Cultivation

Laboratory Animals and Embryonated Chicken Eggs

Prior to the advent of cell culture, animal viruses could be propagated only on whole animals or embryonated chicken eggs. Whole animals could include the natural host—laboratory animals such as rabbits, mice, rats, and hamsters. In the case of laboratory animals, newborn or suckling rodents often provide the best hosts. Today, laboratory animals are

seldom used for routine cultivation of virus, but they still play an essential role in studies of viral pathogenesis.

The use of embryonated chicken eggs was introduced to virology by Goodpasture et al. in 1932 (38) and developed subsequently by Beveridge and Burnet (3). The developing chick embryo, 10 to 14 days after fertilization, provides a variety of differentiated tissues, including the amnion, allantois, chorion, and yolk sac, which serve as substrates for growth of a wide variety of viruses, including orthomyxoviruses, paramyxoviruses, rhabdoviruses, togaviruses, herpesviruses, and poxviruses (57). Members of each of these virus families may replicate in several tissues of the developing egg, or replication may be confined to a single tissue. Several viruses from each of the groups just mentioned cause discrete and characteristic foci when introduced onto the chorioallantoic membrane (CAM) of embryonated eggs, thus providing a method for identification of virus types, or for quantifying virus stocks or assessing virus pathogenicity (Fig. 1). Although the embryonated eggs have been almost wholly replaced by cell culture techniques, they are still the most convenient method for growing high-titer stocks of some viruses, and they thus continue to be used both in research laboratories and for vaccine production. In addition, pock formation on the CAM still provides a specialized method for assay of variants of poxviruses: wild type rabbitpox and cowpox viruses cause red hemorrhagic pocks on the CAM, whereas viruses deficient in specific virulence genes cause white pocks as a result of the infiltration of the lesions with inflammatory cells (34,77) (Fig. 1).

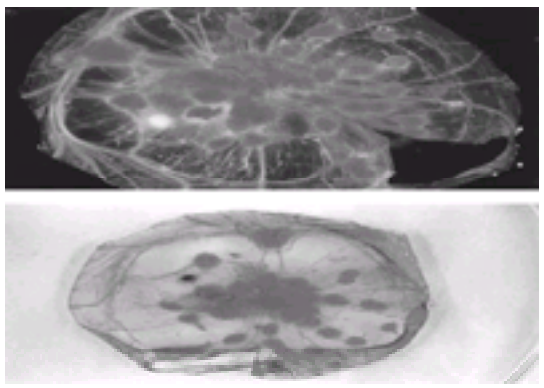

FIG. 1. Cowpox-induced pock formation on the chorioallantoic membrane of chick embryos. The CAMs of intact chicken embryos, 11 days old, were inoculated with cowpox and the eggs were incubated for an additional 3 days at 37.5°C. CAMs were then dissected from the eggs and photographed. The membrane shown at the *top* was untreated, and the membrane at the *bottom* was stained with nitroblue tetrazolium (NBT), an indicator of activated heterophils (34). Wild-type cowpox forms hemorrhagic pocks on the membrane (*top and bottom*). Spontaneous deletion mutants of cowpox virulence genes occur at a high frequency, resulting in infiltration of inflammatory cells into the pock. This infiltration of inflammatory cells causes the pocks to appear white in unstained membrane preparations or dark on NBT-stained membranes. The unstained membrane preparation contains a single white pock; the NBT-stained preparation contains a single dark pock. (Courtesy of Dr. R. Moyer.)

Cell Culture

The maintenance of animal cells *in vitro*, described generally (albeit incorrectly) as tissue culture, can be formally divided into three different techniques: organ culture, primary explant culture, and cell culture. In organ culture, the original three-dimensional architecture of a tissue is preserved under culture conditions that provide a gas–liquid interface. In primary explant culture, minced pieces of tissue placed in liquid medium in a culture vessel provide a source for outgrowth of individual cells. In cell culture, tissue is disaggregated into individual cells prior to culturing. Only cell culture will be discussed in detail here, as it is the most commonly used tissue culture technique in virology.

Cultured cells currently provide the most widely used and most powerful hosts for cultivation and assay of viruses. Cell cultures are of three basic types: primary cell cultures, cell strains, and cell lines, which may be derived from many animal species, and which differ substantially in their characteristics. Viruses often behave differently on different types of cultured cells, and in addition each of the culture types possesses technical advantages and disadvantages. For these reasons, an appreciation of the use of cultured cells in animal virology requires an understanding of several fundamentals of cell culture itself. A detailed description of the theory and practice of cell and tissue culture is provided by Freshney (35), and several additional texts provide excellent summaries of cell culture as it specifically applies to virology (16,28,50).

Primary Cell Culture

A primary cell culture is defined as a culture of cells obtained from the original tissue that have been cultivated *in vitro* for the first time, and that have not been subcultured. Primary cell cultures can be established from whole animal embryos, or from selected tissues from embryos, newborn animals, or adult animals of almost any species. The most commonly used cell cultures in virology derive from primates, including humans and monkeys; rodents, including hamsters, rats, and mice; and birds, most notably chickens. Cells to be cultured are obtained by mincing tissue, and dispersing individual cells by treatment with proteases and/or collagenase to disrupt cell–cell interactions and interactions of cells with the extracellular matrix. With the exception of cells from the hemopoietic system, normal vertebrate cells will grow and divide only when attached to a solid surface. Dispersed cells are therefore placed in a plastic flask or dish, the surface of which has been treated to promote cell attachment. The cells are incubated in a buffered nutrient medium in the presence of blood serum, which contains a complex mixture of hormones and factors required for the growth of normal cells. The blood serum may come from a variety or sources, but bovine serum is most commonly used. Under these conditions, cells will attach to the surface of the dish, and they will divide and migrate until the surface of the dish is covered with a single layer of cells, a monolayer, whereupon they will remain viable but cease to divide. If the cell monolayer is

“wounded” by scraping cells from an isolated area, cells on the border of the wound will resume division and migration until the monolayer is reformed, whereupon cell division again ceases. These and other observations led to the conclusion that the arrest of division observed when cells reach confluency results from cell–cell contact, and it is therefore called contact inhibition. Primary cultures may contain a mixture of cell types, and they retain the closest resemblance to the tissue of origin.

Subcultivation

Cells from a primary culture may be subcultured to obtain larger numbers of cells. Cells are removed from the culture dish and disaggregated by treating the primary cell monolayer with a chelating agent, usually ethylenediamine tetra-acetic acid (EDTA), or a protease, usually trypsin, or both, giving rise to a single-cell suspension. This suspension is then diluted to a fraction of the original monolayer cell density and placed in a culture dish with fresh growth medium, whereupon the cells attach to the surface of the dish and resume cell division until once again a monolayer is formed and cell division ceases. Cultures established in this fashion from primary cell cultures may be called secondary cultures. Subsequently, cells may be repeatedly subcultured in the same fashion. Each subculturing event is called a passage, and each passage may comprise a number of cell generations, depending on the dilution used during the passage. Most vertebrate cells divide at the rate of approximately one doubling every 24 hours at 37°C. Thus, a passage performed with an eightfold dilution will require three cell doublings over 3 days before the cells regain confluency.

Cell Strains

Normal vertebrate cells cannot be passaged indefinitely in culture. Instead, after a limited number of cell generations, usually 20 to 100 depending on the age and species of the original animal, cultured normal cells cease to divide and they degenerate and die, a phenomenon called crisis or senescence (Fig. 2) (45). Starting with the establishment of a secondary culture and until cells either senesce or become transformed (as will be described later), the culture is termed a cell strain, to distinguish it from a primary culture on the one hand, or a transformed, immortal cell line, on the other hand. During culture, cells in a strain retain their original karyotype and are thus called euploid; however, culturing induces profound changes in the composition and characteristics of the cell strain, and these changes are manifested early during the passage history and may continue during passage. Whereas primary cell cultures may contain a mixture of cell types that survive the original plating of cells, only a few cell types survive subculturing, so that by the second or third passage typically only one cell type remains in the cell strain.

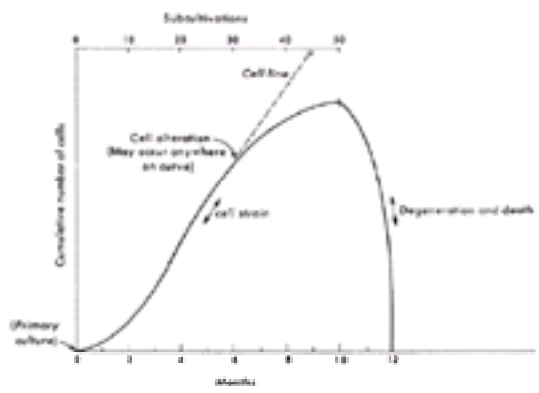

FIG. 2. Growth of cells in culture. A primary culture is defined as the original plating of cells from a tissue, grown to a confluent monolayer, without subculturing. A cell strain (*solid line*) is defined as a euploid population of cells subcultivated more than once *in vitro*, lacking the property of indefinite serial passage. Cell strains ultimately undergo degeneration and death, also called crisis or senescence. A cell line (*dashed line*) is an aneuploid population of cells that can be grown in culture indefinitely. Spontaneous transformation or alteration of a cell strain to an immortal cell line can occur at any time during cultivation of the cell strain. The time in culture and corresponding number of subcultivations or passages are shown on the abscissas. The ordinate shows the total number of cells that would accumulate if all were retained in culture. (From ref. 16 and adapted from ref. 45, with permission.)

Cell strains are usually composed of one of two basic cell types, fibroblast-like or epithelial-like, characterized based on their morphology and growth characteristics (Fig. 3). Fibroblasts have an elongated, spindle shape, whereas epithelial cells have a polygonal shape. Although after only a few passages only one cell type may remain in a cell strain, continued passage may select for faster-growing variants, such that the characteristics of a cell strain may change with increasing passage number. Despite the fact that normal cell strains experience senescence in culture, they may be maintained for many years by expanding the culture to a large number of cells early during the passage history and storing numerous small samples of low-passage cells by freezing. Thus, as a given strain approaches high passage number and senescence, low-passage cells of the same strain may be thawed and cultured.

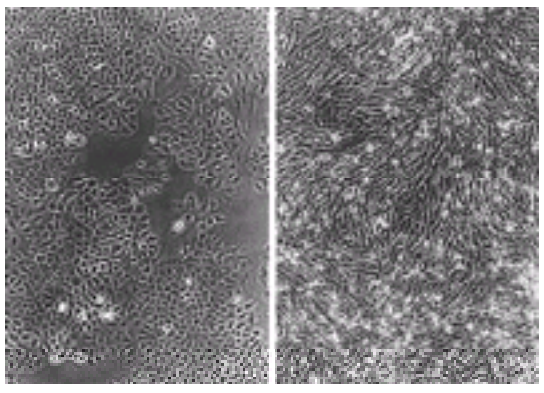

FIG. 3. Cultured cell types. Phase-contrast photomicrographs are shown. *Left*: Epithelial-like cells, A549, a human lung carcinoma cell line, a slightly subconfluent monolayer. *Right*: Fibroblast-like cells, BHK, a baby hamster kidney cell line. (A549 cell culture, courtesy of J. I. Lewis. BHK cell culture, courtesy of D. Holmes and S. Moyer.)

Cell Lines

At any time during the culture of a cell strain, cells in the culture may become transformed, meaning that they are no longer subject to crisis and senescence but can be passaged indefinitely. Transformation is a complex phenomenon, discussed in more detail later and in Chapter 10, but in the context of cell culture, the most important characteristic of transformation is that the transformed cells become immortalized. Immortal cell cultures are called cell lines, or sometimes continuous cell lines, to distinguish them from primary cultures and cell strains. Immortalization can occur spontaneously during passage of a cell strain, or it can be induced by treatment with chemical mutagens, infection with tumorigenic viruses, or transfection with oncogenes. In addition, cells cultured from tumor tissue frequently readily establish immortal cell lines in culture. Spontaneous immortalization does not occur in cultured cells from all animal species. Thus, immortalization occurs frequently during culture of rodent cells, for example in mouse and hamster cell strains, and it has been observed in monkey kidney cells, but it occurs rarely if at all during the culture of chicken or human cells. Immortalization is typically accompanied by genetic changes—such cells become aneuploid, containing abnormalities in the number and structure of chromosomes relative to the parent species, and not all cells in a culture of a continuous cell line necessarily display the same karyotype. Like cell strains, cell lines are usually composed of cells that are either fibroblast-like or epithelial-like in morphology.

As with the propagation of cell strains, continued culture of a cell line may result in selection of specific variants that outgrow other cells in the culture over time, and thus with passage the character of a cell line may change substantially, and cell lines of the same origin cultured in different laboratories over a period of years may have significantly different characteristics. It is prudent, therefore, to freeze stocks of cell lines having specific desirable properties so that these cells can be recovered if the properties disappear during culture. Likewise, it makes sense to obtain a cell line showing certain desired characteristics directly from the laboratory that described those characteristics, because cells from alternative sources may differ in character.

Transformation

Transformed cells are distinguished from normal cells by a myriad of properties, which can be grouped into three fundamental types of changes: immortalization, aberrant growth control, and malignancy. Immortalization refers simply to the ability to be cultured indefinitely, as described in the preceding section. Aberrant growth control comprises a number of properties, several of which have relevance to experimental virology, including loss of contact inhibition, anchorage independence, and tumorigenicity. Loss of contact inhibition means that cells no longer cease to grow as soon as a monolayer is formed and will now grow on top of one another. Anchorage independence means that the cells no longer need to attach to a solid surface to grow. Anchorage independence is often assayed as the ability to form colonies suspended in a semisolid medium such as agar, and a practical consequence of anchorage independence is the ability to grow in liquid suspension. Tumorigenicity refers to the ability of cells to form a tumor in an experimental animal, and malignancy refers to the ability to form an invasive tumor *in vivo.* Of course, malignancy is of vital importance as a phenomenon in its own right, but it has limited application in virology except within the specific discipline of tumor virology (Chapter 10).

It is important to note that the many properties of transformed cells are not necessarily interdependent, and no one property is an absolute prerequisite for another. Thus, transformation is thought to be a multistep genetic phenomenon, and varying degrees of transformation are measurable. Tumorigenicity is often regarded as the most stringent assay for a fully transformed cell, and it is most closely correlated with anchorage independence.

The fact that the various characteristics of transformed cells are not interdependent has important consequences for experimental virology, especially in the assay of tumor viruses. Specifically, a transformed cell line that is immortalized but still contact-inhibited may be used in a viral transformation assay that measures the further transformation to loss of contact inhibition. When cells in a monolayer are transformed by a tumor virus and lose contact inhibition, they grow on top of a confluent monolayer forming a *focus*, or a pile of cells, which is readily distinguishable from the rest of the monolayer. This property forms the basis for quantitative biologic assay of tumor viruses (102), described in more detail later.

Advantages and Disadvantages of Different Cultured Cell Types

The various types of cultured cells just described have specific application to different problems encountered in experimental virology. For most applications, an adherent cell line provides the most useful host cell. Cell lines are

relatively easy to maintain because they can be passaged indefinitely, and adherence is a prerequisite for a plaque assay (described later). A distinct technical advantage of adherent cells is that the culture medium can easily be changed for the purposes of infection or metabolic labeling by simply aspirating and replacing fluid from a monolayer, a process that requires repeated centrifugations with suspension cells. By contrast, relative to adherent cell lines, suspension cell lines are easier to sample than adherent cells, and they produce large numbers of cells from a relatively small volume of medium in a single culture vessel, which has significant advantages for some high-volume applications in virology. Unfortunately, not all viruses will grow on a cell line, and often under these circumstances, a primary cell culture will suffice. This may reflect a requirement for a particular cell type found only under conditions of primary cell culture, or it may reflect a requirement for a state of metabolism or differentiation closely resembling the *in vivo* situation, which is more likely to exist in a primary culture than it is in a cell line.

Last, some viruses do not grow in cell culture at all, and in these cases investigators are reliant either on the old expedients of natural hosts, laboratory animals, or embryonated eggs, or on some more modern advances in tissue culture and recombinant DNA technology. The papilloma viruses, which cause warts, provide an enlightening example of this situation (Chapter 65). Although the viral nature of papillomatosis was demonstrated over 90 years ago, progress on the study of papilloma viruses was seriously hampered in the virology heyday of the mid-20th century because the viruses grow well only on the natural host; they do not grow in culture. The inability to grow in culture is now reasonably well understood: It results from a tight coupling of the regulation of viral gene expression with the differentiation state of the target epithelial cell, which in turn is tightly coupled to the three-dimensional architecture of the epidermis, which is lost in culture. Specialized tissue culture techniques have now been developed that result in the faithful reconstruction of an epidermis by seeding primary keratinocytes on a *feeder* layer composed of an appropriate cell line, and incubating these cells on a *raft*, or grid, at a liquid–air interface. On these raft cultures the entire replication cycle of a papilloma virus can be reproduced *in vitro*, albeit with difficulty (6). In the meantime, it is significant that a large fraction of the genetics and biology of papillomaviruses was determined primarily through the use of recombinant DNA technology, without ever growing virus in culture. Thus, the genetic structure of both the model bovine papillomavirus and many human papillomaviruses has been determined by cloning genomic DNA from natural infections, and regulation and function of many genes can be gleaned from sequence alone, from *in vitro* assays on individual gene products expressed *in vitro*, and from cell transformation assays that use all or parts of a papillomavirus genome. In summary, the inability to grow a virus in culture, while it increases the challenge, no longer presents an insurmountable impediment to understanding a virus.

Recognition of Viral Growth in Culture

Three principal methods exist for the recognition of a virus infection in culture: cytopathic effect, formation of inclusion bodies, and hemadsorption. Cytopathic effect refers to pronounced morphologic changes induced in individual cells or groups of cells by virus infection, which are easily recognizable under a light microscope. Inclusion bodies are more subtle alterations to the intracellular architecture of individual cells. Hemadsorption refers to indirect measurement of viral protein synthesis in infected cells, detected by adsorption of erythrocytes to the surface of infected cells. Cytopathic effect is the simplest and most widely used criterion for infection, but not all viruses cause a cytopathic effect, and in these cases other methods must suffice.

Cytopathic effects, or CPE, comprise a number of cell phenomena, including rounding, shrinkage, increased refractility, fusion, aggregation, loss of adherence, or lysis. CPE caused by a given virus may include several of these phenomena in various combinations, and the character of the CPE may change reproducibly during the course of infection. CPE caused by a given virus are very reproducible and can be so precisely characteristic of the virus type that significant clues to the identity of a virus can be gleaned from the CPE alone (Chapter 18). Fig. 4 depicts different CPE caused by two viruses, measles and vaccinia. Most important to the trained virologist, a simple microscopic examination of a cell culture can reveal whether an infection is present, what fraction of cells are infected, and how advanced the infection is. In addition, because cytopathology results directly from the action of virus gene products, virus mutants can be obtained that are altered in cytopathology, yielding either a conveniently marked virus or a tool to study cytopathology per se.

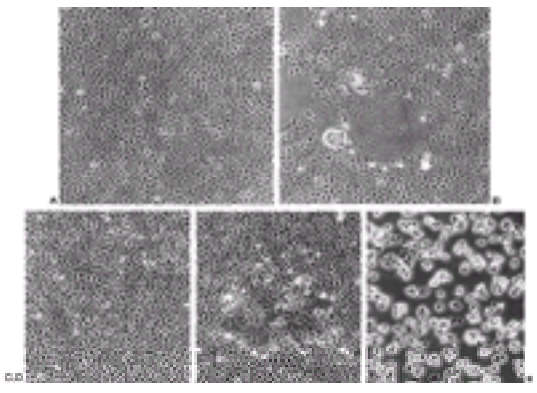

FIG. 4. Virus-induced cytopathic effects (CPE). Phase-contrast photomicrographs are shown. **A:** Uninfected A549 cells, a human lung carcinoma cell line. **B:** A549 cells infected with measles virus at a multiplicity of infection (moi) of less than 0.01 pfu/cell. Individual plaques can be discerned. Measles fuses cells, causing formation of syncytia. In mid field is a large syncytium containing multiple nuclei. Surrounding this area are additional syncytia, including two that have rounded and are separating from the dish. **C:** Uninfected BSC40 cells, an African green monkey cell line. **D:** BSC40 cells infected with vaccinia virus at an moi of less than 0.01 pfu/cell. A single plaque is shown in the middle of the field. **E:** BSC40 cells infected with vaccinia virus at an moi of 10 pfu/cell, 48 hours after infection. All cells are infected and display complete CPE. (Cultures of vaccinia infections courtesy of J. I. Lewis. Cultures of measles infections courtesy of S. Smallwood and S. Moyer.)

The term *inclusion bodies* refers generally to intracellular abnormalities specific to an infected cell and discernible by light microscopy. The effects are highly specific for a particular virus type, so that, as is the case with CPE, the presence of a specific type of inclusion body can be diagnostic of a specific virus infection. Electron microscopy, combined with a more detailed understanding of the biology of many viruses, reveals that inclusion bodies usually represent focal points of virus replication and assembly, which differ in appearance depending on the virus. For example, Negri bodies formed during a rabies virus infection represent collections of virus nucleocapsids (67).

Hemadsorption refers to the ability of red blood cells to attach specifically to virus-infected cells (90). Many viruses synthesize cell attachment proteins, which carry out their function wholly or in part by binding substituents such as sialic acid that are abundant on a wide variety of cell types, including erythrocytes. Often, these viral proteins are expressed on the surface of the infected cell, for example in preparation for maturation of an enveloped virus through a budding process. Thus, a cluster of infected cells may be easily detectable to the naked eye as areas that stain red after exposure to an appropriate preparation of red blood cells. Hemadsorption can be a particularly useful assay for detecting infections by viruses that cause little or no CPE.

Virus Cultivation

From the discussion just presented, it should be clear that ultimately the exact method chosen for growing virus on any particular occasion will depend on a variety of factors, including (a) the goals of the experiment—namely, whether large amounts of one virus variant or small amounts of several variants are to be grown, (b) limitations in the in vitro host range of the virus—namely, whether it will grow on embryonated eggs, primary cell cultures, continuous adherent cell lines, or suspension cell lines, and (c) the relative technical ease of alternative possible procedures. Furthermore, the precise method for harvesting a virus culture will depend on the biology of the virus—for example, whether it buds from the infected cell, lyses the infected cell, or leaves the cell intact and stays tightly cell associated. As a simple example, consider cultivation of a budding, cytopathic virus on an adherent cell line. Confluent monolayers of an appropriate cell line are exposed to virus diluted to infect a fraction of the cells, and the progress of the infection is monitored by observing the development of the CPE until the infection is judged complete based on experience with the relationship between CPE and maximal virus yield. A crude preparation of virus can be harvested simply by collecting the culture fluid; it may not even be necessary to remove cells or cell debris. Most viruses can be stored frozen indefinitely either as crude or purified, concentrated preparations.

Quantitative Assay of Viruses

Two major types of quantitative assays for viruses exist, physical and biologic. Physical assays, such as hemagglutination, electron microscopic particle counts, optical density measurements, and immunologic methods, quantify only the presence of virus particles, whether or not the particles are infectious. Biologic assays, such as the plaque assay or various endpoint methods that have in common the assay of infectivity in cultured cells or *in vivo*, measure only the presence of infectivity, and they may not count all particles present in a preparation, even many that are in fact infectious. Thus a clear understanding of the nature and efficiency of both physical and biologic quantitative virus assays is required to make effective use of the data obtained from any assay.

Biologic Assays

The Plaque Assay

The plaque assay is the most elegant, the most quantitative, and most useful biologic assay for viruses. Developed originally for the study of bacteriophage by d'Herelle in the early 1900s (15), the plaque assay was adapted to animal viruses by Dulbecco and Vogt in 1953 (22), an advance that revolutionized animal virology by introducing a methodology that was relatively simple and precisely quantitative, that enabled the cloning of individual genetic variants of a virus, and that permitted a qualitative assay for individual virus variants that differ in growth properties or cytopathology.

The plaque assay is based simply on the ability of a single infectious virus particle to give rise to a macroscopic area of cytopathology on an otherwise normal monolayer of cultured cells. Specifically, if a single cell in a monolayer is infected with a single virus particle, new viruses resulting from the initial infection can infect surrounding cells, which in turn produce viruses that infect additional surrounding cells. Over a period of days, the exact length of time depending on the particular virus, the initial infection thus gives rise through multiple rounds of infection to an area of infection called a plaque. Photomicrographs of plaques are shown in Fig. 4, and stained monolayers containing plaques are shown in Fig. 5.

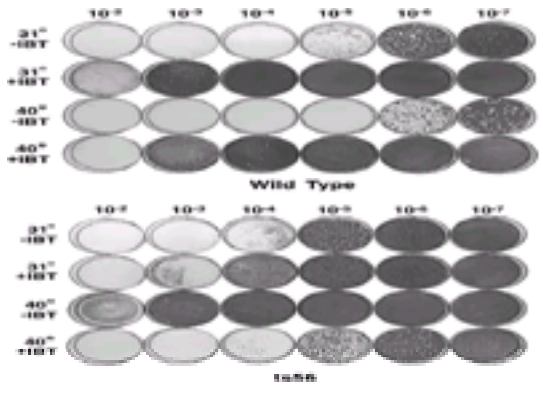

FIG. 5. Plaque assay. Monolayers of the African green monkey kidney cell line BSC40 were infected with 0.5-ml portions of 10-fold serial dilutions of wild-type vaccinia virus or the temperature-sensitive vaccinia mutant, *ts*56, as indicated. Infected monolayers were overlaid with semisolid medium and incubated at 31°C or 40°C, the permissive and

nonpermissive temperatures for *ts*56, in the presence of 45 μM isatin-b-thiosemicarbasone (IBT) or in the absence of drug as indicated, for 1 week. Agar overlays were removed and monolayers were stained with crystal violet. Wild-type vaccinia virus forms plaques at both 31°C and 40°C, but plaque formation is inhibited by IBT. Spontaneous IBT-resistant mutants in the wild-type virus stock are revealed as plaques forming at 10^{-3} and 10^{-4} dilutions in the presence of IBT. The *ts*56 mutant carries a single-base missense mutation in the vaccinia gene G2R (70). G2R is an essential gene that when completely inactivated renders the virus dependent on IBT, hence *ts*56 is not only temperature sensitive, forming plaques at 31°C but not at 40°C in the absence of IBT, but it is also IBT dependent at 40°C, forming plaques in the presence but not the absence of IBT. The *ts*56 mutant is slightly defective at 31°C: It forms smaller-than-wild-type plaques, and it is IBT resistant, forming plaques both in the presence and in the absence of drug, a phenotype intermediate between the wild-type IBT-sensitive phenotype and the null G2R mutant IBT-dependent phenotype. Wild-type, temperature-insensitive revertants present in the *ts*56 stock are revealed as plaques growing on the 10^{-3} plate at 40°C. Based on this assay, the titer of the wild-type stock is 1.6×10^{9} pfu/ml, and the titer of the *ts*56 stock is 2.0×10^{8} pfu/ml. (Plaque assays courtesy of J. I. Lewis.)

The plaque assay can be used to quantify virus in the following manner (Fig. 5). A sample of virus of unknown concentration is serially diluted in an appropriate medium, and measured aliquots of each dilution are seeded onto confluent monolayers of cultured cells. Infected cells are overlaid with a semisolid nutrient medium usually consisting of growth medium and agar. The semisolid medium prevents formation of secondary plaques through diffusion of virus from the original site of infection to new sites, ensuring that each plaque that develops in the assay originated from a single infectious particle in the starting inoculum. After an appropriate period of incubation to allow development of plaques, the monolayer is stained so that the plaques can be visualized. The staining technique depends on the cytopathology, but vital dyes such as neutral red are common. Neutral red is taken up by living cells but not by dead cells, so that plaques become visible as clear areas on a red monolayer of cells. In cases where the virus cytopathology results in cell lysis or detachment of cells from the dish, plaques exist literally as holes in the monolayer, and a permanent record of the assay can be made by staining the monolayer with a general stain such as crystal violet, prepared in a fixative such as ethanol. The goal of the assay is to identify a dilution of virus that yields 20 to 100 plaques on a single dish—that is, a number large enough to be statistically significant yet small enough that individual plaques can be readily discerned and counted. Usually a series of four to six 10-fold dilutions are tested, a number that is estimated to bracket the target dilution. Dishes inoculated with low dilutions of virus will contain only dead cells or too many plaques to count, whereas dishes inoculated with high dilutions of virus will contain very few if any plaques (Fig. 5). Dishes containing an appropriate number of plaques are counted, and the concentration of infectious virus in the original sample can then be calculated by taking into account the serial dilution. The resulting value is called a titer, and it is expressed in plaque-forming units per milliliter, or pfu/ml, to emphasize specifically that only viruses that are capable of forming plaques have been quantified. Titers derived by serial dilution are unavoidably error prone simply because of the additive error inherent in multiple serial pipetting steps; errors of up to 100% are normal, but titers that approximate the real titer to within a factor of 2 are satisfactory for most purposes.

A critical benefit of the plaque assay is that it measures infectivity, but it is important to understand that infectivity does not necessarily correspond exactly to the number of virus particles in a preparation. In fact, for most animal viruses, only a fraction of the particles, as few as 1 in 10 to 1 in 10,000, may be infections as judged by comparison of a direct particle count (described later) with a plaque assay. This low efficiency of plating, or high particle-to-infectivity ratio, may have several causes. First, to determine a particle to infectivity ratio, virus must be purified to determine the concentration of physical particles, and then it must be subjected to plaque assay. If the purification itself damages particles, the particle-to-infectivity ratio will be increased. Second, some viruses produce empty particles, or particles that are for other reasons defective during infection, resulting in a high particle-to-infectivity ratio. Last, it is possible that not all infectious particles will form plaques in a given plaque assay. For example, an infectious virus may require that cells exist in a specific metabolic state or in a specific stage of the cell cycle; thus, if not all cells in a culture are identical in this regard, only a fraction of the potentially infectious virions may be able to successfully launch an infection and form a plaque.

In addition to its utility as a quantitative assay, the plaque assay also provides a way to detect genetic variants of a virus that possesses altered growth properties, and it provides a very convenient method to clone genetically unique variants of a virus (Fig. 5). Genetic variants are considered in detail in the genetics section later, but, in brief, they may comprise viruses that form plaques only under certain conditions of temperature or drug treatment, or form plaques of altered size or shape. Because each plaque results from infection with a single infectious virus particle, unique genetic variants of a virus can be cloned simply by “picking” plaques—that is, literally excising a small plug of agar and infected cells from a plaque using a Pasteur pipette.

The Focus Assay

Some tumor viruses, most notably retroviruses, normally transform cells rather then killing them but can nevertheless be quantified by taking advantage of the transformation cytopathology (94,102). For example, retrovirus-transformed cells may lose contact inhibition and therefore grow in foci, or piles of transformed cells, on top of a contact-inhibited cell monolayer. Dense foci of transformed cells stain more darkly than cells in a monolayer and thus can be quantified by treating an infected monolayer with an appropriate stain. Otherwise, the focus assay is similar to the plaque assay in both technique and function. Photomicrographs of foci and stained monolayers containing foci are shown in Fig. 6.

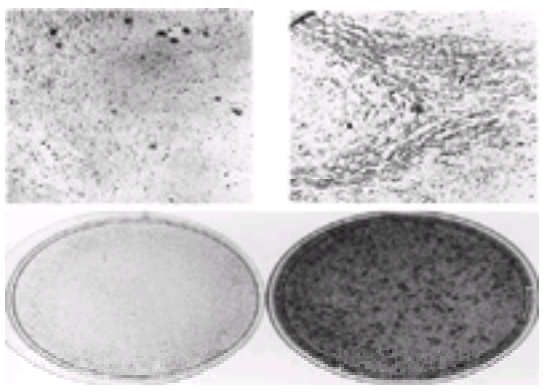

FIG. 6. Focus assay. Monolayers of the NIH3T3 mouse fibroblast cell line were infected with Maloney murine sarcoma virus. The *top two panels* show photomicrographs of uninfected cells (*left*) and a single virus-induced focus (*right*). The *bottom two panels* show stained dishes of uninfected (*left*) and infected (*right*) cells. Foci are clearly visible as darker areas on the infected dish. (Courtesy of D. Blair.)

Pock Formation

As mentioned previously in the discussion of embryonated eggs, many viruses will cause focal lesions on the CAM of eggs. Although cumbersome, this assay can be used to quantify viruses in a fashion similar to a plaque assay. The pock assay found utility before the adaptation of the plaque assay to animal virology, but now it has largely been replaced by other assays utilizing cultured cells and is used only for specialized purposes as noted previously. An example of a pock assay is shown in Fig. 1.

Endpoint Method

Viruses that cannot be adapted to either a plaque or a focus assay, but that nevertheless cause some detectable pathology in cultured cells, embryonated eggs, or animals, can be quantified using an endpoint method. Briefly, virus is serially diluted and multiple replicate samples of each dilution are inoculated into an appropriate assay system. After a suitable incubation period, an absolute judgment is made as to whether or not an infection has taken place. The dilution series is constructed so that low dilutions show infection in all replicate inoculations, high dilutions show infection in none of the inoculations, but some dilutions result in infection in some but not all inoculations. Statistical methods, described in more detail later, have been devised to calculate the dilution of virus that results in infection in 50% of replicate inoculations, and titers are expressed as the infectious dose 50, or ID_{50}. Assay systems are various and include, for example, observation of CPE in cultured cells, yielding tissue culture infective dose 50 (or $TCID_{50}$); cytopathology or embryonic death in inoculated embryonated chicken eggs, yielding egg infectious dose 50 (or EID_{50}); or death of an experimental laboratory animal, yielding lethal dose 50 (or LD_{50}). Like the plaque assay, the focus assay, and the pock assay, the endpoint method has the advantage of measuring infectivity, but it is important to note that the unit of infectivity measured by the endpoint method may require more than one infectious particle.

A sample determination of a $TCID_{50}$ (65) is shown in Table 3. The data for this experiment would be collected by infecting replicate cell monolayers in a multiwell dish with serial dilutions of virus, and after an appropriate incubation period inspecting the wells for the presence of CPE. Inspection of the data reveals that a 10^{-6} dilution of virus results in infection in more than 50% of replicate wells, whereas a 10^{-7} dilution of virus results in infection in fewer than 50% of replicate wells. Thus, the dilution that would yield infection in exactly 50% of samples lies between 10^{-6} and 10^{-7}. The precise dilution required for infection of 50% of the wells can be calculated by either of two methods, the Spearman Karver method (53,92) (not shown) or the Reed-Muench method (83).

Log of virus dilution	Infected test units	Cumulative infected (A)	Cumulative uninfected (B)	Ratio of A/(A + B)	Percent infected
-5	5/5	9	1	9/9	100.0
-6	3/5	4	2	4/6	66.7
-7	1/5	1	6	1/7	14.3
-8	0/5	0	11	0/11	0.0

TCID, tissue culture infective dose.
Source: From ref. 65, with permission.

TABLE 3. *Data for calculation of $TCID_{50}$*

The Reed-Muench method is carried out as follows. First, the cumulative number of infected (column A in Table 3) or uninfected (column B in Table 3) samples is determined by progressively summing the infected samples from the highest dilution to the lowest dilution, and the uninfected samples from the lowest dilution to the highest dilution. Using these numbers, a ratio or percent of infection is calculated for each dilution. The dilution that would yield 50% infected samples is then estimated by determining the proportionate distance between the 10^{-6} and 10^{-7} dilutions that would correspond to 50% infection. This distance is the ratio of the distance between 50% infection and the percent positive above 50%,

divided by the total distance between the percent positive above 50% and the percent positive below 50%:

$$\frac{(\%\text{ positive above }50\%) - 50\%}{(\%\text{ positive above }50\%) - (\%\text{ positive below }50\%)} = \text{proportionate distance},$$

or

$$\frac{66.7\% - 50\%}{66.7\% - 14.3\%} = 0.3.$$

Knowing the proportionate distance between dilutions, the endpoint is calculated taking into consideration the exact dilutions used:

$$(\log \text{dilution above } 50\%) + (\text{proportionate distance} \times \log \text{dilution factor}) = \log \text{TCID}_{50},$$

or

$$(-6) + (0.3 \times 1.0) = -6.3.$$

Therefore the $TCID_{50} = 10^{-6.3}$. The reciprocal of this number is used to express the titer in infectious units per unit volume. Thus, if 0.1 ml had been used for inoculation of each test well in the assay, the final titer would be $10^{7.3}$ $TCID_{50}$/ml.

Physical Assays

Direct Particle Count

The concentration of virus particles in a sample of purified virus can be counted directly using an electron microscope (62,104). Briefly, a purified preparation of virus is mixed with a known concentration of microscopic marker particles such as latex beads, which can be easily distinguished from virus particles with the electron microscope. Samples of the solution containing virus and beads are then applied to an electron microscope grid and visualized after shadowing or staining. The volume of liquid applied to a given area of the grid can be determined by counting the beads. The virus particles in the same area can then be counted, resulting in an accurate determination of the concentration of virus particles in the original solution. An example of an electron microscopic count of vaccinia virus is shown in Fig. 7. Given a solution of virus with a known concentration determined by microscopic particle count, the same solution can be subjected to any number of chemical or spectophotometric analyses to yield a conversion from protein content, nucleic acid content, or simply absorbance at a fixed wavelength to a concentration of virus in particles per unit volume. Thus, once a microscopic particle count has been performed, future quantitative assays of purified virus are greatly simplified. It is important to note that the direct particle count does not distinguish infectious from noninfectious particles.

FIG. 7. Direct electron microscopic particle count. An electron micrograph of a spray droplet containing 15 latex beads (*spheres*) and 14 vaccinia virus particles (*slightly smaller, brick-shaped particles*). (From ref. 23, with permission.)

Hemagglutination

As noted previously in the discussion of hemadsorption, many viruses synthesize cell attachment proteins, which carry out their function wholly or in part by binding substituents such as sialic acid that are abundant on a wide variety of cell types, including erythrocytes. Because these cell attachment proteins decorate the surface of the virion, virions may bind directly to erythrocytes. Because both the virions and the erythrocytes contain multiple binding sites for each other, erythrocytes will agglutinate, or form a network of cells and virus, when mixed with virus particles in sufficiently high concentration. Agglutinated erythrocytes can be easily distinguished from cells that are not agglutinated, and thus hemagglutination can be used as a simple quantitative assay for the presence of a hemagglutinating virus.

In practice, a hemagglutination assay is carried out as follows (Fig. 8). Virus is serially diluted and mixed with a fixed concentration of erythrocytes. The mixture is allowed to settle in a specially designed hemagglutination tray containing

wells with rounded bottoms. Erythrocytes that are not agglutinated are free to roll to the bottom of the well, forming a dense, easily recognizable button, or cluster of cells. Erythrocytes that are agglutinated are not free to roll to the bottom of the well, but instead evenly coat the bottom surface of the well. One hemagglutination (HA) unit is defined as the minimum amount of virus required to cause agglutination, and the titer of the virus solution, expressed as HA units/ml, can be calculated taking into account the serial dilution. It is noteworthy that, like the direct particle count assay, the hemagglutination assay does not distinguish infectious from noninfectious particles. In addition, since it may require many particles to cause a detectable hemagglutination, one HA unit may represent many physical particles.

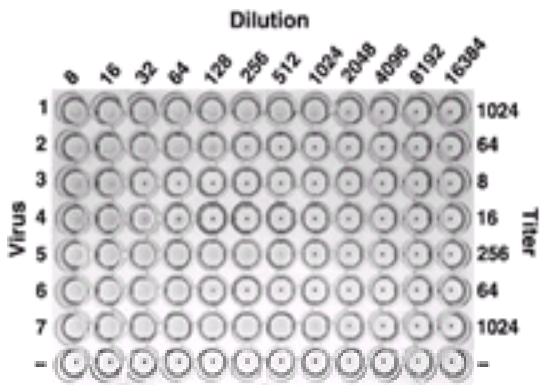

FIG. 8. Hemagglutination assay. Seven different samples of influenza virus, numbered 1 through 7 at the left, were serially diluted as indicated at the *top*, mixed with chicken red blood cells (RBC), and incubated on ice for 1 to 2 hours. Wells in the bottom row contain no virus. Agglutinated RBCs coat wells evenly, in contrast to nonagglutinated cells, which form a distinct button at the bottom of the well. The HA titer, shown at the right, is the last dilution that shows complete hemagglutination activity. (Courtesy of J. Talon and P. Palese.)

Quantitative Considerations in Virus Assay, Cultivation, and Experimentation

Dose Response in Plaque and Focus Assays

With few exceptions, the number of infectious units observed on a given plate in a plaque assay is a linear function of the dilution of the virus, thus the development of plaques follows single-hit kinetics, proving that each plaque results from infection with a single virus particle. Exceptions include the murine sarcoma viruses, assayed in a focus assay, which require co-infection with both a defective transforming virus and a nondefective helper virus, in which case the number of foci observed relative to the dilution used follows two-hit kinetics (40).

Comparison of Quantitative Assays

As noted in the individual descriptions, the various quantitative assays of viruses measure different physical and biologic properties, and a one-to-one correlation between assays cannot be assumed. Table 4 summarizes the titers of an influenza virus preparation as measured by several different assays, providing an example of the magnitude of differences that might be expected between the various assays. Hence, relative to a direct particle count, the efficiency of plating the influenza sample shown in Table 4 is 10^{-1} as assayed in eggs, 10^{-2} as assayed in a plaque assay, and 10^{-7} as assayed in a hemagglutination assay. As indicated in the forgoing discussion, some of the differences result from different properties being measured—for example, physical particles versus infectivity—and some differences result from differences in the sensitivity of the assay—for example, direct particle count versus assay of particles by hemagglutination.

Method	Amount (per ml)
Direct electron microscope (EM) count particles	10^{10} EM
Quantal infectivity assay in eggs	10^{9} egg ID_{50}
Quantal infectivity assay by plaque formation	10^{8} pfu
Hemagglutination (HA) assay	10^{3} HA units

ID, infectious dose; pfu, plaque-forming units.
Source: From ref. 28, with permission.

TABLE 4. *Comparison of quantitative assay efficiency*

Multiplicity of Infection

Multiplicity of infection, often abbreviated moi, measures the average amount of viru $_{s\ added\ per\ c}$ell in an infection. Multiplicity of infection can be expressed using any quantitative measure of virus titer, for example particles per cell, HA units per cell, $TCID_{50}$ per cell, or pfu per cell. Because the efficiency of plating varies depending on the method of quantitation used, some knowledge of the infectivity of the sample or the efficiency of plating is required to correctly

anticipate the consequences of a particular moi. The moi used in different protocols can have a profound outcome on the procedure. For example, some viruses, if serially passaged at an moi of greater than one infectious unit per cell, will accumulate spontaneously deleted defective particles, which are maintained during passage by the presence of complementing wild-type helper virus (103). Passage of the same virus at very low moi, for example 0.01 infectious units per cell, discourages the accumulation of defective particles because few cells will be co-infected with an infectious and a defective particle, and defective particles cannot replicate in the absence of a wild-type helper. On the other hand, most metabolic labeling experiments are done at a high moi, for example 10 infectious units per cell, to ensure that all cells in the culture are infected and that the infection is as synchronous as possible. For such experiments, use of too low an moi may result in an apparently asynchronous infection and a high background because of the presence of uninfected cells in the culture.

The Poisson distribution can be used to predict the fraction of cells in a population infected with a given number of particles at different mois. As applied to virus infections, the Poisson distribution can be written as follows:

$$P(k) = e^{-m}m^k/k!,$$

where P(*k*) = the probability that any cell is infected with *k* particles, *m* = moi, and *k* = number of particles in a given cell.

Note that to determine the fraction of uninfected cells in any experiment, that is when *k* = 0, the equation simplifies to

$$P(0) = e^{-m}$$

For practical purposes, solution of this equation for given values of *m* and *k* (other than 0) is most easily accomplished using published tables (109). Sample solutions are shown in Table 5 for commonly used mois. Inspection of this table and consideration of the error inherent in any virus titration involving a serial dilution leads to some significant practical guides in experimental design. Note first that in a culture infected at an moi of 1 pfu/cell, 37% of cells remain uninfected, an unacceptably high number for an experiment designed to measure a single round of synchronous infection. An moi of at least 3 is required to infect 95% of the cells in culture. Given that titers can easily be inaccurate by a factor of 2, use of a calculated moi of 10 ensures that 99% of the cells in a culture will be synchronously infected even if the measured titer is twofold higher than the actual titer.

	moi (m)			
#/cell (k)	1	3	5	10
0	0.37	0.05	0.01	0.00
1	0.37	0.15	0.03	0.00
2	0.18	0.22	0.08	0.00
3	0.06	0.22	0.14	0.01
4	0.02	0.17	0.18	0.02
5	0.00	0.10	0.18	0.04
6	0.00	0.05	0.15	0.06
7	0.00	0.02	0.10	0.09
8	0.00	0.00	0.07	0.11
9	0.00	0.00	0.04	0.13
10	0.00	0.00	0.02	0.13

Note: See text for definitions of terms and equations.

TABLE 5. *The Poisson distribution: values of* P(k) *for various values of* m *and* k

One-Step Growth Experiment

A classic experiment developed initially for bacteriophage (24) and still frequently used to determine the essential growth properties of a virus is the one-step growth experiment. The goal of the one-step growth experiment is to measure the time course of virus replication and the yield of virus per cell during a single round of infection. The experiment is carried out as follows. Several dishes containing confluent monolayers or an appropriate culture cell are infected simultaneously with virus at a high moi, for example 10 pfu/cell. After an adsorption period, monolayers are washed to remove unabsorbed virus and then incubated in culture medium. At various times after infection, virus from individual dishes is harvested, and at the completion of the experiment, the virus titer in samples representing each time point is determined. The virus yield at each point can be converted to pfu/cell by dividing the total amount of virus present in the sample by the number of cells originally infected in the sample.

The results from one example of a one-step growth experiment, in this case comparing growth of wild-type vaccinia virus and a temperature-sensitive (*ts*) mutant at permissive and nonpermissive temperatures, are shown in Fig. 9. Several features of the growth curve are noteworthy. First, during the first several hours of the wild-type infection or the *ts*56 infection at the permissive temperature, the titer in the cultures decreases and then increases. This dip in the growth curve, called *eclipse*, results from the following sequence: Early during the experiment, the virus that attaches to the cell surface but is not yet uncoated remains infectious. Then, infectivity is lost during the first few hours of infection (the eclipse phase), when the virus is uncoated. Infectivity is then recovered only after new virus is produced. The infection then enters a rapid growth phase, followed by a plateau. The plateau results from the fact that all infected cells have reached the maximal yield of virus, or they have died or lysed, depending on the type of virus infection. The time interval from infection to plateau represents the time required for a single cycle of growth, and the yield of virus at plateau shows the amount of virus produced per cell.

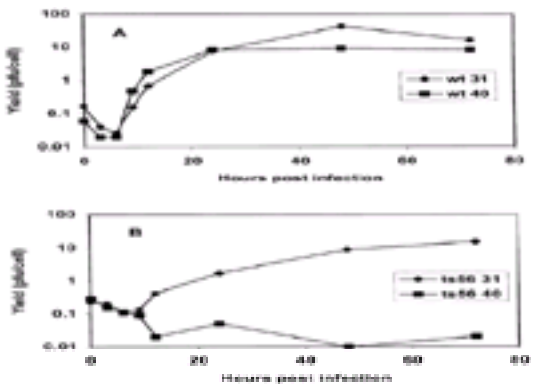

FIG. 9. One-step growth experiment. Monolayers of the African green monkey kidney cell line BSC40 were infected at an moi of 6 pfu/cell with wild-type vaccinia virus (*top panel*) or the temperature-sensitive vaccinia mutant, *ts*56 (*bottom panel*), and incubated at the permissive temperature, 31°C, or the nonpermissive temperature, 40°C, as indicated. Samples were harvested at various times and the titer determined by plaque titration on BSC40 cells at 31°C. The wild-type virus grows equally well at 31°C and 40°C. The *ts*56 grows more slowly than the wild type at 31°C but ultimately produces a yield of virus comparable to wild type. The *ts*56 does not grow at 40°C. (From ref. 41, with permission.)

The experiment in Fig. 9 demonstrates the utility of the one-step growth experiment. As judged by this experiment, wild-type virus grows with identical kinetics and to the identical yields at both 31°C and 40°C, permissive and nonpermissive temperatures for the temperature-sensitive mutant, respectively. The temperature-sensitive mutant, *ts*56 (70), grows more slowly than wild-type virus at 31°C, indicating some defective character even at the permissive temperature, although at plateau the yields of mutant virus at 31°C are equivalent to those of the wild-type virus. The experiment demonstrates conclusively that the mutant does not grow at all at the nonpermissive temperature, 40°C.

Multiplicity of infection is a critical factor in the design of a virus growth experiment. A true one-step growth experiment can be done only at high moi. If the moi is too low and a large fraction of cells are left uninfected, then virus produced during the first round of infection will replicate on previously uninfected cells, and thus multiple rounds of infection rather than one round will be measured. A growth experiment done at low moi has utility in that it measures both growth and spread of a virus in culture, but the time from infection to plateau does not accurately reflect the time required for a single cycle of infection. It is also noteworthy that some mutant phenotypes are multiplicity dependent (5).

VIRUS GENETICS

Viruses are subject to the same genetic principles at work in other living systems, namely mutation, selection, complementation, and recombination. Genetics impacts all aspects of virology, including the natural evolution of viruses, clinical management of virus infections, and experimental virology. For example, antigenic variation, a direct result of mutation and selection, plays a prominent role in the epidemiology of influenza virus and human immunodeficiency virus (HIV) in the human population, and mutation to drug resistance offers a significant challenge to the clinical management of virus infections with antiviral drugs. This section deals primarily with the application of experimental genetic techniques to basic virology.

The ultimate goal of experimental virology is to understand completely the functional organization of a virus genome. In a modern context, this means determination of the structure of a virus genome at the nucleotide sequence level, coupled with isolation of mutational variants of the virus altered in each gene or control sequence, followed by analysis of the effects of each mutation on the replication and/or pathogenesis of the virus. Thus, genetic analysis of viruses is of fundamental importance to experimental virology.

Before the advent of modern nucleic acid technology, that is during a “classical” period of “forward” genetics, genetic analysis of viruses consisted of the random, brute-force isolation of large numbers of individual virus mutants, followed first by complementation analysis to determine groupings of individual mutants into genes, then recombination analysis to determine the physical order of genes on the virus genome, and finally the phenotypic analysis of mutants to determine gene function. This approach, pioneered in the 1940s, 1950s, and 1960s in elegant studies of several bacteriophage, notably lambda, T4, and T7 (Chapter 22), was the primary method for identifying, mapping, and characterizing virus genes. The application of cell culture techniques to animal virology opened the door to classical genetic analysis of animal viruses, resulting in a flurry of activity in the 1950s through the 1970s, during which time hundreds of mutants were isolated and analyzed in prototypical members of most of the major animal virus families (32). Modern nucleic acid technology introduced in the 1970s brought with it a variety of techniques for physical mapping of genomes and mutants, including restriction enzyme mapping, marker rescue, and DNA sequence analysis, which together replaced recombination analysis as an analytic tool. Mutants and techniques from that classical period continue to be of enormous utility today, but recombinant DNA technology has brought with it reverse (or “punk”) genetics, in which the structure of the genome is determined first, using entirely physical methods, and then the function of individual genetic elements is determined by analyzing mutants constructed in a highly targeted fashion.

The genetic approach to experimental virology, or any field of biology for that matter, has the profound advantage of asking of the organism under study only the most basic question, “What genes do you need to survive, and why do you need them?” without imposing any further bias or assumptions on the system. And organisms often respond with surprises that the most ingenious biochemist or molecular biologist would never have imagined. What follows is a summary of the critical elements of both the classical and modern approaches to virus genetics as applied to experimental virology.

Mutants

Wild-Type Virus

It is important to understand that in the context of experimental virus genetics, a virus designated as wild type can differ significantly from the virus that actually occurs in nature. For example, virus genetics often relies heavily on growth and assay of viruses in cell culture, and, as noted previously, natural isolates of viruses may undergo significant genetic change during adaptation to cell culture. In addition, viruses to be designated wild type should be plaque purified before initiating a genetic study, to ensure a unique genetic background for mutational analysis. Last, viruses may be specifically adapted for use in genetic analysis, for example by passage under conditions that are to be restrictive for conditionally lethal mutants so that the analysis can be initiated with a preparation free of spontaneous mutants.

Fundamental Genetic Concepts

Concepts fundamental to genetic analysis of other organisms apply to genetic analysis of viruses, and a clear understanding of these concepts is essential to understanding virus genetics. The most important of these concepts, including distinctions between genotype and phenotype, a selection and a screen, and essential and nonessential genes, are briefly summarized here.

Genotype/Phenotype

Genotype reflects the actual genetic change from wild type in a particular virus mutant, whereas phenotype reflects the measurable manifestation of that change in a given assay system. This distinction is emphasized by the fact that a single genotype may express different phenotypes depending on the assay applied. Thus, for example, the same missense mutation in a virus gene may cause temperature sensitivity in one cell line but not in another, or a deletion in another virus gene may have no effect on the replication of virus in culture but may alter virulence in an animal model.

Selection/Screen

Selection and screen refer to two fundamentally different methods of identifying individual virus variants contained in a mixed population of viruses. Selection implies that a condition exists in which only the desired virus will grow, while growth of unwanted viruses is suppressed. Thus, a drug-resistant virus can be identified by plating a mixture of wild-type, drug-sensitive viruses, and mutant, drug-resistant viruses together on the same cell monolayer in the presence of the inhibitory drug, thereby selecting for drug-resistant viruses, which grow, and selecting against wild-type viruses, which do not grow (Fig. 5). A screen implies that both the desired virus variant and one or several other unwanted virus types grow under a given condition, so that many viruses must be analyzed individually to identify the desired variant. For example, in searching for a temperature-sensitive mutant—that is, a virus whose growth is inhibited relative to wild-type virus at an elevated temperature—no condition exists under which the mutant alone will grow. Therefore, virus must be plated at a low temperature at which both wild-type and mutant virus will grow, and plaques must be tested individually for temperature sensitivity.

Sometimes, a screen can be streamlined by introducing a phenotypic marker into the variant of choice. For example, a knockout virus might be constructed by inserting the beta-galactosidase gene into the virus gene to be inactivated. In the presence of an appropriate indicator dye, viruses containing the insertional knockout produce blue plaques and can therefore be distinguished from unmodified viruses, which form clear plaques, growing on the same plate (107). This example is still a screen, because both wild-type and mutant viruses grow under the conditions used, but the screen is simplified because mutant viruses can be readily identified by their color, obviating the need to pick and test individual plaques. Selections have considerable advantages over screens but are not always possible.

Essential/Nonessential

The terms *essential* and *nonessential* describe phenotypes, specifically whether a given gene is required for growth under a specific condition. Most viruses are finely tuned through selection to fit a specific niche. Not all viral genes are absolutely required for virus replication in that niche; some may simply confer a subtle selective advantage. Furthermore, if the niche is changed, for example from a natural animal host to a cell line in a laboratory, some genes that may have been essential for productive infection in the animal may not be required for replication in cell culture. Genes that are required for growth under a specific condition are termed essential, and those that are not required are termed nonessential. Because as a phenotype, essentiality may be a function of the specific test conditions, the test conditions need to be specified in describing the mutation; for example, the herpesvirus thymidine kinase gene is nonessential for virus replication in cell culture. A genes that is essential or nonessential under a given condition presents unique characteristics for analysis. Thus, mutants in nonessential genes may be easy to isolate because the gene can be deleted, but the function of the gene may be difficult to determine because, by definition, nonessential genes have no phenotype. On the other hand, genes that are essential can be used to study gene function by characterizing the precise replication defect caused by a mutation in the gene, but acquiring the appropriate mutant is confounded by the necessity for identifying a condition that will permit growth of the virus for study.

Mutation

Spontaneous Mutation

Spontaneous mutation rates in viruses are measured by fluctuation analysis (51), a technique pioneered by Luria and Delbruck (61) for analysis of mutation in bacteria, and later adapted to viruses by Luria (59). Fluctuation analysis consists of measuring the proportion of spontaneous mutants with a particular phenotype in many replicate cultures of virus, and applying the Poisson distribution to these data to calculate a mutation rate. It is important to note that, because

spontaneous mutations occur at random and may occur only rarely, the raw data in a fluctuation analysis display enormous scatter, with some cultures containing a high proportion of mutants and some containing no mutants. Thus, from a practical perspective, although the proportion of mutants in a single culture of virus may reflect the mutation rate, it does not necessarily provide an accurate measure of mutation rate.

Both DNA and RNA viruses undergo spontaneous mutation, but the spontaneous mutation rate in RNA viruses is usually much higher than in DNA viruses. In general, the mutation rate at a specific site in different DNA viruses ranges from 10^{-8} to 10^{-11} per replication, whereas in RNA viruses it is at least 100-fold higher, between 10^{-3} and 10^{-6} per replication. The difference in mutation rate observed between RNA and DNA viruses is thought to result primarily from differences in the replication enzymes. Specifically, the DNA-dependent DNA polymerases used by DNA viruses contain a proofreading function, whereas the reverse transcriptases used by retroviruses and the RNA-dependent RNA polymerases used by RNA viruses lack a proofreading function. The difference in spontaneous mutation rate has profound consequences for both the biology of the viruses and for laboratory genetic analysis of viruses. Specifically, RNA viruses exist in nature as "quasi-species" (19)—that is, populations of virus variants in relative equilibrium with the environment but capable of swift adaptation because of their high spontaneous mutation rate (Chapter 13). On the other hand, DNA viruses are genetically more stable but less adaptable. In the laboratory, the high mutation rate in RNA viruses presents difficulties in routine genetic analysis because mutants easily revert to a wild-type virus that can outgrow the mutant virus.

It is noteworthy that whereas the actual mutation rate at a single locus is probably relatively constant for a given virus, the apparent mutation rate to a given phenotype depends on the nature of the mutation(s) that can give rise to that phenotype. For example, spontaneous mutation to bromodeoxyuridine (BUdR) resistance in vaccinia virus may occur at least 10 to 100 times more frequently than spontaneous reversions of temperature-sensitive mutations to a wild-type, temperature-insensitive phenotype. In the case of BUdR resistance, any mutation that inactivates the thymidine kinase causes resistance to BUdR, and thus there are literally hundreds of different ways in which spontaneous mutation can give rise to BUdR resistance. By contrast, a temperature-sensitive mutation is usually a single-base missense mutation: There may exist only one possible mutational event that could cause reversion to the wild-type phenotype, and thus the apparent spontaneous mutation rate for the revertant phenotype is lower than the apparent spontaneous mutation rate to the BUdR resistance phenotype.

From a practical perspective, the apparent spontaneous mutation rate for specific selectable phenotypes may be sufficiently high that induction of mutants is unnecessary for their isolation. Note, for example, that the wild-type vaccinia virus culture titered in Fig. 5 contains numerous spontaneous isatin-beta-thiosemicarbasone (IBT)-resistant viruses that could easily be plaque-purified from assays done in the presence of IBT. However, for most mutants (e.g., temperature-sensitive mutants), in which the desired mutational events are rare and a screen must be used rather than a selection, induced mutation is required for efficient isolation of mutants.

Induced Mutation

Under most circumstances, the incidence of spontaneous mutations is low enough that induction of mutation is a practical prerequisite for isolation of virus mutants. It is usually desirable to induce limited, normally single-base changes, and for this purpose, chemical mutagens are most appropriate. Commonly used chemical mutagens are of two types, *in vitro* mutagens and *in vivo* mutagens (20). *In vitro* mutagens work by chemically altering nucleic acid and can be applied by treating virions in the absence of replication. Examples of *in vitro* mutagens include hydroxylamine, nitrous acid, and alkylating agents, which through chemical modification of specific bases cause mispairing leading to missense mutations. *In vivo* chemical mutagens comprise compounds such as nucleoside analogs, which must be incorporated during viral replication and thus must be applied to an infected cell. One of the most effective mutagens is an alkylating agent, nitrosoguanidine, which, although it is capable of alkylating nucleic acid *in vitro*, is most effective when used *in vivo*, where it works by alkylating guanine residues at the replication fork, ultimately causing mispairing.

The effectiveness of a mutagenesis is often assayed by observing the killing effect of the mutagen on the virus, the assumption being that many mutational events will be lethal, and thus an effective mutagenesis will decrease a virus titer relative to an untreated control. However, killing does not always correlate precisely with mutagenesis, especially with an *in vitro* mutagen that can damage virion structure without necessarily causing mutation. An alternative method for assessing mutagenesis is to monitor an increase in the mutation frequency to a selectable phenotype where possible. For example, in vaccinia virus, mutagenesis causes a dose-dependent increase in resistance to phosphonoacetic acid, a drug that prevents poxvirus replication by inhibiting the viral DNA polymerase (11). In summary, use of mutagens can increase the mutation frequency several hundred fold, such that desired mutants may comprise as much as 0.5% of the total virus population.

Double Mutants/Siblings

The existence of double mutants and siblings can theoretically complicate genetic analysis of a virus. Double (or multiple) mutants may contain more than one mutation contributing to a phenotype. Theoretically, because the probability that a double mutant will be created increases as the dose of a mutagen is increased, there is a practical limit to the amount of induced mutation that is desirable. Double mutants are usually revealed as mutants that are noncomplementing with more than one mutant, or that are impossible to map by recombination or physical methods. Siblings result from replication of mutant virus either through amplification of a mutagenized stock, or during an *in vivo* mutagenesis. The only completely reliable method to avoid isolation of sibling mutants is to isolate each mutant from an independently plaque-purified stock of wild-type virus. In practice, siblings seldom present a problem serious enough to justify the effort required to avoid them.

Mutant Genotypes

There exist two basic categories of mutation, base substitution and deletion/insertion mutations. Both mutation types can occur with consequence in either a protein coding sequence or in a control sequence, such as a transcriptional promoter,

a replication origin, or a packaging sequence. Base substitution mutations consist of the precise replacement of one nucleotide with a different nucleotide in a nucleic acid sequence. In coding sequences, base substitution mutations can be silent, causing no change in amino acid sequence of a protein; they can be missense, causing replacement of the wild-type amino acid with a different residue; or they can be nonsense, causing premature translation termination during protein synthesis. Deletion and insertion mutations comprise deletion or insertion of one or more nucleotides in a nucleic acid sequence. In a coding sequence, deletion or insertion of multiples of three nucleotides can result in precise deletion or insertion of one or more amino acids in a protein sequence. In a coding sequence, deletions or insertions that do *not* involve multiples of three nucleotides result in a shift in the translational reading frame, which almost invariably results in premature termination at some distance downstream of the mutation. In general, nonsense mutations, frameshift mutations, or large in-frame insertions or deletions are expected to inactivate a gene, whereas missense mutations may cause much more subtle phenotypes such as drug resistance or temperature sensitivity.

Mutant Phenotypes

In the context of experimental virology, where the goal is to understand the function of individual virus genes, the most useful mutants are those that inhibit virus replication by inactivating a virus gene. The nonproductive infections with these lethal mutants can be studied in detail to determine the precise aspect of virus replication that has been affected, thus providing information about the normal function of the affected gene. However, one must be able to grow the mutant to conduct experiments, so a condition must be found in which the mutation in question is not lethal, hence the general class of mutant phenotypes called conditional lethal. Conditional lethal mutants comprise by far the largest and most useful class of mutant phenotypes; they consist of host-range, nonsense, temperature-sensitive, and drug-dependent phenotypes, and they will be described individually later. Two additional classes of mutant phenotypes, resistance and plaque morphology, which have very specific application to genetic analysis of viruses, will also be described.

Host-Range

A host-range virus mutant is broadly defined as a mutant that grows on one cell type and not on another, in contrast to the wild-type virus, which grows on both cell types. Two general subcategories of host-range mutants exist, natural and engineered. Natural host-range virus mutants are relatively rare, primarily because they must be identified by brute-force screen or serendipity, in many cases in the absence of a viable rationale for the targeted host range. The existence of a host-range phenotype implies that a specific virus–host interaction is compromised, which also implies that for any specific host-range phenotype, only one virus gene (or a limited number) will be targeted. A classic example of a natural host-range mutants are the *hrt* mutants of mouse polyoma virus, which affect both small and middle T antigens, and which grow on primary mouse cells but not continuous mouse 3T3 cell lines (2). Engineered host-range mutants are constructed by deleting an essential gene of interest in the virus while at the same time creating a cell line that expresses the gene. The engineered cell line provides a permissive host for growth of the mutant virus because it complements the missing virus function, whereas the normal host lacking the gene of interest provides a nonpermissive host for study of the phenotype of the virus. This technology has been useful for studying a variety of viruses, notably herpes simplex virus, where it has facilitated study of several essential virus genes (17,107).

Nonsense Mutants

Nonsense mutants are those that contain a premature translation termination mutation in the coding region of the mutant gene. Nonsense mutants are formally a specific class of conditionally lethal, host-range mutants. Specifically, the permissive host is one that expresses a tRNA containing an anticodon mutation that results in insertion of an amino acid in response to a nonsense codon, thus restoring synthesis of a full-length polypeptide and suppressing the effects of the virus nonsense mutation. The nonpermissive host is a normal cell in which a truncated, nonfunctional polypeptide is made. In practice, most nonsense mutants in existence have been isolated by random mutagenesis followed by a brute-force screen for host range. Nonsense mutants have three distinct advantages for the conduct of virus genetics: (a) Mutants can be isolated in virtually any essential virus gene using one set of permissive and nonpermissive hosts and one set of techniques, (b) the mutations result in synthesis of a truncated polypeptide, thereby facilitating identification of the affected gene, and (c) virus mutants can be relatively easily engineered, as the exact sequence of the desired mutation is predictable.

Nonsense mutants have provided the single most powerful genetic tool in the study of bacteriophage, where efficient, viable nonsense-suppressing bacteria are readily available. Unfortunately, attempts to isolate nonsense-suppressing mammalian cells have met with only limited success, probably because the nonsense-suppressing tRNAs are lethal in the eucaryotic host (89).

Temperature Sensitivity

Temperature sensitivity is a type of conditional lethality in which mutants can grow at a low temperature but not at a high temperature, in contrast to wild-type virus, which grows at both temperatures. Genotypically, temperature-sensitive mutants are usually relatively subtle, single amino acid substitutions that render the target protein unstable and hence nonfunctional at an elevated or nonpermissive temperature, while leaving the protein stable and functional at a low, permissive temperature. In practice, temperature-sensitive mutants are usually isolated by random mutagenesis followed by brute-force screening for growth at two temperatures. Screening can be streamlined by a plaque enlargement technique in which mutagenized virus is first plated at a permissive temperature, then stained and shifted to a nonpermissive temperature after marking the size of plaques, to screen for plaques that do not increase in size at the nonpermissive temperature (91). Replica plating techniques that permit relatively straightforward screening of thousands of mutant candidates in yeast and bacteria have not been successfully adapted to virology, and thus a screen for temperature sensitivity, even when streamlined with plaque enlargement, ultimately depends on the laborious but reliable process of picking and testing individual plaques.

Temperature-sensitive mutants have the profound advantage of theoretically accessing any essential virus gene using a

single set of protocols. Temperature-sensitive mutants have proved enormously useful in all branches of virology, but they have been particularly useful for the study of animal viruses, where nonsense suppression has not been a viable option. Cold-sensitive mutants—that is, mutants that grow at a high but not a low temperature—comprise a relatively rare but nevertheless useful alternative type of temperature-sensitive mutants.

Temperature-sensitive mutants can actually be divided into two subclasses, thermolabile and temperature-sensitive-for-synthesis mutants (108). Thermolabile mutants are those in which the gene product can be inactivated following synthesis by a shift from the permissive to the nonpermissive temperature. Mutants that are temperature sensitive for synthesis display gene dysfunction only if the infection is held at the nonpermissive temperature during synthesis of the mutant gene product; if the gene product is made at the permissive temperature, it cannot be inactivated by raising the temperature. Clearly, the two mutant types can be distinguished by performing appropriate temperature-shift experiments. Thermolability obviously implies that a protein preformed at the permissive temperature is directly destabilized by raising the temperature. Temperature-sensitive-for-synthesis mutations commonly involve multisubunit structures or complex organelles: Theoretically, the quaternary structure of a complex formed correctly at the permissive temperature stabilizes the mutant protein, making the mutation resistant to temperature shift. If a temperature-sensitive-for-synthesis mutant protein is synthesized at the nonpermissive temperature, it may be degraded before assembly or may not assemble properly because of misfolding. For most purposes, the thermolabile and temperature-sensitive-for-synthesis mutant types are equally useful.

Drug Resistance/Dependence

A number of antiviral compounds have now been identified, and virus mutants that are resistant to or that depend on these compounds have found utility in genetic analysis of viruses. A few compounds have been identified that target similar enzymes in different viruses, including phosphonoacetic acid, which inhibits DNA polymerases (44,93), and BUdR, which targets thymidine kinases (21,97). More often, however, antiviral drugs are highly specific for a gene product of one particular virus—for example, guanidine, which targets the polio 2C NTPase (78,79); acyclovir, which targets the herpes simplex virus thymidine kinase and DNA polymerase (8,88); amantidine, which targets the influenza virus M2 virion integral membrane ion channel protein (43); and IBT, which is highly specific for poxviruses and targets at least two genes involved in viral transcription (10,70). Drugs that are most useful are those that inhibit wild-type virus growth in a plaque assay without killing cells in a monolayer, so that resistant or dependent viruses can be selected by virtue of their ability to form plaques on a drug-treated monolayer. Examples of both drug resistance and drug dependence are shown in Fig. 5.

Drug-resistant or -dependent virus mutants have two general uses in virus genetics. First, they can be useful for identifying the target or mechanism of action of an antiviral drug. For example, studies of influenza virus mutants resistant to amantidine were of importance in characterizing both the M2 gene and the mechanism of action of amantidine (80). Second, resistant or dependent mutants provide selectable markers for use in recombination mapping, for the assessment of specific genetic protocols, or for selection of recombinant viruses in reverse genetic protocols. For example, guanidine resistance has been used as a marker for use in three-factor crosses in recombination mapping of poliovirus temperature-sensitive mutants (14); phosphonoacetic acid resistance and IBT dependence have been used in vaccinia virus to assess the efficiency of marker rescue protocols (26,41); and acyclovir resistance and BUdR resistance, resulting from mutation of the herpesvirus or poxvirus thymidine kinase genes, have been used both in herpes viruses and in poxviruses to select for insertion of engineered genes into the viral genome (9,64,74).

Plaque Morphology

Plaque morphology mutants are those in which the appearance of mutant plaques is readily distinguishable from that of wild-type plaques. Most commonly, the morphologic distinction is plaque size—that is, mutant plaques may be larger or smaller than wild-type plaques. However, other morphologic distinctions are possible—for example, formation of clear versus turbid bacteriophage plaques. Most plaque morphology mutants affect very specific virus functions, which in turn affect the virus–host relationship in a fashion that impacts on the appearance of a plaque. Notable examples from bacteriophage research include clear plaque mutants of bacteriophage lambda and rapid lysis mutants of the T-even bacteriophage. Wild-type lambda forms turbid plaques because some percentage of cells are lysogenized and thus survive the infection, leaving intact bacteria within a plaque. Clear mutants of lambda typically affect the lambda repressor so that lysogeny is prevented and all infected bacteria lyse, resulting in a clear plaque (52). Wild-type T-even phages produce small plaques with a turbid halo because only a fraction of infected bacteria lyse during a normal infection, a phenomenon called lysis inhibition. Rapid lysis mutants, which affect a phage membrane protein, do not display lysis inhibition and as a result form large, clear plaques (46). Examples from animal virus research include large plaque mutants of adenovirus and syncytial mutants of herpes simplex virus. The large plaque phenotype in adenovirus results from faster-than-normal release of virus from infected cells (56). Syncytial mutants of herpes virus affect virus surface glycoproteins and result in fusion of infected cells, whereas wild-type virus causes cells to round and clump without significant fusion. Thus, *syn* mutants form large clear plaques readily distinguishable from the smaller dense foci caused by wild-type virus (86). All of these specific plaque morphology mutants have value either in the study of the actual functions affected, or as specific phenotypic markers for use in recombination studies, where they can be used in the same fashion as drug-resistance markers, described previously.

In addition to the existence of specific plaque morphology loci in several viruses, it is noteworthy that any mutation that effects virus yield or growth rate may result in the production of a smaller-than-wild-type plaque, which can be useful in genetic experiments. Thus, many temperature-sensitive mutants form smaller-than-wild-type plaques even at the permissive temperature because the mutant gene may not be fully functional even under permissive conditions, and this property is often useful in mutant isolation or for distinguishing wild-type from mutant virus in plaque assays involving several virus variants. Note, for example, in Fig. 5 that the vaccinia virus temperature-sensitive mutant *ts*56 forms smaller-than-wild-type plaques at the permissive temperature, 31°C. Last, intragenic or extragenic suppressors of conditional lethal virus mutants may grow poorly relative to the wild-type virus and form small plaques as a result, facilitating their isolation from a mixture containing true wild-type revertant viruses (13).

Neutralization Escape

Neutralization escape mutants are a specific class of mutants selected as variant viruses that form plaques in the presence of neutralizing antibodies. Such mutants affect the structure or modification of viral surface proteins. They have been of value in studies of virus structure, antigenic variation, and virus–cell interactions (37,47).

Reversion

Reversion may be defined as mutation that results in a change from a mutant genotype to the original wild-type genotype. Accordingly, revertants in a stock of mutant virus are revealed as viruses that have acquired a wild-type phenotype. For example, Fig. 5 shows that when the vaccinia virus temperature-sensitive mutant *ts*56 is plated at the nonpermissive temperature, a number of plaques with wild-type morphology, probably revertants, are detectable at low dilutions of virus. Spontaneous reversion of missense mutations probably results from misincorporation during replication, as the reversion frequency of different viruses often reflects the error rate of the replication enzyme. Spontaneous reversion of significant deletion mutations occurs rarely if at all, as reversion would require replacement of missing nucleotides with the correct sequence.

Reversion impacts on viral genetics in two ways. First, in any genetic experiment involving mixed infections with two genetically different viruses, wild-type viruses can arise either through reversion or recombination, and in most cases it is important to be able to distinguish between these two processes. This is discussed in more detail later in the sections describing complementation and recombination. Second, as described previously in the description of spontaneous mutation, if the spontaneous reversion rate is extremely high, revertants can easily come to dominate a mutant virus stock, thus obscuring the mutant phenotype and causing serious difficulties in both genetic and biochemical analysis of mutants.

Leakiness

Not all conditionally lethal mutants are completely defective in replication under nonpermissive conditions, and leakiness is a quantitative measure of the ability of a mutant virus to grow under nonpermissive conditions. Leakiness can be quantified with a one-step growth experiment. To quantify leakiness of a temperature-sensitive mutant, for example, cells are infected at a high moi with wild-type or mutant virus, infected cells are incubated at either permissive or nonpermissive temperatures, and maximal virus yields are then determined by plaque titration under permissive conditions so that the growth of mutant and wild-type virus can be quantitatively compared. Ideally, for wild-type virus the ratio of the yield for infections done at the nonpermissive temperature relative to the permissive temperature should be 1—that is, the virus should grow equally well at both temperatures. For mutant viruses, the ratio of the yield for infections done at the nonpermissive temperature relative to the permissive temperature may range from less than 10% to as much as 100%, even for mutants that are clearly defective in plaque formation under nonpermissive conditions. Mutants that are “tight” (i.e., that grow poorly under nonpermissive conditions) are desirable for phenotypic characterization relative to leaky mutants, because leaky mutants will logically display considerable wild-type phenotypic behavior.

In special cases, extreme leakiness is an expected and desirable trait. Specifically, virus mutants that are wild type for replication and production of infectious virions but defective in cell-to-cell spread have a phenotype characterized by defective plaque formation, which requires spread, but 100% leakiness, which does not require spread if assayed in a high moi, one-step growth protocol (4).

Genetic Analysis of Mutants

Complementation

Complementation analysis provides a general method for determining whether two different virus mutants affect the same or different genes. The quantitative test to determine complementation is a two-step procedure in which co-infections are first done to induce an interaction between two mutants, then the results of those infections are quantitatively assessed by plaque titration. The test compares the ability of two mutants to grow in mixed, relative to single, infections under nonpermissive conditions. Specifically, cells are first infected with two different virus mutants at high moi so that all cells are co-infected with both mutants, and infected cells are incubated under nonpermissive conditions where neither mutant alone can replicate, for an interval sufficient to achieve maximal virus yield. Single high-moi infections under nonpermissive conditions are performed as controls. Virus is then harvested, yields are quantified by plaque titration under both permissive and nonpermissive conditions, and a complementation index (CI) is calculated according to the following formula:

$$\frac{\text{yield } (A + B)_p - \text{yield } (A + B)_{np}}{\text{yield } (A)_p + \text{yield } (B)_p} = \text{CI},$$

where A and B represent individual virus mutants and the subscripts p and np represent the conditions, permissive or nonpermissive, under which the virus yields were plaque-titrated. Because both mutant and wild-type viruses will be counted in plaque titrations done at the permissive temperature, the first term in the numerator, yield $(A + B)_p$, measures the yield of all viruses, both mutant and wild type, from the initial high-moi mixed infections done under nonpermissive conditions. The second term in the numerator, yield $(A + B)_{np}$, measures the yield of wild-type viruses, mostly recombinants, from the high-moi mixed infections done under nonpermissive conditions, because only wild-type viruses will be counted in plaque titrations done at the nonpermissive temperature. Subtraction of the wild-type viruses from the total viruses leaves a count of only the mutant viruses in the numerator. The denominator measures the ability of each of the mutants to grow in single high-moi infections done initially under nonpermissive conditions.

If the two mutants, A and B, are in different virus genes, then in the mixed infection done under nonpermissive conditions mutant A can contribute wild-type B gene product and mutant B can contribute wild-type A gene product. Thus, the mutants can help or complement each other, resulting in a high yield of mutant virus in the mixed infection compared to the single infections, and a CI significantly greater than 1. If the two mutants A and B affect the same gene, then the wild-type gene product will be lacking in the mixed infection. In this case, the yield from the mixed infection will be equivalent to the yield from the single infections, and the CI should not exceed 1. In practice, because of error in plaque assays and from other sources, mixed infections with mutants in the same viral gene will often yield complementation indices of slightly greater than 1, and the practical cutoff must be determined empirically for a given viral system.

An example of complementation analysis for several mutants of Sindbis virus is shown in Table 6. Close examination of these data demonstrates both the utility and the vagaries of complementation analysis. For example, *ts*21, *ts*19, and *ts*17 clearly form a complementation group, as they are all noncomplementing with each other, and they complement all other mutants with the exception of *ts*24. The *ts*24 mutant is retained in group A because it is clearly noncomplementing with two other group A mutants, despite relatively high complementation indices with *ts*19 and *ts*17. The *ts*6 mutant is unusual in that it yields, for unknown reasons, relatively high complementation indices with all other mutants. The complementation pattern observed with *ts*11 is erratic; it was originally assigned to an independent group, then to group A, and finally to an independent group called A' to indicate uncertainty in the assignment (76).

Complementation Group	Mutant	Complementation level in mixed infection with: ts21	ts19	ts17	ts4	ts24	ts11	ts6	ts2	ts5	ts18	ts23	ts28
A	ts21	–	0.2	0.1	0.1	1.5	17.6	200	43	27	24	ND	ND
A	ts19		–	0.4	0.5	3.2	100	485	ND	16	19	43	12
A	ts17			–	ND	4.5	32	368	26	31	17	19	23
A	ts4				–	0.36	8.0	127	19	ND	27	ND	8
A	ts24					–	4.2	364	22	25	18	16	5
A'	ts11						–	280	1.8	32	43	ND	12
B	ts6							–	62	93	64	68	21
C	ts2								–	1.3	36	27	5.2
C	ts5									–	345	61	8.9
D	ts18										–	0.7	16.7
D	ts23											–	8.5
E	ts28												–

ND, not determined.
Source: From ref. 76, with permission.

TABLE 6. *Complementation between* ts *mutants of Sindbis virus*

Qualitative complementation tests have also been devised for use with both bacterial and mammalian viruses (7,11,96). These qualitative tests are much easier to perform than quantitative tests and in practice just as reliable. In general, these tests are designed so that bacterial lawns or eukaryotic cell monolayers are infected either singly or with two viruses under nonpermissive conditions and at relatively low moi. The moi must be high enough so that numerous cells are doubly infected in the mixed infection, but low enough so that most cells are uninfected and a lawn or monolayer is maintained. Complementing mutant pairs produce plaques or cleared areas under nonpermissive conditions, whereas noncomplementing mutant pairs do not. An example of a qualitative complementation test is shown in Fig. 10. A disadvantage of the qualitative test is that it theoretically does not discriminate between complementation and recombination, but in practice this deficiency has not been a problem.

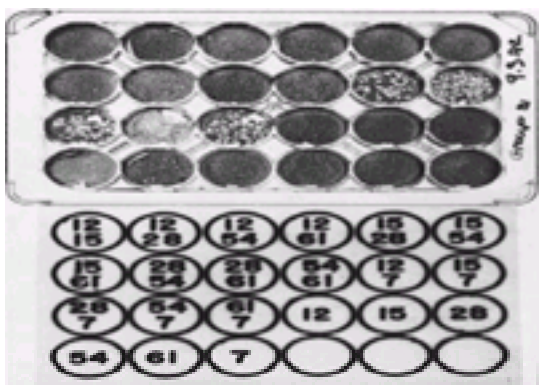

FIG. 10. Qualitative complementation test. This test was done to confirm that five different vaccinia virus temperature-sensitive mutants, *ts*12, *ts*15, *ts*28, *ts*54, and *ts*61, all reside in the same complementation group. Monolayers of the African green monkey kidney cell line BSC40 grown in a 24-well dish were infected at very low moi (0.03 pfu/cell) with individual mutants or mutant pairs. The dish was incubated at a nonpermissive temperature (40°C) for 3 days and stained with crystal violet. The stained dish is shown at the *top*, and a key to the infections is shown at the *bottom*. Mixed infections in the first 10 wells represent all possible pairwise combinations of the five candidate temperature-sensitive mutants, and the absence of plaques in these wells confirms that they all reside in a single complementation group. As a control, each mutant was also tested with another temperature-sensitive mutant, *ts*7, which was known to be in a different complementation group. The presence of plaques in each of the mixed infections containing *ts*7 represents positive complementation and confirms the validity of the test. Each mutant was tested in a single infection as an additional control. (From ref. 12, with permission.)

Complementation analysis has been of tremendous benefit in sorting mutants in most but not all viral systems. A notable exception is poliovirus, where complementation between temperature-sensitive mutants *in vivo* is not observed. The lack

of complementation in picornaviruses may be related to the unique mechanism of viral gene expression, in which all protein products are produced from a polyprotein precursor by proteolytic cleavage. If individual temperature-sensitive mutants affect structure, synthesis, or cleavage of the polyprotein precursor, they may behave as if they all belong to a single complementation group, even though they may map to different protein end products.

As a concept, complementation impacts broadly on virology and is not limited simply to the grouping of conditionally lethal mutants into genes. For example, the growth of engineered host-range deletion mutants in essential virus genes, discussed previously, relies on complementation of the missing viral function by an engineered cell line that expresses the wild-type viral gene product. In addition, the accumulation of defective virus genomes at high-multiplicity passage, also discussed previously, results from a complementing helper function provided by wild-type virus.

Recombination and Reassortment

Recombination describes a process by which nucleic acid sequences from two genotypically different parental viruses are exchanged so that the progeny contain sequences derived from both parents. In viral systems, there exist three distinct mechanisms of recombination, dictated by the structures of the viral genomes. For DNA viruses, recombination occurs by the physical breakage and rejoining of parental DNA molecules through regions of sequence homology, in a fashion similar or identical to the same process in bacteria or higher organisms. Of the RNA viruses containing nonsegmented genomes, only picornaviruses, coronaviruses, togaviruses, and retroviruses display efficient recombination, which is thought to occur during replication via “copy choice”—namely, switching templates during replication so that the newly synthesized genome contains sequences from two different parental molecules (39,48,54,55). For RNA viruses containing segmented genomes, recombination occurs through reassortment of individual parental genome segments into progeny viruses. Historically, recombination has been used to construct genetic maps of virus mutants and to construct novel virus genotypes. Although recombination mapping has been largely replaced by physical mapping techniques such as marker rescue, a technical knowledge of recombination mapping can contribute to an appreciation of the complexity of genetic interactions between viruses.

The methods used to determine recombination frequencies are the same regardless of genome structure or mechanism of recombination. Like complementation, the quantitative test to determine recombination frequency between two mutants, called a two-factor cross, is a two-step procedure, but in this case co-infections are first done under conditions permissive to recombination, and then the fraction of recombinants relative to the total virus yield is quantitatively assessed by plaque titration. Specifically, cells are first infected with two different virus mutants at high moi so that all cells are co-infected with both mutants, and infected cells are incubated under permissive conditions so that both mutants have maximal opportunity for interaction, for an interval sufficient to achieve maximal virus yield. Single high-moi infections under permissive conditions are performed as controls. Virus is then harvested, yields are quantified by plaque titration under both permissive and nonpermissive conditions, and a recombination frequency is calculated according to the followng formula:

$$\frac{\text{yield } (A + B)_{np} - \text{yield } (A)_{np} - \text{yield } (B)_{np}}{\text{yield } (A + B)_{p}} \times 2 \times 100\% = \text{RF},$$

where A and B represent individual virus mutants and the subscripts p and np represent the conditions, permissive or nonpermissive, under which the virus yields were plaque-titrated. The first term in the numerator, yield $(A + B)_{np}$, quantifies wild-type virus emerging from the mixed infection, including both recombinants and revertants, because only wild-type virus will grow in the plaque assay done under nonpermissive conditions. The second and third terms in the numerator, yield $(A)_{np}$ and yield $(B)_{np}$, quantify wild-type virus emerging from the control single infections, providing a measure of reversion in each of the two mutants. Subtraction of the revertants from the total yield of wild-type virus leaves a measure of recombinants only in the numerator. The denominator, yield $(A + B)_{p}$, quantifies the total virus yield from the mixed infection including both wild-type and mutant virus, as all input virus types will grow in the plaque assay done under permissive conditions. The quotient is multiplied by a factor of 2 to account for unscored progeny representing the reciprocal of the wild-type recombinants, namely double mutants, and converted to a percent.

Recombination mapping in DNA viruses relies on the assumption that the frequency of recombination between two genetic markers is proportional to the distance between the two markers. For several DNA viruses, observed recombination frequencies comprise a continuous range from less than 1% up to a theoretical maximum of 50%, allowing the construction of linear genetic maps (32).

In viruses with segmented genomes, recombination between markers on the same segment is extremely rare, but reassortment of segments is extremely efficient, so that recombination is an all-or-none phenomenon, with markers on the same segment displaying no recombination, and markers on different segments displaying very high levels of recombination (82). For these reasons, genetic exchange in segmented RNA viruses is commonly called reassortment rather than recombination. Reassortment analysis for segmented viruses is useful for determining whether two mutants map to the same genome segment, but it cannot be used to determine the order of markers on a given segment. Mutants can be mapped to individual RNA segments by performing intertypic crosses between virus types that differ in the electrophoretic mobility of each RNA segment. Specifically, if crosses are performed between a wild-type virus of one type and a mutant virus of another type and numerous wild-type progeny analyzed, one segment bearing the wild-type allele will be conserved among all the progeny, while all other segments will display reassortment (82).

Marker Rescue

Marker rescue is a physical mapping technique that measures directly whether a given virus mutation maps within a specific subfragment of a virus genome. Use of marker rescue is confined to DNA viruses in which homologous recombination takes place, but it has been of enormous value in these systems. The application of the technique varies somewhat depending on the virus system under study, but the general principles are the same. Specifically, full-length

mutant viral genomic DNA plus a wild-type DNA genomic subfragment, usually a cloned DNA molecule, are introduced into cells under conditions permissive for recombination and for wild-type virus replication. For viruses that contain infectious DNA—for example, herpesviruses (95), adenoviruses (36), and polyomaviruses (58,71)—the mutant genomic DNA and the wild-type genomic subfragment may be co-transfected into cells. For viruses containing noninfectious genomic DNA—for example, poxviruses (99)—the mutant DNA must be introduced into cells by infection with the mutant virus, and this is followed by transfection with the wild-type DNA subfragment. In either case, the protocol allows for homologous recombination between the mutant genome and the wild-type DNA subfragment. If the wild-type DNA subfragment contains the wild-type allele for the mutation, the recombination can exchange the wild type for the mutant sequence in the mutant genome, creating wild-type virus. Conversely, if the wild-type fragment does not contain the wild-type allele for the mutation, no wild-type virus, above a background of revertants, will be created in the experiment.

The presence of wild-type virus can be assayed using either a two-step or a one-step protocol. In the two-step protocol, depending on the nature of the mutation being rescued, infected and/or transfected cells are incubated under permissive conditions to facilitate recombination and replication, or under nonpermissive conditions to select for wild-type recombinants; then wild-type virus yields are quantified by plaque titration under nonpermissive conditions. In the one-step protocol, the infection and/or transfection is done so that only a small fraction of the cells in a monolayer are infected, and cells are then incubated under nonpermissive conditions so that wild-type virus formed during a successful rescue will form plaques on the monolayer (99). In short, regardless of the precise method used, conversion, or rescue, of mutant virus to wild type with a given wild-type DNA fragment means that the mutation maps within that fragment. Initial marker rescue mapping experiments may be facilitated by the use of a few large but overlapping wild-type DNA fragments, and fine mapping may be accomplished with fragments as small as a few hundred nucleotides. Marker rescue mapping has completely replaced recombination mapping as a method for mapping DNA virus mutants, and precise genetic maps of several DNA viruses have now been constructed.

Reverse Genetics

Prior to the advent of recombinant DNA and DNA sequencing technologies, classical genetic analysis, namely random isolation and characterization of virus mutants, was one of the few effective methods for identifying, mapping, and characterizing virus genes, and it was the only method for obtaining virus mutants. With the current ready availability of genomic sequences for virtually all the prototypical members of each virus family and a versatile package of genetic engineering tools, the experimental landscape has changed completely. One can now conduct a genetic analysis with a reasonably complete foreknowledge of the genetic structure of the virus, focus attention on individual genes of interest, and deliberately engineer mutations in genes to study their function. Termed reverse genetics, this process has come to dominate the genetic analysis of viruses.

Reverse genetic analysis involves two distinct considerations: strategies for construction of cloned mutations and strategies for incorporation of mutations into viral genomes. The principles governing these strategies are highly dependent on the structure of a given viral genome and the strategy of virus replication, and thus they vary in the extreme. However, a few general principles can be identified and are discussed later.

Incorporation of Mutations into Viral Genomes

The methods used for incorporation of mutations into viral genomes depend on several features of the individual virus under consideration, including genome size, whether the nucleic acid is infectious, and whether the genome is RNA or DNA. For DNA or RNA viruses with relatively small genomes and for which the nucleic acid is infectious, incorporation of a given mutation into the viral genome is a relatively straightforward matter of cloning the desired mutation in a full-length genomic clone in a prokaryotic vector, then transfecting cells with the mutant nucleic acid. For examples, engineered mutations have been constructed in both picornaviruses (18) and polyomaviruses (1) using full-length genomes.

For DNA viruses that are either too large or too complex to be easily manipulated as a full-length genomic clone, or that contain genomes that are not infectious as nucleic acids, incorporation of mutations into the viral genome requires homologous recombination catalyzed in infected and/or transfected cells, in a fashion virtually identical to marker rescue protocols described previously. For example, *in vivo* recombination has been used very successfully to construct numerous engineered mutations in both vaccinia virus (75) and herpes simplex virus (107).

For RNA viruses whose genomic nucleic acid is not infectious and for which homologous recombination is not an option, the protocols for incorporation of mutations into the genome become more complex and tailored to the particulars of replication of individual viruses. For example, construction of engineered mutations in rhabdoviruses requires construction of a full-length genomic clone which contains the mutation, and which can be transcribed from a bacteriophage T7 promoter. The mutant viral genome is activated by cotransferation with T7 promoter-driven plasmids encoding all required rhabdovirus replication proteins into cells infected with a recombinant vaccinia virus expressing T7 RNA polymerase (84). Similarly, construction of influenza virus recombinants requires co-transfection of multiple plasmids encoding the mutant RNA segment and several influenza replication proteins, and co-infection with a helper virus (81).

The methods used for isolation of the desired mutant virus depend on the nature of the mutation being constructed, and on the method of incorporation into the virus genome. In situations not requiring *in vivo* homologous recombination between cloned mutant DNA and wild-type virus, only the mutant allele of the target gene is present in the construction, all virus recovered from the construction will be mutant, and no selection or screen for mutants is required. This situation applies, for example, to construction of mutations in papovaviruses, picornaviruses, or rhabdoviruses, as described previously. In situations when *in vivo* homologous recombination is used to incorporate the cloned mutation into a wild-type genome, for example in poxviruses or herpesviruses, both mutant and wild-type viruses emerge from the mutant construction protocol, and thus a screen or selection is required to identify the mutant of interest. For mutations in nonessential genes, this may be a relatively straightforward matter of inserting into the target gene a color marker such as beta-galactosidase to facilitate a screen (107), or inserting a dominant selectable marker such as *E. coli* guanine

phosphoribosyltransferase (25), to facilitate a selection. For conditionally lethal phenotypes such as temperature sensitivity, although techniques exist that enrich for recombinant viruses, mutant isolation ultimately relies on a screen of individual mutants for differential growth under permissive and nonpermissive conditions.

Construction of Cloned Mutations

Construction of cloned mutations for use in virology is problematic only if the gene in question is essential, necessitating isolation of a conditionally lethal mutation. For genes that are nonessential, construction of a cloned mutation is a simple matter of engineering a null mutation, for example a deletion, insertion, or nonsense mutation, into the cloned gene sequence. Three basic types of engineered conditionally lethal mutations are currently in use: host-range deletion mutants that rely on the availability of a complementing host cell, temperature-sensitive mutants constructed by clustered charge-to-alanine scanning, and artificially induced gene regulation. For host-range deletion mutants, the primary problem is construction of a host cell that expresses the target gene in a fashion appropriate for complementation of a null mutant in the virus. Once a cell line has been isolated, construction of the cloned mutation in the virus gene follows the same principles governing construction of a null mutation in a nonessential gene. The fundamental problem in creating temperature-sensitive mutations is that it is currently impossible to predict from primary amino acid sequence or even from three-dimensional protein structure what type of mutation will render a protein temperature sensitive. This difficulty has been partially overcome with the use of clustered charge-to-alanine scanning mutagenesis, in which clusters of three or more charged residues in the primary amino acid sequence of a protein are all changed to alanine (105). In theory, charge clusters are likely to reside on the surface of the protein, where they may facilitate protein–protein interactions, and neutralization of the charge by replacement with alanine may weaken such interactions without seriously disrupting the three-dimensional conformation of the protein. In practice, as much as 30% of clustered charge-to-alanine scanning mutants prove to be temperature sensitive *in vivo*, and this mutagenesis technique has been successfully used to construct temperature-sensitive mutants of both picornaviruses and poxviruses (18,42). Last, conditionally lethal mutants have been constructed in poxviruses by placing essential genes under lactose operator–repressor control in the viral genome (85).

Viral Interference, Defective Interfering Particles

Interference refers generally to a phenomenon whereby infection by one virus results in inhibition of replication of another virus. Several distinct types of interference have been described (28), including direct heterologous interference, interferon-mediated heterologous interference, and defective interfering (DI) particle–mediated homologous interference. Direct heterologous interference describes a negative interaction between viruses of two different types, even different families, wherein viral gene products or alterations in the intracellular environment induced by one virus disrupt replication of another virus. The incompatibility of heterologous virus infections is not surprising, given the intricate interplay of virus and host functions in an infection, and the enormous variety of intracellular changes induced by different viruses. In interferon-mediated heterologous interference, discussed in detail in Chapter 12, infection by one virus induces interferon synthesis by the infected cell, and the interferon in turn induces an antiviral state in neighboring uninfected cells. DI particle–mediated homologous interference has been of particular interest to geneticists because of insights into the mechanisms of virus replication provided by defective interfering particles.

Defective interfering particle–mediated homologous interference was first described by von Magnus (103), who noted that serial undiluted passage of influenza virus resulted in a dramatic decrease in infectious titer, whereas the number of particles remained constant. Essentially the same phenomenon was subsequently observed in a wide variety of RNA and DNA animal viruses, and in both plant and bacterial viruses (49). The mechanism of interference in each case is similar: Virus stocks accumulate DI particles, which are virus particles that contain genomes that are grossly altered genetically, usually by significant deletion of essential functions, but that nevertheless retain critical replication origins and packaging signals, allowing for amplification and packaging in co-infections with complementing wild-type “helper” virus. DI particles usually display a replication advantage relative to wild-type virus, resulting from increases in the copy number or efficiency of replication origins. DI particles actively inhibit replication of wild-type virus, presumably by competing for limiting essential replication factors. Study of DI particles has provided significant insight into the viral replication, in particular structure and function of replication origins.

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CHAPTER 3 Principles of Virus Structure

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Virus particles have evolved to transfer genetic material between cells and to encode information sufficient to ensure their own continued propagation. They are, in effect, extracellular organelles. They contain most or all of the molecular machinery necessary for efficient and specific packaging of viral genomes, escape from an infected cell, survival of transfer to a new host cell, attachment, penetration, and initiation of a new replication cycle. In many cases, the molecular machinery works in part by subverting more elaborate elements of the host cell apparatus for carrying out related processes. The principles of virus structure thus arise from the requirements imposed by the functions just outlined on the evolution of viral molecular architecture.

What are these requirements? One of the simplest and most general is the formation of a closed shell to protect viral nucleic acid during its passage from one cell to another. A second is one of genetic economy: the information necessary to specify the structural proteins must not exhaust the coding capacity of the viral genome. A third is the embodiment of specific strategies for exit and entry. The principles of protein folding and assembly, the sources of specificity in protein–protein interactions, and the possibilities for triggered conformational changes have determined what sorts of structures are observed. Indeed, much of our appreciation of these principles and possibilities has come from the study of viruses because they are among the simplest and most accessible of macromolecular machines.

Viruses come in many shapes and sizes. The broadest distinction is between the so-called enveloped and nonenveloped viruses, that is, those that contain or do not contain, respectively, a lipid-bilayer membrane. Most enveloped viruses acquire their envelope by budding through a membrane of the host cell into some extracytoplasmic compartment—either through the plasma membrane itself or through a membrane of the endoplasmic reticulum (ER) or Golgi apparatus. This last step in viral assembly is thus also an important part of the mechanism by which the virus escapes from an infected cell. Enveloped viruses then enter another host cell by fusion of their membrane with a membrane of the cell being infected. Again, this can be either the plasma membrane or the membrane of an internal compartment, such as an endosome. Nonenveloped viruses generally escape by lysis of the cell in which they have propagated, and their mechanism of entry is still incompletely understood.

Further categorization of virus structures depends on details of their molecular organization. The progress made recently in understanding viral architecture in atomic detail now allows us a glimpse of underlying similarities across various families of viruses, and the final section of this chapter attempts to develop some of those categories. We need first, however, to outline how virus structures are studied and to introduce the language needed to describe them. Integrated into this introduction is a synopsis of the principles of virus assembly and a discussion of how observed structural similarities relate to functional homologies in viral entry.

HOW VIRUS STRUCTURES ARE STUDIED

Electron microscopy is the most useful way to determine the general morphology of a virus particle. For examining infected cells and larger, isolated particles, the traditional thin-sectioning methods are used. The thickness of a section and the coarseness of staining methods limit resolution to about 50 to 75 Å, even in the best cases. (*Resolution* means the approximate minimal size of a substructure that can be detected or separated in an image from its neighbor. Recall that one atomic diameter is 2–3 Å; an a-helix, 10 Å; and a DNA double helix, 20 Å.) Negative staining, with uranyl acetate, potassium phosphotungstate, or related electron-dense compounds, gives somewhat more detailed images of isolated and purified virus particles. Viruses embedded in negative stain are often relatively well preserved. The electron beam destroys the particle itself very rapidly, but it leaves the dense “cast” of stain undamaged for much longer. If the particle is fully covered by the negative stain, the image contains contrast from both the upper and the lower surface of the particle, and visual interpretation of finer aspects of the image can be difficult (34).

Recently, methods for preserving viruses and other macromolecular assemblies by rapid freezing to liquid nitrogen or liquid helium temperatures have permitted visualization of electron-scattering contrast from the structures in the particle itself and not just from the cast created by a surrounding of negative stain (3). Moreover, quantitative methods for image analysis, originally developed for studying negatively stained particles, have been applied effectively to such images (3,34). Thus, a three-dimensional reconstruction of the electron-scattering density in a hepatitis B virus capsid has been computed to 7 Å resolution, permitting a tracing of the polypeptide chain later verified by x-ray crystallography at much higher resolution (14,115). An advantage of such cryoelectron microscopy is that regular images can be selected from a heterogeneous field, allowing study of unstable or relatively impure preparations. A gallery of cryoelectron microscopy

images is shown in Figure 1.

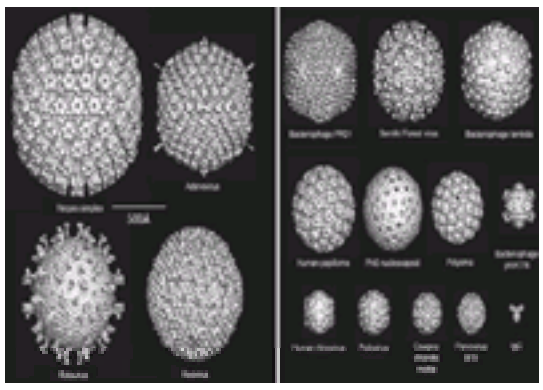

FIG. 1. A gallery of image reconstructions from cryoelectron microscopy of icosahedrally symmetric virus particles. The images illustrate the variety of sizes and specializations, even in these highly regular structures. The particles are labeled; the herpes simplex image is the nucleocapsid only. The image of an immunoglobulin molecule (IgG) is included on the same scale as the virus images, for direct comparison. The scale bar corresponds to 500 Å. (Modified from ref. 3, with permission.)

The detail obtained from even the most elegant of electron microscopy methods still falls short of what is required to understand molecular interactions. A higher-resolution picture can be obtained by x-ray diffraction methods, if single crystals of the relevant structure can be prepared. It has been known since the 1930s that simple plant viruses, such as tomato bushy stunt virus (TBSV), can be crystallized (8), and the first x-ray studies of such crystals were carried out as early as 1938 (11). Crystallization of poliovirus and other important animal viruses showed that the approach could be extended to human pathogens (95). The first complete high-resolution structure of a crystalline virus was obtained from TBSV in 1978 (61), and since then, the structures of a number of animal, plant, and insect pathogens have been determined. Only very regular structures can form single crystals; to study the molecular details of larger and more complex virus particles, it is necessary to dissect them into well-defined subunits or substructures. This dissection was originally done with proteases, by disassembly, or by isolation of substructures from infected cells. For example, the structure of the influenza virus hemagglutinin (114)—the first viral glycoprotein for which atomic details were visualized—was obtained from crystals of protein cleaved from the surface of purified virions (113); the structure of the adenovirus hexon was obtained from excess unassembled protein derived from adenovirus-infected cells (86). Recently, this dissection has more commonly been carried out using recombinant expression (e.g., of a fragment of gp120 from human immunodeficiency virus type 1 [HIV-1]) (65).

VIRUSES AS MOLECULAR PACKAGES

The simplest sort of package is a shell that can assemble spontaneously from a single component. The observation that certain viruses, particularly tobacco mosaic virus (TMV), assemble *in vitro* from dissociated components led Crick and Watson (33) to propose that virus shells would be highly symmetric objects. The arguments behind this proposal were simple: genetic efficiency requires that the viral coat be composed of many identical subunits, and experimentally observed self-assembly requires that the interactions among these subunits be specific and well defined. Identical subunits with specific interactions in general produce symmetric structures.

What is meant by *symmetric*? The rigorous definition of symmetry involves an operation, such as a rotation, that brings an object into self-coincidence. For example, if the ring of three commas in Figure 2 is rotated by 120 or 240 degrees, it will not be possible to recognize that a rotation has occurred (assuming that the commas are truly indistinguishable). The full symmetry of an object is defined by the collection of such operations that apply to it. In the case of protein assemblies, these operations can be rotations, translations, or combinations of the two. A symmetry axis that includes rotation by 180 degrees is called a *twofold axis* or a *dyad*; one with a 120-degree rotation (and, of course, a 240-degree rotation as well) is called a *threefold axis*; and so forth. Note the distinction between shape and symmetry: the *shape* of an object refers to the geometry of its outline, whereas its *symmetry* refers to the operations that describe it. The set of commas in Figure 2 has threefold symmetry; so does an equilateral triangle, the beer-company symbol with interlocked rings, and countless other objects with unrelated shapes.

FIG. 2. Three commas, arranged with threefold symmetry. The threefold rotation axis passes through the solid triangle in the center of the diagram.

Closed shells composed of identical subunits that interact through conserved, specific interfaces and that have a more or less spherical outline can have one of only three symmetries: the symmetry of the regular tetrahedron, the cube, or the regular icosahedron. These shells accommodate 12, 24, or 60 subunits, respectively. The icosahedral shells are obviously the most efficient of the three designs: they use the largest number of subunits to make a container of a given size, and hence they use subunits of the smallest size and the smallest coding requirement. Indeed, many of the viruses of interest to medical virologists contain at least some element with an icosahedrally symmetric shell, and the tetrahedral and cubic symmetries have not appeared in any normal virus assembly. We therefore describe icosahedral symmetry in some detail. Again, note the distinction between icosahedral symmetry and icosahedral shape. Not all objects with icosahedral symmetry have even the vague outline of an icosahedron; conversely, painting a single asymmetric object, like a comma, on each face of an icosahedron, rather than three such objects related by a threefold axis, would destroy the symmetry of the decorated object but would not affect its shape.

Icosahedral Shells

Icosahedral Symmetry

The diagram in Figure 3 shows the operations that belong to an icosahedrally symmetric object. They are a collection of twofold, threefold, and fivefold rotation axes. Placement of a single, asymmetric object on a surface governed by this symmetry leads to the generation of 59 others, when the various rotations are applied. One such object, one sixtieth of the total shell, can therefore be designated as an *icosahedral asymmetric unit,* the fundamental piece of structure from which all the rest can be produced by the operations of icosahedral symmetry. With a typical, compact protein domain of 250 to 300 amino acid residues, close to the upper limit for most single protein domains, what sort of container can we construct? Suppose that the protein is so constructed that 60 copies fit together into a 30 Å thick shell with no significant gaps. The cavity within that shell will then have a radius of about 80 Å, which can contain a 3-kb piece of single-stranded DNA or RNA (ssDNA or ssRNA, respectively), tightly condensed. A few, very simple virus particles indeed conform to this description. The parvoviruses (see Chapter 69) contain a 5.3-kb ssDNA genome, and their shells contain 60 copies of a protein of about 520 residues (Fig. 4). The capsid protein therefore uses up about one third of the genome. (*Capsid,* from the Latin *capsa,* for box, is used to describe the protein shell that directly packages DNA or RNA; *nucleocapsid* is sometimes used to refer to the shell plus its nucleic acid contents.) Likewise, the satellite of tobacco necrosis virus (STNV) contains 60 copies of a 195-residue subunit and a 1120 ssRNA genome, of which over half is used for the coat protein (68). As the name implies, however, STNV is actually a defective virus, and it requires tobacco necrosis virus co-infection to propagate.

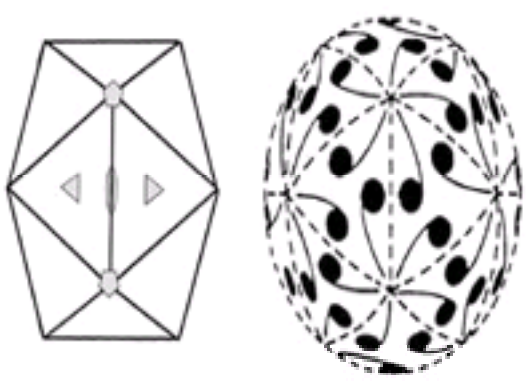

FIG. 3. *Left:* Outline of an icosahedron, showing some of the symmetry axes. Fivefold axes (*vertices*) are represented by pentagons; threefold axes (*faces*) by triangles; twofold axes (*edges*) by ovals. *Right:* 60 commas, distributed with icosahedral symmetry on the surface of a sphere. The *dashed lines* correspond to the edges of an icosahedron, projected onto the spherical surface.

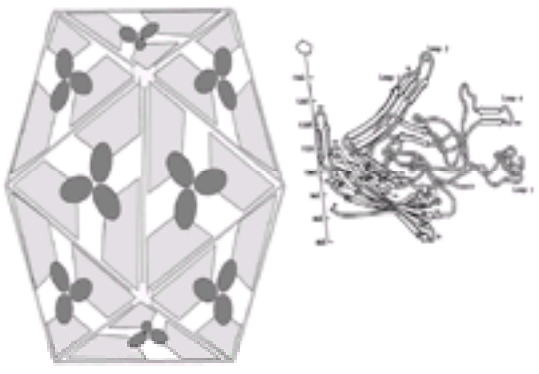

FIG. 4. *Left:* Diagram of the packing of 60 protein subunits in the shell of a parvovirus. The *trapezoids* represent a jelly-roll b-barrel domain (see Fig. 5) that forms the core of each subunit, and the *ovals* represent loops, emanating from the jelly-roll domain, which form contacts about the threefold axes. *Right:* Ribbon diagram of the folded subunit of canine parvovirus, viewed perpendicular to the fivefold and twofold axes, that is, as if viewing from the “southeast” the subunit that is just to the lower right of center in the larger packing diagram. The position and orientation of a fivefold axis, with respect to the subunit, are shown by the line with a fivefold symbol at its end. The jelly-roll b-barrel is at the *lower left* of this ribbon diagram, and the loops extend into the page toward the *upper right.* (From ref. 101, with permission.)

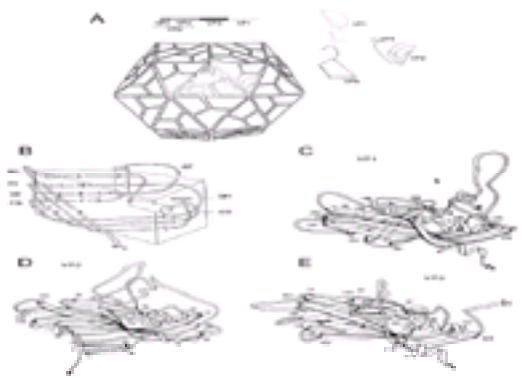

FIG. 5. Organization of the protein shell of picornaviruses. **A:** Subunit packing. *Upper left:* The order of domains in the precursor polypeptide chain. The cleavages between VP0 and VP3 and between VPP3 and VP1 are performed by a viral protease; the cleavage of VP0 to VP2 and VP4 is autolytic. Each subunit contains a jelly-roll b-barrel (*trapezoidal solid*), with highly variable loops and long N- and C-terminal extensions (*curved lines*). Two protomers (VP1, VP2, VP3, and V4) are shown in more detail in the center of the diagram. Distinct line qualities are used for each type of subunit. The diagram on the right is an "exploded view" of the right-hand protomer in the packing diagram. **B:** Schematic diagram of the folding of a polypeptide chain into a jelly-roll b-barrel. The strands are lettered in the order of their sequence in the chain. Strands B, I, D, and G form one sheet; C, H, E, and F, the other. The interstrand loops are designated BC, CD, etc. In picornaviruses and T=3 plant viruses, there are usually a-helices in the positions, shown as a cylinders. **C** to **E:** Ribbon diagrams of the jelly-roll cores of VP1 to VP3 of poliovirus. The numerals show residue numbers in each chain. Note how the loops give each subunit distinct characteristics. There is no evident sequence similarity, despite the similarity of fold. The small, curved arrow in C points to the entrance of a hydrophobic channel in the b-barrel, where various antipicornaviral drugs bind, blocking one or more steps in viral entry.

There are various ways in which viruses have evolved to produce larger icosahedral shells and thus to package larger genomes. The simplest is just to use several different subunits, each of "garden-variety" size, to make up one icosahedral asymmetric unit. The picornaviruses (e.g., polioviruses, rhinoviruses) have 60 copies of three distinct proteins, VP1, VP2, and VP3, each of between 230 and 300 amino acid residues, as well as 60 copies of a small internal peptide, VP4 (Fig. 5; see Chapter 23). The shell has a cavity about 95 Å in diameter, which contains an RNA genome of 7.5 to 8 kb. The picornaviruses thus expend about one third of their genome to encode the structural proteins of the virion. (*Virion* is used interchangeably with *complete virus particle.*) We note here two other important features of picornavirus molecular architecture. First, the folded structures of VP1, VP2, and VP3 are all based on the same kernel—a domain known as a *jelly-roll b-barrel* (see Fig. 5). This is the same fold that is found in the single subunits of the parvoviruses and of STNV. It is a module particularly well suited to the formation of closed, spherical shells, because of its blocklike, trapezoidal outline, but its prevalence among viral subunits may be evidence of a deeper evolutionary relationship. A second noteworthy feature of picornavirus design is the use of armlike extensions of the subunits for tying together the assembled particle (see Fig. 5). The importance of scaffold-like intertwining of subunit arms was first discovered in the simple plant viruses (61). In effect, folding of part of the subunit and assembly of the shell are concerted processes.

Quasi-Equivalence

A conceptually more complex, but evolutionarily simpler and more economical, way to build a shell of more than 60 average-sized subunits was described by Caspar and Klug in 1961 (23). It is illustrated by the diagram of 180 commas in Figure 6. The commas have similar interactions (head to head in pairs; neck to neck in rings of three; tail to tail in rings of five or six), but they fall into three sets, designated *A, B,* and *C.* If the commas are taken to represent proteins, the conformational differences between A and B positions, for example, involve the differences between rings of five and rings of six, for contacts involving the parts of the proteins symbolized by the tails. Caspar and Klug (23) postulated that protein subunits might have the sort of flexibility or capacity for conformational switching needed to accommodate in this way, and that viruses with more than 60 chemically and genetically identical subunits might exhibit the sort of near equivalence seen in the A, B, and C conformers in the comma illustration. Caspar and King (23) called this sort of local distortability, which might conserve the specificity and character of protein contacts, "quasi-equivalence."

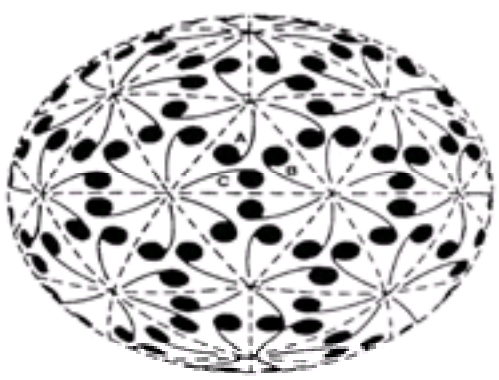

FIG. 6. Quasi-equivalent arrangement of 180 commas, in a T=3 icosahedral surface lattice on a sphere. Compare Figure 3 (*right*), a T=1 arrangement of 60 commas with icosahedral axes oriented similarly.

Quasi-equivalent designs are exemplified by a number of plant and animal viruses, such as TBSV (61) and Norwalk virus (78), as illustrated in Figure 7, Figure 8 and Figure 9. In TBSV and Norwalk virus, there are 180 genetically and chemically identical subunits that compose the capsid. The subunits are actually larger than those of the picornaviruses, but most of the extra size comes from a second, projecting domain that serves functions other than the construction of a closed shell. The size of the *shell domain* (S domain) in both cases is just about 200 residues, and the folded structure of the domain is again a jelly-roll b-barrel. The important feature of the packing of these 180 S domains is illustrated by the TBSV diagram in Figure 8. The contents of an icosahedral asymmetric unit can be described as three chemically identical subunits, with somewhat different conformations. These conformers are denoted A, B, and C, echoing the designation of commas in Figure 6. The differences among the conformers reside principally in an ordered or disordered conformation for part of the N-terminal arm and in the angle of the hinge between the S domain and the *projecting domain* (P domain). The A and B conformations are nearly identical, with disordered arms and similar hinge angles. The C conformation has an ordered arm and a different hinge angle from A and B. The ordered arms extend along the base of the S domain and intertwine with two others around the icosahedral threefold axis. Thus, the whole collection of 60 C-subunit arms forms a coherent inner scaffold.

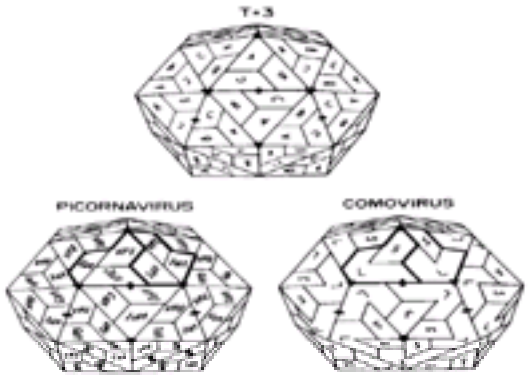

FIG. 7. Comparison of the packing of the jelly-roll b-barrel domains in T=3 plant and insect viruses; vertebrate picornaviruses; and plant comoviruses. In T=3 structures, the A, B and C subunits are chemically identical. In picornaviruses, VP1, VP2, and VP3 are different, but similar in the way the polypeptide chains folds (see Fig. 5). In comoviruses, the large subunit contains two b-barrel domains, in positions corresponding respectively to C and B in T=3 structures or to VP2 and VP3 in picornaviruses, and the small subunit contains one such domain, in a position corresponding to A or VP1. The diagrams are oriented (with respect to the icosahedral axes) like in Fig. 3 and Fig. 5**A**, but tilted slightly upward. The trapezoidal outlines represent subunits; the lettering or numbering on the subunits is oriented to show the rotational relationships among them. The small, solid symbols represent symmetry axes as defined in Figure 3. The heavy outlines in the lower two diagrams surround two protomers, related to each other by the fivefold axes toward the top of the diagrams. In Figure 5**A**, these are the protomers for which the N-terminal extensions of the subunits and VP4 are shown. The protomer on the right in the picornavirus diagram is the one shown in the “exploded” view on the upper right of Figure 5**A**. (Courtesy of J. Johnson.)

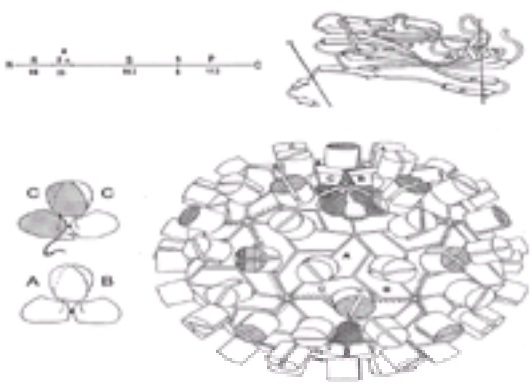

FIG. 8. Molecular architecture of tomato bushy stunt virus (TBSV), illustrating some of the principles of domain organization and quasi-equivalent interaction described in the text. TBSV is an icosahedrally symmetric, RNA-containing particle with 180 identical subunits. The 40-kd coat protein is organized into four regions: a positively charged, N-terminal R-segment; a connecting arm (*a*), and two compactly folded domains—*S* (shell) and *P* (projecting). There is a hinge (*h*) between the S and P domains. The relationship of the domains and connecting segments to the sequence (N- to C-terminus) is shown at the *upper left.* A ribbon diagram of the S domain is shown in the *upper right,* in an orientation corresponding to the similar diagrams in Figure 4 and Figure 5. Note how closely related are the folds of the b-barrels in all the subunits illustrated in these figures. The R segment of TBSV is probably irregularly folded in the particle interior; it interacts with viral RNA. By analogy with work on alfalfa mosaic virus (see text), a short piece of the R segment might be recognized by the RNA packaging signal, but this interaction has not been defined for TBSV. The arm is folded against the S domain on 60 of the subunits (those in positions symmetrically related to *C*), but it extends inward in a more disordered way on the remaining 120 (positions equivalent to *A* and *B*). The folded arms form an internal framework (*dashed lines* in full-particle diagram), with three arms meeting and interdigitating at each of the icosahedral threefold positions (cutaway in full-particle diagram). The shell can be considered an assembly of 90 dimers—30 C/C dimers, with ordered arms and rather little local curvature and 60 A/B dimers, with disordered arms and significant local curvature (*lower left*). The ordered framework of arms forces the bases of the S domains apart, whereas along the line that bisects an A/B dimer (the line that joins a fivefold to an icosahedral threefold), the bases of the S domains come together. The detailed side-chain interactions along the fulcrum of this contact remain essentially unchanged, however. Note that the interdomain hinges are essentially passive followers, so that the pair-wise clustered P domains simply project out along the strict and local twofold axes, and the weak interdomain contacts within a subunit do not significantly influence their

orientation.

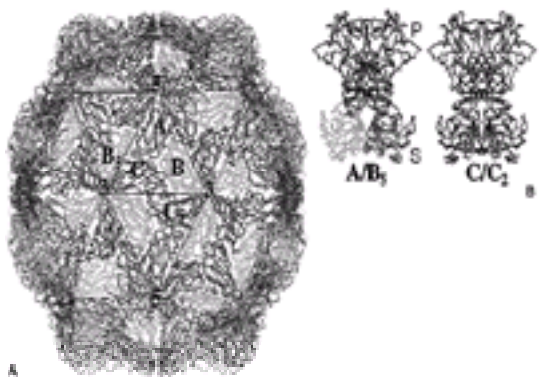

FIG. 9. Molecular architecture of the capsid of Norwalk virus, a human pathogen with a T=3 structure. The subunit arrangement resembles that in TBSV (see Fig. 8). There is a shell (*S*) domain with a jelly-roll b-barrel fold and a protruding (*P*) domain. The packing of S domains is shown in **A**. The P domain in Norwalk virus does not resemble the P domain in TBSV—it is larger, and it has two distinct subdomains—but it also forms strong dimer contacts. By comparing the side-view images of A/B and C/C dimers in part **B** of this figure with those in the lower left of Figure 8, the reader can see that the basic packing principle is similar in the two particles: the A/B contact generates curvature, whereas the C/C contact is relatively flat. The fulcrum around which the S-domain contact rotates in Norwalk virus is somewhat closer to the inward-facing margin of the domain than it is in TBSV. (From ref. 78, with permission.)

How equivalent or nonequivalent are the actual intersubunit contacts in TBSV or in other examples of quasi-equivalent designs? Most of the interfaces are quite conserved, with very modest local distortions that do not significantly change the way individual amino acid side chains contact each other. The interfaces between conformers that do exhibit noteworthy differences are those that include the ordered C-conformer arms in one of their quasi-equivalent locations. Even at these interfaces, however, where a clear switching mechanism takes place, many side-chain contacts are conserved around the “fulcrum” of the differences created by C-arm insertion (see Fig. 8).

The ways of subdividing an icosahedral shell, to create quasi-equivalent subunit packing as in TBSV and Norwalk virus, were enumerated by Caspar and Klug (23). Only certain multiples of 60 subunits can be accommodated in this way; they are given by the formula $T=h^2 + hk + k^2$, where *h* and *k* are any integer or zero. The multiple T is known as the *triangulation number* because, as illustrated by comparison of the 60- and 180-comma structures in Figure 3 and Figure 6, it corresponds to subtriangulations of an icosahedral net on the surface of a sphere. Such nets are known as *surface lattices,* and they are another way of describing the symmetry of a closed-shell arrangement of building blocks. If an icosahedrally symmetric structure is imagined as a folded-up hexagonal net (Fig. 10), 12 uniformly spaced sixfold vertices are transformed into fivefold vertices. The intervening twofold, threefold, and sixfold symmetry axes of the flat net are transformed either into quasi-twofold, quasi-threefold, and quasi-sixfold axes of the icosahedral net (i.e., approximate symmetry axes that apply only locally to the subunits in the immediate neighborhood) or, in some cases, into strict icosahedral twofolds or threefold axes (see Fig. 10). A number of the designs predicted by the Caspar and Klug concepts have been found among various viruses of vertebrates, insects, and plants (see Fig. 1; see also ref. 3).

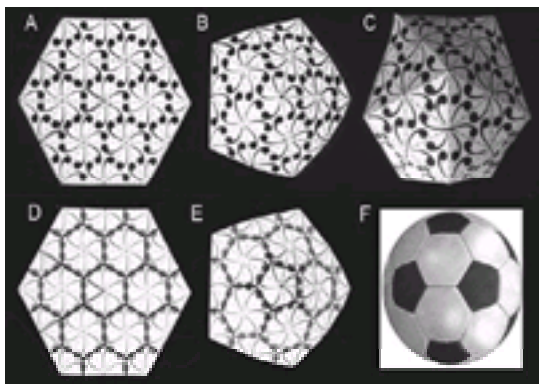

FIG. 10. A: Portion of a hexagonal lattice. Six triangular cells of the lattice meet at each lattice point, and each triangular cell contains three “subunits” (*commas*). Thus, there is a sixfold symmetry axis at each lattice point, a threefold symmetry axis at the center of each triangle, and a twofold axis at the midpoint of each edge. Imagine that the lattice extends indefinitely in all directions. **B:** Curvature can be introduced by transforming one of the sixfold positions into a fivefold (*center*). A 60-degree “pie slice” has been removed from the object in **A** by cutting along the heavy dotted lines, and the cut edges have been joined to generate the curved lattice shown here. **C:** If further cuts are made at regular intervals in an extended lattice, such as the one in **A**, and the edges joined as in **B**, a closed solid can be produced. In the case of the icosahedral solid shown here, vertices of the lattice separated by two cell edges have been transformed into fivefold axes, whereas the intervening lattice points have been left as local sixfold axes, producing a T=4 (h=2, k=0) structure. Notice that the local sixfold axes are actually only approximately sixfold in character; they correspond strictly to the twofold axes of the icosahedral object. **D:** Lines joining the centers of the triangular cells in **A** create a pattern of hexagons. **E:** When a sixfold is transformed into a fivefold, a hexagon becomes a pentagon. **F:** If second nearest-neighbor lattice points are all transformed into pentagons, a “soccer ball” figure results. This is a T=3 structure. A description of the lattice as a network of hexagons and pentagons is complementary to its description as a network of triangles. The representations in Figure 3 and Figure 6 use triangles. The representations in Figure 11 and Figure 12

use hexagons and pentagons. One representation for a given structure can easily be derived from the other.

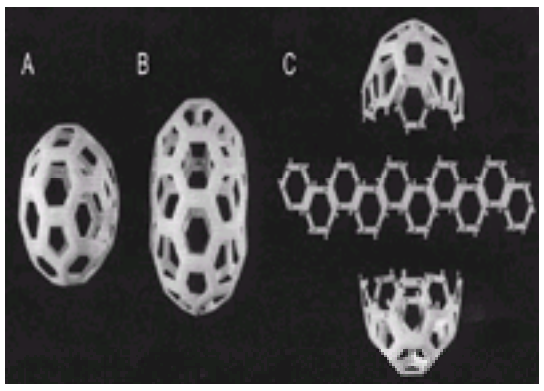

FIG. 11. A: Schematic representation of a T=3 lattice. Compare the soccer ball in Figure 10**F**. In this figure, the lattice is oriented so that there are pentagons at the north and south poles. Altogether, there are 12 pentagons and 20 hexagons (180 subunits) in a T=3 assembly. **B:** Schematic representation of the lattice describing phage f29. There are 12 pentagons and 30 hexagons (240 subunits). Again, there are pentagons at the poles of the structure. **C:** The f29 lattice can be derived by cutting the T=3 structure into two hemispheres, each with a pentagon at its pole, and adding a band of 10 hexagons around the equator. (From ref. 112, with permission.)

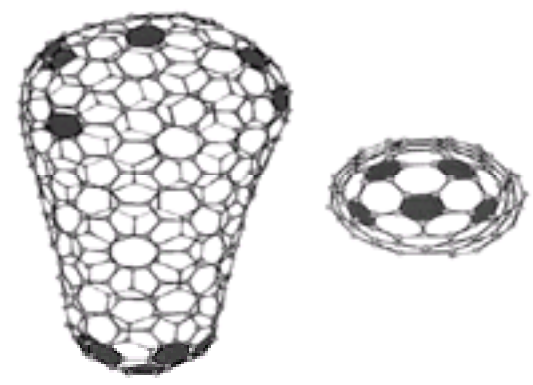

FIG. 12. *Left:* a conelike lattice of hexagons and pentagons, representing the rearranged ("mature") capsid of human immunodeficiency virus type 1. There are seven pentagons in the upper curved surface and five in the lower one. Thus, the upper surface contains a bit more than a full hemisphere and the lower surface, a bit less. *Right:* View from below, showing the five-pentagon surface. Addition of one more pentagon would turn it into a T=3 hemisphere. (From ref. 50, with permission.)

The external appearance of the virus particles in Figure 1 do not in all cases immediately reveal the underlying quasi-equivalent design. At high enough resolution, they will do so, of course, but as seen in the electron microscope at moderate resolution, they reveal variously disposed "bumps" rather than individual protein subunits. The bumps usually correspond to projecting parts of subunits, clustered around local symmetry axes. The 90 bumps in a three-dimensional reconstruction from electron micrographs of negatively stained TBSV, for example, correspond to pair-wise clustered P domains of the protein subunit, projecting at the 30 strict twofold and 60 quasi-twofold positions. In a frequently found arrangement, the intersubunit contacts are particularly tight and prominent around fivefold and quasi-sixfold axes in the surface lattice, as in the images of the herpesvirus capsid, bacteriophage P2, and cowpea chlorotic mottle virus (CCMV) in Figure 1 (*hexamer-pentamer clustering*). In low-resolution electron micrographs, such as those taken of negatively stained particles, the structure of CCMV (see Fig. 1) would simply have 32 smooth bumps, rather than obviously subdivided hexamers and pentamers. In general, hexamer-pentamer clustering produces 10 (T – 1) + 12 bumps in an icosahedral lattice with triangulation number T. Before the molecular principles of virus structure were fully understood, these bumps were called *capsomeres* (meaning the structural units of the capsid). This word is sometimes still used when referring to apparent morphologic units on the surface of a virus shell, but it is best reserved for cases in which all capsomeres are the same and hence represent a defined oligomer, as in the pentameric units of papovaviruses (see later).

Some noteworthy variations on subtriangulated icosahedral designs are nonetheless in keeping with the basic Caspar-Klug notion of quasi-equivalence, namely, that once a stable and specific protein interface has evolved, structures requiring deviations from simple designs generally make use of the possibility for flexing the contact, rather than developing a completely different specificity within the same surface of the folded subunit. One such variation is found in elongated structures with hemi-icosahedral caps (Fig. 11). Near the poles of these caps, the shell looks like a normal icosahedral design. As the lattice approaches the equator, however, the regular interspersion of fivefold and quasi-sixfold axes gives way to sixfold axes only, so that there is a tubular region around the middle of the particle. The tubular region can be of varying extent; in extreme cases, it can be much longer than the caps themselves. A further variation on this theme is found in the shells formed by the CA fragment of the lentivirus Gag protein. Conical structures seen within HIV-1 particles have been shown to be based on the sort of arrangement shown in Figure 12, where one cap has more than six fivefold axes and the other has less, so that the diameters of the two caps are different (50). (Note that

if there are only sixfold and fivefold vertices in a closed surface lattice, there will always be exactly 12 of the latter.)

A key issue in assemblies with quasi-equivalent contacts, like those just illustrated, is positional regulation. Of the different quasi-equivalent interactions, what determines, for any given site, which state is actually present? Consider for definiteness the T=3 design exemplified by TBSV (see Fig. 8). Structures like this one are known to assemble from dimers of the protein subunit. We can think of the dimer as having two states: the A-B state, characterized by unfolded arms and one position for the interdomain hinge, and the C-C state, characterized by folded arms and another position for the interdomain hinge. Experiments show that assembly proceeds by addition of dimers to a growing shell and that the curvature of the growing shell is always nearly correct (94) (Fig. 13). That is, when a dimer adds to a shell, it adopts the correct state, and the characteristics of the site at which the dimer adds determine that state. Assembly mechanisms of this kind depend on getting started correctly—having a correct initial nucleus—and on addition of defined "assembly units." In the case of TBSV, the initial nucleus probably includes an RNA packaging signal, and the assembly unit is the subunit dimer.

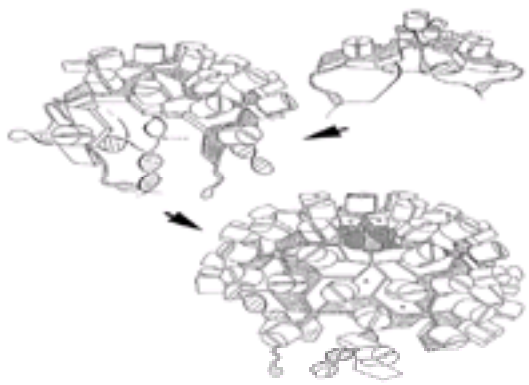

FIG. 13. Model for assembly of a T=3 virus shell (derived from work on turnip crinkle virus; see ref. 94). The key steps are: specific initiation, propagation by addition of a defined assembly unit, and closure. In the case of most T=3 structures that have been studied, the assembly unit is a dimer of the subunit. The initiation structure is shown as a trimer of dimers, nucleated by interaction with a packaging signal on the RNA. All the dimers adopt the C/C (ordered arm) conformation (see Fig. 8). An alternative model, probably not ruled out by the data, would be a pentamer of dimers, all in the A/B conformation (disordered arms), also nucleated by the packaging signal. In either case, the key characteristic of each addition step is that the conformation of the assembly unit that associates with the growing shell is uniquely determined by the nature of the site at which it adds. In the model shown here, the first addition to the trimer of C/C dimers is necessarily an A/B dimer because the interdigitated arms prevent further ordered arms around that threefold, and the assembly unit that slips in must necessarily leave its arms in a disordered state.

More Complex Icosahedral Shells

Pentameric Assembly Units in Papovaviruses

A number of icosahedral shells have structures in which the individual subunits are not in roughly equivalent positions with respect to their neighbors. In papovaviruses, 72 pentamers of the major capsid protein form the viral shell (82). The pentamers are centered on the vertices of a T=7 icosahedral surface lattice (Fig. 14), but of these, only 12 can be on fivefold positions, and the remainder are six-coordinated. That is, 60 of the 72 pentamers are "fivefold pegs in sixfold holes." In the case of the polyomaviruses (such as SV40 and murine polyoma), each subunit has an extended, C-terminal arm that issues from the body of the pentamer and invades a subunit in a neighboring pentamer (67). Thus, each subunit donates and accepts an arm. The interactions made by the arms are essentially invariant: they fit the same way into every target subunit. The essential variability lies in the way an arm bridges from one pentamer to the next, in other words, in the flexibility of the arm as it passes across the boundary between the pentamer from which it emanates and the pentamer into which it inserts. The folded part of the subunit is a jelly-roll b-barrel, just as in the RNA viruses with quasi-equivalent designs, but the long axis of the domain is oriented essentially radially, rather than tangentially, in the shell.

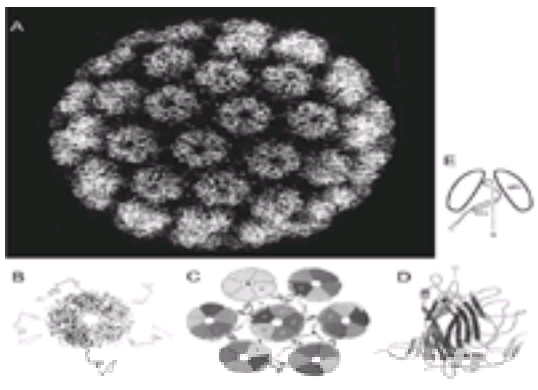

FIG. 14. Organization of the polyomaviruses (67). **A:** Overview of the particle, showing the packing of VP1 pentamers. The diagram is based on a molecular graphics representation in which the polypeptide chain of each subunit is shown as a folded line. The pentamers are all centered on lattice points of a T=7 icosahedral surface lattice. Only 12 of those lattice points are five-coordinated, however; the remaining 60 are six-coordinated. The orientation of the particle corresponds to that of the icosahedron in Figure 3; note the five-coordinated pentamers toward the top and bottom of the image, along the central vertical axis. The intervening pentamers are six-coordinated. **B:** A six-coordinated VP1

pentamer, “extracted” from the model in **A.** The extended carboxyl-terminal arms are shown in the conformations they adopt in the assembled particle; in the free pentamer, they are disordered and flexible. **C:** Pattern of interchange of arms in the virion. The arms “invade” neighboring pentamers, to link the structure together. The body of a pentamer is shown as a five-petalled flower; the arms are cylinders (a-helices) and lines. The *small open circles* mark Ca^{2+} sites. The central pentamer in this diagram corresponds to the one just to the upper right of center in **A. D:** The subunit, viewed normal to the pentamer axis. Strands are represented as *ribbons;* helices, as *cylinders;* loops as *narrow tubes.* The two sheets of the b-barrel domain are in different *dark shades;* the carboxyl-terminal arms are pale; the amino-terminal arms are somewhat darker. The complete carboxyl-terminal arm seen here actually emanates from a subunit in another pentamer; only the initial, a-helical segment of the arm from this subunit is shown because it then extends out of the page. Note how the amino-terminal segment clamps the invading arm in place. The small ball represents the site of a Ca^{2+} ion. **E:** Interaction of VP2 (or VP3) with VP1. The VP1 pentamer is shown in cross-sectional “side” view, with the outside of the virion at the top and the inside at the bottom. The C-terminal part of VP2 (identical with the C-terminal part of VP3) inserts from within the virion, into the conical hollow in the pentamer (4,25).

The assembly unit of the papovavirus shell is a pentamer (91), and just as the assembly mechanism for a T=3 shell described previously involves a unique nucleation event, followed by successive addition of individual dimers, so the likely mechanism for assembly of the papovavirus shell involves a specific initiation step followed by successive addition of pentamers. A scheme can be drawn by which the nature of a site for pentamer addition, at the growing edge of the shell, determines where the C-terminal arms of the new pentamer will insert (96). A clear advantage of the papovavirus design is that there is a single type of preformed assembly unit: the pentamer.

Scaffold Proteins in Phage and Herpesvirus Capsid Assembly

Structures with hexamers and pentamers of the same protein species cannot assemble by simple addition of a single kind of assembly unit, and they consequently require a substantially more intricate assembly mechanism. For example, genuine T=7 structures do exist, with 12 pentamers and 60 hexamers made of the same subunit. The heads of bacteriophages, such as lambda and P22, are examples of such designs (79). Unlike the papovaviruses, however, they cannot be put together from a unique kind of subassembly. Indeed, their assembly pathway is quite complex, involving formation of a precursor particle (the procapsid) with participation of an internal scaffold protein, which is degraded or recycled before insertion of the viral DNA (22). Herpesvirus capsids (see Fig. 1), which have T=16 shells with 12 pentamers and 150 hexamers of the major capsid protein (VP5 in the case of herpes simplex virus type 1 [HSV-1]), also assemble around a scaffold, which is degraded before insertion of the DNA genome (see Chapter 72).

Trimeric Assembly Units, Chemically Distinct Pentons, and “Cement” Proteins in Adenoviruses

Adenoviruses (Fig. 15) exhibit a combination of nonequivalent and quasi-equivalent interactions (98). They have 12 “pentons” on the fivefold positions, and 240 “hexons” on the sixfold positions of a T=25 icoshedral lattice. The pentons are pentamers of the penton-base protein, with an inserted, trimeric fiber (105). The hexons are actually trimers, not hexamers, of the hexon protein, and each face of the icosahedron is triangulated into a net with local threefold (rather than sixfold) symmetry. Within a face, the hexons make essentially equivalent interactions with each other, but at the edges, there are completely different hexon–hexon contacts (see Fig. 15). Additional proteins are needed to cement the hexons in place in the adenovirus shell and to anchor the pentons (47,99). The high-resolution structure of the hexon (Fig. 16) shows that each subunit contains two similar jelly-roll b-barrel domains, so that the threefold symmetric hexon does have a pseudo-sixfold (86). Just as one can imagine taking one subunit of a T=3 quasi-equivalent shell and generating three related but distinct subunits by gene duplication and divergence (e.g., going from a TBSV-like design to a picornavirus-like design), so too can one imagine taking a subunit from a true, quasi-equivalent, T=25 lattice and generating the adenovirus-like structure by gene duplication without separation of the two polypeptide chains.

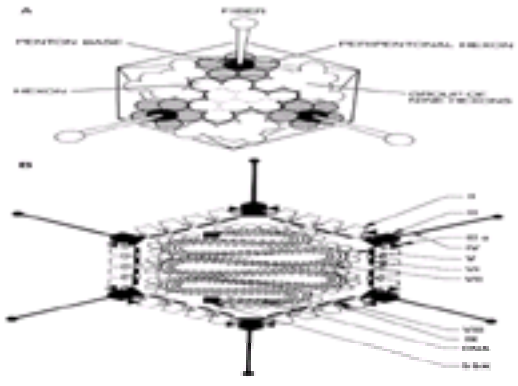

FIG. 15. A: Diagram of the adenovirus particle, showing the location of major protein subassemblies. The nine hexons drawn as a group and the “peripentonal” hexons are trimers of the same polypeptide; they are distinguished only by their location in the structure. (Illustration by John Mack. From ref. 16, with permission.) **B:** Cross-section showing the probable location of the principal polypeptide components and the viral DNA. The assignment for proteins other than II (hexon), III (penton base), and IV (penton fiber) is based on studies of step-wise dissociation and on difference cryoelectron microscopy. (Redrawn from ref. 39, with permission.)

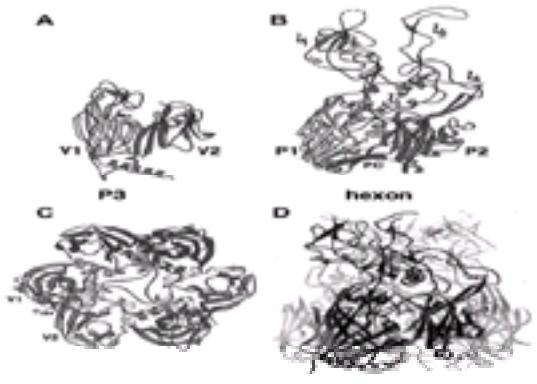

FIG. 16. Comparison of the adenovirus hexon structure and the P3 outer-shell protein from bacteriophage PRD1. **A** and **B:** The polypeptide chains of P3 and adenovirus hexon. The direction of view is perpendicular to the threefold axis. The two b-barrel domains are shown in *gray*; the loops between strands in each domain and between domains are shown in a *lighter shade.* Note that these domains are oriented so that their b-strands would run radially in the virus particle, as in the polyomaviruses, rather than tangentially, as in the picornaviruses. Except for the much more restricted loops of the "tower," P3 closely resembles hexon. **C:** Top view of the PRD1 P3 trimer, showing its pseudo-hexagonal outline. The adenovirus hexon would have, near its base, a very similar outline; this is the part of the trimer that interacts with others in the lattice of the virion shell (compare Fig. 15A). **D:** Side view of the adenovirus hexon trimer. The black subunit, in the foreground, is in the same orientation as the single subunit in **A.** The loops of the individual chains fold extensively with each other. These loops would probably not be ordered in a monomeric subunit, and it is likely that subunit folding and trimer assembly occur coordinately. (**A** to **C** redrawn from ref. 10, with permission; **D** courtesy of R. Burnett.)

Multishelled Architecture of Double-Stranded RNA Viruses

Double-stranded RNA viruses also exhibit both nonequivalent and quasi-equivalent interactions, but in separate protein shells. The core of bluetongue virus, for example, has an inner shell, made of 120 copies of a protein known as VP3, and an outer shell, made of 780 copies of VP7 (57). There are two totally distinct environments for VP3, because 2 is not a permitted triangulation number (Fig. 17). The amino acid side chains on the lateral surfaces of VP3 have different partners, depending on the interface in which they lie. The distortion of the subunit itself, when the two environments are compared, is quite small. By contrast, the VP7 shell has a classic, T=13, quasi-equivalent design. The contacts between VP7 trimers resemble each other closely. Intershell contacts are obviously highly variable because there is no match between the triangulation of the two shells. It is likely that addition of VP7 nucleates at threefold positions, where there is a match in symmetry of the two layers; these strong intershell contacts can then initiate lateral propagation of the VP7 lattice by addition of further trimers.

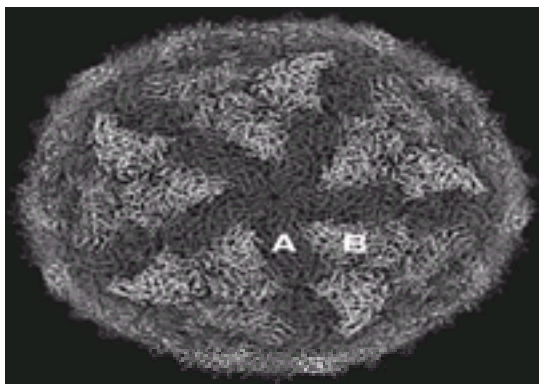

FIG. 17. A structure with 120 identical subunits: the principal shell of the inner capsid particle (core) from reovirus (83). The 60 dark copies of the protein l1 (one of which is labeled *A*) and the 60 lighter copies (*B*) have different interactions within the shell. For example, the "tips" of five A subunits contact each other around a fivefold axis, whereas the corresponding surfaces of B subunits touch different parts of A subunits. Thus, the same molecular surface makes two quite different, yet specific, interactions. A similar arrangement of the protein VP3 is found in the core of bluetongue virus (57).

Rearrangements in Icosahedral Lattices

Icosahedral surface lattices can undergo rearrangements, which preserve the overall symmetry of the structure but change the pattern of specific intersubunit contacts. There is often an accompanying change in the diameter of the shell. The T=3 plant viruses expand, in most cases reversibly, when the calcium cations that stabilize a particular set of subunit interfaces are removed (87) (Fig. 18). This swelling is believed to be the first step in disassembly. Plant viruses are injected by their vectors directly into the cytoplasm of the recipient cell, where they are exposed to a low Ca^{2+} environment. A similar, but transient, expansion occurs when poliovirus binds its receptor (9). The double-stranded DNA (dsDNA) bacteriophages insert their genomic DNA into a preformed "prohead," and the outer shell of the prohead generally expands as its subunits shift around to form the mature structure (22). Herpesviruses likewise package DNA into a procapsid, which undergoes structural changes in the process, but without a change in diameter (100).

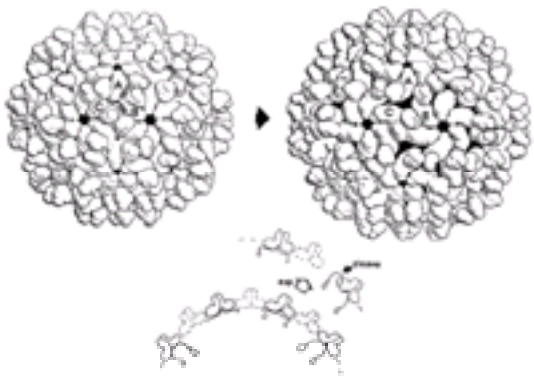

FIG. 18. Expansion of TBSV. The mature, compact particle (*upper left*) expands when Ca^{2+} ions (*small circles*) are removed. The expanded form (*upper right*) is reached by a smooth transition, in which many of the intersubunit contacts are conserved. The contacts that included the ions in the compact state have separated substantially, creating a fenestrated shell. As diagrammed in the lower part of the figure, the arms of A and B subunits, which do not participate in the inner scaffold (see Fig. 8), can loop out through the fenestrations, becoming susceptible to cleavage by proteases.

Symmetry Mismatches

P>The papovavirus structures have already provided us with one example of mismatched symmetry—what we called a "fivefold peg in a sixfold hole." A somewhat different example is the way these same pentamers bind the internal proteins, VP2 and VP3 (4,25). These proteins are the same, except for an about 100-residue N-terminal extension unique to VP2. Each VP1 pentamer binds one internal protein, through interaction with residues near the common C-terminus (see Fig. 14**E**). In the virus particle, all five symmetry-related registers of the VP1-VP2/3 interaction occur, so that the internal protein projecting inward from a given VP1 pentamer adopts one of five equally likely orientations. A similar rotational randomness is likely to characterize the adenovirus fiber, which is a trimeric molecule anchored in a pentameric base (see Fig. 15). Symmetry mismatch appears to be used in rotary motors, such as those that package DNA or RNA in phage heads, to subdivide a full rotation into discrete steps.

Helical Tubes

Isometric virus structures occur much more frequently than tubular ones, perhaps because of the inherently greater efficiency of nucleic acid packaging within a quasi-spherical cavity. There are, however, a number of well-known and widely studied helical and filamentous viruses, and the nucleocapsids (protein-coated nucleic acid) of many enveloped, negative-strand RNA viruses are helical arrays, which coil within the virion and uncoil when released.

Helical structures are organized around a single axis (the *helix axis*). They can be described by the number of units per turn, *u* (not necessarily integral), and the axial rise per unit, *p.* (Axial rise is the increment in "height" along the helix axis between one unit and the next.) The pitch of the helix, *P,* defined as the distance along the helix axis corresponding to exactly one turn, is equal to the product of *u* and *p.* Helical structures can have a rotation axis coincident with the helix axis (e.g., the T4 phage tail, with a sixfold axis) or an array of twofold axes perpendicular to the helix axis (as in DNA). The diagram of TMV in Figure 19 illustrates that a structure with helical symmetry (in this case, having u = 16 $^1/_3$, p = 1.4 Å, and P = 23 Å) can also be described by reference to a surface lattice—the network connecting equivalent units in the curved surface. The lattice lines correspond to different helical paths, as shown in the figure and described in more detail in the caption.

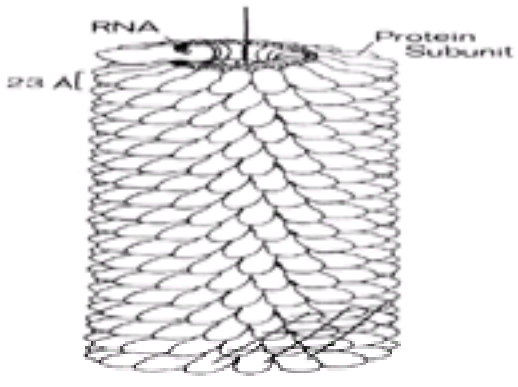

FIG. 19. The helical structure of tobacco mosaic virus (TMV). There are 161/3 subunits per turn of the helix, which has a 23 Å pitch. Three RNA nucleotides fit into a groove on each subunit. There is an about 30 Å diameter hollow along the axis. The surface lattice (four "unit cells") is indicated in the lower part of the figure.

In TMV, and probably in the nucleocapsids of negative-stranded RNA viruses like influenza and vesicular stomatitis virus (VSV), the RNA winds in a helical path that follows the protein. That is, the tubular package does not simply contain the RNA, it co-incorporates it. There are exactly three nucleotides per subunit in TMV, and they fit into a defined groove between the helically arrayed proteins. By contrast, the protein coat of a filamentous, ssDNA phage, such as M13, forms a sleeve that surrounds and constrains the closed, circular genome, without there being a specific way in which each subunit "sees" one or more nucleotides (51). Thus, there can be a non-integral ratio of nucleotides to protein monomers.

Genome Packaging

Incorporation of viral nucleic acid must be specific but independent of most of its base sequence. Therefore, viral genomes often contain a *packaging signal*—a short sequence or set of sequences that directs encapsidation. Recognition of the packaging signal depends on the nature of the genome and on the complexity of the assembly mechanism. The coats of simple viruses usually assemble around the genomes, and in these cases, there is likely to be a direct interaction between genome and capsid protein. More complex viruses sometimes insert nucleic acid into a preformed shell, and genome recognition is a property of the packaging system. If replication and packaging are closely coupled in some way, there may be no requirement for a specific packaging signal at all, as may be true of the picornaviruses.

In positive-stranded RNA viruses, no definite overall secondary or tertiary fold is needed for the genomic RNA, aside from the restriction that it fit within the shell. This restriction is actually quite severe, and the RNA is generally packed very tightly, approximating the density of RNA in crystals (e.g., of tRNA). Even random-sequence ssRNA contains about 60% to 70% of its nucleotides in base-paired stems (55), and to fit efficiently within the interior of a capsid, these RNA stems must pack tangentially, many in contact with the inward-facing surface of the shell. Such packing can be achieved by assembly around the RNA, without defined capsid–RNA interactions. In some viruses, segments of partially ordered RNA appear, packing into shallow grooves on the inner capsid surface (27,42). The ordered structures of these segments may result simply from the shape of the groove and the possible structures that a tightly packed polynucleotide chain can adopt. There do not appear to be any base-specific contacts in these ordered segments.

Recognition of RNA packaging signals has been analyzed in detail in a few cases. In RNA phage such as MS2 and R17, the packaging signal folds into a stem-loop structure (Fig. 20**A**), recognized by a dimer of the coat protein (the assembly unit for this T=3 particle) (20,103,104). Bases of the loop in the packaging signal and a looped-out base in the stem fit into a groove on the inward-facing surface of the subunit dimer, and conserved bases make defined protein contacts. The MS2 subunit has a structure unrelated to those of nonenveloped positive-stranded RNA viruses of eukaryotes, many of which recognize their genomic RNA, not through a groove-like site on the protein, but rather through a flexibly extended, positively charged protein arm, often at the N-terminus. In the case of alfalfa mosaic virus, the arm may fit against a recognition structure formed by the folded RNA packaging signal (2). In a sense, the RNA “recognizes” the packaging signal, rather than the other way around. A similar recognition principle determines the specificity of interactions between the Tat protein of HIV-1 and its cognate site, TAR, on the HIV-1 RNA (80), as well as of those between HIV-1 Rev and its cognate site, the RRE (6). It may also apply to the way the positively charged, N-terminal arm of alphavirus capsid proteins direct specific RNA packaging.

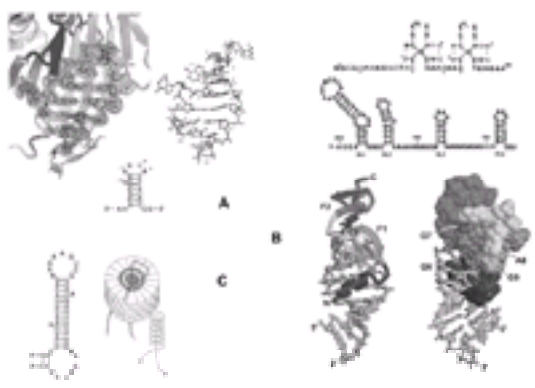

FIG. 20. RNA virus packaging signals. **A:** Recognition of the bacteriophage MS2 packaging sequence (a stem-loop structure, with a sequence shown in the lower part of the figure) by a dimer of the coat protein. The stem-loop fits into a hollow on the inward-facing surface of the protein (*upper left*); three bases in the loop and a looped-out base in the stem are critical for specific recognition; the stem structure presents them to the protein in correct spatial orientation (*upper right*). (Redrawn from ref. 104, with permission.) **B:** Recognition of the human immunodeficiency virus type 1 packaging sequence by the dual “zinc knuckle” element in the NC protein. The amino acid sequence of this 55-residue element is shown at the *top,* using single-letter notation. The nucleotide sequence of the packaging signal (“psi sequence”) in the genomic RNA is shown in the *middle.* Note that it contains four distinct stem-loops. The two zinc knuckles (F1 and F2) fold around the loop at the tip of stem-loop 3 (SL3), and a segment N-terminal to the first Zn knuckle forms an a-helix in the widened major groove of the RNA. The NC peptide itself is ordered only within each Zn-liganding fold (about 16 residues each), and the two knuckles are flexibly linked. The structure of SL3 thus imposes additional structure on the peptide, including the N-terminal helix. (Redrawn from ref. 35, with permission). **C:** Assembly of TMV from an assembly origin that lies just upstream of the coat-protein coding sequence at the 3' end of the genome (17,117). The sequence and likely secondary structure of the assembly origin are shown on the left. The location of this origin, roughly 500 nucleotides from the 3' end of the RNA, implies that the 5' end of the RNA runs back along the axis of the assembling particle, as shown on the right.

Retroviral packaging signals, known as *psi* sequences, are recognized by the nucleocapsid (NC) domain of the Gag protein. The HIV-1 psi element has a stem-loop structure that associates with two “zinc-knuckle” modules in HIV-1 NC (35) (see Fig. 20**B**). The two zinc modules are flexibly linked in unbound NC, but they adopt a defined, three-dimensional organization in complex with the RNA. Thus, the structure of the RNA imparts additional order to the protein element with which it binds.

Helical structures must unwind any base-paired stems of an RNA genome in order to package it. For example, TMV assembly begins at an internal origin sequence, about 1 kb from the 3' end of the genome (102,117). A 75-base

sequence containing a presumptive stem-loop structure is sufficient to initiate specific encapsidation (see Fig. 20**C**), which proceeds by a mechanism that requires the 5' end of the RNA to be drawn through a channel along the axis of the assembling particle (17). This process may be described as an assembly-driven helicase. In negative-stranded RNA viruses such as influenza, the sequences that determine encapsidation by the nucleocapsid protein are probably in the conserved termini of the genome segments (see Chapter 46).

The mechanism of overall condensation of a viral genome may be distinct from the specific recognition just discussed. If there is no regular, repeated interaction between coat protein and nucleic acid, there are various strategies for neutralizing the net negative charge on the nucleic acid. Those icosahedral viruses with inwardly projecting, positively charged arms use most of these arms for nonspecific interactions with RNA and only one or a few for specific recognition. In the picornaviruses, polyamines are incorporated to achieve charge neutralization. Double-stranded DNA viruses use either cellular histone to condense the viral chromosome (polyomaviruses) or basic, virally encoded proteins (adenoviruses). The dsDNA bacteriophages and the herpesviruses “pump” DNA into a capsid precursor, using adenosine triphosphate hydrolysis to drive condensation. The most efficient packing of a double-stranded genome within a hollow shell can be achieved by uniform coiling of the dsDNA or dsRNA, such that in cross-section, adjacent segments are hexagonally arrayed (37,54). DNA is packed this way in herpesviruses and in phage heads (Fig. 21**A**), as is dsRNA in reoviruses and their relatives (83). Reoviruses have segmented genomes, and each segment may form an independent coil (see Fig. 21**B**).

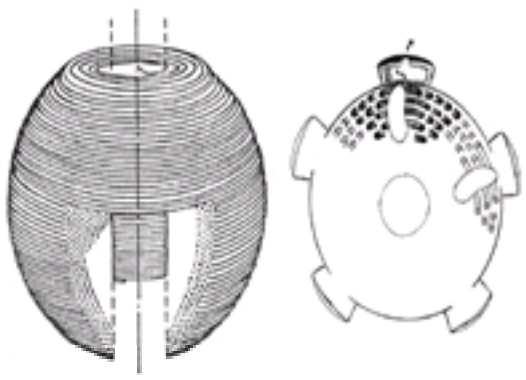

FIG. 21. Packing of double-stranded nucleic acid in virions. **A:** Uniform coiling of DNA within the head of bacteriophage lambda and its relatives (37). The DNA is introduced into a prohead by the action of an adenosine triphosphate–driven rotary motor. In the filled, mature phage head, adjacent, parallel segments of DNA form a local hexagonal packing (*cutaway view toward the bottom of the drawing.*) **B:** Model for coiling of double-stranded RNA (dsRNA) genomic segments within the core of reovirus and other dsRNA viruses (54,83). The negative strand for each segment is generated by the polymerase (*oval outline*), after core assembly in which are packaged one each of the positive-stranded segments. Each genomic segment must be transcribed multiple times, probably by the polymerase around which it is coiled. The transcript exits through a pore along the fivefold axis; in the case of orthoreoviruses, it passes through the chamber of a capping-enzyme complex (*hollow turrets*).

Viral Membranes

Budding

Most enveloped viruses (except for the large and very complex poxviruses) acquire their membrane, a lipid bilayer with associated proteins, by budding through an appropriate cellular membrane—the plasma membrane in many cases, the ER, Golgi apparatus, or nuclear membrane in others (Fig. 22). Using the cell's compartmentalization mechanisms, these viruses direct the insertion of their surface glycoproteins into the relevant membrane of the cell. Budding is driven either by interactions between cytoplasmic tails of the glycoproteins and assembling or preassembled internal structures, by lateral interactions between glycoprotein subunits, or by a combination of both. In some cases, assembly of an internal structure on the cytoplasmic face of the membrane is sufficient to drive budding, even if glycoproteins are lacking. Pinching off from the cell surface, or into the lumen of the ER or Golgi apparatus, appears not to need a cellular activity; assembly of viral components provides the force needed to distort the membrane bilayer. The lipids in the resulting bilayer derive from the cell, whereas the proteins are virally encoded. To a first approximation, the incorporated lipids represent a sample of those in the membrane through which the virus budded, but the significance of potential detailed specificities in lipid incorporation are still poorly understood. Viruses that emerge through the plasma membrane contain phospholipid and cholesterol in characteristic proportions, whereas those that emerge into the lumen of the ER contain almost no cholesterol. Cholesterol tends to increase the thickness of a bilayer, by restricting free rotation around single bonds in the fatty acid chains of adjacent phospholipids (81). The lengths of a-helical transmembrane segments in viral glycoproteins vary accordingly: from about 26 residues in influenza (which buds at the cell surface) to 18 to 20 residues in yellow fever (which buds into the ER).

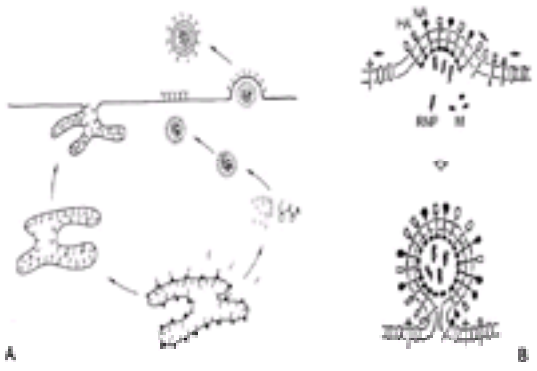

FIG. 22. Budding of enveloped viruses. **A:** Diagram of alphavirus biosynthesis and assembly. The viral structural proteins are synthesized as a polyprotein from a single message. The core protein, a serine protease that acts only on its own polypeptide chain, cleaves itself from the precursor at an early stage. The ribosomes and nascent viral polyprotein then associate with endoplasmic reticulum membranes to complete synthesis of the glycoproteins, which are exported to the cell surface by the standard constitutive route through the Golgi compartments. Core protein and RNA assemble to form nucleocapsids, which associate with glycoprotein patches and initiate budding. **B:** Influenza virus budding. Nucleocapsid protein (N), matrix protein (M), and glycoproteins (HA, NA) are synthesized from independent messages. Glycoproteins arrive at the cell surface by the usual route. Budding is a coassembly at the cell surface of the glycoproteins with M and with RNP segments. Host cell proteins are excluded (*arrows*).

Figure 22 illustrates features of the budding process in two examples—an alphavirus (Sindbis) and an orthomyxovirus (influenza). The structure of the nucleocapsid varies with virus type. It is a compact, spherical particle in the alphavirus; a filamentous, helical nucleocapsid in paramyxoviruses and rhabdoviruses; and a multisegmented helical nucleocapsid in the orthomyxoviruses. The viral glycoproteins are anchored in the cellular membrane by a transmembrane hydrophobic segment (in some cases, a hydrophobic hairpin), and there is a small cytoplasmic domain. In alphaviruses (see Fig. 22**A**), a core particle (nucleocapsid) assembles independently in the cytoplasm. Interactions between the core and the cytoplasmic tail of the glycoproteins then determine the location of budding. Lateral interactions of the glycoproteins also appear to assist the budding process. In orthomyxoviruses such as influenza (see Fig. 22**B**), an M (matrix) protein associates with the nucleocapsid segments and with the underside of the membrane, presumably by interaction with the cytoplasmic domains of the glycoproteins. M organizes both the glycoproteins and the nucleocapsids. Budding then proceeds by coassembly of structures on both surfaces of the membrane. The two patterns of budding shown in Figure 22 are not fundamentally different; rather, they depend on the relative strength of core–core, envelope–envelope, and core–envelope contacts. In at least one case, a mutation has been shown to convert budding from one mode to the other (85). Absolute specificity is sometimes violated in viral budding, leading to cases of phenotypic mixing, in which, for example, SV5 glycoproteins can be found in the membrane of VSV. In retroviruses (Fig. 23), cleavage of core proteins after budding leads to major internal rearrangements.

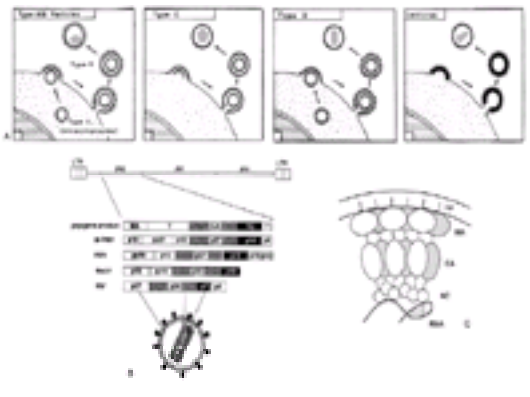

FIG. 23. Assembly and budding of retroviruses. **A:** Diagrammatic representation of the organization of retroviruses of different apparent morphologies in thin section. (Redrawn from ref. 12, with permission.) In at least one case, mutations in *gag* can change the budding pattern from that of type D to that of type C, that is, from preassembly of a core in the cytoplasm to coassembly of core and envelope at the membrane. **B:** Organization of the *gag* region of a retroviral genome and its relationship to the organization of an “immature” (unprocessed Gag precursor) and “mature” (proteolytically processed Gag) virion. *Top:* overall arrangement of genomic information. *Middle:* The subunit products of cleavage of the Gag precursor and their specific names in the case of Mason-Pfizer monkey virus (M-PMV), a type D particle, Rous sarcoma virus (RSV), a type A/B particle, murine leukemia virus (MuLV), a type C particle, and human immunodeficiency virus (HIV), a lentivirus. *Bottom:* Representation of an HIV particle, showing locations of MA, CA, and NC proteins (Redrawn from ref. 64, with permission). **C:** Diagram of the organization of the HIV Gag precursor. The MA domain is myristoylated at its N-terminus, facilitating its association with the membrane. It forms a trimer. The CA domain dimerizes, through its carboxyl-terminal subdomain. This combination of trimer and dimer formation may help drive extended lateral association and budding.

The simplest enveloped virus particles—those of the alphaviruses and the flaviviruses—are icosahedrally symmetric. In these positive-stranded RNA viruses, one-to-one interactions between the envelope glycoprotein and the nucleocapsid subunit appear to ensure coherence between external and internal structures (28,106) (Fig. 24; see Fig. 22**A**). The larger, negative-stranded RNA bunyaviruses also have an icosahedrally symmetric envelope (a T=12 lattice), but their internal structures are probably not icosahedrally organized (108), and the outer lattice is the major determinant of symmetry and stability. The rhabdoviruses have a helically organized shaft with a (probably hemi-icosahedral) cap at one

end. The orthomyxoviruses, like influenza A, bud out as variable elongated structures with no overall symmetry, although there is probably considerable local order. Retrovirus particles also do not appear to have a global symmetry. The fluid character of a lipid bilayer means that the virus can form a closed structure without a perfect surface lattice. Defects in a protein layer that would produce unacceptable holes in a nonenveloped virus are tolerable if the barrier protecting the genome is a lipid membrane rather than a protein shell.

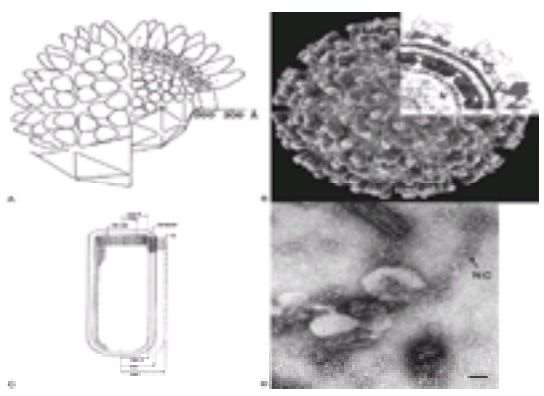

FIG. 24. Organization of two types of enveloped viruses. **A:** Diagram of an alphavirus particle (Sindbis). The pear-shaped objects represent the ectodomains of E1 and E2, closely associated as heterodimers and further clustered into trimers in the T=4 icosahedral lattice that describes the surface organization (107). There are 240 E1:E2 heterdimers in the complete particle. Hydrophobic transmembrane segments of E1 and E2 penetrate the lipid bilayer. A 33-residue internal domain on E1 mediates interactions with the core. The core also has a T=4 icosahedral structure (28). **B:** Reconstruction of the closely related Semliki Forest virus particle from cryoelectron microscopy at 10 Å resolution. Most of the figure shows a surface rendering; the *upper right-hand quadrant* shows a thin cross-section, with a scale to show radius (in angstoms), for comparison with the diagram in **A**. (Redrawn from ref. 69, with permission.) **C:** Diagram of the vesicular stomatitis virus (VSV) particle, showing the relationship of nucleocapsid (N), matrix protein (M), and glycoprotein (G) to the lipid bilayer. The scale of this diagram is about half that of the diagram in **A**, but the VSV genome is only slightly larger than that of Sindbis. The Sindbis RNA, which collapses into stem-loop structures in the interior of the core, is therefore markedly more condensed than the VSV RNA, which winds as an extended strand along the helical nucleocapsid. **D:** Electron micrograph of disrupted VSV, showing partially disassembled "cores." The hollow tubes are helically coiled nucleocapsid (*NC*), fixed into a rigid structure by interaction with M, which has probably dissociated from the more loosely coiled NC (e.g., *upper right*). Paramyxovirus nucleocapsids have a rather similar appearance, after release from disrupted particles.

Internal Structures

As Figure 22, Figure 23 and Figure 24 suggest, the proteins on the internal side of viral envelopes are significantly more varied in design than those in the outer shells of nonenveloped viruses, where most of the known outershell protein structures contain jelly-roll b-barrel domains. The alphaviruses have subverted a serine protease to serve as the principal domain of the capsid subunit (C) (29). The protease is functional in the single step required to cleave C from the nascent polyprotein of these positive-stranded RNA viruses. The core, sealed within the bilayer, can afford to be fenestrated. The hexamer and pentamer clusters of the protease domains do not contact each other, and coherence of the T=4 icosahedral lattice is maintained by interacting N-terminal arms. Thus, these positively charged arms, like those of TBSV (see Fig. 8), appear to knit the core together as well as to recognize and neutralize RNA. The hepatitis B capsid is also an open, almost lattice-like, structure, formed by a largely a-helical subunit that can assemble into either T=3 or T=4 shells (14). The retroviral Gag precursor is usually anchored by an N-terminal myristoyl group to the membrane bilayer, and successive domains are separated by cleavage into radially organized layers (see Fig. 23 and Chapter 57). The structures of the various domains from HIV-1 Gag have been determined (44), as have those of certain domains from a few other retroviruses.

Most viral envelope proteins are so-called type I membrane proteins, with a single transmembrane a-helix linking the ectodomain and the cytoplasmic tail. The transmembrane helices have been resolved in a recent cryoelectron microscopy study of Semliki Forest virus (69). In some positive-stranded RNA viruses, like the flaviviruses, where structural proteins are derived from a polyprotein precursor, the anchor may be an a-helical hairpin that traverses the membrane twice (24). Contacts between the cytoplasmic tails of viral envelope glycoproteins and target sites on the underlying core of matrix subunits generally determine specificity of envelope protein incorporation. These interactions usually involve a short segment of envelope polypeptide, fitting against a site on the internal protein (13,66,75). Individual interactions are weak, and bilayer disruption by nonionic detergents readily dissociates them. But the cooperativity imparted by lateral contacts, both within the core or matrix and within the outersurface layer, is strong, and the ensemble of cooperative, weak interactions drives budding.

Surface Proteins

The proteins on the surface of an enveloped virus must carry out at least two functions: receptor (and co-receptor) binding and fusion. In addition, there may be a receptor-destroying enzyme (e.g., the influenza-virus neuraminidase or the coronavirus esterase), to promote viral release. The membrane of influenza A contains a fourth activity: a proton channel that assists uncoating and transcriptase activation (M2). In some cases (e.g., rhabdoviruses and retroviruses), the receptor-binding and fusion activities are combined in a single protein; in others (e.g., paramyxoviruses), there are two distinct proteins to carry out these functions. A trimeric, coiled-coil fusion-protein structure, found in a wide range of enveloped viruses, is described later under Membrane Fusion.

Viral Entry into Cells

Receptor Binding

There are no simple generalizations about virus receptors and how they bind with viral surfaces (see Fig. 24). We note two broad issues here. The first is that most viruses have evolved a mechanism to avoid "getting stuck" at the cell surface when emerging from an infected cell. Many viruses simply bind weakly to their receptors and thus can dissociate in a reasonable time. The virulence of polyomavirus in mice is inversely related to viral affinity for its sialoglycoconjugate receptor (Fig. 25**A**), demonstrating that spread in the animal host, rather than entry into cells, is the principal correlate of pathogenesis (7). Like polyoma, influenza virus recognizes a sialic acid–containing carbohydrate for cell attachment (77) (see Fig. 25**B**). A receptor-destroying enzyme (neuraminidase) is present on the surface of the virion; its activity allows release of newly assembled virions from the cell surface through which they have budded (76). The neuraminidase is thus required for effective spread of the virus, and the enzyme is the target of antiinfluenza drugs, developed in part by exploiting knowledge of the neuraminidase structure (109). HIV-1 has several mechanisms for down-regulating its receptor (CD4) after infection, both to avoid envelope–receptor interactions within the secretory pathway and to facilitate viral release after budding (see Chapter 59). The second broad issue is that some viruses require a cascade of at least two distinct receptors, in order to enter efficiently. One receptor is required for initial cell attachment and a second for triggering fusion or penetration. The former may be a quite broadly distributed molecule, like sialic acid or other glycans (heparan sulfate for HSV-1), or it may be a quite specific protein, like the adenovirus receptor, CAR, or the HIV-1 receptor, CD4 (see Fig. 25**C**). The latter is often called a *co-receptor,* for example, the chemokine receptors for HIV-1. In the case of HIV-1, CD4 primes the envelope glycoprotein to bind the co-receptor, which in turn induces fusion activation. An obligate order of this sort may turn out to be relatively common.

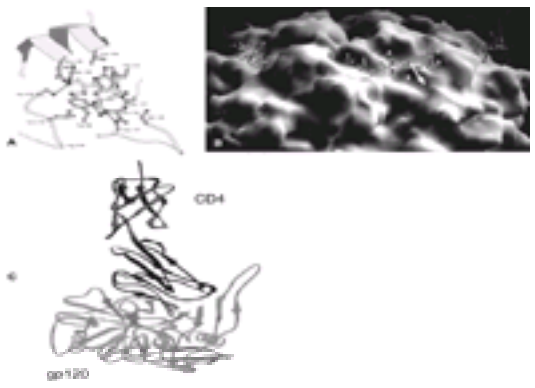

FIG. 25. Virus–receptor interactions. **A:** Influenza virus recognizes the terminal sialic acid on cell surface glycoconjugates, both glycoproteins and glycolipids. The sialic acid–binding site, shown here, lies on the outward-facing tip of the haemagglutinin protein, indicated by a *star* in Figure 26**B** (110). Polypeptide chain segments adjacent to the bound sugar are shown as "*worms*" and as a *helix.* Side chains of residues that contact the sialic acid are shown explicitly. *Dashed lines* indicate hydrogen bonds. **B:** Polyomavirus binds glycans with structures such as sialic acid–a-2,3-Gal-b-1,4-GlcNac, as shown in this view of the outer surface of a VP1 pentamer (compare Fig. 14) (97). The sialic acid fits into a specificity pocket, and the shallow groove that receives the rest of the carbohydrate moiety is complementary to the shape imparted by its particular glycosidic linkages. Pocket 1 receives the terminal sialic acid; pocket 2, the galactose and *N*-acetyl glucosamine; pocket 3 can receive an additional sialic acid, in a-2,6 linkage to the glucosamine. The pockets for sialic acid are structurally unrelated to the influenza sialic acid site. The affinity for an individual receptor group is very low (about 1 mmolar). The virus can probably bind glycan that is attached to any cell surface glycoprotein; the position and direction of the protein linkage is shown by the arrow. **C:** The complex of the core of the human immunodeficiency virus type 1 receptor–binding domain, gp120, with the receptor, CD4 (65). A key phenylalanine on CD4 fits into a hydrophobic pocket on gp120 (*center*).

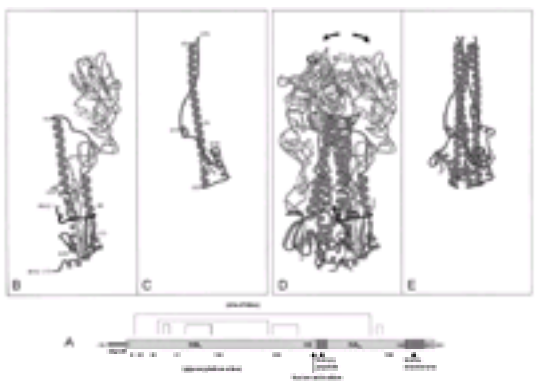

FIG. 26. Influenza-virus haemagglutinin (*HA*). **A:** Schematic representation of the primary structure of the 1968 Hong Kong virus HA, showing the ectodomain, HA_1 + HA_2(1–185), the transmembrane anchor (HA_2 185–211), and the cytoplasmic domain (HA_2 212–221). The cleavage site between HA_1 and HA_2 is labeled *fusion activation.* The secretion signal sequence (removed by signalase), S-S bridges, carbohydrate (CHO) attachment sites, and fusion peptide (N-terminus of HA_2) are shown. The uncleaved precursor is known as HA_0. **B** to **E:** The folded structure of HA and its rearrangement when exposed to low pH. **B:** The HA monomer: HA_1 is *light;* HA_2 is *dark;* the fusion peptide is *black.* Residue numbers in HA_2 are shown at several key positions to assist in visualizing the conformational change that occurs on fusion activation. The receptor-binding pocket in the b-barrel "top" domain of HA_1 is indicated with a *star* (compare Fig. 25**A**). The viral membrane would be at the bottom of this part of the figure. The transmembrane and cytoplasmic segments are not shown. **C:** The HA_2 monomer in the fusion-active form. A short segment of HA_1 (*white*) remains bound

to the rearranged HA_2 in this structure. Note the dramatic conformational change, in which residues 40 to 105 become a continuous a-helix. The introduction of a kink at residue 105 also causes the "bottom" part of the molecule to turn upward, so that the N- and C-termini of the fragment are next to each other. Thus, the fusion peptide, extending from the N-terminus, and the transmembrane segment, extending from the C-terminus, would be spatially adjacent after the full conformational change. **D:** The HA trimer. The subunit toward the right in this illustration is viewed in essentially the same orientation as the monomer in **B.** HA_2 is shaded. Exposure to low pH initiates a conformational change, causing the "top" domains of HA_1 to move apart (*arrows at top of diagram*). **E:** The rearranged HA_2 trimer. Imagine the tops of HA_1 to have spread to either side, and the three HA_2 monomers to have undergone rearrangement to the conformation shown in **C**. (Courtesy of F. Hughson, modified.)

An Irreversible Step Between Assembly and Entry

Assembly of TMV protein and RNA into infectious particles was among the key observations that triggered thinking about viral symmetry. *In vitro* self-assembly of components from the mature virion into complete infectious particles is, however, an exceptional characteristic of the simplest plant and bacterial RNA viruses. A far more general property of virus assembly pathways is a modification, often a simple proteolytic cleavage, that "primes" the particle for large-scale, irreversible events accompanying entry. Loss of a scaffolding protein is a particularly extreme example of such a modification. Picornaviruses such as polio assemble from VP0, VP1, and VP3, but autolytic cleavage of VP0 into VP4 (an internal peptide) and VP2 accompanies assembly (see Chapter 23). When receptor binding triggers expansion of the viral shell, exit of VP4 renders the rearrangement irreversible (9). The receptor acts as a catalyst to lower the energy barrier to an irreversible reorganization. The function of this reorganization is viral entry, and the triggering mechanism has evolved to occur only in an appropriate location. Reoviruses have an outer protein, s3, that caps the penetration protein, µ1 (36). Proteolytic removal of s3 is required to render the particle competent to attach and penetrate (see Chapter 52). There is, in addition, an essential autolytic cleavage of µ1 (73). The hemagglutinin (HA) of influenza virus folds in the ER into a stable, trimeric structure. Cleavage of one peptide bond in HA by the protease, furin, in a compartment late in the secretory pathway, primes the protein to undergo a dramatic, low-pH triggered rearrangement (Fig. 26), which mediates fusion of viral and target cell membranes. In effect, cleavage renders the virion form of HA metastable, but the barrier to rearrangement is so great at neutral pH that no conformational change occurs. Proton binding in the low pH environment of the endosome removes this barrier and triggers a refolding of the HA protein. Protons have the role taken in other cases by a second receptor (e.g., the chemokine receptors for HIV-1). The expression "spring loaded" has been used to describe the state of HA at neutral pH after cleavage to HA1 and HA2 (21). "Jack-in-the-box" might be a comparable image for poliovirus after cleavage of VP0.

Membrane Fusion

The rearrangement of influenza virus HA, shown in Figure 26, has two essential features that also characterize a number of other viral fusion proteins. The first is ejection of a previously protected hydrophobic *fusion peptide,* which is at the N-terminus of HA2 in the case of influenza. The second is a folding back of the fusion protein (HA_2) so that the N-terminus (the fusion peptide) and the C-terminus (the viral membrane anchor) are brought together (92,111). Assuming that the fusion peptide inserts into the target membrane, as generally believed, a possible intermediate state, for which there is indirect evidence in the case of HIV-1 gp41, would be an extended structure with the fusion peptide buried in one membrane and the anchor in the other (Fig. 27). Folding back of this *prefusion intermediate* would bring the two membranes into apposition. As sketched in Figure 27, a ring of rearranged fusion proteins might be required to surround and induce a *fusion pore.* What further molecular events, if any, are needed to cause the two bilayers to fuse remains an important open question.

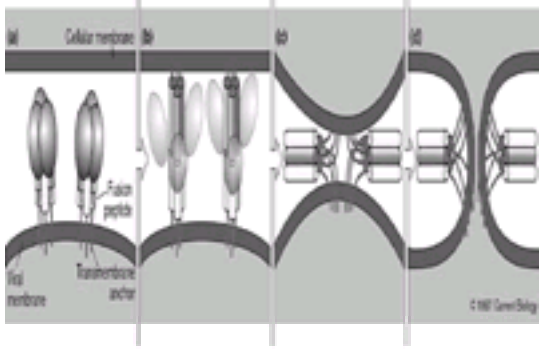

FIG. 27. Fusion. Schematic diagram of membrane fusion, as catalyzed by viral fusion proteins such as influenza virus hemagglutinin (HA). **A:** Neutral pH conformation of HA trimers in the viral membrane (compare Fig. 25D). The fusion peptides are buried. **B:** Initial conformation change triggered by low pH. The receptor-binding domains of HA_1 (ellipsoids) have separated from each other and from the HA_2 stems (although in influenza, they remain attached by a disulfide bond; in retroviruses, the corresponding SU fragment dissociates). The fusion peptides have been thrust by the conformational change into the membrane of the cell to be infected. **C:** The HA_2 stem folds back to form a chain-reversed structure, as seen in Fig. 26**C** and Fig. 26**E**. The fusion peptide and the transmembrane anchor of HA are brought together by the folding back. Because the former lies in the cell membrane and the latter in the viral membrane, the two membranes are forced to approach each other. As suggested by the diagram, more than one HA trimer is needed to form an effective fusion structure. **D:** Putative fusion pore, formed by local rearrangement of the two apposed bilayers. (Adapted from ref. 63, with permission).

The influenza HA is a trimer, organized by a central a-helical coiled-coil (114) (see Fig. 25**A**). HA_1 forms a globular domain at the “top” of the molecule, with a binding pocket for the receptor, sialic acid (110). Binding of protons at low pH leads initially to dissociation of the three HA_1 “heads” from their docked positions (but not to complete dissociation from HA_2, owing to disulfide links between HA_1 and HA_2) (52). This first step liberates HA_2 to undergo the rearrangement shown in Figure 26. The product of this conformational change (see Fig. 25**B**) is a quite simple structure: there is a three-chain, a-helical, coiled-coil inner core, surrounded by three outer-layer helices, derived from the part of each HA_2 chain that immediately precedes the transmembrane anchor (15). The fusion proteins of paramyxoviruses, retroviruses, and filoviruses have all been shown to form similar structures, after triggering by fusion-inducing signals (93).

Are rodlike structures the only way in which molecular fold-back can bring about close approach of two membranes? Apparently not, because the flaviviruses have quite a different structure for their fusion protein, as exemplified by the E protein from tick-borne encephalitis virus (84). In that case, the fusion peptide is an internal loop, and the protein is a platelike rather than a rodlike molecule. Moreover, the E protein itself does not undergo cleavage; rather, proteolytic processing of a second protein, PrM, is the step that primes the virion for a low-pH triggered conformational rearrangement (58). E and M (the truncated product, derived from PrM by removal of most of its ectodomain) form an icosahedral array on the surface of the virus particle, and the fusion-inducing step involves a concerted reorganization of this array (41). The alphaviruses and rhabdoviruses also have fusion proteins with internal loops as their putative fusion peptides (see Chapter 29 and Chapter 38).

Penetration of Nonenveloped Viruses

Nonenveloped viruses must breach a membrane to access the cytoplasm or nucleus of a cell, but unlike their enveloped cousins, they cannot do so by membrane fusion. The molecular mechanism by which a nonenveloped virus particle, bound at the surface of a cell or taken up into an endosome, translocates itself (or its genome) across the intervening lipid bilayer is not fully understood. In general, it appears that binding of a receptor or co-receptor induces a conformational change in the virion and that a consequent exposure of previously buried hydrophobic elements is important for penetration. In several cases, at least one of the exposed hydrophobic elements is an N-terminal myristoyl group: on VP4 in picornaviruses (30), on VP2 in polyomaviruses (90), and on µ1 in reoviruses (74). Myristoyl groups target proteins to membranes, and it is logical to suppose that exposure of the myristoylated protein leads it to associate with membranes and ultimately to contribute to penetration.

One class of models for nonenveloped virus penetration invokes formation of a pore, through which the viral genome is drawn into the cell; another class of models postulates a more extensive, transient disruption of the cell membrane (or of the endosome) in order to admit the virion (in altered form) into the cytoplasm. In the case of adenoviruses, uptake into an endosome and subsequent endosomal disruption have been reported (43,56). The subviral particle admitted to the cytoplasm lacks pentons, as a result of events triggered by receptor and co-receptor binding; this partially stripped virion subsequently disassembles in association with a nuclear pore. The dsRNA viruses must likewise insert an intact, roughly 700 Å diameter inner capsid particle (the *core* in the case of reoviruses; the *double-layered particle* in the case of rotaviruses) into the cytoplasm. Indeed, this particle never uncoats because it contains the enzymes necessary for mRNA synthesis and modification (see Chapter 52 and Chapter 54). In the case of picornaviruses, receptor binding triggers a rearrangement or destabilization of the virion, exposing the myristoylated VP4 as well as a hydrophobic N-terminal segment of VP1 (45,53,59,60,71). Formation of a pore in the endosomal membrane, through which the RNA passes, has been proposed. Such a model requires a mechanism by which secondary structural elements in the RNA are melted out to make translocation possible but for which candidate helicases have not been demonstrated. One possibility would be a ribosome, by analogy with an uncoating mechanism established (*in vitro*) for certain positive-stranded RNA plant viruses. With those viruses, exposure of the 5' end of the RNA (e.g., through expansion of the virion induced by intracellular ionic conditions) leads to association of ribosomes with the still largely packaged RNA genome, and progress of the ribosome along the message-sense genome appears to generate uncoating (114). A similar mechanism could, in principle, draw RNA through a membrane pore as well as through an opening in the viral shell. An alternative picture for picornavirus penetration involves membrane disruption (a “large” pore), either by the altered particle plus VP4 or by the dissociated components; RNA unwinding is not required in this model.

STRUCTURE-BASED CATEGORIES OF VIRAL DESIGNS

A particularly noteworthy consequence of the proliferation of high-resolution structural information about viruses is the frequent discovery of totally unexpected relationships. This sort of surprise came early, when the determination of the structure of southern bean mosaic virus (SBMV) revealed that it closely resembled TBSV (1). Equally striking was the relationship between the picornaviruses and the T=3 plant viruses (62,89). Even the development of sensitive sequence alignment methods has failed to predict structural homologies that are obvious once high-resolution three-dimensional images are available. Thus, the number of strategies that evolution has found, for carrying out the functions that are embodied in virions, is smaller than one might have imagined. In the sections that follow, we attempt to group certain viruses or viral substructures, not by replication strategy or host range, but rather by three-dimensional organization and its functional consequences. The available structural information is by no means sufficient to make a full catalog, but there is reason to believe that in the future, newly determined structures are quite likely to look like something we have already seen in another, perhaps superficially unrelated, virus particle.

Simple, Icosahedrally Symmetric Capsids with Jelly-Roll b-Barrel Subunits

Small, Nonenveloped, Positive-Stranded RNA Viruses

The comparison in Figure 7 shows that picornaviruses and T=3 plant viruses have closely related architectural features, based on 180 jelly-roll b-barrels. Variant packing of the same sort of domain yield T=1 and T=4 structures, such as those found in STNV and in nodaviruses, respectively. Common features in addition to the shell domains themselves are the

formation of an internal network of amino-terminal arms and (in the T=3 and T=4 structures) a specific switching mechanism to ensure accurate assembly. When this switching mechanism is disturbed (e.g., through cleavage of the amino-terminal arms), the same subunit that makes a T=3 structure can instead make a T=1 structure (94). Although the shells have a similar basic architecture, the loops between framework elements of the jelly-roll b-barrel vary enormously from one virus to another. These variations give rise to differences in receptor interactions, antigenic characteristics, and particle stability. The RNA bacteriophages have a T=3 icosahedral shell, but with a very different subunit architecture (104).

Parvoviruses

Parvoviruses have T=1 icoshedral shells (101). The subunit has a standard jelly-roll framework, but substantial insertions into the interstrand loops increase the volume of the capsid it forms. The jelly-roll cores pack neatly around the fivefold and twofold axes, roughly as in the T=1 variants of T=3 structures, but a very large (more than 200-residue) segment between strands G and H makes expanded contacts between threefold related monomers (see Fig. 4). The ssDNA phage fX174 has a shell in which subunits with a jelly-roll framework (protein F) interact very roughly, as in parvoviruses (70). Twelve pentamers of a distinct, jelly-roll subunit (protein G) decorate the surface: the protein G pentamers resemble the polyomavirus VP1 capsomeres in the packing of their subunits. Because there may be a restricted number of ways to organize jelly-roll structures into icosahedral shells, these similarities may not imply close evolutionary relationships.

Double-Stranded DNA Viruses

Papovaviruses

As described earlier (see Fig. 14), the assembly unit of the polyomaviruses and papillomaviruses is a pentamer of the major capsid protein (VP1 in polyomaviruses; L1 in papillomaviruses). The axes of the 72 pentamers that form the viral shell align with the vertices of a T=7 icosahedral lattice, but they do not conform to its symmetry because even the six-coordinated vertices have pentamers of the protein (82). The subunits have jelly-roll b-barrels as a framework, with relatively elaborate loops facing outward (26,67). Both the polyomaviruses and papillomaviruses have an internal protein (VP2/VP3 and L2, respectively). VP2 is probably required for penetration; L2 may have a similar function.

Adenoviruses

The architecture of adenoviruses is based on a shell of hexons, centered at the six-coordinated vertices of a T=25 icosahedral surface lattice. The hexons are trimers with an approximate internal repeat, making them pseudo-hexamers (see Fig. 16). The same fold has been discovered in the principal outershell subunit, P3, of bacteriophage PRD1 (10). The phage protein looks like a “stripped-down” version of the hexon: it lacks the elaborate projecting loops that form the principal antigenic structures in the adenovirus protein. This striking relationship between an animal-virus structural protein and a phage structural protein is evidently more than a simple convergence, but how viruses have made the presumed evolutionary jump is not clear. Phage PRD1 has a separate penton-base subunit and a penton fiber, just like adenoviruses, but it also has a lipid membrane just beneath the outer, adenovirus-like, pseudo T=25 lattice (10). Thus, the common shell can clearly be combined with different internal structures, depending on strategies for assembly, entry, and uncoating.

Herpesvirus Capsids

Herpesviruses are enveloped particles, but the capsid is an independently assembling entity that has many of the characteristics of a nonenveloped particle (100,116). The high-resolution structure of the principal herpesvirus capsid protein has yet to be determined. Moderate-resolution studies of capsid structure and assembly, using electron cryomicroscopy, show that the hexamers and pentamers of the major capsid protein are held together at local threefold positions by a heterotrimeric “triplex” complex, which may serve a function analogous to the cement proteins in adenoviruses. Assembly of the herpesvirus capsid by way of a scaffold-containing procapsid regulates morphogenesis at a different level (72).

Double-Stranded RNA Viruses

All dsRNA viruses of the *Reoviridae* family have a common design for the 120-subunit shell that serves as the major framework of the particle (57,83). The 120- to 140-kd protein that forms this shell—known as l1 in reoviruses, VP2 in rotaviruses, VP3 in orbiviruses, and so forth—has the shape of a flattened teardrop. Five copies radiate from a fivefold axis, like petals of a gently cupped flower, and five further copies insert between them. This decamer may also be the assembly unit. Certain other components (an RNA polymerase or helicase; capping enzymes) are associated with each fivefold, and it is logical to suppose that they are incorporated as part of a 12-decamer assembly mechanism (see Chapter 52). The outer layers of protein, which surround the common framework, differ significantly from one genus of this family to another. Of the nine genera, four have pentameric “turrets” that project outward from each fivefold axis of the framework shell. The structure of the reovirus core (83) shows that the turrets are hollow cylinders, with the active sites of the capping enzymes facing their inner cavity. The mRNA is capped as it is exported through these structures. Members of the other five genera contain a protein with the various capping activities inside the framework shell; it is likely that the capping protein is associated directly with the polymerase. The outermost layers of the dsRNA viruses are specialized for their specific routes of assembly and entry, and the proteins in this layer appear to be the least conserved from one genus to another.

There are three other families of dsRNA viruses, including ones that infect lower eukaryotes as well as the enveloped f6 phage of *Pseudomonas syringae*. Image reconstructions from electron cryomicroscopy of exemplars of each of these families demonstrate structural features in common with *Reoviridae* (3). The fungal and bacterial viruses both have a principal shell with 120 subunits. Thus, it is possible that the designs of all dsRNA viruses, even those of bacteria, are

based on a similar framework.

Enveloped Positive-Stranded RNA Viruses

The alphaviruses and flaviviruses are T=4 and (probably) T=3 icosahedral assemblies, respectively (41,107). There is at present no structural information for pestiviruses or for hepatitis C. The various proteins derive from a single polyprotein; in the alphaviruses, the core and the envelope have the same T=4 surface lattice, as expected from a uniform stoichiometry. Parallels between alphaviruses and flaviviruses are only hinted at by structures currently known. The flavivirus envelope proteins are PrM and E, found in that order in the polyprotein. PrM is a “chaperone” subunit: cleavage of PrM exposes E for low-pH triggered rearrangement. The corresponding envelope proteins in alphaviruses are PE2 and E1; cleavage of PE2 to E2 and E3 (E3 is lost in some cases, not in others) primes E1 for a fusion transition. Appreciation of detailed structural parallels, if any, between flavivirus E and alphavirus E1 must await the crystal structure of the latter (F. Rey, in progress). The flavivirus receptor-binding domain is on E; the alphavirus receptor-binding protein is E2. Transfer of this function from the fusion subunit to the chaperone subunit may parallel the association of receptor binding and fusion on one protein in orthomyxoviruses and the separation of these functions in paramyxoviruses.

Enveloped Viruses with Trimeric, a-Helical, Coiled-Coil Fusion Proteins

A Fundamental Fusion Module

The envelopes of orthomyxoviruses, paramyxoviruses, filoviruses and retroviruses all contain a glycoprotein with a fundamental fusion module, into which may be inserted domains that carry out other functions. Using the HA of influenza virus A as a model (see Fig. 26), the fusion module comprises HA_2 plus the very beginning and the very end of HA_1. The HA_1 N- and C-terminal segments are part of the folded structure of the fusion module in its precursor state; they do not contribute to the rearranged structure that forms after triggering by exposure to low pH. The organization of the hemagglutinin-esterase-fusion (HEF) protein of influenza C (and of coronaviruses) is particularly revealing (88) (Fig. 28). This protein has three activities: receptor (9- *O*-acetylsialic acid) binding (H), receptor hydrolysis (E), and fusion (F). The receptor-binding domain (homologous to the receptor-binding domain in HA from influenza A) is inserted into a surface loop on the esterase domain (homologous to other acetyl hydrolases), which is in turn inserted into the basic fusion apparatus as just defined. Structures have been determined of fusion-module fragments (in the posttriggering state) from members of all four groups of viruses listed previously. They reveal variants of a conserved, trimeric unit (Fig. 29). Although the precursor state (before triggering) is known only for HA of influenza A and HEF of influenza C, it is plausible to suggest that similar final (posttriggering) structures might in all cases be derived from similar initial (pretriggering) structures.

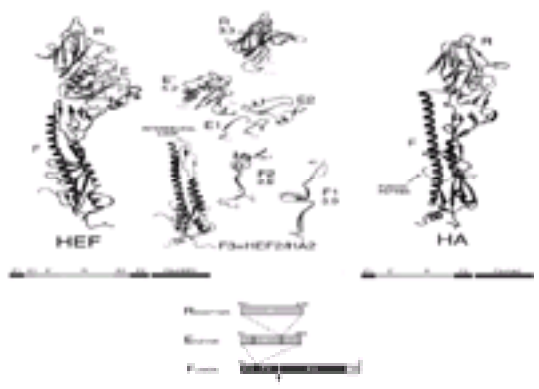

FIG. 28. Comparison of the fusion proteins from influenza C (*HEF*—haemagglutinin-esterase-fusion protein) and influenza A (*HA*) reveals a common core fusion module (*F*), into which evolution appears to have inserted the enzymatic domain (*E*) that catalyzes cleavage of the receptor (in influenza C) and then a receptor-binding domain (*R*). Parts of the E domain have been deleted in the HA of influenza A, leaving R as the only functional part of the insert. (Influenza A has a separate receptor-destroying enzyme, the neuraminidase.) The pattern of insertions is shown in the diagram at the *bottom* of the figure. The core F module thus comprises a segment F1, N-terminal to the insertion point for E (and R), and a segment F2-F3, C-terminal to this point. The fusion-activating cleavage separates F2 from F3 (*arrow in bottom diagram*). F3 is thus known as HEF_2 or HA_2 in influenza C and A, respectively. After triggering by low pH, F3 rearranges. *Top left: Ribbon diagram of one subunit of the HEF trimer from influenza C.* Top right: Similar diagram of a subunit of the HA trimer from influenza A. Compare the view of the HA subunit in Figure 26**B**. Beneath each ribbon diagram is a *bar,* showing the arrangement of segments in the polypeptide chain, *shaded* to correspond to the shading in the ribbon diagram. In the *top center* are ribbon diagrams of the various segments, with their conformations in HEF and HA superimposed. (From ref. 92, with permission; see also ref. 87).

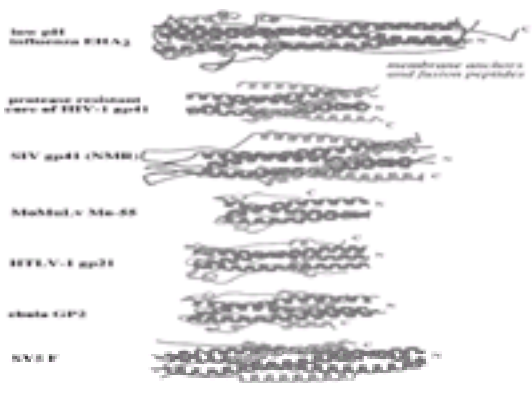

FIG. 29. The core fusion modules from influenza virus (HA_2), human immunodeficiency virus type 1 (HIV-1) and simian immunodeficiency virus (SIV; gp41), Moloney murine leukemia virus (TM), human T-cell lymphotropic virus type 1 (HTLV-1; gp21), Ebola virus (GP2), and SV5 (F), all rearrange after triggering to form related structures. There is a central coiled-coil, formed by three copies of the polypeptide chain segment that immediately follows the fusion peptide, and an outer layer of three helices, formed by the polypeptide chain segments that immediately precede the transmembrane anchor. The fusion peptides and membrane anchors are thus brought together (on the right-hand side of the figure). The fold-back regions on the left have various sizes and characteristics, and they have been deleted in most of the structures actually determined. Note that in all cases, the structures shown here correspond to the "F3" segment of a fusion protein, as defined in Figure 28. (From ref. 92), with permission.)

Diverse receptor-binding domains can evidently be inserted into the common fusion module. The sialic acid–binding domain of influenza virus HA (110) (see Fig. 25**B**), the CD4 and chemokine receptor–binding domain of HIV-1 (65) (see Fig. 25**C**), and the arginine transporter–binding domain of Moloney murine-leukemia virus (40) have unrelated structures. Moreover, some viruses have an additional membrane protein to carry out a function not incorporated into the fusion protein itself. The neuraminidase of influenza A and B, a tetramer with a globular "head" borne on a flexible, extended stalk (32), is unrelated to the esterase domain in the HEF of influenza C (88). The outer surfaces of the larger enveloped viruses can thus exhibit considerable combinatorial variability.

Internal Structures of Large, Enveloped Viruses

Enveloped viruses that share the conserved coiled-coil fusion module can have unrelated internal structures. For example, the Gag proteins of retroviruses and the matrix (M) and nucleocapsid (N) proteins of orthomyxoviruses and paramyxoviruses are completely dissimilar. From the limited structural data available, the following groupings can be discerned.

Retroviral Gag Proteins

When retroviruses bud, the internal protein (Gag) is in the form of a precursor (see Fig. 23). The key domains of this precursor are MA (matrix), CA (capsid), and NC (nucleocapsid); some retroviruses have additional fragments. MA, which trimerizes (probably already before cleavage of the precursor), associates with the inner face of the bilayer. Except in avian retroviruses, it bears an N-terminal myristoyl group, which helps anchor it to the membrane. MA from HIV-1 and from human T-cell lymphotropic virus type II (HTLV-II) have similar structures (31). In HIV-1, MA forms regions of hexagonal lattice, but it does not appear to make a regular icosahedral shell (46). Cleavage of the Gag precursor, subsequent to budding, leads to significant internal rearrangements. In this process, CA assembles into a defined, internal capsid that encloses the two packaged copies of the RNA genome and the associated NC domains. The morphology of the capsid, as seen in the electron microscope, appears to differ from one type of retrovirus to another; a detailed picture is available only for HIV-1 (48,49 and 50). Nonetheless, clear structural similarities among CA proteins from HIV-1, HTLV-I, and Rocus sarcoma virus (19) suggest that the fundamental assembly scheme is likely to be similar in all cases. The NC domain contains one or two (depending on the virus) zinc knuckle domains—small, folded modules stabilized by a Zn^{2+} ion (see Chapter 57). These structures recognize the specific viral packaging signal (see Fig. 20**B**), but they also interact nonspecifically with other segments of genomic RNA (35).

Nucleocapsids and Matrix Proteins of Negative-Stranded RNA Viruses

The genomes of negative-stranded RNA viruses are associated with the viral nucleocapsid protein (N), which remains bound even during transcription. The N proteins of myxoviruses, paramyxoviruses, filoviruses, and rhabdoviruses have similar molecular masses (about 50 kd), and apparent N-protein sequence similarities have been reported among members of the last three of these viral families (5). The ribonucleoprotein complex of Sendai virus has been analyzed by electron microscopy (38). Its most condensed form is a stiff, helical rod, with a pitch of 53 Å and about 13.1 NP subunits per left-handed turn. RNA and protein are wound together, and there appear to be exactly six nucleotides per subunit (18,38). The total length of the rod is 1.1 μm (11,000 ÅÅ); its outer diameter is about 200 Å, and there is a 50 Å diameter hollow core. This form is clearly too stiff and too long to fit into the roughly spherical virions, of about 1000 Å diameter, and indeed much more flexible rods can be seen, corresponding to various degrees of uncoiling of the helix just described (38). The stiff rod could, in principle, represent a transcriptionally active state, if the polymerase were to wind its way down the central hollow and if the bases of the encapsidated RNA were to face inward. Alternatively, it may simply be an intermediate between initial assembly and viral budding.

The VSV ribonucleoprotein complex (RNP) contains eight to nine nucleotides per N-protein subunit. Association with the matrix protein (M) imparts a fixed, tightly coiled structure to the RNP, and this assembly also gives the virion its elongated, bullet-like shape. The dimensions of the viral core are shown in Figure 24**C**. It has a much larger diameter than the paramyxovirus RNP rod just described. The rigid form of the VSV RNP–M complex can be produced either by recombinant coexpression of N and M (which presumably leads to assembly on cellular RNA) or by disassembly of virions. Removal of M produces a far more loosely and flexibly coiled form of the RNP: images from electron microscopy resemble a released spring (Fig. 24**D**). M also mediates incorporation of the envelope glycoprotein (G). Its location with respect to the RNP coil has not, however, been determined unambiguously. Thus, although the structural information currently available hints strongly at parallels between paramyxoviruses and rhabdoviruses, the details remain to be determined.

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CHAPTER 4 Virus Entry and Uncoating

John A. T. Young

IDENTIFICATION AND CHARACTERIZATION OF VIRAL RECEPTORS AND CORECEPTORS

The first step of viral infection requires the binding of viral attachment proteins to specific cell surface receptors and, in some cases, also to coreceptors. Following binding, viruses penetrate a cellular membrane, delivering the viral genome into the host cell cytoplasm. Viral genomes enter cells either as naked nucleic acid molecules, as is seen with a number of plus-strand RNA viruses, or instead as nucleoprotein complexes as with minus-strand RNA viruses, retroviruses, and DNA viruses. Coincident with, or immediately following, their entry into the cell, virions undergo *uncoating* events involving the step-wise disassembly of viral components, leading to genome replication.

This chapter will review some aspects of our current understanding of viral entry and uncoating mechanisms.

Viral Receptors and Coreceptors

Because of their critical involvement in the early steps of infection, viral receptors and coreceptors are major determinants of viral tropism. Viral receptors are defined as cell surface factors that bind directly to native virions and include proteins such as the CD4 receptor for the human and simian immunodeficiency viruses (HIV and SIV, respectively) (130) and polio virus receptor (PVR) for polioviruses (141). In addition to primary binding receptors, some viruses also interact with secondary receptors (coreceptors) that can bind either to native virions or instead to altered forms of virions that are produced as a result of the primary binding step. Viral coreceptors include a variety of CC and CXC chemokine receptors for different strains of HIV and SIV (19), the HveA, HveB, and HveC mediators of herpesvirus entry (82,143,213), and specific integrins used by adenoviruses (222).

Genetic and Receptor-Interference Approaches to Understand Viral Receptors

Genetic and viral receptor-interference studies have been used widely to characterize the nature of virus–receptor interactions. A classical genetic approach is to mate together two parental individuals, one that expresses candidate receptors and the other that does not. The individual progeny of these crosses are then analyzed for their susceptibility to viral infection. These studies can establish the existence of one or more putative viral receptor genes, can determine whether such genes are autosomal or sex-linked, may reveal functionally distinct alleles of these genes, and will establish whether susceptibility to infection is a dominant or a recessive trait.

A second genetic approach involves mapping a putative receptor gene to a specific chromosomal location by analyzing panels of somatic cell hybrids formed between donor cells that express receptors and recipient cells that do not. The chromosomal location of a putative receptor gene can then be deduced by identifying donor cell–derived genomic DNA fragments that are shared between independent somatic cell hybrids that display the appropriate receptor activity. This approach led to the mapping of the putative receptor genes for polytropic and xenotropic murine leukemia viruses (MLVs) and for measles virus, to specific portions of chromosomes 1 from mice and humans, respectively (63,101).

Receptor-interference studies can classify viruses into different receptor usage groups and can indicate whether virus infection abrogates expression of a putative receptor. One approach is based on the principle that cells infected by a given virus are often resistant to superinfection by other viruses that use the same receptor. By contrast, the infected cells remain fully susceptible to infection by viruses that use distinct cellular receptors (Fig. 1A). This technique led to the classification of retroviruses into several distinct receptor usage groups (190).

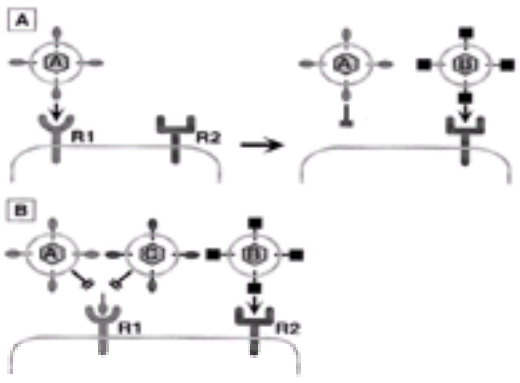

FIG. 1. Viral receptor interference. **Panel A:** Following infection. The cellular receptors for virus type A and virus type B are designated as R1 and R2, respectively. Following infection, expression of the type A viral attachment protein may interfere with the function of R1, leading to a specific block to superinfection by other type A viruses. **Panel B:** Mediated by a soluble viral attachment protein. In this example, type A viruses and type C viruses share the R1 receptor. A soluble form of the type A viral attachment protein may block infection by types A and C viruses, but it will have no effect on the type B virus–R2 interaction.

Although most viruses that establish receptor-interference are thought to do so by forming a complex between newly synthesized viral attachment proteins and the cellular receptor, in most cases the cellular compartment where this occurs is not known. However, in the case of HIV-1, the viral envelope protein (Env) can form a complex with CD4 within the endoplasmic reticulum (ER) and thus interfere with the transport of the receptor to the cell surface (46). HIV-1 encodes two accessory proteins, Vpu and Nef, that also contribute to receptor down-regulation: Vpu causes the ubiquitin-dependent proteolysis of ER-retained CD4 proteins by a mechanism that involves the cellular proteins beta TrCp and Skp1p (132), and Nef seems to serve as an adaptor protein that can target cell surface CD4 for endocytic uptake through clathrin-coated pits (153). Although the Env, Vpu, and Nef proteins of HIV-1 are each capable of reducing cell surface levels of CD4, maximal receptor down-modulation occurs when all three proteins are expressed (37).

A second receptor-interference method used to define viral receptor usage groups relies on the ability of a soluble viral attachment protein to block infection by other viruses that share the same receptor (Fig. 1B). This technique was employed using soluble adenovirus-type 2 (Ad-2) fiber protein to indicate that Ad-2, Ad-5, and Coxsackie B viruses interact with a common receptor (127,160). This notion was later confirmed when the Coxsackie and adenovirus receptor (CAR) that is shared by these viruses was identified (15,203).

Identification of Viral Receptors

Protein Receptors

Various techniques have been employed to identify viral receptors and coreceptors, including the use of specific monoclonal antibodies raised against cell surface proteins, gene and/or cDNA transfer strategies, the virus overlay protein blot assay, and screening the functions of cell surface proteins that are highly related to known viral receptors (Table 1).

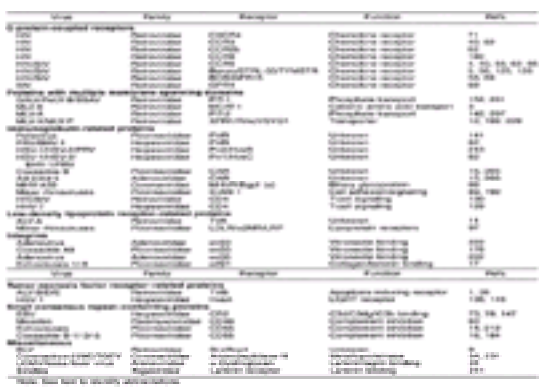

TABLE 1. *Protein viral receptors and coreceptors*

Monoclonal antibodies that bind to specific cell surface proteins and block viral infection have been used extensively to identify viral receptors. Receptors identified by this approach include CD4 for HIV-1 (48,119,130), intercellular adhesion molecule (ICAM)-1 for the major subgroups of rhinoviruses (89,192), MHVR/Bgp1(a) for mouse hepatitis virus A59 (68), a2b1 integrin for echoviruses 1 and 8 (17), CAR for coxsackie B viruses, and adenoviruses (15,203), CD46 for measles virus (63), CR2 for Epstein-Barr virus (73,78,147), CD55 for different subtypes of echoviruses and coxsackie B viruses (14,16,212), aminopeptidase N for human coronavirus-229E and transmissible gastroenteritis virus (54,231), and the high-affinity laminin receptor for Sindbis virus (211). Antibodies specific for the avb3 and avb5 integrins were also used to demonstrate that these cell surface factors can facilitate a post-binding step of adenovirus entry (222).

Gene-transfer and cDNA-transfer methods have been used to clone viral receptor and coreceptor genes. These approaches involve preparing genomic DNA libraries or cDNA libraries from donor cells that express a viral receptor and then introducing these molecules into a recipient cell type that lacks the receptor. Transduced cells that express the

receptor are then identified either (a) because they become susceptible to viral infection or to virus-induced cell–cell fusion, or (b) because they acquire an ability to bind to a viral attachment protein. The viral receptor gene/cDNA can then be directly cloned from these cells.

Viral receptors and coreceptors that were identified by gene/cDNA transfer include PVR for polioviruses (141), PiT-1 for gibbon ape leukemia viruses/subgroup B feline leukemia viruses/simian sarcoma-associated viruses (152), ATRC1/MCAT-1 for ecotropic MLV (3), TVA and TVB for subgroups A, B, and D avian leukosis virus (ALV) (11,26,232), PiT-2 for amphotropic MLVs (142), CXCR4 for X4-tropic strains of HIV-1 (71), and HveA and HveB/Prr2 (poliovirus receptor-related protein 2) for herpes simplex viruses (HSV) (143,213). The use of retroviral cDNA libraries has greatly accelerated the pace of discovering new viral receptors, because putative receptor cDNA molecules can be directly isolated from transduced cells by polymerase chain reaction (PCR) amplification using oligonucleotide primers that are derived from the flanking sequences of the retroviral vector (12,56,168,199,229).

Several viral receptors, including low-density lipoprotein receptor (LDLR)-related receptor proteins for minor subgroups of rhinovirus (97) and a-dystroglycan for lymphocytic choriomeningitis virus and lassa fever virus (31), have been identified using the virus overlay protein blot assay. This assay relies on virion binding to receptors that have been immobilized on a membrane following SDS-polyacrylamide gel electrophoresis.

In some cases, receptors for specific viruses have been identified by testing the activities of either known viral receptors or highly related proteins. For example, the fact that HSV-1 and HSV-2 use Prr2/HveB as a mediator of viral entry led to the finding that these viruses can also use the highly related Prr1 protein for entry (82). Similarly, the identification of the CXCR4/fusin a-chemokine receptor for T-cell tropic strains of HIV (71), coupled with the observation that entry of macrophage-tropic (M-tropic) strains of HIV can be blocked specifically by certain b (CC)-chemokines (43), led to the remarkable discovery that a number of CXC and CC chemokine receptors can serve as coreceptors for different strains of HIV and SIV (5,39,40,55,56,62,65,69,125,126,180).

Carbohydrate Receptors

Certain viruses have evolved to use carbohydrate receptors that are expressed as components of cell surface glycoproteins or glycolipids (Table 2). Because these receptors are generally expressed on cell types from a number of different species, viruses that use carbohydrate receptors tend to have a broad host range.

Virus	Family	Receptor	Refs.
Influenza A	Orthomyxoviridae	Sialic acid-containing oligosaccharides	158
Sendai	Paramyxoviridae	Sialic acid-containing oligosaccharides	93, 133
Reovirus-3	Reoviridae	Sialic acid-containing oligosaccharides	7
Murine polyomavirus	Papovaviridae	Sialic acid-containing oligosaccharides	80
Canine parvovirus	Parvoviridae	Sialic acid-containing oligosaccharides	80
Influenza C	Orthomyxoviridae	9-O-acetylsialic acid	10
Human/bovine coronaviruses	Coronaviridae	N-acetyl-9-O-acetylsialic acid	209
HIV	Retroviridae	Galactosyl ceramide	92
HSV	Herpesviridae	Heparan sulfate	128
Human CMV	Herpesviridae	Heparan sulfate	45

HIV, human immunodeficiency virus; HSV, herpes simplex virus; CMV, cytomegalovirus.

TABLE 2. *Carbohydrate viral receptors*

Carbohydrate receptors can be identified by determining whether the treatment of cells with carbohydrate-destroying enzymes impacts virus binding and infection. Neuraminidase treatment was used to implicate sialic acid–bearing oligosaccharides as receptors for influenza A virus (158), Sendai virus (133), reovirus type 3 (7), porcine rotavirus (177), murine polyomavirus (80), and canine parvovirus (10). Furthermore, virus binding was restored by adding back sialic acid residues to asialylated membranes, confirming the role of this carbohydrate in virus attachment (18,80,93,158,177,198). Similar studies performed using heparinase- and/or heparitinase-treated cells indicated that HSV-1 and human cytomegalovirus (CMV) bind to cell surface heparan sulfate molecules, a result that was confirmed when these viruses failed to attach to cells deficient in heparan sulfate proteoglycan synthesis (45,227).

Carbohydrate-containing receptors have also been implicated using specific monoclonal antibodies that block viral attachment. An antibody that recognizes galactosyl ceramide (GalCer) specifically blocked HIV infection of certain cell types, suggesting that this glycolipid may act as an alternative viral receptor (92). Indeed, GalCer can bind with a high affinity to the HIV-1 Env protein (92,128).

Antibody-Dependent Enhancement of Viral Entry

In addition to classical receptor/coreceptor-dependent mechanisms of entry, viruses may also enter cells when they become complexed with subneutralizing amounts of virus-specific antibodies. Virus–antibody complexes can then be taken up into cells following their binding to immunoglobulin receptors expressed on the cell surface. This type of antibody-dependent enhancement (ADE) of viral infection has been observed through cellular Fc receptors with dengue virus (52), West Nile virus (32,159), influenza A viruses (202), and HIV-1 and HIV-2 (99,137,200). This effect is also seen with Epstein-Barr virus (EBV) that is complexed with polymeric immunoglobulin A (pIgA) specific for the viral gp340 protein, raising the interesting possibility that this pathway of EBV infection may be important at mucosal surfaces of the body where this virus persists and where pIgA is produced (186). The existence of these alternative viral entry pathways indicates that in the absence of a robust immune response that generates high titers of neutralizing antibodies, viruses may be able to bypass their normal requirements for receptors and coreceptors for cell entry, potentially expanding their

in vivo cell-type tropism.

THE ROLE OF ENDOCYTOSIS IN VIRAL ENTRY

After binding viral receptors and/or coreceptors, there are at least two major mechanisms of viral entry that can be distinguished by their requirement for a low pH environment. The pH-independent mechanism of viral entry is proposed to occur at neutral pH, involving viral entry either directly at the plasma membrane or instead from within endosomal compartments following endocytosis of virus–receptor complexes (Fig. 2) (Table 3). By contrast, the pH-dependent mechanism involves the trafficking of virus–receptor complexes to endosomes with an acidic lumenal pH, where the high concentration of protons serves to facilitate viral entry. These distinct mechanisms of viral entry have been distinguished by testing the effect on viral infectivity of treating cells with agents that act to neutralize acidic endosomal compartments such as lysosomotropic agents, carboxylic ionophores, and v-type H^+/ATPase inhibitors such as bafilomycin A1.

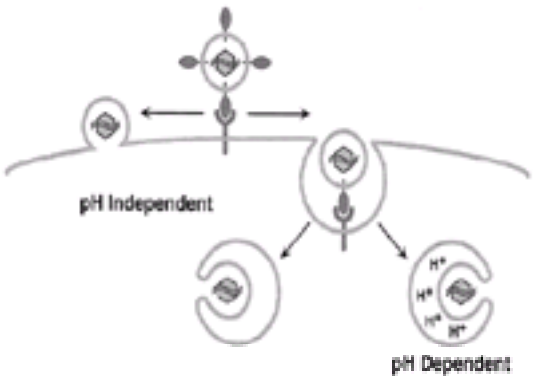

FIG. 2. The pH-independent and pH-dependent mechanisms of viral entry.

pH-Dependent	pH-Independent
Influenza A virus	Retroviruses
Semliki Forest virus	Herpes simplex viruses
Vesicular stomatitis virus	Vaccinia virus
Reoviruses	Rotaviruses
Adenovirus	Paramyxoviruses

TABLE 3. *Examples of pH-Dependent and pH-Independent viruses*

There are two major endocytic pathways that lead to virus uptake from the cell surface, one that is clathrin dependent and the other that is clathrin independent. Electron microscopy has proved useful for uncovering which of these pathways is used by a specific virus—for example, this approach provided strong evidence that influenza A virus enters cells through clathrin-coated pits (135). However, a caveat in using this approach is that RNA viruses usually have a high particle-to-infectivity ratio, so there can be no guarantee that a virus that is imaged represents one that is truly infectious. Therefore, a more definitive approach is to assess the impact on virus infection of expressing dominant negative inhibitors that block cellular endocytic pathways.

Two such inhibitors have been tested for their impact on viral entry, dominant negative forms of the cellular guanosine triphosphatase (GTPase) dynamin and of caveolin. Dynamins constitute a large protein family with at least 27 distinct protein isoforms that are encoded by three separate cellular genes (Dyn1 to Dyn3) (138,182). These proteins are proposed to be “pinchases,” or mechanoenzymes, that can constrict and sever membrane tubules into discrete membrane vesicles (138). Expression of a dominant-negative, GTPase-defective form of Dyn1 inhibits endocytosis through clathrin-coated pits as well as from caveolae, which are microdomains of the plasma membrane that are rich in cholesterol and glycosphingolipids (138,182). The dominant-negative Dyn1 protein blocks the entry of Semliki Forest virus, Sindbis virus, influenza virus, human rhinovirus 14, and adenovirus, indicating that each of these viruses uses a dynamin-dependent pathway for viral entry (58,178,210). By contrast, poliovirus and Moloney murine leukemia virus are apparently unaffected by the expression of this mutant protein, indicating that these viruses use a dynamin-independent mechanism of entry (58,121). A dominant-negative form of caveolin that prevents endocytosis through caveolae has been used to implicate these plasma membrane microdomains as the major sites of SV40 uptake into cells (100,150,157,179).

Factors That Influence Receptor Choice

A comparison of known viral receptors and coreceptors reveals that members of a given virus family have evolved to use a variety of different cell surface factors for viral entry. For example, a cell surface glycolipid, multiple membrane-spanning transporter proteins, and proteins that are members of the immunoglobulin, LDLR, and tumor necrosis factor receptor (TNFR) superfamilies are used as retroviral receptors (see Table 1 and Table 2). Similarly, different picornaviral receptors include immunoglobulin-related proteins, an LDLR-related protein, integrins, and small

consensus repeat–containing proteins that regulate complement activation (see Table 1).

Despite this heterogeneity of viral receptor usage, it is striking that several distinct viruses use precisely the same receptor. Two herpesviruses, pseudorabies virus and bovine herpes virus-type 1, can use the same cellular receptor as poliovirus, a picornavirus (see Table 1). Also, CD4 is a receptor used by retroviruses and by a herpesvirus, whereas CAR and avb3 integrin are used by picornaviruses and adenoviruses (see Table 1).

This apparent convergence of receptor usage may simply be a coincidence. However, it seems much more likely that viruses have evolved to recognize receptors that have properties that are particularly well suited for mediating viral entry. Although these factors remain largely unknown, it intuitively seems that one important property would be the close proximity of the viral attachment site to the host cell membrane. Indeed, by varying the distance between the measles virus attachment site on the CD46 receptor and the host cell membrane, it has been possible to uncouple virus binding from entry (27). An additional factor that could influence receptor choice is the localization of that protein within specific microdomains of the plasma membrane and the subsequent membrane trafficking of that protein after virus binding—for example, SV40 binding leads to a clustering of the major histocompatibility complex (MHC) class I molecule receptors in caveolae, which represent the site of virus uptake into the cell (150,157).

Another factor that can influence receptor choice is the presence of receptor-associated signal transduction pathways that may facilitate steps of viral replication either during or after viral entry. Endocytosis of adenoviruses is stimulated by a signal transduction pathway linked to the integrin coreceptor that activates phosphoinositide-3-OH kinase (PI3K) and cellular Rho GTPases to stimulate the necessary rearrangements of the actin cytoskeleton (123,196). The actin cytoskeleton is also implicated as playing a critical role in the entry mechanisms used by other viruses such as HIV (103) and MLV (117). The interaction between the EBV gp350/220 protein and the CR2 receptor also activates a receptor-associated protein phosphorylation pathway that is important for the early steps of EBV replication (41,174). Similarly, the interaction of the CMV gB protein with an as yet unidentified receptor activates the interferon-responsive pathway, which may provide an optimal cellular environment for the subsequent transcription of viral genes (24).

Receptor interference, which is often observed after virus infection, may represent another selective force on viral receptor usage. For those viruses that assemble and bud at the plasma membrane, this block to cell surface receptor expression is presumably necessary to allow the efficient release of nascent viral particles. The fact that HIV-1 has evolved three separate mechanisms to interfere with cell surface CD4 expression (37) underscores the importance of receptor down-regulation for viral replication.

As discussed, receptor interference can prevent viral receptor expression on the cell surface because newly synthesized receptors and viral attachment proteins can form a complex that is retained within the cell. It therefore follows that the expression level of a given cellular factor may dictate whether it can be used as a viral receptor. Those factors that are expressed at a low level will presumably be more easily titrated out by viral attachment proteins than are factors that are expressed at a higher level.

Receptor interference may also prevent that cellular receptor from interacting with its cognate ligand and thus abrogate the normal receptor function. If the ligand–receptor pair in question plays a critical role in host defenses against virus infection, then this mechanism of interfering with receptor function may provide an additional level of selection on receptor choice. Indeed, this may explain why subgroups B, D, and E of ALV use TVB, a TNFR-related death receptor, as a viral receptor. TVB is most closely related to the human TRAIL (TNF-related apoptosis-inducing ligand) receptors, TRAIL-R1 (or DR4), and TRAIL-R2 (or DR5). TRAIL and its receptors have been implicated in the induction of apoptosis in cell populations infected with several distinct classes of viruses including HIV-1, human CMV, and adenoviruses (113,183,225). Therefore, certain subgroups of ALV may have evolved to use the TVB receptor, at least in part, because after the establishment of receptor interference, the infected cells may be protected against cell death induced by the cognate ligand (26).

ENVELOPED VIRUS ENTRY

Enveloped viruses have a lipid bilayer, or envelope, that surrounds their nucleocapsids. Therefore, the entry of these viruses into cells requires the fusion of viral and cellular membranes by a process that is driven by viral glycoproteins located on the virus surface.

The Influenza A Virus Paradigm and Related Viral Fusion Proteins

The mechanism of virus–cell membrane fusion is best understood in the case of the influenza A virus hemagglutinin (HA) protein. HA is synthesized as a precursor glycoprotein (HA_0) that is assembled into homotrimers within the lumen of the host cell endoplasmic reticulum prior to its export to the cell surface. During its biosynthesis, HA_0 is proteolytically cleaved into two mature subunits, HA_1 and HA_2. This trimer of HA_1/HA_2 heterodimers exists as a metastable trimer on the viral surface (33). HA_1 contains the sialic acid receptor binding site of the viral glycoprotein, and HA_2 contains two hydrophobic regions that are important for the complete fusion of virus and cell membranes, an N-terminal fusion peptide, and a membrane anchor located near the C-terminal end.

Following receptor binding, influenza viruses are endocytosed via clathrin-coated pits and then trafficked to acidic endosomes. The acidic lumenal environment of these endosomal compartments then activates irreversible conformational changes in HA that promote membrane fusion. These structural alterations can be detected as an increased susceptibility of the protein to cleavage by specific proteases such as trypsin and proteinase K (59,163). Also, these structural changes can be detected using conformation-specific antibodies to monitor the appearance and disappearance of certain epitopes of HA. For example, the fusion peptide region of HA_2, which is found buried at the trimer interface in the native HA structure, becomes rapidly exposed following low pH treatment, leading either to aggregation of the viral protein or to its attachment to membranes (59,115,187,219). By contrast, an epitope located at

the interface between two HA_1 subunits in the native trimer is lost following this treatment, indicating that the HA_1 subunits must be dissociated from each other during the fusion reaction (115,219). Indeed, when intermonomer disulfide bonds were introduced into HA_1 subunits to prevent their dissociation from each other, this led to a specific block to the fusion activity of the viral glycoprotein (84,114).

During low pH–induced activation, the HA trimer is converted from its metastable state to one in which HA_2 adopts a highly stable trimeric structure. During this conversion, the hydrophobic fusion peptide regions of HA_2 are predicted to be displayed at the tip of a long, triple-stranded, a-helical coiled coil region that forms the core of the structurally altered viral glycoprotein (29,33,34). X-ray crystal structures of the native form of HA_2, as well as of an apparent postfusion form of this glycoprotein, are described in Chapter 3 and will not be discussed in detail here.

The exposed fusion peptide regions of HA_2 are critical for the membrane fusion reaction and have been proposed to bind directly to target membranes (Fig. 3). The importance of these regions for membrane fusion has been demonstrated using mutant viruses bearing amino acid replacements within this hydrophobic region. Although these altered viral glycoproteins can still bind to sialic acid receptors and are capable of undergoing the characteristic low pH–induced structural changes, their membrane fusion activities are abrogated (51,83,193). In addition, experiments performed with liposomes containing radiolabeled photoreactive lipids have demonstrated that the fusion peptide region is the only domain of HA_2 that becomes associated with a target lipid bilayer following the low pH activation (66).

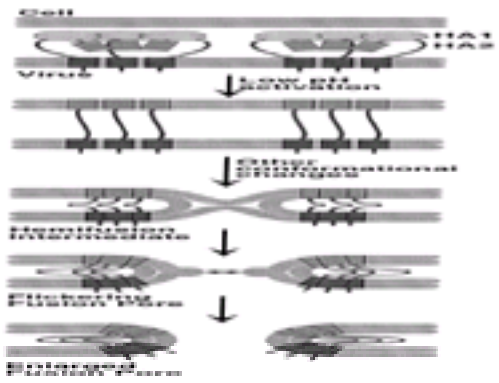

FIG. 3. A model for influenza A virus hemagglutinin (HA) protein–mediated membrane fusion.

It is thought that, following attachment of the HA_2 fusion peptides to the target membrane, this protein undergoes additional rearrangements, bringing the viral and target lipid bilayers into close apposition (see Fig. 3). These rearrangements are thought to lead initially to mixing of lipids between the outer leaflets of membranes, a process known as hemifusion, leading to the formation of a hemifusion diaphragm (see Fig. 3). This step is then followed by the formation of a small fusion pore that flickers open and closed before becoming fully dilated (191). It has been suggested that at least three and perhaps six HA trimers are needed to form the fusion pore (22,50), although a single HA trimer is capable of perturbing membranes (106).

During the membrane fusion reaction, the HA_2 protein is proposed to assume a hairpin-like structure in which the fusion peptide domains and the transmembrane domains of HA_2 become localized in the same lipid bilayer (see Fig. 3). Several independent lines of evidence support this hypothesis. First, in the absence of a suitable target membrane, low pH treatment inactivates virions because the newly exposed fusion peptide domains become inserted in the viral membrane—that is, in the same membrane as the membrane-spanning domain (215,218). Second, the x-ray structure of the low pH form of the core of HA_2 has revealed that the C-terminal end of this protein has an inverted orientation relative to its N-terminal end, and that in fact the N- and C-termini can combine, generating an N-cap structure (38). Whatever the precise mechanism is that leads to membrane fusion, it is clear that the transmembrane region of HA_2 is critical for this process because a glycosylphosphatidylinositol-linked form of HA is capable only of mediating hemifusion (115). It has been proposed that the membrane-anchor region of the HA_2 region might facilitate the complete fusion reaction by imposing a positive curvature on the inner leaflets of the hemifusion diaphragm (139).

Despite the large body of information that is available regarding HA-mediated membrane fusion, there are still several important unanswered questions about this process: (a) What are the precise step-wise structural changes that convert the native HA protein from its metastable form on the virion to the postfusogenic state? (b) At what step during the fusion reaction does the HA_2 hairpin structure form? (c) How many HA trimers are needed to form an active fusion pore and mediate the hemifusion and the fusion reactions? (d) What is the precise role of the transmembrane region of HA_2?

Structural analyses have revealed that the basic architecture of the HA membrane fusion machine is shared by the glycoproteins of a number of other enveloped viruses, including retroviruses, filoviruses, and paramyxoviruses. These glycoproteins include the transmembrane proteins of Mo-MLV (70), HIV (35,217), and SIV (30); the GP2 protein of Ebola virus (131,216); and the F_1 protein of SV5 (8). Like HA_2, each of these viral glycoproteins is the C-terminal cleavage product of a precursor envelope glycoprotein, and each contains a hydrophobic fusion peptide located near the N-terminal end and a single membrane-spanning anchor region located near the C-terminal end. Each of these viral glycoproteins can fold into a rodlike structure similar to that of activated HA_2, with the fusion peptide regions located at the tip of a central, triple-stranded, a-helical coiled, coil. Also, in some of these glycoproteins, three antiparallel helices are packed against the central, triple-stranded coil, coil domain, providing additional support for the idea that the fusion peptide and transmembrane regions of these glycoproteins become located in the same lipid bilayer at a late stage

during membrane fusion (8,30,35,131,216,217).

In addition to the structural similarities observed between retroviral transmembrane and influenza virus HA_2 proteins, there is also biochemical evidence that, like HA, retroviral Env proteins must undergo conformational changes leading to viral entry. However, as most retroviruses are thought to enter cells by a pH-independent mechanism, it is presumed that fusion-activating conformational changes in Env are induced upon receptor/coreceptor binding. Consistent with this hypothesis, CD4-binding can induce conformational changes in the HIV-1 surface protein (SU) that, in lab-adapted viral strains, causes dissociation of the surface protein from the transmembrane protein, increases the exposure of certain antibody epitopes of Env, increases the susceptibility of the viral glycoprotein to protease cleavage, and leads to exposure of the coreceptor binding site of SU (173,181,206,226,228). CD4 and coreceptor interactions also lead to exposure of hydrophobic determinants of HIV Env proteins as measured using a fluorescent probe that binds specifically to hydrophobic groups (108). Additional evidence for receptor-induced structural changes in retroviral glycoproteins has been obtained for a soluble ALV-A Env protein that is induced to undergo structural changes leading to fusion-peptide exposure and to stable liposome binding following its interaction with the TVA receptor (49,96).

Other Types of Viral Fusion Proteins

Although the core structural elements of the membrane fusion machinery described for the influenza HA protein are shared by many viruses, other enveloped viruses use fusion proteins with different architectures to enter cells. For example, the G protein of the rhabdovirus vesicular stomatitis virus (VSV) is a homotrimer of a single protein subunit with an internal hydrophobic fusion peptide region (79,220,233) that becomes labeled by hydrophobic photoactivatable probes under conditions that stimulate viral fusion (67). However, unlike HA, which irreversibly associates with target membranes following low pH-induced activation, VSV-G reversibly associates with these membranes, implying that it is less stably associated with target bilayers (156).

Another type of viral fusion protein is represented by the E protein of the tick-borne encephalitis virus (TBE), a flavivirus that contains two transmembrane segments and has a putative internal fusion peptide (171). The E protein of TBE exists as a dimer that is parallel to the membrane on the virus surface but is converted to a trimer that is active for membrane fusion when incubated under low pH conditions needed for membrane fusion (6,171).

Alphaviruses have yet another type of viral glycoprotein complex. Semliki Forest virus has three envelope proteins: E1 and E2, which are both type 1 membrane glycoproteins, and E3, which is an extrinsic protein. These proteins assemble as E1/E2/E3 heterotrimers. At low pH, the viral glycoprotein complex undergoes conformational changes leading to the formation of a stable E1 homotrimer that mediates membrane fusion (72,94).

More complex viral fusion machines are seen with the herpesviruses. Usually, five glycoproteins of HSV-1 are required for viral entry: gB and gC, which bind to cell surface heparan sulfate molecules; gD, which interacts with herpesvirus entry mediators such as HveA (143); and the gH/gL heterodimer, which participates in membrane fusion (166). Additionally, viral glycoproteins such as the gE/gI complex and gM can facilitate viral spread into certain cell types by interacting with receptors that are expressed at cell–cell junctions (166).

Regulating Viral Fusion Proteins

The membrane fusion activities of viral glycoproteins need to be tightly regulated to prevent their premature activation during viral protein synthesis and assembly. As described, the fusion activities of viral glycoproteins can be controlled by receptor/coreceptor interactions and by exposure to a suitable, low pH–containing environment. A number of viral glycoproteins are also regulated by proteolytic cleavage of their extracellular domains, which converts an energetically stable precursor glycoprotein to a metastable form that is then capable of being triggered for membrane fusion. This form of regulation is seen with the glycoproteins of orthomyxoviruses, paramyxoviruses, and retroviruses (95).

Amino acid residues located within the transmembrane and cytoplasmic tail domains of viral glycoproteins can also regulate their fusogenic activity. For example, the Env proteins of Mason Pfizer monkey virus and Mo-MLV become competent for fusion following viral protease-mediated cleavage at a specific site in their cytoplasmic tail domains (25,165,170). As retroviral proteases become activated at a late stage of viral replication that is coincident with, and/or occurs shortly after, virus budding, this form of regulation ensures that the viral glycoproteins are not prematurely activated within the virus producer cell. Specific mutations introduced into the transmembrane domain of VSV-G or into the transmembrane region or cytoplasmic tail domains of influenza virus HA, have also been shown to selectively block complete membrane fusion without affecting hemifusion (42,140). However, how these residues act to regulate membrane fusion remains to be determined.

Another mode of regulating viral fusion protein activity is seen with paramyxoviruses. For most of these viruses, with the exception of SV5, the fusogenic activity of the F protein is tightly regulated by its association with the homologous hemagglutinin (HN) protein that is responsible for receptor binding. Therefore, the fusion activation of paramyxoviral F proteins generally involves homotypic HN-F interactions following receptor binding (57,195,230). The coordinated action of separate viral attachment and fusion proteins presumably evolved to ensure that the fusion protein would become activated only following viral attachment to an appropriate target cell surface.

NONENVELOPED VIRUS ENTRY

Nonenveloped virus entry into cells involves either the formation of proteinaceous pores in a host cell membrane, as has been suggested for picornaviruses, or processes that disrupt the integrity of a cellular membrane, as seems to be the case for adenoviruses and reoviruses. A common feature of each of these viral entry mechanisms is the orchestrated disassembly of the virion leading to loss of specific capsid proteins and to the interaction of the viral particle with a host cell membrane.

It has been proposed that the positive-stranded RNA genome of picornaviruses enters cells through a proteinaceous core composed of viral proteins. Mature picornaviral virions contain a single copy of plus-stranded genomic RNA and 60 copies each of four viral proteins, VP1, VP2, VP3, and VP4. The VP1 proteins of poliovirus and of rhinovirus contain a deep surface depression, or canyon, located at each fivefold axis of symmetry. This canyon represents the site of receptor interaction on the viral surface (154,164).

During poliovirus entry, the viral particle undergoes a series of structural changes at the cell surface that are predicted to lead to the transfer of the viral RNA genome into the host cell cytoplasm to begin replication. The first change detected is the conversion of mature virions sedimenting as 160S particles to those that sediment at 135S (223). This alteration involves the loss of VP4, which is located within the virion interior, and the buried hydrophobic N-terminus of VP1 is translocated to the virion surface to mediate membrane binding (164,223). Thus, as for a number of enveloped viruses, exposure of hydrophobic viral protein surfaces is a critical event and required for poliovirus entry. A second similarity between polioviruses and the enveloped viruses discussed earlier is that poliovirus virions seem to exist in a metastable state and are converted to more highly stable forms during the process of viral entry (223). At physiologic temperatures, poliovirus is a conformationally dynamic entity, with VP4 and the N-terminal end of VP1 being transiently and reversibly exposed on the virus surface by a so-called breathing mechanism, even in the absence of a target cell (124). Reversible conformational changes in viral capsids have also been noted with rhinovirus (122), and with the nodavirus Flock House virus (23).

The data implicating 135S particles as obligatory intermediates during poliovirus entry are compelling. Receptor binding converts native virions to 135S particles, a result that indicates that the receptor alone may be capable of triggering the first steps of viral uncoating (164,223). However, it is still not clear whether the 135S particles are obligatory intermediates during poliovirus entry or instead are simply dead-end products. In support of their importance, 135S particles are infectious, albeit at much reduced levels compared to mature virions (47). Arguing against their importance, cold-adapted poliovirus mutants seem to enter cells without the need to generate 135S particles (64). These disparate findings might be rationalized if 135S particles are short-lived only in the case of cold-adapted viral strains (223). Additional experimentation is needed to validate the role of the 135S particles during poliovirus entry.

The next stage in poliovirus entry is predicted to involve the conversion of the 135S particle to an 80S form that is devoid of RNA and that probably represents the empty protein capsid (91,204). Furthermore, it has been proposed that an amphipathic helix located at the exposed amino-terminus of VP1, and the N-terminal myristic acid group of the extruded VP4 protein might participate in the formation of protein channels in host cell membranes that could allow translocation of the poliovirus genomic RNA into the cytoplasm (13,91,204). However, it is not yet clear exactly how the viral RNA exits from the virion (13).

Adenovirus is predicted to enter cells by perturbing the integrity of cellular endosomal membranes, thus allowing viral nucleoprotein complexes access to the host cell cytoplasm. Adenovirus is a nonenveloped DNA virus that contains 11 different types of protein, including 12 complexes of penton base, each with an associated fiber protein. Following the binding of the viral fiber protein to the CAR receptor, the viral penton base interacts with specific host cell integrins. Following virus uptake into the host cell by receptor-mediated endocytosis, adenoviruses enter the cytoplasm after the pH-dependent lysis of endosomes, a step that is facilitated by the penton base–integrin interaction (221). Coincident with endosomal lysis, the viral nucleocapsid begins to disassemble with the fiber, penton base, and viral IIIa proteins being shed from the viral particles (88).

Reoviruses are also proposed to enter cells by perturbing a cellular membrane after being trafficked to a low-pH endosomal compartment. However, in contrast to the situation with adenoviruses where low pH is proposed to play a direct role in the membrane perturbation event, the role of low pH in the case of reoviruses is to activate cellular cysteine proteases that cleave the viral outer capsid proteins s3 and µ1/µ1C, giving rise to infectious subviral particles (ISVPs). ISVPs are then capable of infecting cells in a pH-independent manner. It has been suggested that removal of the s3 protein of reoviruses may lead to conformational changes in µ1/µ1C that in turn lead to membrane perturbation (105).

VIRAL UNCOATING

The events that immediately follow viral penetration into the host cell, but precede replication of the viral genome, are commonly referred to as uncoating. These events may be intrinsically coupled with those leading to cellular membrane penetration, as in the case of picornaviruses, which must simply introduce their plus-stranded RNA genome into the host cell cytoplasm for replication to proceed. Other viruses, however, introduce viral nucleoprotein complexes into the cell, and for these viruses a series of disassembly steps that occur after virus entry are required for successful uncoating.

Most RNA viruses are replicated in the host cell cytoplasm, presumably because their replication via the associated RNA-dependent RNA polymerase does not usually require activities provided by the host cell nucleus. By contrast, most DNA viruses, retroviruses, hepadnaviruses, and other RNA viruses such as the orthomyxovirus influenza A virus must access the host cell nucleus for replication to proceed. These viruses require nuclear functions involved in cellular DNA and RNA synthesis as well as in RNA processing. These viruses must traverse the physical barrier imposed by the nuclear envelope in addition to that imposed by the plasma/endosomal membrane.

The nuclear envelope consists of a double membrane, within which are embedded nuclear pore complexes (NPCs) made up of more than 50 different types of nucleoporins that regulate the transport of macromolecules into and out of the nucleus (86). Active transport of proteins and of nucleoprotein complexes through the NPC is mediated by specific transport signals designated as nuclear localization signals (NLSs). The NLS is recognized by importin-a, an NLS receptor, which in turn binds to importin-b, a transport receptor that facilitates binding to, and translocation through, the NPC (86). The importin-a and -b subunits are then exported—shuttled—back to the cytoplasm in a complex with the GTP-bound form of the cellular GTPase Ran (RanGTP) (86). The GTPase activity of Ran becomes activated in the cytoplasm by RanGAP1, giving rise to RanGDP, an event that leads to release of the importin subunits so that they can

engage in a new round of nuclear import. RanGDP is then returned to the nucleus where a guanine nucleotide exchange factor (RCC1) acts to regenerate RanGTP for a new round of nuclear export. It has been proposed that the GTP hydrolysis that is associated with the establishment of the nucleocytoplasmic gradient of RanGTP is the driving force for cellular nuclear import and export pathways (86).

Influenza A viruses and lentiviruses such as HIV-1 seem to employ several distinct NLSs on viral core proteins for efficient targeting of viral nucleoprotein complexes to the host cell nucleus. Influenza A viruses contain eight single-stranded negative-sense RNA segments that encode viral proteins. Within the virion, these segments are found as viral ribonucleoprotein complexes (vRNPs) containing the nucleoprotein NP and the viral polymerase complex, and these vRNPs are associated in turn with the viral M1 protein. An essential step of influenza virus uncoating is the dissociation of M1 from the vRNPs. M1 dissociation occurs under low pH conditions when the virus is trafficked to acidic endosomal compartments, and it results from the translocation of protons through the M2 ion channel located on the virus surface (28,134,161). Thus, this critical step of viral uncoating is linked both spatially and temporally within the cell to that of HA-mediated membrane fusion. Following M1 dissociation, the viral RNPs are trafficked to the host cell nucleus to begin replication by a mechanism that presumably involves nuclear localization signals contained on each of the NP and polymerase proteins (2,107,148,149,214). The NLS of the NP protein has been found to interact with an importin-a/b complex (155).

Lentiviral preintegration complexes (PICs) consist of viral DNA, the matrix protein (MA), nucleocapsid (NC), reverse transcriptase (RT), integrase (IN), and the Vpr accessory protein (194). These viruses differ from most other retroviruses in that they are able to infect nondividing cell types such as macrophages, and thus their entry into the host cell nucleus is not dependent on nuclear envelope breakdown. Several distinct nuclear-targeting signals have been found associated with HIV-1 core proteins. The Vpr protein, which is associated with viral PICs, is an importin-b-like protein that can bind importin-a and is proposed to target the viral core particles to nuclear pores where it interacts with nucleoporins to facilitate viral translocation into the nucleus (77,162,208). Additional nuclear localization signals have been described in the viral integrase protein that mediate interactions with importins (81), as well as in the viral MA protein, although the existence of this latter signal has been the subject of much controversy (76,169).

Posttranslational modifications of viral proteins may regulate their nuclear uptake, as has been seen with the hepatitis B virus core protein, where the NLS is exposed to interact with an importin-a/b complex after protein phosphorylation (110). It has been proposed that this phosphorylation step involves protein kinase C molecules assembled into hepatitis B viral capsids (110). Also, the nuclear transport of HIV-1 PICs can be regulated by the phosphorylation status of the viral MA protein, which is a substrate for the virion-associated mitogen-activated protein kinase (ERK/MAPK) (104).

Viruses have evolved to use components of the host cell cytoskeleton to facilitate their intracellular trafficking to sites of virus replication, as exemplified by adenovirus and by the herpesvirus HSV-1. Intracellular adenoviral complexes are translocated to the NPC through their association with the minus-end-directed microtubule motor complex of dynein and dynactin (197). Adenovirus capsids then dissociate at the NPC so that viral DNA and protein VII can enter the nucleus to begin genome replication (87).

Following HSV-1 entry at the plasma membrane and dissociation of the viral tegument proteins, the capsids associate with dynein, which is proposed to traffic these complexes toward NPCs (189). Once docked at the NPC, HSV-1 DNA is delivered into the host cell nucleus, leaving the empty capsid behind (189). Transport along microtubules probably represents an important event during HSV-1 infection of neuronal cells, where the viral capsids have to travel some distance from the site of virus entry to the nucleus.

INHIBITORS OF VIRAL ENTRY

Specific inhibitors of viral entry have been useful reagents for studying the basic mechanisms of this first step of viral replication and represent attractive antiviral agents because their action does not require that they be taken up into cells. Several different classes of viral entry inhibitor have been described, including neutralizing antibodies, soluble receptors, peptide inhibitors, small molecules, and ligand-based inhibitors that bind to target viral receptors or coreceptors (Fig. 4).

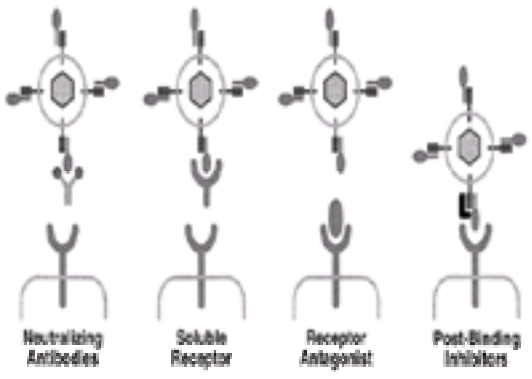

FIG. 4. Inhibitors of viral entry.

Neutralizing Antibodies

One of the ways by which the host organism can restrict viral entry into cells is to produce neutralizing antibodies that bind to and inhibit the function of viral attachment and fusion proteins. These antibodies can block infection by binding directly to the receptor-interaction site located on viral proteins, as has been seen with human rhinovirus 14 (36) and with

influenza virus HA (20). Alternatively, these antibodies can block infection by binding to an epitope that is located outside of the receptor-binding site, where they sterically hinder the receptor association (75). Neutralizing antibodies that block viral association with coreceptors by binding to receptor-induced epitopes located on viral proteins have also been described in the case of HIV-1 (120,228).

Soluble Receptors

Soluble proteins composed of the extracellular domains of viral receptors have been used as reagents to block viral infection. Examples of such soluble receptors include PVR for polioviruses (111,112), ICAM-1 for rhinoviruses (90), and CD4 for HIV-1 (53,74,102,188,205). These agents act by binding to, and occluding, the receptor-binding site of the viral attachment protein. In addition, some soluble viral receptors are capable of triggering a series of conformational changes in virion proteins that are similar to those that occur normally during viral entry and that lead to virus inactivation (85,90).

Although soluble receptors might appear to be suitable antiviral reagents, their efficacy as viral entry inhibitors has been compromised by the fact that independent viral isolates are differentially sensitive to their action. For example, although lab-adapted, T-cell tropic strains of HIV-1 are highly sensitive to soluble CD4-inhibition, primary viral isolates are not (118). An additional problem with the use of soluble receptor–based inhibitors is that viruses that are resistant to the action of these reagents are readily obtained because of the selective pressures they impose on replication. For example, a variety of different soluble receptor–resistant (srr) mutants of poliovirus evolved in the presence of a soluble PVR protein, and most of these viral variants had a reduced binding affinity for receptor and had altered responses to receptor-mediated structural changes (44,112).

Peptide Inhibitors

Different classes of small peptide–based inhibitor have been used to block viral infection. Some peptide inhibitors used are derived from the viral interaction site of a viral receptor. These inhibitors act presumably by binding to the viral interaction site on a viral attachment protein, thus preventing its interaction with cell surface receptors. For example, an oxidized 19–amino acid peptide containing viral interaction determinants of TVA is an effective inhibitor of subgroup A ALV entry (234).

Other classes of peptide inhibitor act to block steps of viral entry subsequent to virus–receptor interactions. For example, peptides derived from two separate a-helical regions of the HIV-1 transmembrane protein that are predicted to interact with each other during the membrane fusion reaction, as described previously, are potent inhibitors of a postbinding step during entry. Presumably, either these inhibitors block the formation of the central coiled coil of the transmembrane protein trimer, or they block the contacts that are formed between this central trimer and the outer-layer a-helices of the transmembrane protein (35,217,224). Peptides corresponding to similar regions of other viral fusion proteins are also effective viral entry inhibitors—for example, in the case of paramyxovirus F_1 proteins (109,167). These inhibitors have been used to define an intermediate “lipid-mixing only” step in the viral fusion reaction, where hemifusion has apparently occurred but complete membrane fusion has not (109,145).

This class of peptide inhibitor has already shown some promise in the clinic, at least when administered at a high dose to HIV-infected individuals (116). However, as with soluble receptors, it seems that one problem that will be encountered with these reagents is the evolution of inhibitor-resistant viral strains with mutations that interfere with inhibitor binding and/or function (172).

Another class of peptide inhibitor that acts to block virus infection after receptor binding are those that antagonize the function of viral coreceptors. For example, the T22 and ALX40-4C peptides that are antagonists of CXCR4 can inhibit entry of T-cell-tropic HIV-1 (61,146).

Small-Molecule Inhibitors

Small-molecule inhibitors of viral entry include the drug amantadine hydrochloride, which was instrumental in revealing the ion channel activity of the influenza A virus M2 protein that is essential for the low pH–induced dissociation of M1 from viral RNPs during viral entry (28,161). Other small-molecule inhibitors act by binding to and inactivating viral proteins that are necessary for entry—for example, the compound WIN 52084 acts to block viral entry when located in a hydrophobic pocket that is located in the canyon floor of the VP1 protein. It does so by preventing dynamic conformational changes in the viral capsid that are necessary for infectivity (122). Also, small-molecule inhibitors of influenza A virus entry have been described that act either by blocking the necessary conformational changes in HA or by prematurely activating these changes (98). Other classes of small molecules have been described that are antagonists of viral receptors and/or coreceptors. These molecules include the bicyclam AMD3100 antagonist of CXCR4 function, which prevents entry of T-cell-tropic strains of HIV into cells (60).

Ligand-Based Inhibitors

In some cases, it has been possible to block viral entry using naturally occurring ligands or ligand derivatives that bind to viral receptors and coreceptors, thus interfering with viral entry. Examples of naturally occurring ligands used as viral inhibitors are the a-chemokine SDF-1, and the b-chemokines RANTES, MIP-1a, and MIP-1b that block viral entry by binding to the HIV coreceptors CXCR4 and CCR5, respectively (4,21,40,43,55,65,151). An example of a chemically modified form of one of these ligands that can still block viral entry without potentiating the normal function of the receptor is amino-oxypentane (AOP)-RANTES (185). However, selection of viral variants that resist inhibition may restrict the utility of this approach, in this case by selecting for viruses that use other HIV coreceptors (144).

CONCLUDING REMARKS

Over the course of the last decade, much has been learned about how virus–receptor interactions lead to viral entry. The exact mechanisms of viral entry into cells are beginning to be revealed through the combined use of biochemical, genetic, and structural analyses of specific viral proteins or whole virions. Also, a number of different cell surface proteins and carbohydrates that serve as viral receptors have been identified and their viral binding determinants defined. The challenge that lies ahead is to understand the step-wise series of events leading to viral entry that occur after virus binding to receptors/coreceptors, and in the presence of other activators such as low pH conditions.

The mechanisms of viral uncoating are also beginning to be understood. Future research in this area should provide valuable new information about this important step of viral replication. It will undoubtedly shed new insights into how viruses exploit host cell processes for their own ends, and it will, we can hope, provide a new set of targets for the development of antiviral strategies.

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CHAPTER 5 Replication Strategies of RNA Viruses

L. Andrew Ball

INTRODUCTION

Replication of genetic information is the single most distinctive characteristic of living organisms, and nowhere in the biosphere is this process accomplished with greater economy and apparent simplicity than among the RNA viruses. To achieve the expression, replication, and spread of their genes, different families of RNA viruses have evolved diverse genetic strategies and life cycles that exploit the biology and biochemistry of their hosts in many different ways. Because the global environments in which viruses replicate and evolve are dictated largely by their hosts, understanding virus replication also illuminates host biology at the molecular, cellular, organismal, and population levels. Each infection represents an encounter between the genetic program of a virus and that of its host, and the ensuing interplay reflects the current status of their ongoing co-evolution. The details not only enrich our understanding of the biosphere in general and virus–host relationships in particular, but also create opportunities for the rational development of antiviral drugs and for domesticating viruses as expression vectors, live attenuated vaccines, and pesticides. This chapter presents an overview of the replication strategies of the major families of RNA viruses that infect animals, attempting wherever possible to identify the general principles that guide and constrain RNA virus replication and evolution. Most of the references cite review articles where citations of the primary literature can be found.

Unique Pathways and Enzymes

RNA viruses are the only organisms known to use RNA as their genetic material. They accomplish this by replicating their genomes through one of two unique biochemical pathways: either RNA-dependent RNA synthesis (RNA replication) or, among the retroviruses, RNA-dependent DNA synthesis (reverse transcription) followed by DNA replication and transcription. Both these pathways require enzyme activities that are not usually found in uninfected host cells and must therefore be encoded in the viral genome and expressed during infection. Furthermore, in some families of RNA-containing viruses, these unique synthetic processes are required right at the start of the infectious cycle. This necessitates copackaging of the corresponding polymerase and other associated enzymes with the viral genome during the assembly of virus particles in preparation for the next round of infection.

Whatever the structure and replication strategy of their genomes, all viruses must express their genes as functional mRNAs early in infection to direct the cell's translational machinery to make viral proteins. The various genetic strategies used by RNA viruses can therefore be organized into a simple conceptual framework centered around viral mRNA (Fig. 1). By convention, mRNA is defined as positive sense and its complement as negative sense. The pathways leading from genome to message vary widely among the different virus families and form the basis of viral taxonomy. Their variety suggests that RNA viruses either had several different origins or that they have diverged expansively since their most recent common ancestors. Although it is generally believed that viruses originated from cellular organisms, it remains possible that some RNA virus components are descended directly from a primordial RNA or ribonucleoprotein world, which may have predated the emergence of DNA and cells.

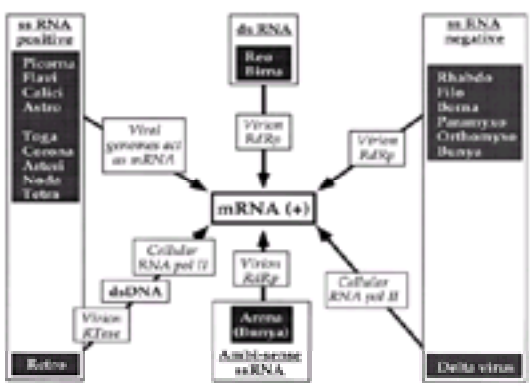

FIG. 1. Pathways of primary mRNA synthesis by RNA viruses of animals. How RNA viruses produce mRNA at the start of infection depends on the nature of the viral genome.

RNA Replication is Error Prone

The polymerases that catalyze RNA replication and reverse transcription have minimal proof-reading activities. As a result, their error rates are about 10 thousand times higher than those encountered during DNA replication, and they approximate the reciprocal of the number of nucleotides in the viral genome (10,19,32). This means that the genome of any individual RNA virus particle will contain an average of one or more mutations from the consensus wild-type sequence for that virus species. This simple fact has far-reaching consequences for the biology and evolution of RNA viruses, because it means that an RNA virus population does not represent a homogenous clone—either in nature or in the laboratory—but rather a molecular swarm of related nucleotide sequences clustered in sequence space around a master consensus sequence (14). This molecular swarm or “quasi-species” provides a fertile source of phenotypic variants that can respond rapidly to changing selection pressures by shifting its composition. As a consequence, RNA viruses can evolve up to one million times faster than can DNA-based organisms (10,14).

Their rapid rates of evolution are not without cost to RNA viruses, however, because high polymerase error rates impose upper limits on genome size. The combination of replicative error rate and genome size defines an “error threshold” above which a virus cannot maintain even the sequence integrity of its quasi-species (14). As a result, few RNA virus genomes contain more than 30 kilobases (kb), and most have between 5 kb and 15 kb. RNA genomes of this size are poised just below their error thresholds, and whereas their genetic diversity inevitably wastes individual progeny that may carry lethal mutations, the cost is offset by the potential for rapid evolutionary response.

Subcellular Sites of Genome Replication

Whereas most DNA viruses of eukaryotes (apart from the poxviruses) replicate their genomes in the nucleus, most RNA viruses replicate in the cytoplasm. However, in addition to the retroviruses, which integrate DNA copies of their genomes into cellular chromosomes, other notable exceptions to this generalization are the orthomyxo- and bornaviruses whose linear negative-sense RNA genomes replicate in the nucleus. The circular RNA genome of hepatitis delta virus, like the viroids of plants, also replicates in the nucleus (Table 1). Each site of replication presents distinct opportunities and challenges in terms of which cellular components and pathways are available to be parasitized, and how the synthesis and trafficking of viral proteins, genome replication, virion assembly, and the release of progeny can be coordinated. For example, RNA splicing occurs only in the nucleus, so this way to access multiple open reading frames in a single transcript can be used only by the retro-, orthomyxo-, and bornaviruses that transcribe there. It is interesting that the paramyxoviruses, which replicate in the cytoplasm, have instead evolved a transcriptional editing mechanism that achieves the same result (8).

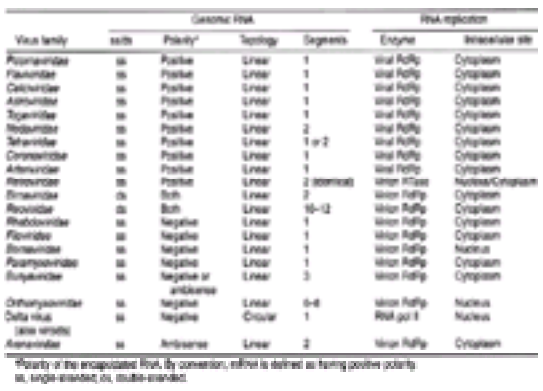

TABLE 1. *Families of RNA-containing viruses that infect animals*

Levels of Segmentation: Genes, mRNAs, and Proteins

Another distinctive feature of eukaryotic cells—besides their partitioning into nuclear and cytoplasmic compartments—has a profound influence on the biology of their viruses. On most mRNAs, eukaryotic ribosomes require a methylated cap structure at the 5' end that plays a critical role in signaling the initiation of protein synthesis. As a result, eukaryotes generally obey the rule “one mRNA, one polypeptide chain,” and with very few exceptions, each message operates as a single translational unit. Similarly, viral RNA-dependent RNA polymerases (RdRp's) generally appear somewhat limited in their ability to access internal initiation sites on RNA templates, and this creates the problem of how an RNA virus can derive several separate protein products from a single genome. Through evolution, different RNA virus families have found three solutions: fragmentation at the level of proteins, mRNAs, or genes, with some viruses using more than one level. For example, RNA viruses in the picorna-, toga-, flavi-, and retrovirus families rely on extensive proteolytic processing of polyprotein precursors to derive their final protein products (13). Others (in the corona-, arteri-, rhabdo-, and paramyxo-virus families, among others) depend on complex transcriptional mechanisms to produce several different monocistronic mRNAs from a single RNA template (8,26,39). Still others (in the reo-, orthomyxo-, bunya-, and arenavirus families, among others) have solved the problem by fragmenting their genomes and assembling virions that contain multiple genome segments, each often representing a single gene (see Table 1) (15,40,45). Among plant viruses, such RNA genome segments are often packaged into separate virions, necessitating co-infection by several virus

particles to transmit infectivity (50), but the genome segments of animal viruses are usually copackaged. In contrast, DNA viruses seldom use either genome segmentation or polyprotein processing. This is likely due to the relative ease with which monocistronic mRNAs can be transcribed from internal promoter sites on double-stranded (ds) DNA and the use of differential splicing of nuclear transcripts to express promoter-distal open reading frames.

STRUCTURES AND ORGANIZATION OF VIRAL RNA GENOMES

Unlike cellular genomes, which uniformly consist of dsDNA, viral RNA genomes provide examples of almost every structural variation imaginable. As shown in Table 1, different families of RNA viruses have genomes that consist of either ds or single-stranded (ss) RNA; of either positive, negative, or mixed (ambi-sense) polarity; of either linear or circular topology; and of either single or multiple RNA segments. Each of these variations has consequences for the pathways of genome replication, viral gene expression, and virion assembly.

Single- and Double-Stranded RNA Genomes

Although all RNA genomes replicate through conventional Watson–Crick base-pairing between complementary template and daughter strands, different RNA virus families encapsidate and transmit different molecular stages of the RNA replication cycle. Families of ssRNA viruses outnumber families of dsRNA viruses by almost 10 to one, roughly the inverse of the ratio found among DNA viruses. In view of the greater stability of ds nucleic acids of both types, this difference calls for an explanation. Two possibilities seem plausible: first, dsRNA viruses must somehow circumvent the translational suppression resulting from the coexistence of equimolar amounts of antisense RNAs. How the dsRNA reoviruses solve this problem is described in Chapter 52. Second, dsRNA is widely recognized by the cells of higher eukaryotes as a signal for the induction of defense mechanisms that act to suppress viral replication, such as the interferon system in vertebrates (27; also see Chapter 12) and gene silencing in plants (30). These two effects probably suffice to explain the relative scarcity of dsRNA viruses.

For these same reasons, it is important even for ssRNA viruses to limit the accumulation of replicative intermediates that contain regions of dsRNA, and the strategies to ensure this differ between positive- and negative-strand viruses. All positive-strand RNA viruses synthesize disproportionately low levels of negative-sense RNA—typically 1% to 5% of the levels of positive-sense RNA—and thereby minimize the potential for dsRNA accumulation. Conversely, negative-strand viruses, which need substantial amounts of both positive- and negative-sense RNAs to use as messages and progeny genomes, respectively, usually prevent them from annealing to one another by keeping the genomic RNA covered with a viral nucleocapsid protein (7,39).

Positive- and Negative-Sense RNA Genomes

The differences between positive- and negative-strand RNA viruses extend beyond the polarity of the RNA assembled into virions. Positive-sense RNA genomes exchange their virion proteins for ribosomes and cellular RNA-binding proteins at the start of infection. Once synthesized and assembled, the virus-specified RdRp and other nonstructural proteins replace the ribosomes to accomplish RNA replication. Virion structural proteins are reacquired during the assembly of viral progeny. In contrast, negative-sense RNA genomes and their antigenomic complements remain associated with their nucleocapsid proteins both within virus particles and throughout the viral replication cycle, even during RNA replication. These fundamentally different adaptations can be attributed to the fact that whereas positive-sense genomes must satisfy criteria for translation that are dictated by the host cell, negative-sense genomes and antigenomes must satisfy only the template requirements of the virus-specified RdRp because they are replicated but never translated. Although it remains unclear how polymerases can copy protein-coated RNA templates, the mechanism was surely refined during the coevolution of negative-strand viral nucleocapsids and their RdRp's relatively free from cellular constraints. Double-stranded RNA genomes are intermediate between these extremes: the parental genome segments remain sequestered in subviral particles, whereas positive-sense precursors to the progeny dsRNAs are initially unencapsidated. These distinctions most likely reflect significant differences in the structures of the viral RdRp–template complexes and in the molecular mechanisms of replication of positive, negative, and dsRNA genomes.

Linear and Circular RNA Genomes

RNA replication not only requires an acceptable error rate, as discussed earlier, but also must avoid the systematic deletion or addition of nucleotides. Genome termini are particularly troublesome in this respect, which has been dubbed “the end problem.” For DNA replication, the end problem is exacerbated by the fact that DNA polymerases cannot initiate daughter strands and must therefore depend on primers, which creates the additional complication of how to replicate the primer-binding site(s). Among several known solutions, the most economical and widespread in nature is to eliminate the ends by covalently circularizing the DNA, as in prokaryotic genomes.

Unlike DNA polymerases, most RNA polymerases do not require primers, so RNA genomes are less susceptible to the end problem. Accordingly, most RNA virus genomes are linear molecules. Covalently closed circular RNAs are found only in hepatitis delta virus in animals (see Table 1), and among the viroids and some other subviral RNA pathogens that infect plants (9,24). Nevertheless, the termini of linear RNAs are especially vulnerable to degradation, and their replication is likely to be particularly error prone. Consequently, every family of RNA viruses has features designed to preserve the termini of the genome. For example, many positive-strand RNA genomes carry the 5'-caps and 3'-poly(A) tracts that protect the termini of eukaryotic mRNAs against degradation, and similar roles are likely played by the Vpg protein that is covalently bound to the 5' end of picornavirus RNAs (36), and by the stable RNA secondary structures found at the 3' ends of flavivirus and other genomes. The 3' ends of many plant virus RNAs form cloverleaf structures that resemble tRNAs so closely that they are recognized by cellular tRNA charging and modifying enzymes (12). In addition to playing protective roles, terminal modifications of positive-sense RNAs also may serve to bring their ends together by binding to interacting cellular proteins such as the poly(A)-binding protein and cap-binding complex, thereby forming noncovalent functionally circular complexes that may promote repetitive translation by ribosomes and repetitive

replication by RdRp's.

Unlike the genomes of positive-sense RNA viruses, negative- and ambisense RNA genomes rarely carry covalent terminal modifications. Instead, these RNAs routinely show some degree of terminal sequence complementarity, which is thought to stabilize the viral nucleocapsid in a panhandle structure and promote RNA replication, perhaps by rendering the template functionally circular, as described for positive-strand RNAs (see Chapter 38, Chapter 41, and Chapter 46). Terminal complementarity also may enable the RNA ends to act as telomeres for one another, with the 5' end serving as a template to restore nucleotides eroded from the 3' end.

In other solutions to the end problem, there is evidence that some ambisense arenaviruses may be able to tolerate a substantial level of sequence variation at the termini of their genomic RNAs, presumably by locating all necessary cis-acting signals internally, well away from the RNA termini (41; also see Chapter 50). Retroviral genomes conversely are terminally redundant, and have direct repeats of between 12 and 235 nucleotides at each end that maintain and restore the integrity of the termini during reverse transcription and virus replication (28; also see Chapter 57).

Segmented and Nonsegmented RNA Genomes

Although genome segmentation facilitates the production of multiple gene products in eukaryotic cells, it also means that the various genome segments must each contain appropriate cis-acting signals to mediate their expression, replication, and assembly into virions. In some virus families with segmented genomes (such as the orthomyxo-viruses and some reoviruses), these signals comprise conserved sequences at the RNA termini, but in others (such as the bipartite noda- and tetraviruses), significant sequence conservation between the segments is undetectable. In these cases, the specificity of RNA replication and assembly is presumably determined by conserved RNA secondary or tertiary structures. However, the mechanisms that coordinate the replication and packaging of multiple genome segments remain poorly understood for any eukaryotic virus.

Evidently the evolutionary barrier between viruses with segmented and nonsegmented RNA genomes is readily traversed, because both genome types occur in members of the alphavirus-like supergroup, a taxonomic cluster based on phylogenetic comparisons of viral nonstructural protein sequences (23). Indeed, among the tetraviruses, segmented and nonsegmented genomes can be found even within the same family (31). Furthermore, the genomes of some toga- and rhabdoviruses, which are naturally nonsegmented, have been experimentally divided in two without destroying viral infectivity, thus confirming the flexibility of RNA virus genomes in this regard. Nevertheless, genome segmentation has major effects on the biology of a virus, because individual segments can often reassort between dissimilar strains in co-infected cells, enabling segmented genome viruses to make substantial evolutionary leaps by horizontal gene transfer. This mechanism underlies the antigenic shifts that produce new pandemic strains of the orthomyxo-virus influenza (see Chapter 47).

Cis-Acting Signals and Specificity

Replication and packaging of viral RNAs display striking specificity; both processes unerringly pick the correct viral molecules from among thousands of cellular RNAs that may be much more abundant. This is generally attributed to the presence of cis-acting signals that selectively channel the viral RNAs into replication and assembly complexes, but in most RNA virus genomes, these signals remain to be clearly identified. Those that have been characterized mostly comprise not linear nucleotide sequences but RNA secondary structures such as bulged stem-loops, tRNA-like cloverleaves, and pseudoknots, which are thought to create distinctive three-dimensional molecular shapes that interact specifically with viral enzymes and structural proteins (11). However, although high-resolution structures have been determined for some RdRp's, reverse transcriptases, and several viral capsids, our understanding of the molecular basis of specificity in RNA replication and virus assembly is limited by our scant knowledge of the three-dimensional structures of the viral RNA and its cis-acting signals. Not infrequently, the structural basis of RNA selectivity during replication and assembly has proved elusive, perhaps because the specificity determinants can be redundant, dispersed, or global properties of the viral genome. Furthermore, in both RNA replication and assembly, specific initiation is followed by less specific RNA–protein interactions that propagate the reactions. The transitions between these different stages are largely unexplored, and much remains to be learned concerning the recognition of cis-acting RNA signals and how they promulgate RNA replication and virus assembly.

Satellite RNAs and Defective RNA Genomes

Occasionally RNA molecules arise that are neither independently infectious nor essential for infectivity but nevertheless contain cis-acting signals that promote their own replication and/or packaging by the proteins encoded by another virus. Such satellite RNAs are parasitic on the parent virus and can modulate its replication and virulence (46). Among the RNA viruses of animals, a prime example is hepatitis delta virus, which packages its genome in virion proteins encoded by the hepadnavirus hepatitis B, and can severely exacerbate its pathogenicity (24; also see Chapter 88). Dependence of an RNA satellite on a DNA virus parent is unusual; more commonly, satellite RNAs are replicated and encapsidated by the proteins of an RNA virus parent with which they share at least some sequence homology. In some instances, satellite RNAs encode their own distinct capsid proteins, or proteins required for RNA replication (as in the case of hepatitis delta virus), but in others, they are translationally silent. Satellite RNAs are much more common among the viruses of plants than in those of animals (see Chapter 19), perhaps because the transmission of animal viruses between hosts generally involves narrower bottlenecks, which select against the spread of satellites.

In contrast to the transmission of viral infection between hosts, the spread of infection within a single animal usually involves successive episodes of localized virus replication, which resemble the conditions of plaque formation and serial high-multiplicity passage in cell culture. These conditions favor the generation and amplification of defective viral RNAs, which can arise from simple deletions of viral genes as well as from more complex genome rearrangements that occur during RNA replication. Like satellite RNAs, defective RNAs parasitize the parent virus and usually interfere with its replication, but because they also depend on it for their own survival, they typically establish a fluctuating coexistence

(46). Most families of animal RNA viruses readily generate defective interfering (DI) RNAs in cell culture, but their influence on viral disease and evolution is less well understood.

EXPRESSION AND REPLICATION OF RNA VIRUS GENOMES

Variety of RNA Virus Genome Strategies

The type of RNA genome largely determines whether the first step of macromolecular synthesis is translation, transcription, or RNA replication. Viruses with positive-sense ssRNA genomes (except the retroviruses) deliver their genomic RNAs directly to cellular ribosomes and begin the infectious cycle with translation (Fig. 2 and Fig. 3). Accordingly, these genomic RNAs are infectious even when completely deproteinized, as was first shown for tobacco mosaic virus RNA in classic work that helped to establish that genes were composed of nucleic acids rather than proteins (17). Positive-sense RNA viruses fall into two groups: those that produce subgenomic mRNAs and those that do not. For viruses in the latter group (like the picorna- and flaviviruses), genomic RNA directs the synthesis of a single polyprotein precursor to both the structural and nonstructural viral proteins, which are therefore produced in equimolar amounts (see Fig. 2). After cleavage by proteases contained within the polyprotein itself, the nonstructural proteins catalyze RNA replication to produce progeny genomes that assemble with the processed structural proteins to form progeny virus particles. For positive-sense viruses in the other group, which include the astro-, calici-, toga-, corona-, and arteriviruses (see Fig. 3), genomic RNA directs the synthesis of a precursor to only the nonstructural proteins, which include the viral RdRp. This enzyme then transcribes one or more subgenomic mRNAs that encode the structural proteins. Their greater transcriptional complexity allows these viruses to produce their proteins in dissimilar amounts.

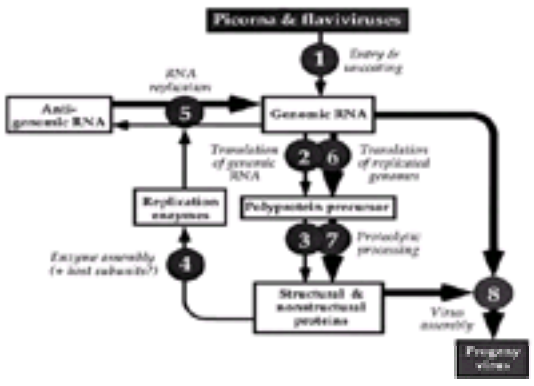

FIG. 2. Replication scheme of positive-strand RNA viruses that make no subgenomic mRNAs. After entry and uncoating (*step 1*), genomic RNA is used directly as the mRNA for both nonstructural and structural proteins (*steps 2 and 3*). The nonstructural proteins catalyze RNA replication via the synthesis of antigenomic RNA (*steps 4 and 5*). Replication produces more genomic RNA for further translation of viral proteins (*steps 6 and 7*) and for assembly with the structural proteins to produce progeny virus particles (*step 8*). For further details, see Chapter 23 and Chapter 32. In this and the figures that follow, *arrow thickness* denotes the relative magnitude of the reactions.

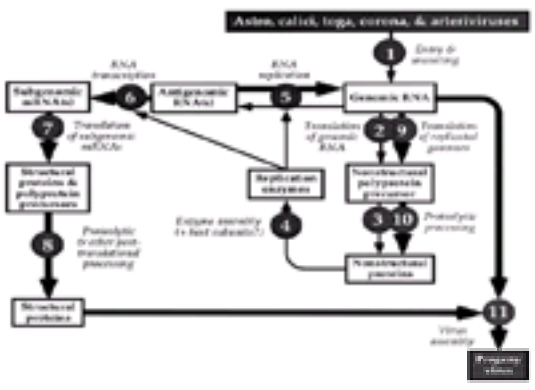

FIG. 3. Replication scheme of positive-strand RNA viruses that make one or more subgenomic mRNAs. After entry and uncoating (*step 1*), genomic RNA is used directly as the mRNA for nonstructural proteins that include the viral RNA-dependent RNA polymerases (RdRp's) (*steps 2 and 3*). The RdRp catalyzes RNA replication through the synthesis of full-length antigenomic RNA (*steps 4 and 5*). It also transcribes one or more subgenomic mRNAs (*step 6*), which direct synthesis of the viral structural proteins (*steps 7 and 8*). Replicated genomes are translated to produce more nonstructural proteins (*steps 9 and 10*), and assembled with structural proteins into progeny virus particles (*step 11*). Note that the corona- and arteriviruses (Chapter 35 and Chapter 37) synthesize their subgenomic mRNAs by a very different mechanism from that used by the toga-, astro-, and caliciviruses (Chapter 27, Chapter 28 and Chapter 29).

Viral genomes that consist of negative- or ambisense ssRNA or of dsRNA are not infectious by themselves because they must begin the infectious cycle by transcribing viral mRNAs, and uninfected cells do not contain an appropriate RdRp. Except in the case of hepatitis delta virus, this reaction is catalyzed by enzymes that are carried into cells by the infecting virions, which transcribe individual mRNAs for the viral proteins (Fig. 4, Fig. 5 and Fig. 6). For negative- and ambisense RNA viruses, the viral RdRp and newly synthesized nucleocapsid protein mediate RNA replication to produce antigenomic nucleocapsids (7,35). Although they are positive sense, antigenomes do not direct protein synthesis but instead are used exclusively as templates for RNA synthesis. For dsRNA viruses, individual mRNAs are transcribed from genome segments that remain enclosed in subviral particles (see Fig. 6). These ssRNAs and their translation products

reassemble into progeny subviral particles, where the synthesis of negative-strand RNAs then recreates the dsRNA genome segments (40,45,47). Uniquely among animal viruses, the genome of hepatitis delta virus, which is a negative-sense circular ssRNA, is transcribed and replicated by the cell's DNA-dependent RNA polymerase II in an uncharacteristic RNA-templated reaction (24; also see Chapter 88). Finally, retroviruses begin their infectious cycles by replicating their ssRNA genomes into dsDNA proviruses by using reverse transcriptase, an RNA/DNA-dependent DNA polymerase that is carried by the infecting virion (28). After integration into a host chromosome, proviral DNA is transcribed by cellular RNA polymerase II to produce unspliced and spliced viral mRNAs that direct viral protein synthesis (Fig. 7).

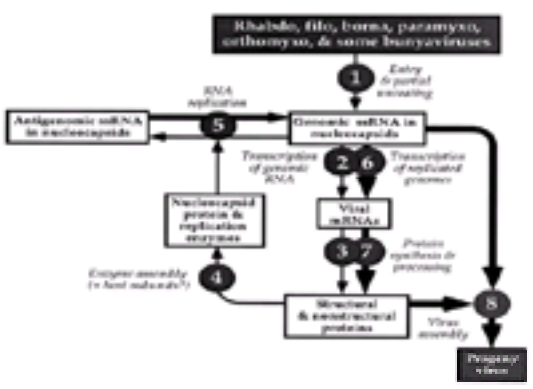

FIG. 4. Replication scheme of negative-strand RNA viruses. After entry and partial uncoating (*step 1*), encapsidated viral RNAs are transcribed by the nucleocapsid-associated RNA-dependent RNA polymerases (RdRp's) to produce mRNAs (*step 2*) for the viral proteins (*step 3*). These catalyze RNA replication through the synthesis of antigenomic nucleocapsids (*steps 4 and 5*). Replication produces more genomic nucleocapsids for further transcription (*step 6*) and translation (*step 7*) of viral genes, and for assembly with the structural proteins to produce progeny virus particles (*step 8*). Orthomyxo- and bornaviruses transcribe and replicate in the nucleus, and some of their mRNAs are spliced (Chapter 46 and Chapter 51). The other virus families listed replicate in the cytoplasm and avoid splicing (Chapter 38, Chapter 40, Chapter 41 and Chapter 48). Some bunyavirus genomes are negative sense and some are ambisense (Chapter 48 and Chapter 49; see Fig. 5).

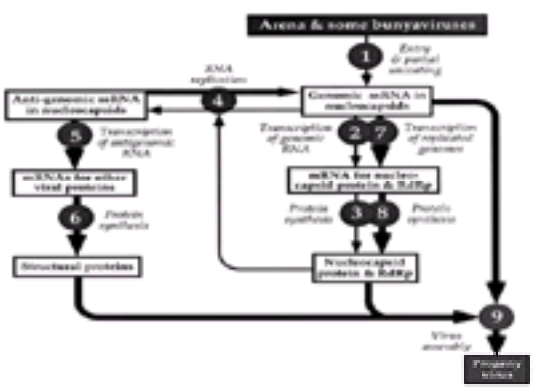

FIG. 5. Replication scheme of ambisense RNA viruses. After entry and partial uncoating (*step 1*), encapsidated viral RNAs are partially transcribed by the nucleocapsid-associated RNA-dependent RNA polymerases (RdRp's) to produce mRNAs (*step 2*) for the nucleocapsid protein and RdRp (*step 3*). These catalyze RNA replication through the synthesis of antigenomic nucleocapsids (*step 4*), which serve both as templates for transcription of mRNAs (*step 5*) for the other viral proteins (*step 6*), and for the production of more genomic nucleocapsids. Replicated genomic nucleocapsids serve as templates for transcription (*step 7*) and translation (*step 8*) of viral genes, and for assembly with the structural proteins to produce progeny virus particles (*step 9*). Some bunyavirus genomes are ambisense and some are negative sense (see Fig. 4). For further details, see Chapter 48 and Chapter 57.

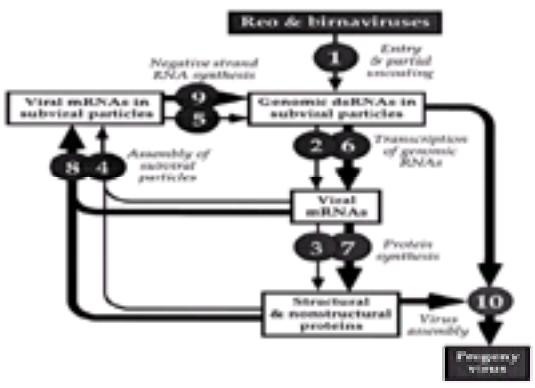

FIG. 6. Replication scheme of double-strand RNA viruses. After entry and partial uncoating (*step 1*), dsRNA segments within subviral cores are transcribed by the core-associated RNA-dependent RNA polymerases (RdRp's) to produce mRNAs (*step 2*) for the viral proteins (*step 3*). These form subviral particles around the mRNAs (*step 4*), which are then copied to produce genomic dsRNAs (*step 5*). Progeny subviral particles contribute to viral gene expression (*steps 6 and 7*) and replication (*steps 8 and 9*), and assemble with outer shell structural proteins to form progeny virus particles (*step 10*). For further details, see Chapter 52, Chapter 54, and Chapter 56.

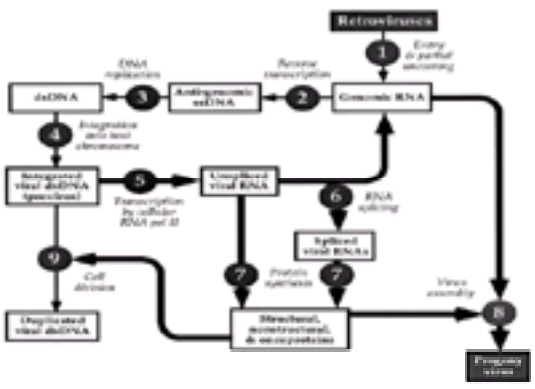

FIG. 7. Replication scheme of retroviruses. After entry and partial uncoating (*step 1*), genomic RNA is copied to dsDNA by reverse transcriptase (*steps 2 and 3*) and covalently integrated into cellular DNA in the nucleus by DNA integrase (*step 4*). Both enzymes are present in the viral core. The integrated viral genome (or provirus) is transcribed by cellular RNA polymerase II (*step 5*) to produce viral RNA transcripts that function both as precursors to mRNAs for the viral proteins (*steps 6 and 7*) and for assembly into progeny virus particles (*step 8*). For further details, see Chapter 58.

Regulation of Gene Expression

DNA viruses typically express different sets of genes before and after DNA replication, predominantly making catalytic amounts of nonstructural proteins during the prereplicative or early phase of infection, and stoichiometric amounts of structural proteins later, after DNA replication has amplified the gene copy-number. In contrast, most RNA viruses show little differentiation between the pre- and postreplicative phases of the infectious cycle, expressing their genes at roughly the same relative levels throughout infection. In cases like the toga- and influenza viruses, in which some temporal modulation of gene expression does occur, the differences are subtle and are accomplished mostly at the transcriptional level through modulation of mRNA levels.

However, posttranscriptional controls and countermeasures dominate the struggle between eukaryotic cells and viruses for the supremacy of gene expression. Apart from the retroviruses, RNA viruses generally inhibit host protein synthesis either directly or indirectly, and some specific mechanisms have been elucidated. For example, influenza virus blocks the splicing and nuclear export of cellular mRNAs, whereas polio- and rhinoviruses degrade mRNA cap-binding protein, but some other viruses monopolize translation simply by flooding the cell with highly efficient mRNAs (1). To counteract virus infection, some cells have evolved antiviral defense mechanisms such as gene silencing in plants and the interferon system in vertebrates, which intervene posttranscriptionally to degrade viral mRNAs and inhibit their translation (27,30; also see Chapter 12). In response, many viruses have acquired mechanisms to neutralize or evade these cellular defenses in ways that provide valuable insights into virus–host coevolution (1). Because retroviruses integrate DNA copies of their genomes into cellular chromosomes, their replication depends on the survival and proliferation of the host cell. Many retroviruses therefore express oncogenes that override cell-cycle controls and drive infected cells to divide (28).

Structural and Nonstructural Proteins

By definition, virus-specified structural proteins are incorporated into virus particles, whereas nonstructural proteins are found only in infected cells. However, negative-, ambisense, and dsRNA viruses assemble their RdRp's and associated enzymes into progeny virions and therefore encode predominantly or exclusively structural proteins. In addition to the polymerase, virus-encoded enzymes often include one or more proteases, an RNA helicase, guanylyl- and methyl-transferases, poly(A) polymerase, sometimes a nuclease, and in the case of the retroviruses, a DNA integrase. However, for several RNA viruses, the evidence that these enzymes are virally encoded is only circumstantial or is based on inconclusive sequence homologies.

The proteases process the primary translation products of which they are a part by cleaving them at highly specific target sequences, and in some picornavirus-infected cells, they also selectively inhibit host cell protein synthesis by cleavage of the cellular cap-binding protein (13). Particularly among the larger RNA viruses, RNA helicases may be required to disrupt inter- or intramolecular base-pairing during RNA synthesis, although some RdRp's are capable of melting RNA duplexes without help (21). Guanylyl- and methyltransferases construct the 5'-caps found on the mRNAs of almost all eukaryotic RNA viruses (42), except for the picornaviruses, which are uncapped, and the orthomyxo- and bunyaviruses, which steal the caps from cellular mRNAs by means of a cap-specific endonuclease (3,15,37). At their 3' ends, most animal virus mRNAs carry untemplated poly(A) tails, although 3' tRNA-like structures are common among plant RNA viruses (12,50). Polyadenylation is usually ascribed to a stuttering side-reaction of the viral RdRp rather than to a separate poly(A) polymerase, as in poxviruses, although the enzymology of the reaction remains to be clearly defined (38).

Comparisons among the amino acid sequences of viral RdRp's establish clear phylogenetic relationships that also reflect other differences in viral genome structures and strategies (23). Moreover, the x-ray crystal structures of the polio- and hepatitis C virus RdRp's (4,18) show unmistakable resemblances not only to one another but also to those of reverse transcriptases, as well as DNA-dependent RNA and DNA polymerases. Although the overall levels of amino acid sequence homology among these diverse polymerases are statistically insignificant, RdRp's share many of the characteristic polymerase motifs that occupy critical positions in the three-dimensional architecture of the polymerase active site (20,34). Taken together, the structural homologies of their RdRp's firmly anchor the RNA viruses to the rest of

the biosphere, and suggest that despite their diversity, these viruses radiated from common ancestors that probably escaped from cellular origins to establish a transmissible parasitic existence (see Chapter 13).

Host-Cell Proteins

Proteins provided by the host undoubtedly play essential roles in RNA virus replication, although different cellular proteins have been implicated in different virus systems (25,44). The clearest example comes from the RNA replicase enzymes of the RNA bacteriophages Qb and MS2, which, in addition to the single phage-specified polypeptide that provides the polymerase active site, contain four cell-specified subunits: the *Escherichia coli* ribosomal protein S1, two translation elongation factors (EF-Tu and EF-Ts), and a strand-specific RNA-binding protein, host factor-1 (2). In view of the similarity of their host components, the distinct specificities of the Qb and MS2 replicases for their cognate RNA templates must be determined by their unique viral subunits. The unexpected recruitment of translation factors to assist RNA replication—despite the evident differences between these two processes—may reflect underlying biochemical similarities in the RNA–protein interactions that are involved. Whether it also reflects a common evolutionary origin for translation and RNA replication is speculative, but the possibility is supported by the discovery that peptide bond synthesis is catalyzed by RNA.

Translation factors also have been implicated in RNA replication in some viruses of eukaryotes. For example, a subunit of the initiation factor eIF-3 binds to brome mosaic virus RdRp and increases its activity, and several other host proteins bind to the termini of some viral RNAs in infected cells. Among these are poly(A)- and polypyrimidine tract-binding proteins, calreticulin, and the snRNA-binding proteins Ro and La (5,29). Although it is often difficult to distinguish fortuitous interactions from those that play functional roles, the development of cell-free systems and recombinant strains of yeast that support the complete replication of certain viral RNAs promises clarification from biochemical and genetic studies, respectively (5).

Host-Cell Membranes

Unlike phage replicases, the RdRp's of eukaryotic viruses are invariably found associated with some type of supramolecular assembly: host-cell membranes for positive-sense RNA viruses, nucleocapsids for negative-sense RNA viruses, and subviral particles for dsRNA viruses. In general, the intracellular membranes of cells infected with positive-sense RNA viruses undergo proliferation and redistribution to form anchor sites for the viral RNA replication complexes. When these complexes are released from membranes during purification, they generally lose the ability to catalyze true RNA replication, although they often retain the more limited ability to copy an RNA template into its complement. In the case of the nodavirus flock house virus, true RNA replicase activity was restored to partially purified RdRp by adding glycerophospholipids to the cell-free extract (49), reinforcing the idea that membrane organization and dynamics play central roles in positive-sense RNA replication. The same conclusion is indicated by the inhibition of poliovirus RNA replication by brefeldin A, which interferes with intracellular membrane trafficking (5). Although the specific role of membranes is unclear, it is thought that they may expedite replication complex assembly by reducing the dimensionality of the process, and perhaps also segregate the RNA products from their templates.

Mechanisms of Replication and Transcription

The simplest mechanism of RNA replication is that used by hepatitis delta virus, in which rolling-circle synthesis by RNA polymerase II makes multimeric RNAs of both positive and negative polarity. Cis-acting ribozymes then cleave linear RNA monomers from these concatamers and covalently circularize them to produce mature antigenomes and genomes, respectively (24). Although this simple mechanism is shared by several viroids and other subviral RNA pathogens of plants (9), delta virus is the only animal RNA pathogen known to replicate by a rolling-circle mechanism. Far more commonly, ssRNA genomes are replicated through the synthesis of complementary RNA monomers produced by successive rounds of strand-displacement during end-to-end copying of linear templates (5). However, both ends of the RNA are generally required for template activity, suggesting that even linear RNAs may function as if they were circular, perhaps to facilitate reiterative replication and to hinder erosion of the termini.

The relative levels of genomic and antigenomic RNAs are regulated in different ways in different virus families. In the case of Qb, negative-strand synthesis is limited by the requirement for an additional host subunit that is not needed for the synthesis of positive-sense RNA (2). In some other systems, it appears that different cis-acting RNA signals are largely responsible for determining the relative template activities of the complementary strands, although it is possible that different host factors are involved here, too. For the togavirus Sindbis, negative-sense RNA is synthesized only by a transient version of the RdRp, an intermediate in the proteolytic processing pathway of the viral nonstructural proteins (43). As infection proceeds, the increased viral protease cleaves this intermediate and thereby switches the template specificity of the RdRp to the synthesis of positive-sense RNA (see Chapter 29).

Unlike the enzymes that replicate DNA, which usually require primers, most RdRp's can initiate RNA synthesis *de novo.* However, there are exceptions: picornavirus RdRp's use a small viral protein (Vpg), which is first covalently uridylated and then used as a primer for all viral RNA synthesis (36). In another mechanism of priming, the enzymes encoded by orthomyxo- and bunyaviruses cleave short capped oligonucleotides from host mRNAs and use them to prime transcription, although for replication, they initiate RNA synthesis *de novo* (37). Arenavirus RdRp's also initiate replication *de novo,* but they start at the penultimate nucleotide of the template and then realign the nascent strand and use it to prime replication of the entire genome, including the 3' nucleotide (16; also see Chapter 50). The ability of some plant virus RdRp's to restore several nucleotides to the 3' ends of their templates suggests that additional novel mechanisms of initiation await discovery (22).

Viral RdRp's also sometimes catalyze unorthodox reactions during RNA chain elongation. For example, some paramyxovirus polymerases edit selected transcripts internally by specifically inserting one or more nontemplated nucleotides, thereby allowing ribosomes access to additional open reading frames and expanding the coding potential of the viral genome (8). Somewhat similarly, negative-strand RNA virus RdRp's generally polyadenylate their transcripts by

stuttering on short runs of U residues in the template (38). Finally, almost all RdRp's occasionally switch templates during replication, thereby producing recombinant progeny molecules that, as satellite or DI RNAs, can influence both the course of infection and the evolution of the virus (6,33,48).

SUMMARY AND PERSPECTIVES

The many different genome strategies of RNA viruses furnish a wealth of unique biochemical pathways found nowhere else in the biosphere. As our understanding deepens, we can look forward to new opportunities for the development of antiviral agents that specifically target these pathways, like the successful antiretroviral protease inhibitors, for example. Furthermore, it is now possible to recover infectivity from cDNA clones of many RNA viruses and thus to manipulate their genotypes and phenotypes in systematic and deliberate ways. This technology has far-reaching potential for the creation of novel reagents, including recombinant vaccines, biopesticides, targeted cytolytic agents, and vectors for gene therapy. Both as foes and friends of mankind, RNA viruses have an interesting and exciting future.

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CHAPTER 6 Replication Strategies of DNA Viruses

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All viruses, whether they contain RNA or DNA genomes, adsorb to cell membranes, enter cells, uncoat, express their genes, replicate their genomes, assemble new virions, and exit from cells. These activities entail interactions with the host cell that range from simple binding to cellular receptors to complex modulation of host processes. In addition, DNA viruses carry out some steps that RNA viruses do not. For most DNA viruses, these steps include transport of virion DNA into the nucleus, initiation of transcription from this DNA and induction of transcription of additional viral genes, preparation of the cell for viral DNA replication, replication of the DNA genome, resolution and packaging of DNA into virions, and egress from the nucleus. In addition, many DNA viruses have evolved interesting mechanisms to evade host defenses, and many DNA viruses can cause tumors in animals. The intimate relationship between viruses and their host cells dictates that viruses exploit key cellular regulatory systems and usurp important cellular processes to carry out successful infection. Thus, studies of various aspects of DNA virus replication have provided fundamental new insights into important cellular processes including gene expression, DNA replication, and cell-cycle control.

This chapter reviews most of the steps just listed that are specific to DNA viruses. Nuclear entry and egress (which are not employed by poxviruses) are discussed in Chapter 7, and virus assembly and packaging of DNA per se are discussed in Chapter 8. Because of space constraints, this review cannot be comprehensive. A limited—and necessarily subjective and arbitrary—set of examples from various mammalian viruses is presented to illustrate unifying themes and interesting exceptions.

TRANSCRIPTION OF VIRAL DNA

Each mammalian cell contains about 6×10^9 base pairs of DNA and $>10^4$ active promoters. This presents a considerable challenge for viral DNA to compete effectively in recruiting the cellular transcriptional machinery to viral promoters. Moreover, once recruited, the machinery must generate viral transcripts efficiently. To meet this challenge, DNA viruses have adopted strategies to permit efficient initiation of transcription at one or more specific promoters on viral DNA shortly after it enters the nucleus. These promoters drive the transcription of what are frequently termed immediate early genes.

Enhancers

To recruit the transcriptional machinery to immediate early viral promoters and activate transcription, many DNA viruses contain enhancers—*cis*-acting regulatory sequences that, unlike promoters, can activate transcription from thousands of base pairs (bps) upstream or downstream of the transcriptional start site. This defining feature of enhancers emphasizes the competitive advantage they confer on viral promoters. Indeed, the high activity of the cytomegalovirus immediate early enhancer/promoter accounts for the popularity of its use in expression vectors. Although cellular genes frequently contain enhancers, the first enhancer to be recognized was that of the papovavirus SV40 (3,30,56). The SV40 enhancer consists of two tandem repeats of 72 bp, which contain binding sites for at least six different transcription factors, including multiple binding sites for certain factors. Although it is possible to create a synthetic enhancer by multimerizing a binding site for a single transcription factor, the multiplicity of factors and binding sites characteristic of natural enhancers appears to be required for optimal enhancer function.

Many enhancers also contain binding sites for “architectural” proteins, which do not necessarily have transcriptional activation functions on their own but rather promote the stereospecific, three-dimensional assembly of enhancer-protein complexes (“enhanceosomes”) (9). These nucleoprotein complexes can then activate transcription by interactions with the RNA polymerase II transcriptional machinery with considerable potency and specificity (29,72,73). Indeed, subtle sequence changes in papovavirus enhancers can drastically affect their function in different cell types (39).

Virion Transcription Factors

In addition to enhancers, some DNA viruses encode proteins that are packaged into virions, enter the cell with the viral DNA, and then facilitate transcription of immediate early genes. An extreme example of this is provided by poxviruses, which replicate in the cytoplasm and make no use of the cellular transcription machinery. Rather, as described in Chapter 84, poxviruses encode and package in virions their own RNA polymerases, capping and polyadenylation enzymes, and

transcription factors. Upon entry into the cell and uncoating, the viral transcription machinery becomes active and synthesizes viral immediate early transcripts.

A more general example is the herpes simplex virus (HSV) transactivator VP16 (also known as aTIF) (58,72,74). HSV VP16 is synthesized late in infection and is packaged into the portion of the virion known as tegument, which lies between the capsid and the envelope. Upon entry of HSV into cells, VP16 is transported to the nucleus and forms a complex with at least two cellular proteins—Oct-1, which is a transcription factor that recognizes an eight-bp DNA sequence, and HCF-1. This tripartite complex binds to specific DNA sequences with the consensus TAATGARAT (R = purine) upstream of HSV immediate early genes, with much of the DNA binding specificity and affinity provided by Oct-1. The complex then serves as a strong activator of transcription. Much of the activation is due to the C-terminal “activation domain” of VP16. This domain, when linked to virtually any sequence-specific DNA-binding domain that binds upstream of a TATA-box element of a promoter, can activate transcription in any eukaryote. Indeed, VP16 has served as a paradigmatic transcriptional activator. The VP16 activation domain can interact with many different proteins in the cellular transcriptional machinery, and it remains controversial as to whether any one protein is most important for its transcriptional activity. Indeed, the ability of VP16 to interact with more than one protein may account for the strength of its activation domain, given the evidence that this domain functions to recruit the transcriptional machinery to promoters rather than to stimulate the activity of any particular transcription protein (e.g., 41). Regardless, in the absence of an intact VP16 activation domain, the expression of HSV immediate early genes and viral replication is greatly impaired (1,64).

Stimulation of Gene Expression by Viral Immediate Early Proteins

As infection proceeds, DNA viruses typically express their genes in a highly ordered sequence. Although expression of viral genomes can be regulated posttranscriptionally, major control is exerted at the level of transcription. Viral immediate early genes generally encode proteins that stimulate expression of viral genes that are expressed subsequently during infection (the early and late genes). This enables early and late promoters, which usually lack enhancers or binding sites for transcription complexes such as those formed by virion proteins like VP16, to compete effectively with cellular promoters for the transcriptional machinery. Expression of some viral genes (e.g., papillomavirus late genes) is restricted to cells in a particular state of differentiation. Tissue-specific expression of essential cellular transcription factors may underlie this restriction. It should also be noted that several viruses also encode proteins that repress expression of certain viral genes at particular times in the virus life cycle. In addition, many viruses affect cellular gene expression. This can entail the induction and repression of specific genes, as well as a more nonspecific shut-off of cellular gene expression. The latter topic is considered in Chapter 7.

There appear to be many mechanisms by which viral immediate early proteins function. Certain of these proteins, such as the Epstein-Barr virus (EBV) BZLF1 protein (also known as Zta or Zebra), can behave like cellular transcription factors in that they bind to specific sequences upstream of genes and activate transcription (66). Other immediate early proteins increase the activity of cellular sequence-specific transcription factors. For example, the adenovirus E1A protein displaces members of the transcription factor E2F family from inhibitory retinoblastoma (Rb) family proteins. This increases transcription of adenovirus early genes that contain E2F binding sites (44) [and also has important consequences on cellular transcription and, indirectly, on viral and cellular DNA replication (see later)]. Other immediate early proteins, such as HSV ICP4, appear to stimulate transcription by direct interaction with the transcriptional machinery without binding to specific DNA sequences in the target promoter (31,36), while still others, like HSV ICP27, appear to stimulate viral gene expression by both transcriptional and posttranscriptional mechanisms (38,51,65). The net result of the activities of these viral proteins is that cells devote most of their gene expression machinery to production of viral gene products.

PREPARING THE CELL FOR VIRAL DNA REPLICATION

Viral Effects on Cell Cycle Progression

During productive viral infection, many DNA viruses carry out prodigious feats of DNA replication, in which a single viral genome gives rise to up to 100,000 or more genome copies in the course of a few days. This requires the action of many proteins, including DNA binding proteins and polymerases, as well as an abundant supply of nucleotides. Replication of some DNA viruses occurs only in cells that are naturally replicating their own DNA, thereby providing the necessary cellular environment for viral DNA replication. Many other DNA viruses also rely largely on the cellular DNA replication machinery, but these viruses encode proteins that induce cells to enter the necessary replicative state. Finally, some of the largest DNA viruses make limited use of the cellular replication machinery but rather encode viral versions of many of the necessary proteins.

The parvoviruses are among the simplest DNA viruses and contain single-stranded linear genomes. Parvoviruses can undergo DNA replication and carry out their full infectious cycle only in cells undergoing cellular DNA replication—that is, only in cells in the S-phase of the cell cycle (70,79). In fact, viral gene expression is not activated until after the cells enter S phase and the incoming DNA genome is converted into a double-stranded form suitable as a template for transcription (15). However, unlike certain other viruses that require cells to be actively replicating cellular DNA, parvoviruses are unable to induce the cells to enter S-phase, and thus they can carry out successful infection only if they encounter cells already committed to DNA synthesis. Some parvoviruses, notably adeno-associated virus (AAV), have even more stringent requirements and can replicate only if helper adenoviruses or herpesviruses provide viral gene products that permit parvovirus gene expression and DNA replication (7,62). Certain forms of cellular stress, such as DNA damage, can also induce a cellular state compatible with AAV DNA replication (62,83,84).

Many other DNA viruses induce cells to enter the replicative state required for viral DNA replication. For these viruses, viral DNA replication is the result of collaboration between cellular replication proteins and viral proteins that have direct roles in replication, such as initiator proteins that localize the replication apparatus to the viral replication origin. These DNA viruses generally induce cells to enter this essential replicative state by encoding proteins that participate in specific

protein–protein interactions with key cellular regulatory molecules, and some of these viral proteins have molecular chaperone activity that enables them to assemble these protein complexes (4,8,67). Often, these interactions lead to the neutralization of cellular tumor suppressor proteins such as p53 and members of the Rb protein family, whereas in other cases, these interactions result in the activation of cellular growth-stimulatory signal transduction pathways, which may indirectly inhibit Rb activity (Table 1).

Neutralization of tumor suppressor function
Direct effects on retinoblastoma expression or activity
Stimulation of cyclin-dependent kinase activity
Activation of growth-promoting signal transduction pathways
Receptor tyrosine kinase pathways
Intracellular signal transduction pathways

TABLE 1. *Mobilization of the host cell replication machinery by DNA Viruses*

The viral proteins that induce the replicative state commonly inactivate Rb family members p105 ^{Rb}, p107, and p130 (13). Rb inactivation relieves repression and releases E2F transcription factors to stimulate expression of numerous cellular proteins required for S-phase, including DNA polymerase a, thymidine kinase, ribonucleotide reductase, and thymidylate synthase (Fig. 1) (17,20,34). Some viral proteins, such as adenovirus E1A and human papillomavirus (HPV) E7, directly bind Rb proteins and inhibit Rb function, thereby activating E2F (2,27,81). Other viral proteins regulate the activity of cyclin-dependent kinases (cdks), which catalyze the phosphorylation of Rb, resulting in release of active E2F and transcription of E2F-regulated genes. Viral proteins that indirectly influence Rb activity in this way include herpesviruses-encoded cyclins that bind and activate cdks (40) and a variety of proteins that impair the expression or activity of proteins that inhibit cdk activity. For example, adenovirus E1B 55K and E4orf6 protein and HPV E6 inhibit the activity of p53, a transcriptional activator (24,61,80). Several viral proteins target the p53 coactivator CBP/p300. Abrogation of p53 function results in decreased expression of the p53-responsive gene encoding the cdk inhibitor p21, thus stimulating cdk activity. Similarly, adenovirus E1A binds the related cdk inhibitor p27, neutralizing its effects (50). SV40 large T antigen is a particularly versatile protein that not only binds and inactivates both Rb and p53 (19,45,49) but also carries out several functions directly required for viral DNA replication (see later) as well as essential roles in other aspects of the virus life cycle. Another mechanism is employed by polyomavirus middle T antigen and bovine papillomavirus E5 protein, which activate growth factor receptor signaling cascades and presumably induce expression of the cyclin D regulatory subunit of the cdks, thereby stimulating cdk activity and Rb phosphorylation (22,23,59). Certain herpesvirus and hepadnavirus proteins also appear to stimulate signal transduction cascades by activating intracellular signal transduction proteins such as NFkB, p21 ^{ras}, and pp60$^{c\text{-}src}$ (23). Although poxviruses encode growth factor homologs, these do not appear essential for viral DNA replication; rather, they are involved in various aspects of viral pathogenesis (6).

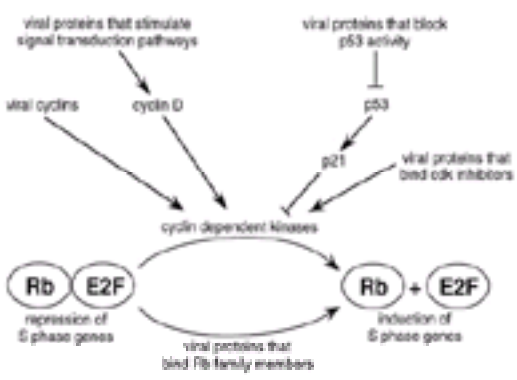

FIG. 1. Inactivation of retinoblastoma family members and activation of E2F by DNA tumor viruses. Viral proteins can cause the release of E2F family transcriptional activators from Rb-E2F repressor complexes by stimulating the activity of cyclin-dependent kinases or by binding directly to Rb family members. See text for details and examples.

Not surprisingly, induction of a panel of cellular DNA replication proteins has profound consequences on the host cell, which is often induced to undergo DNA replication and proliferation as a by-product of this mobilization of the cellular replication machinery. When this proliferative signal is sustained and tolerated, for example in nonpermissive cells that are unable to support viral DNA replication, the cells may undergo stable growth transformation, a topic covered in detail in Chapter 10. Thus, not only do many DNA viruses induce quiescent cells to reenter the cell cycle, but they also transform cells in culture and cause tumors in animals. Viewed in this light, the ability of many DNA tumor viruses to induce transformation is not a feature of normal viral replication but rather an aberrant cellular response to the need of the virus to generate the proper cellular milieu for viral DNA replication. In accord with this view, the parvoviruses, which as noted previously lack the ability to stimulate cellular DNA replication, are one of the few DNA virus groups that do not transform cells. However, the relationship between the ability of viruses to stimulate cellular DNA synthesis and to transform cells is not simple. For example, some herpesviruses stimulate DNA synthesis, others do not, and still others

actually inhibit cell proliferation. Such large viruses, with their large coding capacity, are able to create the proper environment for viral DNA replication without relying on mobilization of the cellular replication machinery.

The cell cycle normally is tightly regulated to prevent excess proliferation and ensure that each step in the cycle is successfully completed before the next step begins. When the cell cycle is disrupted, as is the case when unscheduled cellular DNA synthesis occurs, cells often initiate a suicidal response called *apoptosis*, or programmed cell death. Apoptosis is characterized by specific morphologic changes and biochemical events, including the activation of caspase proteases and the degradation of cellular DNA, and it is regulated by components of the cell cycle machinery. For example, E2F1 can stimulate apoptosis, and p53 appears to play a central role in mediating apoptosis in response to diverse stimuli. Because viruses often target these same cell cycle components, viral proteins can induce or inhibit apoptosis. In fact, DNA virus infection and the DNA synthesis response it elicits often induces host cell apoptosis. Because apoptosis and the resulting premature cell death can curtail virus production, viruses have evolved numerous mechanisms to inhibit apoptosis, including the neutralization of p53 function. Therefore, transformation by some viruses requires the seemingly antagonistic action of two or more viral proteins. For example, adenovirus E1A stimulates both proliferation and apoptosis, in part by inactivating Rb proteins and releasing active E2F1. Stable transformation ensues only if viral E1B gene products prevent E1A-induced apoptosis by interfering with p53 function or by acting downstream of p53 in the apoptotic pathway (18,60). By neutralizing p53, HPV E6 may block a similar apoptotic activity of E7. These relationships suggest that some viral oncogenes have evolved to thwart antiviral host defenses, such as apoptosis or interferon induction, by interfering with cellular pathways that mediate these defense mechanisms (54).

Whether viral oncogenes primarily function to generate the milieu for viral DNA replication or to counteract host defense mechanisms, the fundamental strategy is the same: The virus has evolved proteins that influence intracellular processes to allow vegetative viral replication, and the ability of these proteins to induce cell transformation and tumorigenesis is an unintended consequence of the underlying cellular biochemistry. The papillomaviruses are unusual in that benign tumor formation is an integral part of the virus life cycle. However, even for these viruses, carcinogenic progression is not a normal feature of the virus life cycle but rather a response to defective viruses with deregulated oncogene expression.

Provision of Nucleotides for DNA Replication

As described, parvoviruses require cells to be in S-phase to replicate, and papovaviruses and adenoviruses stimulate cells to enter S-phase. The requirement for S-phase is, in part, a requirement for cells to supply deoxynucleoside triphosphates (dNTPs) for DNA synthesis. Via effects on Rb and E2F family members, papovaviruses and adenoviruses induce the synthesis of enzymes such as ribonucleotide reductase that are required to generate sufficient dNTPs for viral DNA replication (Fig. 2). In contrast, herpesviruses and poxviruses are able to replicate in resting cells. One reason that these viruses can circumvent the requirement for S-phase is that they encode enzymes for dNTP synthesis, both of the *de novo* pathway (e.g., ribonucleotide reductase) and the salvage pathway (e.g., thymidine kinase) (see Fig. 2). In the case of HSV and varicella zoster virus, viral-encoded thymidine kinases provide Achilles heels for antiviral chemotherapy because these viral enzymes phosphorylate nucleoside analogs such as acyclovir more efficiently than do cellular enzymes. Following conversion by cellular enzymes to their triphosphate form, these dNTP analogs selectively impair viral DNA replication (37).

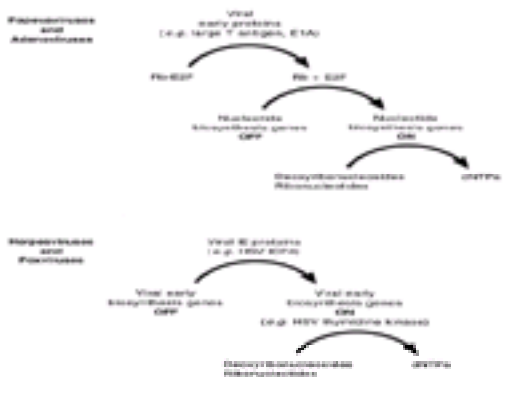

FIG. 2. Mechanisms of provision of nucleotides for DNA replication. **Top:** Papovaviruses and adenoviruses encode viral proteins such as SV40 large T antigen and the adenovirus E1A protein, which displace members of the E2F family of transcriptional activators from the retinoblastoma protein (Rb), as indicated in Fig. 1. The E2F transcription factors are then free to induce expression of numerous cellular genes required for DNA replication, including those that encode proteins that catalyze the formation of deoxyribonucleoside triphosphates (dNTPs) from deoxynucleosides or ribonucleotides. **Bottom:** Herpesviruses and poxviruses encode viral immediate early (IE) proteins such as herpes simplex virus (HSV) ICP4, which induce expression of viral enzymes involved in deoxyribonucleotide biosynthesis, such as HSV thymidine kinase.

VIRAL DNA REPLICATION

Initiation of Viral DNA Replication

Replication of DNA virus genomes generally initiates at specific sites called origins. Unlike most cellular origins, which are active once and only once per cell cycle, a viral replication origin can fire many times during a single replication cycle. Replication proceeds away from the origin either by utilizing classic replication forks with lagging and leading strand synthesis or by displacement synthesis with leading strand synthesis only. Viruses have evolved a number of strategies to assemble the replication apparatus at the origin and initiate DNA synthesis at the correct position. Most commonly, a viral early protein binds directly and specifically to the double-stranded DNA that comprises the origin and

recruits the cellular replication machinery to this site. Other viral genomes have a 3' end base-paired to a template strand that can be extended by polymerase, whereas in some cases unusual protein-priming strategies are used. Despite the diversity of initiation mechanisms, a common theme is the use of specific protein–protein interactions to recruit essential viral and cellular replication proteins to participate in replication initiation.

The role of the SV40 large T antigen in origin-dependent viral DNA replication has been particularly well studied (21,32,48). Large T antigen is the only SV40 protein required for viral DNA replication. It binds tightly and specifically to the SV40 origin, a short segment of viral DNA shown by numerous studies to be the necessary and sufficient *cis*-acting element for initiation of bidirectional viral DNA replication in permissive monkey cells. Elegant genetic studies established that the binding of large T antigen to the origin is required for replication (63). In the presence of ATP, hexameric complexes of large T antigen bind specifically to the origin and carry out several functions essential for replication initiation (Fig. 3). Large T antigen induces local DNA distortion and melting at AT-rich sequences within the origin. Large T antigen then associates with the cellular replication protein A (RPA), a single-stranded DNA binding protein, thereby permitting extensive DNA unwinding catalyzed by the intrinsic ATP-dependent helicase activity of large T antigen (52). DNA polymerase a-primase binds directly to the origin-bound large T antigen, and this replicative polymerase synthesizes short RNA sequences that serve as primers for viral DNA synthesis. The importance of the interaction between large T antigen and DNA polymerase a-primase is highlighted by the finding that the inability of viral DNA to replicate in nonpermissive cells is the result of the inability of large T antigen to recognize DNA polymerase a-primase from these cells (6,25,28,68). The molecular chaperone activity of large T antigen may play an important role in assembling the protein complexes necessary for initiation of replication.

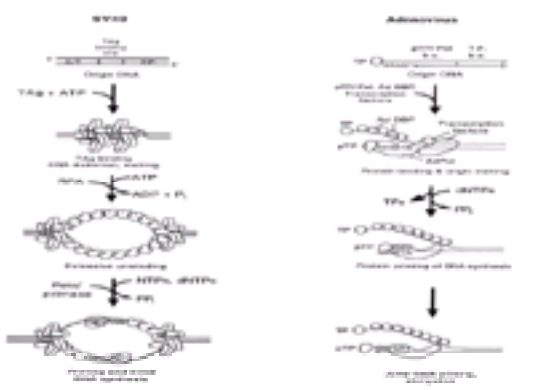

FIG. 3. Two mechanisms of initiation of DNA replication.
Left: A model for initiation of SV40 DNA synthesis. The *top panel* shows the SV40 origin with a binding site for SV40 large T antigen (TAg) flanked by a highly AT-rich sequence (A/T) and a modestly AT-rich sequence known as the early palindrome (E.P.). In the presence of ATP, two TAg hexamers form on the TAg binding site, leading to distortion and melting of the AT-rich sequences. Then, in the presence of the cellular heterotrimeric single-stranded DNA binding protein, replication protein A (RPA), TAg extensively unwinds origin DNA, a reaction driven by ATP hydrolysis. The four-subunit cellular DNA polymerase a/primase (Pola/primase) then binds to each single strand and catalyzes the incorporation of nucleoside triphosphates (NTPs) into RNA primers on each strand (*zigzag line*). The DNA polymerase catalyzes the incorporation of deoxynucleoside triphosphates (dNTPs), extending the RNA primers into newly synthesized strands of DNA. Pola/primase is then replaced at the primer terminus by replication factor C and subsequently the DNA polymerase d complex (not shown; see Fig. 4 for subsequent steps).
Right: A model for initiation of adenovirus DNA synthesis. The *top panel* shows the adenovirus terminal protein TP bound to the 5' end of an adenovirus origin of DNA synthesis at a terminus of adenovirus DNA. Near the end of the DNA is a binding site for the preterminal protein (pTP)-adenovirus DNA polymerase (Pol) complex (pTP/Pol b.s.) and an adjacent binding site for cellular transcription factors (T.F. b.s.). These transcription factors interact with Pol such that pTP/Pol binds to the template strand (sequence 5'-ATGATG-3'OH) with a serine side chain in pTP placed opposite the G, four bases from the 3' end of the viral DNA. This interaction is abetted by the adenovirus single-stranded DNA binding protein (Ad DBP), shown coating the TP-bound strand, thereby favoring the melting of origin sequences. Pol then adds a C to the serine hydroxyl moiety in the pTP protein primer and extends that C two bases in a template-dependent fashion, synthesizing the sequence CAT. The pTP/Pol bound to the newly synthesized CAT sequence then “jumps-back” to the very end of the template strand, and the CAT serves as a primer for elongation of the rest of the daughter DNA strand (see Fig. 4).

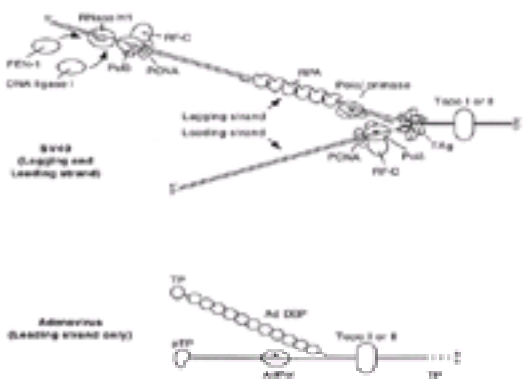

FIG. 4. Contrasting sets of proteins at the replication fork.
Top: A replication fork during SV40 DNA replication. The replication fork is shown as a branched DNA molecule, with 5' and 3' ends indicated. On the right, downstream of the replication fork, either a type I or a type II topoisomerase (Topo I or II) releases supercoiling generated during DNA synthesis. At the replication fork, a hexamer of SV40 large T antigen (TAg), the only viral protein present, serves as a helicase, unwinding DNA in front of the DNA synthetic machinery. The leading strand for DNA synthesis is shown as the bottom of the two branches with the two-subunit DNA polymerase d

(Pold) bound to the processivity factor, proliferating cell nuclear antigen (PCNA; this is trimeric, but it is shown as a ring around the DNA). The heteropentameric replication factor-C (RF-C) is drawn as a large oval bound to Pold and PCNA. RF-C loads the PCNA ring onto DNA, which is then recognized by Pold. The Pold complex synthesizes DNA in a continuous fashion, starting from the RNA–DNA primers laid down by DNA polymerasea/primase at the SV40 origin (see Fig. 3). The lagging strand for DNA synthesis is shown as the top of the two branches. Closest to the replication fork is the four-subunit DNA polymerase a/primase (Pola/primase). The primase subunit lays down RNA primers (*zigzag line*) on the lagging strand, and the DNA polymerase extends the RNA into newly synthesized DNA (*straight line*). To the left of the Pola/primase is replication protein A (RPA), coating single-stranded DNA ahead of Pola/primase. To the left of the RPA is a stretch of RNA–DNA primer previously synthesized by Pola/primase. The Pold/PCNA/RF-C complex, having assembled at the primer terminus, is drawn as extending the DNA strand. Farther to the left, a previously synthesized RNA primer is being digested by RNase H1. The nuclease FEN-1 (also known as MF-1) will remove the last ribonucleotide of the RNA primer. After the Pold complex finishes extending the DNA strand, DNA ligase I will ligate together the two Okazaki fragments. Although events on the leading and lagging strands are shown as occurring separately, there is evidence that they are coordinated within a large macromolecular complex.
Bottom: A replication fork during adenovirus DNA synthesis. The replication fork is shown as a branched DNA molecule. On the *right*, downstream of the replication fork, supercoiling generated during DNA synthesis is released by type I or type II topoisomerase, the only cellular protein present. The top branch is a single strand of parental viral DNA displaced during DNA synthesis, coated with the adenovirus single-stranded DNA binding protein (Ad DBP) and with the 5' end bound to terminal protein (TP). The double-stranded bottom branch is made up of a parental template strand and a newly synthesized strand whose 5' end is bound to preterminal protein (pTP) and whose 3' end is being extended by the adenovirus DNA polymerase. This enzyme synthesizes DNA in a continuous fashion starting from the pTP protein primer, as described in Fig. 3. Events at the adenovirus replication fork are much simpler than those at the SV40 replication fork, largely because there is no lagging strand synthesis.

Similar initiation strategies are used by other viruses with double-stranded origins. However, unlike SV40, in which all the required viral replication functions are contained within a single viral protein, other viruses are not so parsimonious. For example, the papillomaviruses encode two proteins, E1 and E2, required in *trans* for efficient viral DNA replication in rodent fibroblasts (75). The E1 protein appears to be the major viral replication protein, and, like large T antigen, is an ATP-dependent helicase. Both the E1 and E2 proteins bind to specific DNA sequences in the origin region, and they bind to each other (53). The presence of the E2 protein in the complex facilitates the specific, high-affinity binding of E1 to the origin. Moreover, both the E1 and E2 proteins participate in specific protein–protein interactions that localize cellular replication proteins such as DNA polymerase a-primase and RPA to the origin. The presence of more than one viral protein at the origin raises the possibility that different combinatorial multimeric protein complexes may play distinct roles in different phases of the virus life cycle, such as stable maintenance of plasmids at a low copy number during latent infection versus rapid amplification of viral DNA during vegetative DNA replication. Origins also often contain binding sites for cellular transcription factors, which may facilitate replication, perhaps by altering chromatin structure or by recruiting replication proteins. Some of the large DNA viruses, including HSV, have multiple specific origins (55,76). Unlike the case of the polyomaviruses and papillomaviruses, the detailed mechanics of origin recognition and initiation of replication have not been completely worked out for the larger DNA viruses, in part because of the lack of suitable *in vitro* DNA replication systems for these viruses. Indeed, the development and analysis of *in vitro* replication systems for small viral genomes has led to the identification of essential cellular replication proteins and to many important insights into the mechanism of cellular DNA replication (see later) (11,42,48).

The linear DNA packaged by a number of viruses has base-paired 3' ends that are extended as the initial steps in viral DNA replication. In the case of parvoviruses, these base-paired primer-template structures are formed by complicated stem-loop structures occurring at the ends of the viral genome that is otherwise single-stranded (15). These 3' ends are extended by cellular DNA polymerase in a reaction that does not appear to require viral proteins. The resulting double-stranded DNA replication intermediates are then nicked at specific sites by viral NS or Rep proteins to generate primers for ongoing DNA synthesis. Cellular DNA binding proteins collaborate with the viral proteins to ensure efficient nicking. These viral proteins also presumably play an important role in recruiting cellular replication proteins, including DNA polymerase, to these sites. The ends of the linear poxvirus genomes are imperfectly base-paired hairpin loops. Recent evidence indicates that these ends can serve as the sites of replication initiation in the cytoplasm, but the details of initiation of this virus group are obscure (26). Indeed, it is not known whether poxviruses encode all essential origin recognition and replication proteins, or whether the cell also makes a contribution.

The most unusual strategy for initiating viral DNA replication is the use of proteins as primers. The adenoviruses are the prototype viruses that use this strategy (33). Adenovirus DNA replication origins are located at inverted repeats at both ends of the linear double-stranded viral genome. The 5' end of each DNA strand contained within the virus particle is covalently linked to a virally encoded protein (the terminal protein). Immediately adjacent to the terminal protein are specific DNA sequences that comprise a core origin and an auxiliary region that binds cellular transcription factors. Viral DNA synthesis is initiated when a complex of the adenovirus DNA polymerase and the precursor to the terminal protein (preterminal protein, pTP) binds to the origin and melts the origin region in conjunction with a virally encoded single-stranded DNA binding protein (see Fig. 3). The binding of viral proteins to the origin may be assisted by transcription factors bound to the auxiliary sequences and by the bound terminal protein, although these proteins are not absolutely required. The adenovirus polymerase then adds nucleotides directly to pTP by a remarkable template-dependent mechanism that entails a “jump back” so that the first bases added correspond to the sequence of the 5' ends of the genome (12,43). The DNA 3'hydroxyl group of the pTP–oligonucleotide complex is then extended by viral DNA polymerase (see next section).

Provision and Action of Proteins That Act at the Replication Fork

Once viral DNA replication is initiated, the elongation phase of DNA synthesis proceeds via the concerted action of multiple proteins at replication forks. At replication forks in most organisms, one strand (the leading strand) is replicated

by simple 5'-3' extension in a continuous manner. Because the other strand (the lagging strand) must also be synthesized 5'-3', it is replicated discontinuously, by repeated synthesis and joining of short Okazaki fragments. Table 2 lists proteins that act at replication forks, and their functions.

Replication protein	Function
DNA Polymerase	Elongate DNA chains
Polymerase-accessory proteins (e.g., PCNA)	Stimulate polymerase, increase processivity
ss DNA binding protein	Remove hairpins, etc., especially on lagging strand
Primase	Lay down RNA primers on lagging strand
RNases	Remove RNA primers
Ligase	Seal nicks, especially on lagging strand
Helicase	Unwind DNA at fork
Topoisomerase	Remove superhelicity ahead of fork

PCNA, proliferating cell nuclear antigen.

TABLE 2. *Proteins that act at replication forks*

DNA viruses employ different strategies to provide these proteins. For the most part, small DNA viruses use cellular proteins at the replication fork. The best understood of these viruses is SV40 where the replication proteins involved were identified following the development of a cell-free system for replicating SV40 DNA *in vitro* (48) (Fig. 4). Subsequent work led to the purification of 10 proteins that are sufficient to catalyze the replication of SV40 DNA *in vitro* (32,77,78). Nine cellular activities were purified: DNA polymerase a, which is mainly responsible for initiating DNA synthesis at the origin and during lagging strand synthesis; primase, which is associated with DNA polymerase a and primes DNA synthesis at the origin and during the synthesis of Okazaki fragments; DNA polymerase d, which is mainly responsible for leading-strand synthesis and for completing synthesis of Okazaki fragments; proliferating cell nuclear antigen (PCNA), which binds to DNA polymerase d and forms a ring around DNA, thereby increasing polymerase processivity; replication factor-C, which loads the PCNA ring onto DNA and stimulates polymerase d; RPA, a single-stranded DNA binding protein; RNase H, which removes all but one ribonucleotide of the RNA primer; FEN-1 (also known as MF-1), an exonuclease that removes the remaining ribonucleotide; DNA ligase I, which ligates together Okazaki fragments; and either topoisomerase I or topoisomerase II to remove superhelicity during synthesis. Although it is difficult to demonstrate that these cellular proteins perform these functions during SV40 replication in living cells, the importance of their homologs for chromosomal DNA replication in yeast supports this idea. SV40 large T antigen, the only required viral protein, provides the requisite helicase function at the replication fork, in addition to the origin-binding and unwinding activities during initiation alluded to previously.

Other viruses provide almost all of the replication fork proteins. The elongation phase of adenovirus DNA replication *in vitro* requires only the single subunit adenovirus DNA polymerase; the adenovirus single-stranded DNA binding protein, which can increase the processivity of the polymerase; and either cellular topoisomerase I or II (57) (see Fig. 4). This remarkable simplicity is due in part to the unusual nature of adenovirus DNA replication, which does not entail lagging-strand synthesis (see later). Larger DNA viruses provide more activities still. For example, herpesviruses each encode a DNA polymerase, a processivity factor, a primase–helicase complex, and a single-stranded DNA binding protein (10). It is not known whether herpesviruses or poxviruses, whose full complement of virally encoded replication proteins has probably not yet been identified, require cellular proteins at all for synthesis of viral DNA.

STRATEGIES TO ENSURE COMPLETE REPLICATION, GENOME RESOLUTION, AND PACKAGING

It is critical for all organisms to replicate their entire genome. In most organisms, this problem is exacerbated by the semidiscontinuous nature of DNA replication, in which replication of the lagging strand requires laying down a primer; thus, one strand of a linear double-stranded DNA is never completed (the end-replication problem). Eukaryotic cells have overcome this problem by the use of telomeres. DNA viruses utilize several alternate strategies to solve the end-replication problem, which also inform their strategies for resolution of replicating DNA into daughter molecules and their packaging into virions.

Papovaviruses avoid the end-replication problem by being endless—that is, they are circular. Bidirectional replication generates complete circular daughter DNA molecules that are then resolved via the action of topoisomerase II (21). In the virion and in the cell nucleus, papovavirus DNA, like cellular DNA, is wound around nucleosomes, which are disrupted during replication. Daughter DNA molecules then reassemble into nucleosomes that are mixtures of “old” and “new” histones (82); thus, daughter minichromosomes are generated that are the substrates for packaging into new virions.

For other DNA viruses, virion DNA is not a simple double-stranded circle and is not wound around nucleosomes. Herpesvirus DNA is linear in the virion but circularizes when it enters cells. However, instead of generating circular daughter molecules, replication evidently generates long, concatameric molecules via rolling circle and perhaps recombinational mechanisms (10). To regenerate linear progeny DNA molecules with the correct ends, viral proteins cleave the viral DNA at specific sequences during packaging into capsids.

Adenoviruses, poxviruses, and parvoviruses, all with linear genomes, solve the end-replication problem in very different ways from papovaviruses and herpesviruses. Following initiation at one end of the adenovirus genome by protein priming (see earlier), DNA is extended from the protein primer continuously to the other end of the genome, displacing the preexisting parental strand intact (46). The displaced parental single-stranded DNA is replicated after its self-complementary inverted termini anneal, reconstructing a double-stranded origin, which is recognized by the initiation proteins (see earlier), leading to synthesis of another parental-daughter duplex. Thus, each parental duplex can be

replicated semiconservatively. The linear daughter DNAs are substrates for packaging into adenovirus virions, and pTP is cleaved to generate terminal protein. Poxviruses have linear genomes with sealed hairpin ends, so they are effectively circular single-stranded DNAs. They evidently also replicate via leading-strand synthesis only, involving the generation of concatameric, double-stranded genomes that are resolved into unit-length daughter molecules by cleavage at specific DNA sequences by viral proteins. Parvoviruses utilize similar mechanisms involving leading-strand synthesis to generate double-stranded replicative intermediates, but mature unit-length, single-stranded daughter DNA molecules are displaced during the final stages of DNA synthesis (71). These stages of parvovirus and poxvirus replication require complicated genome gymnastics that are described in Chapter 69 and Chapter 84.

HEPADNAVIRUSES: A RUBE GOLDBERG STRATEGY OF VIRAL DNA REPLICATION

Hepadnaviruses have a particularly complicated replication scheme, described in Chapter 86, that incorporates several of the features already outlined here and defies simple categorization. The viral genome in extracellular virus particles is a circular, largely double-stranded DNA molecule that contains a single-stranded gap. In a step similar to that used by parvoviruses, the 3' end of the shorter strand is extended to generate the fully double-stranded circular genome as the first step in viral DNA replication. With the aid of a viral enhancer, cellular RNA polymerase II then transcribes the nuclear double-stranded DNA genome from a specific promoter to generate a terminally redundant linear RNA pregenome that is packaged into immature virus particles. The single-stranded pregenome is later replicated to generate the mature, gapped virion DNA by virion reverse transcriptase (69), which utilizes several mechanisms including protein-priming similar to that employed by adenovirus, template jumping similar to that employed by retroviruses, and scavenging of RNA primers from degraded pregenomic viral RNA. Although this mechanism of genome replication may appear cumbersome, it evidently serves the needs of hepadnaviruses very well, as they often establish persistent infections that last for decades.

EVASION OF HOST DEFENSES AND LATENCY

Cells mount stout defenses against viral infection. Infection can induce the synthesis of interferons, which initiate a complex series of antiviral responses that inhibit virus replication. Cells can also respond to virus infection by undergoing apoptosis. If apoptosis occurs early during the virus life cycle, virus replication is terminated by the premature demise of the host cell, limiting virus yield. (Later in the life cycle, some viruses actively stimulate apoptosis, which might facilitate virus release and dissemination.) Finally, organisms can mount effective immune and inflammatory responses that prevent virus infection or lead to clearance of the virus or of virally infected cells. Spurred on by their evolutionary need to replicate and spread, viruses have evolved a number of mechanisms to overcome these defenses. The study of viral proteins that interfere with the cellular defense machinery is providing new insights into important cellular processes, such as immune recognition and apoptosis, and these viral proteins are being used as tools to identify and characterize key cellular proteins involved in these processes. Details of the interaction of host defense systems and viruses can be found in Chapter 7, Chapter 11, and Chapter 12, and in the chapters on specific viral groups.

Latent Infection

An effective mechanism of evading host defenses is for a virus to withdraw into a latent state in which the viral DNA is present at a low copy number, few viral proteins are expressed, and the cellular defenses are not mobilized. At some later time, perhaps when conditions are more conducive to virus replication, the virus can reenter the lytic, vegetative cycle. Herpesviruses commonly employ this strategy. A striking example is the latency involving varicella zoster virus, a herpesvirus that causes chicken pox during childhood but commonly sets up a latent infection in the neurons in dorsal root ganglia. When immunity has waned decades after the initial infection and acute disease, the virus can reinitiate lytic replication, resulting in lesions termed shingles in the body segment innervated by the affected dorsal root ganglion. The reactivated virus can even be transmitted to a new host.

In general, the mechanisms underlying establishment and release from latency are not well understood. EBV latency in B lymphocytes has been studied intensively (see Chapter 75), and its regulation exemplifies many of the principles discussed in this chapter. Upon infection of B cells, latency is established via an elaborate interplay of host and viral transcription factors acting at various viral promoters to activate or repress transcription. During latency, the viral genome is present in only a few extrachromosomal copies per cell, and the virus evades the immune system by expressing very few proteins, at least some of which are poor immunogens. In latently infected nondividing cells, viral proteins are not even required for replication of EBV DNA. In latently infected dividing cells, EBV DNA is replicated using a specific viral origin sequence and cellular replication proteins, and it is segregated properly to daughter cells by the action of the viral protein EBNA-1. At a low spontaneous rate, or at a high rate in response to certain stimuli such as chemical inducers, there is a dramatic induction of viral DNA synthesis utilizing a different replication origin and viral replication proteins. The viral protein Zta (or Zebra) appears to play the primary role in induction of EBV (16). This DNA-binding transcriptional activator induces the efficient expression of a variety of virus lytic cycle genes, including those required for high-level viral DNA replication. The molecular events responsible for activation of Zta during spontaneous and induced escape from latency are not known.

Smaller DNA viruses, such as papillomaviruses and some parvoviruses, also undergo latent infections. In the case of the papillomaviruses, the basal epithelial cells in virus-induced warts are latently infected: The viral genomes are present at low copy number, late gene products are not expressed, and no virus particles are produced. In the more differentiated cells of the same lesion, latency is disrupted and active viral replication and production of infectious virions takes place. The nature of the switch between latent and vegetative replication is obscure, but it appears to be tightly linked to the state of differentiation of the host cell. In latent parvovirus infection, the viral genome is integrated into the cellular DNA. Superinfection of these latently infected cells with helper adenoviruses or herpesviruses supplies factors required for reentry into vegetative parvovirus replication.

Transmission of Latent Viral Genomes to Daughter Cells

During latent infection, viral genomes must be faithfully segregated during cell division. Some latent viral genomes are integrated into the cellular DNA and are replicated and segregated passively during the appropriate stage of the cell cycle. This is the case for the nonautonomous parvovirus AAV, which in the absence of helper function stably integrates with high efficiency into a specific site in a cellular chromosome and remains latent until helper function is provided (14). Other latent viral genomes are often maintained as relatively low copy number plasmids. For these viruses, unequal segregation would lead to the loss of the viral DNA in some daughter cells. Bovine papillomavirus, which replicates as a nuclear plasmid in transformed rodent fibroblasts, has devised a clever strategy to achieve proper genome distribution during cell division (35,47). The viral E2 replication protein not only binds viral DNA but also associates with mitotic cellular chromosomes. The E2 protein thus tethers the viral DNA to the cell chromosomes as they undergo migration during mitosis and cytokinesis, and the viral DNA hitchhikes a ride into the daughter cells. The EBV protein EBNA1 may carry out a similar function during viral genome segregation during latency. In this fashion, the viruses take advantage of the elaborate cellular machinery that ensures proper chromosome segregation during cell division.

SUMMARY AND PERSPECTIVES

Viruses have evolved many different strategies to effect their replication. The diverse strategies employed for each step in replication are summarized in Table 3. Despite this diversity, there are remarkable similarities in how rather different viruses execute specific steps in their replication. For example, replication of the smallest (parvovirus) and that of the largest (poxvirus) viral DNAs appear to employ similar “rolling hairpin” mechanisms. These similarities presumably reflect the ability of viruses to identify and exploit or interfere with conserved cellular processes to carry out the essential steps of the virus life cycle: viral gene expression, genome replication, evasion of host defenses, and production of progeny virions. Viruses thus continue to be unique and powerful tools to elucidate these fundamental cellular processes. Unfortunately, many intricate and interesting mechanisms utilized by DNA viruses are not outlined in this brief chapter. Some of these mechanisms are described in the chapters on the various viruses in this volume; others remain to be uncovered.

Step	Strategy	Viruses
Initiation of transcription	Strong enhancer(s) Virion protein(s)	Papovaviruses, adenoviruses, hepadnaviruses Herpesviruses, poxviruses
Stimulation of subsequent viral gene expression	Viral-encoded transactivators	All
Modulation of cell cycle	Inactivation of tumor suppressors Stimulation of signal transduction pathways	Papovaviruses, adenoviruses Some papovaviruses, some herpesviruses
Provision of deoxynucleoside triphosphates	Stimulation of cell enzymes Synthesis of viral enzymes	Papovaviruses, adenoviruses Herpesviruses, poxviruses
Initiation of DNA synthesis	Internal origin-recognition proteins Protein-primers plus sequence recognition Preexisting or newly formed primers	Papovaviruses, some herpesviruses Adenoviruses, hepadnaviruses Parvoviruses, poxviruses, hepadnaviruses, some herpesviruses (?)
Provision of replication fork proteins	Mainly cellular proteins Mainly viral proteins	Parvoviruses, papovaviruses Adenoviruses, herpesviruses, poxviruses, hepadnaviruses
Genome completion and resolution	Circles to circles Circles to linear RNAs to circles Linears to linears Linears to circles to linears Hairpins to concatemers to hairpins	Papovaviruses Hepadnaviruses Adenoviruses Herpesviruses Parvoviruses, poxviruses
Evasion of host defenses	Interference with host defense systems Latent infections	Adenoviruses, papovaviruses, poxviruses, herpesviruses Some parvoviruses, some papovaviruses (including papillomaviruses), herpesviruses

TABLE 3. *Strategies utilized during DNA virus replication*

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CHAPTER 7 Virus–Host Cell Interactions

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By definition, viruses are unable to replicate on their own but must enter a host cell and use the host-cell macromolecular machinery and energy supplies to replicate. Thus, in many ways the study of viral replication is a study of the interactions of viruses with their host cells. The study of virus–host cell interactions has impacted several areas of biology and biomedical science. First, investigation of the mechanisms by which viruses replicate in, alter, or take over their host cells has often led to insight into basic cell biologic and molecular biologic mechanisms. Viruses often target critical regulatory events in the host cell; thus, knowledge of the processes subverted by viruses has often highlighted cellular regulatory mechanisms.

Second, during their replication within cells, viruses exploit host-cell molecules and processes at the expense of the host cell, which may result in cell injury or death. These injurious effects of viral replication in cells are one of the basic causes of viral disease. Therefore, precise knowledge of the pathogenic mechanisms by which viruses replicate in specific tissues, spread, and cause disease must come, in part, from studies of the intracellular replication of the virus. Over the past 45 years, increasing understanding of the mechanisms of viral replication has emerged from biochemical and cell biologic studies of virus replication in cultured cells. Investigators of viral pathogenesis have recently attempted to define the molecular events occurring in different cell types during the series of stages that define viral pathogenesis within a host organism.

Third, the definition of antiviral strategies requires knowledge of the replicative mechanisms of viruses and the identification of replicative steps that involve virus-specific processes and not host-cell processes.

This chapter will focus on the interactions of viruses with an individual host cell. Chapter 9 discusses the events that lead to (a) spread of a virus from one cell to another within a host organism, (b) induction of disease, and (c) spread within the environment. Virus infection of a cell can lead to any of several possible outcomes. First, a nonproductive infection can occur in which viral replication is blocked, and the host cell may or may not survive. After the nonproductive infection, the viral genome may be lost from the cell. Alternatively, the viral genetic information may become integrated as DNA in the cellular genome or may persist as episomal DNA in these surviving cells. If the growth properties of the cell are altered to make it oncogenic, this would constitute an oncogenic transformation event (see Chapter 10). The virus may become dormant with limited or no viral gene expression, and a latent infection is established (see Chapter 9). Second, a productive viral infection may result in which the host cell dies and lyses. Third, the cell may survive and continue to produce virus at a low level, resulting in a chronic infection (see Chapter 9).

Which of these possible scenarios is realized is determined by the nature of the interactions between the virus and the

host-cell constituents. For example, a nonproductive infection may result if a host-cell component necessary for viral replication is not present. One of the main goals of this chapter is to describe the types of interactions between virus-encoded macromolecules and the host cell, which may define the ultimate outcome of a virus infection. This chapter will examine (a) the molecular and cell biologic events that allow viral replication, (b) the ways in which viruses modify their host cells to promote their own replication, and (c) the kinds of mechanisms that may have evolved in cells to prevent virus infection. The types of experimental approaches used to obtain evidence for specific virus–host interactions will also be discussed.

The study of virus–cell interactions really started with the growth of viruses in cultured cells (83). Although infection of host organisms had given some indication of cell death resulting from viral infection, there was little clear evidence of other effects of viruses on the host cell before the infection of cultured cells and the identification of cytopathic effect (CPE) of viruses on cells (82). The elucidation of viral replication strategies in the 1950s and 1960s provided the broad outlines of virus replication. More recently, better probes for nucleic acids and proteins have allowed more precise descriptions of the molecular events of viral replication in a host cell. The techniques of molecular genetics and cell biology have begun to define the specific host-cell molecules and cellular compartments with which virus-encoded molecules interact.

In addition to classifying virus–host cell interactions in terms of the final outcome of the infection, virus–cell interactions can also be described in molecular terms with regard to individual replication events. For example, the effects of viruses on the host cell may be mediated by addition or substitution of a virus-specific macromolecule into a cellular complex or structure. Alternatively, the virus may mediate a covalent or noncovalent modification of a host-cell molecule. Virus infection may cause a disassembly or rearrangement of a host-cell complex or structure, or virus infection may lead to the assembly of a new infected cell–specific complex or structure in the infected cell. After defining these molecular interactions, the challenge for us as virologists is to relate this molecular information back to the biology of the virus in the host cell and ultimately the host organism.

CYTOPATHIC EFFECTS OF VIRUS INFECTION

One of the classic ways of detecting virus replication in cells was the observation of changes in cell structure, or CPEs, resulting from virus infection (Fig. 1, Fig. 2 and Fig. 3, and see Fig. 1 in Chapter 18). Some of the most common effects of viral infection are morphologic changes such as (a) cell rounding and detachment from the substrate (see Fig. 1), (b) cell lysis, (c) syncytium formation (see Fig. 2), and (d) inclusion body formation (see Fig. 3). The occurrence of cell morphologic changes resulting from CPE has even led to classification schemes for viruses. In 1954, Enders (1982) proposed classifying viruses into the following groups: (a) those causing cellular degeneration; (b) those causing formation of inclusion bodies and cell degeneration; and (c) those causing formation of multinucleated cells or syncytial masses and degeneration, with or without inclusion bodies. However, as described in Chapter 2, other classification schemes based on virion and genome structure and modes of replication have provided much better ways of classifying viruses.

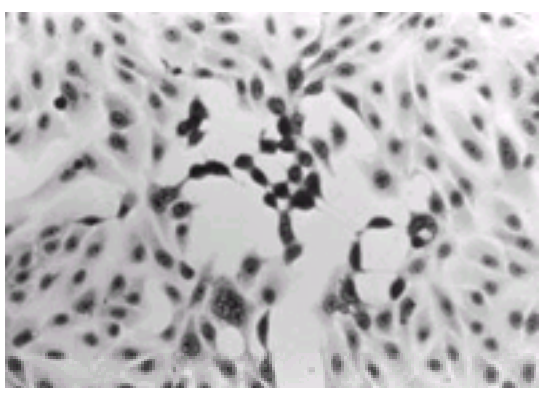

FIG. 1. Cytopathic effect (CPE) resulting from virus infection. The center portion of the figure shows monkey (Vero) cells rounding up and detaching from the substrate after infection with Herpes simplex virus 1 (HSV-1). A normal monolayer of cells is visible around the focus of CPE. The cells were fixed with methanol and stained with Giemsa stain. Micrograph courtesy of M. Kosz-Vnenchak.

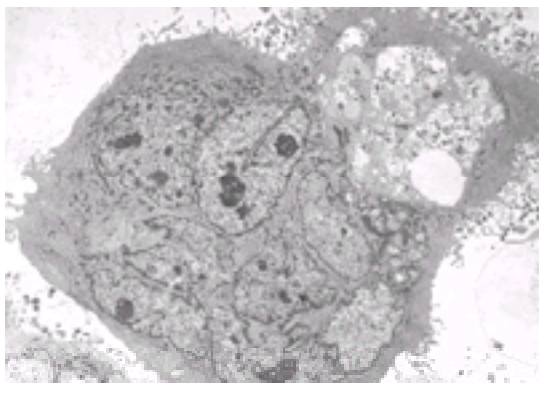

FIG. 2. A syncytium formed by human immunodeficiency virus (HIV) infection of T cells. Electron micrograph courtesy of J. Sodroski.

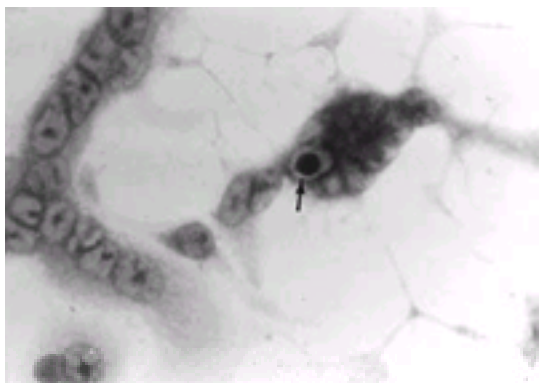

FIG. 3. Inclusion body formation in infected cells. The *arrow* indicates an intranuclear eosinophilic inclusion in a cell infected with HSV-1. The inclusion is surrounded by a clear halo. The cells were stained with hematoxylin and eosin. Micrograph courtesy of M. Kosz-Vnenchak.

The CPE of viruses on cells has also been called cell injury. These terms tend to emphasize the pathology of the host cell; however, from a virologist's point of view, we will see that many of the host-cell alterations by virus infection can now be explained as changes in the host cell that permit necessary steps in viral replication. Thus, many of the CPEs or cell injuries are secondary effects of the virus doing what it needs to do to replicate and are not simply toxic effects of viral gene products on the host cell. Some viral gene products cause toxic effects to the host with no apparent purpose, but as we learn more about the precise mechanisms of virus–cell interactions, many of these effects will likely be explained as aspects of essential steps in viral replication. For example, an adenovirus virion component was shown more than 30 years ago to cause monolayer cells to detach from the culture substrate (87,257), and this was later shown to be caused by the penton base protein (32,328,353). This remained an unexplained toxic effect until it was observed that the penton base protein binds to a_v integrins during adenovirus internalization (368). Thus, penton base protein causes cell detachment by binding through RGD sequences to cell-surface integrins, thereby displacing vitronectin from the integrins. The interaction of vitronectin and integrins is involved in cell attachment and spreading on the culture substrate.

A number of reviews and monographs have examined the various aspects of cytopathology resulting from virus infection (96,175,304). The reader is referred to these sources for detailed discussion and references on these topics. Determining the primary cause of death of a cell resulting from viral infection can be a complex and difficult issue because of the numerous events occurring within the infected cell. It is also apparent now that there are at least two general pathways for cell death: necrosis and apoptosis (programmed cell death). Necrosis involves the death of a cell as a result of physical damage or toxic agents. During necrosis, there is disruption of the mitochondria, swelling of the cell, disruption of organized structure, and lysis. Apoptosis involves cell death in situations controlled by physiologic stimuli during development and hormonal signaling in response to DNA damage. During apoptosis, there is DNA condensation and fragmentation, cell shrinkage, and membrane blebbing. Cell death due to viral infection could result from either mechanism in that viruses may have toxic effects on the host cell, activate programmed cell death pathways, or both.

USE OF HOST CELL MOLECULES AND PROCESSES FOR VIRAL ENTRY

Viruses must enter the host cell to replicate. Therefore, they must cross the cell plasma membrane to gain access to the cellular synthetic machinery in the cytoplasm and, in some cases, the nucleus of the host cell. Virus entry into the host cell has been divided into two events: (a) binding to cell-surface receptors and (b) penetration of the plasma membrane, or entry. These processes are described in detail in Chapter 4; thus, the purpose of this discussion is only to highlight the aspects of the host cell that are utilized during virus entry. Virus particles serve to protect the viral genome during spread from cell to cell or from one host to the next. The lipid envelope and the protein capsid act to protect the genome from nucleolytic attack or chemical reaction in the extracellular environment. As the virus enters a new host cell, these components must be removed to allow the viral genome to be replicated. Thus, viruses have evolved to have a stable, protective particle that can be readily disassembled in a new host cell. For the enveloped virus, cell entry by fusion serves two functions, a means to cross the plasma membrane barrier and a means to remove its outer coat, the envelope.

Viruses utilize a wide variety of cell-surface molecules as their receptors in that they can use protein molecules, carbohydrates, or glycolipids for their initial attachment to cells. Additional molecules or coreceptors may be involved in the fusion event between the viral envelope and the cellular plasma membrane. After binding to its surface receptor, a virus must cross the plasma membrane to replicate. Two general pathways have been defined for virus entry: (a) surface fusion between the viral lipid envelope and the cell plasma membrane and (b) receptor-mediated endocytosis (Fig. 4). Enveloped viruses can gain entry to the cytoplasm by fusion of their lipid envelope with the plasma membrane or endosomal membrane, whereas nonenveloped viruses must use alternative strategies to cross the membrane.

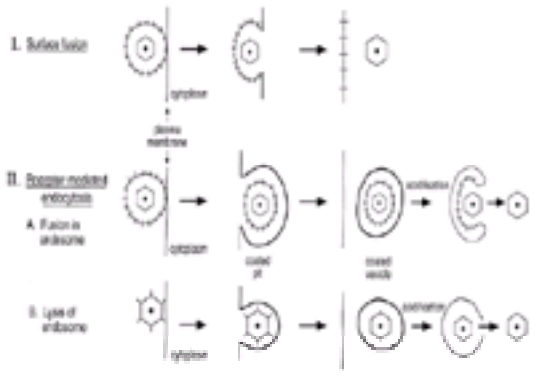

FIG. 4. Pathways for viral entry of the host cell.

Fusion at the surface involves the cellular plasma membrane (see Fig. 4, panel I), and uptake into the cell by endocytosis involves normal endocytotic mechanisms for uptake of molecules into vesicles within cells (see Fig. 4, panel IIA). Cellular proton pumps lower the pH in the endocytotic vesicles, activating membrane fusion between the viral and cellular membranes or activating enzymes that lyse the vesicle (see Fig. 4).

Based on these entry processes, virus–host interactions can be divided into at least seven strategies (Fig. 5). This classification depends on whether the viruses are enveloped or nonenveloped, and in which cell compartment replication and virus assembly or budding takes place. In the case of poliovirus (PV) (see Fig. 5, line 1), the native virion binds to the specific PV receptor and undergoes a receptor-mediated conformational change. The altered (A) particles, which appear to have lost an internal viral protein (VP4), form a pore in the endosome structure, thereby releasing the viral RNA into the cytoplasm. Certain members of the picornavirus group (but not PV) and other nonenveloped RNA viruses, including reovirus, have been shown to require the acidification of endosomes (119).

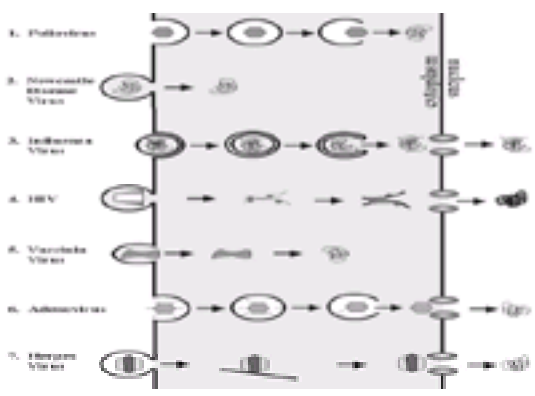

FIG. 5. Entry mechanisms for different classes of viruses. Nonenveloped RNA viruses such as poliovirus (*line 1*) and DNA viruses such as adenovirus (*line 6*) enter the cell by endocytosis at the cytoplasmic membrane. Enveloped RNA or DNA viruses can enter the cell by fusion at the cell membrane [Newcastle disease virus (*line 2*), HIV (*line 4*), vaccinia (*line 5*), and herpes (*line 7*) viruses] or by endocytosis and subsequent fusion of the viral and vesicular membranes [influenza virus (*line 3*)]. Viral replication may ensue in the cytoplasm (*lines 1, 2, 5*), or viral genomes are transported through the nuclear pore into the nucleus (*lines 3, 4, 6, and 7*), where they are replicated. For the sake of clarity, not all viral proteins implicated in entry are shown. Line 7 depicts only one of the two possible routes of HIV egress described in the text.

Even more complex is the entry of adenovirus, another nonenveloped virus (see Fig. 5, line 6). Following attachment to the cell and internalization, the virus undergoes a series of changes in which the viral protein interactions of the capsid are broken down. The acidification of the endosome is a required step in entry as well as the activity of the viral protease (120).

Many enveloped viruses, including paramyxoviruses such as Newcastle disease virus and the Sendai and parainfluenza viruses, fuse directly with the cytoplasmic membrane at neutral pH (see Fig. 5, line 2). For all of these viruses, the first step involves the binding of HN to its sialic acid receptor, which may cause HN to undergo a conformational change. This in turn triggers a conformational change in the F protein, which expresses the fusion peptide and allows it to embed in and perturb the cell membrane. This ultimately leads to the fusion of the cell and viral lipid bilayer (187,363).

A similar mechanism is postulated to mediate entry of retroviruses, including human immunodeficiency virus (HIV) (see Fig. 5, line 4). One of the differences between paramyxoviruses and retroviruses is that the cell binding and fusion activities of the latter are present in the same viral glycoprotein rather than in two different proteins. Also, the entry of HIV has an added degree of complexity in its requirement of coreceptors, which is a defining factor in the fusion process of HIV and target cells (19).

In contrast to the fusion at plasma membranes observed for many enveloped viruses, influenza viruses undergo an acid pH–mediated membrane fusion and entry process (see Fig. 5, line 3). Following binding to sialic acid on the cell surface via the hemagglutinin, the virus–receptor complex is endocytosed and the acidified environment of the endosome triggers a conformational rearrangement of the viral surface protein. The fusion reaction facilitated by the influenza virus hemagglutinin is one of the best characterized processes in virology and it has helped to provide a detailed

understanding of viral entry mechanisms (363).

To promote the release of the influenza viral ribonucleoprotein (vRNP) into the cytoplasm and to conclude the uncoating process, protons in the acidic endosome are pumped into the interior of the virus (see Fig. 5, line 3). The M2 protein in the influenza virion envelope serves as a proton channel (263,332) that exposes the nucleocapsid to low pH conditions, resulting in a conformational change in the M1 protein and its irreversible dissociation from the nucleocapsids (38,210). The antiviral drugs amantadine and rimantadine can block this ion channel and thereby interfere with uncoating of influenza A viruses (but not influenza B viruses).

The alphaherpesviruses make initial contact with the cell by low-affinity binding to glycosaminoglycans (i.e., heparan sulfates; see Fig. 5, line 7). Subsequent interactions with membrane coreceptors facilitate fusion of the viral membrane and the plasma membrane and subsequent entry. In the cytoplasm, the capsid structures undergo a microtubule-mediated transport to the nuclear pore complex (NPC) (322).

TARGETING OF VIRAL GENOMES TO INTRACELLULAR SITES FOR TRANSCRIPTION AND/OR REPLICATION

Once the viral nucleocapsid has gained entry to the cytoplasm, it needs to be targeted to the intracellular site where transcription and/or replication of the viral genome can take place. For most DNA viruses, the viral genome must localize into the nucleus, usually through the nuclear pores. The reverse transcription product of the retrovirus genome also must be transported into the nucleus. In general, trafficking of viral genomes into the cell nucleus involves normal cellular pathways, and, as described later, optimal replication may require targeting to specific sites within the nucleus. In contrast, the poxvirus DNA genome and most RNA virus genomes replicate in the cytoplasm. As we will see, some of these viruses replicate their genome at specific sites within the cytoplasm.

Transport of Viral Genomes into the Cell Nucleus

The cellular mechanisms for transport of macromolecules across the double membranous nuclear envelope involve the recognition of a nuclear localization signal (NLS) on the protein by cellular proteins called importins. This complex then binds to fibrils extending from the NPC and moves to the NPC. Translocation through the nuclear pore involves a complex of the Ran GTP-binding protein and p10 or NTF2.

Most viral nucleocapsids are too large to move through the nuclear pores, so alternative macromolecular complexes containing the viral genome must move through them. Retroviruses, except the lentiviruses, cannot enter the nucleus until mitosis, when the nuclear membrane is disassembled. In contrast, the lentiviruses have an additional gene product, Vpr, that is reported to promote the nuclear entry of the preintegration complex (140). Vpr may interact with importin a (karyopherin a) and the NPC, thus acting as an importin itself (357). Other viral determinants have also been described as contributing to the nuclear uptake of the lentivirus DNA genome. Specifically, the NLSs of the matrix (MA) (95) and of the integrase protein (IN) of HIV-1 (104) have been implicated in the uptake of DNA into the nucleus. However, the significance and contribution of these proteins to the import of HIV DNA into the nucleus remain in debate (98). In fact, HIV-1 nuclear import was recently described as dependent on a cis-acting determinant on the DNA itself. A three-stranded DNA structure, the central DNA flap, was reported to be crucial for nuclear import of the HIV-1 preintegration complex (383).

Influenza virus is unique among RNA viruses in that its RNPs must localize into the nucleus because its transcriptase uses the 5' ends of cellular messenger RNAs (mRNAs) as primers for transcription of the genomic RNAs. The viral RNPs then enter the nucleus through the nuclear pores. All four protein components of the vRNPs (NP, PB1, PB2, and PA) have NLSs and could drive the RNPs into the nucleus (65,243,244,319,360). Nevertheless, the transport of the vRNP depends primarily on the NLSs present in the NP protein. It has been demonstrated that NP binds to the NPI-1 (karyopherin a protein), which then binds karyopherin b, resulting in a docking complex at the nuclear pore (Fig. 6). In studies with permeabilized cells, the influenza virus NP has been shown to be the crucial factor in allowing the import of the incoming RNP from the cytoplasm to the nucleus.

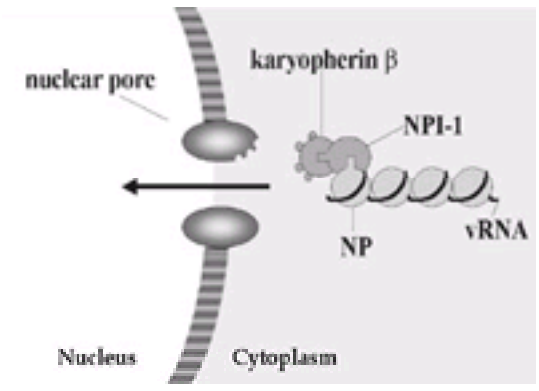

FIG. 6. Influenza viral ribonucleoprotein (RNP) import. Import of influenza viral RNPs is mediated by soluble cellular proteins NPI-1 (karyopherin a) and by karyopherin b. Interaction of the RNP complexes with these cellular factors is mediated by nuclear localization signals (NLSs) located on the viral nucleoprotein (NP). The NLS receptor consists of NPI-1, which binds to the NLS, and karyopherin b, which interacts directly with the proteins of the nuclear pore.

The DNA viruses uncoat their genomic DNA before it enters the nucleus through the nuclear pores. After adenovirus enters the cell by endocytosis and disruption of the endosome, it binds to microtubules that mediate movement to the

nuclear membrane. The adenovirus capsids then bind to the NPC (228), and the viral DNA is transferred into the nucleus in a process that requires metabolic energy (47). The capsid is disassembled during or soon after release of the DNA. Herpesvirus nucleocapsids bind to the dynein motor protein, which transports the nucleocapsid along microtubules to the microtubule-organizing center located near the nucleus (322). These nucleocapsids also bind to the NPC, and the DNA is transferred into the nucleus, but the empty capsid remains at the nuclear pore. Simian virus 40 (SV40) and polyoma viruses are both taken into the cell in endocytotic vesicles, and delivery of the virions from the vesicles to the cytoplasm, the endoplasmic reticulum, and the nucleus has been reported (123,165,217). The precise route of entry of the papovaviruses into the nucleus remains to be defined.

Once within the nucleus, viral DNAs appear to be targeted to specific sites for transcription and possibly initiation of replication. The genomic DNAs of herpes simplex virus (HSV), adenovirus, and SV40 localize to intranuclear sites at the periphery of nuclear domain 10 (ND10) structures, where immediate-early and early gene transcription takes place (155,216). HSV DNA replication proteins localize to these same sites, at which viral DNA synthesis then initiates (216,352). As viral DNA replication progresses, these punctate structures grow into the larger replication compartments in the infected cell nucleus (66). The mechanisms of targeting viral DNA and viral proteins within the cell nucleus remain to be defined, but these viral processes provide some of the best evidence for specific structural organization of the interior of the cell nucleus.

Targeting of Viral Genomes in the Cytoplasm

Much less is known about the localization of viral genomes to specific sites in the cytoplasm for replication. It has long been known that PV replicates on membranes in the cytoplasm (40,62), and other positive-strand RNA viruses also replicate on the cytoplasmic face of internal membranes (13,24,124,308,356,364). In contrast, other viruses such as vesicular stomatitis virus (VSV) and paramyxoviruses replicate on the cytoskeleton (128,131,142).

TAKING OVER THE CELLULAR TRANSCRIPTION MACHINERY

Viral mRNA is needed during replication of viruses to encode viral proteins for replication of the genomic nucleic acid and for virion assembly. In the case of positive-strand RNA viruses, genomic RNA can be used as mRNA directly, so synthesis of viral RNA need not precede initial rounds of translation. However, for negative-strand RNA viruses and DNA viruses, *de novo* synthesis of viral mRNA must occur. If the virion contains an RNA polymerase, then synthesis of viral mRNA may depend on cellular factors to only a limited extent. However, if the virus uses cellular polymerases to synthesize mRNA, specific mechanisms may have evolved to promote transcription of viral DNA or to shift the host-cell RNA polymerase to the transcription of viral DNA. Viruses may even encode a new transcription factor (29).

DNA viruses often exhibit two general phases of transcription during infection of the host cell. First, they must ensure efficient transcription of at least part of their DNA genome immediately upon entry into the cell, a process known as immediate-early or early gene transcription. This has been accomplished by packaging a new polymerase in the virion (poxviruses), packaging a transcriptional adaptor molecule in the virion (HSV), or having a strong transcriptional enhancer upstream from the promoter for the immediate-early genes (e.g., adenovirus, papovaviruses, and cytomegalovirus). Second, they must be capable of efficient transcription of the later viral genes, called early, delayed-early, and late genes. The strategy used by DNA viruses to achieve this last aim is to encode a trans-activator protein as an immediate-early or early viral gene product that activates later viral gene expression after the viral DNA template has increased in number. The following sections describe how viruses inhibit host-cell transcription and ensure efficient immediate-early transcription and later viral gene transcription.

The general process of transcription in eukaryotic cells can be described as follows: Preinitiation complexes are formed by the binding of the general transcription factors, primarily transcription factors II-D (TFII-D), TFII-B, and TFII-I, to the promoter, and this complex then positions RNA polymerase II (pol II) near the start site of transcription. The pol II molecules that initiate transcription are not phosphorylated on the C-terminal domain (CTD), which consists of 52 repeats of a heptapeptide sequence (YSPTSPT). Molecules bound to the CTD also interact with activators bound to the DNA, and these interactions increase the number of initiation events. The general transcription factor TFII-E is bound to pol II and binds TFII-H, which contains DNA helicases and the CDK-activating kinase. The helicase activities open the double-stranded DNA so that it can be transcribed by pol II, and the kinase activity phosphorylates the CTD, which destabilizes the binding of the mediators and activators. Elongation factors, which include capping enzymes, splicing factors, and polyadenylation machinery, can then bind to the CTD and promote elongation. Viruses target several of these steps to inhibit pol II transcription or to divert it to their own advantage.

Inhibition of Cellular Transcription

Infection with many viruses leads to an inhibition of transcription of cellular protein-coding genes by host RNA pol II. However, little is known about how DNA viruses cause an inhibition of host-cell transcription, except for possible competition for RNA pol II and cell transcription factors. For RNA viruses, which do not use host-cell RNA polymerases for their replication, the presumed advantage conferred by this inhibition would be to provide larger pools of ribonucleoside triphosphate pools for viral RNA synthesis. Possible mechanisms for inhibition of host transcription have been formulated for cells infected with the RNA viruses VSV and PV. VSV infection causes rapid inhibition of host RNA synthesis, and this inhibition requires transcription of the viral genome (213,362). Two viral components have been implicated in the process of RNA pol II inhibition. Earlier data suggested that the leader RNA—a short (approximately 50 nucleotides long) transcript synthesized from the 3' end of the virion genome—has this activity (125). However, Dunigan et al. (80) cast doubt on this interpretation because the kinetics of expression of the leader RNA in infected cells did not correlate with the timing of the shut off of host mRNA transcription. The second component of VSV that has been shown to inhibit host transcription is the M (matrix) protein (26). This activity of the M protein appears to be independent of that required for viral assembly at the cytoplasmic membrane (93). The inhibition of RNA pol II–dependent transcription by the VSV M protein is not cell-type specific. Although the mechanism by which the VSV M protein exerts its activity is not well defined, recent data suggest that the TFII-D is altered, resulting in an inhibition of host transcription (379). This

inactivation of TFII-D is probably not mediated through a direct protein–protein interaction, but rather through indirect effects of the VSV M protein in the nucleus. Yet another mechanism of modulation of TFII-D by viruses has been observed. Infection by PV results in the proteolytic cleavage of TATA-binding protein (TBP) through the viral protease 3C (53).

Transcription factor II-D is not unique in being a target for virus-mediated inhibition of transcription. Inhibition of host mRNA transcription may involve cleavage of capped host mRNAs through a viral polymerase complex. It appears that influenza viruses inhibit the expression of host genes at least in part through the viral activity of cap snatching, which is localized to the nucleus of infected cells (182). In addition, it has been reported that the influenza A virus NS1 protein targets the poly(A)-binding protein II (PAB-II) and the cleavage and polyadenylation specificity factor (CPSF) of the cellular 3'-end processing machinery (48,235). When both host activities are inhibited by the viral NS1 protein, this should result in a severe reduction of the level of functional host mRNA transcripts. Whether the induction of a proteolytic activity (301) in influenza virus-infected cells is a third major factor contributing to the remarkable ability of influenza viruses to shut off host transcription remains to be seen.

On the other hand, many RNA viruses—including negative-strand RNA viruses such as Sendai virus—do not appear to have an appreciable effect on host transcription. Although these viruses can cause cytopathic and cytolytic effects, they may do so by mechanisms that do not involve the inhibition of cellular transcription.

Mechanisms to Ensure High-Level Transcription upon Viral Entry

DNA viruses have several general mechanisms to promote efficient transcription of their DNA immediately after entry into cells, and many of these mechanisms involve some form of subversion of the host transcriptional apparatus.

Packaging a Virally Encoded RNA Polymerase in the Virion

Poxviruses contain a double-stranded DNA genome but replicate in the cytoplasm. These viruses have evolved their own transcriptional and DNA replication machinery, allowing these processes to occur in the cytoplasm, almost completely independent of the host cell nucleus. The poxvirus particle contains a virally encoded RNA polymerase (166) capable of transcribing about one half of the viral genome (30,250). The presence of the RNA polymerase in the virion ensures efficient transcription of the viral genome upon entry into the cell.

Packaging a Transcriptional Activator in the Virion

The best-known example of a virion trans-activator is the HSV virion protein 16 (VP16). HSV VP16 (also called aTIF or Vmw65) is assembled into the virion and, when introduced into the cell, becomes a part of a transcriptional activation complex that specifically stimulates viral immediate-early (a) gene expression (41,271). VP16 binds to host-cell proteins (221,272); specifically, it is thought to bind to at least one host factor, the HCF protein complex (180), and this complex binds to the transcription factor Oct-1 bound to DNA (112). One portion of VP16 binds to HCF and Oct-1, whereas another activates transcription (346) by binding to basal transcription factors. Thus, HSV provides for adequate transcription of its immediate-early genes by carrying in its virion a viral trans-activator that binds to a cellular protein, which in turn binds to specific DNA sequences located in immediate-early gene promoters, thereby stimulating transcription and causing increased transcriptional initiation on immediate-early viral gene promoters that contain this specific sequence.

Use of an Enhancer to Ensure High-Level Immediate Transcription

An alternative mechanism by which DNA viruses promote efficient transcription of viral genes immediately after infection is to have an enhancer sequence located upstream of these genes. Enhancers are blocks of DNA sequences containing multiple binding sites for transcription factors acting in synergy to promote high-level transcription from a nearby gene. They act in a position- and orientation-independent manner. Enhancers were, in fact, first identified in viral genomes (12,17). For example, the early transcriptional units of SV40 virus (12,17), polyoma virus (351), human papilloma virus (58), the adenovirus E1A transcriptional unit (139), and the human cytomegalovirus IE1/IE2 transcriptional unit (31,343) all contain enhancers. These enhancer sequences provide for utilization of multiple cellular transcription factors in the transcription of the earliest genes of these viruses immediately after infection.

Mechanisms for Activation of Later Viral Genes

Many DNA viruses encode trans-activators among their earliest gene products. The SV40 large T-antigen stimulates late transcription, both by promoting viral DNA replication and by binding to host-cell transcription factors (127). The adenovirus E1A, the HSV ICP4, and the papilloma virus E2 proteins all serve to stimulate later viral gene transcription by altering the activity of host-cell RNA pol II. The mechanism of trans-activation has been most extensively studied with the adenovirus E1A proteins (23,160). The E1A gene encodes two mRNAs, 13S and 12S, by differential splicing, and much of the trans-activation activity is caused by the 13S gene product. The E1A proteins increase the expression of pol II–transcribed genes (121,152,237) and pol III–transcribed genes (20,109,144). The promoter requirements for E1A trans-activation coincide with basal-level promoter elements (22,238). The E1A proteins do not bind to DNA specifically or efficiently, and there is now considerable evidence that E1A stimulates transcription through various effects on cellular transcription factors (191,316,374). The numerous specific effects of E1A are described in detail in Chapter 67, but the general mechanisms are relevant here. The E1A protein activates transcription by interacting with basal transcription factors, by interacting with activating proteins that bind to upstream promoter and enhancer elements, and by interacting with other regulatory or DNA-binding proteins. E1A protein binds directly to the TBP of TFII-D to activate transcription (145,192), and it also binds to p53 and Dr1 and relieves their repressive effects on transcription (146,179). E1A can also bind to TAF subunits of TFII-D and TFII-F (110,198,218).

E1A can activate transcription of certain genes by binding to the pRb protein, releasing E2F to activate transcription at its

cognate sites (8). E1A can also activate transcription by binding to ATF transcription factors, by binding to YY1 to relieve its repressive effects, and by binding to AP1 to activate transcription. E1A activates pol III transcription of the adenoviral VA genes by increasing the amount of the IIIC2a transcription factor.

Promotion of RNA Polymerase II Elongation on Viral Templates

Transcription of the HIV genome by pol II is very inefficient in the absence of prior viral gene expression. However, when the HIV Tat protein is expressed, it serves as a novel transcription activator in that it regulates elongation rather than initiation of transcription and acts by binding to the nascent transcript rather than to DNA (337). Pol II initiates transcription in the HIV long terminal repeat (LTR) promoter but synthesizes only short RNA products. Tat binds to a specific sequence called the trans-activation response (TAR) element on the nascent RNA molecule and promotes elongation of transcription. This is accomplished by Tat interacting with the cyclin subunit of the positive elongation factor b (P-TEFb) and with TAR. Tat also interacts with cyclin T1 and recruits the cyclin-dependent kinase 9, which phosphorylates the CTD of pol II. This phosphorylation activates pol II for elongation along the proviral genome. Thus, HIV Tat promotes transcription of the HIV genome by a novel mechanism, and elucidation of this mechanism has provided insight into the molecules regulating transcriptional elongation.

VIRUSES CAN REGULATE CELLULAR SIGNAL TRANSDUCTION PATHWAYS

Viruses often modify the host cell signal transduction pathways for their own benefit. The effect may be exerted by the binding of virions to a surface receptor on the cell surface, by a new protein encoded by modifying the activity of a cellular protein, or by a viral protein mimicking a cellular protein. For example, binding of human cytomegalovirus to the cell surface is believed to activate NF-kB (34), which would induce expression of cellular regulatory proteins, some of which would block the interferon response (386). Similarly, binding of Epstein-Barr virus to its receptor, CR2, activates B-cell signaling pathways (286). As a third example of this mechanism, binding of adenovirus to a_v integrins activates mitogen-activated protein (MAP) kinase signaling pathways (236). An example of a different mechanism is human herpesvirus 8, which is associated with Kaposi's sarcoma, encoding a protein, vIRF, that heterodimerizes with the host interferon regulatory factor, IRF-1, and blocks its action in interferon responses (see later).

TAKING OVER THE CELLULAR RNA PROCESSING AND TRANSPORT MACHINERY

RNA splicing and transport to the cytoplasm are cellular pathways often used by viruses for maturation of their mRNA from nucleus to cytoplasm. In fact, the first evidence for RNA splicing came when the adenovirus late mRNAs were mapped on the viral DNA by R-loop hybridization (21,51). These studies showed that adenoviral late mRNAs are encoded by noncontiguous portions of the genome. Splicing of the viral mRNA precursors is accomplished by cellular enzymes recognizing splice donor and acceptor sequences in the viral RNA. However, some viruses use the cellular splicing mechanisms but regulate the extent to which the full-length transcript is spliced. For example, influenza and retroviruses have transcripts that are infrequently spliced (see Chapter 46 and Chapter 57). In cells infected with influenza virus, the viral NS1 and M1 RNAs are spliced to yield the NS2/NEP and M2 mRNAs, respectively, at a frequency of 10% (186,188). Although splicing of the NS1 RNA is inefficient, formation of the spliceosome complex involving the snRNPs U1, U2, U4, U5, and U6 is efficient (2). Thus, the block seems to occur after formation of the spliceosome complex. The block may be mediated by the structure of the RNA itself or by a virus-encoded protein. This appears to be a situation in which virus infection determines the extent of splicing of one of the viral RNAs, thereby regulating the levels of one of its own gene products. The NS1 gene product has been postulated to be responsible for this regulatory event (181). However, a mutant influenza virus lacking the entire NS1 open reading frame (ORF) was shown to have normal levels of splicing of the viral mRNAs (106).

Adenovirus inhibits maturation of cellular mRNA at a different stage. In adenovirus-infected cells, cell transcripts are synthesized and processed but do not accumulate in the cytoplasm (15). The adenoviral E1B-55kD and E4-34kD proteins are required for inhibition of transport of cellular mRNA as well as for promotion of cytoplasmic accumulation of viral mRNA (7,130,262). The E1B and E4 proteins have been observed to localize within and around the nuclear inclusions where viral transcription is believed to occur (245). Thus, these proteins may redistribute a cellular factor involved in mRNA transport. This system may provide important insights into the regulation and specificity of mRNA transport.

A similar observation has been made in cells infected with HSV, in which transport and processing of host-cell RNA is inhibited (359). In HSV-infected cells, small nuclear ribonucleoprotein particles become aggregated (211), and this may be related to impaired transport or splicing. The HSV IE protein ICP27 or IE63 has been reported to be necessary and sufficient for this effect (133,134,259), possibly by affecting phosphorylation of SRP proteins. Only four of the more than 70 HSV genes contain introns. Thus, inhibition of host-cell splicing should contribute to host shutoff, but the mechanisms for specific retention of viral RNA splicing, if any exist, are unknown.

The regulation of RNA maturation may also provide a mechanism for temporal regulation of viral gene expression. HIV encodes several regulatory gene products from spliced mRNAs. One of these regulatory gene products, the *rev* gene product, stimulates the cytoplasmic accumulation of unspliced viral mRNAs that encode the viral structural proteins (90,173,207,294,323,341). The Rev protein seems to promote the export of newly synthesized viral transcripts to the cytoplasm so that the splicing pathway is avoided (208). The Rev protein enters the nucleus using a classic NLS and binds to the viral RNA via a Rev-responsive element (RRE) (61). The Rev-RNA complex is then recognized by the nuclear export machinery. Specifically, it appears that the nuclear export signal (NES) of rev binds to the CRM1 exportin (94), which in turn interacts with members of the nucleoporin family. The NES-mediated interaction of Rev with CRM1 is inhibited by the drug leptomycin B (371). Lentiviruses, including HIV and human T-cell lymphoma virus (HTLV)-1, have developed this mechanism of a viral protein (Rev and Rex, respectively) that binds to viral RNAs and chaperones them through the NPC.

Other retroviruses also transport unspliced or partially spliced viral mRNAs from the nucleus into the cytoplasm.

However, they do this by relying on a different mechanism. Many oncovirus RNAs possess so-called constitutive transport elements (CTEs), which interact directly with the cellular RNA export machinery without the mediation of a rev-like viral protein. Tang et al. (336) showed that a cellular RNA helicase A binds to the CTE from simian retrovirus (SRV)-1 and thus facilitates the export process. Similar CTE-like elements are found in all oncovirus RNAs, and they are thought to allow unspliced (or partially spliced) viral mRNAs to be effectively transported from the nucleus to the cytoplasm. Like the retroviruses, the hepatitis B viruses produce RNA transcripts in the nucleus that possess a cis-acting posttranscriptional regulatory element (PRE) that facilitates interactions with the nuclear export machinery (289).

Influenza viruses face yet another RNA maturation/transport problem. Not only does transcription of viral mRNAs occur in the nucleus, but also the entire genome of the virus replicates in infected cells. In fact, one incoming genome complement (consisting of eight RNA segments) may result in the amplification of up to 100,000 copies, which must then be transported out of the nucleus. The work of many groups now indicates that the newly amplified virion (v) RNAs are not in naked form in the nucleus but exist as complexes with the viral NP and the polymerase (P) proteins. In addition, the presence of the viral M1 protein appears to be necessary for the formation of mature nucleoplasmic vRNP structures destined for export into the cytoplasm (366). Finally, the influenza virus nuclear export protein (NEP) (formerly the NS2 protein), which had no previously assigned function, has been shown to facilitate the nuclear export of virion RNAs by acting as an adaptor between vRNP/M1 complexes and the nuclear export machinery of the cell. NEP interacts with cellular nucleoporins and CRM1 and can functionally replace the effector domain of the HIV-1 Rev protein. A functional domain on the NEP with characteristics of an NES was mapped: It mediates rapid nuclear export when cross-linked to a reporter protein. Microinjection of anti-NEP antibodies into infected cells inhibited nuclear export of vRNPs, suggesting that the Rev-like NEP mediates this process. In the model shown in Figure 7, NEP acts as a protein adaptor molecule bridging influenza vRNPs and the NPCs of infected cells. This novel export mechanism for genomic viral RNA differs from that mediated by Rev for HIV-1 RNA in that the influenza viral NEP does not interact directly with the virion RNA. Rather, the NEP functions as a chaperone for the newly formed influenza virus ribonucleoprotein complexes.

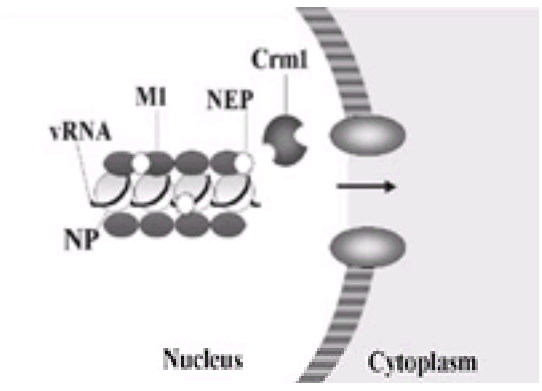

FIG. 7. Influenza viral ribonucleoprotein (RNP) nuclear export. The nuclear export of amplified influenza viral RNP complexes is mediated by the viral nuclear export protein (NEP). NEP interacts with a cellular export factor, Crm1, and with the viral RNPs via the viral matrix (M1) protein. By interacting with the cellular export machinery, NEP mediates the transport of the viral genome from the nucleus to the cytoplasm via the nuclear pore.

Influenza virus intervenes in and takes advantage of the host-cell mRNA maturation pathway in another novel way. Influenza mRNA transcription from the genomic RNA segments occurs in the host cell nucleus (141). Cellular nascent transcripts are cleaved by a virus-encoded endonuclease, and the 5' end of the host transcript is used as a primer for synthesis of viral mRNA from the viral genome (33,266). Thus, influenza virus transcriptional complexes subvert the host mRNA maturation pathway to obtain primer molecules for the viral transcriptional process, and then the virus most likely uses the cellular mRNA export machinery to transport its viral mRNAs out of the nucleus.

TAKING OVER THE TRANSLATIONAL MACHINERY: STRATEGIES FOR OPTIMIZATION OF VIRAL PROTEIN SYNTHESIS AND ANTAGONISM OF CELLULAR PROTEIN SYNTHESIS

Animal viruses utilize the host translational machinery for production of their own proteins. The biochemical pathway of mRNA translation in animal cells is summarized in Figure 8. Several strategies are utilized by viruses to optimize their own protein synthesis, and to antagonize cellular protein synthesis, by altering the host translational machinery. For some virus–cell combinations, the efficiency of mRNA translation (that is, the amount of protein produced per unit of mRNA) varies during the infective process. Multiple biochemical mechanisms account for the translational control observed in virus-infected cells. Many, but not all, are operative at the initiation phase of mRNA translation, the phase generally believed to be rate-limiting.

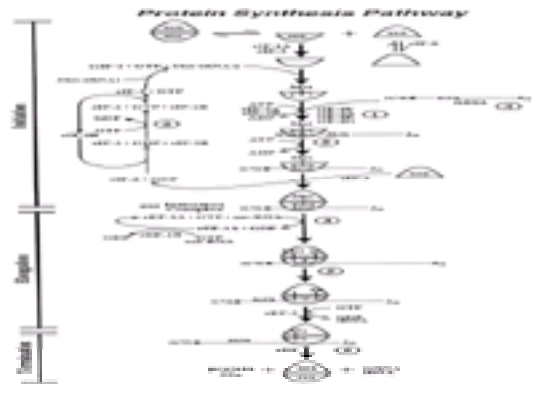

FIG. 8. The cellular protein synthesis pathway and examples of the modification of the process by viruses. **1:** Modification of eIF-4F initiation factor function involved in recruitment of the 40S subunit to mRNA. Picornaviral 2A protease catalyzes cleavage of the eIF-4G component of the eIF-4F complex and also the poly A binding protein; phosphorylation of the eIF-4E component is decreased in cells infected with adenovirus, influenza virus, and encephalomyocarditis virus. **2:** Modification of eIF-2 initiation factor function involved in binding of tRNA to the 40S subunit. Phosphorylation of the a subunit of eIF-2 is antagonized by several viral RNAs (including adenovirus VA1 and Epstein-Barr virus EBER) and proteins [including reovirus s3, vaccinia E3L and K3L, hepatitis C virus NS5A and E2, influenza virus NS1, and Herpes simplex virus (HSV) g34.5 and Us11]. **3:** Elongation factor function is altered in cells infected with human immunodeficiency virus, HSV, and vesicular stomatitis virus. **4:** Host-cell mRNA is degraded in cells infected with HSV, influenza virus, and poxvirus. **5:** Unconventional strategies used for viral protein synthesis include leaky scanning and ribosome shunting during initiation, frameshifting during elongation, and suppression of termination.

Two steps of translation regulated during initiation are the recruitment of the small (40S) ribosomal subunit to mRNA, and the binding of the initiator transfer RNA (met-tRNAi) to the 40S subunit. Most cellular and viral mRNAs found in animal cells possess a 5'-terminal cap [7mG(5')ppp(5')N] structure and a 3'-terminal poly A tail (224,258,293). The 5'-cap and 3'-poly A tail act synergistically to enhance 40S ribosomal subunit recruitment to mRNA during the initiation phase of translation (258,293). Initiation factor eIF-2 functions in a complex with GTP and met-tRNAi to deliver the initiator transfer RNA to the 40S ribosomal subunit (224). The 40S small ribosomal subunit is recruited to the mRNA 5'-cap structure by protein synthesis initiation factor eIF-4F in a process that requires ATP. The eIF-4F is composed of three subunits: eIF-4A (which is an RNA-dependent ATPase), eIF-4E (which interacts directly with the cap structure), and eIF-4G (formerly known as p220). The eIF-4G factor appears to function as a scaffold, binding several initiation factors during the assembly of the mRNA-ribosome initiation complex including eIF-3, -4A, and -4E, as well as the poly(A)-binding protein PABP (224,227,258).

Modification of Initiation Factor Function Involved in Recruitment of the 40S Ribosomal Subunit to mRNA

For many members of the *Picornaviridae* family, infection leads to a rapid and efficient inhibition of cellular protein synthesis. One of the best-characterized examples of the selective shutoff of host cell translation in infected cells is provided by poliovirus. Cellular protein synthesis is drastically reduced within about 1 to 2 h after PV infection (256). PV and other picornavirus mRNAs lack the 5'-cap structure (14,293). Their translation initiation occurs by a cap-independent mechanism, in which the 40S subunit binds directly to an internal ribosome entry site (IRES) located in the 5'-UTR region of the picornavirus RNA (158,255,347). Translation of 5'-capped cellular mRNAs is inhibited at the initiation step in picornavirus-infected cells because of proteolytic inactivation of the eIF-4G subunit of the cap binding protein complex eIF-4F (14). Two functional homologs of the eIF-4G factor have been identified, eIF-4GI and eIF-4GII. Both eIF-4GI and -4GII are cleaved in PV-infected (117) and rhinovirus-infected (333) cells. The cleavage of eIF-4GII coincides with the shutoff of host protein synthesis and, at least for PV and HRV-14, this eIF-4GII cleavage appears to be the rate-limiting step in the shutoff process. The molecular basis for the differential susceptibility of eIF-4GI and -4GII to proteolytic degradation, and the physiologic significance of the differential sensitivity in the context of the virus–host interaction, are not yet fully resolved. In addition, whether this cleavage pattern is general for all picornavirus infections is not yet clearly established. The picornaviral 2A proteases ($2A^{pro}$) catalyze the cleavage of eIF-4GI and II (189). However, eIF-4G is not the only target of picornavirus $2A^{pro}$. For example, Coxsackie virus $2A^{pro}$ also catalyzes the cleavage of PABP (170). This may represent an additional mechanism for shutoff of host protein synthesis in picornavirus-infected cells. The eIF-4G subunit binds PABP and functions in poly(A)-dependent translation (258). Thus, the possibility of a synergistic reduction in host protein synthesis arises in picornavirus-infected cells by cleavage of two different factors by viral 2A proteases, eIF-4G and PABP, that interact during the process of initiation of translation to synergistically enhance 40S ribosomal subunit recruitment to mRNAs.

In group A rotavirus-infected cells, the nonstructural viral protein NSP3A appears to play an important role in the efficient expression of viral proteins by disruption of cellular eIF-4G factor function. NSP3A is bound to the 3' end of viral mRNAs. NSP3A also interacts *in vivo* with the initiation factor eIF-4GI, an association that evicts the cellular PABP from eIF-4GI (264). The translation of the viral mRNA, which is not polyadenylated, thus is enhanced at the expense of the translation of the cellular polyadenylated mRNA by the action of NSP3A. Despite binding on the same site on eIF-4GI, the PABP and NSP3 do not have strong sequence similarities even in their respective eIF-4GI binding domains.

Phosphorylation of the mRNA 5'-cap binding protein eIF-4E correlates with increased translation rates (115,171). Initiation factor eIF-4G recruits MAP kinase–activated protein kinase Mnk1 to phosphorylate eIF-4E (361). The extent of eIF-4E phosphorylation is often decreased in virus-infected cells, including cells infected with adenovirus, influenza virus, PV and, encephalomyocarditis (EMC) virus, but not with Semliki Forest virus or reovirus (172). For example, in adenovirus-infected cells, the vast majority (>95%) of the eIF-4E factor is dephosphorylated. A late adenovirus gene function impairs the phosphorylation of eIF-4E, an effect that coincides with the inhibition of cell protein synthesis. Presumably, the relative amounts of eIF-4E required for efficient translation of the viral and cellular mRNAs differ, possibly because of differences in relative structures among the RNAs. The function of eIF-4E is also regulated through binding to a family of translational repressor proteins designated as 4E-BPs. Binding of 4E-BPs to eIF-4E depends on the phosphorylation status of 4E-BP; thus, the 4E-BPs act in their unphosphorylated form as inhibitors of eIF-4E function. Dephosphorylation of 4E-BP1 following infection with picornaviruses such as PV and EMC virus has been observed, and in the case of EMC virus, the time course of 4E-BP dephosphorylation generally coincides with the shutoff of host protein synthesis (115). Thus, both 4E-BP1 dephosphorylation and eIF-4GII cleavage may contribute to picornavirus-mediated shutoff of host protein synthesis.

Influenza virus infection results in a dramatic shutoff of cellular protein synthesis. This shutoff is concomitant with the selective translation of viral mRNAs, mediated in part by the cellular RNA-binding protein GRSF-1, a positive-acting

translation regulatory factor that binds to specific G-rich sequences within the 5'-UTR region of influenza virus mRNA (251). Influenza virus mRNAs contain the cellular 5'-cap structure and about a dozen nucleotides from host mRNA sequences at their 5' end and are translated by a 5' cap-dependent mechanism. The precise mechanism by which GRSF-1 enhances viral mRNA translation is not yet known, but it may involve interaction with eIF-4G, which is known to interact with the PABP. Dephosphorylation and inactivation of eIF-4E also plays a role in the shutoff of host cell translation in influenza virus–infected cells (115,172). Influenza virus mRNAs display a decreased requirement for eIF-4F relative to host mRNAs, thus allowing them to be translated in the presence of low levels of the cap-binding complex factor.

Modification of Initiation Factor Function Involved in Binding of Initiator Transfer RNA to the 40S Ribosomal Subunit

Initiation factor eIF-2 is composed of three subunits (a, b, and g). Phosphorylation of eIF-2a correlates with decreased translation rates and suppression of cell proliferation (55,298,334), and phosphorylation of the a subunit mediates an inhibition of translation. Among the protein kinases that phosphorylate eIF-2a is the RNA-dependent protein kinase PKR (298). PKR plays a central role in the regulation of protein synthesis in virus-infected cells (55,295,297,298). PKR possesses a double-stranded RNA binding domain that consists of two tandem copies of a conserved dsRNA-binding motif (dsRBM). Binding of structured RNAs of viral origin to the dsRBM motifs occurs in a sequence-independent fashion and leads to either activation or inhibition of kinase enzymatic activity. Following catalytic activation by an RNA-dependent intermolecular autophosphorylation, PKR then catalyzes the phosphorylation of eIF-2a at serine residue 51. This modification of eIF-2a leads to an inhibition of protein synthesis, because it blocks the eIF-2B catalyzed guanine nucleotide exchange reaction of eIF-2:GDP (55,298).

During virus infection, PKR may become activated by viral RNA molecules and then in some cases later inhibited, thereby reversing any PKR kinase–mediated translational block that had been established. The mechanism by which PKR-catalyzed phosphorylation of eIF-2 is antagonized differs among viruses and can involve either RNA or protein modulators. The best-characterized viral RNA inhibitor of PKR is the adenovirus virus–associated VAI RNA (214). VAI RNA is produced in large amounts at late times after infection, and it subsequently impairs activation of PKR and phosphorylation of eIF-2a (214,215). Viral mutants that lack functional VAI RNA multiply poorly because of the dominant effect of virus-specific RNAs that activate PKR, thereby causing an inhibition of late viral protein synthesis. Adenovirus VAI RNA is bound by the dsRBMs of PKR, the same motifs that also bind activator RNAs (219). Modulation of eIF-2a phosphorylation is the primary function of viral VAI RNA, at least in cell culture, because substitution of serine 51 with alanine not only eliminates the phosphorylation site on the eIF-2a substrate but functionally complements the deletion of VA from the virus genome. Epstein-Barr virus–encoded RNAs (EBER), which functionally can substitute for adenovirus VAI RNA *in vivo*, also inhibit PKR activation (55).

The fundamental importance of PKR in the modulation of the translational pattern in virus-infected cells is further demonstrated by the large number of viruses that encode proteins that affect PKR function and subsequently the biochemical activity of initiation factor eIF-2 (102,215,298). The s3 major capsid protein of reovirus was among the first animal virus proteins identified that antagonize PKR function by sequestering activator dsRNAs (299). The preferential translation of reovirus mRNAs and the inhibition of cellular protein synthesis appear to be mediated in part by a s3-dependent mechanism that depends on both the subcellular localization of s3 (303) and the interaction of s3 with µ1c, the other major outer capsid protein of reovirions (380). Proteolytic cleavage of s3 results in enhanced dsRNA binding activity (299), but the association of s3 with µ1c eliminates dsRNA-binding activity and the ability to antagonize PKR function and stimulate protein synthesis (380). Reovirus mRNAs also can activate PKR. For example, the poorly translated S1 mRNA that encodes the minor capsid protein s1 is an efficient activator of PKR, whereas the efficiently translated S4 mRNA that encodes s3 is a poor PKR activator (298,299).

In influenza virus–infected cells, changes in the translational machinery mediated by down-regulation of PKR occur by two different strategies. One involves the viral nonstructural RNA-binding protein NS1 (102,137) and the other involves the activation of the stress-related cellular P58 ^{IPK} inhibitor of PKR (223). NS1, an abundant protein in influenza virus–infected cells, performs several regulatory functions during the viral infective cycle including regulation of synthesis, transport, splicing, and translation of mRNAs. NS1 prevents activation of PKR by binding to dsRNA activators. Temperature-sensitive mutant influenza A viruses with an RNA-binding defective NS1 protein cannot block activation of PKR in infected cells. These ts mutant virus strains exhibit temperature sensitivity in virus protein synthesis at the translational level, in a manner that correlates with increased activation of PKR and phosphorylation of eIF-2a. P58 ^{IPK}, a constitutively expressed cellular protein, inhibits PKR activity through direct protein–protein interaction with PKR. Influenza virus infection activates P58 ^{IPK} by promoting dissociation from its negative regulator, heat-shock protein 40; thus, cellular P58^{IPK} is believed to be targeted with hsp70 to PKR, thereby inhibiting kinase function in influenza virus–infected cells (223). The baculovirus PK2 gene product also inhibits PKR by a dimerization mechanism. Baculovirus PK2 protein is a truncated viral kinase that forms a heteromeric complex with the cellular PKR, thereby blocking kinase autophosphorylation and eIF-2a phosphorylation. Insect cells infected with wild-type baculovirus, but not PK2-deleted virus, exhibit reduced phosphorylation and increased translational activity (72).

Hepatitis C (HCV) and vaccinia viruses provide examples of animal viruses that encode multiple proteins that act synergistically to disrupt PKR function. Two HCV-encoded proteins, the nonstructural protein NS5A (101) and the envelope protein E2 (338), have been reported to prevent activation of PKR. NS5A of HCV interacts with a region of the PKR catalytic domain that also serves as a dimerization domain, thereby disrupting PKR dimerization and PKR-mediated eIF-2a phosphorylation (101). The HCV envelope protein E2 also appears to bind to and inhibit PKR (338). Interestingly, E2 contains a sequence identical to the phosphorylation sites of the PKR kinase and the eIF-2a substrate, but the contribution of E2 as a potential pseudosubstrate to the antagonism of PKR in natural infections is unknown, given the different subcellular localizations expected for PKR and this portion of E2. Inhibition of PKR by the NS5A and E2 proteins of HCV may facilitate survival and proliferation of the infected host, ultimately leading to HCV-associated hepatocellular carcinoma (342).

Vaccinia virus also encodes two gene products, E3L and K3L, that stimulate translation through inhibition of the PKR kinase (55,298). E3L and K3L function by different mechanisms. The viral E3L protein is a dsRNA binding protein that both sequesters dsRNA activators of PKR (314) and interacts directly with the eIF-2a substrate-binding region of PKR (309), thereby down-regulating PKR autoactivation and eIF-2a phosphorylation. The vaccinia K3L protein is a virus-encoded homolog of the eIF-2a substrate. The K3L pseudosubstrate inhibits PKR-catalyzed phosphorylation of eIF-2a (167).

TAR, a highly structured RNA leader element located at the 5' end of all HIV transcripts, activates the PKR kinase (55,206). HIV uses two proteins to counter this TAR-mediated activation of PKR. PKR function is down-regulated by the cellular TAR-RNA binding protein TRBP, which forms an inactive heterodimer with PKR (16), and by the viral trans-activating Tat protein, which inhibits autophosphorylation of PKR and competes with eIF-2 (35). HIV Tat protein also activates transcription factor NF-kB through a process that requires the function of the PKR kinase (69). The relative roles during the HIV infective process of the negative (35) and positive (69) effects of Tat protein on PKR kinase activity are not yet fully resolved.

In HSV-infected cells, the viral g34.5 protein affects phosphatase function, thereby blocking the shutoff of protein synthesis mediated by activated PKR. In the absence of the g34.5 gene, eIF-2a is phosphorylated and protein synthesis is impaired beginning at about 5 hours after infection; however, in the presence of g34.5, protein synthesis continues even though PKR is activated. The g34.5 protein binds to the protein phosphatase 1a and redirects it to dephosphorylate eIF-2aP (138). The HSV U_s11 protein can substitute for the g34.5 protein if it is present early in infection before PKR is activated. U_s11 protein blocks the shutoff of protein synthesis by binding to PKR and blocking the subsequent phosphorylation of eIF-2a (43).

Modification of Elongation Factor Function in Virus-Infected Cells

Viral proteins also interact with the translation elongation factors eEF-1A (formerly known as EF-1a) and eEF-1B (52,64,168,169,376). Factor eEF-1A is the protein synthesis elongation factor involved in the delivery of aminoacylated transfer RNAs to the ribosome A site as an eEF-1A:GTP:aa-tRNA ternary complex during the process of polypeptide chain elongation (see Fig. 8). The matrix MA protein and the $Pr55^{gag}$ polyprotein of HIV-1 both interact with eEF-1A (52). This interaction appears selective, as eEF-1A does not associate with nonlentiviral MA proteins. RNA, possibly the eEF-1A:aminoacyl-tRNA complex, is involved in the eEF-1A interaction with MA/Gag. The interaction between eEF-1A and MA/Gag leads to an inhibition of translation (52). Elongation factor eEF-1B catalyzes the exchange of GDP for GTP on the eEF-1A factor. The second coding exon of the HIV-1 Tat protein interacts with the d subunit of eIF-1B and specifically inhibits cellular translation, without significantly affecting the efficiency of viral translation (376). Interaction of viral proteins with eEF-1 delineates a possible mechanism for HIV-1–mediated modulation of host translation.

In addition to the functions that translation elongation factors play during the process of protein synthesis, the eEF-1B factor also is implicated as a component of the RNA replication complex of VSV (64). Purified VS virions include packaged eEF-1B, which interacts with the L protein of VSV to give an active viral RNA polymerase, in striking similarity to earlier observations made with Qb RNA phage where the bacterial homolog of eEF-1B is involved in viral RNA replication. The eEF-1A factor has been shown to bind RNA genomes of some viruses, for example West Nile virus genomic RNA (28) and tRNA-like structures from plant viral RNAs (113), and, like eEF-1B, it may play a role in RNA replication.

Degradation of Cellular mRNA in Virus-Infected Cells

Degradation of the host mRNA following virus infection provides an additional mechanism to modulate the utilization of ribosomes and factors of the translational machinery for preferential translation of viral mRNAs and also to delay the host response to infection. For example, following infection with HSV, influenza virus, or poxvirus, inhibition of cellular translation occurs and a decrease in intact host-cell mRNA is observed (92,153,242,282). For viruses such as these that have a lytic life cycle, an overall reduction in the level of cellular mRNAs, together with high rates of transcription of a comparatively small number of viral genes, creates an environment where highly abundant viral mRNAs can effectively out-compete relatively more scarce cellular mRNAs for the translational machinery.

An important question concerns the origin of the nuclease(s) that catalyze the degradation of cellular mRNAs in infected cells. Probably the earliest HSV gene that plays a significant role in abating or delaying the host response to infection through degradation of mRNA is U_L41, which encodes a protein that is introduced into the cell in virions at the time of infection (278). Although the HSV virion host shutoff (vhs) activity of the U_L41 protein reduces the synthesis of host proteins by causing the destruction of mRNA, it apparently does so in a nonspecific manner, as the half-lives of both host and viral mRNAs are reduced by the U_L41 protein (185,246,382). U_L41 has the biochemical properties of an RNase, but it has not been unequivocally resolved whether U_L41 alone possesses ribonucleolytic activity or whether an accessory component is required. Interestingly, the U_L41 function of HSV-2 strains generally leads to shutoff of the host more rapidly and completely than that seen with HSV-1 strains (88,91). Because there is no apparent discrimination between host and viral mRNA in the degradative process, in addition to providing a means for inhibiting host translation, it is conceivable that the U_L41 viral function promotes the shutoff of immediate-early and early gene expression in the HSV lytic cycle.

Another cellular RNA degradation pathway that can be activated by viral infection, which is also up-regulated by interferons, is the 2-5A system. Interferon treatment of cells induces a family of RNA-dependent 2'-5' oligoadenylate synthetases (279,297,315). These enzymes synthesize novel 2'-5'-phosphodiester-linked oligoadenylates (2-5A) that function to activate an endoribonuclease, designated as RNase L. The 2-5A dependent RNase L degrades single-stranded RNAs by preferentially cleaving uridylate-rich sequences. Although RNase L is constitutively present in most types of mammalian cells, because the 2-5A effector of the nuclease is unstable, the effects of 2-5A are transient.

Among the small structured viral RNAs that can activate 2-5A synthetase are the EBER-1 RNA of Epstein-Barr virus, the VAI RNA of adenovirus, and the TAR RNA of HIV-1 (71,206,310).

Unconventional Translational Strategies Used for Viral Protein Production

Most viral mRNAs found in the cytoplasm of infected cells possess the following five features: (a) a 5'-terminal cap structure; (b) a 5'-untranslated sequence; (c) an ORF that typically begins with an AUG codon and ends with an UAA, UAG, or UGA codon; (d) a 3'-untranslated sequence; and (e) a poly A tail. Following attachment of the small ribosomal subunit at or near the 5' end of the mRNA and identification of the functional AUG initiation codon by the scanning 40S subunit, the host translational machinery reads the single ORF of the mature viral mRNA to produce a single protein product (178). In addition to this conventional strategy, viruses also utilize a number of other strategies in specific instances to optimize the production of their protein products, strategies that include leaky scanning, ribosome shunting, translational frameshifting, and suppression of termination, as well as posttranscriptional RNA editing to alter the expression of viral genetic information.

Leaky Scanning

Some animal virus families include members that encode functionally polycistronic mRNAs in which translation initiation occurs alternatively at one or more AUG initiation sites in the same mRNA (296). The efficiency of translational initiation at a given AUG codon is thought to be modulated in part by the position and context of the start codon. The leaky scanning model provides a conceptual framework for understanding the process of initiation of translation on those viral mRNAs that utilize more than one AUG codon for initiation (178). According to the model, 40S ribosomal subunits bind at or near the 5' end of the mRNA and advance linearly until an AUG codon in a favorable context is reached, at which point assembly of complete 80S ribosomes takes place and initiation of polypeptide chain synthesis begins. If the 5'-proximal AUG codon is in an optimal context with purines in the –3 and +4 positions relative to the A of the AUG, initiation is typically unique and efficient. If the context is suboptimal, initiation at that codon is inefficient, scanning is leaky, and consequently some of the 40S subunits bypass the first suboptimal AUG and begin initiation at a downstream AUG positioned in a more favorable context. Thus, leaky scanning provides a mechanism by which multiple viral proteins may be produced from a single mRNA.

For several of the polycistronic viral mRNAs, the AUG codon of the 5'-proximal upstream cistron is present in a suboptimal context, whereas the initiation codon of the internal downstream cistron is often, but not always, present in an optimal context (178,296). Thus, some of the scanning 40S ribosomal subunits would presumably “leak” past the suboptimal initiation site and begin translation at the downstream initiation site, which may occur either in the same or different ORF (296). The relative efficiency of utilization of the upstream and downstream initiation codons may be of possible regulatory importance, as certain polycistronic mRNAs appear to be organized so that the relative abundance of the proteins encoded by the different cistrons correlates with the relative strength of the initiation codon.

A number of animal virus genes encode mRNAs in which different in-frame AUG initiation sites are alternatively utilized to generate related forms of a protein that differ in their amino terminal sequences. This is exemplified by the SV40 late 19S mRNAs encoding capsid proteins VP2 and VP3. The less abundant VP2 capsid protein is synthesized from the 5'-proximal AUG, which is in a suboptimal context, and the more abundant VP3 capsid protein is synthesized from an in-frame downstream AUG codon in a more favorable context (307). About 70% of the scanning ribosomes are estimated to bypass the VP2 initiator AUG and instead initiate translation at the downstream VP3 translation start site. Several examples of polycistronic viral mRNAs exist in which two proteins are produced from different, overlapping reading frames, as exemplified by the reovirus S1 mRNA, which encodes the minor capsid protein s1 and the nonstructural protein s1ns, also known as s1S (296). The steady-state distribution of translating ribosomes on reovirus S1 mRNA is not uniform, and interactions may occur between ribosomes in different reading frames. Ribosome pausing occurs at sites of initiation and termination of translation, both for the simple S4 mRNA in which a single ORF is utilized to produce the major capsid protein s3 and for the more complex S1 mRNA, where two overlapping ORFs are utilized. For the polycistronic S1 mRNA, the rate-limiting initiation event at the downstream favorable AUG utilized for ORF2 initiation of s1ns synthesis leads to ribosome stacking and elongation arrest in the upstream s1 ORF1 that encodes the minor capsid attachment protein (78).

Ribosome Shunting

Ribosome shunting refers to the process by which intervening regions of an mRNA are bypassed without scanning by ribosomes during the translation process. Examples in which ribosome shunting or jumping occurs include animal cells infected with adenovirus (381) and papilloma virus (281) and plant cells infected with cauliflower mosaic virus (76). In the case of adenovirus late mRNAs that are preferentially translated during infection, ribosomes are recruited to the 5' end of the tripartite leader RNA and then are translocated to a downstream AUG codon without scanning the intervening sequences (381). The tripartite leader confers preferential translation by reducing the requirement for initiation factor eIF-4F, the rate-limiting cap binding complex. Adenovirus inhibits cell protein synthesis, in part, by inactivating eIF-4F. The tripartite leader directs both 5' linear scanning and ribosome jumping when eIF-4F is abundant, but it exclusively uses the jumping mechanism at late times after infection when the level of eIF-4E phosphorylation is reduced and eIF-4F function is impaired (381).

Translational Frameshifting

In addition to the conventional process of mRNA translation where a single ORF is read by an elongating ribosome, some retroviruses, coronaviruses, toroviruses, arteriviruses, and astroviruses exploit the ability of the host cell ribosomes to shift from one reading frame to another in response to signals programmed by the mRNA (36,89). The elongating ribosome typically slips backward by one nucleotide (–1), resulting in a change in reading frame. For many retroviruses, production of the polymerase gene products occurs following a –1 frameshift during translation. The *pol* gene partially overlaps and is in the –1 reading frame with respect to the upstream *gag* gene. As exemplified by Rous sarcoma virus

(157) and HIV (156), a small fraction of the ribosomes (about 5%) shift reading frames during translation to produce a Gag-Pol fusion protein, whereas the majority of ribosomes (about 95%) terminate at the end of the *gag* ORF. This provides a mechanism for viruses to synthesize, from a single mRNA, relatively large amounts of an upstream product (e.g., Gag structural proteins) compared to the downstream product (e.g., reverse transcriptase). Two cis-acting viral mRNA signals are involved in the process of –1 ribosome frameshifting: a slippery sequence where the ribosome changes reading frame, and a stimulatory structure located downstream (36,89). Pseudoknot structures involved in frameshifting of various retroviral RNAs and infectious bronchitis coronavirus RNA are believed to impose a thermodynamic barrier to elongating ribosomes, resulting in ribosome pausing, which increases the opportunity that ribosome-bound tRNAs will slip into the –1 reading frame at the slippery sequence because of specific interaction between the viral mRNA and host protein synthesizing machinery (49,233). Small amounts of HSV thymidine kinase enzyme can be produced by drug-resistant strains utilizing a translational recoding mechanism of +1 (or –2) frameshifting (147). Thus, this provides a general mechanism whereby specific viral RNA sequences cause ribosomal slippage so that small amounts of an essential viral protein can be synthesized.

Suppression of Translational Termination

The UAG, UGA, and UAA codons typically specify translation termination. However, termination can sometimes be leaky. Expression of limited amounts of a viral protein can be achieved by suppression of a stop codon, resulting in the synthesis of a carboxy-terminally extended protein. For certain retroviruses, as exemplified by murine leukemia viruses (MuLV), this is the mechanism used to express the *pol* gene as a Gag-Pol fusion protein by reading through the *gag* stop codon to produce viral reverse transcriptase (261). The amber UAG codon of MuLV is decoded as glutamine, thus allowing read-through to permit expression of downstream Pol ORF sequences at about 5% to 10% efficiency (4,261). Suppression of translational termination is also observed with some alphaviruses, as exemplified by Sindbis virus, where read-through of an opal UGA codon occurs to produce nsp4, a nonstructural protein required for viral RNA polymerase activity (195). No cases of read-through are yet known for UAA, a commonly used translation termination codon. Like frameshifting, the suppression of translational termination by ribosome read-through requires cis-acting sequences on the viral mRNA to interact with the host translational machinery to make the nonsense stop codon an inefficient terminator, and thus subject to read-through to allow the regulated expression of downstream viral ORFs (4,195).

Posttranscriptional RNA Editing

RNA editing is a posttranscriptional process by which RNA transcripts are covalently modified in a manner that potentially alters the function of the encoded product. Site-selective conversion of adenosine to inosine represents one such editing process, catalyzed by RNA-specific adenosine deaminases (ADAR). This A-to-I RNA editing modifies the expression of genetic information by changing codons, as inosine is read as guanine instead of adenine by ribosomes and polymerases. Two types of A-to-I editing processes have been described in viral RNAs. First, modifications characteristic of adenosine deamination are found at multiple sites, as exemplified by the extensive adenosine modifications present on the viral antisense RNA late in polyoma virus infection (183) and by the biased hypermutations observed in negative-stranded RNA virus genomes during lytic and persistent infections, as in the case of measles virus (44). Second, highly site-specific A-to-I modifications have also been identified at one or a few sites in certain viral and cellular RNA transcripts, as demonstrated by the editing of hepatitis delta virus (HDV) RNA (270) and pre-mRNAs encoding the glutamate GluR and 5HT-2c serotonin receptor channel subunits (200), respectively. Editing of these viral and cellular RNAs results in codon changes and thus mRNAs that encode protein products with altered functional activities. In the case of HDV infections, RNA editing plays an essential role in the production of two forms of hepatitis delta antigen (HDAg) from the same ORF. A-to-I editing at adenosine 1012 of HDV antigenomic RNA has the effect of converting an amber UAG termination codon to a UGG tryptophan codon in the mRNA (270), thereby extending the ORF. The two HDAg proteins encoded by the ORF have different functions in the viral life cycle. Small HDAg is required for RNA replication, and large HDAg, which inhibits replication, is required for packaging of viral RNA. Interestingly, the human ADAR gene is regulated by two promoters, one of which is interferon inducible and one of which is not inducible (111). The possible roles of RNA editing by ADAR in viral pathogenesis are numerous but so far have not yet been studied extensively.

NUCLEAR LOCALIZATION OF DNA REPLICATION AND ASSEMBLY PROTEINS

During viral infection, proteins encoded by certain viruses must localize from the cytoplasm into the nucleus to replicate viral genomes, to regulate viral transcription and RNA processing, and to assemble progeny viral capsids. In general, viral proteins use cellular machinery for localization through the nuclear pores into the nucleus (reviewed in ref. 366). In fact, the first NLS was identified as PKKKRKV in the SV40 large T-antigen (162). The importin a/b complex binds to this sequence and attaches to fibrils that extend to the nuclear pore. Once at the nuclear pore, the complex binds to the nucleoporins and the protein is transported into the nucleus. Some proteins, such as the SV40 large T-antigen, can localize to the nuclear compartment independently of other viral proteins and assemble into higher-order complexes after they enter the nucleus. Other proteins, such as the HSV helicase–primase complex (209) or capsid proteins (241,285), must assemble into a complex in the cytoplasm prior to localization into the nucleus.

TAKING OVER THE CELLULAR DNA REPLICATION MACHINERY

Inhibition of Host-Cell DNA Replication

Both RNA and DNA viruses cause the inhibition of host-cell DNA synthesis. There are several possible reasons for viral inhibition of cell DNA synthesis: (a) to provide precursors for viral DNA synthesis, (b) to provide host-cell structures and/or replication proteins for viral DNA synthesis, or (c) as a secondary effect of inhibiting cellular protein synthesis. The possible mechanisms by which virus infection might inhibit cellular DNA synthesis are discussed individually.

A Secondary Effect of Inhibiting Cell Protein Synthesis

There appears to be a small pool of an essential cell DNA replication protein (or proteins), as suggested by the decrease in rate of DNA chain growth seen within minutes after inhibition of protein synthesis (265,329). It has been proposed that some viruses, such as the herpesviruses, inhibit cellular DNA synthesis as a consequence of inhibiting cellular protein synthesis (164), and indeed, the two shutoff functions do map together on the HSV genome (91). HSV and adenovirus DNA synthesis do not require the limiting cellular factor, or the factor is not limiting in infected cells because their DNA synthesis continues independently of whether protein synthesis is ongoing or not (148,287).

Displacement of Cellular DNA from Its Normal Site of Replication

Herpesvirus infection has been variously reported to displace cellular DNA from the nuclear membrane (240) or to cause the displacement of cellular chromatin to the periphery of the nucleus (63,305). In either case, the cell DNA could be displaced from its normal site for replication because cell DNA synthesis has been reported to occur on the “nuclear cage” (220), or the nuclear matrix (18). The exposed sites on the nuclear matrix may provide a structural framework for viral DNA replication and late transcription (174).

Recruitment of Cell DNA Replication Proteins to Viral Structures

Several studies have shown that HSV infection leads to a redistribution of the host-cell DNA replication apparatus (66,369). This type of event could serve the dual function of providing cellular factors for viral DNA replication and inhibiting cell DNA synthesis, thereby reserving deoxynucleoside triphosphates for viral DNA synthesis.

Degradation of Cellular DNA

Infection by vaccinia virus leads rapidly to a marked inhibition of cell DNA synthesis. A virion-associated DNase enters the host cell nucleus and acts on single-stranded DNA (ssDNA) (267,268). This inhibition is mediated by the action of a virion component on the host cell.

Mechanisms by Which DNA Viruses Ensure the Availability of a DNA Replication Apparatus

DNA viruses need a replication apparatus for amplification of their genomic nucleic acid. Although the host-cell enzymes may be used for DNA replication, they may not always be available for use by the virus. Thus, several strategies have evolved to ensure the availability of DNA replication machinery. These range from encoding an entirely new replication apparatus to inducing host cell DNA synthesis to allow the virus to use the cellular machinery.

Viruses Can Encode a New Replication Apparatus

The poxviruses replicate entirely in the cytoplasm. Indeed, poxviral DNA synthesis can occur in enucleated cells. Therefore, they must encode all (or nearly all) of the proteins needed for replication of their DNA in the cytoplasm. Similarly, retrovirions contain an enzyme capable of copying the genomic RNA into DNA. This step is not possible in cells without the virion enzyme, because a cellular reverse transcriptase is not available to copy the viral RNA. For these viruses, DNA synthesis is usually not restricted in different cells, because the enzymes are virally encoded.

The herpesviruses encode several proteins that form a major part of the replication complex. For example, HSV encodes seven viral proteins required for and directly involved in viral DNA replication (46,373). In addition, HSV encodes several other enzymes involved in providing deoxynucleoside triphosphate precursors for viral DNA synthesis: thymidine kinase, ribonucleotide reductase, and dUTPase. HSV replicates in the resting neuron and has apparently evolved to encode these DNA replication proteins because they are expressed at low levels in resting neurons. Some cellular proteins may be involved in HSV DNA replication. Although the identity and role of specific cell proteins in HSV DNA replication have not been defined, the cellular DNA replication machinery is redistributed after HSV infection so as to co-localize with viral proteins in the cell nucleus (66). HSV DNA replication proteins also bind to the nuclear matrix (274), so cellular proteins may be required to anchor DNA replication complexes and thereby promote optimal HSV DNA synthesis. However, HSV blocks cells from progressing into S phase (66), so any cellular proteins required for HSV DNA synthesis may be present in non-S phase cells.

Viruses Can Induce Cellular DNA Synthesis

Some viruses, especially the DNA tumor viruses, rely on the host-cell DNA replication machinery, and to ensure its availability, they induce S phase in their host cells if they are not cycling or are in G0 phase. SV40 and polyoma viruses encode one protein, large T-antigen, which essentially inserts itself into the host-cell replication complex and directs it to replicate viral DNA. The SV40 large T-antigen (a) binds to the SV40 DNA sequences that serve as the origin of DNA replication (344), (b) interacts with the cellular a-DNA polymerase (318), and (c) acts as a helicase (324). Through these and possibly other functions, T antigen promotes replication of the SV40 chromosome. T antigen could be viewed as an origin-binding protein that substitutes for an analogous cellular protein. The specific interaction of T antigen with host proteins is apparent in other ways. SV40 DNA replication can occur in extracts prepared from only certain cell types (196), and the interaction between T antigen and the a polymerase–primase complex seems to define or play a role in defining this specificity (230). Thus, the permissivity of a cell for viral growth may be defined by the ability of a viral DNA replication protein to interact with the cellular DNA replication apparatus.

However, the levels of several critical components of the cellular DNA replication apparatus are limiting, except during S phase. Thus, the host DNA replication machinery would not be available at all stages of the cell cycle. Polyomaviruses have long been known to induce S phase in resting (G0) cells (79), and the mechanisms for this effect are now understood. SV40 T antigen forms complexes with pRb and related proteins (68), releasing transcription factors of the E2F family that are important for expression of cellular DNA replication and cell cycle proteins such as c-fos, c-myc, DNA pol a, dihydrofolate reductase, thymidine kinase, thymidylate synthetase, and cdc-2 (239). Thus, activation of E2F

contributes to the preparation of the host cell for viral DNA replication.

Adenovirus encodes a DNA polymerase, a terminal protein, and an ssDNA-binding protein for viral DNA replication (see Chapter 67), but the remainder of the replication proteins and nucleotide metabolism enzymes are host-cell gene products. Thus, adenovirus also induces its host cell to enter S phase. Like SV40 T-antigen, the E1A proteins bind to pRb (367), releasing E2F and activating transcription of cell cycle genes. It is also becoming clear that adenovirus infection disrupts a number of protein complexes that are involved in regulation of the cell cycle. The adenovirus E1B-55K protein binds to the cellular p53 protein (302), inactivating the G1 cell cycle block imposed by p53. This also contributes to activation of the host-cell S phase and the optimal conditions for viral DNA synthesis.

Maintenance of Viral DNA Within the Host Cell

Viruses whose DNA genomes persist within growing cells have evolved mechanisms for their genomes to be maintained stably in these cells via association with cellular chromosomes. There are two types of mechanisms by which viral DNA is stably maintained within the host cell in association with chromosomes. First, the viral DNA can be integrated into the cellular chromosome and propagated as part of the cellular DNA. For example, retrovirus DNA is integrated into the cellular genome after its synthesis by the reverse transcriptase (see Chapter 58). Integration is promoted by the viral integrase function, but host functions can modulate the process. For example, the mouse Fv-1 gene mediates a postpenetration block to MuLV DNA integration (135,197,330). Thus, this host gene can define the host range of MuLV in mouse cells.

Second, viral DNA can be maintained as an extrachromosomal circular molecule in the infected cell. For example, Epstein-Barr virus (EBV) DNA is maintained in latently infected B lymphocytes as an episomal molecule (1) and requires a specific sequence, oriP, for replication and maintenance in latently infected B cells (377). Epstein-Barr nuclear antigen 1, EBNA-1, is also required for EBV DNA maintenance (377). Although EBNA-1 was thought to serve as an origin-binding protein, recent studies have shown that it plays a role in maintenance of the DNA rather than in its synthesis (3). EBNA-1 binds to specific sequences on viral DNA (277) and also binds to cellular chromosomes (126). Thus, EBNA-1 is thought to maintain EBV DNA in growing B cells by linking the viral DNA to cellular chromosomes. The related gammaherpesvirus, human herpesvirus-8 or Kaposi's sarcoma-associated herpesvirus also encodes a latency-associated nuclear antigen that tethers viral DNA to cellular chromosomes (11,56). Similarly, the papillomavirus genome contains sequences (called plasmid maintenance sequences) needed to maintain and replicate the DNA as an extrachromosomal element (149,203). Long-term maintenance of these plasmid genomes depends on attachment to host chromosomes, which is mediated by the viral E2 protein (151,193,317). In addition, these extrachromosomal elements appear to be subject to the normal copy number control existing in the normal cell because there is a constant number of episomal copies of these viral DNA molecules per cell as the cells divide. In contrast, HSV DNA can apparently persist during latent infection of neurons in a nonreplicating form because the host cell is not dividing.

ASSEMBLY OF FACTORIES FOR NUCLEIC ACID REPLICATION AND VIRION ASSEMBLY

Viral replication complexes, transcriptional complexes, replicative and assembly intermediates, and nucleocapsids and virions often accumulate in specific locations within the host cell and form structures called factories or inclusion bodies. The location of these structures obviously reflects the site of replication of a given virus. They may form in the nucleus, as do the inclusion bodies or factories in cells infected with herpesviruses (Fig. 9) or adenoviruses, or in the cytoplasm, as do the factories in reovirus-infected (Fig. 10) or poxvirus-infected cells or the Negri bodies in rabies virus–infected cells. Inclusion bodies have been useful in diagnostic virology because areas of altered staining can be detected at specific locations in the cell with specific staining properties, either basophilic or acidophilic, characteristic of individual groups of viruses. Crystalline arrays of capsids or nucleocapsids may accumulate in these inclusions (see Fig. 10). The assembly of these new structures in the cell may alter or displace host-cell components and lead to one form of CPE.

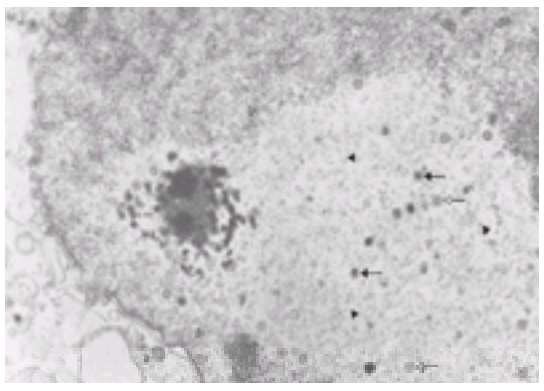

FIG. 9. Electron microscope visualization of nuclear inclusion areas. The *filled triangles* denote the electron-translucent nuclear inclusion area in a human HEp-2 cell infected with HSV-1. The *filled arrows* indicate full capsids, and the *unfilled arrows* indicate empty capsids. The *unfilled triangles* indicate host chromatin compressed to the periphery of the nucleus, apparently by the nuclear inclusion. Micrograph courtesy of D. Furlong and B. Roizman.

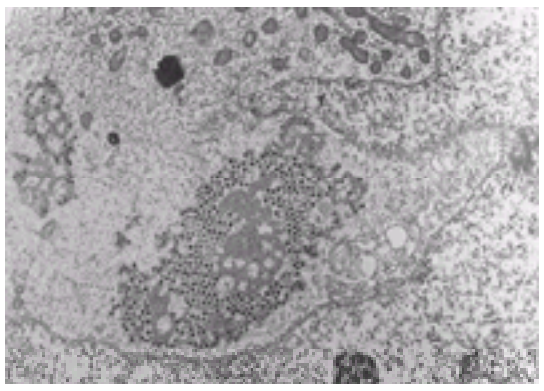

FIG. 10. Electron microscope visualization of cytoplasmic “factories” in a cell infected with reovirus. Micrograph courtesy of B. Fields.

Much as other viral proteins have provided probes for important cell biologic questions, HSV DNA replication proteins and their localization to intranuclear inclusions have provided a situation in which to study intranuclear localization mechanisms. Initial studies characterized the nuclear inclusions of herpesvirus-infected cells and their molecular components. Electron microscopy (EM) of herpesvirus-infected cells showed electron-translucent intranuclear inclusions surrounded by marginated and compacted cell chromatin (63) (see Fig. 9). Light microscopic observation of nuclear inclusions shows an hourglass appearance of the inclusions at early times and an eosinophilic staining at later times (232,321) (see Fig. 3). Immunofluorescence experiments using antibodies specific for HSV DNA replication proteins have shown that viral DNA replication proteins accumulate in intranuclear foci by 3 hours postinfection and that these foci enlarge into globular nuclear structures called replication compartments (Fig. 11) (66,274). The replication compartments are likely to be equivalent to (a) the translucent nuclear inclusions seen by EM (see Fig. 9) and (b) the early nuclear inclusions seen by light microscopy.

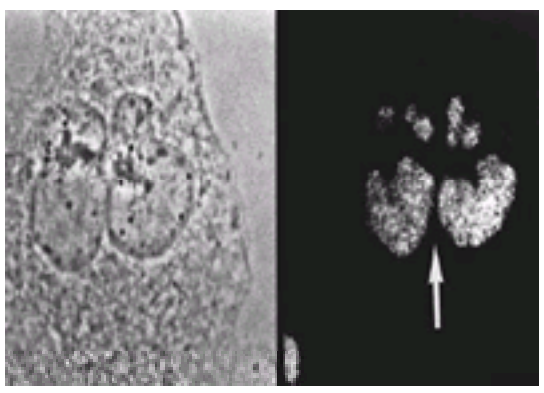

FIG. 11. Immunofluorescence detection of HSV replication compartments in binucleate cells. Micrograph courtesy of A. de Bruyn Kops.

These replication compartments are located in the interior of the nucleus as shown by EM (63,306) (see Fig. 9) and by confocal microscopy (67). Bromodeoxyuridine pulse labeling followed by immunofluorescence detection of bromodeoxyuridine-substituted DNA has shown that viral DNA synthesis occurs in the replication compartments (66). This technique allows the determination of the cellular location of DNA synthesis. Similarly, *in situ* hybridization with a viral DNA probe has shown that progeny HSV DNA accumulates in replication compartments (Fig. 12). The initial sites of formation of these compartments most likely takes place at specific locations in the nucleus defined by the availability of host-cell proteins or structures (67), near ND10 sites in the nucleus (216,352). The accumulation of progeny DNA, the probable template for late gene transcription and the viral transcriptional trans-activator protein ICP4 (177,276), pol II (283), and pulse-labeled viral RNA (260) in replication compartments argue that late gene transcription takes place in the replication compartments. Thus, late gene transcription is likely compartmentalized in infected cell–specific nuclear structures. Empty capsids appear to be assembled around dense bodies or in the inclusions within the infected cell nucleus, whereas encapsidation of viral DNA appears to occur within the inclusion body itself (see Fig. 9). Therefore, these intranuclear compartments in HSV-infected cells are sites of viral DNA replication, late gene transcription, and nucleocapsid assembly.

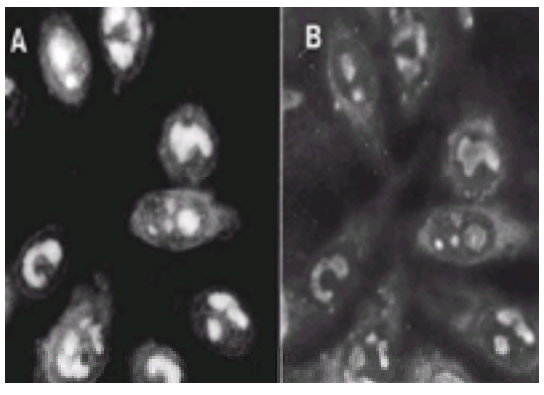

FIG. 12. *In situ* hybridization detection of the location of viral nucleic acids in infected cells. Monkey cells infected with

HSV-1 were fixed in formaldehyde, permeabilized in acetone, and incubated with biotin-labeled HSV DNA. The cultures were heated to denature the cellular DNA and the probe, and the culture and solution were allowed to cool. The cells were then reacted with mouse anti-ICP4 monoclonal antibody followed by rhodamine-conjugated goat antimouse immunoglobulin antibody and fluorescein-conjugated avidin. **A:** Rhodamine fluorescence showing the location of the HSV trans-activator protein ICP4. **B:** Fluorescein fluorescence showing the location of HSV DNA in the infected cells. Micrograph courtesy of S. Rice.

Studies of the nuclear inclusions in cells infected with HSV have raised several important cell biologic questions related to the assembly of nuclear complexes and structures and compartmentalization of nuclear metabolic processes. First, what signals target viral and cellular proteins to these compartments? Intranuclear localization mechanisms have not been studied extensively. Both viral and host proteins localize to these compartments. The HSV replication proteins, including the ICP8 DNA-binding protein, DNA polymerase, polymerase accessory protein, and helicase-primase complex (39,66,116,199,202,274) localize to compartments. These proteins undergo multiple interactions with each other; thus, their targeting could involve binding to other proteins in the replication complex, binding to cellular proteins, or other targeting mechanisms. Cellular proteins, including pRb, p53, and DNA pol a (369) and SV40 large T-antigen (378), also localize to replication compartments in HSV-infected cells.

Second, is there a defined assembly pathway for viral protein complexes in these compartments? Experiments examining protein localization in cells infected with mutants defective for each of the HSV DNA replication proteins have shown that there is a functional order for assembly of HSV DNA replication proteins into nuclear structures (199). The helicase-primase complex of U_L8, U_L42, and U_L52 proteins, the U_L9 origin-binding protein, and the ICP8 ssDNA-binding proteins are the minimal HSV proteins needed to assemble into nuclear structures. The polymerase-U_L42 complex then joins the structure.

Third, is there a receptor on the nuclear framework that anchors viral protein complexes? No cellular molecule has been identified as a receptor for the HSV replication proteins, and nuclear receptors remain an important problem in the targeting of nuclear proteins. One of the few situations where saturable binding of a viral protein to the nuclear matrix has been demonstrated is with the adenovirus preterminal protein (97). The adenoviral preterminal protein interacts with the nuclear matrix, specifically with CAD, a pyrimidine biosynthesis enzyme (5). The identification of a nuclear molecule or receptor that anchors specific proteins in the cell nucleus remains as an important confirmation of the concept of the nuclear matrix, a nuclear structure believed to consist of nuclear lamins under the nuclear envelope and internal fibrous elements.

Fourth, what defines the intranuclear location at which compartments are formed? In binucleate cells infected with HSV, replication compartments often show nearly identical patterns of compartments along a twofold axis of symmetry between many of the sister nuclei (see Fig. 10) (67). Therefore, the location of HSV replication compartments is defined by preexisting nuclear architecture, probably the internal nuclear matrix or receptors thereon. Although the parental HSV genome and DNA replication proteins localize to sites near ND10 sites in the nucleus (216,352), the precise molecular sites of localization remain to be defined.

Nuclear viral inclusions in adenovirus-infected cells (280,331,358) are also sites of viral DNA synthesis and accumulation of progeny DNA (37,212,358) and probably late viral transcription (212,372). Adenoviral inclusions may have an added level of complexity over the HSV nuclear inclusions in that the E1B-55kD/E4-34kD complex of proteins is located in and around the inclusions and is believed to recruit critical components of the host-cell RNA transport process to the sites of late viral RNA synthesis (245).

USE OF THE HOST CELL FOR MATURATION AND BUDDING

As described previously, viruses use the host-cell translational machinery to synthesize the structural proteins for progeny virus production. In addition, viruses may use a cellular polymerase or a virus-encoded polymerase to replicate their genomes. In addition, viruses use cellular proteases, membranes, glycosylation machinery, kinases, and chaperone proteins for their protein maturation (175). Because the sites of viral genome replication—as well as of maturation in general—are different from those where viral protein synthesis takes place, the intracellular trafficking of viral components is crucial for infectious particle formation. Furthermore, it appears that structurally intact cells are essential for efficient virus replication.

The site of assembly and mechanism of progeny virus release from the infected cell depends on the structure of the virus (Fig. 13). Enveloped viruses exit from the infected cell surface either by budding through the plasma membrane (Fig. 14) or by fusion of secretory vesicles containing virus particles with the plasma membrane (326). Thus, nucleocapsids can bud through the plasma membrane (e.g., orthomyxoviruses, paramyxoviruses, rhabdoviruses, and retroviruses), directly producing extracellular virions, or through internal membranes such as the endoplasmic reticulum (rotaviruses) and/or Golgi apparatus (coronaviruses and bunyaviruses) or inner nuclear membrane (herpesviruses).

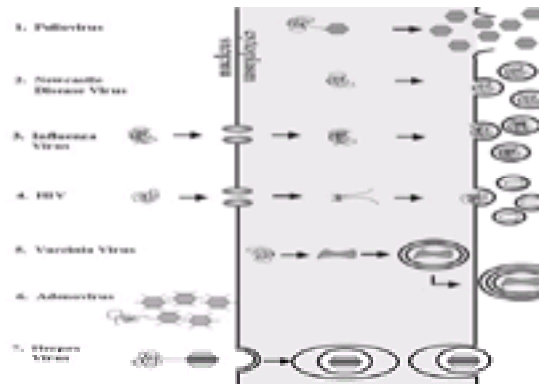

FIG. 13. Pathways of exit from cells for different viruses. Nonenveloped RNA viruses such as poliovirus (*line 1*) and DNA viruses such as adenovirus (*line 6*) leave the cell upon cell lysis. Enveloped viruses that replicate in the cytoplasm such as Newcastle disease virus (*line 2*) exit the cell by budding from the cytoplasmic membrane. Influenza viruses (*line 3*) also leave the cytoplasm in this manner, but the amplified viral ribonucleoprotein (RNP) complexes must first exit the nucleus via the nuclear pore. The amplified genome of HIV (*line 4*) exits the nucleus in a manner similar to influenza virus via the nuclear pore, and the virus buds from the cytoplasmic membrane. Vaccinia virus (*line 5*) replicates in the cytoplasm where it acquires its membrane(s), and in general it remains cell associated. Herpes viruses (*line 7*) acquire envelopes as they bud through the inner membrane of the nuclear envelope. The viral particles are transported within intracellular vesicles to the cytoplasmic membrane, where fusion of the vesicular and cytoplasmic membranes allows release of the virus from the cell. For the sake of clarity, not all viral proteins implicated in exit are shown.

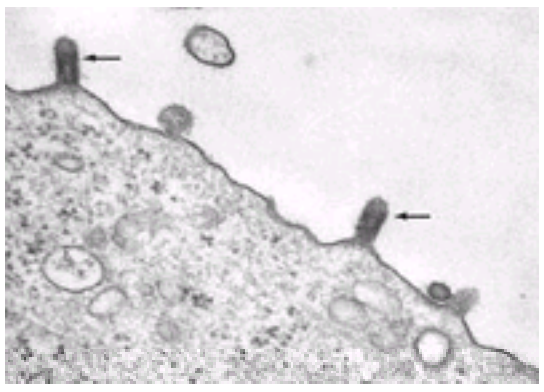

FIG. 14. Electron micrograph of enveloped virus particles budding from the infected cell surface. The *arrows* indicate VSV particles budding from the surface plasma membrane of infected Chinese hamster ovary cells.

Assembly Within the Cytoplasm

Some viruses, including the picornaviruses, replicate exclusively in the cytoplasm, and infectious progeny virus particles can be produced in enucleated cells (85,269). PV, for example, shows a cytoplasmic localization of viral protein synthesis, of viral RNA replication, and of particle assembly. Perhaps this virus is the most “host independent,” because its replication appears to rely the least on trafficking within an intact cell. In a landmark paper (226), Wimmer's group showed that an extract of uninfected HeLa cells permitted cell-free translation of naked PV RNA, synthesis of virion RNA, and assembly of infectious PV particles. However, the successful formation of infectious particles required the presence of membranes in the HeLa cell extract (59) and possibly the presence of chaperones such as heat-shock protein 70 (204). Although the efficiency of the cell-free virus replication was low, this experiment revealed that even such a complex process as viral RNA replication and PV encapsidation may be achieved outside a living cell. It has been generally assumed that unenveloped viruses are released by lysis of the cells. However, PV is released almost exclusively from the apical surface of polarized human intestinal epithelial cells (350). Thus, there may be cellular mechanisms used for viruses for active release from the cytoplasm in a polarized fashion (see Fig. 13, line 1).

Assembly Within the Nucleus

The prototypic viruses restricted to assembly within the nucleus are adenovirus and SV40. For adenovirus, synthesis and subsequent transport of the hexon and penton components into the nucleus are essential for virus formation. The transport from cytoplasm to nucleus involves the presence of NLSs on the adenoviral proteins and is accompanied (or followed) by the assembly of the viral components. Although the presence of a viral nonstructural component (the L1 52/55kD protein) appears to be required for encapsidation (129), little is known about which proteins (structures) are involved in the assembly of adenoviruses. Either an empty protein shell is made first, into which the viral DNA is introduced, or the packaging of DNA occurs along with assembly of the viral proteins. Whatever the precise mechanism turns out to be, it is likely that the formation of infectious adenovirus particles requires an intact nucleus (see Fig. 13, line 6).

Even the assembly of the much simpler SV40 virus is quite complex. Despite numerous efforts, the formation of stable SV40 particles through an *in vitro* assembly system has not yet been achieved (300), and, again, efficient packaging seems to require an intact host nucleus. For both adeno- and SV40 viruses, great quantities of virus particles can accumulate in the nucleus, leading eventually to lysis of the cell and release of virus. Like PV, SV40 may also undergo polarized released from epithelial cells (54).

Assembly of Enveloped RNA-Containing Viruses

Many of the RNA viruses that derive their lipid-containing envelopes from the cell's plasma membrane contain single-stranded RNA as their genome. Newcastle disease, vesicular stomatitis, and influenza viruses are prototype viruses of the negative-strand RNA virus group, the study of which has provided us with much of the information presently known about intracellular trafficking of viral glycoproteins and assembly in the plasma membrane. Viral glycoproteins, especially the influenza HA and VSV glycoprotein (G), have been used extensively as prototypes for the study of biogenesis of plasma membrane proteins. These proteins use the signal receptor particle, endoplasmic reticulum enzymes, Golgi apparatus enzymes, and transport mechanisms to realize their proper structure and cell-surface location.

Part of the evidence for the concept that transmembrane proteins are synthesized on membrane-bound polyribosomes and translocated cotranslationally across the endoplasmic reticulum membrane while peripheral membrane proteins are synthesized on free polyribosomes came from the study of virus membrane proteins (176,229). Detailed studies of *in vitro* insertion of viral membrane proteins into the endoplasmic reticulum membrane came from studies of the VSV G protein (292). Detailed genetic study of the VSV G protein has provided evidence for the loop model for signal sequence insertion into the endoplasmic reticulum (311). Transit along the secretory pathway is well described for the glycoproteins of these three viruses, although the ultimate fate of specific viral proteins may depend on specific signals present in the molecule. For example, epithelial cells have apical and basolateral surfaces that can be targeted by specific viral glycoproteins. Influenza virus buds apically and VSV buds basolaterally. This selective localization may be determined by specific transport vesicles that recognize signals in the viral proteins (184,234).

It should also be noted that the neuraminidase cleaves off sialic acid from the host cell and from budding virus particles, thereby preventing binding of the hemagglutinin to cell surfaces and viral surfaces, respectively. Viruses with a temperature-sensitive neuraminidase aggregate on cell surfaces when grown at the nonpermissive temperature or inhibited by neuraminidase inhibitors (190,247,248 and 249). Inhibiting this interaction of the viral neuraminidase with the host sialic acid receptor is the basis for drugs recently approved to fight influenza virus infection.

Although the role of the glycoproteins in the efficient assembly of enveloped viruses is well established, it is also thought that the M protein of these viruses may be involved in viral assembly and release. For example, when the matrix (M) protein of VSV is expressed in mammalian cells, it is subsequently released from the cells in the form of lipid vesicles that bud through the plasma membrane (161). Recently it was demonstrated that a highly conserved amino acid motif (PPXY, where P is proline, Y is tyrosine) present within the M protein of VSV and other rhabdoviruses, including rabies virus, may play a functional role in budding. It was found that mutating the PPXY motif in the VSV M protein prevented the release of the protein from cells in this functional budding assay. Interestingly, the PPXY motif within rhabdoviral M proteins was shown to be a ligand for proteins that contain a WW (tryptophan–tryptophan) sequence (136). The hypothesis is that cellular WW proteins interact with the viral M proteins and function as “budding” proteins, facilitating the assembly and release of the viral particles. No such hypothetical budding partners have as yet been identified *in vivo.*

The properties of the PPXY motif in the M proteins of rhabdoviruses are similar to those of the late (L) budding domain identified in the gag-specific protein p2b of Rous sarcoma virus, an avian retrovirus (108,370). In a functional budding assay, the region of the Rous sarcoma virus gag protein (57) containing the proline-rich motif can be replaced by the homologous region of the VSV M protein. However, these PPXY-mediated interactions between viral and cellular proteins are not sufficient to allow virus particles to bud from the cytoplasmic surface. The M and gag proteins appear to possess membrane-association domains (different from the PPXY motifs) that are required for the localization to the cytoplasmic membrane (50,375). The M and gag proteins may also interact with the respective viral glycoproteins, which could lead to an enhanced membrane binding of these viral components. Furthermore, the viral RNAs in the form of ribonucleoprotein complexes must bind to these structures in specific ways to prevent packaging and budding of cellular RNAs rather than viral ones. Many questions remain in this area of study because the precise steps leading to the formation and extracellular release of enveloped RNA viruses remain obscure.

The mouse hepatitis virus, a positive-strand RNA virus, follows an unusual pathway toward infectious particle formation. Rather than budding from the plasma membrane, it appears that murine hepatitis virus replication occurs on late endosomal and/or lysosomal membranes (355). We do not know how the mouse hepatitis virus replication components are targeted to the correct membranes or what the advantage to the virus is of localization in these cytoplasmic compartments. It will be interesting to find answers to these questions in the future.

Assembly of Vaccinia Viruses

Vaccinia virus is known to replicate in the cytoplasm, and it does so within specific regions called factories. What is unusual about the use of the host by vaccinia virus is that the infectious intracellular particle derives two membranes from the Golgi network. This “wrapping” mechanism for forming the intracellular mature virions is not well understood, nor are the cellular components involved in this process well characterized. Some of these virus particles become engulfed by another envelope derived from a post-Golgi or endocytic compartment, which allows fusion with the plasma membrane and eventual release. Thus, there are two forms of infectious particles—one inside the cell and one that is released (see Fig. 13, line 5). In another remarkable process, the intracellular enveloped virions can also become associated with actin tails (60,99). It was found that the viral protein A36R in the viral envelope becomes tyrosine-phosphorylated by cellular Src-family kinases. This phosphorylation results in the recruitment of the cellular adaptor protein Nck and of N-WASP next to the vaccinia particle. The latter two proteins are involved in actin polymerization at the plasma membrane. It thus appears that vaccinia virus mimics the receptor tyrosine kinase signalling observed during actin-based motility of (uninfected) cells. Vaccinia virus particles appear to sit on the tips of actin tails and thereby gain mobility that allows the virus to spread from cell to cell. The actin tails decorated by vaccinia virus (or the virulent smallpox virus) push the virus along and may dip into neighboring cells, thus transferring the virus to a new cell.

Assembly of Herpesviruses

Cellular cytoskeletal structures are exploited by yet another DNA virus for trafficking of viral components. Sodeik et al. (322) showed that the incoming herpesvirus capsids are rapidly transported along microtubules with the help of dynein, a microtubule-dependent motor. Transport of the viral capsids proceeds along microtubules from the cell periphery to the nucleus (see Fig. 5, line 7), involving an interaction of capsid proteins with dynein and microtubules. All herpesvirus capsids acquire a membrane from the inner leaflet of the nuclear membrane when they leave the nucleus. In the reenvelopment model of egress, this enveloped particle loses its envelope after transport into the cytoplasm, and naked capsids travel along (reorganized) microtubules (81,84). What determines directionality of this transport remains undefined. Also, this microtubule-mediated transport may be of great importance for the axonal transport in neurons, where large distances have to be overcome by the virus and its components, and it may be less relevant in other herpesvirus-infected cells (see Fig. 13, line 7a).

A second model describing the interactions of herpesvirus and cellular components during egress is the lumenal model. According to this model, viral capsids acquire their envelopes from the inner leaflet of the nuclear membrane and bud from the cell following the host's secretory pathway without leaving the lumenal spaces of the cell (see Fig. 12, line 7b). Thus, the naked capsids observed by EM, for example, would be “dead end” structures (118) and exocytosis would be independent of the organization of microtubules and/or the organization of the Golgi apparatus (6). The relative importance of these two pathways for herpesvirus egress remains to be defined.

Targeting and Release in Polarized Cells

Finally, release of viruses may be asymmetrical in polarized epithelial cells. Polarized epithelial cells have differentiated apical and basal surface plasma membranes. Thus, viruses budding through the plasma membrane or vesicles containing virus particles can traffic specifically through either membrane. For example, orthomyxoviruses and paramyxoviruses bud at the apical surface, whereas VSV and retroviruses bud at the basal surface. Viral glycoproteins have an intrinsic ability to localize to specific surfaces of polarized cells (159,273,290,327), and this is thought to be an important factor in deciding the site of virus budding in polarized epithelial cells. Sorting of proteins destined for the different surfaces appears to occur within the Golgi apparatus (326). Studies of the targeting of chimeric glycoproteins expressed from recombinant clones indicate that these targeting signals are complex (349). Some studies indicated that the ectodomain contains the targeting signal (222,291), whereas other studies found that the ectodomain is not sufficient (10,150,273,326). However, targeting of some viruses is not determined by the surface glycoprotein (205).

Once the progeny viruses have been released, they can initiate infection in new cells, and a whole new round of virus replication and interaction with a host cell can begin.

HOST CELL RESPONSES TO VIRAL INFECTION AND HOW VIRUSES COMBAT THESE RESPONSES

As host organisms for virus infection, we have evolved responses that block the replication and spread of viruses. This is apparent at the system level in the immune responses that are made against viruses and other foreign agents. However, host responses are apparent even at the cellular level, and in fact much of the innate immune response to viruses is initiated by host responses at the cellular level.

Interferon

One of the major host responses to viral infection is the production of interferons, components of the innate immune response. Isaacs and Lindenmann (154), during their seminal studies on virus interference, and Nagono and Kojima (231) observed that virus-infected cells produce a secreted factor which mediates the transfer of a virus-resistant state, and furthermore, that the resistance was displayed against both homologous and heterologous viruses. This secreted factor with antiviral activity was called interferon (IFN). It is now known that IFNs comprise a family of cytokines that play a centrally important role in the host response against viral infection.

The IFNs are grouped into two types: type I IFN (viral IFN, IFN-a/b) that includes the large number of a (leukocyte) IFNs and the single b (fibroblast) IFN, all of which act through the IFN-a/b receptor; and, type II IFN (immune IFN, IFN-g), of which there is a single species that acts through a cognate IFN-g receptor (297). The IFN response represents an early host defense, one that occurs prior to the onset of the immune response.

Pathogenesis studies utilizing knock-out mice in which the type I IFN-a/b receptor function has been eliminated by targeted gene-disruption illustrate the central importance of the interferon response; IFN-a/b receptor-null mice are unable to establish an antiviral state. These mutant animals are highly susceptible to infection by certain viruses despite the presence of an otherwise intact immune system (106,122,354). IFN-g possesses unique immunoregulatory activities. Like IFN-a/b, IFN-g plays an important role in mediating protection against viral infection, especially long-term control of viral infections, as revealed from studies of knock-out mice lacking the type II IFN-g receptor (42,201). Mice lacking both the type II IFN-g receptor and the type I IFN-a/b receptor are especially sensitive to viral infection (354).

The discovery that a number of viruses encode gene products that antagonize the antiviral actions of IFNs further illustrates the fundamental importance of the interferon system as a host defense against viral infection. Numerous mechanisms are utilized by viruses to impair the IFN response (Fig. 15). In most cases, the virus-encoded products antagonize one of two stages of IFN action: (a) the IFN signal transduction pathway that leads to transcriptional activation of the 30 or so cellular genes whose products are responsible for the biologic activities of the IFNs, or (b) the biochemical activity of one or a few specific IFN-induced cellular proteins responsible for inhibition of virus replication. A third possible stage of antagonism of the interferon response by viruses is the inhibition of IFN production. However, the viral gene products and biochemical mechanisms responsible for this level of inhibition are generally less well

characterized than those responsible for the antagonism of either the signal transduction pathway or the action of IFN-induced proteins.

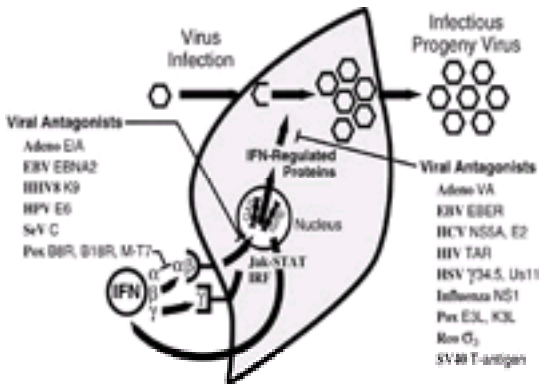

FIG. 15. Antagonism of interferon action by viruses. Virus-coded products that antagonize the IFN signal transduction pathway leading to transcriptional activation of IFN-regulated genes include adenovirus E1A; EBV EBNA2; HHV8 K9; HPV E6; poxvirus B8R, B18R, and M-T7; and Sendai virus C proteins. Virus-coded RNA and protein products that antagonize the biochemical activity of IFN-induced cellular proteins include adenovirus VA, EBV EBER, HCV NS5A and E2, HSV g34.5 and U_S11, influenza virus NS1, and SV40 T-antigen.

Several human viruses, including adenovirus, EBV, Sendai virus, and papillomavirus, encode viral proteins that inhibit the IFN-mediated transcriptional activation of cellular genes. The Jak-STAT signaling pathway utilized by IFN is blocked by the adenovirus E1A oncoprotein. The step affected by E1A appears to reside upstream of the activation of ISGF3, the major transcription factor complex formed of Stat1, Stat2, and IRF-9 (formerly known as p48) in response to IFN-a/b treatment (325), as the DNA-binding ability of ISGF3 is inhibited by the E1A protein (194). Overexpression of the IRF-9 component of ISGF3 restores IFN responses and the transcription of interferon-stimulated response element (ISRE)–driven genes in adenovirus infected cells (194). The Epstein-Barr virus EBNA2 protein also impairs the induction of genes by IFN-a/b but apparently by a mechanism different from that of the adenovirus E1A protein, as formation of the ISGF3 transcriptional activator complex is not significantly affected by EBNA2 (163). Sendai virus, a paramyxovirus that replicates in the cytoplasm of the host, circumvents the IFN-induced antiviral response by interfering with the transcriptional activation of IFN-inducible cellular genes. The C proteins of Sendai virus play a role, because infections with two different C gene mutants eliminate the ability to prevent establishment of an antiviral state against VSV (107). Impairment of the IFN-induced antiviral response appears to be a key determinant of Sendai virus pathogenicity (74,107).

Insight into the biochemical basis of the antagonism of the IFN signal transduction process by impairment of the function of signaling factors other than ISGF3 has been gained from studies of human papillomavirus (HPV) and also adenovirus. IRF-3, a key transcriptional activator affected by viral infection, is constitutively expressed in many cells and tissues. IRF-3 is directly activated by dsRNA or by virus infection, and subsequently plays a role in the activation of the IFN-a and IFN-b promoters and IFN-a/b responsive genes (143). The HPV E6 oncoprotein binds selectively to IRF-3 but only very poorly to other cellular IRFs including IRF-2 and IRF-9. The association of E6 with IRF-3 inhibits the trans-activation function of IRF-3, thereby providing HPV with a mechanism to circumvent the antiviral response (288). Adenovirus E1A protein also inhibits IRF-3–mediated transcriptional activation by a mechanism that depends on the ability of E1A to bind p300. Overexpression of CBP/p300 partially reverses the E1A-mediated inhibition, which is not observed with mutant E1A protein that does not bind p300. CBP/p300 and PCAF histone acetyltransferase (HAT) enzymes are coactivators for several transcription factors including Stat1a, p53, and nuclear hormone receptors; the viral E1A protein represses HAT activity and inhibits p300-dependent transcription and nucleosomal histone modifications by PCAF (45).

There are several known examples in which viruses mimic one or more of the cellular components of the IFN signal transduction pathway. In some instances, this molecular mimicry leads to an antagonism of the IFN signaling process and subsequent impairment of the development of an antiviral state. Virus-coded homologs of IFN signal transduction pathway components include homologs of type I and type II IFN receptors, a viral ISRE-like promoter element that functions as a target of cellular IRF transcription factors, and a viral homolog of the interferon regulatory factor (IRF) family of transcription factors that represses the ISRE element function of IFN-inducible promoters. Soluble viral IFN receptor homologs (vIFN-gRc) encoded by poxviruses are secreted from virus-infected cells and bind IFNs, thereby preventing them from acting through their natural receptors to elicit an antiviral response (320). The first poxvirus receptor homolog identified, the M-T7 protein, was found in myxoma virus–infected cells and acts as a decoy to inhibit the biologic activity of rabbit IFN-g. The vIFN-gRc gene product is a critical virulence factor for poxvirus pathogenesis. Myxoma virus lacking the M-T7 gene is highly attenuated; infected rabbits survive and do not develop myxomatosis. Other poxviruses also encode soluble IFN-g receptor homologs, including vaccinia virus in which the B8R gene encodes the IFN-gRc homolog. Vaccinia virus and several other orthopoxviruses also express a soluble vIFN-a/bRc protein that, in the WR strain of vaccinia, is encoded by the B18R gene. The viral type I IFN receptor homolog of vaccinia virus binds several different IFN-a subspecies as well as IFN-b and blocks IFN-a/b signaling activity.

A viral ISRE-like element (vISRE) has been identified in the Q promoter (Qp) of Epstein-Barr virus (384). Qp is used for the transcription of EBV nuclear antigen 1 (EBNA1) during the highly restricted type I latent infection, but it is inactive in type III latency. Constitutive activation of EBV EBNA1 gene transcription is mediated in part by IRF-1 activation of the Qp promoter. Another IRF factor, IRF-7, binds to the vISRE-like sequence in Qp and represses transcriptional activation by both IFN and IRF-1. Expression of IRF-7 is high in type III latency cells but almost undetectable in type I latency, corresponding to the activity of the Qp of EBV in these latency states (384). The Jak-STAT signaling pathway positively regulates Qp activity, and the Zta up-regulation of p53 leads to p53-mediated interference with Jak-STAT activation of Qp (48). Conceivably, the binding of IRFs and Stats at Qp may be mutually exclusive. Interestingly, EBNA2, which is

expressed in type III but not type I latency, is reported to have an anti-IFN effect, greatly impairing the induction of IFN-inducible genes (163).

Human herpesvirus 8 (HHV8), a gammaherpesvirus associated with Kaposi's sarcoma, encodes an IFN regulatory factor homolog (vIRF) that functions as a repressor of transcriptional activation induced by a, b, and g IFNs (105,143). The vIRF product of the K9 ORF of HHV8 forms heterodimers with cellular IRF-1 and competes with binding of IRF-1 to the coactivator p300, thereby down-regulating transcriptional activation by the cellular IRFs. Thus, the HHV8 vIRF protein potentially plays an important role in HHV8 pathogenesis and neoplastic transformation by antagonizing IFN and IRF-mediated transcriptional control. Expression of vIRF antisense RNA in HHV8-infected cells increases IFN-mediated transcriptional activation and down-regulates expression of HHV8 genes. Overexpression of the viral vIRF in NIH3T3 cells induces transformation resulting in morphologic changes, focus formation, and growth in low serum concentrations (105), characteristics similar to the cellular IRF-2 repressor, which likewise mediates transformation of NIH3T3 cells and displays oncogenic potential in the nude mouse model (143).

Several viruses have evolved strategies to block the function of the IFN-inducible, RNA-dependent protein kinase PKR. Antagonism of PKR and the subsequent phosphorylation of eIF-2a have been directly linked to inhibition of the IFN-induced antiviral response of the host (55,102,215,298). Vaccinia virus, for example, can rescue IFN-sensitive viruses such as VSV and encephalomyocarditis virus from the antiviral effects of IFN by antagonizing the biochemical activity of PKR. The vaccinia E3L protein is a potent inhibitor of the PKR kinase and also the 2',5'-oligoadenylate synthetase (OAS), two IFN-inducible RNA binding proteins (284,297,314). E3L acts in part by sequestering activator RNA of PKR and 2'5'OAS. Deletion mutants of vaccinia virus lacking the E3L gene are sensitive to the antiviral effects of IFN, but RNA-binding proteins (including the reovirus s3 protein, RNaseIII, TRBP, rotavirus NSP3, and E3L mutants that retain RNA binding activity) complement the E3L gene deletion and reverse the IFN-sensitive phenotype. Vaccinia mutants that express E3L proteins unable to bind dsRNA are IFN sensitive. Deletion mutants of vaccinia virus lacking the K3L gene, the eIF-2a homolog, are more sensitive to the antiviral effects of IFN than wild-type virus. Complementation studies suggest that the E3L gene product is most likely responsible for the vaccinia virus–mediated rescue of VSV from the antiviral effects of IFN, and that the K3L gene product is at least partially responsible for the rescue of EMC virus (313).

Adenovirus, influenza virus, PV, and SV40 provide four examples of viruses that antagonize the IFN-induced PKR kinase by different strategies. Adenovirus VAI RNA antagonizes the antiviral state of IFN by preventing activation of PKR; deletion mutants that do not express VAI RNA display IFN sensitivity, whereas wild-type virus that produces large amounts of VAI RNA is relatively resistant to type I IFN (214). The influenza virus NS1 protein, which binds RNA and prevents activation of PKR, plays a crucial role in inhibiting IFN-mediated antiviral responses of the host against influenza A virus (102,106,137). PV infection leads to a degradation of PKR (27), although the protease responsible for the down-regulation has not yet been identified. Translation of SV40 early RNA is relatively resistant to the antiviral effects of IFN (297). SV40 large T-antigen antagonizes PKR function but downstream of the kinase activation step (275). The mechanism is not resolved, but one possibility is a T-antigen–mediated enhancement of the phosphatase activity responsible for the dephosphorylation of eIF-2a similar to that observed in HSV-infected cells. In HSV infection, eIF-2a phosphorylation is reduced even though PKR is activated, because of the interaction of the HSV g34.5 protein with phosphatase 1a (138).

Studies of the genetic evolution of HCV suggest that IFN-a therapy alters virus–host interactions as a result of changes in the nature of circulating HCV quasispecies (253). HCV genotype 1b resistance to IFN-a therapy is associated with changes in the HCV NS5A gene central region quasispecies (254). The HCV second envelope hypervariable region (HVR1), an 81-nucleotide sequence located at the 5' end of the E2 gene, shows changes in HVR1 quasispecies major variants during and after IFN therapy in patients who failed to clear HCV RNA. Although the underlying mechanisms are unknown, one intriguing possibility is an IFN-mediated selection for resistant viruses mediated by editing of HCV viral RNA catalyzed by the IFN-inducible RNA-editing enzyme ADAR1 (252).

Apoptosis

Apoptosis, or controlled cell death, can serve as a defense mechanism for the host cell to combat viral infection. The apoptotic response involves a cascade of intracellular signals initiated in response to a wide variety of stimuli, including viral infection. Morphologic changes associated with apoptosis of virus-infected cells include condensation of chromatin and vacuolization of the infected cell's cytoplasm, and biochemical changes including activation of cellular proteases and nucleases and degradation of cell DNA to nucleosome-sized fragments. The fundamental importance of apoptosis as a component of the host response to viral infection is illustrated by the fact that a number of viruses encode gene products that either induce or inhibit apoptosis (86,132,312,339). Untimely early destruction of infected host cells may greatly reduce virus yields. Hence, viruses have evolved strategies to antagonize the apoptotic response by encoding viral gene products that target effector components in the apoptotic cascade. Suppression or delay of apoptosis can extend the period of time available following infection to produce new virions prior to destruction of the host biosynthetic pathways utilized by the virus. The opposite also occurs. Viral gene products have been identified that function as inducers of apoptosis, possibly to facilitate release and spread of progeny virions from the infected host (86,132,312,339).

A wide variety of strategies is used by viruses to affect the balance between growth and death of the infected host cell. The adenovirus E1A gene product stabilizes p53 and induces p53-dependent apoptosis (312,339). This effect of E1A is countered by the adenovirus E1B-19kD, E1B-55kD, and E3 proteins, each of which can antagonize the apoptotic process. The adenovirus E3 gene product promotes the degradation of Fas which prevents apoptosis (345). The E1B-55kD–mediated block of apoptosis involves antagonism of p53 function (340), whereas the E1A-19kD protein is a Bcl-2 homolog that forms heterodimers with proteins such as Bax (365). Bcl-2 is an apoptosis suppressor that prolongs cell survival (25). Among the viral Bcl-2 homologs identified are the LMW5-HL protein of African swine fever virus that functions to antagonize apoptotic cell death, and the BHRF1 protein of EBV that protects human B cells from programmed death (132). The E6 protein of human papilloma virus and SV40 large T-antigen also impair p53-mediated apoptosis (132,312,339).

Cowpox virus blocks apoptosis by encoding CrmA, a serpin family protease inhibitor that blocks the interleukin-1b-converting enzyme (ICE) protease (100). ICE, a cysteine protease, is a key component of the apoptotic response in mammalian cells. The vaccinia virus B13R gene product is homologous to CrmA and inhibits apoptosis induced by Fas or TNFa (75). The baculovirus p35 protein, which prevents apoptosis during infection of insect cells, likewise inhibits the ICE family of proteases (225). Apoptosis via the Fas and TNF death receptors involving death domain (FADD) signaling cascade involves recruitment of several proteins including the procaspase 8 protease FLICE. Lymphotropic g-herpesviruses (including HHV8, herpesvirus saimiri, equine herpesvirus-2, and bovine herpesvirus-4) encode FLICE-inhibitory proteins known as vFLIPS that are recruited to the activated receptor, but vFLIPS lack proteolytic activity and thus do not activate death signals (348).

Type I IFNs are essential mediators of apoptosis. Primary mouse embryonic fibroblasts undergo apoptosis when infected with EMC virus, VSV, and HSV, but apoptosis induced by these viruses is inhibited by anti-IFN-a/b antibodies and in homozygous null cells lacking either the IFN receptor or the Stat1 signaling factor (335). IFN alone does not induce apoptosis unless the treatment is combined with double-stranded RNA. Two important mediators of the IFN-induced antiviral response, the PKR kinase (334) and the 2'-5'A-dependent RNase L (73,385), play key roles as effectors of apoptosis. Overexpression of wild-type PKR but not catalytically inactive PKR causes apoptosis in the presence of dsRNA (9). Induction of apoptosis by PKR involves eIF-2a and NF-kB, because overexpression of the S51A mutant of eIF-2a or the S32,36A mutant of IkBa led to an inhibition of apoptosis (114). Interestingly, an up-regulation of Fas receptor mRNA accumulation correlates with PKR activation and subsequent apoptosis (77,114). Apoptosis is substantially suppressed following treatment with dsRNA, TNF-a, or lipopolysaccharide of cells from mice with a targeted disruption of either the PKR gene (70) or the RNase L gene (385). Suppression of apoptosis in the PKR null cells was associated with defects in activation of IRF-1 and in Fas mRNA induction (70). Several viral antagonists of PKR function have been described (102,298); presumably, repression of PKR by these viral products would impair the apoptotic response. For example, the antiapoptotic and oncogenic potentials of HCV virus are linked to IFN resistance by viral repression of the PKR protein kinase (103). Resistance to apoptosis is attributed to an HCV NS5A-mediated block in eIF-2a phosphorylation; furthermore, cells expressing NS5A exhibit a transformed phenotype.

SUMMARY

The major points of this chapter can be summarized as follows:

1. The ability of a virus to replicate in a host cell can be determined by the availability of specific macromolecules in that type of cell. These molecules may be external, such as a receptor, or internal, such as a transcription or replication factor.

2. Some of the cytopathic effects of virus infection on a host cell are caused by specific alterations in host-cell metabolism or structure that allow viral replication events. These cytopathic effects are usually not simply toxic effects of virus infection. Instead, viruses have evolved to manipulate the environment of the host cell for their own optimal replication.

3. Interactions of viruses with host cells may involve subtle changes in the host cell, and an understanding of the nature of the interactions between viral gene products and the host-cell molecules often provides insight into the metabolic processes and critical regulatory events of the host cell.

4. The host cell often responds to viral infection, such as by synthesis of IFN and induction of apoptosis. Viruses have evolved to counter these defenses, and this evolutionary chess game continues.

Thus, our challenge continues to be to attempt to relate this information about the interactions of viruses with their host cells to the biology of viruses in their host organisms, and to apply this knowledge to the design of effective antiviral strategies.

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CHAPTER 8 Virus Assembly

Eric Hunter

Virus assembly, a key step in the replication cycle of any virus, involves a process in which large numbers of chemically distinct macromolecules are transported, often through different transport pathways, to a point within the cell where they are assembled into a nascent viral particle. A diversity of strategies and intracellular assembly sites are employed by members of the various virus families to ensure the efficient production of fully infectious virions. Nevertheless, a virus, irrespective of its molecular structure (membrane enveloped or nonenveloped) or the symmetry with which it assembles (icosahedral, spherical, or helical), must be able to take advantage of the intracellular transport pathways that exist within the cell if it is to achieve this goal. The end product of this selection process is assembly of each virus at a defined point within the cell. This chapter focuses on the cell biology of these intracellular targeting events, the intermolecular interactions that mediate targeting, and the assembly steps themselves.

Most viruses encode a limited number of gene products. They are, therefore, dependent on the cell not only for biosynthesis of the macromolecules that constitute the virus particle but also for the preexisting intracellular sorting mechanisms that the virus uses to achieve delivery of those macromolecules to the sites of virion assembly. Because these are the same sorting mechanisms that the cell uses to delineate its subcellular organelles, the viral macromolecules must possess targeting signals similar to those of the components of those organelles. For a virus such as adenovirus, which assembles its nonenveloped capsids in the nucleus, this means that, after translation in the cytoplasm, each of the structural proteins of the mature virus must have the necessary protein-targeting information to be efficiently routed through the nuclear membrane to the assembly site. The situation is more complicated for a membrane-enveloped virus, such as influenza virus. For this virus, which assembles and releases virions from the apical surface of the epithelial cells that it infects, there is a necessity to ensure that the surface glycoproteins of the virus are correctly sorted by the secretory pathway of the cell to the apical surface. In addition, nucleocapsids, assembled in the nucleus, must be transported into and through the cytoplasm to the same intracellular location. As will be discussed below, the intracellular site at which the final phase of assembly and budding of an enveloped virus takes place is most often defined by the accumulation of the viral glycoproteins at a specific point in the secretory pathway of the cell. This also implies that there is specific molecular recognition of the virally encoded, membrane-spanning envelope components by the cytoplasmic nucleocapsids in order for a productive budding process to occur. Thus, interactions between proteins of viral and cellular origin, between viral proteins and nucleic acids and lipids, as well as between the viral proteins themselves are at the heart of the assembly process.

PARTITIONING OF PROTEINS WITHIN THE CELL

For an actively growing eukaryotic cell, there is a constant need to transport proteins and nucleic acids from their site of synthesis to the specific intracellular domains where they must function (Fig. 1). Proteins synthesized on cytosolic ribosomes, for example, must be transported to specific regions of the cytoplasm, to mitochondria, and into the nucleus, whereas those synthesized on membrane-bound ribosomes enter the secretory pathway and are targeted to specific organelles along the way. At the same time, messenger RNAs (mRNAs) and integral RNA components of the ribosome, ribosomal RNAs (rRNAs), must be transported out of the nucleus. Proteins that participate in these intracellular trafficking processes have evolved to contain specific motifs that ensure the correct localization of the protein within the cell. Viruses have similarly evolved to take advantage of these preexisting pathways to accumulate the necessary components for assembly of a nascent virus in specific locations.

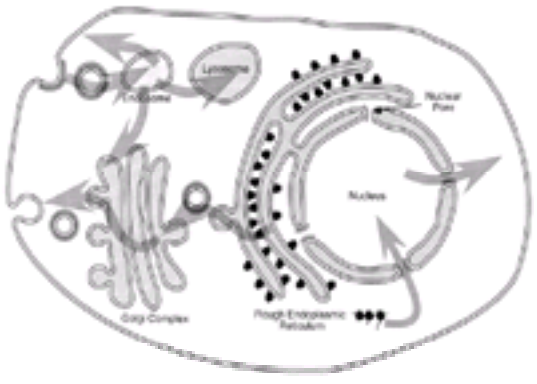

FIG. 1. Protein localization in a mammalian cell. Proteins destined for the plasma membrane traverse the secretory pathway of the cell. They associate with the endoplasmic reticulum (ER) cotranslationally and are translocated across the ER membrane through a proteinaceous pore: the translocon. Proteins are transported from the ER to the Golgi complex and on to the plasma membrane unless they contain specific amino acid motifs that localize or retain them at an intermediate location. Transport from one compartment to the next is by coated vesicles. Some membrane-spanning (integral membrane) proteins contain endocytosis motifs in their cytoplasmic domain that facilitate incorporation into clathrin-coated endocytic vesicles. Such proteins can be sorted back to the plasma membrane, to the *trans*-Golgi compartment of the secretory pathway, or to a lysosome for degradation. Proteins destined for the nucleus contain nuclear localization signals. These are short amino acid sequences that allow interaction with the nuclear pore machinery for transport across the nuclear membrane. Some proteins shuttle between the nucleus and cytoplasm and contain, in addition, a nuclear export signal.

Nuclear Import and Export of Proteins and Nucleic Acids

The Nuclear Pore Complex

Because the nucleus is segregated from the cytoplasm by an inner and outer membrane, access to and egress from this subcellular domain is mediated by specialized structures termed *nuclear pore complexes* (NPCs) (reviewed in 23). There are about 3,000 NPCs on the nuclear envelope of an animal cell, and each provides a proteinaceous channel between the nucleus and cytosol. The NPC itself is a large octagonal structure with a molecular mass exceeding 120 × 10^6 daltons—about 30 times larger than an 80S ribosome. NPCs can be released from nuclear membranes by detergents and show eightfold symmetry. Negative stain and cryoelectron microscopy reconstructions of these complexes reveal a cagelike structure with upper and lower rings (corresponding to the inner and outer faces of the nuclear membrane) linked by radial spokes that form an inner ring with a diameter of about 48 nm (1,49) (Fig. 2). Inside this is located the transporter, which forms a cylindrical pore of about 10 nm. Attached to both faces of the central framework are peripheral structures, cytoplasmic filaments, and a nuclear basket assembly, which interact with molecules that transit the NPC (54). Small molecules and proteins less than 60 kd in mass may be able to diffuse passively through the eight outer channels formed by the spokes and rings of the NPC (see Fig. 2). Larger proteins and macromolecular assemblages must be actively moved through what is presumably a dynamic, malleable transporter structure that has the capacity to accommodate macromolecular complexes up to about 20 nm in diameter.

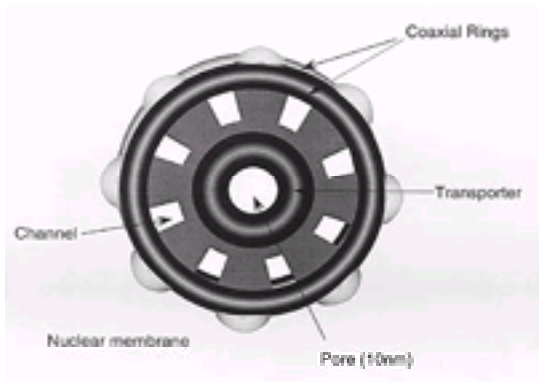

FIG. 2. Schematic representation of a nuclear pore complex. The nuclear pore complex is composed of two ringlike structures that are linked through eight spokes to a central transporter structure. The coaxial rings appear to interact with the outer and inner nuclear membranes. In negative-stain electron microscopy, the pores have a distinct eightfold symmetry. On the cytoplasmic side of the structure, filaments extend outward into the cytoplasm; whereas on the nuclear side, a basket-like structure is formed from the eight filaments that extend from the coaxial ring. Small proteins (5–50 kd) can diffuse into the nucleus, perhaps through the small channels formed by the spokes, whereas larger proteins must be actively transported through the pore.

Nuclear Localization Signals

Proteins that are actively transported into or out of the nucleus are characterized by the presence of amino acid motifs that allow them to interact with the nuclear transport machinery. For import into the nucleus, these motifs are termed *nuclear localization signals* (NLS), and for export, *nuclear export signals* (NES). NLS motifs, such as that first identified in the SV40 T antigen (57), are not only necessary for nuclear localization of the proteins in which they are present but are also sufficient to actively direct large foreign proteins, such as b-galactosidase, into the nucleus. Although there is no

conservation of sequence in different NLS motifs, they are generally short (less than 20 amino acids), rich in basic amino acids, and frequently preceded by proline residues (Fig. 3). Some NLS motifs, such as that in the adenovirus DNA-binding protein, are bipartite and require two separate short clusters of basic residues to be functional (137).

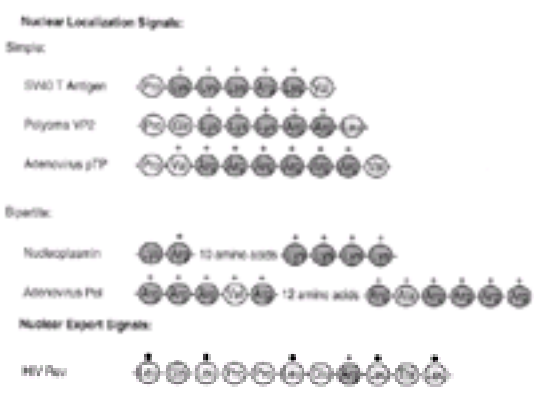

FIG. 3. Nuclear localization and export signals. The amino acid motifs that direct proteins into the nucleus are termed *nuclear localization signals* (NLS). Simple NLS sequences, such as those present in the SV40 virus T antigen or polyomavirus VP2, often contain a proline residue followed by a stretch of basic residues. This short sequence can relocate large proteins, such as b-galactosidase, into the nucleus. Bipartite NLS sequences, such as those present in nucleoplasmin or the adenovirus polymerase, are characterized by two stretches of basic amino acids separated by a variable spacer sequence. These NLS sequences are recognized by import receptor molecules such as importin-a. Proteins that shuttle into and out of the nucleus, such as Rev, possess a second motif, the nuclear export signal (NES). These motifs are characterized by a pattern of conserved leucine residues.

To be exported from the nucleus, proteins contain an NES. This nuclear transport signal is also short (about 10 amino acids) and contains a pattern of conserved leucines (83) (see Fig. 3). Some proteins, such as Rev of human immunodeficiency virus type 1 (HIV-1), possess both an NLS and an NES and appear to shuttle back and forth between the cytoplasm and the nucleus (75).

Nuclear Transport Pathways—In and Out

Nuclear import is a two-stage process. In the first stage, the newly synthesized, NLS-containing, protein interacts with cytosolic carrier proteins that then bind to the cytoplasmic side of the NPC; a process referred to as *docking.* This complex is then translocated, in an energy-dependent process, through the nuclear pore into the nucleus, where the complex is disassembled, allowing the transported protein to become functional (39).

The best characterized carrier protein is importin-a or karyopherin-a. After binding its NLS-containing cargo, importin-a interacts with importin-b, which then mediates docking with the NPC through interactions with members of the nucleoporin family of proteins (79). Other import systems use a single molecule, such as transportin or importin-b3, that mediates both binding of the cargo molecule and the docking process (84). Active transport of large molecules in either direction across the nuclear pore involves a family of proteins, known as *nucleoporins,* as well as a small guanosine triphosphate (GTP)-binding protein termed Ran (78) (Fig. 4). The directionality of transport is regulated by whether Ran is complexed with guanosine diphosphate (GDP) or GTP. In the cytosol, Ran-GDP, together with importin-ab and the cargo, forms a stable transport complex that is subsequently disassembled in the nucleus where GTP replaces GDP. Similarly, stable export complexes that form in the nucleus with Ran-GTP disassociate in the cytosol where a GTPase activity is active. The exact mechanism by which the Ran-GDP and importin–cargo complex is carried through the nuclear pore is not known. It is possible, however, that the process involves a series of docking and release cycles with nucleoporin proteins that make up the transporter machinery.

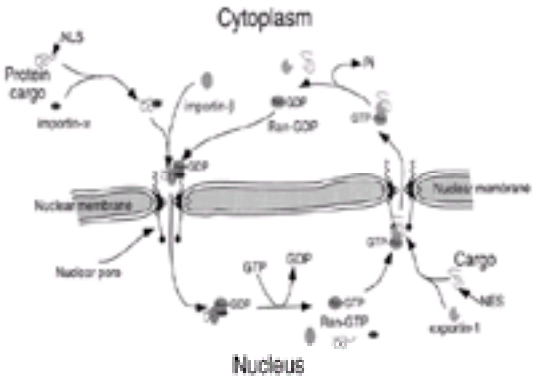

FIG. 4. Nuclear import and export pathways. A protein bearing an NLS is recognized and bound by importin-a. Importin-b binds to this complex and carries it to the cytoplasmic side of the nuclear pore where it binds initially to nucleoporins in the cytoplasmic filaments. Translocation of the protein complex into the nucleus requires the involvement of the guanine nucleoside–binding protein Ran in the form of Ran–guanosine diphosphate (GDP). Ran binds specifically to certain nucleoporins and is thought to mediate the transfer of the complex from the cytoplasmic face of the nuclear pore to the nuclear face. In the nucleus, a Ran-specific guanine nucleotide exchange protein (Rcc) converts Ran-GDP to Ran–guanosine triphosphate (GTP) and the complex dissociates delivering the protein cargo into the nucleus. Proteins destined for export out of the nucleus bind to exportin-1 through their NES. This complex, together with Ran-GTP, binds to nucleoporins localized to the nuclear basket of the pore complex and initiates translocation into the cytoplasm. Once there, Ran-GTP is converted to Ran-GDP by a Ran-GTPase–activating protein (RanGAP-1), causing the

cargo-exportin-1-Ran complex to dissociate, thereby delivering the cargo to the cytoplasm.

Export from the nucleus of large proteins and ribonucleoprotein (RNP) complexes involves interactions with an export carrier protein such as exportin-1 (32,118). This molecule binds to the NES sequence of the cargo protein and to Ran-GTP, which in turn mediates interactions with the nucleoporins of the NPC (see Fig. 4).

The Secretory Pathway of the Cell

Proteins of both viral and cellular origin that are destined for the outer membrane of the cell travel along a highly conserved route known as the *secretory pathway.* This complex series of membrane-bound subcellular compartments, through which proteins pass sequentially, includes the endoplasmic reticulum (ER), an intermediate membrane compartment, and the *cis*-, medial-, and *trans*-compartments of the Golgi apparatus (see Fig. 1). Proteins that traverse this pathway, such as the envelope glycoproteins of viruses, enter through the ER. Insertion of proteins into the ER occurs during translation, through a process termed *translocation.* The ER network of tubules and sacs defines a unique environment in which protein modification and folding can occur isolated from the cytoplasm. Regions of the ER membrane where polybosomes are bound tightly and in the process of translating proteins that are translocating into the secretory pathway are known as *rough ER*.

Translocation

Translating ribosomes are directed to the ER membrane by a short sequence in the nascent polypeptide known as the *signal sequence.* In most proteins, this 15– to 30–amino acid sequence, which contains a core of hydrophobic amino acids, is located at the N-terminus of the protein. Shortly after the signal peptide emerges from the ribosome, it is bound by an RNP complex known as the *signal recognition particle* (SRP) (131). Binding of the SRP transiently arrests further translation and directs the ribosome to an SRP receptor on the ER (Fig. 5). Both the SRP and its receptor have GTP-binding components, and the presence of this nucleotide is essential for efficient targeting.

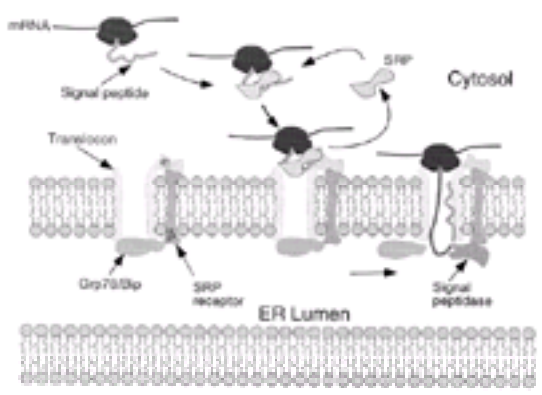

FIG. 5. Protein translocation into the secretory pathway. Translation of a protein destined for the secretory pathway proceeds until the signal peptide exits the ribosome. The signal recognition particle (SRP) binds to the signal peptide and the ribosome and arrests translation. The ribosome–SRP complex moves to the endoplasmic reticulum (ER) membrane where SRP binds to its receptor (*SRP receptor*). This interaction, with concomitant hydrolysis of bound GTP, releases SRP and mediates a tight interaction between the ribosome and a proteinaceous channel, the translocon. The release of SRP and binding of the signal peptide to components of the translocon allows resumption of translation to occur. Grp78/Bip, which appears to block the lumenal side of the translocon before and early in translocation, now allows the extrusion of the nascent polypeptide into the ER as translation continues. The signal peptidase complex removes the signal peptide cotranslationally from those proteins that have a cleavable signal peptide. Secreted proteins continue to traverse the translocon until they are completely located in the lumen of the ER. In contrast, for integral membrane proteins, translocation stops after introduction of the hydrophobic anchor domain into the translocon.

After the initial docking of the translationally arrested complex, the ribosome becomes tightly associated with the ER membrane through the translocon, a gated, aqueous, protein channel that spans the ER membrane (115). Concomitant with this process, the SRP and its receptor are released, the signal peptide is introduced into the channel, and translation resumes. The components of the translocon have been identified through biochemical approaches in mammalian cells and genetic approaches in yeast and are composed of a heterotrimeric complex known as the Sec61p (composed of Sec61 a-, b-, and g-chains). The complex forms a cylindrical structure, with a diameter of 85 Å and a central pore of 20 Å, which spans the ER membrane and is made up of three or four heterotrimers (44). Additional proteins (e.g., the translocating chain-association membrane [TRAM] protein) are necessary for optimal translocation. It is unlikely that the central pore of the translocon ever allows free diffusion between the cytosol and the lumen of the ER because these compartments are chemically distinct (18). More likely, GRP78/Bip, a member of the Hsp70 family of chaperone proteins, seals the luminal face of the translocon pore until the ribosome forms a tight seal on the cytosolic side. The chaperone is then poised to facilitate the folding of the nascent polypeptide chain as it emerges from the pore, although this binding is not essential for translocation to proceed. The signal peptide, in those proteins with a transient N-terminal sequence, is cleaved from the rest of the polypeptide by a complex of five proteins called the *signal peptidase* shortly after it enters the lumen of the ER (see Fig. 5).

For secreted proteins, translocation of the polypeptide through the translocon continues until the entire protein is present in the lumen of the ER. In contrast, integral membrane proteins, such as the envelope glycoproteins of viruses, contain a

stop-transfer, membrane-anchor sequence that is generally located toward the C-terminus of the protein. After the translation of this short (25 amino acids), mostly hydrophobic sequence, the translocon undergoes a conformational change that allows the membrane-spanning domain to be associated directly with the lipid bilayer. The product of this process is a type-I integral membrane protein (Fig. 6), such as the influenza virus hemagglutinin (HA), in which the N-terminal ectodomain is in the lumen of the ER and the C-terminus is in the cytoplasm. In some proteins, such as the influenza virus neuraminidase, a longer N-terminal signal peptide also functions as the membrane anchor. In this case, the signal peptide is not cleaved from the polypeptide chain and the sequences C-terminal to it are translocated into the ER lumen, resulting in a type-II orientation. Multiple membrane-spanning proteins, such as the M protein of the coronaviruses, appear to possess hydrophobic sequences that are alternatively recognized as signal and stop-transfer sequences.

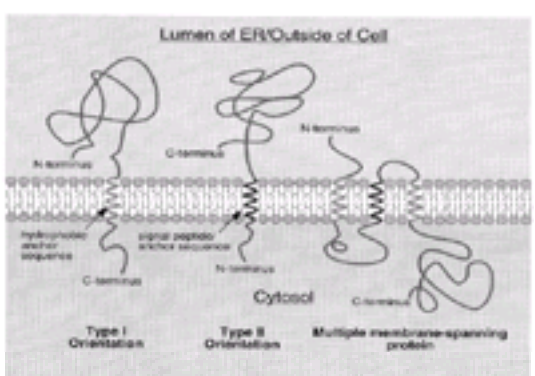

FIG. 6. Protein topology. Proteins with type-I orientation generally have a cleavable N-terminal signal peptide that is removed cotranslationally from the nascent protein. The protein continues to be transferred into the lumen of the endoplasmic reticulum (ER) until a hydrophobic anchor sequence is translated and enters the translocon. Translocation then stops, and the protein transitions from the protein pore into the lipid bilayer. Thus, type-I integral membrane proteins, such as the hemagglutinin of influenza virus, have their C-terminus in the cytoplasm and their N-terminus in the lumen of the ER (topologically equivalent to outside the cell). In type-II proteins, such as the influenza virus neuraminidase, the signal peptide forms the membrane anchor domain, so that at the end of translation, the C-terminal sequences are translocated into the lumen of the ER, leaving the N-terminus in the cytoplasm. For multiple membrane-spanning proteins, such as the M protein of the coronaviruses, translocation is initiated at the first signal peptide sequence and continues until the first anchor domain. It is reinitiated after translation of a subsequent signal sequence and stopped again after translation of a second anchor. The exact mechanism by which this is accomplished without dismantling the translocon at intermediate steps in the process is not understood.

Posttranslational Modifications

Protein Folding and Quality Control

Proteins enter the lumen of the ER in an unfolded state and the process of folding into a transport-competent conformation is facilitated by interactions with molecular chaperones and folding enzymes located there. This collection of proteins includes BiP, calnexin (Cnx), calreticulin (Crt), GRP94, and protein disulfide isomerase (PDI). In addition to assisting in folding the nascent molecules, these proteins retain incompletely folded molecules in the lumen and act as a quality control system for the secretory pathway (27). Oligomeric proteins, such as the receptor and fusion proteins of enveloped viruses, also assemble into their quaternary conformation in this compartment. For many of these molecules, oligomerization appears to be a prerequisite for transport out of the ER.

Glycosylation

Most proteins that traverse the secretory pathway are modified by the addition of oligosaccharide side chains either to the amino group of asparagines (*N*-linked glycosylation) or through the hydroxyl group of serines or threonines (*O*-linked glycosylation). *N*-linked moieties are added cotranslationally in the lumen of the ER, where mannose-rich oligosaccharides are transferred from a lipid (dolichol) carrier to asparagine residues present in NXS/T motifs (where X is any amino acid but proline) within the protein. Trimming of terminal glucose and mannose residues from the branched oligosaccharide occurs in the ER and is closely linked with the Cnx- and Crt-mediated quality control process (reviewed in 27). Further trimming of mannose residues followed by addition of other sugars (*N*-acetylglucosamine, galactose, fucose, and sialic acid), to yield complex oligosaccharide structures, occurs in the Golgi complex. *O*-linked oligosaccharides are also added in this organelle.

Transport Through the Secretory Pathway

Transport of soluble and membrane-spanning proteins from one compartment of the secretory pathway to the next is mediated by the formation of coated membrane vesicles that travel to and fuse with the target organelle. Thus, once the process of protein folding and quality control has been completed in the ER, proteins are sequestered into these transport vesicles before transit to the Golgi complex. The processes of cargo protein selection, budding, targeting, and fusion are probably all mediated by the specific protein coats that define the different transport vesicles involved in shuttling proteins between components of the secretory pathway (107). In the case of ER to Golgi transport, the vesicles have COPII coats (3), whereas retrograde transport of vesicles from the Golgi to the ER, as well as anterograde transport through the Golgi, is mediated by COPI coats (69,93). COP is an acronym for coat protein. Budding is initiated when a small myristylated protein (SAR/ARF) is converted to the GTP-bound form, allowing it to bind to the membrane and recruit coat proteins (coatomers) in a stoichiometric manner (3). Formation of the coat itself induces membrane curvature

and vesicle budding. The N-ethylmaleimide-sensitive factor (Nsf) and the soluble NSF attachment proteins (SNAPs) appear to play a crucial role in subsequent vesicle fusion events. Membrane receptors that mediate docking of the transport vesicle with the target organelle are also incorporated into the coat and appear to define the specificity with which the cargo protein is delivered. These molecules are termed SNAP receptors or *SNAREs* (117). A target-specific SNARE (t-SNARE) that interacts only with a vesicle specific SNARE (v-SNARE) identifies the target membrane. After docking, Nsf and soluble Nsf attachment proteins SNAPs bind to the SNARE complex. Vesicle membrane fusion is initiated when Nsf hydrolyzes adenosine triphosphate (ATP) and induces a conformational change in the complex (reviewed in 107).

Protein Localization

Subcellular localization of proteins within the secretory pathway appears to be determined by a combination of sorting and targeting signals that mediate interactions with the coat complex for inclusion in a transport vesicle and of retention signals that localize the protein to a specific compartment within the secretory pathway. Localization is enhanced by the interplay of anterograde and retrograde transport that allows retrieval of proteins inadvertently transported beyond their target location. The classic example of this is the KDEL peptide sequence found on soluble proteins that are localized to the lumen of the ER (81). Proteins containing this sequence are efficiently retrieved from the *cis*-Golgi by the KDEL receptor, which is incorporated into COPI vesicles for trafficking back to the ER. Similarly, membrane-spanning proteins localized to the ER have at the C-terminus of the cytoplasmic domain a KKXX amino acid motif that ensures their efficient retrieval from the Golgi complex (53). This type of motif is used by the primate foamy viruses to concentrate the envelope glycoprotein complex (gp80 and gp48) in the ER intermediate compartment, where virus budding occurs (37). For integral membrane proteins, retention signals often appear to be associated with the membrane-spanning domains of the protein, as is the case for the coronavirus M protein, and may reflect preferred association with specific lipid compositions of the membrane within a particular component of the secretory pathway (see later).

The Golgi Complex

The Golgi complex represents a unique organelle within the secretory pathway in that it is composed of a series of membrane-bound compartments that are the sites for specific biochemical modifications to proteins and oligosaccharides, as well as locations where specific protein sorting decisions are made. Proteins transported from the ER enter the Golgi complex through the *cis*-Golgi network, and after traversing the *cis*-, medial-, and *trans*-cisternae, exit through the *trans*-Golgi network (107). Each of the compartments provides a spatially distinct site for maintaining an ordered set of enzymes involved in the process of oligosaccharide maturation. They are also the sites at which proteins undergo *O*-linked glycosylation, through the addition, at certain serines and threonines, of monomeric sugar residues. It is in the *trans*-Golgi and *trans*-Golgi network where viral glycoprotein precursors, such as the Env polyprotein of the retroviruses, are cleaved to their mature forms through the action of members of the furin family of proteinases—enzymes that normally function to process cellular substrates, such as polypeptide hormone precursors. This cleavage event is crucial to the generation of a biologically functional glycoprotein and thus to virus infectivity (60).

INTRACELLULAR TARGETING AND ASSEMBLY OF VIRION COMPONENTS

Viruses can be nominally divided into two groups based on the presence or absence of a lipid bilayer envelope. The nonenveloped viruses can assemble in the cytoplasm or nucleus and generally, for those that propagate in animal cells, exhibit icosahedral symmetry (see Chapter 3). For these viruses, the viral structural proteins and genomic nucleic acid must be targeted to or retained at the subcellular domain at which assembly occurs. Enveloped viruses, by their nature, must acquire a lipid bilayer from one of the cell's membranes during the process of assembly. In a few viruses, such as the herpesviruses and some retroviruses, this envelopment step takes place after the assembly of an intact capsid shell, whereas for others, the processes of envelopment and capsid assembly occur concomitantly. Some viruses undergo transient envelopment and, in certain cases, reenvelopment during the process of assembly.

For nonenveloped viruses, the tightly assembled structure of the icosahedral shell forms a protective coat that prevents degradation of the genome by environmental factors. For enveloped viruses, the integrity of the nucleocapsid structure is less crucial because the membrane provides a barrier to external degradative enzymes.

Assembly of Nonenveloped Viruses in the Nucleus

Adenoviruses are nonenveloped icosahedral viruses, 70 to 100 nm in diameter that have a protein shell surrounding a DNA core. The protein shell (capsid) is composed of 252 capsomeres, of which 240 are hexons and 12 are pentons. Each penton consists of a five-subunit base (polypeptide III) and a trimeric fiber (polypeptide IV) that extends out away from the shell. The hexon capsomeres are composed of trimers of three tightly associated molecules of polypeptide II (Fig. 7).

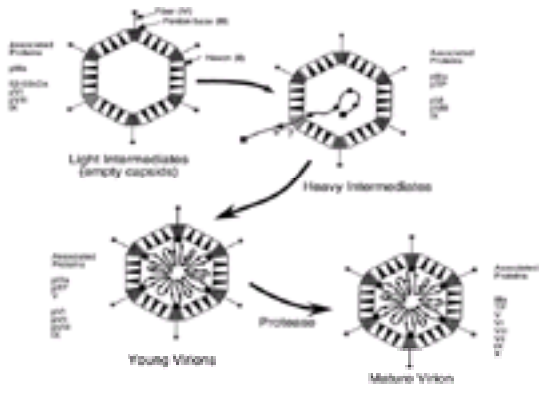

FIG. 7. Assembly pathway for adenoviruses. After transport into the nucleus, the hexons and pentons are proposed to

assemble into *empty capsids* (previously known as *light intermediates of assembly*). This stage of assembly appears to require the L1 region 52- to 55-kd scaffolding proteins. These proteins are lost on association with viral DNA, which is inserted into this structure through a packaging sequence at the left end of the genome. The mechanism of insertion is not known. *Heavy intermediate* forms of the capsid probably represent those in which DNA packaging is incomplete and the DNA is fragmented. Precursor core proteins are packaged into the empty capsid along with the genome to form *young virions.* Proteolytic cleavage of the precursor proteins by the viral proteinase yields the mature virion.

For a nonenveloped virus such as adenovirus, which replicates exclusively in the nucleus, there is a strong dependence on nuclear targeting and transport pathways to export newly synthesized mRNAs out of the nucleus and to import structural proteins back into the nucleus. Nuclear import of the major capsid protein, polypeptide II, depends on the involvement of a second adenovirus protein, the pVI (precursor) polypeptide (59), which provides the necessary NLS for transporting the trimer into the nucleus. Trimer formation is in turn dependent on yet another virus-encoded, chaperone-like protein, L4 100K, which transiently binds to the newly synthesized hexon monomer and mediates its association with two additional monomers (9,10). Thus, the most abundant structural protein of the adenovirus capsid needs to interact with two additional virus-encoded factors to attain the correct tertiary structure and subcellular location for assembly.

The two other proteins that form the 12 vertices of the capsid, penton, and fiber appear to assemble independently in the cytoplasm. Mutations in the C-terminus of the penton that block assembly into pentamers do not prevent transport into the nucleus (58), indicating that each monomer has an active NLS. In contrast, the fiber must form trimers to be efficiently transported to the nucleus (91), even though this protein has an active NLS located at its N-terminus (50). It seems likely that the oligomerized penton base and fibers are transported independently into the nucleus and assemble into the intact penton at the site of assembly.

Two distinct pathways for adenovirus capsid assembly have been postulated from a large body of work in this area. Results from experiments that combined kinetic labeling with temperature-sensitive replication mutants yielded an assembly scheme shown in Figure 7, where, in a manner similar to that observed with DNA phages, an empty capsid is first formed. DNA and associated core proteins are then subsequently packaged into these empty shells to yield “young virions” that then undergo proteolytic maturation. Additional viral products appear to act as scaffolding proteins around which shells are assembled and which facilitate the encapsidation process. These proteins are present in the intermediate capsid-like particles but are absent once DNA has been encapsidated (reviewed in 20). Disruption of adenovirus capsids with denaturants results in the release of groups of nine hexons that are associated with the faces of the icosahedron and that lack the peripentonal hexons. Under acid conditions, these nanomers can reassemble to form icosahedral shells that lack the 12 vertices, which would normally be composed of the penton and the five peripentonal hexons, raising the possibility that hexon nanomers are intermediates of adenovirus capsid assembly.

The empty-capsid intermediate assembly pathway has, however, been brought into question by the observation that DNA packaging appears to be intimately linked with capsid assembly. A mutation in the DNA sequence necessary for genomic packaging, which results in inefficient DNA–capsid interactions, blocks assembly of empty capsids and results in the accumulation of nonassembled capsid precursors in the nucleus (45,46). This would argue that capsid assembly does not occur spontaneously in the absence of an interacting DNA, that DNA–protein interactions play a crucial role in initiating the assembly process, and that assembly might proceed around the DNA-containing core.

Assembly of Enveloped Viruses in the Nucleus

Two enveloped animal virus families, both of which use cell components that are located within the nucleus in their replication, initiate their assembly within that compartment. These are the herpesviruses and the orthomyxoviruses. In addition to importing into the nucleus the necessary components for assembly, these viruses must also export large nucleoprotein complexes back out into the cytoplasm.

In some respects, the herpesviruses represent a hybrid between a nonenveloped virus, such as adenovirus, and a more conventional enveloped virus, such as a retrovirus, in that they use the secretory pathway to transport large capsid structures from the nucleus to the outside of the cell. This group of large viruses assemble, within the nucleus, an icosahedral capsid shell that is 160 Å thick and 1,250 Å in diameter. The major component of this protein shell is VP5, which forms both the pentameric and hexameric capsomeres necessary to assemble the icosahedral structure. Associated with the outer surface of the hexamers is the abundant small protein VP26. Two additional proteins, VP19C and VP23, in a 1:2 ratio, form heterotrimeric triplexes, which fit between and link together adjacent capsomeres (87). Scaffolding proteins are essential for herpes simplex virus type 1 (HSV-1) capsid assembly and maturation, and in their absence, incomplete and aberrantly shaped capsids are assembled. As with adenovirus, the major capsid protein lacks a nuclear targeting signal, and its transport into the nucleus requires an interaction with either the scaffolding protein (VP22a) or the triplex protein VP19C, either of which can presumably provide the necessary NLS. VP23 similarly requires VP19C for nuclear localization, whereas VP26 appears to be directed there by its interaction with VP5 (100).

Studies of virus-infected cells, together with *in vitro* assembly studies, have provided valuable insights into the assembly process (88). These studies point to a pathway in which VP5, VP19C, and VP23 assemble around a scaffold to form an icosahedral, but predominantly spherical “procapsid”—the “B-capsids” identified by electron microscopy. The size and integrity of this structure is defined by the triplex proteins, which mediate the interactions between capsomeres (108,127). Cleavage of the scaffolding protein at a site near its C-terminus removes a 25–amino acid sequence that is necessary for binding to the major capsid protein, VP5. The resulting disassociation and release of scaffold allows for packaging of the viral DNA genome and induction of maturation of the capsid into a more angular icosahedral structure, the previously identified “C-capsids.” In the absence of an active proteinase, the scaffolding proteins remain associated with the protein shell preventing packaging of the viral DNA, and the procapsid is unable to mature (89,101). Several aspects of this

morphogenic pathway parallel the assembly and maturation of the bacteriophage P22 (61).

Unlike adenoviruses, which accumulate in the nucleus and are released on lysis of the cell, herpesviruses exit the nucleus by budding through into the lumen of the nuclear membrane. It is in this way that a herpesvirus initially enters the secretory pathway of the cell; the complexities of this interaction are discussed later in this chapter.

For influenza virus to take advantage of its unusual capacity to “steal” the capped 5' ends of host cell mRNAs to initiate its own mRNA synthesis, transcription and viral RNA replication must occur in the nucleus (see Chapter 7 and Chapter 46). Genomic (minus sense) RNAs are replicated by a different mechanism to yield templates for mRNA synthesis as well as progeny viral genomes. The eight viral RNA segments of this virus are packaged into individual RNPs containing the four proteins of the transcriptase complex (PB1, PB2, PA, and NP, each containing a functional NLS sequence) but are not exported into the cytosol until late in infection when the viral matrix (M1) protein begins to be synthesized (71). Under conditions in which matrix synthesis is inhibited, the viral RNPs (vRNPs) accumulate in the nucleus, tightly associated with the nuclear matrix. The block to export can be relieved by expression of matrix from an independent vector (8). Matrix association with the vRNPs also appears to be important for preventing their reentry into the nucleus because conditions such as acidification or mutations that promote dissociation of M1 allow the RNPs to be reimported (reviewed in 133). Thus, the matrix protein of influenza virus is a key modulator of vRNP transport into and out of the nucleus.

Assembly of Viruses in the Cytoplasm

Although targeting and import of proteins into the nucleus or secretory pathway involves well-characterized motifs on the proteins involved, the processes by which proteins are targeted to destinations within the cytoplasm remains, for the most part, obscure. Most viruses, even those that are nonenveloped, initiate or complete their assembly in association with membranes of the secretory pathway. Nevertheless, the intracellular pathways that function to transport their capsid components and genomes to these sites have not been defined. Reoviruses are the only animal viruses that appear to complete their assembly entirely in the cytoplasm without the involvement of membranes. Both genome replication and virus assembly occur in specialized areas of the cytoplasm known as *virus factories.* Little is known about the establishment and retention of proteins in these sites, but this specialized structure may be a mechanism to avoid the complexities of transporting virion components to a separate cytoplasmic assembly site after translation.

Intracytoplasmic Transport and Assembly of Retroviral Capsids

Retroviruses are enveloped viruses that, for the most part, complete their assembly by budding through the plasma membrane of the infected cell. The immature capsid of the virus is assembled from polyprotein precursors that must be transported through the cytoplasm to the underside of the plasma membrane. The membrane-spanning viral glycoproteins, on the other hand, must be transported through the secretory pathway of the cell to the cell surface, where they co-localize with the nascent, membrane-extruding capsid. All replication competent retroviruses contain four genes that encode the structural and enzymatic components of the virion. These are *gag* (capsid protein), *pro* (aspartyl proteinase), *pol* (reverse transcriptase and integrase enzymes), and *env* (envelope glycoprotein) (see Chapter 57). However, the product of the *gag* gene has been shown to possess all the necessary structural information to mediate intracellular transport, to direct self-assembly into the capsid shell, and to catalyze the process of membrane extrusion known as *budding* (109). In most retroviruses, the nascent Gag polyproteins are transported directly to the plasma membrane, where assembly of the capsid shell and membrane extrusion occur simultaneously (Fig. 8). Viruses that undergo this type C form of morphogenesis include members of the *Alpharetroviruses* and *Gammaretroviruses*. Lentiviruses and *Deltaretroviruses* assemble their capsids in a similar fashion. In the second morphogenic class of viruses, the type B/D class, the Gag precursors appear to be targeted first to an intracytoplasmic site, where capsid assembly occurs. These preassembled immature capsids are then transported to the plasma membrane where they undergo budding and envelopment (see Fig. 8). Viruses that undergo this process of assembly and release include members of the *Betaretroviruse*s. Members of the *spumavirus* genus also assemble immature capsids in the cytoplasm, but they are targeted to the ER for envelopment.

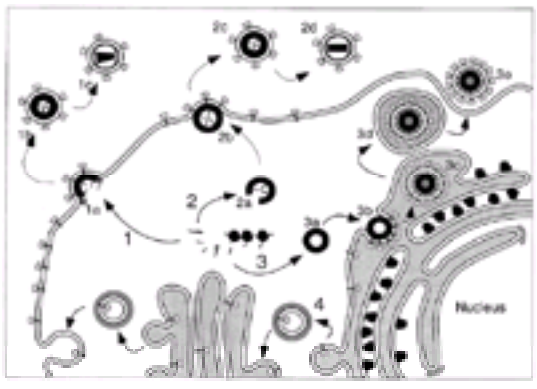

FIG. 8. Assembly of retroviruses. The assembly pathways of retroviruses that exhibit C-type morphogenesis (*Pathway 1*) , B- and D-type morphogenesis (*Pathway 2*) and that of the foamy viruses (*Pathway 3*) are shown. The envelope glycoproteins are translated on membrane-bound polysomes and, for most retroviruses, are transported to the cell surface through the cell's secretory pathway (*Pathway 4*). For all morphogenic classes, the Gag proteins are synthesized on free polysomes. In the case of the C-type morphogenic viruses (i.e., Rous sarcoma virus and human immunodeficiency virus), the Gag and Gag-Pol proteins migrate either individually or in small multimers to the plasma membrane, where immature capsid assembly and envelopment occurs concurrently (*1a*). At some ill-defined point in this pathway, the viral genomic RNAs associate with the Gag and Gag-Pol precursors and are incorporated into the developing capsid. In the case of the B- and D-type viruses, the Gag and Gag-Pol precursors are first transported to an intracytoplasmic assembly site (*2a*), where they assemble into immature capsids. The immature structures are then transported (*2b*) to the plasma membrane, where they associate with the envelope glycoproteins and induce viral budding. For both classes of retroviruses, the capsids of the nascent immature particles appear as doughnut-shaped

structures in thin-section electron microscopy and contain unprocessed Gag and Gag-Pol precursors (*1b* and *2c*). The mature virus particles contain electron-dense cores with morphologies characteristic of the virus (*1c* and *2d*). The maturation step is required for infectivity and is the result of the activation of the viral protease, which cleaves the Gag and Gag-Pol precursors into the internal structural and enzymatic proteins of the virus. For the foamy viruses, Gag and Pro-Pol precursors assemble into immature capsids in the cytoplasm (*3a*). Budding primarily occurs at the endoplasmic reticulum membrane where the viral glycoproteins are retained (*3b*). The enveloped virion is presumably transported to the plasma membrane by transport vesicles (*3d*). Maturational cleavage of the immature core is limited to removal of 4 kd from the C-terminus of the precursor; the mature infectious virion maintains an immature morphology (*3e*).

Although the size and protein content of the precursor varies between different retroviral families, at least three *gag*-encoded proteins are found in all retroviruses; these are the matrix protein (MA), the capsid protein (CA), and the nucleocapsid protein (NC). In addition to these functionally conserved domains, the Gag precursor can, depending on the virus encoding it, contain additional peptide sequences (Fig. 9), whose function in virus assembly and future cycles of infection is only now being resolved. Some of these additional domains appear to allow the correct folding of the precursor protein for assembly and subsequent processing into the mature virion proteins.

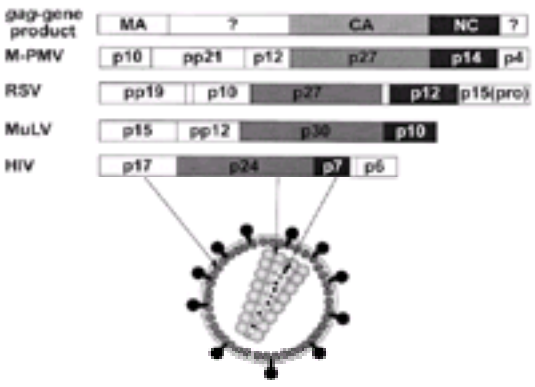

FIG. 9. Organization of the retroviral Gag precursor. The Gag precursor polyproteins of all retroviruses contain, beginning at the N-terminus, matrix (MA), capsid (CA), and nucleocapsid (NC) domains, linked in this order. The *gag* gene products of Mason-Pfizer monkey virus (M-PMV), Rous sarcoma virus (RSV), murine leukemia virus (MuLV), and human immunodeficiency virus (HIV) are shown. The *unshaded boxes* represent regions of the Gag precursor for which no common functions or locations in the mature virion have been established. However, for these three representatives of the alpha-, beta-, and gamma-retroviruses, a “late domain” function required for pinching off of the virus particle from the cell is located between the MA and CA domains. The specific name associated with the Gag cleavage products is derived from their respective apparent molecular weights ($\times 10^{-3}$).

The detailed mechanisms by which the capsid precursor proteins are directed to the site of assembly remains for the most part unknown, but the process is mediated primarily by the MA domain of the Gag precursor. In most retroviruses, the matrix protein contains two elements involved in plasma membrane targeting. The first of these is an N-terminal myristic acid, which is thought to insert into the hydrophobic lipid bilayer. The second is a surface patch of basic amino acids that are hypothesized to mediate the initial interaction of Gag with the negatively charged, phospholipid head groups of the membrane. The bipartite signal created by these two elements appears to be important for intracellular targeting to the plasma membrane because mutations that interfere with either myristylation or the charged residues can abrogate plasma membrane targeting. In some instances, Gag precursors mutated in these sequences are targeted to internal membranes (reviewed in 33).

In the *Betaretroviruses*, where capsid assembly and virus budding are discrete events, a recent genetic dissection of this process suggests that Gag-containing precursors of these viruses express a dominant sorting signal, identified as a surface loop of the MA domain, that targets the proteins to the initial assembly site. A point mutation within this region of the Mason-Pfizer monkey virus (M-PMV) MA domain abrogates intracytoplasmic targeting and results in the type C–like transport of precursors to the plasma membrane, where efficient capsid assembly occurs. In addition, transfer of the protein motif to the Gag of the C-type virus murine leukemia virus (MuLV) results in its retention and assembly in the cytoplasm (11). These results suggest that the *Betaretroviruses* have evolved to use a transport pathway of the cell to target precursors for intracytoplasmic assembly.

By what pathways might preassembled immature capsids of the *Betaretroviruses* or the Gag-containing precursors of the C-type viruses be transported to the plasma membrane? Several studies have suggested that cytoskeletal elements might be involved in this process, but direct evidence for this has not been obtained (reviewed in 62). Recent experiments indicate that the transport process is an active rather than a passive one since it appears to require energy. In cells treated with agents that block ATP synthesis, M-PMV immature capsids are unable to migrate to the plasma membrane from sites deep within the cytoplasm (132).

Irrespective of the assembly site, Gag precursor proteins must associate in a reproducible fashion to assemble into the nascent capsid. Mutational analyses of *gag* genes, as well as *in vitro* assembly studies, have shown that the CA and NC domains of Gag play a crucial role in assembly; MA is dispensable for this process (reviewed in 17 and 33). NC binding to RNA may act to nucleate the capsid assembly process, whereas CA forms a symmetric network of proteins. *In vitro,* the CA protein alone can assemble into tubes that exhibit local sixfold symmetry (hexamers), but cryoelectron microscopy studies of immature capsids has revealed a large heterogeneity in particle size and no evidence of icosahedral

symmetry. It is possible, therefore, that the immature capsid has a fullerene-like structure similar to that proposed for the mature core (34,86).

For most retroviruses, capsid assembly drives the process of membrane extrusion known as budding. As discussed later, in order for an infectious virus to be formed, the Gag precursors (or for other viruses, NPs) and surface glycoproteins of the virus must be targeted to the same region of the same membrane. In this way, during virus budding, a proper complement of glycoproteins can be incorporated into the nascent virion.

Assembly of Enveloped Viruses at Cellular Membranes

For most enveloped viruses, the location within the cell at which envelopment takes place is determined by the targeting to or retention of the viral glycoproteins at that site. Indeed, with the exception of the retroviruses, the efficiency of virus budding and particle release is highly dependent on the presence of the envelope glycoprotein, and in its absence, few particles are produced. Because the glycoproteins define the site of virus budding, specific interactions between the viral NP and the glycoproteins, sometimes mediated by a matrix protein, must take place to ensure that the genome of the virus is incorporated. For each of the viruses that assemble at intermediate points within the secretory pathway, fully assembled viruses must traverse the remainder of the pathway to be released from the cell (Fig. 10). Glycoproteins on these released viruses have complex oligosaccharides and are most likely modified by the Golgi-localized enzymes on the way to the cell surface.

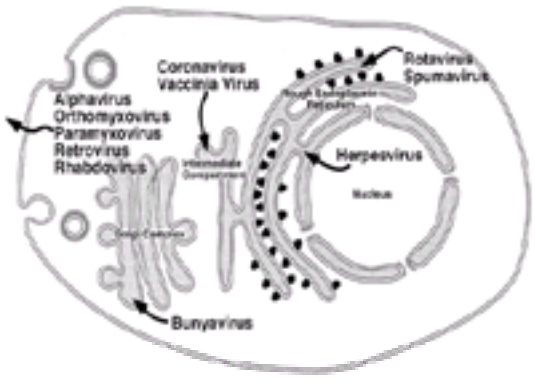

FIG. 10. Viral assembly at cellular membranes. Schematic representation showing the intracellular locations at which enveloped virus assembly takes place. For each virus that is enveloped at organelles within the secretory pathway, the virions must traverse the remainder of the pathway to be released from the cell. For the herpesviruses, two morphogenic pathways have been proposed (see also Fig. 12). In the first, the virus buds into the lumen of the nuclear membrane and is then transported to the cell surface through the secretory pathway. In the second, the virus also buds into the lumen of the nuclear membrane, but then the nascent virus fuses with the outer nuclear membrane to release the capsid, which is then enveloped by membranes derived from the Golgi complex. Rotaviruses appear to use the endoplasmic reticulum (ER) membrane as a scaffold for assembly of virion proteins—the capsids that form during the assembly process are only transiently enveloped, and nonenveloped particles accumulate in the lumen of the ER. Coronaviruses localize the three membrane proteins (E, M, and S) in the ER intermediate compartment, in which virus budding occurs. In contrast, the vaccinia virus nucleocapsid appears to be wrapped by a double membrane derived from this compartment but remains free in the cytoplasm so that it can then be further enveloped by Golgi-derived membranes. The G1 and G2 glycoproteins of bunyaviruses co-localize in the *trans*-Golgi compartment to direct budding of nucleocapsids at this location. For the several families of viruses that assemble at the plasma membrane, the final location of the viral glycoproteins also appears to be important in defining where assembly occurs. Even for the retroviruses, which can assemble and release virus particles in the absence of envelope glycoproteins, these proteins direct virus budding to the basolateral plasma membrane of polarized epithelial cells.

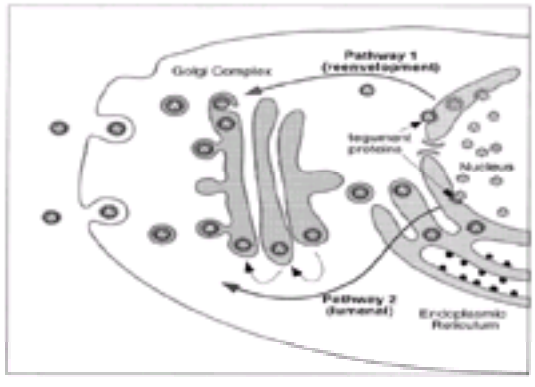

FIG. 12. Herpesvirus assembly. Two pathways are proposed for herpesvirus assembly and release from the cell. In the reenvelopment pathway (*Pathway 1*), nascent assembled capsids bud into the lumen of the nuclear membrane using a subset of virally encoded glycoproteins. These enveloped particles then fuse with and are released from the outer nuclear membrane into the cytoplasm. There, they associate with the tegument proteins and are transported through the cytoplasm to be enveloped by Golgi-derived membranes that contain the full complement of virion glycoproteins. In the lumenal pathway (*Pathway 2*), the nascent capsids associate with tegument proteins in the nucleus before budding through the inner nuclear membrane, which must contain a full complement of virion glycoproteins. The enveloped virus particle now traverses the secretory pathway, and the glycoproteins are modified to their mature form during transit through the Golgi complex.

Assembly at the Endoplasmic Reticulum Intermediate Compartment

The coronaviruses are positive-stranded RNA viruses with large (30 kb) genomes packaged in a helical nucleocapsid. The nucleocapsid acquires its envelope by budding into the lumen of a pre-Golgi compartment of the secretory pathway termed the *endoplasmic reticulum–Golgi intermediate compartment*. Coronaviruses invariably encode three envelope proteins. The spike protein (S), which determines the host range of the virus, is a type I glycoprotein that forms the distinct bulbous peplomers of the virus. Expressed independently of the other glycoproteins, S is transported to the plasma membrane and so is unlikely to determine the site of virus assembly. The most abundant virion protein is the membrane (M) glycoprotein. M spans the lipid bilayer three times, exposing a short N-terminal domain outside the virus and a long C-terminus inside the virion. Because of its abundance and because M is transported to the Golgi complex but not to the surface, it was initially thought to define the site at which this family of viruses were enveloped. Recent studies, however, have shown that the small envelope protein (E), which is only a minor component of virions, is the key to defining the site and nature of coronavirus envelopment. E is a hydrophobic type-I membrane protein that localizes to the intermediate compartment of the ER and induces the formation of tubular, convoluted membrane structures characteristic of virus infection (15,98). How E is retained in the intermediate compartment is not known. Coexpression of E, M, and S results in the assembly and release from cells of virus-like particles containing these three viral membrane proteins. The enveloped particles produced by this system form a homogeneous population of spherical particles indistinguishable from authentic virions in size and shape (129). Only M and E are required for efficient particle formation, and expression of E alone can mediate particle release (68). The E protein plays a key role in morphogenesis because mutations in this protein can result in morphologically aberrant viruses (30). The S glycoprotein is thus dispensable for virus particle assembly but is retained in the Golgi by the M protein, which appears to direct its assembly into virions. M protein also directs the incorporation of nucleocapsids containing the genome length RNA into virions, and nucleocapsids associated with newly synthesized M protein have been localized to the budding site in the intermediate compartment (85).

Although most of the retroviruses are enveloped at the plasma membrane of the cell, members of the spumavirus genus bud into the ER intermediate compartment region of the secretory pathway. Expression of the primate foamy virus Env complex, gp80-gp48, in the absence of other structural proteins, results in its localization to the ER. A dilysine ER retrieval motif at the C-terminus of gp48 is responsible for this location, and mutation of either lysine results in efficient transport of the protein to the plasma membrane. Although a greater fraction of capsids bud from the plasma membrane in mutant virus–infected cells, envelopment of capsids at the ER is still observed (37). A second distinguishing feature of foamy viruses is the dependence of capsid envelopment on Env expression. As with the *Betaretroviruses*, immature foamy virus capsids assemble in the cytoplasm. They are then transported to the ER (or plasma membrane) for envelopment. In the absence of foamy virus Env, these preassembled capsids do not associate with membranes or initiate budding (31). Recent experiments have shown that it is the posttranslationally cleaved (148 amino acids long), membrane-spanning, signal peptide domain of the foamy virus Env that mediates capsid membrane association and envelopment and that this protein is incorporated into the virus in the process.

Assembly in the Golgi Complex

Bunyaviruses are negative-stranded, enveloped viruses with a segmented genome that bud into the Golgi complex (see Fig. 10). The glycoprotein spikes of the best-characterized member of this family, Uukuniemi virus, are composed of two type I glycoproteins, G1 and G2, that determine the site of virus budding. G1 and G2 are cotranslationally cleaved from a single precursor protein by signal peptidase, which cleaves after the internal signal sequence that mediates translocation of G2. The two proteins have been shown to fold with distinctly different kinetics, but once properly folded, they form a G1-G2 heterodimer that is transported to the Golgi complex. G2 expressed in the absence of G1 is retained in the ER, whereas G1 expressed alone is targeted to the Golgi (reviewed in 42). The Golgi localization signal of G1 has been mapped, through analysis of mutations and glycoprotein chimeras, to the membrane proximal half of the 98–amino acid long cytoplasmic tail of the protein. Surprisingly, a region of 81 amino acids, between the putative transmembrane domain and the G2 signal peptide at the C-terminus of the G1 cytoplasmic tail, expressed as a soluble protein or as a green fluorescent protein (GFP) fusion, could localize to the cytoplasmic face of the Golgi complex (2). Glycoprotein retention in this case, therefore, appears to depend on interactions between the cytoplasmic tail of G1 in the G1-G2 heterodimer, with components residing on the cytoplasmic side of the Golgi membrane.

During infection by bunyaviruses, the helical nucleoproteins, consisting of the three single-stranded genomic RNA segments and the associated nucleocapsid (N) protein, accumulate in the region of the Golgi and presumably interact with the G1 cytoplasmic domain to initiate the budding of virus particles into the Golgi lumen. The region of the secretory pathway at which G1-G2 heterodimers accumulate clearly defines the budding site because, in the presence of brefeldin, a drug that redistributes Golgi components to the ER, virus budding occurs into the ER (42).

Assembly at the Plasma Membrane

Members of several virus families undergo their envelopment at the plasma membrane. These families include the togaviruses, rhabdoviruses, paramyxoviruses, orthomyxoviruses, and retroviruses. In each of these cases, the viral glycoproteins, either as hetero-oligomeric complexes or homo-oligomers, have traversed the entire secretory pathway to be delivered to the plasma membrane of the cell. Assembly at the plasma membrane obviates the need for the assembled virus to navigate additional compartments of the secretory pathway because virions are released directly into the external milieu of the cell. Alphaviruses are perhaps the best characterized of these different viral systems because a combination of biochemical, genetic, and structural information has been amassed to shed light on the complexity of this assembly process. The major glycoproteins E1 and E2 of the alphaviruses are translated from a subgenomic 26S RNA as a p62, 6K, E1 precursor complex that is transported to the Golgi, where p62 is processed to E2. Stable trimers of E1-E2 heterodimers are then transported to the plasma membrane, where they associate with assembled core structures composed of the capsid (C) protein and genome-length RNA (reviewed in 35). Recent cryoelectron microscopy analyses of mature alphavirus particles have revealed for the first time a detailed structure of this enveloped virion (see Chapter 3 and Chapter 29). These studies have shown that both the envelope and the core display icosahedral symmetry.

Surprisingly, however, the trimers of E1 and E2 are located at the threefold and quasi-threefold symmetry axes of the pentameric and hexameric order of the nucleocapsid (Fig. 11). Moreover, the heterodimers of each spike splay out above the membrane in a skirtlike fashion, traverse the lipid bilayer individually, and interact with three underlying capsid (C) proteins that belong to three separate capsomeres. This creates a complex network of molecular interactions, where the glycoprotein–capsid protein interactions not only mediate the binding of the nucleocapsid to the spikes but also stabilize the connections between the capsomeres. Above the membrane, the skirts formed by the E1-E2 heterodimers form lateral connections that mimic the pentameric and hexameric arrangement of the capsid. These interactions may facilitate the process of budding by providing a multivalent binding site for the capsid as well as by providing a force for membrane bending (35). Budding, however, requires a cooperative interaction between the glycoproteins and the capsid protein because, in the absence of either, budding does not occur. Moreover, mutations in the cytoplasmic tail of the E2 protein alone can abrogate budding. It has been proposed that alphavirus budding is initiated by NC binding through hydrophobic interactions between the cytoplasmic domain of E2 and the capsid protein to a cluster of E1-E2 trimers at the plasma membrane. The recruitment of additional clusters of glycoprotein heterodimers into the complex by cooperative trimer–trimer and heterodimer–capsid protein interactions would continue until envelopment is completed (35). This model does not, however, take into account a role for the 6K protein, which can clearly influence the budding process and may be present at the cell surface as part of the glycoprotein complex.

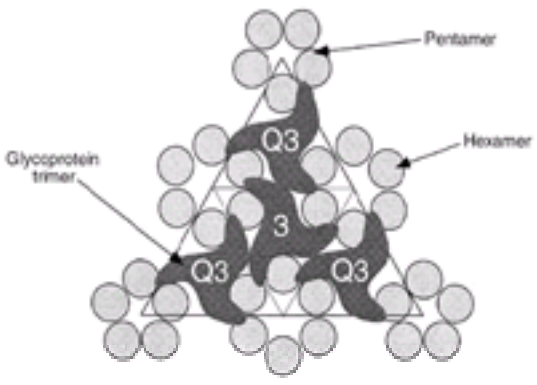

FIG. 11. Alphavirus glycoprotein–capsid interactions. Schematic representation of the interactions between the glycoprotein spikes at the threefold (*3*) and quasi-threefold (*Q3*) axes of the nucleocapsid structure and the capsid proteins of the hexameric and pentameric capsomeres of that structure. The spikes are involved in extensive lateral interactions near the lipid bilayer through their skirts, and through interactions with the capsid protein, they stabilize interactions between capsomeres. (Modified from ref. 35, with permission.)

For the negative-stranded RNA viruses, orthomyxoviruses, paramyxoviruses, and rhabdoviruses, an additional protein, the matrix protein, mediates the interactions between the viral glycoproteins and the RNP and appears to play a key role in envelopment. These proteins are able to bind to membranes through hydrophobic domains or, as with the retroviruses, through a cluster of positively charged residues that initiates electrostatic interactions with the plasma membrane. Cross-linking studies have demonstrated that these M proteins form homo-oligomers in the virus and can self-associate *in vitro* or when expressed at high levels in cells. As discussed earlier, the M1 protein of influenza virus acts to mediate the transport of RNPs from the nucleus to the site of virus assembly on the plasma membrane. Little is known about the intracytoplasmic transport pathways involved in targeting this complex; nevertheless, the RNPs appear to accumulate at regions of the plasma membrane enriched for M1. The importance of the M protein in the paramyxovirus budding process was inferred from the defective measles viruses found in subacute sclerosing panencephalitis (SSPE) that are unable to assemble virus particles and that have mutations in the M protein–coding region (6). However, the recent development of reverse genetics systems for the negative-stranded viruses, in which mutations can be reintroduced into the viral genome, has allowed a more rational approach to examining these questions. Construction of rhabdovirus genomes that lack the M coding region resulted in a dramatic (more than 105-fold) decrease in the release of virus particles, and those that were released lacked the characteristic bullet-shape morphology. The defect could be complemented by expression of M in *trans* (74). Thus, in the rhabdoviruses, the M protein condenses the helical RNP into its characteristic shape and mediates the envelopment process.

Similar approaches have begun to shed light on the role of the G glycoprotein in rhabdovirus assembly. Viruses that lack the G protein–coding domain do assemble and release bullet-shaped particles but at only 3% the efficiency of wild-type viruses (73). Initial studies with G proteins lacking a cytoplasmic tail suggested that this domain might be important in the budding process; however, recent studies have shown that although there is a general requirement for at least a short cytoplasmic tail, it is amino acid sequence independent. Thus, the basis for M protein interactions with G is unresolved. Furthermore, foreign glycoproteins can be efficiently incorporated into rhabdovirus particles, but they do not stimulate budding (102). This is consistent with recent experiments that have identified a domain within the extracellular membrane proximal stem (GS) of vesicular stomatitis virus (VSV) G that is required for efficient VSV budding. Recombinant viruses encoding glycoprotein chimeras with 12 or more membrane proximal residues of the GS, as well as the G protein transmembrane and cytoplasmic tail domains, produced near wild-type levels of particles. In contrast, those with shorter regions produced 10- to 20-fold fewer particles. It is possible, therefore, that this region of the G protein membrane proximal domain modifies the membrane to facilitate the budding process (103).

In influenza virus, the fact that the cytoplasmic domains of both the HA and the neuraminidase (NA) are highly conserved in all subtypes of the virus pointed to a role for this domain in assembly of enveloped virus. Early studies indicated that HA proteins with foreign cytoplasmic tails are not incorporated into virions, although HAs lacking this domain can be incorporated at levels 50% that of wild-type viruses (82). Recent studies using reverse genetics have confirmed these findings and have shown that the short (6 amino acid) cytoplasmic domain of NA also plays an important role in morphogenesis. Viruses encoding the truncated NA protein were released less efficiently and were larger and more filamentous. However, in viruses encoding tail-less versions of both HA and NA, a 10-fold reduction in virus release was

observed, and morphogenesis was drastically altered. Virus particles released from these cells were greatly elongated, with an extended irregular shape and a reduced level of viral RNA. Thus, it appears that for influenza virus, the interactions between M1 and the viral glycoproteins are so important for envelopment and morphogenesis that the virus has developed redundant interaction domains in both HA and NA (55,136).

As described earlier, with the exception of the spumaviruses, glycoprotein–capsid interactions are not required for the assembly and release of enveloped retrovirus particles; the Gag precursor alone contains the information necessary to be specifically targeted to the plasma membrane and to drive budding. Moreover, there is contradictory evidence regarding the role of the cytoplasmic domain in directing the envelope glycoprotein into budding virions because Env proteins lacking a cytoplasmic domain can be efficiently incorporated into retrovirus particles and can effectively mediate virus entry into target cells (reviewed in 51). Nevertheless, for members of the lentivirus family, such as HIV and simian immunodeficiency virus (SIV), that encode envelope glycoproteins with a long (150 amino acids or more) cytoplasmic domain, a specific interaction between the tail of TM and the MA domain of the Gag precursor appears to be necessary for Env incorporation. For these viruses, mutations in MA or in the cytoplasmic tail can abrogate Env incorporation (reviewed in 33). Further evidence for this interaction is derived from polarized epithelial cells, in which retroviral glycoproteins define the plasma membrane domain at which virus budding and release occur (see later).

Targeting of Viral Glycoproteins in Polarized Epithelial Cells Defines the Site of Budding

Many viruses initiate their infection of a host by interacting with cells of an epithelial surface. Individual cells within an epithelial layer are tightly connected by junctional complexes that form a barrier to diffusion of molecules throughout the cell membrane and divide the cell surface into two distinct plasma membrane domains: the apical domain, which faces the exterior; and the basolateral domain, which faces the interior. As a result of differential targeting of lipids and protein components to apical and basolateral membranes, epithelial cells in tight monolayers are highly polarized, with each plasma membrane domain having a distinct lipid and protein composition (105). The assembly and release of many viruses from epithelial cells is also highly polarized, occurring selectively at either the apical or basolateral surface. Influenza virus releases newly assembled virions from the apical surface of polarized epithelial cells, whereas VSV and many retroviruses are released from the basolateral membrane. In the absence of other viral proteins, VSV G protein and retroviral Env proteins are transported to the basolateral surface, whereas HA and NA are targeted to the apical membrane. This delivery to specific membranes is consistent with viral glycoproteins directing the site of virus assembly and envelopment (reviewed in 14). Sorting of proteins to one or the other domain occurs in the *trans*-Golgi network, and recent evidence suggests that proteins destined for the basolateral surface contain within their cytoplasmic domain motifs that direct the protein to that membrane. In the VSV G protein, a tyrosine-based motif within the cytoplasmic tail appears to be crucial to basolateral targeting (124). Similarly, tyrosine-based endocytosis motifs in the cytoplasmic domain of both the MuLV and HIV Env proteins are important for their polarized expression and for basolateral budding of their cognate viruses. In the absence of this signal, Env proteins are delivered to and virus buds from both membranes with equal efficiency (65).

Apical transmembrane proteins appear to contain two signals that probably act cooperatively in targeting to the apical surface. Thus, glycosylation in the ectodomain and a signal in the membrane-spanning domain (MSD) function together to ensure association with sphingolipid-cholesterol–enriched membrane domains or rafts. Rafts have been proposed to mediate apical transport in polarized epithelial cells, and the influenza virus HA partitions into these detergent-insoluble glycolipid-rich membrane fractions through sequences in its MSD (111,112). Polarized virus assembly and release may be important in determining the pathogenesis of viral infections because it can influence in a major fashion the pattern of virus spread in the infected host (14).

Complex Interactions with the Secretory Pathway

Although most enveloped viruses undergo assembly and envelopment at a single site within the secretory pathway, some viruses have a more complex interaction that can involve deenvelopment or reenvelopment as part of their assembly pathway.

Rotavirus Assembly Within the Endoplasmic Reticulum

Rotaviruses are nonenveloped viruses that undergo transient envelopment at the ER as an essential step in the formation of the mature double-shelled (triple-layered) virus that is retained within the lumen of the ER until cell lysis. As with the reoviruses, the nucleocapsid, in this case containing 11 double-stranded RNA segments, is assembled in electron-dense areas of the cytoplasm, located close to the ER membrane, known as *viroplasm* (29,90). These double-layered nucleocapsids have an outer icosahedral shell assembled from VP6, the most abundant protein of the virus, surrounding an inner core (see Chapter 54). They appear to bud directly into regions of the ER that contain the two outershell proteins VP7 and VP4. A nonstructural protein NSP4, which forms hetero-oligomers with the two outershell proteins, mediates the interaction of the immature particle with the ER membrane (77). A hydrophobic N-terminus anchors this integral membrane protein in the ER, whereas the C-terminal 20 amino acids acts as a receptor for the VP6-containing protein shell. A region of NSP4 near the C-terminus, distinct from the receptor domain, adopts an a-helical coiled-coil structure and mediates the oligomerization of the virus-binding domains into a homotetramer. In this way, presumably, a multivalent receptor for the rotavirus single-shelled particle is connected to an a-helical coiled-coil stalk, which projects from the ER membrane, such that membranes containing NSP4 can bind double-layered particles *in vitro* (92,123).

The exact topology of VP7 in the ER is not known. The mature protein has a cleaved signal peptide but is retained in the ER as if it were an integral membrane protein (56,119). Sequences at the N-terminus of the mature protein, which include the highly conserved LPITGS sequence, are important for its retention (67). VP4 is thought to associate with VP7 and NSP4 just before budding of the nucleocapsid into the ER. Enveloped particles can be observed in the lumen of the ER, which then undergo a process of calcium-dependent deenvelopment, forming in the process an outer icosahedral shell of VP7 and VP4 (97). Thus, this nonenveloped icosahedral virus appears to use the ER membrane transiently as a scaffold

on which to assemble an icosahedral shell.

Herpesvirus Transport from the Nucleus

As described earlier, the end product of herpesvirus capsid assembly is a large icosahedral structure that is too large to transit the nuclear pore. To release virus to the exterior of the cell, herpesviruses have evolved to use portions of the secretory pathway. This presumably provides the virus with the ability to propagate progeny virions, whereas the cell and its nucleus remain intact. The precise pathway taken by herpes capsids to the exterior of the cell remains controversial mainly because the bulk of the evidence has come from electron microscopic observations of infected cells (42,99). Nevertheless, there is consensus that the first stage involves budding of the capsid through the inner nuclear membrane into the luminal space. Morphologically, the sites of budding show thickening of the membrane at sites to which viral glycoproteins appear to be targeted (38). Some of these glycoproteins, such as the gB protein of human cytomegalovirus (HCMV), appear to have a novel amino acid motif in their cytoplasmic domain responsible for this intranuclear targeting (76). Little is known, however, about the cellular factors or viral components involved in this envelopment step.

It is equally unclear which of two proposed routes the initially enveloped capsid takes to the exterior (Fig. 12). In the first (Pathway 1: reenvelopment), the membrane of the newly enveloped virus fuses with the outer nuclear membrane to release the capsid into the cytoplasm. It is here that *tegument proteins,* a collection of proteins including transcription factors located between the viral capsid and its lipid bilayer in the mature particle, are proposed to associate with the capsid before its reenvelopment by Golgi-derived membrane vesicles. In the second proposed route (Pathway 2: lumenal transport), the virion retains the membrane derived from budding into the lumen of the nuclear membrane and is released into the cytoplasm within a vesicle formed from the outer nuclear membrane or contiguous ER. In this latter pathway, a full complement of viral glycoprotein and tegument proteins must be incorporated into the virus during the nuclear budding step. Maturation of the viral glycoproteins might then be expected to occur after vesicular transport of virions through the Golgi complex.

Although differentiating between the two pathways might seem straightforward, this has not been the case. However, a recent reanalysis of the evidence for each pathway concludes that the balance favors the reenvelopment pathway (28). Some of the clearest evidence comes from the beta-herpesviruses, HCMV, and human herpesvirus type 6 (HHV-6). In these viruses, the tegument is a dense structure that can be observed in electron micrographs. Enveloped virions in the lumen of the nuclear membrane display no evidence of tegument but acquire this layer after loss of the nuclear-derived envelope and release into the cytoplasm (106,125). Studies of the glycoproteins of the gamma-herpesvirus, Epstein-Barr virus, also support this model. Distribution of the viral glycoproteins gp110 and gp350/220 is consistent with the former being incorporated in virions at the nuclear membrane then lost on budding into the cytoplasm, whereas the latter is acquired only during reenvelopment in the Golgi (38). Similarly, studies of the lipid composition of extracellular HSV-1 virions indicate that it is similar to that of the Golgi complex and distinct from that of the nucleus (128). Thus, it appears likely that herpesviruses have evolved to make use of a two-step pathway of release from the cell. It remains to be seen whether different sets of glycoproteins are used to mediate the envelopment of capsid at each of these steps and what the molecular interactions are that drive each event.

Poxvirus Acquisition of Multiple Membranes

Poxviruses, exemplified by vaccinia virus, exhibit an equally complex interaction with the secretory pathway. These large DNA viruses, which encode all of the machinery necessary for genomic replication and transcription, propagate entirely in the cytoplasm in specialized areas designated virus factories (see Chapter 84). The assembly and envelopment of these viruses is particularly complex because they apparently can be enclosed by as many as four lipid bilayers. Initial electron microscopy studies suggested that the crescent membranes that wrap the immature virions are formed *de novo* within the virus factories (21). Subsequently, it was shown that these envelopes, composed of two closely apposed membranes, are derived from the intermediate compartment of the secretory pathway (116). Co-localization of intermediate compartment resident cellular proteins with several vaccinia virus membrane proteins (A17L, A14L, and A13L) known to be crucial to the formation of immature virion membranes supported this model (63,110). The process by which the nucleoprotein and transcription machinery is targeted into these immature envelopes is unclear. Maturation of the immature virion to the infectious intracellular mature virus (IMV) involves a series of proteolytic cleavages of vaccinia structural proteins (80). The mature virus particles are transported out of the assembly areas toward the periphery of the cell, where they become wrapped by additional membranes that are derived from the *trans*-Golgi or early endosomal compartments to form the intracellular enveloped virus (IEV). The membranes of these compartments contain vaccinia proteins that are present in the external enveloped virus (EEV). Wrapping requires the participation of at least one protein present on the IMV and two EEV membrane proteins and thus appears to be driven by interactions between vaccinia-encoded membrane proteins (7,104). The IEV thus contains four concentric membranes, two derived from the intermediate compartment and two from the Golgi and endosome. These particles are then transported through a reorganized actin cytoskeleton (so called actin tails) to the plasma membrane (19), where membrane fusion releases the infectious three-membrane EEV form of the virus.

Modification of the Secretory Pathway

Transcription and Assembly of Poliovirus on a Disassembled Secretory Pathway

Poliovirus is a nonenveloped positive-sense RNA virus that modifies the host cell extensively during its replication. Once introduced into a target cell, the genomic RNA is translated into a long polyprotein precursor that contains both structural and replicative proteins of the virus (Fig. 13; see Chapter 23). Early in the replication of the virus, membranous vesicles appear in the cytoplasm that are found first near the nucleus and then throughout the entire cytoplasm. Viral RNA transcription occurs in close association with these vesicles, and both viral RNA and viral proteins known to be required for RNA replication have been shown by electron microscopy to be associated with their cytoplasmic surface (5,126). Similarly, priming of RNA synthesis by the protein primer, VPg, appears to require membranes (122). Electron microscopy and inhibitor studies initially suggested that the induced vesicles are derived from the host cell secretory

pathway because they appear to be connected to ER membranes and both their formation and poliovirus replication are blocked by brefeldin A (5,52). More recent studies have shown that a single poliovirus protein, 3A, which also appears to be responsible for attaching VPg to membranes as a 3AB precursor, induces the formation of these ER-derived vesicles and disassembly of the Golgi complex (26). It is hypothesized that 3A interferes with the assembly of COPII vesicles so that newly synthesized membranes, and those recycled from the Golgi, accumulate in the ER (26).

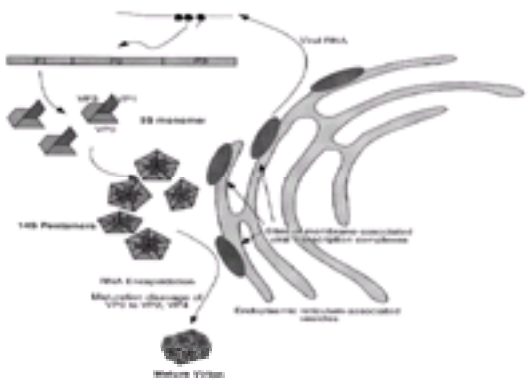

FIG. 13. Poliovirus assembly. The P1 precursor protein is cleaved through an autocatalytic event from the nascent polyprotein and is then cleaved twice by the 3CD-protease to yield VP1, VP3, and VP0. This cleavage is essential for assembly of the 5S monomers into 14S pentamers. It is likely that 12 of these pentamers associate directly with nascent genomic RNA that is being transcribed from membrane-bound transcriptional complexes to assemble an immature 150S provirion. Maturation cleavage of VP0 to yield VP2 and VP4 results in conversion of these immature provirions into the 160S mature virion.

The formation of vesicular platforms for transcription may also be necessary for efficient capsid assembly. The poliovirus capsid appears to be assembled in a sequential process in which the 5S protomers containing VP1, VP3, and VP0 assemble to form 14S pentamers, which in turn are assembled into virus capsids (see Fig. 13). Pentamers may associate with newly synthesized genomic RNA on intracytoplasmic vesicles, then assemble to form RNA-containing capsids upon completion of the RNA. This assembly pathway is supported by immunoelectron microscopy studies showing that pentamers associated with the replication complex can, if released by detergent, rapidly assemble into empty capsids—arguing that the vesicle prevents assembly of the capsid and keeps the pentamer in an appropriate configuration for association with the RNA (95). In this instance, then, poliovirus appears to have circumvented the problems of intracytoplasmic localization of replicative and structural components by using a membranous organizing center.

Modification of Post-Golgi Vesicle pH by the Influenza Virus M2 Protein

The influenza HA is synthesized as a precursor protein (HA_0) that, in the case of the avian influenza viruses, is cleaved in the *trans*-Golgi by furin-like proteinases to the biologically functional HA_1-HA_2 heterodimer, three of which in turn form the trimeric HA complex (see Chapter 3 and Chapter 46). The influenza virus HA present on an infecting virion is activated to its fusogenic state by the low pH of the endosome. Thus, the similarly low pH of the *trans*-Golgi network and post-Golgi vesicles poses a problem for the virus during the assembly phase of its life cycle, if it is to maintain HA in a functional prefusogenic form. This is apparently solved by the action of the M2 protein, a small tetrameric protein that forms cation-selective ion channels that can increase the pH of the *trans*-Golgi network by pumping out hydrogen ions (96,120).

INCORPORATION OF THE NUCLEIC ACID GENOME DURING THE ASSEMBLY PROCESS

Although the processes involved in the assembly of structural components of a virus are complex and interplay intimately with those of the host cell, they merely provide a mechanism by which the genomic information of the virus can be packaged into a protective environment for transfer to additional host cells. As such, the assembling virus must have evolved to select its genomic nucleic acid efficiently out of the pools of RNA or DNA that are present at the assembly site. Packaging of viral genomes generally involves a cis-acting sequence in the nucleic acid (the so-called packaging sequence) and a structural components of the virus that can recognize and bind this element.

DNA Viruses

As we described earlier, questions remain about the linkage of adenovirus DNA packaging to the process of capsid assembly. Nevertheless, early studies indicated that incomplete virus particles contain subgenomic-length viral DNA that have a large overrepresentation of left-end sequences. Subsequent studies showed that a cis-acting packaging sequence is located within the left 390 base pairs. This region is highly conserved between different adenovirus subtypes and, in Ad 5, overlaps with two distinct enhancer elements for E1A transcription. Deletion of the packaging domain abrogates viability, but infectivity can be restored by substitution of the left-end sequences at the right end of the genome (47). The packaging sequence only functions if it is within 600 base pairs of the inverted terminal repeat (ITS). Mutational analyses showed that this region contains at least seven functional adenosine-thymine (AT)-rich units called *A repeats.* Of the seven, elements I, II, V, and VII have been found to be dominant, and associated 11 base pairs downstream from each of these is a CG motif that also appears crucial to function. Overexpression of an A-repeat multimer results in a dramatic decrease in viral yield without affecting DNA replication or late-transcription (41,114), suggesting the involvement of a limiting viral protein component in packaging. Although this protein has yet to be identified, more recent studies have shown that encapsidation of DNA requires the presence of the nonstructural L1 52/55K protein: empty capsids form in its absence (43). How it might mediate packaging is unclear.

Packaging of the herpesvirus genome is equally complex. As with the large DNA phages, herpesvirus DNA replicates to yield concatamers of genomes in a head-to-tail arrangement, and so packaging must be linked to the generation of genomic length units of DNA. In this virus, two packaging sequences, *pac1* and *pac2,* located within the terminal A repeats, appear to be important for both recognition and cleavage of the DNA (see Chapter 72 and ref. 25). As with the DNA phages, a combination of cleavage recognition sequences and “head-full” packaging appears to ensure the incorporation of unit-length genomes into the procapsid. Studies using a temperature-sensitive protease mutant of HSV-1, which accumulates procapsids (containing an uncleaved scaffolding protein) at the nonpermissive temperature, but which can remove the scaffold and package DNA upon temperature shift to the permissive temperature, have shown that ATP is necessary for DNA packaging to occur but that DNA synthesis is not (12,22). At least seven viral proteins are required for cleavage and packaging of HSV-1 DNA. Sequence analysis reveals that one of these, encoded by the *UL15* gene, shares homology with gp17, the large catalytic subunit of the bacteriophage T4 terminase (134). Thus, the product of this gene may play a direct role in the cleavage of viral DNA replication intermediates into monomers. Moreover, it raises the possibility that herpesviruses assemble a portal and terminase structure, similar to phages such as P22 or l, for DNA packaging.

RNA Viruses

For retroviruses, genomic packaging represents a particularly difficult problem because the assembling capsid must select genomic RNAs from a large pool of viral and cellular mRNAs that are also present in the cytoplasm. Viral RNAs to be specifically packaged are identified by the presence of an RNA sequence named the packaging signal or y (psi) and are selected from the cytoplasmic pool by a specific interaction with the NC domain of the Gag precursor (reviewed in 4). The cis-acting RNA element is located at the 5' end of the viral genome and appears to be composed of a series of stem-loop structures that each contribute to the strength and specificity of the signal. The best characterized, although perhaps most complex, y sequence is that of HIV-1, which is now known to span the first 360 nucleotides of the genome. This region, which can confer packaging specificity on foreign RNAs, includes the TAR sequence that is important in Tat-mediated transcriptional control, the adjacent poly(A) loop, and four stem-loops designated SL1 to SL4. Gag has been shown to bind specifically to the latter region, with SL1, SL3, and SL4 each providing independent high-affinity binding sites. It is likely, although less well documented, that Gag can bind to the 5' stem-loops also. Disruptive and compensatory mutations within the TAR, poly(A), SL1, and SL3 elements indicate that it is the structure rather than the sequence of the stem-loops that is important for packaging (13,72). Recent nuclear magnetic resonance analyses of SL3 in the presence and absence of the NC protein have begun to provide insights into how Gag might bind this region (24,135).

Given its role in specifying genomic RNA packaging, one might expect the y sequence to be located downstream of the splice donor site for subgenomic mRNAs. This is the case for the MuLV and reticuloendotheliosis y sequences but is not the case for HIV-1, as shown previously, or for Rous sarcoma virus (RSV). In the case of HIV, the SL3 stem-loop is located in this downstream domain and deletions in this region alone do decrease packaging 5- to 10-fold. In the case of the avian leukosis viruses, however, the entire 5' y sequence is present in both genomic and subgenomic mRNAs; thus, the basis for packaging specificity in this case is unknown (113).

Genomic RNA packaging signals in other RNA viruses are less well defined. In the alphaviruses, this appears to involve direct binding of genomic RNA by the capsid protein. A short (132-nucleotide) segment of RNA in the region of the genome encoding the nonstructural protein NS1 is crucial to this interaction and for packaging of genomic RNA. Like the retroviral y sequence, it has also been predicted to form a series of stem-loop structures. The N-terminal third of the capsid protein is basic and is unstructured in the current crystal structure. Nevertheless, a 32–amino acid region (residues 76 to 107) has been shown to be essential for RNA binding in a gel shift assay (113).

For segmented viruses such as the orthomyxoviruses or the reoviruses, genomic packaging requires that a complete set of segments be packaged together in a single virus particle. For influenza virus, reverse genetic approaches have shown that the signals for transcription, replication, and packaging of an RNA segment are located in the 22 5'-terminal and the 26 3'-terminal nucleotides of the RNA (66). These studies do not, however, address how a full complement of RNAs is selected during assembly. Selective packaging of the eight genomic RNAs would presumably require complex recognition systems and molecular interactions. Although there is some evidence to support such a mechanism, the bulk of the evidence appears to be most compatible with a model in which infectious influenza virus particles are formed from random packaging of RNA segments (reviewed in 113).

POSTASSEMBLY MODIFICATIONS AND VIRUS RELEASE

Proteolytic Cleavage and Virus Maturation

Virus-encoded proteolytic enzymes play important roles in the process of assembly for many viruses and in a postassembly maturation steps that are required for the development of an infectious particle for others. These cleavage events can act as molecular switches that introduce flexibility as well as irreversibility into the assembly process.

Proteolytic cleavage in the case of the alphaviruses, for example, is used to allow protein domains, translated from a single mRNA, to enter different transport pathways in the cell. The C protein, which is encoded at the 5' end of the subgenomic 26S mRNA, folds cotranslationally into an active serine proteinase that cleaves itself from the growing polypeptide chain. This releases the C protein into the cytoplasm for capsid assembly while freeing up the N-terminus of the signal peptide for the p62 glycoprotein precursor, which can then bind SRP and direct the ribosome to the ER.

In the herpesviruses, proteolytic cleavage of the scaffolding protein occurs after assembly of the procapsid is complete and is a prerequisite for DNA packaging. The scaffolding proteins of HSV-1 are encoded by a pair of overlapping genes, *UL26* and *UL26.5*, in which the open reading frame of *UL26* is an in-frame N-terminal extension of that of *UL26.5*. The product of the smaller gene is the abundant scaffolding protein pre-VP22a, whereas the larger gene encodes a protease

precursor, which cleaves itself internally to give the proteins VP24 (protease) and VP21. Temperature-sensitive mutants in *UL26* that have an inactive proteinase at the nonpermissive temperature are blocked at the procapsid stage of assembly (reviewed in 99).

A somewhat more complex proteolytic pathway is used by the picornaviruses to regulate the release of both structural and replicative protein components from the single polyprotein precursor that is translated from its positive-stranded RNA genome (see Fig. 13). The initial event in this proteolytic cascade is the primary cleavage in which the P1 structural protein precursor is separated from the P2-P3 precursor. This reaction is catalyzed by the 2A proteinase, which hydrolyzes a Tyr-Gly bond at its own amino-terminus. Although cleavage of the P2-P3 region is carried out by the 3C proteinase at Gln-Gly bonds, the efficient cleavage of the structural protein precursor at the VP0-VP3 and VP3-VP1 boundaries is catalyzed specifically by the 3CD precursor that includes the viral polymerase. Cleavage of the P1 precursor appears to be a prerequisite for entry of the capsid proteins into the assembly pathway. The final processing step is cleavage of VP0 to VP4 and VP2. This autocatalytic, maturational cleavage occurs late in the assembly pathway and appears to be linked to encapsidation of the viral RNA genome. It is likely that the structural alterations that accompany the cleavage event both stabilize the capsid and irreversibly commit the virus to the conformational changes induced on receptor binding that are necessary for productive infection of cells (reviewed in 48).

A similar maturational cleavage is required after assembly and envelopment of retroviral capsids. As described earlier, the retroviral capsid is assembled from polyprotein precursors that are encoded by the *gag* gene. The viral aspartyl proteinase is encoded by the *pro* region of the genome and is translated from unspliced, genome length RNA by a ribosomal frame-shift mechanism as a Gag-Pro or Gag-Pro-Pol precursor protein (see Chapter 57). The presence of the Gag sequences ensures that this key enzyme is targeted to and incorporated into the assembling capsid. The mechanism by which the protease is activated late in the assembly process (most probably after envelopment is complete) is not known. Because the active enzyme is a homodimer of two protease subunits, it has been postulated that dimerization of proteinase precursors in the nascent capsid might allow a functional enzyme to form. However, in the *Betaretroviruses*, the immature capsid contains a full complement of proteinase precursors, which are not activated until late in envelopment at the plasma membrane.

Cleavage of the Gag precursors in the immature capsid is accompanied by a major morphologic rearrangement in which the mature NC protein condenses the genomic RNA inside a CA shell (see Fig. 8)—the electron-dense core structure of the virus seen in electron micrographs (36). Recent evidence suggests that cleavage at the N-terminus of CA induces a conformational change in the protein, which allows it to assemble the core shell structure (130). Maturational cleavage, as in the case of poliovirus, results in an irreversible commitment to the entry pathway. It prevents a simple reversal of the assembly process and, presumably through the formation of the core, allows the rearrangement of RNA and the reverse transcriptase into a conformation that is optimal for reverse transcription of the genome. In the absence of cleavage, reverse transcription cannot occur, and the virions are noninfectious (reviewed in 121).

Adenoviruses, like retroviruses, undergo a maturational cleavage after assembly that is essential for infectivity. The young virions formed after DNA packaging contain five precursor proteins that must be processed before the mature infectious virion is produced. These include components of the core and the preterminal protein (pTP) used to initiate DNA synthesis. The 23-kd adenovirus proteinase, which is encoded by the L3 region of the genome, is inactive in its purified form. It requires an 11–amino acid peptide present at the carboxyl-terminus of pVI and viral DNA as cofactors for its activity. This unique use of DNA as a cofactor ensures that proteolytic maturation of the virus cannot take place until packaging of DNA is complete (reviewed in 20).

Budding—Role of Viral and Cellular Proteins in Membrane Extrusion

It is clear from the discussions in previous sections that different strategies are used by different enveloped viruses to mediate the process of membrane extrusion that we have termed budding. Envelopment by a lipid bilayer fulfills two functions for a virus: it provides a protective outer layer into which can be embedded the necessary machinery for target cell attachment and entry; and, in most cases, it releases the virus into an environment that is topologically equivalent to the exterior of the cell. The strategies used to drive the budding process can be classified into three general mechanisms (35). In the first, membrane extrusion is driven by envelope glycoproteins alone. This is exemplified by the E protein of the coronaviruses that is capable of inducing the release of virus-like particles in the absence of an RNP. The second strategy is that used by the retroviruses in which the capsid precursors, in the absence of the viral glycoproteins, are able to induce membrane extrusion and particle release efficiently. In this instance, it is hypothesized that the tight interaction between the MA domain of Gag and the lipid bilayer, coupled with the force of Gag-Gag interactions in assembly, drives the budding process. The third mechanism is that of the alphaviruses, which involves interactions between the viral glycoprotein spikes and the assembled capsid. In this case, neither the glycoproteins nor the capsid alone can mediate budding. It appears that lateral interactions between the glycoproteins, coupled with cytoplasmic domain–capsid connections, progressively bend the membrane to form the spherical particle (35,70).

For all of these mechanisms, the final step of budding, the process of pinching off, requires a membrane fusion event. Although for most enveloped viruses, there is no evidence that a viral component is specifically required for pinching off to occur, in the retroviruses, it is clear that this is a separable event that requires the involvement of both viral and host components. Experiments in HIV showed that deletion of the p6 domain at the C-terminus of Gag results in a block to virus release at the pinching-off stage (40). It was later discovered that species within several retrovirus genera encode a similar Gag “late function” domain that is required for efficient release of virions (94). The mechanism by which these “late” domains mediate the pinching-off step is not known, but recent studies of the Yes protein tyrosine kinase have provided a clue. During signal transduction, a PPPY motif on Yes mediates binding through a second amino acid motif called a WW domain—characterized by two widely spaced tryptophan residues—on its partner protein Yap. In the alpha-, beta-, and gamma-retroviruses, the late function of particle release depends on the presence of a PPPY-containing motif that may mediate interactions with WW-containing cellular proteins. The p6 domain in HIV-1 and the late domain of equine infectious anemia virus each appear to bind different cell proteins but can functionally replace the PPPY motif of the *Alpharetroviruses* (reviewed in 121). Interestingly, a similar functional PPPY motif has been found

in the matrix protein of VSV, pointing to a similar role for host cell proteins in the release of VSV (16).

Mechanisms to Facilitate the Release of Nascent Particles

If they are to travel efficiently to an uninfected target cell, viruses face one last hurdle after envelopment and release. This is the need to avoid rebinding to receptors on the producing cell. For viruses such as the orthomyxoviruses and paramyxoviruses, which use sialic acid as a receptor on the cell surface, this problem is circumvented by the incorporation of a receptor-destroying enzyme, a sialidase, that can remove these residues during transit through the secretory pathway. For influenza virus, the neuraminidase is crucial to virus release because, in its absence, nascent virus binds to both the cell surface and other virions in large aggregates (64).

Retroviruses have similarly evolved mechanisms to facilitate release and thereby reduce superinfection of a cell. Cells infected with one retrovirus are generally resistant to subsequent infection by a second virus of the same receptor class. The presumed mechanism for this viral interference is the synthesis of viral glycoproteins in large excess over the levels that are incorporated into virions with the effect that cellular receptors that are transported to the cell surface are already occupied by their ligand. This results in removal or down-regulation of the receptor from the cell surface. For HIV-1, down-regulation of the primary receptor CD4 seems to be crucial because the virus has evolved two additional mechanisms to ensure this. Two of the accessory proteins encoded by HIV, Vpu and Nef, act by binding CD4 in the ER and at the cell surface, respectively, and targeting the receptor for degradation (see Chapter 59). In the context of infected tissue, this down-regulation of an abundant receptor could be essential to facilitate spread to adjacent target cells.

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CHAPTER 9 Pathogenesis of Viral Infections

Kenneth L. Tyler and Neal Nathanson

INTRODUCTION

Viral pathogenesis is the process by which viruses produce disease in the host (350,553). Much of our existing knowledge of viral pathogenesis is based on experimental studies using animal models of natural human infections. These are at best imperfect analogs to human infections, but they allow observations and experiments that are not possible in humans and are invaluable in understanding how viruses produce human diseases.

The production of disease, which is the focus of pathogenesis, is in fact a relatively unusual outcome of viral infection of a host (Fig. 1). The virulence of a virus is its capacity, when compared with other closely related viruses, to produce disease in a host. Virulence depends on a variety of viral and host factors, such as the dose of virus, its route of entry, and the host's age, sex, immune status, and species. With many “virulent” viruses, subclinical or inapparent infections often outnumber cases of symptomatic illness. During epidemics of mosquito-borne encephalitis, for example, the ratio of serologically documented infections may exceed cases of acute encephalitis by several hundred fold. However, for certain classic viral infections such as measles, smallpox, rabies, and influenza, almost all infected individuals develop disease.

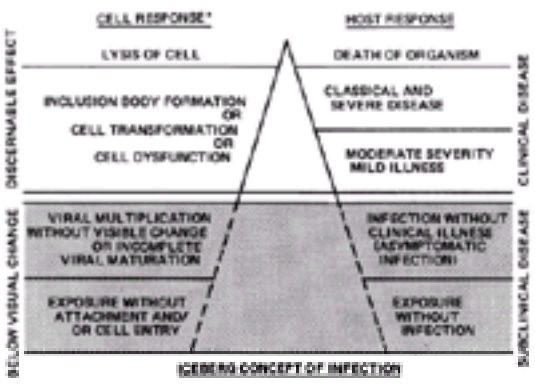

FIG. 1. Varieties of host and cellular responses to virus infection. (From ref. 143, with permission.)

In addition to acute infection, the interaction between a virus and a host may lead to a variety of other outcomes including the development of persistent or latent infection (see later) and cellular transformation (see Chapter 10). As viral infection proceeds, the virus encounters a constant series of obstacles within the host. Among the most important of these are the various components of the host's specific and nonspecific immunity (see Chapter 11 and Chapter 12). Ultimately the interaction between a virus and cell may lead to alterations in host cell function, which are discussed in more detail elsewhere (see Chapter 7).

A virus must survive in the environment until it encounters a susceptible host. It must then enter the host, through one of a number of available portals (e.g., the respiratory or the gastrointestinal tract). Once inside the host, the virus undergoes initial replication, typically at a site near its original portal of entry. Viruses that produce localized infections may be released from surface mucosal epithelium or analogous sites and subsequently shed from the host (e.g., in stool or other bodily fluids or through coughing and sneezing). After entering the environment, these viruses may then reinitiate the cycle of virus–host interaction by transmission to a new host.

Viruses that produce systemic rather than localized infections must cross mucosal barriers and spread to distant sites in the host, using defined pathways of spread such as the bloodstream, lymphatics, or nerves. After spreading, or at the site of local infection, viruses are targeted to specific populations of cells within individual organs or tissues (tropism). Each of these stages in viral pathogenesis is discussed in more detail in the following sections.

Each stage of pathogenesis poses a series of biologic obstacles that the virus must overcome to proceed to the subsequent level. The successful completion of all the stages in pathogenesis results in disease, whereas failure to complete these stages results in either abortive, nonproductive, or failed infection. Viral structure has evolved to enable the virus to overcome obstacles successfully at particular stages in pathogenesis. The structure of the virus must enable it to remain infectious after being exposed to conditions such as temperature and humidity that are present in the ambient environment. For many viruses, environmental stability is determined by the envelope or by outer capsid proteins. Once a virus has entered the host, the parts of the virus that ensure environmental stability or promote transmission become nonessential and are often removed as the virus proceeds through subsequent stages in pathogenesis. For example, the proteolytic removal of capsid proteins may, at this stage, actually enhance infectivity rather than attenuate it (381).

ENTRY

The predominant routes by which viruses enter the host include through the skin, or by way of the respiratory, gastrointestinal, or genitourinary systems (377,378) (Fig. 2).

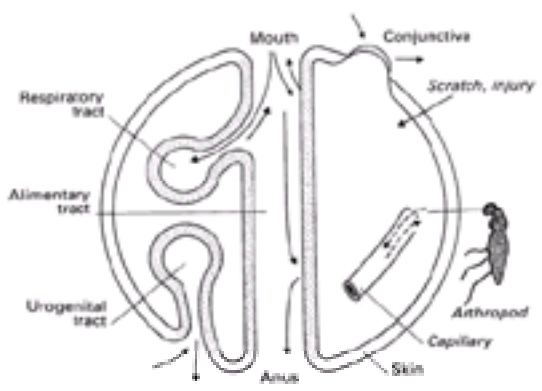

FIG. 2. Portals of entry of viruses into the host, and sites of shedding from the host. (Modified from ref. 350, with permission.)

Skin

Breaks in the integrity of the skin or the mucosa of the respiratory, gastrointestinal, or genitourinary tracts facilitate entry of viruses. The outer layer of the epidermis is composed of the dead keratinized cells of the stratum corneum, which do not support viral replication (553). Underneath the stratum corneum are the living cells of the stratum malpighii. Some viruses, such as papillomaviruses—which produce human warts—enter the skin at sites where scrapes or abrasions have resulted in loss of epithelial integrity. Certain animal papillomaviruses are mechanically transmitted by arthropod vectors, and then replicate in epithelial cells at the site of inoculation (see Chapter 66). Primary infection begins in the basal cells, and virion replication and maturation occur as these cells differentiate. Mature virions are found in the granular layers and are shed with the dead cells of the stratum corneum.

Sheets of keratinocytes, similar to those used for epithelial cell skin grafts, have been used to study the entry and tropism of viruses that can infect the skin. For example, studies of viral infection of epithelial grafts suggest that cell-free inoculums of human immunodeficiency virus (HIV) do not productively infect keratinocytes, which apparently lack HIV receptors, but cell-associated HIV can initiate productive infection of these cells (415).

Because the epidermal layer is devoid of blood vessels, lymphatics, and nerve fibers, viruses that initiate epidermal infection are typically restricted to the site of entry and only rarely disseminate to produce systemic infections. The system of skin-associated lymphoid tissue (SALT), which includes antigen-presenting Langerhans cells, T cells, and cytokine-producing keratinocytes, may provide a pathway for spread of viruses that can initiate systemic infection after inoculation into the skin. T cells and dendritic cells (Langerhans cells) can circulate between the skin and regional lymph nodes. Both HIV and herpes simplex virus (HSV) can infect Langerhans cells, which can emigrate out of the epidermis to

enter regional lymph nodes (342). When macrophage-tropic strains of HIV are inoculated onto abraded epidermis of skin explant grafts, virus can be detected in both epidermal and dermal emigrant cells, which include both T cells and dendritic cells (430). The importance of early dendritic cell infection in the pathogenesis of HIV also can be inferred from the predominance of “macrophage-tropic” viral strains isolated during primary HIV infection (581). Dermal replication in macrophages and fibroblasts also occurs when mouse-, cow-, or rabbit-pox viruses enter the host through traumatic skin lesions in the epidermis (154).

The molecular basis for viral infection of skin remains largely unknown. In the case of varicella-zoster virus (VZV) and HSV, the gC glycoprotein is a critical determinant (354). This suggests that the interaction between viral attachment proteins and specific receptors on skin cells may be critical to initiating epidermal infection.

The bites of arthropod vectors including mosquitoes, mites, ticks, and sand flies allow subcutaneous inoculation of many arthropod-borne viruses (see Chapter 30 and Chapter 33). Transmission of virus may be purely mechanical, as illustrated by transmission of myxomavirus by the mouthparts of biting insects, or may require replication of the virus within the gut or salivary system of the arthropod vector (205,369,376). Most of the arthropod-borne toga- and flaviviruses, which include the agents of epidemic encephalitis and yellow fever, appear to replicate in the gut of the insect vector, with subsequent spread to salivary tissue. Virus is then inoculated into the host during feeding, and part of the inoculum enters the general circulation, while part remains in the local tissue. Once a virus has reached the dermis, it has access to blood vessels, lymphatics, and dendritic cells, including macrophages present in the skin.

Some viruses are inoculated directly through the epidermis and dermis into deeper tissues. Deep inoculation, into the subcutaneous tissue and muscle, can follow hypodermic needle injections, acupuncture, ear piercing, tattooing, or animal bites. Hepatitis B virus (HBV) may gain access to the host through needle inoculation of various types (313). Infection with both rabies and herpesvirus simiae is characteristically initiated by the bites of infected animals (18,557).

Respiratory Tract

Inhibition of viral entry through the skin depends primarily on the mechanical integrity of the epidermal barrier. By contrast, more complex host defense systems are involved in inhibiting viral entry through mucosal surfaces (1,569). In the respiratory tract, goblet cells secrete mucus that is propelled by the action of ciliated epithelial cells and acts to clear foreign material from the respiratory tract. Clara cells, a type of nonciliated secretory cell, produce trypsin-like proteases that may act to cleave viral proteins such as the hemagglutinin (HA) of influenza virus (269).

Both humoral and cellular immune mechanisms also act to protect the host against mucosal infection (1). Bronchial-associated lymphoid tissue (BALT) includes collections of lymphocytes and macrophages in the tonsils and adenoids as well as in the subepithelial tissue of the nasopharynx and trachea. Specialized epithelial cells known as M cells participate in generating mucosal immunity by delivering antigens to underlying lymphoid tissue (470). Viruses and other microbial pathogens can exploit these cells as transport pathways, enabling them to traverse mucosal surfaces. This has been elegantly demonstrated for reoviruses (360) (reviewed later in the discussion of the analogous pathway in the gastrointestinal tract).

Cytokines also appear to play a critical role in preventing systemic dissemination of some viruses that initiate infection through the respiratory tract. For example, knock-out mice with disruptions in the STAT1, interferon (IFN)a-receptor, or IFNg genes all show markedly enhanced severity of influenza A infection and systemic dissemination compared with that in their wild-type counterparts (168). Similarly, mice with targeted mutations inactivating the IFNa/b receptor show enhanced spread of measles virus to the lungs and more prominent inflammation than wild-type mice (366).

An additional factor in host defense is the cool temperature of the upper respiratory tract, which acts to inhibit the replication of many viruses (569), but is optimal for the replication of rhinoviruses, a major cause of upper respiratory infections (see Chapter 25). The temperature differential between the nose and the body has been exploited in viral vaccine development. Temperature-sensitive (ts) mutants of viruses such as influenza, parainfluenza, and respiratory syncytial virus can replicate in the upper respiratory tract and generate host immune responses, but will not survive higher body temperatures and therefore do not disseminate (569). Despite the nature of host barriers to respiratory infection, a number of viruses including herpesviruses, adenoviruses, myxoviruses, paramyxoviruses, and rhinoviruses can initiate either local or systemic infection via this route.

Viruses that enter the respiratory tract do so primarily in the form of either aerosolized droplets, saliva, or through spread from infected nasal secretions to hands and then subsequently back to the nose (nose–hand–nose transmission). Site of penetration is influenced by droplet size, with only the smallest inhaled droplets reaching the alveoli of the lungs. Initial infection occurs predominantly at the epithelial surface with subsequent spread of virus from cell to cell or by inhalation.

Receptors (see later) for viruses that initiate spread via the respiratory route are predominantly ubiquitous cell-surface molecules such as sialic acid (influenza), and cell adhesion molecules (rhinovirus). In some cases these receptors may be distributed preferentially on certain surfaces of polarized respiratory epithelial cells. For example, the CAR receptor for adenoviruses 2 and 5 and coxsackie B is located predominantly on the basolateral surface of epithelial cells (542), whereas the measles virus receptor CD46 is found preferentially at the apical surface (42). The subsequent propensity of some viruses to produce disease predominantly confined to respiratory mucosal surfaces (e.g., influenza, respiratory syncytial virus), whereas others induce systemic infection after primary replication in respiratory mucosa (e.g., measles, varicella), may be determined in part by the pattern of viral release from infected cells (polarized infection, see later) (98,521). For example, viruses such as influenza and respiratory syncytial virus (436), which are released predominantly from apical surfaces, tend to produce infection confined to the respiratory mucosal surface. Conversely, release of viruses, such as certain murine coronaviruses (447), from the basolateral surface of epithelial cells may facilitate systemic spread (see polarized infection later).

A unique situation occurs in the superior portion of the nares, which contains olfactory receptor cells. These cells have

distal rods that extend beyond the outer epithelial surface and proximal axon processes (fila olfactoria), which join to form olfactory nerve fibers. The olfactory nerve fibers penetrate the arachnoid space and form synapses with mitral cell neurons in the olfactory bulb. A variety of experimental studies using polioviruses, herpesviruses, coronaviruses, togaviruses, and rhabdoviruses have shown that these viruses can spread to the central nervous system (CNS) through the olfactory nerve (396,496). This situation, although postulated to explain some cases of human rabies and HSV infection, is probably an extremely unusual route of natural viral infection (251).

Experimental models of natural aerosol infection have been developed, as exemplified by aerosol inoculation of bovine respiratory syncytial virus into calves, and seem to be reasonable facsimilies of natural infection (567). Explant organ cultures, such as those of fetal trachea, and cell cultures of nasal epithelial and adenoidal cells also have been used to study early events in respiratory infection (561,569).

Coughing and sneezing can generate enormous numbers of small aerosolized particles that may travel up to 5 feet at velocities that approach 100 ft/sec. Coughing generates even higher aerosol velocities that can approach 850 ft/sec (569). Particles smaller than 5 µm in diameter can remain airborne for long periods, a property that can be dramatically enhanced by the presence of air currents. The distribution of inhaled aerosol particles within the respiratory tract is largely a function of particle size (277). Large particles rarely escape the filtering action of the nasal turbinates, whereas small particles (<5-µm diameter) are capable of reaching the alveolar spaces.

Environmental factors including air temperature and humidity also influence the stability of aerosolized viral particles (277). The physicochemical properties and structure of virion particles also can influence their stability as aerosols. For example, enveloped viruses are less susceptible to desiccation than are their nonenveloped counterparts. This may provide one explanation for the fact that enveloped viruses (e.g., influenza, parainfluenza, respiratory syncytial virus) are more common causes of lower respiratory tract infections than are their nonenveloped counterparts (e.g., adenovirus, rhinovirus).

The proper functioning of the mucociliary transport system appears to be an important determinant in resistance to viral infection of the respiratory tract. Drugs that inhibit this transport process increase host susceptibility to a number of viral infections (21). Susceptibility to Sendai virus infection in mice has been linked to a genetic polymorphism in mucociliary transport function (58). Patients with HIV infection often have decreased rates of mucociliary clearance, which also facilitates recurrent upper respiratory and sinus infections (347). Acquired ciliary defects, which inhibit host antiviral defense, appear to be a common feature of many other viral upper respiratory infections, even in immunocompetent hosts (75).

Gastrointestinal Tract

Many viruses enter the host via the gastrointestinal (GI) tract, where they may either initiate local infection (e.g., rotaviruses, coronaviruses, adenovirus, and Norwalk agent) or cross the mucosal layer to invade underlying tissues and spread within the host (e.g., enteroviruses, hepatitis A).

The physicochemical environment in the upper GI tract would appear to be extremely inhospitable to invading microorganisms (103). The secretion of acid by gastric parietal cells may reduce the intraluminal pH to 2.0 or lower. In addition, a variety of proteases are secreted by gastric and pancreatic cells, and bile salts enter the duodenum from the biliary tract. Mucus is secreted by both gastric and intestinal cells and may contain both specific [e.g., immunoglobulin A (IgA)] and nonspecific inhibitors of viral infection. To initiate infection via the GI route, a virus would ideally have the properties of acid stability, resistance to loss of infectivity in the presence of bile salts, and resistance to inactivation by proteolytic enzymes. In fact, the majority of viral pathogens that enter via the GI tract generally exhibit all these properties.

Almost all picornaviruses are capable of producing enteric infections in humans. A notable exception to this rule is the rhinoviruses. Unlike other picornaviruses and rotaviruses, rhinoviruses are extremely acid labile and undergo complete inactivation with loss of infectivity at low pH (Chapter 25). The pH-induced inactivation appears to result from the loss of the VP4 surface protein and resulting leakage of the viral RNA, which produces noninfectious “empty capsids.” Interestingly, many of the enteroviruses undergo an analogous type of degradation under alkaline conditions (263). Similar investigations with reoviruses suggest that under certain circumstances, exposure to alkaline pH can result in the loss of the viral cell-attachment protein from the viral outer capsid (129,130). Changes in the outer capsid structure of rotaviruses also have been observed at both extremely acidic or alkaline pH (Chapter 54).

The effect of proteolytic enzymes, such as those present in the GI tract, on the infectivity of enteric viruses also has been investigated. Trypsin, pancreatin, and elastin have all been shown to enhance rotavirus infectivity (92,141) and are routinely incorporated into procedures to isolate and cultivate these viruses. Likewise, inhibition of proteolysis may result in attenuation of rotavirus infection (536), and the importance of proteolytic processing of virions *in vivo* has been demonstrated for reoviruses (24,45). Reovirus particles present in the intestinal lumen are converted into infectious subviral particles (ISVPs) by proteolytic processing of their s3 and µ1C outer capsid proteins. ISVPs appear to be the form of the virion responsible for the subsequent initiation of intestinal infection, and inhibition of intraluminal proteolytic digestion dramatically inhibits intestinal infection by reoviruses (7,24,45).

Studies with a number of viruses indicate that host proteases act primarily to alter the viral outer capsid or envelope proteins. For example, proteolytic digestion of rotaviruses with trypsin, pancreatin, or elastase results in cleavage of the outer capsid protein VP4 and the appearance of two cleavage products, VP8 and VP5 (141). Enhancement of coronavirus infectivity also occurs in the presence of proteolytic enzymes (489) and may be due to the cleavage of the E2 peplomer glycoprotein (the viral cell-attachment protein) from an inactive precursor into its active form. Genetic studies with reovirus reassortants derived from parental viruses that differ in their susceptibility to proteolytic digestion indicate that the reovirus major outer capsid protein µ1c is the major determinant of protease sensitivity (450).

Detailed studies of the mechanism of action at a molecular level of proteolytic digestion of viral proteins have been made with influenza viruses and Sendai virus (169,174,294,460,559). These studies provide insights into how proteolytic processing of viral proteins can influence viral pathogenicity. Cellular exopeptidases cleave the HA of influenza into two disulfide-bonded subunits (HA_1 and HA_2). After virus enters target cells through the process of receptor-mediated endocytosis, the amino terminus of the HA_2 protein undergoes a pH-dependent conformational change that facilitates the subsequent fusion of the influenza virus envelope with the inner membrane of the endocytic vesicle (473). This in turn results in the escape of the viral nucleocapsid into the target cell's cytoplasm, where replication occurs. If cleavage of the HA does not occur, then influenza virus still binds to cell-surface receptors but is unable to initiate a productive infection. Inhibition of HA cleavage by administration of protease inhibitors results in attenuation of influenza virus infection *in vivo* (502,559).

In the case of measles virus, the fusion glycoprotein gene encodes an inactive precursor protein (F_0) that is cleaved within a region containing a stretch of basic amino acids (108,109,110,111 and 112) to generate two disulfide-linked active fusion proteins (F_1 and F_2). Cleavage occurs within the trans-Golgi network (TGN) and involves host-cell enzymes belonging to the family of proprotein convertases. Furin has been identified as the likely candidate (46).

Thus many viruses have evolved so that proteolytic processing actually facilitates rather then inhibits viral infection. The mechanisms by which this facilitation occurs are gradually becoming better understood. As a general principle, proteolytic processing of these viruses usually results in partial cleavage of specific viral surface proteins, which in turn facilitates specific events, such as membrane fusion or transcriptional activation, in the viral replicative cycle. Conversely, viruses that are easily inactivated by proteolytic processing are unlikely to initiate infection via sites such as the GI tract where proteolytic enzymes are a prominent part of the internal milieu.

The presence of bile salts in the intestinal lumen also may play an important role in determining the types of viruses that are capable of initiating GI infection. Viral envelopes are derived from host-cell lipoprotein bilayer membranes and are particularly susceptible to dissociation by bile salts. Conversely, the protein capsids of nonenveloped viruses are quite resistant to the action of bile salts. This may explain why, with the exception of coronaviruses, enveloped viruses do not initiate infection via the upper GI tract. Coronaviruses are susceptible to inactivation by bile salts in vitro, and the factors that enable them to survive inactivation *in vivo* remain unknown.

Viruses associated with gastroenteritis can infect a variety of cell types within the GI tract. Some viruses such as adenoviruses, calciviruses, rotaviruses, and coronaviruses infect predominantly the mature nondividing absorptive enterocytes. Other viruses have a predilection for the dividing cells of the crypts (e.g., animal parvoviruses) or infect specialized epithelial cells such as the M (microfold cells) overlying Peyer's patches (e.g., poliovirus, HIV, and reovirus) (103). Factors that determine the capacity of viruses to infect intestinal cells are becoming better understood. In the case of coronaviruses, a critical determinant is the presence of cell-surface receptors such as members of the carcinoembryonic antigen (CEA) family including human biliary glycoprotein and aminopeptidase N (83,227,512,513). Other enteric viruses use more ubiquitously distributed receptors such as sialic acid residues (rotavirus). Some viruses, such as the rotaviruses, produce enteric infections that are typically limited to the intestinal epithelial cells (Chapter 55). Local involvement of intestinal epithelial cells also occurs during infection with adenovirus, astrovirus, calcivirus, coronavirus, and parvovirus (110,455).

The factors that determine whether viral infection remains limited to the GI tract or spreads to produce systemic illness are largely unknown. Increasing interest has focused on the role of gut-associated lymphoid tissue (GALT) in viral entry. Several enteric viruses, including reoviruses, HIV, and poliovirus, spread from the lumen of the small intestine to collections of submucosal lymphoid tissue known as Peyer's patches (362,470). The early events in the spread of reoviruses after intestinal infection have been investigated extensively using electron microscopy. Virus initially adheres to the surface of specialized intestinal epithelial cells (“M cells”) that overlie the surface of Peyer's patches. Reovirions can be sequentially visualized at the surface of M cells, being transported in vesicles through these cells, and subsequently free in the extracellular space of Peyer's patches (26,564,565) (Fig. 3). The M cell pathway also is used by poliovirus and HIV to penetrate the intestinal epithelial barrier (6,282,469), suggesting that this may be a general pathway for the entry of viruses that spread beyond the mucosal surface. Once cells have traversed the epithelial barrier, the macrophages and lymphocytes, present within GALT, may play a role in further dissemination, as has been suggested for reo- and rotaviruses (362).

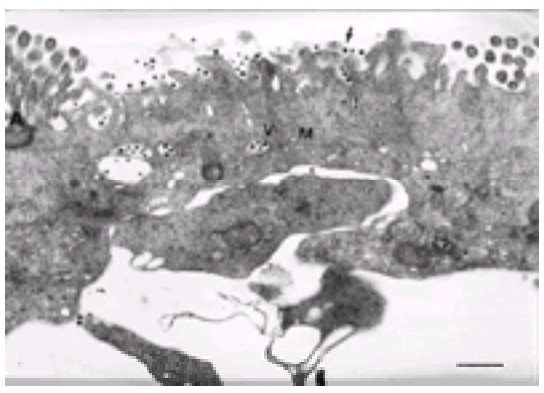

FIG. 3. Reovirus serotype 1 strain Lang virions on the surface (*arrow*) and within vesicles (*V*) of an M cell in the ileal epithelium overlying a Peyer's patch. (From ref. 565, with permission.)

Additional factors favoring localized infection may be the pattern of release of virus from intestinal epithelial cells with

apical release (toward the intestinal lumen) favoring local cell-to-cell spread, as exemplified by rotaviruses (256) and basolateral release facilitating systemic invasion (see later). For example, infection with the porcine coronavirus transmissible gastroenteritis virus (TGEV) occurs at the luminal (apical) surface of epithelial cells, as does release. This facilitates local cell-to-cell spread but reduces the probability of systemic infection. Conversely, the mouse coronavirus mouse hepatitis virus (MHV)-A59 enters cells at the apical surface, but may be preferentially released from the basolateral surface, facilitating systemic spread (447).

The recognition that anal intercourse is a major risk factor for the transmission of HIV infection also has led to the recognition that some viruses that produce systemic disease may gain entry to the host through the lower GI tract. HIV has been detected in bowel epithelium and enterochromaffin cells of patients with HIV, and can infect human colonic cell lines in culture (305,379,397). The mechanisms by which HIV infection is further spread from the lower GI tract remain to be established.

Genitourinary Tract

In addition to the respiratory and GI tracts, other mucosal surfaces may provide sites for viral entry into the host. Sexual activity may produce tears or abrasions in the vaginal epithelium or trauma to the urethra, allowing viral entry via the genitourinary route. Sexually transmitted viruses in humans include HIV, herpes simplex, human papillomaviruses 11, 16, and 18 (the agents responsible for genital warts or condyloma acuminata), and hepatitis B and C (456). In the case of HIV, infection may be facilitated by breaks in the genital epithelial barrier, but the virus also may productively infect cervical epithelial cells and dendritic (Langerhans) cells within the vaginal epithelium (295,296,501). HHV-6 (85) and human papillomavirus (HPV) 16 and 18 also have been shown to infect cervical epithelial cells in culture (566). As might be expected, for sexually transmitted viruses, the degree of sexual promiscuity and the number of sexual partners are risk factors for transmission (456).

The sequence of events that follow vaginally initiated virus infections has not been comprehensively investigated. In a macaque model, after intravaginal inoculation of a simian immunodeficiency virus (SIV)/HIV chimeric virus, virus is first detected in T lymphocytes in the submucosal epithelium, followed by the pelvic and mesenteric lymph nodes within 2 days of inoculation, and virus-infected cells are found in the thymus and spleen within 4 days (248).

Acute hemorrhagic cystitis in young boys has been associated with adenovirus 11 and 21 (Chapter 68); however, it is not known whether these viruses reach the bladder directly through the urethra or indirectly via another site of entry. Other viruses, including polyomaviruses, can produce viruria, but this is generally not associated with symptomatic disease. In some of these cases, virus appears to reach the kidney, presumably through the bloodstream, and then enters the urine (Chapter 64). Little is known about the nature of host factors that influence viral invasion by these routes. The nature of the cervical mucus, the pH of vaginal secretions, and the chemical composition of the urine all may play a role in host defense against infection.

Conjunctiva

The conjunctiva also may provide a route for the entry of viruses that produce either local disease (conjunctivitis) or more rarely disseminate from this site to produce systemic infection (e.g., paralytic illness with enterovirus 70 and experimental Ebola infection). Direct inoculation of virus onto the conjunctiva during ophthalmologic procedures (e.g., foreign-body removal and tonometry) can produce keratoconjunctivitis, and many cases have been attributed to adenoviruses 8, 19, and 37. Similarly, the conjunctivitis that follows swimming in public areas (“swimming pool conjunctivitis”) appears to be frequently due to direct infection most often with adenoviruses (see Chapter 68). Conjunctivitis also can occur in association with enteroviral infections (e.g., echo 7, 11; coxsackie A24, B2) (112). Coxsackie-adenovirus receptor (CAR)-mediated binding of adenoviruses to conjunctival cells via the distal domain of the fiber protein has recently been demonstrated. Mutations at amino acid 240 within the fiber protein can either facilitate or abrogate binding of adenovirus strains to these cells (235).

Systemic spread of virus after conjunctival entry is rare, although as noted, it is an important feature of the acute hemorrhagic conjunctivitis produced by enterovirus 70. This virus can spread from the conjunctiva to the central nervous system, where it can produce poliomyelitis, radiculomyelopathy, or cranial nerve palsies (448). Ebola virus also can be transmitted effectively via the conjunctival route in nonhuman primates, suggesting that this, like the oral route, could be a potential mode of transmission (247).

Conjunctivitis also may occur as part of a systemic viral infection in which virus reaches the conjunctivae after the induction of viremia rather than directly from the external surface. The conjunctivitis associated with acute measles infection is usually considered in this category, but recent experimental studies indicated that systemic measles infection can be produced in rhesus monkeys after conjunctival inoculation with measles, indicating that the conjunctiva also may serve as a portal of entry (335).

SPREAD IN THE HOST

Localized Versus Systemic Infection

Some viruses produce tissue injury predominantly near the site at which they enter the host. This pattern of infection characterizes the upper respiratory infections of influenza, parainfluenza, rhinoviruses, and coronaviruses; the GI infections caused by rotaviruses; and the skin infections of the papillomaviruses. In these cases, spread of virus occurs primarily as a result of contiguous infection of adjacent cells. Virus rarely spreads beyond the epithelial cell layer, although in some cases, involvement of regional lymph nodes and even systemic invasion can occur.

Polarized Infection of Epithelial Cells

Certain viruses preferentially bud from either the apical or basal surfaces of polarized epithelial cells, and this pattern may be an important determinant of whether infection remains localized or becomes systemic (98,99,521) (Fig. 4). Apical release of virus from epithelial cells lining mucosal surfaces favors the development of localized infection and facilitates cell-to-cell spread along the epithelial layer, whereas basolateral release directs virus toward underlying deeper tissues, facilitating mucosal invasion and the development of systemic infection. This pattern may be further reinforced by differences in the susceptibility of polarized epithelial cells to infection at their apical and basolateral surfaces (427). Finally, in some cases, viruses may preferentially enter cells on one surface and be released from another. This is illustrated by the coronavirus MHV-A59, which enters polarized murine kidney epithelial cells through the apical membrane, whereas progeny virions are released preferentially from the basolateral domain (447). Changes in the patterns of polarized release may dramatically alter viral virulence. For example, wild-type Sendai virus buds from the apical surface of polarized bronchial epithelial cells and produces localized bronchial infection, but mutant viruses with the capacity to bud from basolateral as well as apical surfaces have extended tissue tropism and enhanced virulence in vivo (503).

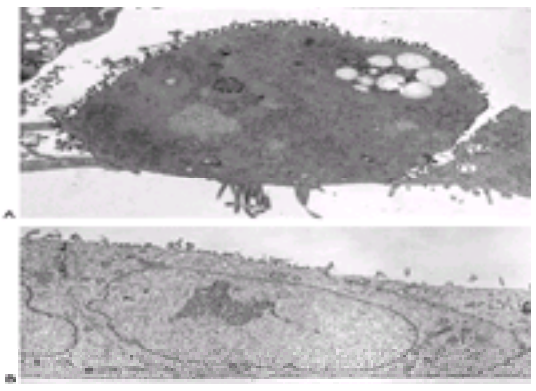

FIG. 4. A: Polarized budding of SV5 and vesicular stomatitis virus (VSV) virions from different surfaces of the same Madin–Darby canine kidney (MDCK) cell. Filamentous SV5 buds from the apical surface, while bullet-shaped VSV particles assemble at the basolateral domain of the cell. (From ref. 100, with permission). **B:** Budding of influenza virus from the apical surface of an MDCK cell. (From ref. 442, with permission.)

Examples of viruses with polarized patterns of release from epithelial cells include vaccinia, vesicular stomatitis virus (VSV), and certain retroviruses that are assembled at and bud from the basolateral cell membrane, and influenza, parainfluenza, SV40, poliovirus, rotavirus and Sendai virus which use the apical surface (98). Not all viruses exhibit polarized patterns of infection and release, and some, such as poliovirus, are capable of bidirectional entry into polarized epithelial cells (42,522). Viruses within the same family also may differ in their patterns of release. For example, the Hantavirus Black Creek Canal shows predominantly apical release from polarized epithelial cells, as opposed to the basolateral release characteristic of most other bunyaviruses (427).

In cases of polarized infection by enveloped viruses, envelope glycoproteins are inserted into the appropriate region of the cell membrane before viral budding. This suggests that it is the initial targeting and localization of these proteins to specific regions of the cell membrane that determine the subsequent polarity of viral budding. The information required for appropriate targeting of these proteins to the cell surface is contained in specific regions of the targeted proteins, and even single amino acid substitutions in the appropriate domain can alter polarized delivery (49,236,290,308,506,524).

In the case of retroviruses, including HIV, a tyrosine-based motif located in the intracytoplasmic domain of the envelope glycoprotein is highly conserved and functions as a critical basolateral targeting signal (309). Measles virus may be an exception to the general rule that sorting signals are contained in envelope glycoproteins, as its capacity to bud from apical surfaces of polarized epithelial cells does not appear to depend on prior localization of the H and F surface glycoproteins, which are transported in a nonpolarized fashion (323).

Not all membrane glycoproteins play an equivalent role in determining the site of polarized release. For example, the polarized release of coronaviruses is not determined by the spike (S) glycoprotein, but rather by the membrane (M) and hemagglutinin-esterase (HE) envelope glycoproteins (446).

Patterns of polarized infection not only depend on viral factors, but also are influenced by specific host-cell transport proteins. Transport of proteins to the plasma membrane uses specific signal-mediated constitutive pathways through the trans-Golgi network that involve a variety of “cargo” proteins that form transport vesicles. Distinct cytosolic proteins appear to be involved in the assembly of apical and basolateral transport vesicles. Interruption of these processes or interference with specific plasma membrane proteins may disrupt polarized sorting and release of virions. For example, depletion of MAL/VP17 protein, a nonglycosylated cellular membrane protein, from polarized epithelial cells dramatically reduces transport of influenza HA to the apical surface of infected Madin-Darby canine kidney (MDCK) cells (418).

Hematogenous Spread

Viruses that produce systemic disease typically spread from their initial site of entry to distant target tissues through either the bloodstream or inside nerves. Direct inoculation of virus into the bloodstream can occur with the bite of arthropod vectors, through iatrogenic inoculation with a contaminated needle, or by the transfusion of contaminated blood products (“passive viremia”). If the inoculum size is sufficient, a passive viremia may be adequate to initiate infection in target organs without requiring preceding replication. A more common sequence of events, exemplified by viruses such as flaviviruses, measles, and polio, is for virus to undergo primary replication near the site of entry, followed by spread to

regional lymph nodes. From the regional lymph nodes, virus can gain access to efferent lymphatics, the thoracic duct, and the systemic circulation. Some viruses can even replicate in lymphatic endothelial cells, further facilitating the development of viremia.

In almost all cases, some degree of replication at a primary site near the site of entry in the host appears to precede the onset of viremia. In some cases, primary replication is followed by a transient low-titer viremia (“primary viremia”), which serves to spread virus to sites such as the reticuloendothelial system, where additional replication leads to a more sustained and higher titer (“secondary viremia”; Fig. 5). Fenner (153) elegantly defined these events in a series of classic studies of the pathogenesis of ectromelia (mousepox; Fig. 6). Although viremia occurs in many human infections (*vide infra*), it is often difficult to demonstrate discrete phases of primary and secondary viremia.

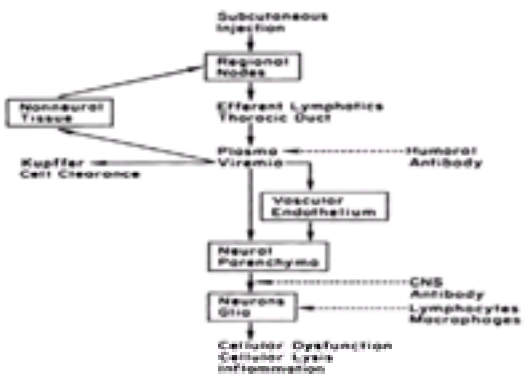

FIG. 5. Stages in the pathogenesis of flavivirus infection. (From ref. 376, with permission.)

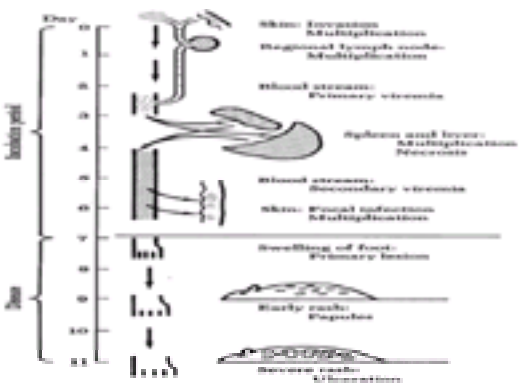

FIG. 6. The pathogenesis of mousepox (ectromelia). (From ref. 153, with permission.)

Once virus has reached the circulation, it can travel either free in the plasma or in association with cellular elements (Table 1). Cell-associated viruses can replicate in monocytes and T or B lymphocytes [see (377) for review and additional references]. Colorado tick fever virus (CTFV) provides an unusual example of a virus that is found in association with circulating erythrocytes (red blood cells). CFTV infects bone marrow erythroblasts, which are subsequently released into the circulation as mature erythrocytes. Platelets, their precursor cells (megakaryocytes), and neutrophils do not typically support virus replication and do not appear to be a significant source of circulating virus *in vivo*. Circulating monocytes can be infected by viruses belonging to a number of viral families (see Table 1) including retroviruses (e.g., HIV), herpesviruses [e.g., cytomegalovirus (CMV)], flaviviruses (e.g., dengue), and togaviruses (e.g., measles). Examples of viruses that replicate in lymphocytes include HIV, human T-cell lymphoma virus type I (HTLV-I), and human herpesviruses 6 and 7 (T lymphocytes) and Epstein-Barr virus (EBV; B lymphocytes). Some of the most comprehensive studies of cell-associated viremia have involved HIV. It has been estimated that the percentage of HIV-infected $CD4^+$ T lymphocytes increases from 0.2% to 10% in asymptomatic individuals to as high as 60% in some patients with acquired immunodeficiency syndrome (AIDS). Based on these figures, the blood of asymptomatic HIV^+ individuals contains 5,000 infected cells per milliliter.

Free in plasma	Togaviruses, picornaviruses
Red cell associated	CTFV
Platelet associated	HSV, LCMV, retroviruses
Lymphocyte associated	CMV, EBV, HBV, HHV 6 & 7, HIV, HTLV-I, JC & BK, LCMV, mumps, measles, rubella
Monocyte/macrophage associated	African swine fever, CMV, dengue, HIV, lentiviruses, LCMV, measles, mousepox (ectromelia), polio, rubella
Neutrophil associated	Influenza

When a virus family is listed, this indicates that one or more members of the family may spread in association with these cellular elements. Association of a virus with a formed element of the blood does not necessarily imply replication or a pathologic effect. See text and ref. 378.

Abbreviations: CMV, cytomegalovirus; CTFV, Colorado tick fever virus; EBV, Epstein-Barr virus; HBV, hepatitis B virus; HHV, human herpesvirus; HIV, human immunodeficiency virus; HSV, herpes simplex virus; HTLV, human T-lymphotropic virus; LCMV, lymphocytic choriomeningitis virus.

TABLE 1. *Selected examples of viruses associated with or replicating in circulating blood cells*

The magnitude and duration of viremia reflect the outcome of a series of interactions between the virus and the host. Viral replication at the primary site, and subsequently in a variety of other tissues, provides a continuing source of virus input into the circulation. Important sites of primary replication for viruses that spread through the bloodstream include skeletal muscle for togaviruses, connective tissue, muscle, endothelial cells, and reticuloendothelial organs for flaviviruses, and brown fat for enteroviruses (193,368,376).

At the same time that virus is entering the blood compartment from these sites, a variety of clearance mechanisms are actively removing virus from the circulation. The magnitude of viremia varies as a result of the dynamic interrelationship between the amount of virus entering the blood compartment and the efficiency with which it is removed. The extent to which virus input into the bloodstream exceeds the rate of host clearance determines the magnitude of viremia. Viremia persists for as long as virus input into the bloodstream equals the host's capacity to clear virus from the circulation. The turnover rate for infectious virus in the bloodstream (as opposed to the duration of viremia) varies for different viruses. For example, exogenously infused SIV and HIV particles have been reported to have a plasma half-life that may be as low as 3 (580) to 26 (242) minutes.

Phagocytic cells in the reticuloendothelial system (RES) and serum factors including complement and antibody act in concert to facilitate clearance of virus from the bloodstream. One of the most important determinants of clearance is particle size (351). Rapid clearance (>99.99% of an intravenous inoculum cleared within an hour) is seen after inoculation of a large virus such as vaccinia into the bloodstream of a mouse. A similar pattern of clearance is seen with small viruses opsonized by antibody or complement. The coating of virus with antibody or complement may facilitate clearance by phagocytic cells such as macrophages because of the presence of receptors on these cells for the Fc portion of antibody molecules and for complement (C3 receptor). For example, the plasma half-life of infused cell-free HIV particles is reduced from a range of 13 to 26 minutes in HIV nonimmune macaques to 3.9 to 7.2 minutes in animals with high-titer HIV-specific neutralizing antibodies (242).

The nature of the protein(s) on the outer surface of the virus also may be an important determinant of the pattern of clearance. For example, the sialic acid content of the envelope of Sindbis virus appears to influence the degree of viral clearance (217). The net charge on a viral particle also may influence its clearance.

The nature of the interaction between virus and the macrophages of the RES seems to be an important determinant of the development of viremia (351). The capacity of a virus to avoid phagocytosis by macrophages facilitates the maintenance of viremia. Inhibition of the phagocytic capacity of macrophages can serve to amplify viremia and potentiates the severity of experimental viral infections caused by viruses as diverse as yellow fever virus, Semliki Forest virus, and HSV (244). Infection of macrophages with one pathogen may facilitate their infection with another. For example, infection of macrophages with opportunistic pathogens such as *Mycobacterium avium* and *Pneumocystis carinii,* or their exposure to bacterial products such as lipopolysaccharide (LPS), may promote HIV-1 replication through modulation of macrophage cytokine expression and alteration in cell-surface distribution of HIV chemokine coreceptors (361).

Certain viruses may replicate in macrophages, which serves to amplify viremia further rather than terminating it. Viruses known to replicate in macrophages include herpesviruses (e.g., HSV, CMV) (471), togaviruses (e.g., lactate dehydrogenase–elevating virus, Sendai), flaviviruses (e.g., dengue) (87), poxviruses (e.g., ectromelia), lentiviruses (e.g., HIV, visna, equine infectious anemia virus, Aleutian disease virus) (387,398), coronaviruses (e.g., mouse hepatitis virus, human coronavirus OC43) (97), arenaviruses (e.g., lymphocytic choriomeningitis virus [LCM]), reoviruses, picornaviruses (500), rhabdoviruses (428), and myxo- and paramyxoviruses (e.g., influenza, measles) (299). Because macrophages have receptors for the Fc portion of antibody, these cells may more readily take up viruses coated with antibody. In some cases this facilitated uptake actually leads to an enhanced replication of these viruses in macrophages ("antibody-mediated enhancement"). Antibody-mediated enhancement may play an important role in the pathogenesis of viral infections such as dengue fever and foot-and-mouth disease (27).

Differences in the susceptibility of macrophages to viral infection may account for variation in the age-related susceptibility of animals to certain types of viral infection (216) (see section on host factors). For example, alveolar macrophages derived from young mice will support the replication of HSV. Transfer of adult macrophages to young mice increases their resistance to HSV infection (216,359). More recently it has been suggested that certain HIV strains may differ in their capacity to infect neonatal monocytes as compared with placental macrophages and adult adherent macrophages. This difference may result from maturity-related changes in the levels of HIV coreceptor CCR-5 on macrophages, in combination with differences in HIV gp120 V3 loop sequences (151). Differences in the capacity of Theiler's virus and CMV to infect macrophages after infection also have been correlated with the state of macrophage differentiation (477).

As noted earlier, specific viral proteins may influence the capacity of viral strains to infect macrophages, and this may in turn influence viral pathogenicity and disease expression. Determinants of macrophage infection have been found on envelope proteins, such as the V3 loop of HIV gp120 (151), and on nonenvelope proteins such as the L* protein of Theiler's virus (500) and HIV accessory protein Vpx (218). Examples of macrophage tropism correlating with patterns of pathogenesis have been extensively investigated for HIV (Chapter 59 and Chapter 60) and lentiviruses such as visna (510) as well as for the Theiler's virus (500).

The interaction between viruses and macrophages also is influenced by both cytokines and chemokines (see Chapter 12 for review and references). Some cytokines, such as g-IFN, tumor necrosis factor (TNFa), interleukin 2 (IL-2), and IL-16 inhibit replication of viruses such as HIV in macrophages. These cytokines may be secreted by infected macrophages and act via autocrine pathways or be produced by other activated leukocytes including neutrophils, natural killer (NK) cells, or T and B cells. Paradoxically, cytokines also may facilitate the capacity of some viruses to infect macrophages productively. For example, certain opportunistic infections may promote macrophage infection by HIV by modulating chemokine receptor expression. Not surprisingly, viruses have adopted a wide variety of strategies (reviewed in Chapter

11) to circumvent the antiviral defenses of macrophages, including down-regulation of HLA antigens and inhibition of the production of antiviral molecules such as nitric oxide (NO).

Vascular endothelial cells also appear to play an important role in the maintenance of viremia for certain viruses. The capacity of viruses to infect endothelial cells has been studied both *in vitro* and *in vivo* for a large number of viruses (107,157,161,297,334,405,532,541,582). Viruses may differ in their capacity to infect endothelial cells derived from vascular beds in different organs, which may be a factor in their organ-specific tropism and invasion (156). This may be of particular importance in influencing viral invasion of the CNS. For example, mouse hepatitis virus A59 binds to receptors expressed on the endoluminal surface of liver-derived endothelial cells, but fails to bind to endothelial cells derived from brain, which in turn prevents entry of virus into the CNS after intraperitoneal (but not intracerebral) inoculation (179). Conversely, the capacity to infect the CNS and the pattern of disease induced by some strains of adenovirus correlate with their ability to infect cerebral vascular endothelium (81). Viruses that infect circulating cells may enhance their capacity to spread subsequently to endothelial cells by up-regulating integrins such as LFA-1, which facilitate binding of circulating cells to vascular endothelium. This suggests that, in some circumstances, cell-to-cell spread may be an important mechanism of initiating endothelial infection (238).

There is a general correlation between the capacity of blood-borne neurotropic viruses to generate a high-titer viremia and their neuroinvasiveness 376). Similarly, attenuated virulence may reflect incapacity to generate sufficient viremia, even for viruses that are fully virulent after direct CNS inoculation (181). For example, mutants of Semliki Forest virus (SFV) generate high-titer viremia but, unlike their wild-type counterparts, are unable to invade the CNS (13). Thus the capacity to generate a viremia of adequate magnitude and duration may be necessary for neuroinvasiveness, but is not always sufficient. A similar relationship between the degree of virus present in the plasma and clinical outcome has been repeatedly noted in HIV infection, in which plasma viral load at diagnosis correlates with subsequent risk of progression to AIDS and death (341).

Tissue Invasion

The factors that determine the capacity of blood-borne virus to escape the vascular compartment and invade specific organs are poorly understood. The problem has probably been most extensively investigated as it applies to invasion of the CNS by blood-borne viruses (Fig. 7) (251). In most regions of the CNS, tight junctions (zona occludens) join capillary cells of the cerebral microvasculature, and there is an underlying dense basement membrane. A notable exception to this pattern occurs in the choroid plexus, where the capillary endothelium is fenestrated and the basement membrane is generally sparse. This was originally postulated as a potential site of invasion for polioviruses, and subsequently established as an important site of infection for lentiviruses such as visna (307). Recent studies suggest that HIV also may infect the choroid plexus (147), and that choroid plexus dendritic cells, which are similar to antigen-presenting dendritic cells elsewhere in the body, may serve as a reservoir and source for CNS disease (203).

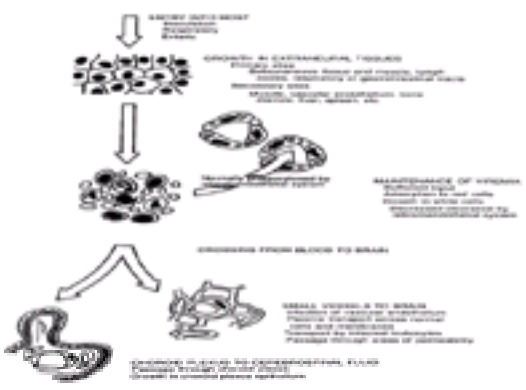

FIG. 7. Pathways of entry of blood-borne viruses into the central nervous system. (From ref. 251, with permission.)

Some viruses invade the CNS from the bloodstream by entering the stroma of the choroid plexus through the fenestrated capillary endothelium and then either infecting or being passively transported across the choroid plexus epithelial cells into the cerebrospinal fluid (CSF). Once in the CSF, virus can infect the ependymal cells lining the walls of the ventricles and can then invade the underlying brain tissue. This pattern of infection has been shown for a number of viruses including mumps, arboviruses, and rat virus (251). Visna, a lentivirus, can be shown to infect cultured choroid plexus epithelial cells *in vitro* (372), suggesting that this pathway may be used by this virus *in vivo* as well. In some cases, direct infection of choroid plexus may not be demonstrable, although the sequential appearance of virus in CSF and ependymal cells suggests the possibility that virus has used this route initially to reach the CSF.

A number of viruses that invade the CNS do so by either directly infecting or being passively transported across capillary endothelial cells. Ability to infect endothelial cells has been demonstrated *in vitro* for many viruses (107,161,297,532,541). Infection of endothelial cells has been proposed as a mechanism for CNS invasion of such disparate groups of viruses as picornaviruses (107,582), togaviruses (376), bunyaviruses (252), parvoviruses, and murine retroviruses (336,411). In the case of some murine retroviruses, there is a close association between neuropathogenicity and capacity to bind to brain endothelial capillary cells *in vitro* (334).

Transendothelial transport of viruses including measles, mumps, canine distemper virus, togaviruses, and lentiviruses may provide another route of penetration of the endothelium and may occur via the diapedesis of infected monocytes, leukocytes, and lymphocytes (159,199). This process may be facilitated by viral modulation of the activity of LFA-1 and other cell-surface adhesion molecules (14,211). Finally, factors that increase vascular permeability, such as vasogenic

amines, also may facilitate viral invasion of sites such as the CNS (465).

Neural Spread

Another important route for viral spread is through nerves (251,285) (Fig. 8). Although this was first postulated as a mechanism for the spread of rabies as early as the 18th century (251), it was not clearly demonstrated until the elegant work by Pasteur and his contemporaries (382). During the first half of the 20th century, experiments by Goodpasture, Hurst, Sabin, Bodian, and others established that herpesviruses, polioviruses, and certain arboviruses also were capable of neural spread (183,234,453). These early observations have been repeatedly confirmed in more modern studies (108,177,291,293,389,432,495,517,527). The group of neurally spreading viruses has now expanded to include reoviruses (157,363,525), pseudorabies virus (71,74,573), coronaviruses (23,407,408), and Borna disease virus (68,358).

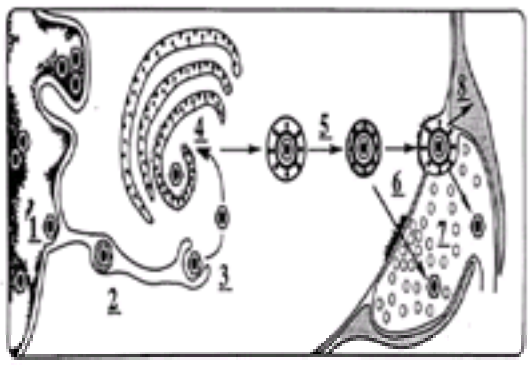

FIG. 8. Significant events that lead to replication and transneuronal passage of pseudorabies virus. Virions replicate in the cell nucleus and acquire a membrane envelope as they bud from the nuclear membrane (*1*), traverse the endoplasmic reticulum (ER) (*2*), and are subsequently released into the cytoplasm after a fusion event between the virion membrane and ER (*3*). Virions acquire an envelope at the Golgi apparatus (*4*), which consolidates and becomes denser (*5*). Transneuronal passage of virus occurs after fusion of the virion envelope with the postsynaptic membrane (*6*); the nucleocapsid can subsequently undergo retrograde axonal transport toward the neuronal cell body (*7*). Alternatively, the virus particle can be released into the extracellular space to infect other cells in the neural parenchyma (*8*). (From ref. 71, with permission.)

Classic experiments on the spread of poliovirus through the sciatic nerve of the monkey by Bodian and Howe (234) established several important principles of neural spread, which have been repeatedly confirmed in other systems (17). They demonstrated that primary replication of virus in extraneural tissues was not an absolute prerequisite for neural spread by showing that poliomyelitis could develop in monkeys after immersion of the cut distal end of the sciatic nerve into a solution containing virus. They also established that the incidence of subsequent neurologic disease, which reflected the efficiency of neural spread, depended on a variety of factors including the amount of nerve exposed to virus, the duration of this exposure, and the concentration of virus present. Under circumstances of natural infection, direct exposure of a nerve to virus is presumably unusual, and primary replication of virus at peripheral sites, although not essential for subsequent neural spread (108,467), may facilitate this process by amplifying the size of the initial inoculum.

Techniques for localizing viral antigens have been used to document the sequential progression of viral infection along neural pathways (251,291). The direct visualization of herpesvirus and rabies virus particles within axons provided further evidence for intra-axonal transport (214,245,285,367,420). A natural corollary of these observations was the finding that axonal transport could also be demonstrated in cultured neurons *in vitro* (318,319,518,519), as well as in isolated nerve preparations (327), and that inhibitors of axonal transport also inhibited the spread of neurally spreading viruses *in vivo* and *in vitro* (39,76,77,283,285,319,516,525).

Both kinetic studies and experiments using pharmacologic inhibitors indicated that most viruses that spread neurally do so via fast axonal transport (285,327,389,525). *In vivo* studies, in which the rate of viral transport is generally measured by dividing the distance traveled by the elapsed time between inoculation and detection at a distant site, have resulted in calculated transport values of 2 to 16 mm/day for viruses as diverse as polio, rabies, herpes simplex, and reovirus T3 Dearing (234,245,285,525). These rates are faster than those associated with slow axonal transport, but far slower than the rate of fast axonal transport (>100 mm/day). The discrepancy may be due to the fact that viral transit times also reflect the time required for a variety of events in addition to transport (e.g., adsorption, penetration and uncoating, and in some cases, replication). The rate of viral transport in *in vitro* systems is often (318,518,519) but not invariably (319) considerably faster than that calculated from *in vivo* experiments.

Further confirmation of the use of fast axonal transport by viruses comes from pharmacologic studies. For example, selective inhibition of fast axonal transport with colchicine, but not the inhibition of slow axonal transport with imidodiproprionitrile (IDPN), inhibits the neural spread of reovirus T3 (525). Colchicine also has been repeatedly shown to inhibit the neural spread of both rabies virus and HSV both to the CNS (39,77) and within the CNS (76). Similarly, taxol, nocodazole, and vinblastine, agents that disrupt the microtubules that serve as the structural guides for fast axonal transport, can inhibit neural spread of viruses *in vivo* and *in vitro* (90,285,319). These results are all consistent with morphologic studies showing a close association between neurally transported viruses and microtubules (258). A selective inhibitor of retrograde axonal transport, erythro-9-3-(2-hydroxynonyl)adenine, also has been shown to inhibit transport of HSV (283), as has an inhibitor of axonal transport in sensory neurons (capsaicin) (482).

The exact mechanism(s) by which axonal transport of viruses occurs has not been established. As described earlier, in the case of several enveloped viruses, such as rabies and herpes simplex, both mature virions and naked nucleocapsids

have been visualized within axons. Fast axonal transport typically involves the movement of material contained in vesicles along microtubules. It is easy to see how viruses that initially enter nerve cells via receptor-mediated endocytosis, and thus appear in vesicles, could be transported to the perikaryon in a vesicle-associated fashion. In some cases, virus might be inserted into vesicles via the Golgi apparatus, in a fashion akin to the sorting of certain cellular macromolecules. However, it remains unclear whether virus is transported in this fashion. Some viruses, including reoviruses and adenoviruses, are capable of binding to isolated purified microtubules (16), suggesting that movement of extravesicular virus also could occur.

In vivo studies clearly indicated that viruses can cross the neuromuscular junction and travel transneuronally at both synaptic and nonsynaptic sites (72,284,285,483,490,527), with the predominant route depending on the virus. In some cases, transneuronal spread may be facilitated by the pattern of viral release from the nerve cell or myocytes. Polarized sorting of viral glycoproteins to the axon and dendrites of cultured neurons, a process analogous to that seen in epithelial cells (*vide supra*), has been described (127), and undoubtedly plays a role in the patterns of viral release and transport in neurons. Rabies virus accumulates in myocytes at the neuromuscular junction (367) and may bind to the nicotinic acetylcholine receptor at the neuromuscular junction (see receptors) (62,202). The synaptic terminals of nerve cells may contain increased numbers of receptors for certain viruses including herpes simplex and rabies (318,319), perhaps explaining how entry at the distal axon terminal is facilitated. Rabies virions also bud from peripheral nerve cells along their axons at the nodes of Ranvier, at the boundary between opposing myelin-producing Schwann cells (367). A related virus, vesicular stomatitis virus (VSV), will preferentially bud from synaptic sites in the presence of antiviral antibody, suggesting that the synaptic region of the axon terminal may have unique structural features that facilitate the exocytosis of some viruses (134).

Viral factors that influence neuron-to-neuron spread are still poorly understood. In the case of herpes simplex virus, it has been suggested that a heterodimer of the envelope glycoproteins gE and gI may be required to facilitate efficient neuron-to-neuron transmission through synaptically linked neural pathways (125).

Different strains of the same virus may show a predilection for retrograde, anterograde, or bidirectional systems of fast axonal transport. This direction-specific predilection has been seen with certain strains of herpes simplex virus and pseudorabies virus (70). The capacity of viruses to spread along discrete neuronal pathways and transneuronally across synapses has made them valuable tools for defining neuroanatomic pathways. Viruses have been used to map synaptically linked interneuronal circuits both within the CNS and between the peripheral and central nervous systems. Injection of virus into a particular target tissue, and sequential study of its central transit, may allow the peripheral and central innervation of these structures to be mapped with exquisite fidelity. HSV and pseudorabies virus have both been used extensively for this purpose (69,71,72,229,230,291,311,384,483,490).

It is important to recognize that viremia and neural spread are not mutually exclusive processes. Some viruses may use different pathways to reach different organs or different pathways at different stages in pathogenesis. For example, it has been suggested that certain flaviviruses may spread via the bloodstream to reach the neurons of the olfactory bulb and then travel retrogradely via neural spread within these specialized neurons to reach the CNS (355). VZV disseminates to the skin via the bloodstream to produce the classic exanthemal rash of chickenpox but then travels up the axons of sensory neurons to reach sensory ganglia where the virus remains latent. Reactivation of VZV is associated with spread of virus via nerves to reach the skin and the appearance of the dermatomal lesions of shingles (Chapter 79).

As discussed earlier, different strains of the same neurotropic virus may differ in their capacity to spread at synaptic versus nonsynaptic sites and to use anterograde or retrograde systems of fast axonal transport. Different strains of the same virus also may differ in the predominant pathway (neural or hematogenous) they use to spread in the host. For example, under experimental conditions, some strains of poliovirus spread to the CNS via nerves (389,432), whereas others do so via the bloodstream (145,234,374,454). Similar differences have been noted for reoviruses, with reovirus serotype 1 strain Lang spreading to the CNS predominantly through the bloodstream, whereas reovirus serotype 3 strain Dearing uses predominantly neural spread (525).

Molecular and Genetic Determinants of Viral Spread

Although the capacity of viruses to spread via different routes in the infected host has been recognized for some time, only recently have the specific viral genes involved in determining this process been identified. Thus several studies with reoviruses indicated that their pattern of spread in the infected host is determined by the nature of the viral S1 gene and the outer capsid protein (s1) that it encodes. After intramuscular inoculation, reovirus T1 Lang (T1L) spreads to the CNS via the bloodstream, whereas T3 Dearing (T3D) spreads via nerves. The viral S1 gene, which encodes the outer capsid protein s1, determines the capacity of T1L × T3D reassortants to spread via either the bloodstream or the nerves to reach the CNS (525). T1 and T3 also show different patterns of spread after oral inoculation into mice. T3 does not disseminate to extraintestinal organs, but T1 does. The pattern of spread of T1 suggests that virus initially spreads via local lymphatics and then via the bloodstream. Studies with T1L × T3D reassortants indicate that the capacity of T1 to spread extraintestinally also is determined by the S1 gene (Chapter 53).

Genetic factors that influence the capacity of HSV to spread to and invade the CNS also have been investigated. There appear to be numerous factors that influence HSV neuroinvasiveness, neurovirulence, and latency (486). The region of the HSV genome between 0.25 and 0.53 map units (m.u.) appears to contain genes that are critical in allowing HSV-1 to spread to the CNS after footpad inoculation in mice (“neuroinvasiveness”) (507). An area contained within this region (0.31–0.44 m.u.) also has been shown to influence the capacity of intertypic HSV-1 × HSV-2 recombinants to spread to the CNS via nerves after corneal inoculation in mice (116). A number of viral proteins including the DNA polymerase, thymidine kinase, the nucleoprotein p40, and the gB envelope glycoprotein are encoded by genes contained in these regions. Mutations in gB may be particularly important in influencing the neuroinvasive capacity of certain HSV strains including KOS and ANG (578). Another genetic mapping study has implicated the DNA polymerase (0.413–0.426 m.u.) (116). The mechanisms by which alterations in the polymerase gene enhance or attenuate neural spread remain speculative. It would appear likely that the effect is an indirect one having to do with replication competence rather than a direct effect on axon transport of virus, because it can be shown that recombinants with differences in this region of the

genome vary dramatically in their capacity to replicate in peripheral nervous system tissue.

Mutations within the gD envelope glycoprotein can also influence HSV neuroinvasiveness. For example, the neuroinvasive HSV 1 ANG-path strain differs from its nonneuroinvasive parent by only a single amino acid substitution within gD (246). Finally, as noted earlier, the glycoproteins gE and gI play an important role in determining the capacity of HSV to spread transsynaptically between neurons (125).

These studies suggested that, in the case of HSV, neuroinvasiveness may be affected by mutations involving a number of steps in pathogenesis. Mutations in envelope glycoproteins such as gB and gD may affect viral attachment or penetration. Polymerase mutations and mutations in viral immediate early proteins may hamper viral replication.

Strain-specific patterns in the transport of herpesviruses have been investigated for both HSV and pseudorabies virus (PRV). For example, the PRV-Becker, PRV-Bartha, and PRV-91 strains all differ in their pattern of neuronal uptake and subsequent neural spread (70,74). The PRV-Becker and PRV-91 strains differ only in the deletion of the gene encoding the PRV gI and gp63 glycoproteins, suggesting that the complex formed by these proteins may be critical in determining differences in neuronal uptake and spread (73,272,551).

A number of studies in the bunyavirus system have identified viral genes that are important in the capacity of these viruses to spread from the periphery to the CNS (“neuroinvasiveness”). Comparisons between nonneuroinvasive and neuroinvasive strains indicate that the viral M genome segment, which encodes the two envelope glycoproteins (G1, G2), is the major determinant of the neuroinvasive phenotype (Chapter 9). Subsequent studies with monoclonal antibody–resistant bunyavirus variants suggested that the viral G1 glycoprotein may be the important determinant.

Studies with monoclonal antibody–resistant mutants of the flavivirus, Japanese encephalitis virus (JEV) indicated that mutations in the E envelope glycoprotein can reduce the neuroinvasiveness of these variants without altering their virulence after intracranial inoculation (78). Thus studies in a number of viral systems including reoviruses, togaviruses, herpesviruses, and bunyaviruses indicated that viral surface glycoproteins may be critical determinants of the capacity of these viruses to reach the CNS from peripheral sites.

TROPISM

The pattern of systemic illness produced during an acute viral infection depends in large part on the specific host organs infected and in many cases on the capacity of viruses to infect discrete populations of cells within these organs (tropism). The tissue tropism of a virus is influenced by the interaction between a variety of host and viral factors. Although a great deal of attention has focused on the importance of viral cell attachment proteins and specific viral receptors on target cells, it also has become clear that a variety of other virus–host interactions can play an important role in determining the ultimate tropism of a virus (207,385). This fact is clearly indicated by experiments that demonstrate that the mere presence of a functional viral receptor may be insufficient to allow viral infection of target cells, as has been shown with HIV, polio, rotaviruses, measles, and MHV (88,91,206,383). For example, intracellular factors and the route of viral entry may be important determinants of tropism (see later). Increasing attention also has focused on the importance of “coreceptors” in mediating viral binding and entry. For example, entry of HIV-1 into target cells requires the presence of both CD4 and a second coreceptor protein that belongs to the G-protein–coupled seven-transmembrane receptor family and includes the chemokine receptor proteins CCR5 and CXCR4 (123). As a result, cells expressing CD4 but not the coreceptor are resistant to HIV infection (see earlier).

Binding to Target Cells and Penetration of Cell Membranes

The importance of viral receptors in determining tissue tropism was initially emphasized for polioviruses (224). Since that time, there has been an explosion of knowledge concerning the nature of viral cell-recognition proteins and their target cell receptors. A full discussion of each viral system and its target cell receptor is beyond the scope of this chapter, and readers are directed to the chapters dealing with individual viral families for more detailed information and more complete references. A partial list of virus families and examples of possible associated host cell receptor(s) can be found in Table 2.

TABLE 2. *Cell-surface molecules proposed to play a role in virus binding and entry*

As the list of potential receptors for specific viruses becomes more and more complete, it is possible to identify certain common themes. Different viruses may use the same cellular structures as receptors. For example, sialic acid residues have been reported to be components of the receptor for certain coronaviruses (human coronavirus OC43, bovine coronavirus) (533), orthomyxoviruses (influenza A and B) (546), Sendai virus (571), and reoviruses (certain serotype 3

strains and rotaviruses) (172,406). Similarly gangliosides have been implicated as potential receptors for rotaviruses (SA11, OSU) (497), paramyxoviruses (Sendai) (329), and rhabdoviruses (rabies) (498). Sometimes several different viruses belonging to the same family use the same receptor, as appears to be the case with heparan sulfate proteoglycan molecules that are used by HSV-1, human cytomegalovirus, pseudorabies, and bovine herpesvirus-1 for initial attachment. Interestingly, heparan sulfate proteoglycans may also serve as receptors for nonherpesviruses including Sindbis (64) and adeno-associated virus type 2 (AAV-2) (494), demonstrating that the same surface molecule can potentially serve as a receptor for distinct families of viruses. Another example of this shared receptor use is represented by the CAR, which, as its name suggests, serves as a receptor for adenoviruses 2 and 5 as well as Coxsackie B viruses (33). In some cases, even though the receptor molecules are not identical, they may share enough structural similarity to merit inclusion together as part of superfamily. For example, many rhinoviruses, poliovirus, and HIV-1 bind to molecules that are members of the immunoglobulin superfamily (190,343,484,509,556).

Certain types of cell-surface molecules appear to be used as viral receptors far more frequently than would be expected by chance. Among this group are the human membrane cofactor proteins, which include regulators of complement activation such as the measles receptor, CD46 (371), which binds C3b and C4b, the EBV receptor, CD21 (155), which serves as a C3d receptor, the coxsackievirus and enterovirus 70 receptor CD55, another complement regulatory protein also known as decay-accelerating factor (DAF) (261), and the canine distemper virus receptor CD9 (312). HIV-1 and certain other retroviruses bind to CD4 (113).

Major histocompatibility complex (MHC) antigens have been implicated as putative receptors for CMV (196), SFV (208), and lactate dehydrogenase elevating virus (LDEV) (243), although in some cases, the assignment remains controversial (395).

Other cell-surface antigens, including the members of the CEA family (coronaviruses) (138) and the erythrocyte P antigen (parvovirus B19) (57), also serve as viral receptors. Integrins also have been identified as receptors or coreceptors for a large and diverse group of viruses including adenovirus type 2 (558), AAV-2 (493), hantaviruses (171), and papillomaviruses (142).

Receptors for neurotransmitters, growth factors, and extracellular matrix molecules including the acetylcholine receptor (rabies) (197), the epidermal growth factor (EGF) receptor (vaccinia, reovirus T3D) (492), and the laminin receptor (Sindbis) (544) may be involved in virus–cell recognition, although controversy again exists about the validity of certain of these assignments.

Among the new categories of proteins that have been implicated as potential receptors are permeases (Gibbon leukemia virus) (249), aminopeptidases (coronaviruses) (513), amino acid–transporter proteins (Mahoney leukemia virus) (270), a-dystroglycans (66), and lipoprotein receptors (356).

A number of general principles have emerged from studies of virus–receptor interactions (207). Many viruses appear capable of interacting with more than one receptor molecule (see Table 2). HIV-1 binding to different receptors by a single virus may be mediated by either a single cell-attachment protein, or by different proteins. For example, HIV-1 uses the gp120 envelope glycoprotein to bind to both CD4 and to galactosylceramide (see Chapter 59), although different domains of the protein appear to be involved. Adenovirus 2 binds to certain integrin molecules through the penton base, which contains an Arg-Gly-Asp (RGD) sequence of the type recognized by cell-adhesion receptors of the integrin family (19,558). For initial entry into target cells, adenovirus also attaches with high affinity to cell-surface receptors via the fiber protein (186). Similarly, Sindbis virus binds to the high-affinity laminin receptor on many mammalian cells (544), but appears to use a distinct receptor for binding to mouse neuronal cells (526). Single amino acid substitutions in the Sindbis E2 protein can alter binding to neuroblastoma cells but not to BHK cells, suggesting that distinct receptor-binding domains may exist on the E2 spike.

It appears that attachment is often a multiphasic interaction between viral attachment protein(s) and several host-cell receptors. In the case of the alphaherpesviruses, it appears that internalization is better understood in terms of a cascade of events that involve different glycoproteins and different cell-surface molecules at different stages (166,207,385,479). Different cell-surface proteins may be used for initial attachment and entry into target cells and for cell-to-cell spread across closely apposed populations of cells (124). An initial HSV attachment step appears to involve the interaction between gC, and possibly gB, with heparan sulfate molecules (210,287). This is followed by a stable attachment phase that involves gD, and possibly gH, and occurs independent of heparan sulfate binding (166). Other glycoproteins including gE and gI may facilitate cell-to-cell spread across contiguous cell layers (124). An analogous series of events has been postulated to occur with other alphaherpesviruses including pseudorabies (259), human CMV (101), and bovine herpesvirus (BHV-1) (391).

Even for viruses that may use only a single receptor, a progression from weak initial binding to secondary stable attachment (“adhesion strengthening”) may occur, as a result of factors such as temperature shifts, or recruitment of receptors to allow multivalent binding (207,385).

For all viruses, initial attachment serves as the first in a series of steps that ultimately delivers the genome to a site in the cell where it can begin replication. For enveloped viruses, penetration of the cytoplasmic membrane takes place after fusion between the viral envelope and the cell membrane. For nonenveloped viruses, penetration of the cytoplasmic membrane is still not completely understood, but involves different strategies (220,331,555). In some cases, as discussed earlier for alphaherpesviruses, initial attachment and subsequent fusion may be mediated, entirely or in part, by different viral proteins. In other cases, the interaction of a virion cell-attachment protein (VAP) with its target cell receptor may initiate conformational changes in other virion proteins that may in turn be essential for subsequent steps in virus–cell interaction including fusion. In some cases, an envelope glycoprotein, such as the vaccinia HA, may have fusion-inhibitory activity, with removal of the protein activating the fusion protein(s) (390). As another example, binding of the alphaviruses Sindbis and SFV to host cells results in structural reorganization of the envelope glycoproteins on the virion surface, which expose previously hidden hydrophobic fusogenic domains (56,158,538). In the case of SFV,

dissociation of the E1/E2 heteromer is followed by the appearance of E1 homotrimers that initiate subsequent fusion events (538). In the case of HIV, a conformational change in the gp120 envelope glycoprotein, which serves as the cell-recognition protein, may increase exposure of the N-terminus of the transmembrane (TM) protein, which contains a stretch of nonpolar amino acids that are essential for fusion activity (339). Conformational changes in the envelope glycoproteins required for fusion also may occur after initial receptor binding has resulted in internalization of the virion into acidified vesicles, as occurs with influenza (419,555) and lymphocytic choriomeningitis virus (LCMV) (122). Given these results, it is not surprising that alterations in fusion proteins can dramatically alter viral host range and cell tropism, independent of changes in viral cell-recognition proteins (165,253).

Fusion events may occur under pH-independent or pH-dependent conditions (294,555). The fusions of the envelopes of herpesviruses, coronaviruses, paramyxoviruses, and certain retroviruses with the plasma membrane of target cells are all examples of pH-independent fusion. Conversely, fusion activity may occur only after endocytosis has delivered virions into the acidic environment of endosomal vesicles. This type of pH-dependent fusion is characteristic of orthomyxoviruses, togaviruses, rhabdoviruses, bunyaviruses, and arenaviruses (47,555). It is important to recognize that the type of entry pathway used by a virus may depend on the cell type. For example, EBV entry into lymphoblastoid and epithelial cells seems to occur by direct fusion of the viral envelope with the target cell plasma membrane, whereas entry into normal B cells involves initial internalization into intracellular vesicles (349). In some cases, a single protein serves both as the viral cell-recognition and fusion protein (e.g., the HA of influenza A), whereas in other viruses, these activities are separated (e.g., paramyxoviruses, HIV).

A number of general features of virion fusion proteins have been identified (554,555). For enveloped viruses, typical fusion proteins have external N-termini and internal C-termini, with most of their mass lying outside the viral envelope. Many of these proteins exist as oligomers, typically trimers or less frequently tetramers. This is perhaps best exemplified by the influenza HA, which is a trimer composed of three identical disulfide-linked subunits, each of which is involved in fusion. Many fusion proteins contain a distinctive stretch of apolar amino acids referred to as a fusion sequence, although in some cases, proteins capable of fusion do not have obvious “fusion sequences.” Fusion sequences vary from short stretches of three to six amino acids to longer sequences 24 to 36 amino acids in length, and often appear to have a predicted secondary structure either of amphipathic helices with hydrophobic amino acids on one helical face and nonpolar residues on the other, or of stretches of nonpolar amino acids. Because fusion proteins are typically inserted into viral envelopes via apolar transmembrane anchoring segments, the presence of a second apolar region (the fusion peptide sequence) presumably facilitates their simultaneous interaction with the target cell membrane. The fusion sequence may be located at the N-terminal of the fusion protein, as is the case with orthomyxovirus HAs, paramyxovirus F proteins, and many retroviral envelope fusion proteins.

Nonenveloped viruses, like their enveloped counterparts, undergo conformational changes consequent to receptor binding. Intracellular delivery of the viral capsid also may be a complex event involving several viral proteins. For example, in the case of reoviruses, the s1 outer capsid protein serves as the viral cell-recognition protein (see Chapter 52). Proteolytic removal of other outer capsid proteins, such as occurs during natural infection in the lumen of the intestine, is associated with a change in the s1 cell-recognition protein from a compact to an extended fibrous form (132). This transition may facilitate subsequent receptor binding. Proteolytic digestion also exposes a fragment on another outer capsid protein, μ1, that is proposed to have a role in membrane penetration (228,511).

During the entry of polioviruses into target cells, conformational transitions also occur that are dependent on binding of the virion to its cell-surface receptor (364). The VP4 capsid protein and the amino terminus of VP1 are extruded from virion interior (160,364). These proteins appear to play a role in membrane-binding interactions that are important postattachment steps in poliovirus entry.

Viral Gene Expression

For some viruses, the interaction between VAP and host-cell receptors is the principal determinant of tropism (see Table 2). However, as more cellular receptors become identified, it has become apparent that the interaction between a virus and its receptor is not the only determinant. Other important factors, especially for retroviruses and papovaviruses, are the elements in the viral genome that regulate the transcription of viral genes in a cell-, tissue-, or disease-specific fashion, including enhancers and transcriptional activators. A full review of the many examples of viral transcriptional regulatory elements is beyond the scope of this chapter, and readers should refer to the individual chapters dealing with specific viruses for more complete discussion and references.

Both SV40 and polyoma have enhancer elements that show some degree of cell-type specificity (see Chapter 63 for review and references), although they also behave in a “promiscuous” fashion and stimulate viral gene expression to some degree in many varieties of differentiated cells. When the SV40 enhancer and early promoter are linked to a marker gene (CAT, chloramphenicol acetyltransferase), gene expression is about fivefold greater in monkey kidney cells as compared with murine cells. The SV40 enhancer consists of tandemly repeated sequences that contain a number of functionally independent elements, that have distinct patterns of cell-type specificity, which can be mimicked by synthetic oligonucleotides corresponding to the nucleotide sequences of the individual elements.

Wild-type polyomavirus does not replicate in undifferentiated mouse embryonal carcinoma cells (ECs), and there is no early gene expression. Polyoma virus mutants that can infect EC cells have point mutations and duplications within the polyoma enhancer. Polyoma enhancer sequence alterations can alter enhancer-specific viral DNA replication in an organ-specific way and affect acute and persistent infections differently (438). It appears that certain types of EC cells make a negative regulatory factor that represses activity of the wild-type polyomavirus enhancer, but not that of the mutant enhancer. Aging and age-specific transcription factors also affect organ-specific transcription in polyoma virus–infected mice (562).

Another striking example of enhancer-related cell specificity occurs with JC papovavirus, the agent responsible for the neurologic illness progressive multifocal leukoencephalopathy (PML). When the JCV enhancer is coupled to the gene for

CAT and this construct is tested for activity in human fetal glial cells and HeLa cells, the enhancer is active only in the glial cells (267). This corresponds well with the fact that, within the CNS, JCV productively infects only oligodendroglia.

Further information on the tissue specificity of papovavirus enhancers has come from studies using transgenic mice (50,474). The offspring of transgenic mice made with early region genes (promoter, enhancer, and T-antigen genes) from JCV develop a neurologic disorder characterized by tremor and seizures. Pathologically, they have abnormalities in CNS myelin due to dysfunction of the myelin-producing oligodendrocytes. Oligodendrocyte dysfunction correlates with the expression of JCV DNA and the presence of high levels of JCV-specified messenger RNA (mRNA) in these cells. It has been suggested that the cell specificity of JCV gene expression is due to the viral enhancer and that cell dysfunction itself is due to expression of virally encoded T antigen.

Transgenic mice made by using the early control region and early genes of SV40 virus often develop choroid plexus epithelial cell tumors. Deletion of the SV40 enhancer region from the microinjected DNA results in a dramatic decrease in the incidence of these tumors, suggesting that the enhancer region may play an important role in the choroid plexus specificity of tumor development, with large-T antigen expression accounting for tumor development (50,399,530). Further studies with polyoma virus indicated that enhancer requirements determine differences in cell-specific control of DNA replication in normal versus transformed pancreas cells (439).

Papillomaviruses also contain enhancers with both inducible and constitutive elements (178). In the case of human papillomavirus 11, the enhancer may play an important role in tissue tropism, as it can be shown that the enhancer is specifically active in keratinocytes (485). Replication and gene expression of papillomaviruses increase as skin cells differentiate from basal cells to mature keratinocytes (29).

The role of enhancer elements in tissue and disease specificity also has been investigated extensively with avian and murine retroviruses and HIV (see Chapter 57 and Chapter 59 for review and references). Enhancer elements within the long terminal repeats (LTRs) play a key role in the capacity of avian and murine retroviruses to produce both neoplastic and nonneoplastic diseases. For example, certain strains of avian leukosis virus induce lymphomas, whereas other strains produce osteopetrosis in avian hosts. The capacity of these viruses to produce these different disease patterns is determined by enhancer regions with the LTR region of the viral genome, although non-LTR gene sequences also may play a role. Enhancer regions also determine the disease specificity of murine leukemia viruses. After inoculation into mice, Friend murine leukemia virus (F-MuLV) produces erythroleukemia, and Moloney murine leukemia virus (M-MuLV) produces T-lineage lymphoid cell neoplasms (Chapter 57). When recombinant viruses are made containing the enhancer regions of either F-MuLV or M-MuLV, the pattern of disease production correlates with the enhancer type. Similarly, replacement of the M-MuLV enhancer sequences in the LTR with the enhancer sequences from SV40 results in loss of the capacity of recombinant viruses to produce T-cell lymphomas and the appearance instead of B-lineage lymphomas and acute myeloid leukemia in infected mice. Recombinant polyoma virus that has its B enhancer element replaced with the 72–base pair repeat enhancer from Moloney leukemia virus genome has strong specificity for the pancreas. The LTRs also appear to play a role in the induction of neurodegenerative disease by certain murine retroviruses (404). A variety of cellular factors regulate HIV gene expression and may influence viral load and disease progression (402). These findings indicate that tissue specificity can be achieved by elements within many viral genes.

Enhancer regions also are present in the genomes of herpesviruses (see Chapter 72 for references). EBV virus contains at least two relevant enhancer domains. The A domain is constitutively active in fibroblasts, epithelial cells, and certain myeloid lineage cells but not in B lymphocytes. The B domain is active in B lymphocytes but requires the presence of a viral transactivating factor for activity (Chapter 74).

Viral enhancers also have been found within the hepatitis B virus (HBV) genome (Chapter 86). Some HBV enhancer elements are more active in liver-derived cells (575) compared with nonhepatic cell lines. Transgenic mice injected with the entire HBV genome (15,61) also show a tissue-specific pattern of HBsAg expression, although antigen expression is not exclusively confined to liver tissue.

Site of Entry and Pathway of Spread

The tropism of a virus may depend on both its site of entry and pathway of spread in the infected host. This principle has clearly been illustrated for a number of neurotropic viruses. For example, in a series of classic studies, Howe and Bodian (234) inoculated the Rockefeller MV strain of type 2 poliovirus into rhesus monkeys via various routes and then studied the distribution of lesions within the CNS. Some areas of the brain, including the sensorimotor cortex and certain thalamic nuclei, were always infected regardless of the route of viral inoculation. Other areas, such as the hippocampus and striate cortex, were never involved. Finally, a number of areas were selectively involved only after viral inoculation via specific routes (e.g., the septal nuclei and olfactory bulbs after intranasal inoculation). Similar results have been obtained with a variety of viruses that depend exclusively on neural spread to travel from the site of inoculation to distant target tissues including VSV (453), HSV (8), herpes suis (332), and rabies (18) viruses (see section on neural spread).

The dependence of neural localization on the site of viral inoculation also is illustrated by the appearance of neurally spreading viruses such as herpes simplex, rabies, and reovirus T3D in the region of the spinal cord innervating the skin and muscles at the site of inoculation. The ultimate lesions depend on the neural pathways innervating the site of inoculation.

The effect of portal of entry on the ultimate tropism of viruses has not been extensively investigated for hematogenously spreading viruses. For polyomaviruses, the route of inoculation appears to determine the site of primary replication and the eventual site of persistent replication. The mechanism for this effect has not been clearly established, although the viral VP1 protein type appears to be involved in this effect (133).

For viruses whose ultimate tissue tropism depends on the generation of viremia, it is difficult to understand how route of entry should alter tropism unless inoculation at different sites results in differences in the magnitude or duration of

viremia. An interesting and unexplained exception to this rule may be the “provoking effect” described in studies with poliomyelitis. It was shown that if intracardiac injection of poliovirus serotype 1 into a monkey was preceded by an intramuscular injection, paralysis tended to occur more frequently in the injected limb (hence the so-called provoking effect of trauma or intramuscular injection on the subsequent development of paralysis) (234). It was suggested that local trauma could selectively increase the permeability of blood vessels in the region of the spinal cord innervating the traumatized site. This in turn would allow blood-borne virus to localize specifically in that region of the spinal cord and produce preferential paralysis of the traumatized limb. Direct evidence for this sequence of events is lacking, and the mechanism of the provoking effect is still under study (194).

The provoking effect has been suggested as a possible mechanism to explain the high incidence of paralysis in the inoculated limb of individuals injected with incompletely inactivated lots of poliovirus vaccine (375). The provoking effect also has been suggested as a possible mechanism to explain reports of a higher than expected incidence of paralytic poliomyelitis associated with the oral polio vaccine when it was administered within 30 days of intramuscular inoculations (491). The provoking effect may represent an example of the dependence of the tropism of a hematogenously spreading virus on the site of viral inoculation. However, it remains unclear how intramuscularly inoculated poliovirus in humans reaches the CNS. If virus spreads from the site of inoculation to the CNS via nerves, a similar preferential distribution of paralysis also might be expected.

For some viruses, the pathway of spread may vary depending on the site of inoculation, and this difference may in turn influence tropism. This possibility is illustrated by studies in mice with the neurotropic NWS strain of influenza (431). After intraperitoneal inoculation, virus spreads through the bloodstream, and viral antigen is found in the meninges, choroid plexus, ependymal cells, and in perivascular locations in the brain parenchyma. After intranasal inoculation, virus spreads through nerves, and antigen is noted primarily in the olfactory bulbs, trigeminal ganglia, and brainstem. In this instance, the site of inoculation influences the mechanism of viral dissemination, which then determines the ultimate tissue tropism.

PATTERNS OF VIRAL INFECTION

As noted earlier, viral infection of a susceptible host can result in the production of acute infection that may be clinically symptomatic or asymptomatic. Clearance of initial acute infection may be permanent, or conversely, the host may develop periodic reactivations of infection with intervening disease- and symptom-free intervals. Viruses that produce recurrent infection, exemplified by the herpesviridae, have developed strategies for establishing, maintaining, and reactivating from the latent state (117). If the host fails to clear the initial acute infection, it may become chronic or persistent. In persistent active infections, virus is continuously present in infected tissues and may be continuously shed from the infected host. Selected examples of viruses known to cause persistent infections of humans and animals and their major sites of persistence within the host are shown in Table 3, Table 4 and Table 5. Viruses capable of producing persistent infections include both enveloped and nonenveloped viruses, viruses with DNA and RNA genomes, and members of many viral families.

TABLE 3. *Viral strategies for evading the immune system*

Category	Status of Viral Genome	Virus–Cell Interaction	Antiviral Immune Response	Example
Latent	Latent, recurrent activation	Lytic, latent	Present, normal	HSV, VZV
“Overshoot”	Active replication (continuous high level)	Noninfectious	Impaired or absent to some viral Ags)	HBV
“Breakthrough”	Active replication (continuous but low level)	Lytic, latent	Present (may be reduced or elevated)	SSPE, HIV

Abbreviations: Ags, antigens; HBV, hepatitis B virus; HIV, human immunodeficiency virus; HSV, herpes simplex virus; SSPE, subacute sclerosing panencephalitis; VZV, varicella-zoster virus.

TABLE 4. *Selected examples representing major categories of persistent viral infections in humans*

Recognition System	Target Molecule	Mechanism of Viral Control
Antibody (B-cell product)	Surface glycoproteins Outer capsid proteins Viral glycoproteins expressed on infected cell membranes	Neutralization Opsonization of virus particles ADCC, ADMC Down-regulation of intracellular viral gene expression
CD4+ T cells	Viral peptides presented by MHC class II (10–20 mer peptides derived from exogenous proteins)	Release of antiviral cytokines Activation/recruitment of Mϕ Help for Ab production Help for CD8+ CTL responses Killing of virus-infected cells
CD8+ T cells	Viral peptides presented by MHC class I (8–10 mer proteins derived from endogenous proteins)	Killing of virus-infected cells Release of antiviral cytokines Activation/recruitment of Mϕ

Abbreviations: Ab, antibody; ADMC, antibody-dependent complement-mediated cytotoxicity; ADCC, antibody-dependent cell-mediated cytotoxicity; CTL, cytotoxic T cell; Mϕ, macrophage; MHC, major histocompatibility complex.
(Modified from ref. 3 and reproduced with permission.)

TABLE 5. *Antiviral B-cell and T-cell immune responses*

Persistent Infections

Reduced CPE

For a virus to establish persistent infection [see (84) for review], it must be able to limit its cytolytic effects, maintain its genome within host cells over time, and avoid elimination by the host's immune system (3). It has recently been recognized that numerous viruses are able to induce apoptotic cell death in target cells (429). Conversely, many viruses have developed an extensive variety of strategies to limit or restrict apoptosis in target cells (176) (see also previous references). It has been suggested that inhibition of apoptosis may favor the development of persistent rather than lytic infection (302). Additional factors that may influence the balance between lytic and nonlytic infections include the state of cellular differentiation (301), or the specific type of cell involved. In the case of Sindbis virus, lytic infection occurs in neuroblastoma cells, but differentiation of these cells into mature neurons in the presence of nerve growth factor favors persistent infection. Conversely, CMV causes productive infection in differentiated teratocarcinoma cells, but restricted gene expression in their undifferentiated counterparts. Retinoic acid–induced differentiation of the undifferentiated cells results in a conversion from nonlytic to lytic CMV infection. In a more biologically relevant system, CMV tends to produce nonproductive infection with restricted gene expression in monocytes, but productive infection results when these cells are induced to differentiate into macrophages (504). Like CMV, LDEV also fails to induce permission infection in macrophage precursors, but does so in differentiated cells.

Some viruses, for example, arenaviruses, have evolved to induce predominantly nonlytic infections in their natural hosts (117). For viruses that are normally lytic, persistent infection generally requires restriction of viral gene expression or the generation of attenuated viral variants. For many viruses, the appearance of persistent infection is associated with the generation of defective interfering (DI) particles that are subgenomic deletion mutants lacking one or more components of the wild-type viral genome. These mutant viruses may be unable to generate productive infection, or may act by modulating infection of associated wild-type virus. Viral gene mutations involved in the establishment and maintenance of persistent infection have been studied in reoviruses. Mutations in the S1 and S4 genes, which encode the virus-attachment protein and a major surface protein, result in alterations in early stages of viral replication and in viral effects on host cell macromolecular synthesis that favor the establishment and maintenance of persistent infection (550). Similarly, coronaviruses, which produce persistent infections in cell culture, frequently contain mutations within the S glycoprotein and show delayed cell penetration (180), and mutations in the Sindbis virus envelope glycoproteins reduce the virus' capacity to induce lytic neuronal infection (301).

In cell culture, persistent infections result from a dynamic interplay between mutations in the infecting virus and alterations in the target cell, as has been reported for reoviruses, coronaviruses, and picornaviruses and parvoviruses (120). This coevolution of cells persistently infected with viruses has been associated with alterations in the expression of cell-surface receptors, or changes in intracellular vesicles.

It has been suggested that a critical factor in the restriction of viral cytolytic effects may be infection of target cells that are only semipermissive. This type of restricted gene expression is characteristic of latent HSV infection of sensory neurons, EBV infection of B cells, and papillomavirus infection of basal skin cells (3). It is important to recognize that noncytolytic persistent infections may be associated with loss of a variety of physiologic cell functions including hormone, cytokine, or neurotransmitter synthesis that may have deleterious effects on the host, even though the infected cells appear anatomically normal (117).

Obviously persistence requires that the viral genome be maintained in infected cells. This is not an issue in nondividing cells, such as mature neurons. Persistent infection of dividing cells requires replication of the viral genome during cell division. Retroviruses, such as HIV (see later), and dependoviruses like AAV (106) integrate their genome directly into the host chromosome, so that each cycle of cell replication also replicates the viral genome. Some viruses, including the herpesviruses HSV and EBV and some papovaviruses, maintain their genome as a circular extrachromosomal “episome” that is readily maintained during latent infection (4).

Evasion of the Immune Response

In addition to developing strategies for avoiding induction of cytolysis, and maintaining their genomes, viruses that persist must also evade host immune responses. The interaction between viruses and the immune system is discussed in detail in Chapter 11, and has been comprehensively reviewed elsewhere (84), but some of the basic strategies used by viruses to avoid host immunity are described later (see Table 4).

Obviously one of the most effective strategies to avoid host immunity is to induce a state of virus-specific immune

suppression within the host, by infecting T or B cells and inhibiting their function, by inducing tolerance by deleting virus-specific T cells within the thymus or peripheral lymphoid organs, or by destroying antigen-presenting cells. These mechanisms are discussed in detail in Chapter 11 [see also (84)] and are not considered further. Viruses also have developed specific strategies to evade host cell immunity. Among the more important of these are restricted expression of viral genes and proteins in infected cells, infection of immunoprivileged sites in the host, interference with the effector arm of cellular immunity through down-regulation of MHC molecules or cell-adhesion molecules, interference with antigen processing and presentation, inhibition of cytokine activity, antigenic variation and the development of escape mutants, and spread through syncytial fusion (3).

Latency

Perhaps the best-studied example of limited gene and protein expression is that of HSV (see later). During latent infection, the only viral transcripts that are detected are the latency-associated transcripts (LATs), and no proteins are expressed. Similarly, latent EBV infection is associated only with expression of the EBNA-1 protein. In less dramatic fashion, many viruses appear to show decreased expression of their glycoproteins in persistently infected cells (394).

Privileged Sites

Persistent infection of immunologically privileged sites is perhaps best exemplified by viral infections of the brain, testes, and parts of the eye (488). In the case of the CNS, this may have to do with the specialized nature of the lymphocyte surveillance and trafficking system within the CNS (549). In addition, the absence or markedly reduced expression and induction of MHC molecules on neurons hampers their capacity to serve as effective targets in cytotoxic T lymphocyte (CTL) responses (232,423). In other tissues, including the eye, testes, and possibly CNS, immune privilege also may be maintained in part by the apoptosis of infiltrating activated lymphocytes bearing Fas (CD95) receptor that interact with resident cells bearing Fas ligand on their surface (365,437). In experimental HSV infection, Fas-triggered apoptosis of antiviral lymphocytes may contribute to immune evasion and persistence of HSV (422). The kidney, although not classically considered an immunologically privileged site, is frequently involved in persistent infections (3). This may reflect the fact that although lymphocytes traffic through the kidney, they may have only limited access to epithelial cells because of the presence of a dense basement membrane (9). In some immunologically privileged sites, MHC class I expression may be low or absent, and low levels of transporter proteins may be necessary for antigen processing (see later) (254).

Escape Mutants

Viruses, especially those with RNA genomes, generate mutations resulting in the creation of viral variants with high frequency (225); the potential biologic significance of these viral “quasispecies” is unclear (see 126). Certain quasispecies may replicate better in specific types of cells, as has been shown for LCMV quasispecies in neurons (126). These variants might also facilitate development of persistent infection by avoiding either antibody neutralization (antibody-resistant variants) or by escaping from T-cell recognition (reviewed in 392). The appearance of antibody-resistant variants also is a characteristic of many lentivirus infections including those caused by equine infectious anemia virus, visna virus, caprine arthritis encephalitis virus, and both SIV and HIV. Although the appearance of these variants was once considered a key feature in the pathogenesis of persistent infections, more recent studies suggest they are not in fact essential to the generation of disease (84,317) (see Chapter 61).

Just as viruses can escape recognition by the humoral immune system, they can also escape T-cell recognition (392). CTL escape mutants have now been identified in a wide variety of viral systems including arenaviruses (LCMV), herpesviruses (EBV), retroviruses (HIV and SIV), and reoviruses (281). The significance of these viruses in the generation of persistent infections, like that of antibody-escape variants, is difficult to establish. At least in the case of SIV and HIV, these mutants do not appear essential for viral persistence, nor do they typically become the predominant viral species in infected hosts (82).

As noted, one strategy for viruses to evade host antiviral CTLs is to develop mutations within regions of their proteins (epitopes) recognized by virus-specific CTLs (CTL escape mutants) in the context of MHC and the T-cell receptor (TCR). In some cases, viral mutants are still recognized by the TCR, but fail to induce a stimulatory signal, and instead inhibit T-cell responses and induce T-cell anergy (TCR antagonism) (36). HIV variants of this type have been identified with mutations within the gag protein (275).

Decrease in Expression of MHC Molecules

In addition to anergy induced by TCR antagonism, suppression of virus-specific host T-cell responses can result from down-regulation of cell-surface MHC class I (for $CD8^+$ cells) or class II (for $CD4^+$ cells) molecules or other cell-surface molecules essential for efficient induction of T-cell responses including integrins and adhesion molecules (434).

In the MHC class I pathway, viral proteins synthesized within infected target cells are ubiquitinated and then processed within 26S proteosomes into peptide fragments, which are transported to the endoplasmic reticulum (ER) in association with transporter associated with antigen processing (TAP) proteins. These transporter proteins are encoded by two genes (TAP1, TAP2) within the MHC, and have homologies to the adenosine triphosphate (ATP)-binding family of transmembrane transporter proteins. Deficiency of TAP proteins results in a failure to load peptides onto class I MHC molecules and a resultant defect in MHC class I restricted antigen presentation. Once inside the ER, peptide antigens bind to the MHC molecules and the antigen/MHC I complex is then exported through the Golgi and trans-Golgi apparatus to reach the cell surface (162).

In the MHC class II pathway, extracellular soluble viral proteins are taken up by endocytosis and processed within endosomes into peptide fragments. Endosomes containing these peptide antigen fragments fuse with vacuoles containing newly synthesized MHC class II molecules derived via the Golgi and trans-Golgi apparatus from the ER.

These antigen/class II complexes are then transported to the cell surface.

Viruses known to down-regulate MHC class I include HSV, CMV (murine and human), EBV, measles, retroviruses including HIV, and adenoviruses (434). A variety of mechanisms, affecting virtually all steps in the MHC class I processing pathway, have been implicated.

Viral proteins, exemplified by EBV EBNA-1, can inhibit ubiquitin-proteosomal protein processing of antigens (303). Similarly, a CMV matrix protein with kinase activity appears to modify other viral proteins in a way that limits their proteolytic processing (175). Viruses also can interfere with the processing and export of the MHC class I molecules. In the case of adenoviruses, the adenovirus E3/19K protein localizes to the ER, where it binds directly with MHC class I molecules and inhibits their terminal glycosylation and processing and blocks their transport out of the ER, so that class I/peptide complexes never reach the surface of infected cells.

Cells infected with human CMV show rapid degradation of the MHC class I molecular complex. Deletion of a viral fragment that encompasses eight genes in the unique short region of the CMV genome abrogates the effects of CMV on MHC expression. These genes appear to encode proteins that act to inhibit the intracellular transport of MHC complex molecules, promoting their retention in the ER and Golgi apparatus and subsequent degradation (162). CMV also encodes a homolog (UL18) of the MHC class I heavy (H) chain protein (146). This protein associates directly with b_2-microglobulin, and was originally postulated to decrease MHC class I expression in CMV-infected cells. More recent studies suggested that this protein may actually result in the appearance of a “decoy” MHC molecule on the cell surface that does not activate CTLs, but does decrease the likelihood that NK cells will recognize the infected cell (119,146,149,162,286,413).

The HSV protein ICP47 binds directly to the TAP molecule within infected cells and prevents the translocation of processed antigen peptides to the ER (213). Down-regulation of TAP also has been seen in cells infected with adenovirus 12 (449). Another strategy is exemplified by HIV, in which the viral tat protein has been shown to decrease MHC class I mRNA transcription. Another HIV protein, Nef, causes down-regulation of MHC class I expression in infected cells by causing the accumulation MHC class I molecules in the Golgi apparatus and then subsequently in clathrin-coated vesicles, where they undergo degradation (298).

Down-regulation of MHC class II has been described after infection with CMV, coronaviruses (JHM), measles, and retroviruses including HIV (434). Down-regulation of MHC class II molecules may involve either the reduction of MHC class II expression or interference with the induction of increased cell-surface expression of class II that usually accompanies virus-induced production of g-IFN and other cytokines (40). Some viruses encode soluble homologs of cytokine receptors including TNF-R (e.g., poxviruses), or encode cytokine homologs such as IL-10. In both instances, the action of antiviral cytokines such as IL-2 and g-IFN is inhibited (e.g., EBV) (475). Myxomaviruses encode a secretory protein that binds to g-IFN and prevents its interaction with its cell-surface receptor. Vaccinia, reovirus, adenovirus, and HIV all encode proteins with double-stranded (ds)-RNA binding activity. These proteins effectively “trap” dsRNA molecules and prevent them from acting to stimulate g-IFN induction in infected cells (182).

Latency: The HSV Example

An exhaustive discussion of the mechanisms by which viruses establish, maintain, and reactivate from latency is beyond the scope of this chapter. However, the basic principles are well illustrated by HSV latency (537) and HIV latency and persistence (see later).

Primary infection with HSV-1 typically involves epithelial cells of the oropharyngeal mucosa producing gingivostomatitis. Productive infection of epithelial cells at the primary site involves the orderly transcription of a (immediate early), b (early), and g (late) genes and their cognate proteins. Replication of the viral DNA and structural proteins is then followed by assembly of progeny virions. Progeny virions and/or their nucleocapsids bind to and enter the distal terminals of sensory neurons innervating the oropharynx, and spread through retrograde axonal transport to reach the perikarya of these neurons within the trigeminal ganglion. A similar sequence of events occurs after primary HSV-2 infection of the epithelial cells of the mucosa of the genital tract, with virus spreading to reach sacral dorsal root ganglia.

Once virus reaches the sensory ganglia, it may either produce acute infection with subsequent neuronal cell death or become latent. Acute infection is associated with virus that is easily detectable on direct culture of infected tissue. By contrast, during latent infection, no infectious virus is detectable on direct tissue culture, but virus can be detected when tissues are cocultivated with permissive cells. During the latent state, there is restricted gene expression limited essentially to the production of what are referred to as LATs (43), and minimal or absent expression of any viral proteins. Viral DNA is maintained as a nonreplicating circular episome (340), and probably occurs as between 10 and 1,000 viral genomes per latently infected neuron (212). It has been calculated that 10% or fewer neurons are latently infected (189), although some estimates are substantially higher (424).

The mechanism of reactivation of latent HSV is poorly understood. Reactivation is associated with neuronal injury, stress, fatigue, hormonal changes such as those occurring during menses or lactation, immunosuppression, malnutrition, and exposure to ultraviolet light. Recurrences may be clinical or subclinical, and can occur as frequently as monthly (31). It is presumed that these events trigger the activation of as yet unidentified signal-transduction transcription and kinase pathways within neurons. Reactivation results in initiation of gene transcription and translation, assembly of progeny virions, and their transport through anterograde axonal transport back to mucosal surfaces where active infection results in herpes labialis (cold sores, fever blisters) or herpes genitalis (genital herpes). Under unusual circumstances, reactivated virus may spread to nonmucosal sites including the meninges (herpes meningitis), CNS (herpes encephalitis, myelitis), or eye (herpes keratitis).

The mechanisms responsible for establishment of HSV latency are not yet completely understood. It is possible that special characteristics of neuronal cells, as compared with their epithelial counterparts, may be necessary for HSV to

establish latency (280). The key determinant may be the quantity of immediate early and early proteins present within particular neurons. If sufficient quantities of viral a gene products are present, then neurons undergo lytic infection, whereas if only low levels are present, virus becomes latent. This suggests that restriction of HSV a gene transcription within sensory neurons may be a critical determinant in initiating latency (487). This restriction in turn may result from the presence of cellular inhibitors of HSV gene transcription such as the octamer binding proteins Oct-1 and Oct-2 (266), or the absence of cellular transcription factors required for HSV gene transcription (233). In an alternative model, HSV-encoded proteins such as ICP4, 8, or 27 have been suggested to serve as negative regulators of HSV a gene expression in infected neurons (see Chapter 72 for review of HSV replication).

The commitment by virus to latent neuronal infection, as opposed to productive infection (328), appears to occur at an early stage in the virus replication cycle, and does not require an initial cycle of completed viral replication (464). No specific viral product absolutely required for HSV to establish latency has been identified. Viruses lacking functional thymidine kinase, immediate early regulatory genes, and even LATs can all establish latent infection (see Chapter 72), although LAT mutants may be less efficient at establishing latency than are their wild-type counterparts (458).

During latency, no infectious virus can be detected in sensory ganglia by direct culture. Viral DNA is present in a nucleosome-associated circular episomal state (440), and can be detected in infected cells by *in situ* hybridization and polymerase chain reaction (PCR) (264). Neurons are postmitotic cells, and therefore maintenance of latent episomal HSV DNA does not require ongoing DNA replication. Maintenance of the latent state is associated with gene expression limited to the LAT transcripts (43). No protein products of LATs or other HSV genes are detectable in latently infected neurons.

Stimuli that induce reactivation of HSV from latency are well documented, but the mechanism has defied analysis (537). It has been suggested that an early event may be stimulus-induced expression of ICP0, which in turn may lead to a cascade of events starting with the expression of other a gene products including ICP4 and ICP27 (300). ICP0-minus HSV mutants can still reactivate from the latent state, but appear to do so with markedly reduced efficiency compared with their wild-type counterparts (300). LAT-minus mutants also show a similar reduction in efficiency and frequency of reactivation, suggesting a possible role for LATs in this process (515). It appears that frequent reactivation from latency in sensory ganglia can occur without inducing significant neuronal cell loss (105), suggesting that HSV must also have a mechanism for limiting neuronal cell death during reactivation. It is possible that the ICP34.5 protein, which has antiapoptotic activity (89), plays a role in this process.

Persistence: The HIV Example

Another example of persistent infection is provided by HIV (337). In HIV infection, unlike latent HSV infection, high levels of virus are continuously being produced in the infected host. Estimates suggest that patients with advanced AIDS may produce more than 10^7 virions/day (219). The major source of new virus appears to be recently infected cells, rather than chronically infected cells or latently infected cells undergoing reactivation (93). Initial exposure to HIV frequently causes an acute viral syndrome with high levels of HIV cell-free and cell-associated viremia. These levels subsequently decline by 100- to 1,000-fold, but the virus does not disappear, and patients enter a clinically asymptomatic stage of infection (410). During this phase, high levels of virus are detectable in lymphoid organs and extralymphatic follicular dendritic cells, even when plasma viremia is of only modest proportions (139,401).

Although HIV induces a persistent infection associated with continuous production of new virus, latently infected $CD4^+$ lymphocytes also can be detected. After infection of target cells, HIV preintegration complex is transported through nucleopores into the cell nucleus. In nondividing (G_0 phase) T lymphocytes, HIV does not undergo proviral integration, but remains as a nonintegrated extrachromosomal replicative intermediate. In this state of “preintegration” latency, the genome is vulnerable to degradation, and is often rapidly lost from infected cells (337). More commonly, proviral integration occurs, and may then lead to a latent state without production of progeny virions, which has been referred to as “postintegration latency” to distinguish it from preintegration latency (337). After initial inoculation of HIV at skin or mucosal surfaces, the associated local activation of $CD4^+$ T cells, facilitated by their interaction with local skin and mucosal dendritic cells, enhances the likelihood of integration of proviral DNA (416). Stimulation of T cells harboring integrated provirus—by antigen, cytokines, phorbol esters, and other activators of transcription—may then result in active viral production (187). The *nef* gene and its product may play a role in regulating this process, as patients with HIV isolates containing mutations within this gene appear to have markedly reduced levels of productive infection from latently integrated genomes (273).

It has been suggested that there are three distinct broad populations of infected T lymphocytes in lymphoid organs of HIV-infected hosts (337). One population encompasses cells that are actively producing virus, and the other two populations include latently infected cells harboring integrated proviral genomes. Of this latter group, one population includes cells harboring “defective” proviral genomes that appear to be blocked in their capacity to produce virions, and another population carries latent proviral genomes that can produce progeny virions. The body load of HIV presumably reflects a balance between the number of actively infected cells and the rate at which virus spreads to uninfected cells, counterbalanced by the host's ability to destroy productively infected cells. The reservoir of latently infected cells probably plays a minor role except under special circumstances, such as treatment with antiviral drugs. Studies suggested that differences in these various parameters may be one factor accounting for differences in disease progression among infected individuals (67). Based on mRNA expression and RNA-splicing pattern in infected cells, it appears that the proportion of latently infected—as opposed to productively infected—cells declines with advancing disease (346). These shifts from latent to productive infection may reflect factors that trigger proliferation of latently infected cells such as cytokine exposure (414), or may even result from direct transactivation of the latent HIV genome by co-infection of latently infected cells by opportunistic pathogens (262).

TRANSMISSION OF VIRAL INFECTIONS

After infecting a susceptible host, the virus is shed into the environment, where it encounters a new susceptible host and

the infectious cycle begins anew. Many of the basic issues and concepts in the transmission of viral infection are reviewed in Chapter 14 and are only briefly covered here. Virus transmission typically begins with dissemination from the infected host through respiratory, enteric, or genitourinary secretions. However, there are a number of important exceptions to this general pattern. Arbovirus infections typically involve the ingestion by an arthropod vector of a blood meal from a viremic host, and transmission occurs when the vector feeds on another susceptible host. The transmitting fluid, blood, is never really “shed” from the body. A similar pattern of transmission can occur when contaminated tissues or blood products are removed from an infected individual and transplanted, transfused, or inoculated into a susceptible host. This pattern is exemplified in the transmission of HIV and hepatitis B virus infection by blood, blood products, or between drug addicts sharing blood-contaminated needles. It is important to recognize that both patients and physicians are at risk for viral transmission resulting from needle-stick exposure and surgical procedures (314).

A number of factors influence the subsequent likelihood of blood-borne transmission of viral infection. These include the titer of virus in the blood, the duration of the viremic state, the amount of material transmitted, and the route of transmission. Before the advent of adequate tests for screening donated blood, HBV infection was a serious potential complication of transfusion. This reflected the incidence of infection in certain populations (e.g., professional blood donors), the frequency of a chronic carrier state with persisting viremia, and high virus titer (up to 10^{7} infectious doses of virus per milliliter) (22). By contrast, posttransfusion infection with hepatitis A virus is an extremely rare event, due to the short duration of viremia and the low virus titer in the blood (226).

Skin

Viral infection of the skin is a hallmark of many types of viral infection including those produced by measles, rubella, certain enteroviruses, herpes simplex, varicella-zoster, various poxviruses, and the HPVs (350). In many of these cases, even though infectious virus can be demonstrated in skin lesions, this does not appear to be a significant source of viral transmission. Exceptions to this rule include the spread of genital herpes simplex and occasionally herpes labialis from infected skin lesions (114), rare cases of chickenpox contacted after exposure to shingles, and the mechanical transmission of HPVs. In the case of certain poxviruses and papillomaviruses of animals, skin lesions provide a source of virus that can infect biting arthropods, which in turn transmit the virus to new hosts (553). Smallpox virus remained infectious in dried skin crusts for extensive periods, and fomites provided an important route of transmission for this disease before its eradication.

Respiratory Tract

Viral transmission from the upper and lower respiratory tracts depends on aerosols generated during coughing or sneezing, or in infected saliva. Some of the factors that influence the stability of viruses in aerosols are reviewed in the section dealing with viral entry. Transmission via infected secretions, which can contain large amounts of infectious virus (400), is important in the genesis of upper respiratory infections, as well as a number of systemic viral infections (e.g., measles, varicella) that enter the host via the respiratory route.

Viruses isolated from saliva include rabies (18,109,152), EBV (315), CMV (316), HIV, and HTLV (2), human herpesviruses 6, 7, and 8 (316), and hepatitis viruses (86). Rabies is the most widely recognized example of salivary transmission of virus (18). Inoculation of infected saliva through the bite of a rabid animal appears to be the predominant mode of rabies viral entry (109). Infected saliva plays an important role in the transmission of EBV, either through intimate oral contact or possibly by salivary residues left on cups, food, toys, or other objects. Mumps virus is shed in the saliva and can be transmitted after nasal or buccal mucosal inoculation of virus.

Salivary excretion of CMV can frequently be demonstrated, but the importance of oral secretions in disease transmission remains unknown. Hepatitis B, C, and G have all been isolated from saliva, but experimental transmission studies with HBV-infected saliva have demonstrated transmission of hepatitis only after saliva is parenterally inoculated, suggesting that transmission via this route is unusual (321). Saliva has also been shown to contain HIV and HTLV, but salivary secretions have never been shown to play a significant role in viral transmission. Certain murine retroviruses can be isolated from saliva, which may play a role in male-to-male viral transmission when infected saliva is parenterally inoculated during fighting (417).

Enteric Tract

Many viruses are shed in feces. This route of transmission is central to the pathogenesis of enterovirus infection (e.g., polio) (234) and also occurs with rotavirus (433) and hepatitis A virus (577). Enteroviral excretion in feces is prolonged, magnifying the opportunity for person-to-person spread. Transmission to susceptible hosts typically occurs by way of fecal–oral transmission, which is particularly common among young children or institutionalized patients. Colonoscopy also has been implicated as a unusual potential source for transmitting hepatitis C virus between patients (55). Infected stool also can contaminate water supplies when sewage and waste-disposal conditions are substandard. Some viruses, such as HIV (528), that can be detected in feces are not readily transmitted by the enteric route.

Urine

A number of viruses including CMV, hepatitis A, HBV, mumps, polyomaviruses, and HIV have been isolated from urine (11), but are not thought to be transmitted by this fomite. However, urinary excretion is central to zoonotic transmission of many arenaviruses and hantaviruses via human exposure to aerosolized urine-contaminated material.

Sexual Transmission

Sexual transmission plays a critical role in the spread of HIV, HSV, papillomaviruses, and hepatitis B and C viruses (239). Viral infection of semen occurs with HIV (63), herpesviruses including HSV (540), CMV (326), and HHV 8 (44), HPVs (292), and HBV (463). In the case of CMV, virus can be detected in about 5% of cryopreserved semen samples collected

for donor insemination (326), although this may not be representative of the prevalence of this virus in the population at large. HSV has been detected in about 3% of semen samples from men with a history of genital herpes, and probably occurs in even higher frequency at the time of clinical reactivation (539).

HIV-infected semen appears to be important in disease transmission. HIV has been found both in cell-free seminal fluid and as cell-associated virus (63) in 85% of HIV^+ men (135). In up to 35% of HIV infections, viral titers in semen are equal to or exceed those found in blood plasma (135). The genotype of HIV found in semen may be genetically distinct from that HIV strains found in blood, suggesting that in some instances, semen and blood compartments are distinct (63,104). HIV-1 can be detected in semen of HIV^+ men who received highly active antiretroviral therapy (HAART), even at times when plasma HIV is undetectable (579), showing that the genital compartment may harbor residual virus. Male-to-female heterosexual transmission of HIV-1 is facilitated by breaks in the cervical, vaginal, or urethral epithelium often associated with sexually transmitted disease, although infection of intact epithelial cells also may occur. Cases of HIV infection related to insemination of women with HIV^+ donor sperm also have been reported (568). Many aspects of HIV transmission parallel events described for the horizontal transmission of murine retroviruses (304).

Several viruses, including HIV and HSV, also have been isolated from the genital secretions and cervix of infected women (415,535,539,563). Infected secretions play a role in female-to-male heterosexual transmission of HIV infection as well as in perinatal vertical transmission from pregnant infected mothers to their neonates (10). Virus shed in vaginal secretions also may play a role in the perinatal transmission of papillomaviruses (505).

Milk

Several viruses, including CMV (201), mumps, rubella, HIV and other retroviruses (478), human herpesviruses (164), hepatitis C (289), certain flaviviruses, and minute virus of mice (MVM), have been isolated from human or animal milk or colostrum. Infected breast milk provides a source of transmission of CMV from mother to child during the perinatal period. In one study (534), it was estimated that up to 85% of CMV-seropositive mothers excrete CMV in breast milk. Nearly 60% of preterm infants born to these mothers will develop CMV infection. Infection of preterm infants born to CMV-seropositive mothers was significantly lower if these infants were not breast fed, indicating that this is an important mode for viral transmission. Hepatitis B and C viruses also can be transmitted from mother to child during the perinatal period. Although this type of transmission is well documented, and virus can be found in the milk of infected mothers, it is unclear what role infected milk plays in disease transmission. In the case of hepatitis C, it appears that the risk of maternal–infant transmission occurs predominantly with symptomatic HCV-infected mothers who breast feed their infants as compared with breast-feeding mothers who were asymptomatic carriers (289). This may reflect the fact that symptomatic mothers had dramatically higher viral loads than did their symptomatic counterparts (289). Maternal milk also has been implicated in the vertical transmission of visna maedi and may play a role in human transmission of HTLV-I.

HIV RNA is detectable by quantitative competitive reverse transcription–PCR (RT-PCR) in the milk of 40% of HIV-1–seropositive women (306). Virus may be either cell-free (306) or present in the T cells and macrophages found in colostrum and milk (478). Recent data showed that 5% to 20% of infants born to HIV-infected mothers may be infected by breast feeding, with transmission occurring up to age 18 months (478). Although mumps virus can be found in human milk, and rare cases of perinatal mumps infection occur (255), infected milk is not important in viral transmission.

Maternal–Child Transmission

Maternal–child transmission can result from intrauterine infection, infection during delivery from virus present in the maternal birth canal, of from neonatal exposure to infected breast milk. Maternal–child transmission of infection has been documented for a number of viral infections (324) as exemplified by HIV, hepatitis B and C, CMV, HSV, human herpesviruses (472), HPVs (505), varicella, measles, mumps, rubella, and human parvoviruses (B19) (279). In the case of HPV, the vertical transmission rate from infected mothers to their infants born vaginally may be as high as 30% (505). Premature rupture of membranes appears to be an important risk factor, with increasing rates of maternal–child transmission being directly correlated with the time elapsing between rupture of membranes and delivery (505). The risk of transmission of parvovirus B19 infection has been estimated to be as high as 50%, but is fortunately only rarely associated with symptomatic disease (279). The risk of transmission for hepatitis C appears significantly lower (2–11%), but may be increased in mothers with associated HIV infection, HCV viremia, and elevated liver-function tests (185).

Perhaps the most extensively studied example of maternal–child viral transmission is that of HIV infection. The risk of transmission of HIV infection from an infected mother to her child appears to be directly proportional to maternal HIV-1 viral load (102,167,353). The risk may be as small as 5% or less in mothers with fewer than 1,000 copies/mL and can reach 40% or higher in those with more than 100,000 copies/mL (102). In another study, the HIV viral load was found to be more than 65,000 copies/mL in mothers who transmitted infection to their children, as compared with 5,139 copies/mL in nontransmitters (412), again indicating a general correlation between the degree of maternal viremia and the risk of transmission. It has been suggested that for each 10-fold increase in maternal HIV RNA level, there is an approximately twofold increased risk of maternal–child transmission (265). Premature delivery, prolonged duration between premature rupture of membranes and delivery, and the presence of genital warts have all been reported to increase the risk of intrapartum transmission of HIV infection (288).

The mechanism by which HIV infection is transmitted transplacentally may involve the presence of maternal chorioamnionitis (529). Infection of placental chorionic villi has been documented in HIV infection (344), although only a subset of the HIV quasispecies found in maternal blood are detectable in chorionic villi (344). This suggests that the capacity of certain HIV strains to infect the placenta may be an important factor in maternal–child transmission. Interestingly, certain HIV-1 p17 matrix protein amino acid sequence motifs appear to be found in nontransmitting HIV isolates as compared with those associated with maternal–child transmission. For example, the p17 matrix protein amino acid sequence K_{103} IEEQN$_{109}$ was found in all HIV B subtype isolates from mothers who had transmitted infection to children, whereas nine of 17 nontransmitting mothers had a valine $_{104}$ in place of the isoleucine $_{104}$ (373).

Organ Transplantation

Organ transplantation has provided an entirely novel method for iatrogenic transmission of viral infections. A full discussion of this subject is beyond the scope of this chapter (192). Transplanted bone marrow, kidneys, livers, and hearts have provided the source for infection with CMV (215), EBV and other herpesviruses (192), and retroviruses including HIV and HTLV-I (409). In one case, HTLV-I–associated myelopathy was attributed to acquisition of infection during cardiac transplantation (184). There also are reports of rabies and hepatitis B transmission resulting from corneal transplantation (222). Increasing use of animal-derived tissues for transplantation (xenotransplantation) has raised fears that these tissues may be the source for transmission of infectious agents (80), although no viral infections have yet been reported.

Little is known about the role played by specific viral genes and the proteins they encode in determining the transmissibility of viral infections (268) (see earlier discussion of maternal–child transmission of HIV). In the case of reoviruses, the capacity of certain strains of virus to spread from an infected mouse to its uninfected littermates is related to both the magnitude of viral growth in intestinal tissues and the amount of virus shed in the stool (268). Studies with reassortant viruses derived from low- and high-transmission reovirus strains indicated that the viral L2 gene, which encodes the core spike protein l2, is the major determinant of transmission (268). Because this gene also is a major determinant of the capacity of reoviruses to grow in intestinal tissue after peroral inoculation (Chapter 53), it may facilitate transmission by increasing viral growth and shedding.

Studies also have been made of the aerosol transmission of influenza virus between mice (462). Although a highly transmissible influenza strain (Jap/305) and a poorly transmissible one (Ao/NWS) grow to equivalent titer in the lungs, the amount of Jap/305 is considerably higher in bronchial secretions and in exhaled air. It was suggested that the neuraminidase protein might account for differences in the amount of virus released from respiratory epithelial cells into bronchial secretions and expelled air, although direct evidence is lacking.

HOST FACTORS

The outcome of viral infection of a particular host depends not only on viral factors but also on a variety of host factors. The role played by host factors in infection has been comprehensively reviewed (553), and interested readers are referred to these sources for more detailed information. The impact of host variation on the epidemiology of viral infection also is discussed in Chapter 14.

Experience with human infections has repeatedly shown that when a large population is exposed to the same viral pathogen, such as occurs, for example, during epidemics of encephalitis, the result is a range of outcomes varying from asymptomatic infection to significant and even fatal disease. The same pattern has been reproduced in inadvertent natural experiments such as the inoculation of HBV-contaminated lots of yellow fever virus vaccine into 45,000 military personnel (459). Clinical hepatitis occurred in only 2% of those vaccinated (914 cases), and of this group, only a small minority (4%) developed severe disease. The variation in outcomes appears to have been due to differences in host susceptibility, because the amount of HBV inoculated can be presumed to have been fairly uniform among those vaccinated.

In 1955 nearly 120,000 grade-school children were vaccinated with improperly inactivated lots of poliovirus type 1 prepared by Cutter Laboratories (375) (“Cutter incident”). It was subsequently estimated that about 50% of those vaccinated were susceptible to infection (the remainder had preexisting antibody), and of this group, at least 10% to 25% became infected (as estimated by the presence of minor illness or fecal excretion of virus). However, only 60 cases of paralytic poliomyelitis were ultimately documented among those vaccinated. Thus the Cutter incident again emphasizes the fact that when a large population of individuals is inoculated with approximately similar doses of virus, there is a wide range of possible outcomes that presumably depend on a variety of factors specific to the host.

The importance of host factors in determining the outcome of viral infection has been repeatedly demonstrated in animal models (51). Classic studies with VSV infection of mice clearly illustrated the importance of host factors including immune status, genetic background, age, and nutrition in determining the outcome of infection (453). The role of the host immune response and mediators such as cytokines in influencing viral infection are discussed separately (see Chapter 11 and Chapter 12).

Genetic Determinants

The genetic constitution of the host is undoubtedly one of the most important factors influencing the outcome of viral infection, and has been extensively investigated using inbred strains of mice (20). It was recognized as early as 1937, by Webster (545), that it was possible to breed selectively strains of mice that differed in their resistance to flaviviruses, including St. Louis encephalitis virus and louping ill. This observation was later extended to a number of other flaviviruses (115) including yellow fever virus and West Nile virus (195). In general, susceptibility correlated with increased levels of viral replication in tissues. In some cases resistant mice appeared to be more susceptible to the antiviral action of IFN (204), although at least one resistance gene (Flv^r) confers flavivirus-specific resistance by an IFN-independent mechanism (52).

Since the early studies with flaviviruses, genetic factors influencing susceptibility to infection have been found for herpesviruses (5), poxviruses (386), papovaviruses (53), rhabdoviruses (310), coronaviruses (48,278), retroviruses (53), myxoviruses (200), arenaviruses (393), and Borna disease virus (451). In almost all cases, the genes determining resistance to different types of virus appear to segregate independently, suggesting that a variety of mechanisms are involved rather than a single universal type of resistance or susceptibility.

Although it would seem logical that genetic resistance to infection would be linked to host genes controlling the immune

response (e.g., H-2 in the mouse), this is surprisingly infrequent. An exception to this rule may be human infection with HTLV type I in which HLA class II alleles are associated with different patterns of disease expression and may influence whether infected individuals develop adult T-cell leukemia (ATL) or HTLV-associated myelopathy (HAM) (325). Transmission of HIV-1 from infected mothers to their offspring also has been linked to the infant's MHC class II allelle (560). Recently it was observed that certain MHC class II haplotypes appear to influence the outcome of SIV infections in rhesus macaques, and that HLA alleles may influence viral load and rate of progression to AIDS after HIV infection in humans (209,452).

Susceptibility to virus infection may be associated with the presence or absence of the appropriate host-cell receptors on target cells. This mechanism may explain the susceptibility of some strains of mice to intestinal infection with MHV (48). However, some strains of mice remain resistant to MHV infection despite the presence of functional virus receptors (576), indicating that other factors also are involved. The recognition of chemokine coreceptors in HIV infection has documented one determinant of susceptibility to HIV infection. Individuals homozygous for a deletion (D32) within the chemokine receptor 5 (CCR5) gene have a markedly decreased incidence of HIV infection compared with those with a functional CCR5 gene (209) [see (94) for review]. Polymorphisms within the CCR2 allele also may influence the rate of HIV progression in infected individuals (476). However, factors other than CCR genotype are clearly involved in susceptibility to HIV (370).

Another example of a host gene influencing susceptibility to human viral infection occurs with the TNFa gene encoded within the MHC locus. Polymorphisms within the promoter for this gene have been linked to susceptibility to the development of chronic active hepatitis C infection (223).

The genetic basis for resistance of certain strains of mice to infection with influenza viruses has been extensively investigated. A2G mice are resistant to infection with influenza A. Resistance is inherited as an autosomal dominant trait that is associated with the Mx allele on mouse chromosome 16 (481). Resistance is mediated by the actions of IFN-g and IFN-b that induce a 72-kd protein (Mx protein) in cells derived from resistant mice. The Mx protein accumulates in the nucleus of cells and inhibits viral replication by inhibiting viral mRNA synthesis (131). Treatment of A2G mice with antibody to interferon renders them susceptible to infection (200). Transfection of cells derived from susceptible mice with cDNA encoding the Mx protein confers protection against influenza A infection *in vitro* (481). Mice that are susceptible to infection appear to have either deletions or nonsense mutations in the Mx gene (480).

Other Physiologic Determinants

Human viral infections provide numerous examples of the correlation between the age of the host and the severity of viral infection. Some viruses tend to produce less severe infection in infants (e.g., varicella, mumps, polio, EBV, hepatitis A), whereas others are more severe (e.g., rotaviruses, RSV, WEE, and measles). Age-related susceptibility to virus infection also occurs in a variety of experimental viral infections in mice (30,188,191,257,320,562). Conversely, the case/infection ratio is higher in older persons for a few infections such as St. Louis encephalitis (see Chapter 14).

The basis for the age dependence of viral infection is poorly understood. Some types of enhanced resistance to viral infection with increasing age may reflect the maturation of both specific and nonspecific components of the host's immune system including phagocytosis, NK activity, cell-mediated cytotoxicity, and antibody production. Organ-specific differences in the immune response may explain why some tissues in adult animals become resistant to viral infection, whereas others do not (562). Finally, age-specific restrictions may reflect changes in the maturity and state of differentiation of target cells, as has been suggested for SFV infection of neurons (396).

For Sindbis (alpha) virus encephalitis, the state of neuronal maturity appears to be an important factor in determining age-related susceptibility (388), whereas immunologic factors play little or no role in this process (191,520). It has recently been shown that changes in the Sindbis E2 glycoprotein can compensate for age-dependent neuronal restriction of replication (520). Dependence of viral infection on the state of cellular differentiation also has been described with coronaviruses (37), herpesviruses (137), polyomaviruses (29,163,322), parvoviruses (198), retroviruses (111), and arenaviruses (118).

Some viruses preferentially infect dividing cells, and differences in the organ distribution of these cells during development and maturation may influence the pattern of disease. For example, prenatal infection of cats with feline parvovirus results in destruction of the germinal cells of the cerebellum and subsequent cerebellar aplasia. Conversely, infection of adult cats involves primarily dividing bone marrow and intestinal epithelial cells and does not result in neurologic illness (250). Induction of cellular damage or injury also may trigger responses that make previously nonpermissive cells able to support viral replication (12).

Hormones also can influence the outcome of viral infection. This may provide one explanation for the frequent observation that male mice are more susceptible to a variety of viral infectious than are their female counterparts (310). A number of infections, including those with polio, hepatitis A and B, and smallpox virus are commonly more severe during pregnancy, although it is not known whether this reflects hormonal alterations or other factors. Reactivation of polyoma virus infection also occurs more commonly during pregnancy, although this typically occurs without associated clinical disease (96). Pregnant mice are more susceptible to intravaginal infection with HSV but not to intraperitoneal or intranasal infection. It has been suggested that this may be due to local hormonal effects. In experimental studies, the administration of both steroid and thyroid hormones can be shown to affect adversely the outcome of certain types of viral infection (reviewed in 350).

The nutritional state of the host also can exert a marked influence on the outcome of viral infection (79). For example, protein malnutrition dramatically exacerbates the severity of measles infection, perhaps by depressing host cellular immunity (79). A similar increase in susceptibility to coxsackievirus and flavivirus infection occurs in experimental infection of malnourished mice. It has been suggested that dietary-induced oxidative stress resulting from deficiency of vitamin E and selenium can increase the severity of certain viral infections, as exemplified by the potentiation of

coxsackievirus B3 myocarditis in selenium– and vitamin E–deprived mice (28). Deficiency of vitamin A may result in enhanced severity of respiratory syncytial virus infection in children (380). As a result of these studies, it has been suggested that supplementation with vitamins A, B, C, D, and E may provide an inexpensive form of supplemental therapy for HIV infection, although data directly supporting this approach are still lacking (150). Vitamin A also has been shown to reduce the rate and severity of complications and the duration of hospitalization in children with measles infection (241), and may facilitate healing in HSV keratitis. At the opposite extreme, hyperalimentation and the induction of hypercholesterolemia also may be associated with increased severity of viral infection (65).

Other physiologic factors also may play a role in the severity of viral infection. For example, the severity of influenza is increased in chronic smokers (260), related to the deterioration in mucociliary clearance. Preceding vigorous exercise may worsen the severity of a subsequent bout of poliomyelitis (234), although the mechanism for this has never been clearly established. Experimentally, exercise also can be shown to increase the severity of coxsackievirus infection in mice (170).

The role of host responses such as fever and inflammation in combating viral infection has been examined (435). Classic studies of myxoma infection in rabbits clearly demonstrated that increasing body temperature increased protection against infection and that decreasing temperature increased severity of infection. Similar results have been found with ectromelia and coxsackievirus infections in mice. Blocking the development of fever with drugs (e.g., salicylates) increases the mortality of vaccinia infection in rabbits and increases the shedding of influenza by ferrets (240), providing additional evidence for an antiviral effect of fever. Virus infection exacerbates host stress responses, which in turn may contribute to the pathogenesis of disease. For example, mice infected with Sindbis virus have dramatically increased levels of IFNa/b, TNFa, adrenocorticotropic hormone (ACTH), and corticosteroids. Stress also is associated with high levels of circulating catecholamines including norepinephrine (514). In one recent study, norepinephrine was shown to accelerate HIV replication through its action on protein kinase A–dependent pathways and the subsequent reduction in host levels of several antiviral cytokines including IL-10, IFN-g, TNFa, IL-1b, and IL-2, -4, and -6 (95).

The importance of host-cell enzymes in influencing the outcome of viral infection was discussed earlier in this chapter. It is well exemplified in the role played by host-cell enzymes in the pathogenesis of myxovirus infection (Chapter 46 and Chapter 47). Influenza requires cleavage of the viral hemagglutinin from an inactive precursor (HA_0) form into two disulfide-bonded subunits (HA_1 and HA_2) to become infectious. This cleavage is mediated by host-cell trypsin-like proteases, and without this cleavage, virus is avirulent. The HA proteins of mammalian influenza viruses and nonpathogenic avian viruses are susceptible to proteolytic cleavage in a limited number of cell types, whereas the HAs of many pathogenic avian influenza viruses are cleaved by proteases present in many types of host cells (276). Differences in the susceptibilities of these HAs to cleavage is due to the presence of only a single arginine residue at the cleavage site in the mammalian and nonpathogenic avian viruses, whereas the pathogenic avian viruses typically have several basic amino acids present at the cleavage site (276). Mutations in the influenza HA near the cleavage site can dramatically alter both the host range and pathogenicity of influenza viruses (121).

A host-cell protease-mediated cleavage similar to that required for influenza virus is required for activation of the fusion activity of paramyxoviruses such as Sendai virus. Virus containing an uncleaved fusion (F_0) protein does not replicate after inoculation into mice. However, virus will replicate if the F protein has previously been cleaved *in vitro* into the disulfide-bonded F_1 and F_2 subunits by trypsin. Mutant viruses whose F protein can be cleaved by chymotrypsin but not by trypsin also do not produce disease unless the protein is cleaved *in vitro* (352).

Host-cell enzymes also may play an indirect role in susceptibility to Sindbis virus infection. The envelope of the virus contains sialic acid residues derived as the virus buds from host-cell plasma membranes. If the membranes are rich in sialic acid, virus is a more potent activator of complement. Complement activation and subsequent opsonization of virus facilitate viral clearance and thus decreases viral virulence (217). In this example, the biochemical composition of host-cell membranes alters the subsequent virulence of virus.

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CHAPTER 10 Cell Transformation by Viruses

Joseph R. Nevins

GENERAL FEATURES OF VIRUS-INDUCED CELL TRANSFORMATION AND ONCOGENESIS

The science of virology includes studies directed at understanding the mechanisms by which pathogenic viruses cause disease. In addition, viruses serve as simple model systems to explore complex cellular events. Also, the ability of the oncogenic viruses to deregulate or disrupt cellular biochemical pathways has led to a better understanding of the role of these pathways in cell growth and differentiation.

Viruses that induce cell transformation and tumor formation can be found in several taxonomic groups. All RNA tumor viruses belong to the retrovirus family and in general represent the generation of viruses that have developed an oncogenic capacity by acquiring and modifying cellular genetic material. There are exceptions, such as the viruses that induce tumors by inserting next to key cellular genes and human T lymphotropic virus-I (HTLV-I), in which the oncogenic activity is due to the action of an essential viral gene. But in the majority of cases, the RNA tumor viruses can be seen as cloning events that select for cellular genes with the capacity to transform when deregulated. The replication of oncogenic retroviruses is not cytocidal, and therefore oncogenic transformation is compatible at the cellular level with the production of infectious progeny virus. However, virus production is not a prerequisite of oncogenesis (115,241).

The DNA tumor viruses come from seven different groups encompassing both nonenveloped and enveloped viruses (Table 1). Regardless of taxonomic provenance, the oncogenic potential of the DNA tumor virus is tightly linked to the genetic strategy of virus replication. In general, the synthesis of infectious progeny is linked to cell death; hence oncogenic transformation can occur only if the viral life cycle is aborted (354).

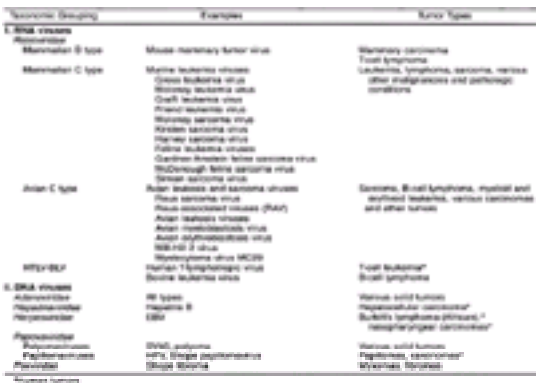

TABLE 1. *Oncogenic viruses*

Some viruses can act as carcinogens in the natural setting, whereas others reveal their oncogenic potential only in experimental systems. The potency of viruses as inducers of tumors varies widely. The most effective retroviruses can initiate tumorous growth in virtually all infected animals within a matter of days. Most other viruses, however, require a much longer latent period, and only a small fraction of the infected hosts eventually develop the virus-induced malignancy. All human tumors associated with virus infections belong in this category. The fact that tumor formation is not an inevitable consequence of virus infection reflects the multistep nature of oncogenesis, in which each step constitutes an independent and irreversible genetic change that incrementally contributes to the deregulation of cell growth. Viral infection represents one of these steps; only if the others occur in the same cell does a cancer develop. In cell culture, viruses can transform cells to a fully oncogenic phenotype. Such cells display all the altered properties of the tumor cell and are capable of initiating a cancer in a susceptible host animal. In some instances, however, the

transformation is only partial, discernible in culture by changes in morphology and growth properties but not capable of inducing a tumor *in vivo.*

Virus-induced transformation in cell culture has played a major role in the conversion of modern cancer research into a genetic science. Much of what we know today about molecular mechanisms of oncogenesis has its origin in the study of tumor viruses. These studies have led to an almost universal consensus among cancer researchers that the oncogenic phenotype results from discrete changes in key cellular control genes. Detailed mechanisms of virus-induced oncogenic transformation diverge widely, but all have important characteristics in common. These include a single infectious virus particle is sufficient for transformation: virus-induced transformation is a “single hit” process; all or part of the viral genome persists in the transformed cell, although there is often no production of infectious progeny virus; in virtually all cases of virus-induced transformation, at least part of the viral genome is expressed in the transformed cell; transformation results from corruption of normal cellular growth signals; and reversion of the transformed cellular phenotype can be achieved by specific interference with the function of viral effector molecules (e.g., by transdominant negative mutants of oncogenes).

Viral mutants that are temperature sensitive in their transforming potential can be used to compare cellular properties at the permissive temperature, when the cell is transformed, with the nonpermissive temperature, when the cell is not transformed, but all viral functions not required for the maintenance of transformation are still active. Expression vectors carrying single viral oncogenes have likewise been instrumental in determining the effects of these genes on cellular properties. The alteration of cellular parameters accompanying transformation can be divided into changes in cell growth and changes in cell morphology; the latter affect the cytoskeleton, the cell surface, and extracellular matrix (Table 2). Both kinds of change are interrelated, but not all of the changes are seen in every type of transformed cell.

Cellular Property	Oncogenic Change or Component
Growth	Increase in saturation density
	Reduced growth factor requirement
	Increased nutrient transport
	Anchorage independence of growth
Cytoskeleton	Loss of “stress fibers”
	Redistribution of microfilaments
Cell surface	Increased agglutinability by lectins
	Increased lateral mobility of transmembrane protein
	Increased production of surface proteases
	Decreased adhesion to solid substrates
Extracellular matrix	Decreased levels of fibronectins

TABLE 2. *The transformed cellular phenotype*

Alterations in cell growth affect three parameters: saturation density, growth-factor requirements, and anchorage dependence. When normal cells replicate to form a confluent layer, covering the available solid substrate surface, they stop dividing, even if ample nutrients are supplied. This saturation density of a culture is significantly higher when the same cells are transformed, reflecting an enhanced ability of the transformed cells to pile up and to grow in multiple layers (20). If a monolayer of normal cells is infected and transformed by a few individual virus particles, these changes in cellular growth behavior lead to the outgrowth of microtumors, referred to as transformed cell foci (349). Normal cells also are highly dependent for their growth on the presence of growth factors in the culture medium. Viral transformation often abrogates or reduces these growth-factor requirements.

Normal fibroblasts and epithelial cells must have a solid glass or plastic surface on which to spread before they can grow and divide. Transformation leads to a loss of this anchorage dependence. Transformed cells are able to form colonies if suspended in a semisolid gel nutrient medium. This anchorage independence allows the selection of a few transformed cells occurring within a population of mostly normal cells (219). The latter will survive in the nutrient gel for a few days, but will not grow and form colonies. In contrast to fibroblasts and epithelial cells, normal hematopoietic cells can often form colonies in semisolid medium, provided the appropriate growth factors are supplied.

As the name implies, transformation also leads to changes in cell morphology. These changes affect the cytoskeleton, the cell surface, and the extracellular matrix (354,374). Cytoskeletal changes are detectable by light-microscopic inspection and show up as a loss of “stress fibers.” At the electron-microscopic level, they are seen as a rearrangement and redistribution of microfilaments. Several proteins are involved in this reorganization. The major structural components are actin, myosin, and tropomyosin, and near the plasma membranes, a-actinin, vinculin, and talin (50,341). For instance, actin cables prominent in normal fibroblasts disappear on transformation; the actin becomes diffusely distributed in the cytoplasm. Particularly important in the process of maintenance of cell morphology through the interaction with the extracellular matrix is the action of the focal adhesion kinase, a nonreceptor tyrosine kinase that is intimately involved in the processing of signals from the cell–matrix interaction involving recruitment of the Src kinase (66,153).

Changes in cell-surface glycoproteins make transformed cells agglutinable by lectins such as wheat germ agglutinin or concanavalin A (276). Viral transformation also stimulates the production of surface bound and secreted proteases (59,62,251). These have a mitogenic effect on the cell and facilitate tumor invasion and metastasis in the animal. Finally, cells synthesize macromolecules that form an extracellular matrix important in the attachment, spreading, and movement of the cell. Major components of this matrix are fibronectin, laminin, and collagen. Matrix molecules bind to specific cell-surface receptors called integrins, heterodimeric transmembrane proteins that form a connection between the extracellular matrix and the cytoskeleton (162). Transformed cells show decreased levels of fibronectin because of the action of extracellular proteases, reduced fibronectin synthesis, and reduced expression of fibronectin receptors

(61,161,162,249).

VIRUS-INDUCED TRANSFORMATION: MODELS FOR CELL-CYCLE CONTROL AND SIGNAL-TRANSDUCTION PATHWAYS

Much of our present understanding of the cell cycle and cell growth control events derives from an elegant combination of genetics and biochemistry that the budding and fission yeast have provided (143). The ability to identify critical regulatory activities through mutation, to identify the genes that specify these activities, and then to understand the biochemical mechanisms of action of these gene products has been enormously productive. Although the study of the cell cycle in mammalian cells clearly suffers from the limited genetic analysis possible in mammalian systems, studies of the oncogenic action of the proteins encoded by the tumor viruses, particularly the DNA tumor viruses, have provided important insights into the mechanisms controlling mammalian cell growth. Such studies have helped to elucidate roles for the retinoblastoma and p53 tumor-suppressor proteins as regulators of mammalian cell growth (Fig. 1). Each of these proteins controls the progression of cells through the G_1 phase of the cell cycle. The p53 gene plays an additional role in triggering programmed cell death (apoptosis) in response to various signals. In each case, DNA tumor virus oncoproteins act to inhibit the action of these two key tumor-suppressor proteins, thereby driving an otherwise quiescent cell to enter S phase. Indeed, this is the key to understanding the link between the replication strategy of the DNA tumor viruses and their oncogenic potential. The natural host cell for the viruses is a quiescent, differentiated cell that presents an environment that is not conducive to viral DNA replication. Thus the strategy of these viruses is to alter this environment, by deregulating the normal cell-cycle control of entry to S phase to induce the genes encoding activities necessary for DNA replication. In the normal replicative infection by these viruses, this cell-cycle deregulation is of no consequence because the infected cell eventually dies. However, when the infection is in some way impaired, then the events causing deregulation of the cell cycle contribute to oncogenic transformation.

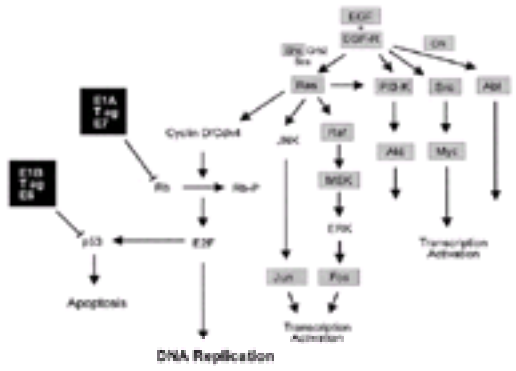

FIG. 1. Cell growth-regulatory pathways, activated by growth factor–receptor activation, which are targets of viral oncoproteins. The DNA tumor virus oncoproteins (*black boxes*) target the Rb and p53 tumor suppressors, leading to an inactivation of their function and deregulation of the G_1 cell-cycle pathway and the apoptotic response pathway. In contrast, the transforming genes found in the RNA tumor viruses (*gray boxes*) represent activated oncogenes, encoding various proteins that function in growth factor–signaling pathways.

In many respects, the virus-mediated disruption of a cell growth control pathway is analogous to the genetic analysis afforded by yeast. For instance, the inactivation of p53 function through the action of the adenovirus E1B protein is, in principle, equivalent to the isolation of a p53 mutant cell; in essence, the viral protein is a mutagen. Moreover, the ability to understand this “mutation” in the context of a viral infectious cycle provides an additional physiologic context, the evolution of a viral replication strategy, in which to view the mutation.

Likewise, the analysis of the oncogenes recovered in the RNA tumor viruses has been particularly important for the elucidation of signal-transduction pathways linking the cell surface with the regulation of the genetic apparatus in the nucleus. Unlike the DNA tumor viruses that have adopted a strategy of inactivation of key tumor-suppressor proteins, the RNA tumor viruses have identified oncogenes, the products of which function to drive cell proliferation when deregulated. Perhaps most striking is the fact that the oncogenes recovered in the RNA tumor viruses encode proteins involved in virtually every step in the pathway of signal transduction (see Fig. 1). Indeed, the study of these viral oncogenes has identified many of the participants in these complex pathways. Unlike the oncogenes of the DNA tumor viruses, the RNA tumor virus oncogenes are not essential viral genes but rather, in most cases, are cellular genes that have been acquired during viral replication and selected based on transforming function. As such, the study of these genes cannot be placed in the context of a strategy of viral replication. The presence of these genes usually renders the virus defective for replication. Nevertheless, the real utility of the RNA tumor viruses is the facility with which cellular genes encoding proteins that participate in cell growth control are trapped within the viral genome, altered in the process, and converted to a form that can radically change cell growth properties. The power of this approach is enormous, as evidenced by the sheer number of cellular genes so identified.

Without question, the study of these tumor viruses has been of immense value in elucidating events of cell growth control, providing reagents for further dissection of pathways, and in providing a perspective in which to view the action of various regulatory events. The contributions of the DNA tumor viruses and the RNA tumor viruses are distinct, each group providing unique advantages and approaches to the elucidation of mechanisms of oncogenesis. Indeed, the nature of the oncogenic activities of the two groups of viruses defines the two distinct events of oncogenesis seen in cancer cells: the RNA tumor viruses create oncogenes, whereas the DNA tumor viruses encode proteins that inactivate tumor suppressors. Like the combination of yeast genetics and biochemical analysis that has propelled the analysis of the cell cycle, the combination of these two viral oncogenic systems has provided a wealth of information and afforded an enormously valuable approach to the study of cell growth control and differentiation and the disruption of this control that

is evident during oncogenic transformation.

MECHANISMS OF CELL TRANSFORMATION BY THE RNA TUMOR VIRUSES

Since early in the 20th century, retroviruses have been known to cause cancer in animals. Lymphoid tumors in chickens were among the first diseases to be recognized as having a viral etiology; shortly thereafter, virus-induced sarcoma in fowl was discovered (106,295). But these tumors were regarded as somewhat of an oddity, having uncertain relevance for our understanding of cancer in higher animals, let alone cancer in humans. Only when mammary tumors, leukemias, and sarcomas in mice were traced to virus infections did the idea of virus-induced tumors gain some measure of acceptance and respectability as a worthwhile and relevant subject matter for cancer research (34,118,134,137,142,183,233).

The single most important development to open the field of tumor virology as a quantitative science was the advent of cell culture, making it possible to induce oncogenic transformation of individual cells inoculated with cell-free virus. These *in vitro* transformation assays, first established for Rous sarcoma virus (220,349), provided the basis for cellular and molecular studies that distinguished RNA from DNA tumor viruses and set RNA tumor viruses apart as a unique group of microbes (206,357,375). The salient features of the RNA tumor virus life cycle include reverse transcription of the single-strand RNA viral genome into double-strand DNA; integration of this DNA into the host chromosome; and expression of the integrated provirus under the control of viral transcriptional regulatory sequences. It is the process of reverse transcription that has given this viral group the name retroviruses. Integration into the host genome, together with an absence of cytocidal action, is the basis for the genetic permanence of retroviral infection. Retroviral genomes that become established in the germline are then transmitted as a set of mendelian genetic elements. Of particular importance for oncogenesis are two additional features of the retroviral growth cycle that are direct consequences of integration into the host genome: the ability to acquire and transduce cellular genetic material and the insertional activation (and occasional inactivation) of cellular genes by the integrated provirus. The analysis of retrovirus-induced transformation in cell culture and in animals led to the discovery of a set of cellular genes, called oncogenes, that function as effectors of viral carcinogenesis and play key roles in the control of cell growth and differentiation (for reviews, see refs. 31,46,282,358,370,371). Retroviruses, through their interaction with oncogenes, have provided independent and often exclusive access to these important regulatory elements of the cell. Today, retroviruses are recognized as significant natural carcinogens in several animal species: fowl, mice, cats, cattle, and monkeys. A rare but aggressive form of human leukemia has been linked to infection with a retrovirus, HTLV-I (122,232,263).

Retroviruses transform cells and induce tumors by three distinct mechanisms (Table 3). Most retroviruses effect oncogenesis through the action of cellular oncogenes. This majority comprises two well-defined groups: retroviruses that carry a cellular oncogene within their genomes, called *transducing retroviruses,* and retroviruses that lack cellular information but transform by integrating in the vicinity of a cellular oncogene, the *cis-activating retroviruses.* Almost all of the transducing retroviruses have lost some viral coding information in exchange for cellular sequences. Consequently they are defective with respect to the production of progeny virus and depend on the co-infection by a closely related helper virus for reproduction. They also are highly efficient carcinogens that transform cells in culture and cause tumors with short latent periods, often within days after infection. All of the cis-activating retroviruses retain the full complement of viral genes and multiply efficiently in solitary infection. Characteristically, they induce tumors more slowly, within weeks or months. In cell culture, these cis-activating retroviruses fail to cause oncogenic transformation. Although the establishment of infection by transducing and by cis-activating retroviruses is dependent on viral coding sequences, such viral coding information does not play an essential and direct role in the processes of oncogenesis induced by these viruses. Transducing retroviruses often have none of their basic coding domains intact, and in cis-activating retroviruses, these also become expendable once the provirus integrates near a cellular oncogene.

Virus Category	Tumor Latency Period	Efficiency of Tumor Formation	Oncogenic Effector	State of Viral Genome	Ability to Transform Cultured Cells
Transducing retroviruses	Short (days)	High (per weight of animal)	Cell-derived oncogene carried in viral genome	Viral-cellular chimera, replication defective	Yes
Cis-activating	Intermediate (wk, mo)	High to intermediate	Cellular oncogene activated in situ by provirus insertion	Intact, replication competent	No
Trans-activating	Long (mo, yr)	Very low (<5%)	Virus-coded transcriptional regulatory protein	Intact, replication competent	No

TABLE 3. *Retroviruses: Mechanisms of Oncogenicity*

Although only poorly understood, a third mechanism of retroviral oncogenesis involves the action of nonstructural viral regulatory proteins that function primarily to enhance transcription from the viral long terminal repeat (LTR). An example is seen in the HTLV-I virus, associated with the development of adult T-cell leukemia, where the viral *Tax* gene, responsible for activation of viral LTR-dependent transcription, has been implicated in oncogenesis. Such a mechanism, involving the action of a viral protein that is essential for viral replicative growth, is analogous to transformation by the DNA tumor viruses. However, in contrast to the DNA viruses in which expression of the viral gene can be observed in the tumor, HTLV-I *Tax* expression is not found in tumors. Moreover, the precise mechanism of action of *Tax,* particularly with respect to how it might contribute to cell transformation, is not understood.

Retrovirus-Mediated Oncogenesis Defines Components of Cellular Regulatory Systems That Control Growth and

Differentiation

The vast majority of the oncogenes found in the genomes of retroviruses are derived from the cell. Most cellular proto-oncogenes or c-oncogenes are conserved over long evolutionary distances. For instance, the *ras* oncogene and parts of the *jun* oncogene are found in eukaryotes ranging from yeast to humans. This evolutionary conservation of proto-oncogene sequences suggests that they are indispensable for a broad spectrum of life forms, fulfilling some fundamental function that permits little change. All known oncogenes encode protein products, referred to as *oncoproteins.* There are no cell-derived oncogenes that consist only of noncoding, regulatory sequences. Oncoproteins must, by definition, harbor the potential for inducing oncogenic cellular transformation.

The carcinogenic action of oncogenes is usually a corruption, or deregulation, of their normal physiologic function. To understand oncogene-mediated viral carcinogenesis, it is therefore important to uncover the normal functions of cellular oncogenes. In many instances, the function of the normal cellular gene counterpart has been determined, whereas for a significant fraction of oncogenes, information on physiologic roles remains general and tentative. In general, these genes are involved in the regulation of cell growth, representing components of signaling networks that receive input from outside the cell and propagate the signal to the nucleus, where the information is converted into patterns of gene activity.

A typical growth signal may arrive at the cell surface in the form of a polypeptide growth factor and bind to its specific receptor (see Fig. 1). The receptor, often an integral membrane protein with tyrosine-specific kinase activity, becomes activated by binding to the ligand. It may dimerize, leading to activation of kinase activity and autophosphorylation of specific cytoplasmic residues of the receptor molecule. The signal is further propagated by sequential and specific protein–protein interactions involving numerous participants and ending in the nucleus. The ultimate recipients of incoming signals are transcription factors that control the expression of specific sets of genes.

Of special importance in the transduction of cellular signals are two protein domains that were first recognized in the Src oncoprotein: SH2 (Src homology region 2) and SH3 (Src homology region 3). These domains occur on several protein components of signal-transduction chains; they mediate specific binding of interacting proteins. The SH2 domains bind to phosphotyrosine-containing sequences. The binding specificity is determined by the SH2 sequences in one molecule and the amino acid context of the phosphotyrosine in the other. SH3 domains have an affinity for proline-rich sequences. Proteins carrying SH2 or SH3 domains often also have an enzymatic activity, such as the Src protein tyrosine kinase, but some of these proteins are devoid of any catalytic activity; they function as linker–adaptor molecules and as regulators in binding to and controlling the activity of key enzymes (37,229,236,307).

Table 4 lists the principal retroviral oncogenes, arranged according to function. The *sis* oncogene is derived from the gene encoding the platelet-derived growth factor (PDGF) (95,369). The *int-2* oncogene, which has not been found in a retrovirus genome but which can be cis-activated by mouse mammary tumor virus (MMTV), codes for a protein related to fibroblast growth factor (FGF) (91). *ErbB, fms, kit,* and *mpl* code for altered growth-factor receptors with known ligands (97,124,245,328,337); several other oncogenes produce proteins that have the general structure of a polypeptide growth-factor receptor, but specific ligands remain to be identified. The products of the *ras* genes belong to a family of small guanosine triphosphatases (GTPases) that occupy central positions in signal transduction (38,42,128,228,330). The precise functions of *src, abl,* and other genes coding for nonreceptor tyrosine kinases are not clear; they may be active in the amplification stage of growth signals (49,70,72,262,316).

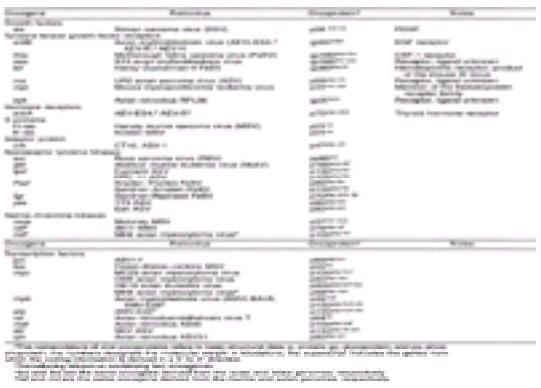

TABLE 4. *Oncogenes recovered in transducing retroviral genomes*

Another class of signaling molecules are those involved in phosphoinositol signaling, initiated by the activation of phosphatidylinositol 3-kinase (PI-3K) after growth factor–receptor activation. PI-3K activity is stimulated in many cell types in response to extracellular signals, generating a variety of phosphoinositides. One downstream target for PI-3K signaling is the Akt protein kinase, a serine/threonine kinase that is activated by phosphoinositides. A number of lines of study have implicated Akt in signaling pathways that control cell fate, in part through control of GSK-3 activity, but likely other targets as well. Although not initially identified by retroviral transduction, both PI-3K and Akt have been recovered in transforming retroviruses (7,55).

A number of oncogenes code for cytoplasmic kinases that are either tyrosine (*fps*) or serine/threonine specific (*raf, mos*). The oncogene, *crk,* is of interest because it lacks enzymatic activity but contains both SH2 and SH3 domains. Similar to the Grb-2 protein, it belongs to a class of linker–adaptor molecules that connect various catalytic steps of signal transduction (227). Finally, a large number of oncogenes encode proteins that control transcription. Notable are the Jun and Fos proteins that make up the AP-1 transcription factor, Rel, a member of the NF-kB family, and Myc, of interest because of its frequent involvement in human tumors (5,29,39,75,125,319). Other oncogenes are related to the control of development, such as *wnt*-1 and *qin. Wnt*-1 is frequently activated in MMTV-induced tumors. It shows a striking

tissue-specific distribution during mammalian embryogenesis and is homologous to the *Drosophila* gene *wingless,* which participates in the control of segmentation polarity in *Drosophila* embryos (286). Qin is a transcription factor with homology to the *Drosophila* forkhead gene product, which controls fore- and hindgut development in the early embryo (208). It is self-evident that qualitative or quantitative alterations of these growth regulatory genes carry the risk of uncontrolled growth.

Cellular Transformation by Retroviral Transduction of an Oncogene

With a combination of genetic and biochemical studies, mutants of Rous sarcoma virus (RSV) were isolated that are temperature sensitive for transformation; additional mutants were isolated that nonconditionally lost transforming capacity (222,224,355,363). The temperature-sensitive transformation mutants fail to induce or maintain oncogenic transformation at the nonpermissive temperature, while remaining fully oncogenic at the permissive temperature. Their replication is not temperature sensitive. These temperature-sensitive mutants provide unequivocal evidence for the dependence of oncogenesis on viral genetic information. The properties of these mutants also show that viral information must be expressed continuously to maintain the transformed phenotype. The nonconditionally transformation-defective RSV represents a deletion mutant that has lost about 20% of the genome. This loss does not affect the replicative properties of the virus; the deleted sequences, although essential for transformation, are therefore dispensable for virus production. This nonessential characteristic of transforming information for the viral growth cycle presaged the cellular origin of retroviral oncogenes. In temperature-sensitive and transformation-defective mutants, the same gene is affected: the two types of mutants cannot complement each other or form wild-type virus by recombination (25). The gene of RSV initially defined by these mutants is now known as the *src* gene. The deletion of *src* sequences in transformation-defective RSV also allowed the production of the first nucleic acid probe specific for *src.* A cDNA transcript of wild-type virus was generated and then was hybridized to the genome of the transformation-defective deletion mutant lacking the *src* gene. The nonhybridizing single-strand cDNA sequences of this reaction were separated from the double-strand hybrids. These single-strand sequences represented the viral oncogenic information. The cDNA *src* probe was found to hybridize to cellular DNA, providing conclusive evidence that *src* is not a viral but a cellular gene (340).

The basic characteristics discovered with the *src* gene apply to transduced retroviral oncogenes in general (32,282,358,370). All are derived from the cellular genome, and all are nonessential for virus replication. The virus serves as a vehicle of transduction, but it also exercises transcriptional control over the transduced oncogene. The cellular origin of retroviral oncogenes is of far-reaching significance because the transforming potential of a cellular oncogene can become activated independent of any viral intervention. Point mutations, gene amplification, and chromosomal rearrangements that can affect transcriptional regulation, as well as altering protein structure and function, can uncover the oncogenic potential of an otherwise normal gene. Activated oncogenes play important roles in nonviral, as well as viral, carcinogenesis; they may be the common denominator of all kinds of cancer.

In several cases, isolated temperature-sensitive as well as nonconditional mutants convincingly show that the gene is required for the initiation and maintenance of the transformed cellular phenotype (27,28,180,222,258,331,355,388). Transduced oncogenes also can be excised from their native viral genomes and inserted into an expression vector (156,157). They then confer oncogenicity on this new vector. In the transducing retrovirus, the cell-derived oncogene is the only coding information that is needed for oncogenicity. Most or all retroviral coding information may be either nonfunctional or deleted without affecting the oncogenicity.

Transduced retroviral oncogenes cause transformation within a very short time; moreover, in appropriate target cells, transformation is an inevitable consequence of infection and of expression of the oncogene. With some retroviruses (e.g., RSV), it is possible to transform all cells of a culture synchronously with one inoculum in the first round of infection. Because transformation occurs with high frequency, tumors induced by transducing retroviruses are usually polyclonal in origin. Unlike most cancers that result from a multistep transformation process, the tumors caused by transducing retroviruses probably result from single-step carcinogenesis. Some retroviruses are vectors for two oncogenes; for instance, avian erythroblastosis virus strain R carries the *erbA* and *erbB* genes, and MH2 avian carcinoma virus transduces *raf* and *myc* (33). In these cases, one of the two oncogenes is sufficient to induce transformation (*erbB* or *myc* in the earlier examples). The second enhances oncogenicity of the virus and conveys greater nutritional and growth-factor independence on the tumor cells. This is interesting in the case of MH2, which carries the *mil* gene together with *myc,* in that biochemical experiments have shown that the stability of the Myc protein is significantly extended by the action of Raf (the murine equivalent of avian Mil), leading to an enhanced accumulation of active Myc (314).

Mechanism of Acquisition of Cellular Sequences by Retroviruses

Because incorporation of an oncogene into a retroviral genome is a rare event, it can be studied only after the fact. A plausible model involves the integration of a retrovirus upstream of a potential cellular oncogene. The provirus has a deletion that includes its 3' LTR and is integrated in the same transcriptional orientation as the adjacent oncogene (67,131,343). Transcription from the 5' LTR will extend through the oncogene, creating a chimeric RNA that contains the packaging signal located near the 5' terminus of the viral genome. Complete introns are removed from this transcript, and the processed RNA can be exported in virus particles. The model also envisages infection of the same cell by a second virus that has not had a deletion and replicates complete progeny RNA as well as all structural proteins. Because retroviruses are diploid, such a cell will produce heterozygous progeny virus containing a complete viral genome and the defective chimeric RNA. In the next round of infection during reverse transcription of such a heterozygous particle, nonhomologous recombination between the two virion RNAs may take place, possibly as a result of template switching by the polymerase.

This model proposes two formal steps of recombination. The first step involves the integration of the defective provirus near the cellular oncogene, the formation of the 5' junction of cellular with viral sequences, and the generation of the packageable chimeric RNA. This recombination event occurs at the DNA level. The second recombination event takes place during reverse transcription and involves the formation of the 3' junction of cellular sequence with viral information. The resulting recombinant is a provirus with two LTRs and 5' coding information derived from the chimeric RNA; 3' sequences come from the nondefective retrovirus. Recent evidence supports this model (109,110). If chicken neuroretina

cells are infected with the avian leukemia virus RAV-1, their growth is stimulated, and the culture releases a virus that transduces the *raf* oncogene. The identifiable steps in this acquisition of a cellular gene are in accord with this model. However, other possible models, suggested by exceptional provirus structures, have not been ruled out. RNA splicing of viral and cellular sequences would be an alternative mechanism for recombination (164,225). A third possibility is that viral–cellular recombinants derive from packaged read-through transcripts of a nondefective retroviral genome and an oncogene located downstream (150). It also is possible that transducing retroviruses can be generated by more than one mechanism.

The defectiveness of transducing retroviruses is caused by the replacement of viral coding sequences with cellular oncogene sequences. There are only rare exceptions including some strains of RSV, in which the *src* oncogene is added to a complete viral genome (Fig. 2). In contrast, the MC29 virus has acquired the *c-myc* gene at the expense of viral *pol* and part of *gag.* The defective viruses go through a partial life cycle that includes reverse transcription, integration, RNA synthesis, and translation. The expression of the defective provirus leads to oncogenic transformation; however, because one or several of the essential virion proteins cannot be synthesized, no infectious progeny are made. Only if the same cell is infected by a replication-competent retrovirus that supplies the missing protein(s) in *trans* is infectious transforming virus released. This transforming progeny virus remains, however, genetically defective and helper dependent.

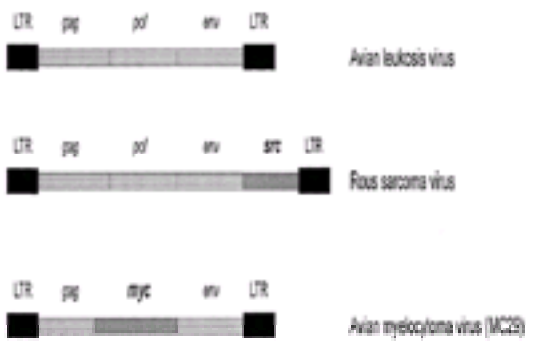

FIG. 2. Genomic structure of avian leukosis virus (ALV) and two transducing retroviruses. In addition to the long terminal repeat (LTR) sequences that provide transcriptional regulatory elements, the normal genome of ALV contains three major coding regions including *gag, pol,* and *env. Gag* encodes structural proteins of the virus, *pol* encodes enzymes involved in reverse transcription and integration, and *env* encodes the virion surface glycoproteins. In Rous sarcoma virus, the cellular *src* sequences are added to an otherwise intact retroviral genome. In contrast, in the MC29 virus, the addition of *myc* sequence is at the expense of the entire *pol* gene and parts of both *gag* and *env*.

It is interesting that cellular oncogenes have been found only in one subfamily of retroviruses, appropriately called the oncornaviruses. They have not been seen in the genomes of the lenti- and spumaviruses, although the steps of replication that are thought to favor acquisition of cellular sequences are the same for all retroviruses. A possible explanation is the greater complexity of the lenti- and spumaviral genomes. Oncoviruses efficiently replicate without virus-coded regulatory proteins, whereas lenti- and spumavirus replication depends on nonvirion proteins that are generated from the provirus by way of multiply spliced messages. Generation of these messages and their cis-acting targets would be destroyed by a cellular genetic insert, essentially inactivating the virus. Oncornavirus genomes, conversely, can accept cellular inserts without such debilitating effects (31,33,40,282,361,370).

Activation of the Transforming Potential of Oncogenes by Retroviral Transduction

Part of the process of turning an otherwise normal cellular gene into an oncogene is simply the process of viral transduction, resulting in misexpression of the gene due to control by the viral LTR in a cell when it would normally not be expressed. There are proto-oncogenes that, if sufficiently overexpressed, can transform cells. The *mos* gene is an example of this category (385). For such oncogenes, structural changes of the transduced or insertionally activated oncogene are not essential to bring forth the oncogenic potential, although they may contribute to it. Increased dosage is then the main factor in oncogenesis.

In many cases, the mere insertion of a proto-oncogene into a retroviral vector, resulting in expression at levels comparable to the viral gene, is not, in and of itself, sufficient to transform cells. Thus specific structural changes in the viral version must be responsible for eliciting the transforming potential. For example, the cellular *src* gene, overexpressed in a retrovirus vector, does not induce oncogenic transformation (163,255,320). As outlined later, specific changes in *c-src* are necessary to reveal its oncogenic trait. Another example is the ErbB oncoprotein, derived from the avian erythroblastosis virus, in which the ligand-binding domain of the EGF receptor has been deleted and replaced with a fused viral *gag* sequence (Fig. 3).

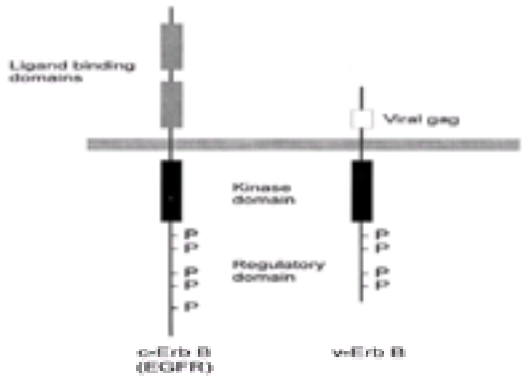

FIG. 3. Structural comparison of the cellular Erb B protein (the epidermal growth factor receptor, EGFR) with the transduced retroviral version, the v-Erb B protein of avian erythroblastosis virus H (AEV-H). The oncogenic v-Erb B lacks a regulatory phosphorylation site in the carboxyl terminal of the protein and has lost the extracellular ligand-binding domain. It functions as a constitutively activated EGFR.

Various approaches have defined oncogenically relevant structural changes for a number of oncogenes (e.g., *src, ras,* and *fms*) (282,370). These changes are always found in the oncogene proper; the viral sequences that are sometimes fused to it may facilitate oncogenicity by increasing the stability of the oncoprotein, determining the cellular localization, or enhancing efficiency of translation, but they do not participate directly in the transformation process.

It also is true that a comparison of activated oncogenes and their cellular counterparts often reveals structural and functional changes. Transduced oncogenes are usually truncated at one or both ends (for reviews, see refs. 282,370). The remaining coding sequences carry point mutations and sometimes deletions. They are often fused with viral sequences that are themselves truncated. Commonly, the viral sequences constitute the 5' end of the chimeric transcription unit, providing splice acceptor site and initiation codon. Occasionally, the oncogene sequences also may be fused to other, unrelated cellular sequences as, for instance, in viral *src* (345).

Examples of Oncogenes Identified by Retrovirus-Mediated Transduction

It is now clear that deregulation of virtually any step in the process of growth factor–mediated signal transduction can represent an oncogenic event. This can be seen from the identification of mutations in tumors but also from the recovery of oncogenic retroviruses. The isolation and analysis of transforming retroviruses has illuminated many of the details of signaling pathways critical for cell growth. Starting from the extracellular environment and working through the cell to the nucleus, examples will illustrate the process.

Sis: *Growth Factor and Autocrine-Mediated Transformation*

Sis is the oncogene of simian sarcoma virus (SSV) (for reviews, see refs. 289,377,383); it is an insert that was acquired from the genome of the woolly monkey, a New World monkey. A homologous oncogenic determinant derived from the genome of a cat was discovered in the Parodi Irgens feline sarcoma virus (FeSV). The amino acid sequence of the SSV Sis protein shows about 88% identity with the sequence of the B-chain of the human PDGF. The discovery of this homology provided the first direct and exciting link between oncogenes and cellular growth signals. The immediate cellular precursor of the *sis* gene is most likely the PDGF-B gene of the woolly monkey genome, with the deviation from the human sequence being a species difference.

Sequence analysis of the SSV genome shows that *v-sis* is expressed as a fusion product containing, at its N-terminus, sequences derived from the retroviral envelope (Env) protein, including the Env signal sequence that effects the translocation of the Env-Sis protein into the vesicles of the endoplasmic reticulum. The initial *v-sis* product is a 28-kd glycoprotein that is rapidly dimerized to a gp56 dimer and then proteolytically processed to become a p24 homodimer, similar in structure to the homodimer of the PDGF-B chain. This Sis protein is firmly associated with cellular membrane fractions, but only about 1% is secreted by the SSV-transformed cell. Primary and processed *v-sis* products show PDGF-like activity: they react with anti-PDGF antibodies, bind to PDGF receptors, induce internalization of the receptor–ligand complex, induce phosphorylation of the receptor on a tyrosine residue, and stimulate the mitogenic response of the cell. This biologic activity of the *v-sis* product suggests an autocrine transforming mechanism for SSV-infected cells. Additional support for this suggestion comes from the following observations: SSV transforms only those cells that express a PDGF receptor. Transformation appears to be dependent on the interaction of the *v-sis* product with the PDGF receptor. In addition, the nascent *v-sis* product must be translocated into the endoplasmic reticulum to be transforming. Deletion of the Env signal sequence from the Env-Sis fusion protein abolishes transforming activity. Because the v-Sis protein is functionally equivalent to PDGF, the cellular PDGF gene also can function as an oncogene by autocrine mechanisms similar to those of v-Sis: The PDGF B-chain expressed under the control of a strong retroviral promoter induces oncogenic transformation in cells expressing the PDGF receptor.

The transforming signal of Sis could be generated by binding to receptors at the cell surface, but intracytoplasmic receptor binding also may play a role in transformation. The PDGF receptor is processed along the same cellular compartments as is the v-Sis protein, and it is conceivable that the two interact before reaching the cell surface. SSV-transformed cells, exposed to antibody against Sis, sometimes assume a near-normal phenotype. This observation was interpreted to suggest that the relevant interaction between growth factor and receptor, leading to transformation, is a cell-surface event. However, if a signal sequence (KDEL) that effects retention in the endoplasmic reticulum is added to the PDGF-B chain, the transforming potential for PDGF receptor–expressing cells is not abolished. Therefore PDGF may interact with the receptor intracellularly and then become translocated to the cell surface as a complex.

ErbB *and* Fms: *Growth Factor Receptors and Constitutive Mitotic Signals*

The oncogene *erbB* has been studied in two avian retroviruses that cause erythroblastosis and fibrosarcoma in chickens: avian erythroblastosis virus strain H (AEV-H) and strain ES4 (for reviews, see refs. 146,223,359). AEV-H contains *erbB* as its only oncogene. AEV strain ES4 carries, in addition to *erbB,* the *erbA* oncogene derived from a thyroid hormone–receptor gene (373). However, *erbB* alone is both necessary and sufficient to induce the pathogenic effects associated with AEV infections, erythroblastosis, and fibrosarcoma.

The nucleotide sequence of *erbB* shows close homology to that of the epidermal growth-factor receptor (EGFR) gene (95). The *erbB* of AEV strains H and ES4 is derived from the chicken *EGFR* gene. The EGFR contains four major structural and functional domains: an amino-terminal extracellular ligand-binding domain, a transmembrane segment, a kinase domain, and a carboxyl-terminal regulatory segment with at least five tyrosine phosphorylation sites: P1 through P5 (see Fig. 3). In *erbB,* most of the growth-factor–binding domain is deleted. Furthermore, carboxyl-terminal deletions have removed P1 from the *erbB* of AEV-H and have removed P1 as well as P2 from *erbB* of AEV ES4.

The mechanism of *erbB*-induced oncogenesis probably includes elements of the same mitotic signal that is initiated by the binding of epidermal growth factor (EGF) to its receptor (see Fig. 1). Ligand binding appears to induce an allosteric change and oligomerization of the receptor (305,306 and 307). The kinase activity of EGFR is elevated, and the protein becomes autophosphorylated on tyrosine residues at its carboxyl-terminal regulatory sites. EGFR substrates of potential importance in mitotic signaling include the SHC proteins (containing an SH2 domain but no recognizable catalytic domain), GAP (GTPase activating protein), PI-3K, and phospholipase C. At least one of the phosphotyrosines in the cytoplasmic tail of EGFR is recognized and bound by the SH2 domain of a small adaptor protein called Grb2 that also binds with its SH3 domains to the nucleotide releasing factor Sos. Sos, in turn, interacts with the product of the proto-oncogene *ras,* leading to an exchange of the Ras-bound guanosine diphosphate (GDP) for GTP, thereby activating the Ras protein. From Ras the signal is propagated through a kinase cascade involving the product of the proto-oncogene *raf* and the MAP kinase (mitogen-activated protein kinase) among other kinases. Translocation of the signal into the nucleus and activation of the ultimate recipient of the signal pathway, presumably a transcription factor, are still poorly understood. The tyrosine-specific kinase activity of EGFR is essential for the mitogenic effect of EGF. Binding of EGF to the receptor is followed by internalization of the receptor–ligand complex. This process removes available EGFR from the cell surface and results in a down-regulation of EGF responsiveness. Reduced EGF responsiveness also can be caused by an activated protein kinase C, probably through phosphorylation of a threonine residue near the EGFR kinase domain.

The transforming activity of *erbB* has been analyzed with the help of several conditional and nonconditional mutants of AEV (133,244). Transforming activity of ErbB requires localization of the protein at the plasma membrane as well as truncation of the carboxyl-terminal regulatory domain. The ErbB protein is glycosylated. Intracellular forms are gp65 and gp68 proteins; the mature cell-surface molecule is a gp74. The *erbB* coding information lacks a conventional signal sequence, so targeting to the plasma membrane appears to be a function of the transmembrane domain. Temperature-sensitive mutants of AEV ES4 that prevent membrane localization at the nonpermissive temperature also are temperature sensitive for the transformed cellular phenotype. Deletions from the carboxyl-terminal end of the c-ErbB protein have shown that loss of the P1 phosphorylation site is important for both erythroblast and fibroblast transformation. As the size of the carboxyl-terminal deletion increases, there is a loss of erythroblast transformation, while still retaining fibroblast transformation.

A somewhat different picture emerges from the study of AEVs that have recently acquired *erbB* (230,273,274) (see section entitled cis-activation of oncogenes). These newer *erbB*-transducing agents carry a version of c-*erbB* that lacks the ligand-binding domain of EGFR, but some have an intact carboxyl terminus and yet can induce erythroblastosis in a highly susceptible chicken line. They do, however, fail to transform fibroblasts. A separate analysis of fibroblast transformation using mutants of AEV ES4 has shown that an intact kinase domain is essential for this biologic effect. These observations are compatible with the proposal that ErbB transforms cells as a constitutively activated EGFR. Membrane localization and kinase activity are essential.

Ligand-independent activation of receptor tyrosine kinase also plays an important role in the oncogenicity of v-*fms* (for reviews, see refs. 283,292,293,326,327). *Fms* is the oncogene of the McDonough strain of feline sarcoma virus (SM FeSV). It also has been found as the oncogenic insert in the Hardy Zuckerman 5 (HZ5) FeSV. *Fms* is derived from the gene of the receptor for the macrophage colony-stimulating factor CSF-1 (328). In SM FeSV, it replaces most of the viral *gag* and practically all of the *pol* gene, whereas the *env* gene of the virus has remained intact. *Fms* is fused in frame to the partial *gag* sequences. The crossover point in the *fms* gene is located in the 5' untranslated region of c-*fms* messenger RNA and preserves the signal sequence of c-*fms*. The 3' recombination point in *fms* is located 120 nucleotides upstream of the c-*fms* termination codon. SM-FeSV and HZ5-FeSV induce fibrosarcomas in cats but no hematopoietic malignancies, notwithstanding the normal function of CSF-1 as a hematopoietic growth factor.

The CFS-1 receptor has a structure similar to that of growth-factor receptors such as the PDGF receptor, the Kit protein and Flt (26,151,329,390). The extracellular domain consists of five immunoglobulin-like repeats; the three amino-terminally located of these repeats form the ligand-binding region. The fourth immunoglobulin-like domain functions in receptor dimerization; the point mutations contributing to the oncogenicity of v-*fms* are in or near this domain. Important cytoplasmic regions of Fms are the tyrosine kinase domain and the C-terminal tail, which contains targets for negative regulation. Activation of Fms by ligand or by mutation results in autophosphorylation of several tyrosines in the cytoplasmic domain of the receptor. These phosphotyrosines are then bound by the SH2 regions of effector molecules including PI-3 kinase, members of the Src family of proteins, and Grb2. From Grb2, propagation of the signal continues via SOS-1, Ras and Raf, and the MAPK cascade. An alternative pathway to the nucleus may involve the interferon-stimulated gene factor 3, but molecular details of this signal chain are still sketchy.

The v-*fms* genes of SM-FeSV and of HZ5-FeSV differ from c-*fms* by several point mutations leading to amino acid

substitutions and by a deletion that removes the 50 carboxyl-terminal amino acids from the protein. The deleted residues are replaced in SM- and HZ5-FeSV by 14 and 11 unrelated amino acids, respectively. The oncogenicity of the v- *fms* genes can be attributed to two point mutations in the extracellular domain and to the carboxyl-terminal deletion. The deletion removes phosphorylation sites that negatively regulate the kinase activity of the receptor, and the point mutations in the extracellular domain lead to a ligand-independent activation of the receptor.

Src *and* Abl: *Membrane-Bound Nonreceptor Tyrosine Kinases*

Src was the first retroviral oncogene discovered and defined (for reviews, see refs. 130,316). It was also the first whose origin from the cellular genome was recognized and the first for which a protein product was identified (49,340). The discovery of the *src* gene product was soon followed by observations that showed that this protein is a tyrosine-specific protein kinase, providing suggestive evidence for a regulatory function of Src and raising hopes for an immediate understanding of its oncogenic action (68,160,205). Throughout this period of rapidly expanding work on oncogenes, *src* has served as an important and pace-setting model. Yet despite this paradigmatic role and despite much additional data on structural and functional properties of cellular and viral *src,* the mechanism by which Src transforms cells is only now emerging. *Src* codes for a 60-kd phosphoprotein that is bound to cytoplasmic membranes. (This protein has been termed $pp60^{src}$; we will refer to it as Src protein, to remain consistent with the terminology applied to other oncoproteins.) The cellular Src protein contains several well-defined functional domains: an amino-terminal myristilation domain, followed by a region that diverges among related nonreceptor tyrosine kinases, an SH3 and an SH2 domain, a catalytic domain, and a C-terminal regulatory region. The viral Src protein shows the same general structure but has had a deletion in the carboxyl-terminal regulatory domain. Because c-Src expressed at levels comparable to transduced v-Src does not transform cells (163,255,320), structural differences between the cellular and viral version of the Src protein must activate oncogenic potential. The oncogenicity of Src requires binding to the plasma membrane and is correlated with increased kinase activity. The kinase activity of Src is controlled positively by phosphorylation of tyrosine 416 in the catalytic region and negatively by phosphorylation of tyrosine 527 in the C-terminal regulatory region. Tyrosine 527 is the target of Csk (C-terminal Src kinase), which appears to be a specific regulator of Src family kinases. The phosphotyrosine 527 is most likely bound intramolecularly by the SH2 domain of the Src protein. This folding of the Src protein is believed to affect the catalytic domain and to turn off kinase activity. Significantly, the viral Src lacks tyrosine 527 and is constitutively active as a kinase. Activation of Src in the presence of tyrosine 527 may involve dephosphorylation of this residue by a specific phosphatase. However, access to tyrosine 527 may depend on an allosteric change of the molecule that includes dissociation of phosphotyrosine 527 from the SH2 domain. Consequently, such allosteric interactions could target the SH2 and SH3 domains of Src that connect to other members of the signal transmission chains. Accordingly, oncogenic activation of Src can result from mutations in SH3, SH2, the kinase domain, and the C-terminal tail (69,256,348), suggesting that all of these may be important in the allosteric transitions that affect Src kinase activity.

Over the years, numerous proteins have been identified that can serve as downstream targets and substrates of Src. Most of these are probably not relevant in oncogenic transformation. Exceptions appear to be an SH2-containing protein (SHC) and focal adhesion kinase (FAK) (66,154,229,278,294). SHC is an adaptor protein that is phosphorylated by Src and related kinases and then binds to Grb-2; the SHC–Grb-2 complex then interacts with the SOS, making a nucleotide exchange factor that activates Ras. FAK is bound by the SH2 domain of Src, and this interaction appears to keep FAK in the activated state. The resulting overphosphorylation of cytoskeletal proteins may be responsible for the poor adhesion and altered shape of Src-transformed cells.

Src is highly expressed in some cell types such as blood platelets, neurons, and osteoclasts. Src-deficient mice develop osteopetrosis, the result of dysfunctional osteoclasts, but show no other major abnormalities in development and growth, possibly because the Src defect can be compensated by related kinases (336).

The product of the oncogene *abl* shares with the Src protein two important properties: attachment to the cytoplasmic face of the plasma membrane by a myristic acid residue and tyrosine-specific protein kinase activity (for reviews, see refs. 281,287,384). *Abl* was first identified as the oncogene of the Abelson murine leukemia virus (A-MuLV). More recently, it also has been found as a cell-derived oncogenic insert in the Hardy–Zuckerman HZ2 FeSV. In A-MuLV (with which most of the work on *abl* has been carried out), the *abl* gene is merged to partial *gag* sequences and is expressed as a Gag–Abl fusion protein. The *abl* insert in A-MuLV is truncated at its 5' terminus as compared with the cellular gene, but the *abl* coding sequences in p160v-abl begin downstream of the SH3 domain. Point mutations also are found in the *abl* sequences in v-abl. The v-Abl protein contains three domains: the amino-terminal Gag domain, an SH2 and SH3 domain mediating interactions with other signal-transduction proteins, and a catalytic domain that is homologous to the corresponding domain of other tyrosine kinases. Mutational analysis of the *abl* gene shows that for oncogenic transformation to occur, two properties are essential: membrane association through myristilation and elevated tyrosine kinase activity. In the v-Abl protein, the Gag domain provides the myristilation signal and therefore becomes indispensable for transformation—in contrast to the Gag domain of most other oncoproteins, which can be deleted without affecting oncogenicity. The Gag sequences of v-Abl also increase the half-life of the protein in hematopoietic cells. The c-Abl protein localizes to both the cytosol and the nucleus, and it is phosphorylated in a cell cycle–dependent manner (181,182). In some cell types (for example, primary neuronal and epithelial cells), c-Abl is primarily cytoplasmic, whereas in other cell types (for example, hematopoietic cells), the protein is largely nuclear. Recent observations suggest that it may function as a negative regulator of cell growth; transdominant negative mutants of c- *abl* disrupt the control of the cell cycle and enhance cellular susceptibility to oncogenic transformation (302). The SH2 and SH3 domains of Abl have complex regulatory functions. The SH3 domain appears to be the target of a negative control mechanism; deletion of this domain activates the oncogenic potential of Abl. The SH2 domain of Abl may have multiple functions—controlling the kinase activity of Abl by intramolecular binding to phosphotyrosine residues and interacting with signal-transduction proteins apparently in a phosphotyrosine-independent manner. Replacement of the Abl SH2 domain with a heterologous SH2 domain also may activate the oncogenic potential of this nonreceptor kinase (65,236,237). A-MuLV is an effective transforming agent for early B cells. Other hematopoietic cells can be transformed, but special conditions and additional selective pressure are required to reveal these less frequent transforming events. A-MuLV also can transform fibroblasts. The HZ2 FeSV induces only sarcomas and does not transform hematopoietic cells either *in vitro* or *in vivo.* An activated *abl* oncogene also is found in human chronic myelogenous leukemia. The leukemic cells carry a chromosomal translocation that gives rise to the marker Philadelphia chromosome and joins *abl* sequences of chromosome 9 to *bcr*

(breakpoint cluster region) sequences of chromosome 22 (149,333). *Bcr* and *abl* sequences are found in a single, fused transcript in leukemic cells. Although the junction point between *bcr* and *abl* varies in different patients, the regularity with which this translocation occurs in chronic myelogenous leukemia strongly suggests that the Bcr-Abl protein conveys a growth advantage to the leukemic cells.

Ras: *Growth-Regulatory GTPase*

Mammalian cells contain three *ras* genes: H-*ras*, K-*ras*, and N-*ras* (for reviews, see refs. 38,44,45,204). All three are composed of four coding exons that contain information for a protein of about 190 amino acids, or 21 kd, plus an upstream noncoding exon. The amino-terminal halves of these cellular p21 proteins are virtually identical. About two thirds of the carboxyl-terminal halves are also closely related, and only the most 25-carboxyl-terminal amino acids show divergence. Two of the mammalian *ras* genes—H-*ras* and K-*ras*—have been found transduced by retroviruses; N-*ras* has not been detected in a naturally occurring virus. More than half a dozen independently isolated retroviruses that transduce a *ras* gene; the best studied are Harvey and Kirsten murine sarcoma viruses (H-MuSV and K-MuSV). Their *ras* inserts are derived from the rat genome and carry sequences of an endogenous rat retrovirus VL30.

Ras proteins are GTPases that act as nodal points in cellular growth control, receiving inputs from several signal-transmission chains and propagating the signal through kinase cascades toward the nucleus. Ras proteins belong to a superfamily of low-molecular-weight GTPases that also encompasses the Rho proteins, important in the control of the cytoskeleton, and the Rab proteins, which function in membrane protein trafficking. Other related proteins with GTPase activity and homology to Ras are the heterotrimeric G proteins involved in signal transduction and the elongation and initiation factors of the protein-synthesizing machinery.

Ras proteins must be anchored in cellular membranes for proper functioning. They are posttranslationally modified by covalent attachment of prenyl groups to cysteines near their C-terminus, providing a lipophilic tail. Ras proteins can be considered as binary switches that are either on (bound to GTP) or off (bound to GDP). They are regulated by at least three types of proteins; the guanine nucleotide exchange factors (GEFs), the GTPase-activating proteins (GAPs), and guanine nucleotide dissociation inhibitors (GDIs) (38). GEF proteins activate Ras through exchange of Ras-bound GDP for GTP. An example of a GEF is the SOS protein, which is instrumental in transmitting growth signals from tyrosine kinases to Ras. Gap proteins have an attenuating effect on Ras. They stimulate the conversion of active GTP Ras to the inactive GDP Ras. They are represented by p120 GAP and by NF1 (neurofibromin). The GDI proteins are the least understood regulators of Ras. They bind to GDP Ras, thereby interfering with the action of GEF activators and possibly also with GAP-like attenuators.

Oncogenicity of Ras is greatly elevated by specific point mutations, notably in codons 12, 13, and 61. These mutations inhibit the binding of GAP proteins to activated Ras and thus prevent the stimulation of Ras GTPase activity. The mutant Ras proteins remain bound to GTP and are constitutively active, contributing to oncogenic transformation. Retroviral Ras proteins carry these activating mutations. Additionally, the transforming potential of Ras can be elicited by changes in upstream nontranslated regions; the endogenous VL30 sequences also can have an enhancing effect on transformation.

Activated Ras with a mutation in one of the critical codons also is found in human cancers, notably in carcinomas of the bladder, colon, and lung. A significant minority of the primary tumors in these sites contain the mutated oncogene. Normal tissue surrounding the tumor is free of the mutation. The possibility that activated Ras plays an important role in human tumor development also is supported by animal studies. Virtually all tumors induced by certain chemical carcinogens contain an activated *ras* gene (19,342). Examples are mammary carcinomas in the rat induced by nitrosomethylurea and skin carcinomas in the mouse caused by dimethylbenzanthrazene.

Mos *and* Raf: *Cytoplasmic Serine/Threonine Kinases*

The *mos* oncogene was originally found in Moloney sarcoma virus as a cell-derived insert into the retroviral *env* gene (for reviews, see refs. 40,318,394). Moloney sarcoma virus induces rhabdomyosarcomas in mice and transforms cultured murine fibroblasts. The viral Mos protein is an Env–Mos fusion product; it has a molecular mass of 37 kd, and its first five codons are derived from the 5' end of the retroviral *env* gene. The v-Mos protein is localized in the soluble portion of the cytoplasm. Several strains of the Moloney murine sarcoma virus differ by point mutations and deletions. The HT1 strain, which is probably closest to the original isolate, carries a *mos* insert that is identical to c-*mos* if amino acid sequences are compared. Another *mos*-containing retrovirus, myeloproliferative sarcoma virus, has a frameshift mutation and appears to initiate *mos* translation from an internal site downstream of this mutation.

The cellular *mos* gene can transform cells in culture with virtually the same efficiency as the viral gene, suggesting that the transforming potential of this gene can be activated by deregulated expression of the cellular product (385). *Mos* shows homology to cyclic adenosine monophosphate (AMP)-dependent protein kinase and to the Src family of tyrosine kinases. The Mos protein is a serine–threonine kinase; the cellular *mos* is an intronless gene that is expressed at very low levels (less than one copy of *mos* RNA per cell) in most tissues. Notable exceptions are germ cells, where *mos* is highly expressed (132,269,299). Recent investigations show that the cellular Mos protein is a regulator of meiotic maturation. It is required for meiosis, and as a component of the cytostatic factor (CSF), it is essential for the normal arrest of meiosis at metaphase II. Mos also stabilizes maturation-promoting factor (MPF p34 ^{cdc2}) in meiosis. It further associates with and phosphorylates tubulin and can activate MAP kinase. One hypothesis of *mos*-induced oncogenic transformation suggests that Mos imposes a mitotic program onto the interphase cell and thus contributes to reduced adhesiveness, cell rounding, and cytoskeletal reorganization, all part of the transformed phenotype (394).

The *raf* oncogene has been found to be the sole oncogenic determinant in murine sarcoma virus 3611 but has also been isolated as a second oncogene, together with *myc,* from the genome of the MH2 avian carcinoma virus (for reviews, see refs. 147,277). This avian homolog is still sometimes referred to as *mil.* In the mammalian genome, other *raf*-related genes have been identified, among them A-*raf* and B-*raf*. Cellular *raf* sequences inserted into a retroviral vector induce oncogenic transformation. These sequences are truncated at the 5' end, a modification that is probably instrumental in

activating the oncogenic potential. In cells infected with the 3611 murine sarcoma virus, Raf is expressed as a 79-kd Gag–Raf fusion protein. This protein is myristilated in its Gag domain and is phosphorylated on serines and threonines. Because the murine Gag protein also occurs in a glycosylated form, there is a corresponding Gag–Raf glycoprotein in cells transformed by virus 3611. In contrast to the mammalian *raf* genes, the avian homolog *mil* does not induce transformation on its own. The *mil* insert of the MH2 avian carcinoma virus also is expressed as a fusion product with Gag. The 100-kd Gag–Mil and the 70-kd Gag–Raf proteins are both cytoplasmic serine–threonine kinases, and they show some homology to the Src family of tyrosine kinases. Raf is emerging as a second important nodal point besides Ras for the propagation of growth signals. Raf is distal to Ras but upstream of the MAP kinase cascade. Activated Ras binds to the amino terminal regulatory domain of Raf, and there is a suggested association of Raf with MAP kinase kinase.

Fos, Jun, *and* Myc: *Transcriptional Regulatory Proteins*

Finally, a large number of oncogenic retroviruses have been recovered that encode nuclear proteins that act as transcription factors. In this category belong *myc, myb, fos, rel, ski, jun, erbA, maf,* and *qin* (6,76,77,80,102,108,152,253,267,280,339,359,365).

Myc is the oncogenic determinant in avian retroviruses MC29, CMII, MH2, and OK10 (108). Besides c-*myc*, the human genome contains several other *myc*-related genes including N-*myc* and L-*myc*: the latter two have not been found as oncogenes in retroviruses. *Myc* plays an important role in a number of human cancers. In all Burkitt lymphomas, *myc* is translocated into the vicinity of an immunoglobulin gene and, unlike the nontranslocated allele, is actively expressed in B cells (139). An understanding of the Myc protein has been advanced with the discovery of a small Myc dimerization partner, the Max protein (36,216,266) and a variety of other interacting proteins that determine the function of Myc and related complexes (Fig. 4). Max associates with Myc by a helix–loop–helix and a leucine zipper motif. Genetic experiments show that dimerization with Max is required, not only for Myc DNA binding (consensus sequence CACGTG) but also for oncogenic transformation and for Myc-induced apoptosis (3,4). Through the use of protein-interaction assays, an additional Max-interacting partner was termed the Mad protein (11). Further studies have shown that, depending on the growth and differentiation state of a cell, there exists an equilibrium between homodimers of Max and heterodimers of Myc–Max or Mad–Max (10). Whereas the Myc–Max heterodimer is associated with transcription activation, no unique activity has been attributed to the Max–Max homodimer. In contrast, various experiments have now shown that the Mad–Max heterodimer functions as a transcription repressor through the recruitment of the Sin 3 protein and a histone deacetylase (12,195).

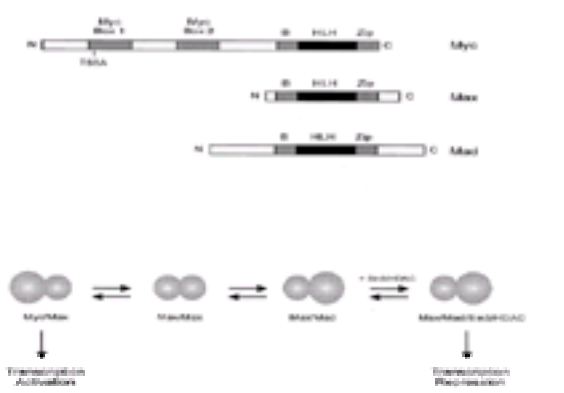

FIG. 4. Comparison of the Myc protein, the dimerization partner Max, and the alternate Max partner Mad. Myc and Max, as well as Max and Mad, associate through the leucine zipper helix–loop–helix domains. The heterodimers bind to the sequence CACGTG. Association with Max is necessary for transcriptional regulation and for transformation by Myc. Myc box-1 and -2, and the acidic region represent sequences conserved in various *myc* genes. The threonine 58 residue (corresponding to threonine 61 in avian Myc), which is a site for phosphorylation, is the most frequent site of mutation in oncogenic forms of *myc* recovered in viruses and is often found in Burkitt's lymphomas (1). Shown is a schematic depiction of the relationship between the various interacting proteins, changing the function from one of transcription activation (Myc–Max) to one of transcription repression (Mad–Max).

Myb is the oncogene of avian myeloblastosis virus AMV BAI-1 and of the avian leukemia virus E26; it binds to a specific DNA sequence, and the viral oncogenic version of the gene may transform by interference with normal transcriptional control (29,93,217,324). The cellular *myb* gene is preferentially expressed in hematopoietic tissues, controls the expression of myeloid-specific genes, and is required for normal fetal hematopoiesis (235,272,280,312).

The *rel* oncogene of avian reticular endotheliosis virus belongs to the NF-kB transcription factor family. The viral version of this gene corrupts normal transcriptional regulation by NF-kB (210,285). The *ets* oncogene is the second oncogene of E26 AMV. It is a member of an important transcription factor family (167,253,368) and can be activated by insertional mutagenesis as well as by transduction (21). *Fos* is the oncogene of the FBJ and FBR murine osteosarcoma viruses. It is a component of the cellular AP-1 transcription factor complex, and like other transcription factor oncoproteins, it transforms by altering normal transcriptional controls (77,113). The product of the *maf* oncogene is structurally and functionally related to the AP-1 family of transcription factors. *Maf* was originally found in avian retrovirus AS42; it can interact with AP-1 proteins (178). *Qin,* the oncogene of avian sarcoma virus 31, codes for a transcription factor of the fork head/HNF-3 family. Fork-head proteins are important regulators of embryonal development (208).

Jun is the cell-derived insert in the genome of avian sarcoma virus 17 (6,77,365). In cells transformed by this virus, *jun* is expressed as a 65-kd Gag–Jun fusion protein concentrated in the nucleus. Together with Fos, the cellular Jun protein is a component of the AP-1 transcription factor complex (5,39). It belongs to a multigene family together with the related

genes *junB* and *junD* (154,296). Areas of homology among these genes roughly correspond to functional domains: a carboxyl-terminal leucine zipper dimerization domain, an adjacent basic region that makes contact with DNA, and an amino-terminal domain required for transcriptional activation (77,196,248). Jun proteins can form homodimers, but under physiologic conditions are usually found in heterodimers with Fos family proteins, with proteins of the ATF transcription factor family, and with other leucine zipper proteins. In the heterodimers, the leucine zipper of Jun joins with that of the partner protein in a coiled structure (64,126,138,187,310,356). The DNA contact domain extends from the leucine zipper, forming a fork that interacts with the major groove for the DNA double helix. The principal functions of Jun show a hierarchic dependence: dimerization is a prerequisite for DNA binding and transactivation, and DNA binding is required for transactivation. All three domains, for dimerization, DNA binding, and transactivation, are needed in oncogenic transformation.

Transformation by Jun reflects a disturbance of normal transcriptional controls (364), but the molecular details of this process are still unknown. Viral Jun differs from cellular Jun by a 27-amino-acid deletion in the transactivation domain, by two amino acid substitutions affecting important regulatory sites of the molecule, and by the loss of the 3' untranslated region, which appears to be responsible for the short half-life of *jun* messenger RNA (Fig. 5). All these mutations contribute to the oncogenic potential of *jun. Jun* acts as a single oncogene in transforming avian cells, and there appears to be no requirement for auxiliary genetic changes. In mammalian cells, *jun* transforms only in cooperation with a second oncogene such as activated *ras* or another growth-stimulatory gene. Transactivation by the Jun protein is regulated through phosphorylation. Phosphorylation of amino-terminal serines and threonines increases transactivation potential, and phosphorylation of a serine and a DNA-binding domain inhibits transactivation. Transformation depends on the presence of the transactivation domain, but it is not correlated with a general increase in AP-1–dependent transcription that is controlled by AP-1 consensus sequences. The transforming potential of Jun reflects more subtle changes that may involve altered preference for heterodimer partners, for DNA consensus sequences, and hence for target genes. Although numerous target genes responsive to AP-1 are known, the target genes important in the oncogenic action of Jun have not been identified. The possibility that the oncogenic change in Jun reflects a loss of function rather than a gain of function also has not been ruled out.

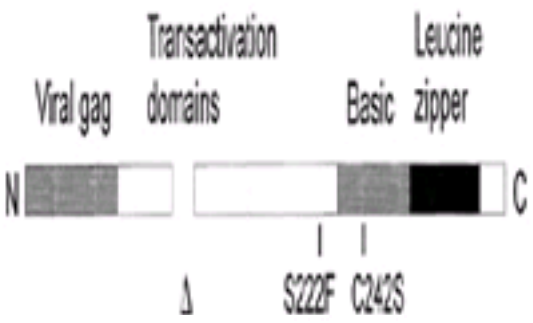

FIG. 5. Alterations in the *jun* sequences recovered in avian sarcoma virus 17. The viral *jun* differs from the cellular progenitor found in chicken cells in the following ways: v- *jun* has fused at its amino terminal partial Gag sequences derived from an avian retrovirus. It has a 27-amino-acid deletion affecting the transactivation domains. Two amino acid substitutions interfere with normal posttranslational regulation, and an absence of 3' untranslated sequences in the message contributes to the greater stability of the v-Jun mRNA. With the exception of the Gag sequences, all changes seen in viral Jun contribute to its enhanced transforming activity as compared with cellular Jun.

An entirely different regulator of transcription is the product of the oncogene *erbA* (for reviews, see refs. 80,133). *ErbA* occurs as the second oncogene in AEV strains ES4 and R, which also carry the *erbB* oncogene. *ErbB* alone is sufficient for the induction of erythroblastosis and sarcoma. In cultured erythroblasts, expression of the v- *erbB* oncogene induces a high rate of self-renewal without completely blocking a low incidence of spontaneous differentiation to erythrocytes. V-*erbA* does not induce proliferation in erythroblasts, but it blocks spontaneous differentiation and allows cell growth in a wide range of Na^+ concentrations and pH. Erythroblasts transformed by *erbB* and *erbA* together are nutritionally less fastidious than are erythroblasts transformed by *erbB* alone. Through its inhibitory effect on differentiation and by inducing nutritional vigor, *erbA* augments the oncogenicity of *erbB. ErbA* is the homolog of the thyroid hormone receptor (300,372,373,397). It belongs to a protein family that also includes receptors for glucocorticoid hormones, retinoic acid, and estrogen. These receptor proteins are related in structure and mechanism of action. They contain a ligand-binding domain, a transactivation domain, and a DNA-binding domain with two zinc fingers. They bind to similar, but not identical palindromic DNA consensus sequences. Initially the homology of v- *erbA* to the gene encoding thyroid hormone receptor led to the suggestion that the oncogenic effect of the *erbA* is due to its interference with the thyroid receptor function. Indeed, the v-ErbA protein can act as a repressor of thyroid hormone–dependent transcription, functioning as a transdominant negative mutant. However, recent investigations have shown that the oncogenic potential of v- *erbA* is primarily due to its inhibitory effect of the retinoic acid receptor rather than the thyroid hormone receptor (309,322). The transformation-related target genes that are retinoic acid (and possibly thyroid hormone) dependent and affected by the v-*erbA* still remains to be determined.

Oncogene Activation by Retrovirus Insertion

Many retroviruses that do not carry an oncogene are nevertheless capable of inducing tumors in animals (for reviews, see refs. 192,193,247). The kinds of tumor seen with these viruses are similar to the ones caused by transducing retroviruses. They include sarcomas, various forms of leukemia, and carcinomas. However, a uniform difference with respect to the tumors induced by transduction is the latent period of tumor formation. Nondefective retroviruses lacking a transduced oncogene induce tumors only after a long latent period of several weeks to several months. None of these viruses induces oncogenic transformation in cell culture. These differences largely reflect the mechanism by which

viruses induce transformation. As discussed in detail later, transformation results from the cis-activation of a proto-oncogene by the insertion of the retrovirus. Thus the long latency *in vivo,* as well as the absence of transformation *in vitro,* is due to the infrequency with which an insertion occurs adjacent to a potential oncogene.

The tumors caused by these nontransducing retroviruses have several common properties that are important in understanding their origin. They all contain an integrated provirus. The proviral sequences are found in the same chromosomal site in all cells of a given tumor; hence each tumor is monoclonal, having originated from a single transformed cell. Although infection is initiated with a nondefective virus that undergoes multiple rounds of replication, the proviruses seen in tumors are usually defective, containing only part of the viral genome. The part that is always present and conserved is at least one LTR, whereas a portion or all of the viral coding sequences may be missing. This observation suggests that viral coding sequences are not required for the maintenance of the transformed state. Most important in the genesis of these tumors is the fact that viral sequences are integrated at preferred sites of the cellular genomes. In several tumor systems, these sites are located in the immediate vicinity of a cellular oncogene that has also been found in transducing retroviruses. As a result of retroviral integration nearby, the transcription of the cellular oncogene is elevated in the tumor cells. This is referred to as insertional activation, or cis-activation, of an oncogene.

The active replication of a nontransducing competent retrovirus in a susceptible host and the presumably accidental insertional mutation or cis-activation of a cellular oncogene are not the only factors important in tumorigenesis by nontransducing retroviruses.

A systematic genetic analysis of the nontransducing retrovirus genomes for oncogenic potential has revealed a surprising multiplicity of important sequences and has demonstrated the complexity of their interactions. Among both avian and murine retroviruses, there are strains of high and low leukemogenicity. Recombinants between these strains, as well as mutants of these strains, show that the LTR is a major determinant (but not the sole one) of oncogenicity (48,89,290,291). The coding information for *gag, pol,* and *env* can be involved to varying degrees as well. In some murine leukemia viruses (e.g., Moloney leukemia virus), viral replication during the latent period of the disease gives rise to recombinants with endogenous retroviral information in the *env* gene (67,142). These recombinant “MCF” viruses multiply more actively in hematopoietic target tissue than does parental virus, and this increased replication is an important factor in leukemogenicity. The multiplicity of contributing factors suggests that every step of virus replication—from efficient entry into specific target cells to active transcription of the provirus and maturation of highly infectious progeny—is important in increasing the probability of cis-activation.

A number of questions on the mechanism of cis-activation remain to be answered. For instance, although the insertional activation of the cellular oncogene is a rare event, it does not account for the fixed minimal duration of the latent period, suggesting that some sort of threshold condition has to be met. The monoclonality of the tumors indicates that the transformation to the oncogenic phenotype is rare. It is compatible with the idea that two or more independent events are necessary to convey tumorigenicity to the infected cell. The possibility of more than one necessary event also is suggested by the fact that retroviruses that cause cancer through cis-activation of a cellular oncogene have not been found to induce transformation in cell culture. If a single cis-activating event were sufficient, it should be detectable *in vitro.* Another puzzling aspect in cis-activation is the tissue tropism of oncogenesis. It is not clear why RAV activates the *myc* gene in some chicken lines and the *erbB* gene in others or why some mouse strains develop B-cell and others T-cell or myeloid lymphomas in response to infection with the same murine leukemia virus.

Two principal mechanisms of cis-activation have been identified: promoter insertion and enhancer insertion. In promoter insertion, a chimeric mRNA is generated that combines R and U5 sequences of the viral LTR with sequences of the cellular oncogene. If the transcripts originate in the 5' LTR of the provirus, they also may contain partial viral coding sequences. However, transcription starts in the 3' LTR are more common; in these cases, the provirus has usually had extensive deletions that extend into, and may include, the 5' LTR. In fact, the activation of the 3' LTR promoter may depend on the inactivation of the 5' promoter. The preferred integration sites of promoter insertions may lie within the cellular oncogene and either truncate the coding sequences or effectively remove noncoding domains that contain negative regulatory elements. For enhancer insertion, the provirus need not be integrated in the transcriptional orientation of the cellular oncogene, and its position may also be downstream of the cellular gene. In enhancer insertion, the oncogene transcripts do not contain viral sequences. To illustrate the essential aspects of insertional oncogenesis, selected representative examples will be discussed.

Myc *and* ErbB *Are Activated by Insertion of Avian Leukosis Virus*

The Rous-associated viruses types 1 and 2 (RAV-1 and RAV-2) are replication-competent avian retroviruses that do not carry a cell-derived oncogene. In young chickens, these avian leukosis viruses induce B-cell tumors originating in the bursa of Fabricius. The tumors arise after a prolonged latent period, they are monoclonal with respect to proviral integration site, and they overexpress c-*myc*. Most contain a defective provirus that has suffered a 5' deletion. The provirus is integrated in the vicinity of the cellular *myc* gene (147,258). The exact integration site varies from tumor to tumor. Most commonly, the integrations are located between exon 1 (which is noncoding) and exon 2 of c-*myc* or within exon 1 (Fig. 6). The cis-activation of c-*myc* then occurs by promoter insertion, generating a chimeric viral–cellular fusion transcript that starts in the 3' viral LTR. In some tumors, the provirus is found downstream of *myc,* and it may also be integrated in the opposite transcriptional orientation. These situations suggest that viral enhancer activity elevates *myc* transcription; the *myc* transcripts do not contain viral sequences. Although provirus insertion often leads to a truncation of the *myc* gene, the deleted sequences are noncoding. Their removal or inactivation seems to contribute to the elevated expression of c-*myc*. The Myc protein in avian B-cell lymphomas has been found to contain point mutations, but there is no evidence that these are necessary for transforming activity. The part played by *myc* in avian lymphomagenesis requires only overexpression of the normal cellular gene. Insertional activation of c-*myc* also is seen in murine and in feline leukemias induced by nontransducing, replication-competent retroviruses.

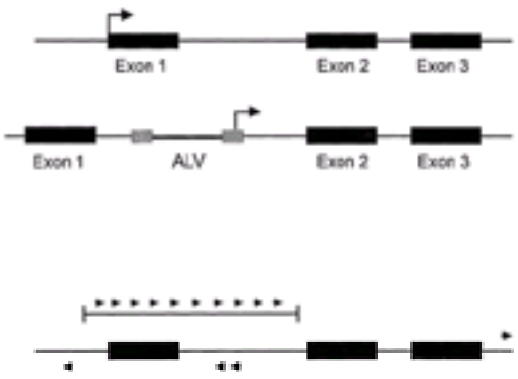

FIG. 6. Integration sites (*black triangles*) in avian B-cell lymphoma. The majority of integrations (*bracketed region*) are clustered in the noncoding exon 1 of the cellular *myc* gene and in intron 1. Most of the proviruses are integrated in the orientation of myc transcription (*triangles pointing to the right*), but some are found in opposite orientation.

In certain lines of chicken, notably those with the B5 MHC haplotype, RAV-1 induces erythroblastosis instead of lymphoma (119,230,273). These tumors also appear after a long latent period of almost 3 months, and they are monoclonal. Unlike the lymphomas, however, they contain an intact, nondefective provirus, integrated near the cellular *erbB* gene, and *erbB* expression is elevated in the tumor cells. The provirus integration sites in *erbB* are tightly clustered in a region that corresponds to the carboxyl terminus of the ligand-binding domain of the EGF receptor. Primary read-through transcripts are synthesized containing the entire proviral sequences and the truncated c-*erbB* sequences. RNA processing then creates an mRNA of uniform size with R, U5, partial *gag, env,* and *erbB* sequences. This overexpressed *erbB* mRNA codes for an EGF receptor that has deleted the ligand-binding domain at the same site as transduced viral *erbB.* The mechanism of transformation is probably similar to that of v-*erbB*: a constitutive mitogenic signal originating from the truncated EGF receptor. However, unlike v-*erbB*, the cis-activated gene contains the complete 3' terminus, and the resulting protein retains the regulatory tyrosine phosphorylation sites of the EGF receptor. The absence of some of these sites in the *erbB* insert of AEV is therefore not the only factor determining increased tyrosine kinase and oncogenic activity. In RAV-1–induced erythroblastosis, the activated *erbB* gene also is incorporated into the viral genome with comparatively high frequency. This results in the generation of new *erbB*-transducing viruses that now induce polyclonal erythroblastosis within a short latent period.

Activation of Wnt-*1 by Mouse Mammary Tumor Virus Integration*

Another retrovirus that does not carry an oncogene in its genome and induces tumors by insertional activation of a cellular oncogene is the MMTV. MMTV has several preferred integration sites in tumor target cells. Those most frequently used are close to three cellular genes *int*-1, *int*-2, and *int*-3 (for reviews, see refs. 193,246,247,259). These genes are located on different chromosomes; furthermore, despite the identical names, there is no sequence relationship between the three. The integrated MMTV provirus enhances transcription of the adjacent cellular *int* locus in the mammary tumors, and this increased expression probably plays a key role in tumor development. Although none of the *int* sequences has been found as transduced oncogenes in naturally occurring retroviruses, there are good reasons to believe that these cellular loci represent oncogenes.

Int-1, now referred to as *wnt*-1 (an abbreviation derived from the name of the homologous *Drosophila* segment polarity gene *wingless* and *int*), belongs to a gene family that fulfills important functions in pattern formation during embryonal development from *Drosophila* to mammals (193,286). *Wnt*-1 codes for a glycoprotein that is secreted but remains closely associated with the cell of origin. It is probably a component of a short-range developmental signal. Ectopic expression of *wnt*-1 in mammary cell lines induces at least partial oncogenic transformation (47).

Int-2 codes for a protein that belongs to the FGF family (91). It also appears to be a secreted protein and may act as a growth factor. Another member of this family, *hst,* is occasionally activated in mammary tumors. *Hst* also has been isolated from human tumors (Kaposi's sarcoma and stomach cancer) as a transforming oncogene (87,344). The *int*-3 oncogene also codes for a protein with presumed developmental function and belongs to the notch family of developmental regulators (288).

Transformation by Molecular Mimicry and Insertional Mutagenesis: Friend Leukemia Virus

The Friend leukemia virus occupies a unique position among retroviruses with respect to mechanism of oncogenic transformation. Key factors in this process are an altered viral envelope protein and preferred sites of provirus integration that lead to cis-activation. However, Friend virus also inactivates the cellular p53 tumor-suppressor gene, and some of this inactivation occurs by insertional mutagenesis. Friend leukemia virus causes erythroid malignancies that result from a two-step transformation (for reviews, see refs. 21,173). Virus stocks contain two agents: a replication-competent retrovirus and a replication-defective virus called spleen focus-forming virus (SFFV), with the former acting as a helper virus for the latter. Infection of mice with SFFV and its helper leads first to a polyclonal proliferation of infected erythroblasts, causing pronounced splenomegaly. The replicating erythroblasts are neither immortal nor transplantable in syngeneic animals. The pool of dividing cells is maintained and increased through recruitment of new target erythroblasts by replicating virus. The second step of transformation in Friend disease occurs in only one or a few cells in an infected animal. It results in immortality and tumorigenicity and is due to a discrete genetic change.

A molecular analysis of this two-step leukemogenesis has revealed complex interactions between SFFV, helper virus, and target cells and tissues. Helper-free stocks of SFFV have been produced in packaging cell lines. These cell lines contain helper virus information necessary to produce infectious SFFV, but cannot synthesize infectious helper virus. Helper-free SFFV infects cells but does not produce infectious progeny. Its pathogenicity appears to be restricted to the

first stage of Friend disease. Mutant analysis of the SFFV genome indicates that this initial, polyclonal proliferation of erythroblasts is caused by the expression of the altered Env protein of SFFV, gp55. Gp55 is the only gene product of SFFV, and it acts as a mitogen for erythroblasts, by binding to and activating the erythropoietin receptor (78,207). As we shall see later in the discussion of the DNA tumor viruses, this is not a unique situation—in many respects, the suspected action of the SFFV gp55 protein is analogous to the action of the E5 product of bovine papillomaviruses (see Fig. 11). The E5 protein, a transmembrane protein that interacts with the PDGF receptor, has been to shown to activate the PDGF receptor–mediated signaling by causing a ligand-independent dimerization of the receptor.

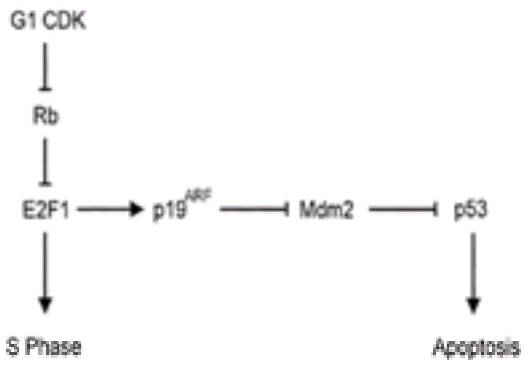

FIG. 11. The E2F1/ARF/Mdm2/p53 pathway. The accumulation of E2F1 protein, after inactivation of Rb by G_1 cyclin–dependent kinase action, leads to the activation of transcription of the $p19^{ARF}$ gene. The ARF protein has been shown to interfere with the ability of Mdm2 to target p53 for ubiquitin-mediated degradation. Thus as a result of activation of the ARF pathway, p53 levels increase due to reduced turnover.

The second step in Friend virus–induced leukemia is due to a rare event that leads to the development of a monoclonal tumor consisting of transplantable, immortal, transformed erythroblasts. Second-stage Friend virus–induced malignancies have two important properties in common: the provirus is integrated in either one of two preferred loci, referred to as *fli*-1 and *spi*-1, and a high percentage of Friend virus–induced leukemias also show inactivation of p53. The cellular *fli*-1 and *spi*-1 genes code for members of the Ets family of transcription factors; the Fli-1 and Spi-1 proteins are functionally distinct; their activation is associated with different variants of Friend virus disease. Some of the p53 inactivation events are the result of proviral integration in one of the p53 alleles with consequent loss of wild-type function and reduction to inactivated homozygous state (21,169). The activation of Ets-related transcription factors and inactivation of p53 represent rare genetic changes probably contingent on continuous helper virus–dependent replication that increases the probability for critical integration events.

Oncogenesis Mediated by Essential Retroviral Proteins

Although the vast majority of oncogenic retroviruses mediate transformation by either transducing an oncogene or activating a gene after insertion, there is at least one example whereby oncogenesis is the consequence of action of an essential viral gene product. Specifically, the *tax* gene product of HTLV-I has been implicated in the development of adult T-cell leukemia (ATL) (for review, see refs. 122,240,311). The fundamental features of HTLV-I in ATL are as follows: all cases of ATL harbor the HTLV-I provirus; the provirus is found in the same chromosomal site in all cells of a given case of ATL, and hence ATL is a monoclonal disease; integration of HTLV-I does not occur in preferred sites of the host genome; HTLV-I does not carry a cell-derived oncogene within its genome; the HTLV-I provirus is not expressed in ATL cells; and if ATL cells are placed in culture, viral gene expression is turned on.

The presence of HTLV-I provirus in all cases of ATL indicates an etiologic association between HTLV-I and ATL. The monoclonality of the tumors indicates that oncogenic transformation is a rare, and by no means inevitable, consequence of infection. It also suggests the necessity for second and possibly third contributing event. Fewer than 0.1% of all individuals infected with HTLV-I later develop ATL; the latent period can be in excess of 20 years. The absence of a preferred integration site for HTLV-I in ATL shows that there is no cis-activation of a cellular oncogene; because there is no transduced oncogene in the HTLV-I genome, ATL must be caused by a unique mechanism of viral transformation. The lack of viral expression distinguishes ATL from most other retroviral tumors and implies that although a viral gene product may be required for the initiation of transformation, it is not required for the maintenance of the transformed phenotype. The absence of viral gene expression in the fully developed leukemia also supports the probable necessity for additional events that make the growth of the leukemic cell independent of continuous viral intervention.

The HTLV-I genome contains coding information for several nonstructural proteins (Fig. 7). The best studied of these is the p40 product of the *tax* gene (58). This protein functions as a transcriptional activator for the HTLV-I genome, probably in conjunction with cellular transcription factors. The target sequences are located in the LTR of the virus. The possibility that Tax could affect the transcription of cellular genes has received support by work with transgenic mice carrying *tax* as a transgene under the control of the HTLV-I LTR. A group of these animals expressed p40 in muscle tissue. These mice developed multiple soft-tissue sarcomas of independent origin (239). The Tax protein also has been shown to transform fibroblasts in culture together with Ras (265). Similar results have also been obtained with transgenic mice that carry the *tat* gene of human immunodeficiency virus (HIV). These animals develop Kaposi's sarcoma (362). Could *tax, tat,* and other regulatory genes of retroviruses function as oncogenes? Because *tax* is a viral gene without a cellular homolog and is required for efficient replication of HTLV, the situation is formally similar to that of DNA-virus–induced transformation, where the oncogenic effectors also are essential viral genes. The data on ATL and on transgenic mice are compatible with the proposal that the Tax protein affects the expression of some key cellular regulatory genes. In the case of ATL, important targets appear to be the transcription factors CREB/ATF and NF-kB, which become stably up-regulated. Inhibition of NF-kB in leukemic cells with antisense constructs leads to tumor regression in the nude mouse model

(184,334).

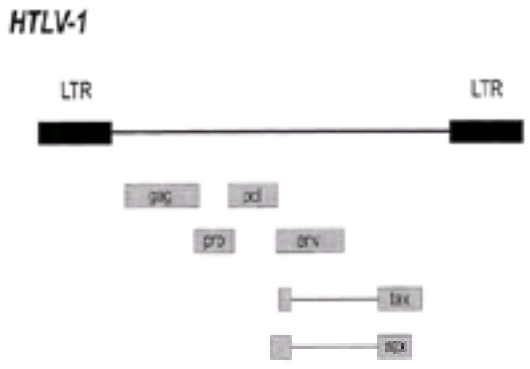

FIG. 7. Coding organization of human T-lymphotrophic virus I (HTLV-I). Gag and Env code for the major structural virion proteins; Pro for a protease, and Pol for the reverse transcriptase. Two nonstructural proteins are Tax and Rex. Tax is suspected of playing a role in HTLV-induced leukemogenesis.

MECHANISMS OF CELL TRANSFORMATION BY DNA TUMOR VIRUSES

Each major DNA virus family, except the parvoviruses, includes viruses that have the capacity to elicit cellular transformation (see Table 1). This represents an extremely diverse group of viruses with very different structures, genome organizations, and strategies of replication. In some cases, the DNA viruses have been shown to be responsible for inducing tumors in their natural hosts, including the induction of human cancers, whereas in other cases, they are known only to induce transformation of cells in culture and form tumors in experimental animal systems. For instance, a variety of data strongly implicate various types of the human papillomaviruses (HPVs) with the development of almost all cervical carcinomas (400). Epstein-Barr virus (EBV) has long been associated with the development of Burkitt's lymphoma as well as nasopharyngeal carcinoma, and epidemiologic data provide compelling evidence for a role of hepatitis B virus in the development of hepatocellular carcinoma (401). In contrast, although adenoviruses can form tumors in rodents and the viral genes E1A and E1B can transform human cells in culture, there is no evidence that adenoviruses are responsible for any human cancers (218). Likewise, SV40 and polyomavirus, which are clearly capable of forming tumors in experimental settings, have not been shown to be oncogenic in a natural setting. Nevertheless, it is these small DNA tumor viruses, which includes the adenoviruses, polyomaviruses, and papillomaviruses, that have been studied in greatest depth and that have provided much of our current understanding of virus-mediated oncogenic transformation. This is principally due to the small genome size and utility of these viruses as model systems for the study of gene expression and DNA replication, to name but two examples. The understanding of the oncogenic process has been aided by the concurrent elucidation of molecular mechanisms of action of the tumor virus oncoproteins. Because of the role these viruses have played in uncovering many of the details of cell-growth control, and the elucidation of the mechanisms that couple cell proliferation with cell-fate determination, the discussion focuses on the properties of cell transformation by the small DNA tumor viruses. For further discussion of the particulars of oncogenesis by either EBV or hepatitis B virus, please refer to Chapter 74, Chapter 75, and Chapter 87.

Transformation by the DNA Tumor Viruses: The Relationship to the Viral Replication Cycle

With the exception of HTLV-I, the oncogenic properties of the retroviruses (RNA tumor viruses) are unrelated to the requirements for productive viral growth. In the vast majority of cases, the oncogenic retroviruses are defective for replication because of the loss of essential viral sequences during the formation of the oncogenic virus. In sharp contrast, the oncogenic properties of the DNA tumor viruses are intimately associated with the ability of these viruses to carry out a productive infection. Whereas the oncogenes of the retroviruses are cellular genes that have been acquired as a consequence of the integration of the viral genome into the host cell chromosome, and then selected by virtue of their oncogenic activity, the DNA tumor virus oncogenes are essential viral genes that bear little or no relationship to cellular counterparts. Thus infection of a cell that is permissive for viral replication will generally result in a productive infection, liberating increased numbers of viral particles, and eventual cell death. A transformation event that may have been initiated would never be scored. It is only when the viral infection takes place under nonpermissive circumstances, in which the viral replication process is aborted, that a transformation event can be observed.

The frequency of cellular transformation after infection of a nonpermissive cell by adenovirus, SV40, or polyomavirus is very low, usually less than 10^{-5}. In large part, this inefficiency reflects the lack of a specific mechanism for integration of the DNA tumor virus genome into the host cell chromosome. Thus the transformation process is extremely inefficient because of the low probability that the viral genes that are essential for transformation will integrate intact and in a state that will allow transcription at appropriate levels. The lack of a system for analyzing a productive papillomavirus infection precludes such determinations for this virus group.

The oncogenic events mediated by the DNA tumor virus oncoproteins reflect the ability of these viruses to stimulate a quiescent, nongrowing cell to enter the cell cycle. For instance, a normal target for infection by adenovirus is a terminally differentiated epithelial cell that lines the upper respiratory tract. Because this cell is not in the cell cycle, the essential substrates for the replication of both viral and cellular DNA are in short supply and thus limiting. In particular, the levels of deoxyribonucleotides are tightly regulated during the cell cycle, and increase to non–rate-limiting levels only during S phase (351). The ability of the virus to stimulate such a cell to enter S phase, creating an environment for DNA replication, is thus critical for the efficient replication of the virus. This virus-mediated S-phase induction is dependent on the viral genes that also elicit transformation in other contexts. Thus if the infection does not proceed to completion, either because of a nonpermissive cell type or as a consequence of a viral mutation that blocks viral growth, the

disruption of cell-growth control that was intended to prepare the cell for viral DNA replication may instead lead to the development of a transformed cell. It is the elucidation of the mechanisms of action of these viral proteins, and in particular the cellular pathways that are targeted by the action of these viral proteins, that has propelled much of the knowledge of mammalian cell-growth control.

The DNA Tumor Virus Oncogenes

A viral infection can be divided into an early and late phase, as defined by the timing of viral gene expression relative to viral DNA replication. In general, the early genes encode proteins that prepare the infected cell to replicate viral DNA, whereas the late gene products include the structural components of the virion. For the DNA tumor viruses, these early gene products, or at least a subset of the early products, are responsible for oncogenic transformation. What follows is a general description of the adenovirus, polyomavirus, and papillomavirus genes that encode the transforming activities. For a more detailed description of these viral genes and gene products, refer to the appropriate sections in the chapters on the specific viruses.

Adenovirus

The identification of the adenovirus genes responsible for oncogenic transformation was accomplished by the mapping of viral sequences that were retained in an integrated state in transformed cells (94,121), as well as by mapping the viral transcripts present in the transformed cell (323). Such analyses indicated that a full range of viral DNA sequences could be recovered from transformed cells but that it was the left end of the viral chromosome that was common in every case. Transfection assays provided a direct demonstration that these viral sequences could indeed mediate transformation (135,136). Subsequent studies identified two distinct transcription units within this region that encode stable mRNAs found in the cytoplasm of transformed cells, as well as during the early phase of a productive infection (24). The two transcription units, termed E1A and E1B, each encode two major messenger RNAs through alternative splicing of the two primary transcripts (Fig. 8).

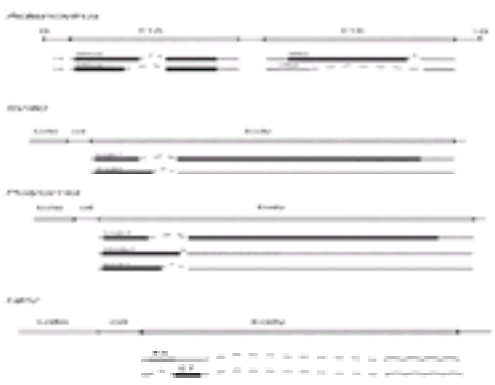

FIG. 8. The DNA tumor virus transforming genes. The left end of the adenovirus chromosome (0 to 10 map units) includes the E1A and E1B transcription units. The major mRNA products of each region are depicted, as well as the coding sequences contained within each (*black boxes*). The E1A mRNAs are commonly referred to by their sedimentation coefficients (13S and 12S), and the protein products by the amino acid residues. The E1B products are generally identified by their molecular weights. The early regions of SV40 and polyomavirus are depicted, together with the major mRNA products. The region between the early and late coding sequences referred to as “ori” contains the origin of DNA replication as well as transcriptional regulatory sequences. Coding sequences for large, middle, and small T antigen are shown by the *boxes*. The splice acceptor for polyoma large T and middle T are distinct, resulting in a change in the reading frame. The early region of the human papillomavirus genome is depicted, together with the structures of the major mRNAs that encode the E6 and E7 products. Both RNAs derive from a common promoter that starts transcription at nucleotide 97. The *wavy lines* representing 3' sequence in the mRNAs reflect the use of splice acceptors found in cellular sequences at the site of integration of the viral DNA.

The major products of the E1A gene include proteins of 289 and 243 amino acids, encoded by the so-called $E1A_{13S}$ and $E1A_{12S}$ mRNAs, respectively, that are identical in sequence except for the additional 43 amino acids found in the middle of the $E1A_{13S}$ product. The $E1A_{13S}$ product is critical for the activation of viral transcription during a lytic infection (23,171), dependent on the CR3 sequences unique to this protein (284). This activity is not important, however, for transformation. Rather, the ability of distinct domains of the protein to interact with a series of cellular proteins, including the retinoblastoma gene product (Rb), does coincide with the transforming activity. The two major E1B gene products, a 55-kd protein of 495 amino acids and a 19-kd protein of 175 amino acids, are encoded by alternatively spliced mRNAs. The two E1B proteins do not share sequence, and they appear to perform distinct although related functions. The 55-kd E1B protein, in concert with a 34-kd product of the early region 4 gene, functions to facilitate transport of viral mRNA from the nucleus to the cytoplasm (13,201,261). In addition, the 55-kd protein interacts with at least one cellular protein, the p53 tumor suppressor (301), and it is this latter activity that correlates with transforming function (395).

Polyomaviruses

Although this group includes a large number of viruses that infect a variety of cell types and host species, the two best studied viruses include the mouse polyomavirus and the monkey SV40 virus. These two viruses are very closely related in genome structure and DNA sequence, but they differ with respect to the organization and function of the early genes that carry the transforming activity of the virus (see Fig. 8). Both viruses encode a multifunctional protein, termed large T antigen, directly involved in the initiation of viral DNA replication through specific binding to the origin of replication (88,170). Although the origin binding capacity, as well as associated ATPase (127,353) and helicase (82,338) activities,

are essential for DNA replication and a productive infection, these activities are not important for transformation (9,129,174,221,268). Rather, the ability of the viral protein to bind to a variety of cellular proteins is critical for transformation. In this regard, the function of large T antigen of SV40 and polyomavirus overlap but also are distinct. Specifically, although both bind to Rb, only SV40 large T antigen binds to p53.

In addition, both viruses encode a low-molecular-weight protein termed small t antigen that contains sequence in common with the amino terminus of large T antigen, in addition to a unique sequence. Two activities have been associated with the small t antigen: the ability to bind to the PP2A cellular protein phosphatase (252,367), inhibiting activity for some substrates (392), and the capacity to activate polymerase II– and polymerase III–dependent transcription (212). At least one target for this transcription activation is the E2F transcription factor (213), which also is a target for activation by adenovirus E1A, SV40 large T antigen, and the HPV E7 protein (242). Although small t antigen does not appear to be essential in a lytic infection of tissue culture cells, it does contribute to transformation efficiency in conjunction with large T antigen (30,43).

Unlike SV40, polyomavirus encodes a distinct third early protein, termed middle T antigen, that is the principal transforming activity of the virus. Middle T antigen also shares sequence with the N terminus of large T antigen and small T antigen, but then as a result of the use of an alternative splice acceptor, the reading frame is changed from that used to encode the large T antigen (see Fig. 8). Unlike the other polyomavirus and adenovirus transforming proteins, which are localized in the nucleus, the polyoma middle T antigen is an integral membrane protein primarily associated with the plasma membrane (166,317). As we shall see later, middle T antigen binds to and activates *src* family tyrosine kinases as well as the phosphatidylinositol-3 kinase, PI-3 kinase (74,185,186,240,347).

Papillomavirus

Although the papillomaviruses are grouped within the general category of the papovaviruses, they are quite distinct from the polyomaviruses with respect to structure, gene organization, and nucleotide sequence. The majority of initial work directed at papillomavirus-mediated transformation focused on the bovine papillomaviruses, particularly BPV-1. Attention has now tended to shift to the study of the HPVs, particularly given their association with human cervical carcinoma, as well as the realization of the mechanistic similarities with the adenovirus and SV40 oncogene products.

The papillomavirus genetic information is read from only one strand, with distinct transcripts being produced by alternative transcription start sites, alternative splicing, and alternative polyadenylation (refer to Chapter 65). The genes responsible for oncogenic transformation have been identified through transfection assays using *in vitro* cell-culture systems (99,215), as well as from the identification of viral sequences that are retained and expressed in tumor cells (16,308,313,335). These studies have shown that the E5 gene of BPV encodes the major transforming activity of this virus. The E6 and the E7 gene products also contribute to transformation, but E5 is clearly the major activity. In contrast, E6 and E7 gene products of the HPVs (see Fig. 8) are primarily responsible for eliciting transformation and are always found to be expressed in tumor cells. In addition, the integrated papillomavirus DNA found in human tumor cells usually is disrupted in the E2 gene, the product of which negatively regulates transcription of the E6,E7 promoter (16).

Common Cellular Targets and Strategies for the DNA Tumor Virus Oncoproteins

Although the adenoviruses, polyomaviruses, and papillomaviruses are quite distinct and do not share a common evolutionary relationship, the actions of the transforming proteins of the three groups of viruses are remarkably similar and have defined a shared strategy that relates to the common need of these viruses to prepare the host cell for efficient viral replication. That is, the host environment in which each of these viruses must replicate is a quiescent, nondividing cell that represents a poor environment for viral DNA replication. It is the common ability of these viruses to alter this environment, through the action of the proteins that are also oncogenic, that relates replicative growth to oncogenicity.

Much of the current understanding of the actions of the DNA tumor virus proteins has derived from the analysis of physical associations of these viral proteins with a variety of cellular polypeptides. Initial studies, directed at the function of SV40 large T antigen, led to the identification of a 53-kd cellular protein that was found in T antigen immunoprecipitates (197,209). Further work demonstrated a specific interaction between T antigen and this cellular protein, which became known as p53, and subsequent studies have shown that most of the small DNA tumor viruses encode a protein that interacts with p53 (Table 5). The one clear exception among the small DNA tumor viruses is polyomavirus, which shows no evidence of encoding a protein that binds to p53. Most important, the ability of the adenovirus 55-kd E1B protein, the HPV E6 product, and SV40 T antigen to associate with p53 coincides with the transforming activity of the viral proteins (197,209,376,395).

Virus	Gene Product	Cellular Target
Adenovirus	E1A	Rb
	E1B	p53
SV40	Large T antigen	Rb, p53
Polyomavirus	Large T antigen	Rb
	Middle T antigen	Src, PI 3-K
Papillomavirus	E7	Rb
	E6	p53
	E5	PDGF receptor

TABLE 5. *Cellular targets of the DNA tumor virus oncoproteins*

Similar studies identified a large number of cellular polypeptides associated with the adenovirus E1A product (140,393). Analysis of E1A sequences essential for binding to these cellular proteins revealed two domains of interaction. One, involving N-terminal sequence and a portion of the CR1 domain, bound to a 300-kd protein, whereas the other, including the CR1 and CR2 domains, bound to a series of proteins ranging in molecular mass from 130 kd to 33 kd. Two subsequent findings with respect to these E1A interactions were of enormous significance, with respect to developing an understanding of the action of both the DNA tumor virus oncoproteins and normal mechanisms of cell-growth control. First, one of the E1A-associated proteins, a polypeptide of 105 kd, was identified as the product of the retinoblastoma susceptibility gene (382). This simple finding had immediate and far-reaching implications, because this was the first suggestion that the action of a DNA tumor virus transforming protein could be viewed as inactivating the function of a cellular growth-suppressing protein.

Second, it soon became evident that the binding of the adenovirus E1A protein to the Rb tumor-suppressor protein was an activity shared with the other DNA tumor viruses (see Table 2). SV40 and polyomavirus large T antigen, as well as the E7 product of the HPVs, were all found to bind to Rb (84,101). Moreover, the ability of each viral protein to bind to Rb was found to be dependent on viral sequences that were also important for oncogenic activity. Comparison of sequences of the viral proteins revealed a short region of homology that included the sequence important for binding to Rb (111) (Fig. 9). Although the homology between the viral proteins is limited to these sequences and is not consistent with an evolutionary relationship among the viruses, it does suggest that the viruses have each acquired a common activity.

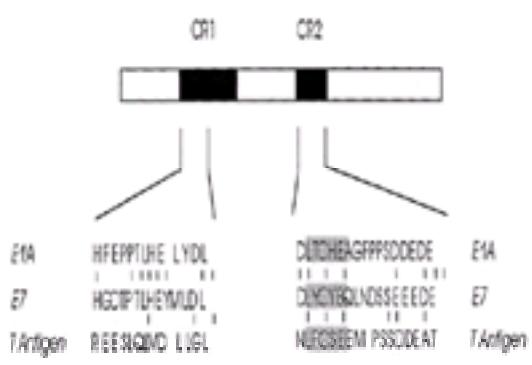

FIG. 9. Homology in the viral sequences involved in binding to the Rb family of proteins. The regions in E1A that exhibit homology with sequences in SV40 T antigen and HPV E7 are depicted (111). The L-X-C-X-E motif found in the CR2 region of E1A, that is also shared with various cellular proteins (85) including the D-type cyclins (227,390), is indicated by the *hatched box*.

Given that the ability of the various DNA tumor virus proteins to interact with Rb and p53 coincides with the transforming activity of the viral proteins, together with the fact that it is the loss of Rb and p53 gene function that is associated with the development of tumors, implies that the viral proteins achieve the same result by binding to the cellular regulatory proteins. This point is further emphasized by the relationship between HPV gene expression and the state of the Rb and p53 genes in cervical carcinoma cell lines. The vast majority of cervical carcinomas are associated with the so-called high-risk types of HPV, primarily HPV 16 and HPV 18 (400). An analysis of cell lines derived from cervical carcinomas revealed the presence of normal, wild-type Rb and p53 genes in those cells that were HPV positive and that expressed the E6 and E7 viral gene products, whereas mutations were found in the Rb and p53 genes in cell lines that were HPV negative (303). It is apparent from these results that loss of Rb and p53 function is a common event in the development of these human tumors, either as a result of mutation or through the action of the viral proteins.

Common Mechanisms of Action of the DNA Tumor Virus Oncoproteins

The physical association of the DNA tumor virus proteins with the same two cellular growth-regulatory proteins implies that these otherwise diverse viruses share a common need. Although the inactivation of Rb and p53 is most often viewed in the context of oncogenesis, the targeting of these proteins by DNA virus regulatory proteins implies a role in facilitating a productive infection. Given the common properties of the DNA tumor virus oncoproteins in the binding to key cellular growth-regulatory proteins, we discuss the common aspects of their function rather than discussing each individual viral protein in isolation.

Inactivation of Retinoblastoma Protein Function

As described earlier, adenovirus E1A, SV40 large T antigen, and HPV E7 physically interact with the retinoblastoma protein. A variety of studies have now shown that these interactions involve viral protein sequences that also are critical for transforming activity, and thus it is clear that this association is part of the transforming function. A very large body of work has now elucidated the role of the Rb protein in normal cell-growth control and provided a rationale for the strategy of the DNA tumor virus oncoproteins in disrupting this function (see Fig. 1).

Studies directed at the action of the E1A proteins in viral transcription activation during a lytic infection led to the identification of a cellular transcription factor, termed E2F, that was important for transcription of the adenovirus E2 gene (188). Subsequent studies revealed that this cellular transcription factor was normally complexed to other cellular proteins in most cell types, that these interactions prevented the activation of E2 transcription, and that the E1A protein possessed the capacity to disrupt these complexes, releasing E2F that could be used for E2 transcription (14). The ability of E1A to carry out this dissociation was found to be dependent on viral sequences that were known to be important for oncogenic activity, including binding to the Rb protein (279). This finding suggested the relationship depicted in Fig. 10A,

in which the interaction of E1A with a cellular protein such as Rb, as previously described by immunoprecipitation assays, results in disruption of the E2F complex. A variety of experiments have now shown that the Rb protein, along with the majority of the other proteins previously identified as E1A-binding proteins, is a component of the E2F complexes (15,17,18,53,57,90,332). Moreover, not only does E1A have the capacity to release E2F from interactions with Rb and Rb-family proteins, but so also do the other DNA tumor virus oncoproteins including T antigen and E7 (56).

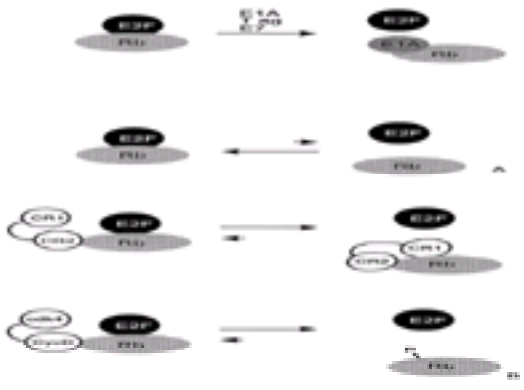

FIG. 10. Inactivation of Rb-mediated control of E2F activity. **A:** Depicted is the process of E1A (and T antigen and E7) mediated release of E2F from the complex with Rb. This action leaves the viral protein firmly associated with Rb, releasing a transcriptionally active E2F. **B:** A mechanism for E1A or D/cdk4-mediated disruption of E2F–Rb interaction.

The realization that E2F was a target for control by Rb, together with the observation that Rb function was controlled by the action of G_1 cyclin-dependent kinases, led to the eventual delineation of a G_1 regulatory pathway that ultimately controls the accumulation of E2F activity. The G_1 cyclin-dependent kinase–mediated phosphorylation abolishes the ability of Rb to interact with E2F proteins; thus the physiologic control of Rb function is mediated by G_1 CDK phosphorylation. The viral oncoproteins E1A, Tag, and E7 effect an inactivation of Rb through a direct physical interaction, independent of phosphorylation. Interestingly, the mechanism for recognition of Rb by either the viral oncoproteins or the G_1 cyclin may be similar. As depicted in Fig. 10B, the E1A-mediated disruption of the E2F–Rb complex would appear to involve first the recognition of Rb by the CR2 domain of E1A, followed by interaction of the CR1 domain with a site on Rb involved in E2F interaction (165). The conserved LXCXE sequence element found in CR2, which is shared in the other DNA tumor virus oncoproteins (see Fig. 8), also is found in the cyclin D1 protein and has been shown to be important for the interaction of cyclin D1 with Rb (96). Thus the LXCXE motif may be responsible for targeting either a viral protein or a kinase to Rb to achieve an inactivation of function.

The consequence of inactivation of Rb, either by phosphorylation or by binding of a viral oncoprotein, is to allow the accumulation of E2F activity. A large body of work has now shown that the genes subject to E2F control include the majority, if not all, of the genes encoding proteins essential for DNA replication (100,243). These include components of the enzymatic machinery such as DNA polymerase a and PCNA as well as the enzymes generating the necessary deoxyribonucleotide substrates such as ribonucleotide reductase, thymidine kinase, and dihydrofolate reductase. Moreover, it also is now clear that the majority of genes encoding proteins that function at the origins of replication, including the proteins of the origin recognition complex (ORC), the Mcm proteins, and Cdc6 are all E2F targets (200). Thus the Rb/E2F pathway, which is normally controlled by the action of G_1 cyclin-dependent kinases in response to a growth stimulus, or deregulated by the action of the DNA tumor virus oncoproteins, is responsible for the synthesis of the apparatus of DNA replication.

Numerous previous experiments have shown that the infection of quiescent cells by adenovirus, SV40, and polyoma results in the activation of S phase in these infected cells and that this coincides with the induction of the activities normally associated with entry into S phase and that are now known to be E2F targets (98,116,177,198,391). Very likely, this activation reflects the need of the virus to create an environment appropriate for viral DNA synthesis. Thus when a DNA tumor virus infects a quiescent, nongrowing cell, which is the normal target for infection by these viruses, the environment for DNA replication, whether it be viral or cellular, is not suitable. For instance, the levels of deoxynucleotides are very low in quiescent cells and they only increase as a consequence of the induction of ribonucleotide reductase activity when such cells are stimulated to grow and enter S phase (35,107). Thus through the ability to activate genes encoding the enzymes that create the appropriate environment, the virus is then able to achieve efficient replication of viral DNA.

Inactivation of p53 Function

After the discovery of p53 as a protein in association with SV40 T antigen (197,209), various analyses suggested that p53 functioned as an oncoprotein (105,168,254). Thus early speculation centered on a role for the association of the viral protein with p53 in stimulating or somehow augmenting the action of this oncoprotein. A radical change in thinking occurred when it was realized that the p53 gene used for the initial studies of transformation was in fact a mutated version and that the transforming activity of the previously assayed p53 gene was dependent on this mutation (104,113). Moreover, assays of the normal wild-type version of p53 revealed that it possessed characteristics of a tumor-suppressor molecule (112). It is now clear that the loss of p53 function, as a result of mutation or deletion of the gene, occurs during the development of a large number of human cancers (202). These studies suggest that loss of p53 function may be an event involved in the majority of human tumors.

Once again, each of the DNA tumor viruses encodes a protein that has the ability to interact with p53 and, as a consequence of this interaction, inactivate p53 function. In contrast to the targeting of Rb, however, the viral protein sequences involved in these interactions, as well as the result of the interaction, appear to be distinct among the different

viruses. That is, an examination of amino acid sequences known to be involved in the targeting of p53 in adenovirus E1B, SV40 T-antigen, and HPV E6 show no evidence for sequence similarity. Moreover, it would appear that distinct domains of the p53 protein are recognized by the viral proteins. Finally, it also is apparent that the targeting of p53 represents a point of divergence in the polyomavirus group, in that the primate polyomavirus SV40 has evolved a protein to target the p53 molecule, whereas the mouse polyomavirus lacks this function and shows no evidence of perturbing the function of p53.

Although the actual mechanism by which E1B, T antigen, and E6 target p53 does not appear to be conserved, the ultimate consequence of the interaction of these viral proteins with p53 is the same: a loss of p53 function. For instance, whereas T-antigen interaction with p53 appears to stabilize the p53 protein, presumably in an inactive state, the interaction of the HPV E6 protein with p53 triggers a ubiquitin-mediated degradation of the p53 protein (304). The process of E6-mediated degradation of p53 has been studied in some detail because these events can be assayed in cell-free extracts. These studies have led to the identification of an additional cellular protein, termed E6-AP (E6-associated protein), that functions in concert with E6 to target p53 for destruction (158,159). E6-AP functions as an E3 ubiquitin protein ligase and may be targeted to p53 through the interaction with E6, because E6-AP does not appear to be involved in p53 degradation in the absence of E6 (346).

What normal function of p53 is disrupted by these viral oncoproteins? Most likely, it is the ability of p53 to function as a transcription factor that is targeted by the viral proteins. The p53 protein has been shown to bind to DNA in a sequence-specific manner (180) and, as a consequence of this binding, activates transcription if the binding site is part of a promoter element (120,396). The *WAF1* gene, which encodes the p21 cyclin kinase inhibitor (141), was originally identified as a target for p53 transcriptional activation (103). Thus the p53-mediated suppression of cell growth may be due in part to the inhibition of G_1 cyclin-dependent kinases, the activity of which is critical for G_1 progression and cell growth.

p53 Has been extensively characterized as a transcription factor that can contribute to the activation of many genes as well as the repression of others. The transcriptional activity of p53 appears to underlie its ability to induce growth arrest, and at least in some contexts, its ability to induce apoptosis. p53 Binds DNA as a tetramer, recognizing a DNA consensus consisting of two inverted repeats of 5'-PuPuPuC(A/T)-3'. p53 Directly regulates, through p53 binding sites in the promoters, the expression of several genes involved in cell-cycle control, apoptosis, and the response to cellular stress, including p21, Bax, GADD45, IGF-BP3, and 14-3-3g (185,203). p53 Also can repress the expression of other genes lacking p53 binding sites in their promoters, including the antiapoptotic *Bcl2* gene, apparently by interacting with the TATA-containing basal promoter (185,203). Transcriptional repression by p53 may be important for its proapoptotic function, as the expression of *Bcl2* or E1B 19K blocks p53-dependent apoptosis and transcriptional repression without affecting transcriptional activation (297,325). As a further indication that p53-dependent growth arrest and apoptosis are genetically separable, p53 that is deleted of its proline-rich domain (amino acids 62–91) retains the ability to induce growth arrest, but is deficient in the induction of apoptosis (360). Interestingly, this p53 mutant can still transactivate some genes such as *mdm2*, *p21*, and *bax*, but fails to activate the PIG3 redox enzyme gene or to repress the transcription of genes normally repressed by p53.

The importance of transcriptional control by p53 is highlighted by the fact that most mutational hotspots found in human cancers map to the DNA-binding domain of p53 (185). Nonetheless, DNA damage-induced p53-dependent apoptosis can occur in the presence of inhibitors of RNA and protein synthesis (51,366), and overexpression of transactivation-defective p53 can induce apoptosis in HeLa cells (145). In other contexts, however, such as apoptosis induced by the adenoviral E1A oncoprotein, apoptosis is abrogated when the expressed p53 possesses mutations that reduce DNA binding or transactivation (8,298). Thus p53 appears to mediate both transcription-dependent and transcription-independent apoptosis.

The Actions of the DNA Tumor Virus Proteins Suggest a Functional Link Between the Rb and p53 Pathways

Although the combined action of adenovirus E1A and E1B leads to the oncogenic transformation of susceptible cells in culture, E1A expression alone most often leads to cell death as a result of apoptosis (275,380). Expression of the adenovirus E1B gene suppresses this E1A-induced apoptosis response, allowing the cells to become stably transformed. This phenomenon was first observed in cells infected by adenovirus E1B mutants, in which the infection led to the degradation of both viral and cellular DNA (260,379), a hallmark of the apoptosis response.

Further studies have shown that the induction of apoptosis in an adenovirus infection not only is dependent on E1A expression but also involves an induction of the p53 tumor-suppressor protein. In particular, there is no such apoptotic response in cells that are lacking a functional p53 gene, but the response can be restored on transfection of the wild-type p53 gene (83). It is now evident that the action of E1A to induce apoptosis is tightly linked to its ability to deregulate the Rb pathway. Direct evidence for this has come from studies of E1A mutants, in which it is clear that the ability of E1A to promote apoptosis and induce p53 accumulation is dependent on the domains involved in Rb binding (214). As discussed previously, the inactivation of Rb leads to deregulation of E2F. Indeed, overexpression of the E2F1 protein, a key target for Rb control, leads not only to an induction of S phase but also to an induction of apoptosis, which is largely p53 dependent (190,270,321,387). Further work has shown that the ability of E2F to induce apoptosis when expressed in quiescent cells is an activity unique to E2F1 (86). Moreover, E2F1, but not E2F2, also induces an accumulation of p53 protein (189). Thus one can now envision the apoptotic response and the induction of p53 accumulation as a response to the deregulation of the Rb pathway, with the E2F1 protein providing a signal to trigger p53 accumulation.

The inactivation of p53 function by the viral oncoproteins may mimic the normal activity of the product of the cellular *mdm2* gene. *Mdm2* was originally identified as a gene amplified in various tumors, primarily sarcomas (52). Subsequent studies revealed that the Mdm2 protein could physically interact with p53 and inhibit the transactivation function of p53 (60,250). In addition, it is now evident that Mdm2 functions to target p53 for ubiquitin-mediated degradation (144,191). Thus like the HPV E6 protein, Mdm2 can control the accumulation of p53 by controlling the stability of the protein.

A variety of studies have now elucidated a pathway that links the deregulation of the Rb pathway with the induction of p53 accumulation. A key component in this pathway is the $p19^{ARF}$ gene product. ARF (alternate reading frame) was identified as an alternate reading frame contained within the locus encoding the p16 cyclin kinase inhibitor (271). ARF has now been shown to interact with and control the activity of Mdm2; specifically, ARF prevents Mdm2 from targeting p53 for degradation (81,175,264,400,401). Moreover, other work has demonstrated that the $p19^{ARF}$ gene is activated by E2F1 (86), thus linking the deregulation of the Rb pathway with the activation of ARF, inactivation of Mdm2, and thus the accumulation of p53 protein (see Fig. 11).

Mdm2 may play a normal role in regulating the action of p53, possibly during normal growth stimulation (386). Although it is straightforward to envision the E2F1–ARF–Mdm2 pathway as a mechanism to monitor the integrity of the Rb pathway, triggering cell death when mutations or viral oncoproteins disrupt the normal control of the pathway, it also is possible that the balance of these two regulatory pathways (Rb and p53) plays an important role in normal cell growth and tissue homeostasis. Perhaps the most striking observation relating Mdm2 to p53 in the context of normal physiology is the analysis of mice bearing deletions of the p53 or Mdm2 locus. Targeted deletion of the Mdm2 gene leads to an early embryonic lethality, with mice not surviving beyond embryonic day 6. Although loss of p53 is without discernible effect during embryonic development, combining the p53 deletion with the Mdm2 null leads to a complete suppression of the Mdm2 lethality (172,234). Thus under circumstances in which there is likely to be no DNA damaged–induced p53 response, or oncogene activation, p53 and Mdm2 are seen to function in relation to one another. Given the fact that normal proliferation leads to the accumulation of E2F1 as a consequence of Rb phosphorylation, it may be necessary to block the ARF–Mdm2–p53 pathway each time cells are stimulated to grow.

The paradigm established by the analysis of the DNA tumor virus oncoproteins, whereby the dual inactivation of Rb and p53 can be seen as a mechanism to deregulate cell proliferation (inactivation of Rb) and block the response to this deregulation (inactivation of p53), may reflect events occurring during the development of human tumors. With the exception of colon cancer, virtually all human cancers exhibit a deregulation of the Rb pathway. This might take the form of amplification of a D-type cyclin gene, amplification of the cyclin-dependent kinase gene, loss of a cyclin kinase inhibitor, or loss of Rb. Even in colon cancer, the pathway may be deregulated, because recent data have shown that loss of the APC tumor suppressor, which controls the b-catenin/TCF transcription factor, leads to an activation of Myc (148) as well as D cyclin transcription (350). Certainly, a role for TCF in the activation of D cyclin transcription provides one direct connection to the Rb/E2F pathway, but, in addition, other experiments provide evidence for a direct role for Myc in the activation of E2F genes (199,315).

In addition to the fact that virtually all tumors exhibit a loss of Rb pathway control, roughly half of all human tumors exhibit an inactivation of p53, resulting from either inactivating mutations or generation of dominant negative mutant forms of p53. Amplification of the Mdm2 gene is seen in a fraction of those tumors that appear to be p53 wild-type, and recent data indicated that loss of ARF is frequently seen in p53 wild-type tumors. Thus as is the case for the Rb pathway, one way or another there is a loss of p53 function. One might speculate that the deregulation of the Rb pathway, leading to loss of proliferative restraint, coupled with loss of the p53 pathway that abolishes the response to loss of Rb, are events that will always be found in the course of tumorigenesis.

Other DNA Tumor Virus–Transforming Activities

Although the inactivation of Rb and p53 function is an activity that is common to the small DNA tumor viruses, at least two other virus-specific activities have evolved that do not appear to be shared in common among the DNA tumor viruses, but that do clearly contribute to the transforming capacity of the viruses. In one instance, the activity may represent an alternate path to p53 inactivation.

Although the bovine papillomaviruses (BPVs) show considerable homology and related function to the human viruses, the major transforming activity of BPV is the product of the E5 gene, a 44-amino-acid transmembrane protein. Studies of the mechanism of action of the E5 protein has revealed a role in activating tyrosine kinase signaling. In particular, the E5 homodimer interacts with, and promotes dimerization of, the PDGF receptor, leading to the activation of kinase activity of the receptor and thus the signaling cascade normally activated by binding of PDGF (see Fig. 12) (for review, see ref. 92). As such, the E5 protein can be seen to act as a molecular mimic of a growth factor, providing a growth stimulus in the absence of the normal proliferative signal. This action of E5 is quite analogous to the action of the SFFV gp55 protein, also a transmembrane protein that leads to a ligand-independent activation of the Epo receptor.

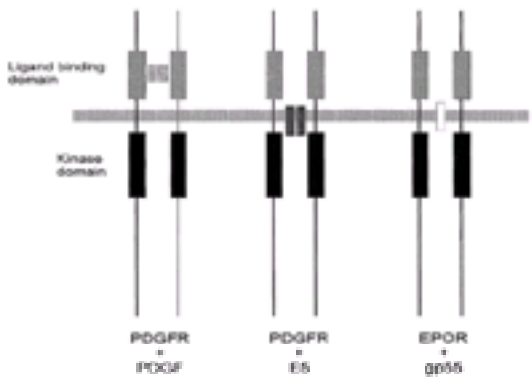

FIG. 12. Oncogenesis by molecular mimicry. The platelet-derived growth factor (PDGF) receptor is normally activated by PDGF-mediated receptor dimerization, which then activates the tyrosine kinase activity of the receptor and initiates a signal-transduction cascade. The E5 transmembrane protein of bovine papillomavirus provides a mimic of the PDGF signal by inducing a ligand-independent dimerization of the receptor through a direct interaction. Although not proven in detail, the gp55 protein of SFFV may act similarly to activate the EPO receptor independent of erythropoietin.

A second example of virus-mediated activation of a kinase signaling pathway, independent of normal proliferative signals, can be seen in the analysis of the polyomavirus transformation. The polyomavirus middle T antigen, a product unique to polyoma, revealed the association of this viral protein with the c- *src* proto-oncogene (73,74). The interaction of middle T with the c-*src* product results in an activation of the tyrosine kinase activity of the Src protein (41,71), as a consequence of the prevention of a phosphorylation-mediated inhibition of the Src kinase (54,71). Subsequent experiments have revealed that middle T can complex with other members of the Src kinase family including the Yes and Fyn kinases (63,155,186,194). In addition, formation of the middle T antigen/Src kinase complex leads to a recruitment of the PI-3 kinase (176,381), resulting in the formation of a ternary complex involving middle T, Src, and the PI-3 kinase. The formation of this complex is dependent on the phosphorylation of middle T at tyrosine 315 by the Src kinase (347). One role for the middle T–mediated activation of Src kinase activity for a lytic viral infection would appear to be the promotion of phosphorylation of the late viral capsid protein VP1, an essential modification for viral morphogenesis (123).

The interaction of middle T with Src family kinases and the PI-3 kinase appears to be a unique feature of polyomavirus, because an equivalent interaction is not found with proteins encoded by SV40, adenovirus, or papillomavirus. Moreover, this association and concomitant activation of Src kinase activity appear to be sufficient for transformation by polyomavirus. Given that this activity does mediate transformation, together with the observations that the common aspect of the other small DNA tumor viruses appears to involve an ability to drive quiescent cells into a cell cycle, one suspects that the action of middle T in activating the Src kinase pathway achieves a similar result: the creation of a proliferating cell environment that is conducive to viral replication. Indeed, analysis of a polyomavirus mutant that is defective in binding and activating PI-3 kinase suggests that this activity of middle T is important for the ability of polyomavirus to prevent apoptosis (79).

A number of previous experiments have identified polyomavirus mutants that exhibit a host range and transformation defective phenotype (hr-t) (22) that have mutations in the small and middle T specific sequences. These mutants are unable to induce a permissive state for virus replication in certain cell types, presumably a reflection of the requirement for middle T function in altering the growth properties of the target cell. Although in most instances there is a tight link between the two phenotypes, isolate middle T mutants continue to grow but fail to transform (123).

The analysis of middle T antigen–mediated transformation and tumor induction *in vivo* has shown that the presence of the src kinase is not essential for this activity because infection of src-negative mice with a middle T–expressing retrovirus led to the formation of tumors with a frequency nearly the same as that for Src-positive mice (352). Nevertheless, although the association of middle T with Src may not be absolutely critical, it is certainly possible that other Src family kinases compensate for the loss of Src because middle T is found in association with *yes* and *fyn.* These results do raise the question, however, of the nature of the underlying specificity in the interaction of middle T with one of the kinases.

Finally, although the action of middle T antigen is clearly sufficient to transform cells, one suspects that the ability of polyoma large T antigen to bind to and inactivate the Rb protein may well contribute in some circumstances. For instance, although polyomavirus mutants that are defective in large T function with respect to Rb binding are still capable of transformation and induction of tumor formation, they are defective in immortalization assays (117). Thus it may well be true that it is the combination of large T function in inactivating Rb together with the action of middle T in activating the Src kinase pathway that is necessary to facilitate fully the creation of a suitable environment for polyomavirus replication.

EVOLUTION OF COMMON STRATEGIES OF THE DNA TUMOR VIRUSES

Given that the small DNA tumor viruses represent a diverse group of viruses with little or no evolutionary relationship, it is striking that they share common targets and strategies for replication and transformation. Although the inactivation of Rb and p53 by the DNA tumor virus oncoproteins is generally considered in the context of oncogenic transformation, these events must be important for the normal process of a productive infection by these viruses, because it is the ability to replicate that provides the driving force for the evolution of these viral genes. Clearly, these evolutionarily distinct viruses share a common need and have developed a common strategy, not to transform cells, but to replicate.

As discussed previously, these otherwise diverse viruses do have a common need to induce a quiescent, nondividing cell to enter S phase so as to create an environment that is favorable for viral DNA replication. The inactivation of Rb function through the action of E1A, T antigen, or E7 would appear to facilitate this process by activating the E2F transcription factor and possibly the D type G_1 cyclins. At least for E2F activation, this would lead to an induction of various genes that create the environment for DNA replication.

The virus-mediated inactivation of p53 function also appears to facilitate entry to S phase, because expression of p53 can result in a G_1 arrest, particularly in response to DNA-damaging events (211), or to initiate a pathway of programmed cell death in response to various proliferative signals (378). As discussed in previous sections, the ability of E1A to stimulate S phase also coincides with the induction of apoptosis. The mechanism for this response remains to be determined, but it does involve the p53 tumor-suppressor protein. Indeed, there is an induction of p53 expression in cells expressing E1A. The E1B 55-kd protein, SV40 T antigen, and HPV E6 all block the action of p53 and thus block the apoptosis pathway. Thus one might view these actions as “allowing” the E1A-mediated process of S-phase induction to continue.

Given the common activities exhibited by adenovirus, SV40, and HPV, it is perhaps equally striking to find a distinct activity that is unique to polyomavirus, the middle T antigen–mediated induction of tyrosine kinase activity. Presumably polyomavirus has evolved a mechanism to accomplish the same result, the creation of a favorable environment for viral replication, without the need of eliminating the Rb and p53 suppression events. Nevertheless, it also is true that polyoma large T antigen does target Rb, and given the pairwise relationship between Rb inactivation and p53 inactivation seen

with the other viral oncoproteins, one wonders if the ultimate action of middle T might lead to the same result.

Finally, if the small DNA tumor viruses have evolved a common mechanism to drive a quiescent cell into S phase to allow efficient viral DNA replication, one might also anticipate that other DNA viruses that must replicate DNA at a high level, such as the herpesviruses or the poxviruses, would also find this to be a beneficial event, yet there is no compelling evidence that any of the herpesviruses or poxviruses encode proteins that inactivate Rb or p53. It is striking, however, that many of the genes whose products create the S-phase environment, and that are suspected to be activated as a consequence of the release of E2F from inhibition by Rb, are also found within the genomes of the large viruses of the herpesvirus and poxvirus family (2). Although it is clear that the entire complement of genes is not found within every virus of these groups, it is nevertheless true that each virus contains a ribonucleotide reductase, the rate-limiting enzyme in deoxynucleotide biosynthesis (351). Thus a common strategy of the DNA viruses, whether oncogenic or not, may be to induce activities that create the appropriate environment for viral DNA replication to take place in an efficient manner. If this involves the disruption of normal cell-growth control events to force a quiescent cell into S phase, then transformation can result if the infection does not go to completion.

CONCLUSIONS

The study of virus-induced oncogenic transformation has defined paradigmatic changes that differentiate cancer cells from their normal progenitors. These changes affect the components of signal-transduction pathways and circuits that control the cell cycle. RNA tumor viruses mutate the sequence information of growth-regulatory genes. These mutations are causative events in oncogenesis. DNA tumor viruses, conversely, disrupt various growth regulatory events as a consequence of the interaction of viral proteins with cellular regulatory proteins, temporarily inactivating the function of the cellular protein. The genes encoding these cellular proteins remain unaltered.

We have seen from the study of the RNA and DNA tumor viruses that the induction of oncogenic transformation results from the targeting of two distinct classes of cellular genes and their products: oncogenes and tumor-suppressor genes. Oncogenes encode components of cellular signal transduction, and their activation leads to a constitutive growth signal. Most often, it is this process that is seen in the retroviruses. By contrast, tumor-suppressor genes encode negative regulators of cell growth, particularly the cell cycle, and their inactivation removes such attenuating controls. The oncogenic activities of the DNA tumor viruses most often target the tumor suppressors.

The activation of cellular oncogenes can occur by all manner of mutagenic events, which include, besides viral intervention, point mutation, and chromosomal translocation and amplification. DNA-transfection techniques have identified numerous genes that can confer oncogenic properties of recipient cells. Some of these genes are closely related to the oncogenes that have also been identified in retroviruses. Many others, however, have no known association with viruses. However, the normal functions of all these genes are related to the control of cell growth, providing strong evidence for their oncogenic potential as altered control elements.

The genetic inactivation of tumor-suppressor genes clearly plays an important role in the heritable forms of human cancer. Although the action of the tumor virus proteins in achieving a phenotypic inactivation of these gene products is clearly not relevant to the understanding of the genetic basis of human cancer, it is true that somatic mutations of tumor-suppressor genes are also increasingly recognized as critical factors in sporadic cancers. Virus-induced and genetic inactivation of tumor suppressors have the same net effect as most dramatically seen in the case of cervical carcinomas: the loss of tumor-suppressor activity and thus the study of the events after inactivation by viral proteins has been and will continue to be an important approach for the study of human cancer.

Finally, despite strong evidence for the genetic etiology of cancer, it is likely that epigenetic factors also play important, but much less well defined and understood roles. In certain circumstances, such factors can exert a dominant effect, thereby converting tumor cells into normal cells. A dramatic demonstration of such epigenetic reversion is seen with murine embryonal carcinoma cells that, when injected into a normal mouse blastocyst, become subject to developmental control and contribute to the development of normal mouse tissues (231). Because molecular biology is biased in favor of genetic analysis and interpretation, it is especially important to keep a watchful eye for such epigenetic phenomena. Although the influence of these events on the development of a tumor is often difficult to measure, it is nevertheless true that these epigenetic factors also will influence the development of virally induced tumors, and thus one might expect that use of tumor viruses will play a role in the understanding of these more subtle events.

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CHAPTER 11 The Immune Response to Viruses

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Virus infections have long been a major cause of human morbidity and mortality, at times substantially affecting the course of human history (167). However, due mainly to the astonishing benefits of antiviral vaccination in the past 50 years, smallpox has been eliminated, worldwide polio eradication is on the horizon, and at least in the “developed” countries, many of the childhood illnesses (measles, mumps, rubella, chickenpox, etc.) appear under control. Therefore as we reach the millenium, it is tempting to become sanguine, to conclude that we are close to completing the catalog of viruses and their associated diseases, and perhaps even to suggest that virus infections no longer pose a significant threat to human health. Nothing could be further from the truth. First, viruses still exact a heavy toll in human suffering, increasingly so with the spread of human immunodeficiency virus (HIV), which in 1998 became the fourth leading cause of death worldwide (behind ischemic heart disease, cerebrovascular disease, and acute lower respiratory tract infections). Second, new (or at least newly recognized) diseases continue to appear, most tragically demonstrated by the mushrooming acquired immunodeficiency syndrome (AIDS) epidemic, first recognized 18 years ago, and for which the causative virus was identified in 1983 (11). More recently, an outbreak of a “mysterious virus” occurred in Malaysia, killing more than 100 people (1). This virus is related to, but distinct from, Hendra virus, an equine morbillivirus first isolated in stables in Hendra, Australia, which had high pathogenicity in humans (214). Whereas Hendra virus is associated with horses, and may be spread by cats and bats (276), the Malaysian paramyxovirus is associated with pigs. Third, diseases that in the past have been geographically constrained may plausibly be spread worldwide by rapid intercontinental travel (e.g., Ebola, Lassa). Fourth, we must be continually wary of the reappearance of old foes; there were 90 deaths from measles in the United States in 1990 (200) and over 150,000 cases in 1998, 1999, and 200 in Japan, as a result of reduced vaccine compliance; and the 1997 outbreak (fortunately limited) of influenza in Hong Kong had a 30% fatality rate (219). Fifth, it is becoming increasingly clear that virus infections are associated with various cancers; for example, human papillomavirus with cervical carcinoma; human herpesvirus 8 with Kaposi's sarcoma; and hepatocellular carcinoma may be causally associated with hepatitis B virus (149), which infects hundreds of millions. Finally, there remain many diseases whose causes are undefined, and for which undiscovered viruses remain prime etiologic candidates.

To counter the continuing threat, it is vital that we understand the antiviral immune response. Studies of antiviral immunity can help us evaluate clinical problems (at the most basic level, our knowledge of the kinetics of the antibody response has been the basis of many viral diagnostic procedures, at least until recently). Furthermore, in the last decade we have begun to uncover the mechanisms that underlie the success of antiviral vaccines, and with this knowledge in hand, we may now be able to design better vaccines. In addition, viruses are powerful tools with which to probe the workings of the host immune system, and the knowledge that accrues may yield benefits in diverse fields. For example, the relationship between virus infection and subsequent immunity was noted more than 200 years ago by Jenner (85), whereas virus-induced immunosuppression was first suspected more than 90 years ago by von Pirquet (260). Major histocompatibility complex (MHC) restriction—described later in this chapter—was discovered with a viral model system (286); so pivotal was this finding to almost all aspects of antigen-specific immunity (not only in virology, but also in tumor immunology and autoimmunity) that its discoverers were awarded the 1996 Nobel Prize in Medicine.

OVERVIEW OF THE IMMUNE RESPONSE TO VIRUS INFECTION

Virus infections are countered by host immune responses. Acute virus infections represent a race between viral replication and host immunity; the usual outcome is either death of the host or termination of infection followed by recovery. In chronic virus infections, the time scale is lengthened, and both ongoing virus replication and a continuous host immune response may be present. In both acute and chronic infections, the antiviral immune response can itself be harmful to the host; this is termed immunopathology. For example, host responses may kill virus-infected cells, perhaps eradicating the agent, but at the cost of local tissue destruction. In addition many of the systemic clinical signs and symptoms manifested during viral infection are a consequence not of virus replication *per se,* but rather of the host immune reaction; cytokines released in response to infection may cause systemic symptoms (fever, headache, myalgia, anorexia, weight loss, etc.) (248,255). Furthermore, particularly during chronic infection, viral antigen–antibody complexes may form with subsequent manifestations of immune complex disease. Finally, the immune response triggered by the virus may be aberrant, focusing not on the virus but instead on self proteins, and possibly causing autoimmune disease (166,273).

The immune response can be subdivided in several ways, the two most useful of which are shown in Fig. 1. Immune responses may be categorized either by the *nature of the effector function* or by the *antigen specificity of the response.*

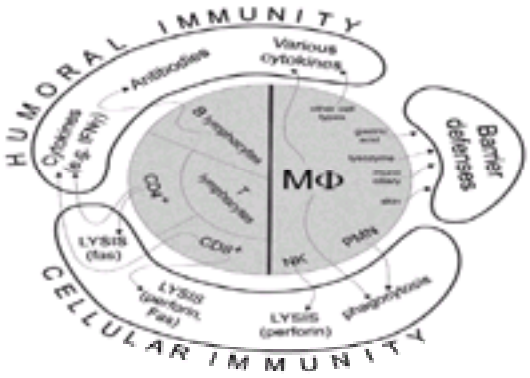

FIG. 1. Cutting the immunological cake. The central gray circle represents the immune response, which can be subdivided into antigen-specific responses (*left half*) and innate or non-specific immunity (*right half*). The correlations of these responses with an alternative means of classifying immunity (cellular, humoral, and barrier) are shown, as are some of the effector functions involved (lysis, cytokine release, etc.). *M*F, macrophages; *NK*, natural killer cells; *PMN*, polymorphonuclear leukocytes; *IFN*, interferon.

By effector function. Effector functions carried out directly by cells [e.g., natural killer (NK) cells, T cells] are classified as "cellular" immunity. When the effector functions are fluid-borne, they are termed "humoral" immunity; this term is often wrongly thought to be synonymous with antibodies, but in fact it encompasses all fluid-borne effector molecules (antibodies, cytokines, chemokines, complement, etc.). These divisions are obviously overlapping; cellular immunity can involve cytokine secretion, which is itself humoral. Finally, physicochemical defenses are termed "barrier" immunity.

By antigen specificity. Immune responses may be termed antigen specific (adaptive) or non–antigen specific (innate); as the names indicate, the former (mediated by lymphocytes) recognize specific antigenic motifs, as described later, whereas the latter (e.g., interferons, natural killer cells, mucociliary responses) are incapable of doing so. However, the key distinction between antigen-specific and nonspecific immunity is that only the former has "memory." On first exposure to a virus, both nonspecific and antigen-specific responses are activated, and the infection is (we hope) cleared. However, on second exposure to the same agent, the antigen-specific response occurs much more rapidly, and is much stronger, because of amplification of memory cells, whereas the nonspecific response occurs almost precisely as before. The beneficial effects of vaccination rely on the induction of memory cells.

Both innate and adaptive immunity are important in clearing virus infection. Innate responses, and their effector functions, are described later in the chapter; we focus first on adaptive (antigen-specific) immunity. As shown in Fig. 1, this relies on lymphocytes, of which there are two classes, T (thymus-derived) lymphocytes and B lymphocytes (which give rise to antibody-producing plasma cells). Antibodies act mainly to diminish the infectivity of free virus, whereas T cells recognize (and often kill) infected cells. Thus antibodies and T lymphocytes act in a complementary manner: antibodies reduce the infectivity of free virus in fluid phase, thereby decreasing the number of infected cells that T lymphocytes must deal with, and T lymphocytes kill infected cells before virus maturation has occurred, minimizing the release of infectious virus and thus easing the load on antibodies. Primary virus infection (when the host is first exposed to the wild virus or to a vaccine containing viral antigens) in many cases induces readily detectable antiviral antibody and T-cell responses. At the peak of the T-cell response (usually 7–10 days), some 50% to 60% of $CD8^+$ cells are virus specific (26,147); this number decreases (often in parallel with the resolving virus infection), and by 3 to 4 weeks after infection, the cell numbers have decreased, although a significant proportion (1–10%) of splenic $CD8^+$ cells are virus-specific (memory) cells. Antibody responses usually peak later than T-cell responses; antibodies are often barely detectable in the acute (symptomatic) stage of infection, but increase in number over a period of 2 to 4 weeks to a readily measurable level that often lingers for weeks or months, depending on the host and virus. Antibodies to some viruses (e.g., yellow fever and measles) can linger for a lifetime. Because antibody molecules have half-lives measured in weeks, the lifelong presence of antibody implies ongoing production, which may be stimulated by reexposure to related viruses circulating in the population. Long-term antibody production can be effected by long-lived plasma cells (224). After a second exposure to the same antigen(s) (i.e., reexposure to the wild virus, or initial exposure to wild virus after appropriate vaccination), both classes of lymphocytes display antigen-specific memory. The classic "anamnestic" or memory antibody response is seen, with

antibody levels climbing more rapidly and higher than before, and usually being maintained for a longer time. Secondary cytotoxic T-cell responses, too, are brisk, because of the presence of the memory T cells, which proliferate on antigen contact. The molecular requirements for T-cell memory remain somewhat controversial. Some workers argue that specific antigen must persist to restimulate memory T cells continuously (157), but persuasive evidence indicates that memory cells can persist in the absence of specific antigen (81,114), perhaps being sporadically stimulated in a nonspecific manner by local cytokine release during other, antigenically unrelated, immune reactions.

THE IMMUNE SYSTEM HAS BEEN SHAPED BY THE TYPES OF MICROBES THAT CHALLENGE IT

The vertebrate immune system fights a ceaseless battle against infectious agents, and both of these opposing forces have been shaped by the constant conflict. Immunological pressures bear on mutable microbes, selecting those best able to escape the unwelcome attentions of the immune system. Microbial evolution is thus driven, at least in part, by the efforts of the immune response to eradicate infection. These enforced microbial changes are mirrored in the vertebrate hosts: the immune system is molded by the nature of the microbes that it faces. For example, hosts whose immune systems are less able to counter a specific infectious agent may die; those that live (and their progeny) are better able to resist similar organisms. Hence, the vertebrate gene pool is altered by exposure to specific microbes. This battle for evolutionary supremacy places the virus in a difficult situation. If the virus is too readily eliminated by host immunity, and is in consequence unable to maintain itself in the host population, it becomes extinct. Conversely, if a virus is too virulent and cannot be controlled by host immunity, it may result in eradication of the host and thus, theoretically, viral extinction. Thus from the virus' viewpoint, it is best to be sufficiently virulent to allow transfer from an infected to an uninfected host, but to temper its pathogenicity to ensure that the host will survive long enough to permit the transfer. At either extreme, the virus may be eliminated. Frequently the initial virulence of a virus is diminished by mutation to a less virulent form, and this, in concert with the selection of more resistant hosts, results in a fluctuating equilibrium, in which both host and microbe coexist. Many examples of microbial and host changes have been documented. For example, influenza virus is the quintessence of antigenic variation in the face of host immunity, and those influenza viruses best able to evade host responses have fueled outbreaks of epidemic or pandemic proportions. The complementary outcome, in which the microbes select for survival those hosts best able to counter their pathogenic effects, is best demonstrated when the offspring have enhanced survival prospects. Such improved resistance in the progeny may, of course, be in part conferred by passive (maternally derived) immunity rather than by genetic fitness, but there are clear examples of the latter. For instance, wild Australian rabbits that survived the first onslaught by myxoma virus (a poxvirus deliberately administered in an attempt to control the exponential increase in the rabbit population) bred hardy offspring more resistant to the agent (46). Natives of the New World were devastated by smallpox and measles, introduced by the European invaders, and those who remained were better able to survive infection (135); and the appearance in dolphins (12,251) and seals (177,258) of a morbillivirus related to canine distemper virus caused high initial morbidity, which now appears to be decreasing. It has been suggested that a contemporary example of such an evolving host–parasite relationship may be that between HIV and its human host. Many host gene systems are responsible for determining the ability of the individual host to respond to an infectious agent, and several have been characterized in experimental model systems; some of them are discussed later. In summary, the immune system has been shaped at least in part by the nature of the challenges it has faced. We cited some specific examples of such evolutionary sculpture, but can we discern a more global effect whereby different classes of organisms (e.g., viruses compared with bacteria) have caused the immune system to evolve in a particular way? We shall argue later that this is indeed the case; that the two different facets of the antigen-specific immune response (antibodies and T cells) have evolved to control infection, depending on the replication site of the infecting organism (i.e., whether it is intracellular or extracellular); and that the deciding factor in determining which component of the immune response will predominate (i.e., antibody or T cell) is which of the two antigen-presentation pathways the microbial antigens best can enter.

THE ROLES OF ANTIBODIES AND T CELLS IN THE CONTROL OF MICROBIAL INFECTIONS: POINTERS FROM EXPERIMENTS OF NATURE

Antibody responses have long been recognized as important indicators of recent virus infection. Historically, a patient with an acute illness had blood drawn (acute-phase serum sample), and at a later date, often 2 to 4 weeks later, after clinical recovery, a second sample was gathered (convalescent-phase serum sample). For various candidate viruses, the levels of specific antibodies in the two samples were compared, and an increase of more than fourfold was usually considered diagnostic of recent infection. Diagnosis of a current infection requires virus isolation and growth or, nowadays, polymerase chain reaction (PCR)-based identification. The ready detection of antiviral antibodies, and the demonstration that some of them were capable on inactivating the virus, led to the reasonable assumption that antibodies were important in combating primary virus infections. This position was proven by many studies that showed that several acute virus infections could be modified or prevented by transfer of serum from an immune source. Passive antibody therapy can protect against or modify the course of several human virus infections. For example, infusion of antibodies specific for the Junin arenavirus is beneficial in Argentinian hemorrhagic fever (42,130), whereas postexposure rabies prophylaxis relies on vaccination and concurrent administration of virus-specific immunoglobulins (Igs) (122). Furthermore, in experimental models, antibodies can reduce viral titers and modulate disease. For instance, recovery from ocular herpesvirus infection is hastened by administration of anti-HSV antibody (115). Similarly, the role of antiviral antibodies in vaccination has long been unquestioned. It is important to note that the studies cited earlier showed convincingly that antibodies are sufficient for protective immunity against some viruses; they did not show that antibodies are required. Nevertheless these findings, along with the excellent correlation between vaccine immunogenicity (as judged by induction of high levels of specific antibody) and vaccine efficacy (as judged by the vaccine's protective effect) has led some workers to the misguided conclusion that antibodies alone contribute to vaccine-induced protection. “Experiments of nature” in humans strongly suggest that, for some viral infections, antiviral antibody responses can be dispensed with at little cost to the host. For example, children born with genetically determined deficiencies in antibody production, with no detectable Igs, do not show an increased susceptibility to most viral diseases, with the exception of rare enteroviral meningitides, caused most often by picornaviruses such as coxsackievirus (58,76) and echovirus type 9 or 11 (134,142). Indeed the picornavirus family is the exception that proves the rule. Before the disorder was diagnosed, a patient with common variable immunodeficiency syndrome (a defect in antibody formation) was given oral (live) polio vaccine; the patient developed paralytic disease, and continues to shed infectious poliovirus many years later (102). In addition, coxsackievirus infection of mice lacking B cells leads to a lifelong chronic infection, confirming that antibodies

are required to control picornaviruses (136). However, for most viruses, the incidence of viral disease, and disease severity, are similar in antibody-deficient individuals and normal children. These data suggest that—even though they are capable of conferring antiviral protection—antibodies are expendable in resolving some virus infections. In contrast to their ability to clear virus infections, agammaglobulinemic children show a marked increase in susceptibility to bacterial infection (60,61). Similarly, children with genetic defects in components of the complement cascade (a final effector mechanism in some antibody-mediated responses) are less able to cope with bacterial, particularly meningococcal, challenge, but their resistance to virus infection and disease appears normal (133,176). Thus antibodies are required primarily to control bacterial, not viral, infection. In the absence of antibodies, the host has an alternative means of controlling viruses: T cells. That T cells can play a critical part in eradicating human viral infections is suggested by several observations. The frequency and severity of virus infections are markedly increased in humans with impaired T-cell responses [for example, in patients with Di George's syndrome (congenital thymic aplasia), AIDS, leukemia, or recipients of immunosuppressive therapy] (60). In many of these cases, however, there also is some impairment of antibody response, and so it is difficult to draw firm conclusions. In animals, models have been developed with clean mutations in various aspects of antibody or T-cell function; these are discussed later in this chapter. However, measles infection provides an excellent example of the importance of T cells in controlling virus infections in humans. In normal children, the disease is typified by the characteristic rash, and complete recovery is usual. In contrast, in T cell–deficient children, the disease is often fatal (64,148,221). The rash itself is T-cell mediated and does not develop in severely immunosuppressed children; indeed, the presence of a rash in an immunosuppressed child (e.g., in a child with leukemia who contracts measles) is considered a positive prognostic indicator (101). In agammaglobulinemic children, the rash develops normally, and the course of the disease is unaltered by the absence of antibody responses. Furthermore, agammaglobulinemic children are subsequently immune to measles; thus antibodies in this instance are required neither for control of the primary infection nor for resistance to secondary disease (61). In summary, as outlined at the beginning of this section, overwhelming evidence shows that antibodies can control many virus infections; nevertheless, they are sometimes expendable.

Thus although both antibodies and T cells play important roles in the control of virus infection, disruption of the former has less effect on the host's ability to control virus infections but dramatically reduces the host's capacity to counter bacterial challenges. What is the molecular explanation for this phenomenon? To understand this question, it is necessary to review how antibodies and T cells see virus antigens on the virus particle or at the surface of an infected cell and to describe in some detail how these virus antigens get there. We approach this issue in the form of an overview, and then describe in greater detail the individual genes and gene families involved.

THE ROLES OF ANTIBODIES AND T CELLS IN THE CONTROL OF MICROBIAL INFECTIONS: THE IMPORTANCE OF HOW ANTIGENS ARE RECOGNIZED

Antigen Recognition by Antibodies

Antibodies recognize antigen by using regions of hypervariable sequence. The antibody–antigen union has been subjected to crystallographic analyses (7,199), which indicated that the union is more “hand-in-glove” (in which components can, to some extent, alter their conformation to accommodate one another) than “lock and key” (in which both elements are fixed, each unable to modulate to the other). Antibodies recognize intact proteins. Thus antibodies can recognize free bacteria and viruses, as well as viral proteins (most often glycoproteins), expressed on the surface of infected cells. It is therefore easy to imagine how antibodies can play a major role in the control of viruses and bacteria, by inactivating them as free extracellular entities.

However, what happens when a microbe can evade antibody recognition by locating itself within a host cell? Few bacteria can accomplish this, but viruses are obligate intracellular parasites; their replication is intracellular, often relying on subversion of host machinery. How can antibodies exert an antiviral effect at this stage? One obvious mechanism is the recognition of viral proteins on the surface of infected cells. However, many viruses delay the display of cell-surface proteins until late in the infective cycle, by which time viral maturation may have occurred, and antibody-mediated cell lysis would simply release the infectious progeny.

How can a host detect an infected cell early in the infectious process, thus maximizing its immunological advantage? Here antibodies are less effective, being limited by their recognition requirements, whereas T cells play a critical role. T cells, as will be detailed later, can recognize almost any viral protein, including those made immediately on infection; when this early-recognition capacity is allied to the ability to destroy such infected cells, T cells become a formidable component of the antiviral armamentarium.

Antigen Recognition by T Cells

T cells can be subdivided by the surface marker proteins (CD4/CD8) they express. The majority of $CD8^+$ cells are cytotoxic T lymphocytes (CTLs), although some $CD8^+$ cells exert their antiviral effects mainly by cytokine release (204), whereas most $CD4^+$ cells are helper cells that secrete cytokines to assist B-cell maturation.

T cells recognize antigens via a cell-surface heterodimer, the T-cell receptor (TcR). This molecule is structurally reminiscent of the Fab portion of an antibody molecule, but the nature of T-cell recognition differs from that of antibody recognition in one critical aspect: whereas antibodies recognize antigen in isolation, T cells react to antigen in the form of a short peptide presented by a host glycoprotein encoded in the MHC. This phenomenon, named MHC restriction, was discovered by analyses of host responses to virus infection (285,286). There are two major classes of MHC molecule (class I and class II), and there is a tight relationship between the class of MHC/peptide complex recognized by a T cell and the surface marker (CD4 or CD8) borne by the T cell. MHC class I molecules are the “classic” molecules associated with graft rejection (the phenomenon that gave the MHC its name); they are expressed on most somatic cells [neurons being a rare exception (88)], and they interact with T cells bearing the CD8 surface marker. In contrast, MHC class II molecules, previously termed Ia or Ir (immune response gene) molecules, have a much more restricted expression, being found only on specialized antigen-presenting cells (APCs; e.g., macrophages, B lymphocytes, dendritic cells), and they

interact with T cells carrying the CD4 surface marker. The relationships between surface marker, MHC class, and cell function are summarized in Table 1. The CD4/II and CD8/I relationships are determined by direct interactions between the respective molecules: CD4 binds to a specific conserved region of MHC class II (109,256), and a similar interaction occurs between CD8 and class I (57,212).

	CD8+ T cells	CD4+ T cells
MHC class that presents the antigen to T cells	MHC class I	MHC class II
Cell types that bear the MHC molecule and thus can present antigen to the T cells	Almost all nucleated cells, with the exception of neurons	Specialized antigen-presenting cells (e.g., macrophages, dendritic cells, B lymphocytes)
Source of antigen recognized	Usually endogenous antigen, made within the presenting cell	Usually exogenous antigen, taken into the presenting cell by endocytosis
Such an antigen will be made during infection by	Intracellular organisms (e.g., viruses), a few bacteria (e.g., Listeria, Mycobacterium), and some parasites	Viruses, bacteria, and other parasites; infection results in microbial proteins being shed into the fluid phase
Effector functions	Usually cytotoxic, killing target cells. Other mechanisms of antiviral activity (e.g., cytokine released)	Usually provide "help" in the form of cytokines to B cells, thereby helping in antibody production

TABLE 1. *Recognition requirements and functions of CD4+ AND CD8+ T Cells*

So evolution has produced two classes of T cells, and the major feature distinguishing them is which of the two MHC/peptide complexes each can recognize. It is clear how T cells distinguish between class I and class II (using the CD4/8 proteins); but what is the importance of their being able to make this distinction? Presumably there is some vital and fundamental difference between the class I and class II complexes, and the ability of the immune response to detect and respond to this class I/class II difference is of prime importance.

At the target cell surface, class I and class II molecules are similar in overall structure. The molecules are heterodimers, with four domains: two Ig-like domains at the cell membrane and two distal domains. The class I heterodimer comprises the class I heavy (H) chain (three domains) closely complexed with a non–MHC-encoded protein, b_2-microglobulin (b_2M). Stable expression of the class I H chain on the cell surface usually requires b2M. The class II heterodimer consists of two similar chains, a and b, each with two domains and encoded in the MHC. For both class I and class II, the two membrane-distal domains together form a structure graphically described as a “Venus fly trap,” the “groove” of which binds the antigenic peptide in a sequence-specific manner and displays it on the cell surface for the perusal of T cells (18,19). The MHC molecule is hence said to “present” the antigenic peptide. MHC molecules (both I and II) are polymorphic, and many alleles exist in the population. Much of the polymorphism lies at or near the peptide-binding groove; as a result, different MHC molecules bind different peptides. Because MHC differs among different individuals, different people will present different parts (epitopes) of a given antigen (e.g., a virus) and therefore will mount T-cell responses of different epitope specificities. Thus there are many common features between class I and class II complexes: both have four domains, both have a groove that presents antigenic peptide, and both are polymorphic around this groove.

What is the evolutionary benefit of producing two such apparently similar molecular complexes? Presumably it relates to their interactions with the specific T-cell subsets. What, then, is the essential difference between the class I/peptide complex and the class II/peptide complex, and why is it important that T cells can distinguish between them? The critical difference between the two complexes lies not in the complex itself but instead in how the MHC/peptide complex reaches the cell surface. The two classes of MHC reflect two different antigen-processing pathways (described later), one of which (class I) is optimized to present intracellular antigen and the other (class II) to present extracellular antigen. Thus when T cells distinguish between a peptide/class I complex and a peptide/class II complex, they are really discriminating based on the source of the peptide: from protein made within the APC, or from protein taken up from the extracellular milieu.

ANTIGEN PROCESSING: CLASSICal AND NON-CLASSICal PATHWAYS

There are two “classical” pathways of antigen presentation: (a) the class I pathway, in which endogenous antigen (usually made within the target cell) is processed, eventually to appear as a peptide fragment in the class I groove on the cell surface, and (b) the class II pathway, in which exogenous antigen, usually soluble protein applied to the outside of the presenting cell, is internalized and eventually also finds itself presented on the cell surface in an MHC/peptide complex. The key here is the location of the antigen; endogenous antigens are presented by class I MHC, and exogenous antigens, by class II MHC. As with most generalizations, this division is not absolute; there are examples of *de novo* synthesized antigen that enters the class II pathway, and of protocols to introduce exogenous antigens into the class I pathway. By and large, however, this generalization holds. Figure 2 summarizes the two pathways. A cell is shown, bisected by a dotted line; this hypothetical cell expresses both classes of MHC (it could therefore be, for example, an activated macrophage or dendritic cell). Below and to the right of the dotted line, the class I antigen processing/presentation pathway is outlined, and above and to the left of the line, the class II pathway is shown.

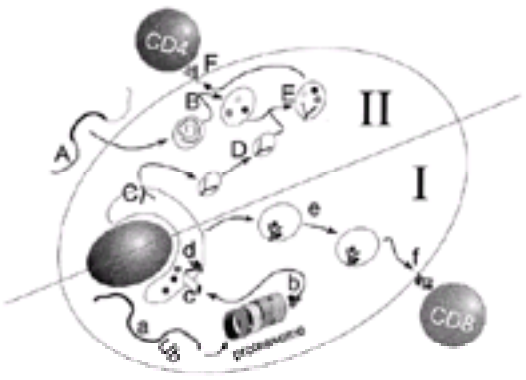

FIG. 2. Antigen-processing pathways. A cell capable of presenting antigen via both class I and class II pathways is shown, bisected by a line which separates the pathways. The letters identify various stages in the pathways, described in the text. A–F, class II; a–f, class I; Ub, ubiquitin (see text).

Antigen Presentation by Class I MHC

The thick line (a) in Fig. 2 represents a virus protein, newly synthesized in the cell cytoplasm. A certain proportion of all intracellular proteins are “tagged” for destruction by the covalent attachment of the 76-amino-acid cellular protein ubiquitin (Ub in Fig. 2). Ubiquitin covalently attaches, via its C-terminal glycine residue G76, to a lysine on the target protein; a second ubiquitin then attaches to a lysine residue on the first, and then a third ubiquitin attaches to the second, and so on. The doomed protein is therefore marked with a poly-ubiquitin chain, and this complex is taken to the proteasome (b), where it is degraded to generate shorter peptides (150). The ubiquitin system can be manipulated to increase protein degradation, resulting in enhanced induction of MHC class I–restricted T-cell responses (203,205,245,278). The peptides are next passed into the endoplasmic reticulum (ER) by a transporter mechanism, the TAP (transporter associated with antigen presentation) proteins (c), which belong to the ABC transporter family and are encoded within the MHC (100,186,249). Three peptides are shown in the ER, where they encounter the heterodimeric MHC class I complex (d) [comprising the class I protein and an accessory protein b_2-microglobulin (b_2m)]; one peptide is selected by the class I MHC heterodimer (on the basis of binding affinity in the MHC groove), and assembly of the class I/peptide complex occurs. These events have been recently reviewed in some detail (48,202). The complex passes into the Golgi apparatus (e), finally reaching the cell surface (f), where the peptide/MHC complex is recognized by a $CD8^+$ T cell–bearing TcR of the appropriate specificity. Note that peptides can reach class I molecules by a TAP-independent route, not shown in the figure; signal sequences, when clipped by signal peptidases in the ER, can be degraded, and the resulting peptides are available for MHC class I loading (73). For example, one of the CTL epitopes of lymphocytic choriomeningitis virus (LCMV) (272) is located within a putative signal sequence in the viral glycoprotein. Furthermore, recent work suggests that some class I proteins can present modified peptides (for example, glycopeptides) to T cells (230).

Antigen Presentation by Class II MHC

The thick line (A) in Fig. 1 represents a protein, but this time outside the cell. The protein is taken up by endocytosis and eventually reaches an acidic compartment, where it is degraded by proteases, generating peptides (B). Meanwhile, the class II molecule is synthesized within the ER (C) and is transported out through the Golgi/trans-Golgi (D). The class II vacuole fuses with the endosome containing the fragmented internalized protein (E), and a peptide is bound in the MHC class II groove, again selected on the basis of affinity. The mature complex is passed onto the cell membrane (F), where it is recognized by the TcR of a $CD4^+$ T cell. Note that the class II molecule in the ER (class II, C) does not bind peptides introduced by the transporters (class I, d) because the class II molecule in the ER is attached to another protein, the invariant chain (not shown), which prevents peptide binding. The invariant chain is lost as the class II molecule undergoes transport, so when fusion occurs with the endosome (class II, E), the class II molecule can bind to the peptide fragments therein (240). A recent review of presentation of exogenous antigen is available (156).

Biological Importance of the Difference Between the Two Classical Antigen-Presentation Pathways

From this it can be seen that T-cell recognition distinguishes between intracellular and extracellular sources of antigen; $CD8^+$ T cells, usually CTLs, recognize peptides from endogenously synthesized proteins, and $CD4^+$ T cells, usually helpers, detect peptides derived from exogenously applied proteins. Why is it important for the different T-cell subsets to respond differently, depending on the source of antigen? Organisms that replicate intracellularly (viruses, as well as some bacteria and other parasites) will feed antigens into the class I pathway, as well as into the class II pathway (when antigens are shed, e.g., during cell death). Conversely, obligate extracellular organisms (most bacteria) will be limited to introducing antigen mainly into the class II pathway. Therefore bacteria (extracellular) will induce mainly $CD4^+$ T-cell and antibody responses, but few $CD8^+$ T cells; whereas the response to viruses will include $CD8^+$ T cells in addition to $CD4^+$ T cells and antibodies. These considerations allow the experiments of nature to be viewed in a more molecular light. The absence of antibodies in patients with agammaglobulinemia will markedly reduce the host's ability to control bacterial infection, because this ability relies primarily on antibodies, and there is no “backup” available if antibodies are absent. In contrast, agammaglobulinemia will have much less effect on most antiviral responses because the $CD8^+$ T-cell response, effective against intracellular organisms, remains intact. Furthermore, if $CD8^+$ T cells have evolved in part to control infections by intracellular organisms, then the removal of the T-cell arm might be expected to most severely disable the host's capacity to combat such infections; this is the case. However, there are no clinical syndromes in which the class I presentation pathway alone is defective, and so it is difficult to infer how well antibody and $CD4^+$ T-cell responses could cope with virus infection in the absence of $CD8^+$ T cells. This question has been addressed experimentally in several transgenic mouse knock-out models. For example, mice lacking b_2M express little or no MHC class I on their cell surfaces

and, because $CD8^+$ T-cell maturation requires thymic expression of MHC class I, these mice develop very few $CD8^+$ T cells. Such mice mount extremely limited $CD8^+$ CTL responses, but develop readily detectable $CD4^+$ CTLs (80,146). The outcome of virus infection in these $CD8^-CD4^+$ mice varies with the virus used, but in general, there is delayed clearance of several viruses (50). These studies support the contention that $CD8^+$ CTLs play an important role in controlling virus infection, because their ablation reduces the host's ability to clear virus; however, the second pathway ($CD4^+$ T cells and antibodies) provides a certain degree of antiviral protection.

Thus there is an evolutionary relationship between the type of invading microbe, the MHC pathways that present its antigens, and the T cells that respond. Evolution appears to have honed the interactions to ensure that, upon infection, the immune response which predominates is that best suited to deal with the specific type of organism being faced. The fulcrum upon which this decision balances is the MHC antigen-presentation pathway. Most bacterial (extracellular) infections will present antigen through the class II pathway and in consequence will induce a predominantly $CD4^+$ T-cell and antibody response; antibodies can recognize intact antigen, and use various effector mechanisms (e.g., opsonization, agglutination, and complement fixation) to destroy the bacteria. In contrast, during bacterial infection, $CD8^+$ T cells, which recognize antigen only if associated with MHC class I, are infrequently induced and of limited value. Conversely, viral (intracellular) infections are susceptible to both antibodies and $CD8^+$ T cells, both of which are induced, and play important roles in recovery from infection. The explanation advanced earlier focuses on the induction phase of the immune response. However, the situation is not quite as straightforward as it at first appears. T-cell responses can be efficiently induced only by APCs, and the induction of virus-specific responses therefore requires that virus proteins be presented by the MHC class I pathway in this cell population. It has been argued that most viruses cannot infect specialized APCs, although in fact many types of virus can do so; for example, dendritic cells can be infected by retroviruses including HIV (179); RNA viruses such as measles virus (96), LCMV (20), and influenza virus (13); and DNA viruses such as pox viruses [e.g., ectromelia (232)] and African swine fever virus (65). However, many viruses do have quite tightly restricted tissue tropisms, and it appears unlikely that all viruses can infect APCs; how, then, can $CD8^+$ T cells be induced? Here we encounter the phenomenon of “cross priming,” in which exogenous proteins can be taken up by APCs and introduced into the MHC class I pathway. Several studies have clearly demonstrated that cross-priming can occur in specialized cells (201), and it has been suggested that cross-priming, far from being unusual, may be the major mode of induction of virus-specific $CD8^+$ T cells (29). Extending this cross-priming idea, it has been suggested that endogenous synthesis is important only in the recognition phase of the immune response, rather than in the induction phase (216). By this argument, antiviral $CD8^+$ T cells can be induced by exogenous protein taken up by specialized APCs (i.e., by cross priming). However, the vast majority of infected cells are not specialized APCs, and therefore cannot be “cross primed”; therefore, if the $CD8^+$ cells are to exert their effector functions, these cells must be able to present the internal antigens, and they do so via the pathway of endogenous synthesis. A recent study using transgenic mice provides strong support for the hypothesis (222), although it remains open to interpretation. Many questions remain about the true biological relevance of cross priming. For example, bacteria shed abundant protein into the extracellular milieu and are phagocytosed by APCs; if cross priming is so efficient, why do we not see brisk $CD8^+$ T-cell induction during bacterial infections?

Certain aspects of the $CD8^+$ CTL response appear particularly well suited to combat viral infections. First, the MHC allows the cell to present a fragment from a viral protein made at a very early stage of infection. Thus a cell can signal its infected status before the virus has had a chance to replicate, and lysis of the cell by CTLs is beneficial to the host, as potential virus factories are eliminated. In contrast to the ability of CTLs to recognize early transcribed (usually nonstructural) antigens, antibodies usually recognize viral cell-surface proteins, often made late in the viral life cycle during virus maturation. Second, $CD8^+$ T cells are more likely to be of direct antiviral benefit than are their $CD4^+$ counterparts because almost all cells [excepting neurons (88)] express class I molecules. Conversely, most cells are class II negative, and virus in these cells would be “invisible” to $CD4^+$ T cells. These evolutionary lessons have implications for the design of antiviral vaccines.

Non-classical Antigen-Presentation Pathways

The two classical pathways described earlier allow the host to present short peptides for the perusal of the cellular immune system. However, evolution often provides variety, and growing evidence points to the existence of alternative antigen-presentation pathways which expand the epitope universe against which the host can respond. For example, as described later, the MHC class I genes can be subdivided into two groups, class Ia genes (encoding the classical class I proteins discussed earlier) and class Ib genes which encode proteins similar to, but structurally distinct from, class Ia. Somewhat surprisingly, deletion of the class Ia genes does not completely eradicate the ability of the host to mount $CD8^+$ T-cell responses (261), and class Ia knock-out mice cope well with certain microbial challenges (213), in part because some of the class Ib proteins appear able to present modified peptide antigens to $CD8^+$ T cells (178). In mice, the class Ib MHC molecule H-2M3 is able to bind and present a peptide from the bacterium *Listeria monocytogenes* (178). It has been suggested that this molecule may present *N*-formylated peptides, characteristic of prokaryotes (110), but viral proteins too may be presented by class Ib MHC molecules (141). No equivalent of H-2M3 has yet been identified in humans. The class Ia and Ib genes are described in more detail later.

For many years, antigen presentation at the cell surface has been seen as the sole purview of the MHC locus. However, a fascinating new family of antigen-presentation molecules, not encoded in the MHC locus, has been identified. This CD1 family comprises proteins which are structurally related to classes I and II but, rather remarkably, present non-peptide antigens to T cells. CD1 glycoproteins are—like MHC class I—expressed on the cell membrane in association with b_2M but, like MHC class II, they find their way into acidic endosomal compartments where they encounter their non-peptide antigens (190). The antigens include lipids and glycolipids, which bind in the CD1 groove and are specifically recognized by $CD8^+$ T cells. CD1 proteins can present microbial antigens or self antigens. In the former case, glycolipids (from, for example, mycobacteria) are presented by CD1 to $CD8^+$ T cells bearing an apparently “normal” TcR, which confers dual specificity for the lipid antigen and the CD1 gene product (63). Certain CD1 proteins present a self molecule, glycosylceramide, which is recognized by the TcR of NK T (NKT) cells, an unusual class of mononuclear cell which expresses the NK1.1 surface marker (characteristic of NK cells) along with a TcR of very limited polymorphism (99,137).

Mouse model studies show that NKT cells may be important in tumor rejection (35), and similar cells have been identified in humans (155).

SOME HOST GENES INVOLVED IN THE IMMUNE RESPONSE

Current immunological mechanisms have evolved in response to the variety of pathogenic challenges imposed on their predecessors; implicit in this assumption is the existence of primordial genes from which present-day genes have evolved. Molecular analyses of the host genes described in the following section show that many of these genes are evolutionarily related, containing domains of similar protein folding. This has led to the inclusion of many of the genes in an Ig superfamily. The primitive gene(s) from which this superfamily has descended remains unidentified but presumably resided in a less complex organism, perhaps subserving an intercellular recognition function.

This Ig superfamily contains several families of proteins involved in the generation of an effective immune response. These include the class I and class II glycoproteins of the MHC; the CD1 glycoproteins, which present lipid antigens; the TcR proteins, present in the membranes of T cells and responsible for the antigen-specific recognition of peptide in the context of the earlier mentioned MHC and CD1 glycoproteins; proteins present in the TcR complex and necessary for T-cell function but which are not antigen specific; and of course, the Ig molecules themselves, the mediators of specific humoral immunity. Other genes, not members of the Ig superfamily but important to the immune response, also are discussed later. These genes encode the TAP transporter proteins, which lie in the MHC and are responsible for shuttling peptides from the cytoplasm into the ER, and proteins involved in antigen processing and cleavage.

The MHC Locus

The MHC loci of mice and humans, although not identical, bear strong similarities both in the overall organization of genes within the complexes and in the structure and sequences of individual genes. The murine MHC locus (on chromosome 17) comprises two regions (H-2 and Tla), whereas the human MHC locus (four megabases on chromosome 6, encoding >200 genes) is termed human leukocyte antigen (HLA). The loci have not been fully sequenced, and the cloning of the complete mouse MHC on a yeast artificial chromosome (4) doubtless will allow many interesting secrets to be unveiled. The genes within the MHC locus are divided into three classes on the basis of structure and function. The first two MHC classes, I and II, were introduced earlier and are described in more detail later. Although structurally unrelated to the class I and class II MHC genes, some of the additional genes within the MHC complex encode immunological functions (e.g., complement proteins, tumor necrosis factor, transporter proteins) and are loosely termed “class III” genes. The linkage of the class III genes to the class I/II families may be incidental or may reflect evolutionarily selected cosegregation.

MHC Class I Genes

Many texts refer to the MHC class I genes as a homogeneous group, but in fact, the group supports at least one further subdivision, into classes a and b. Class Ia proteins, by far the most studied class I molecules (and those referred to whenever the generic term class I is used) are the transplantation antigens, which led to the discovery of the MHC. These are the proteins that control MHC restriction, a phenomenon central to the specificity of cellular immune responses not only to transplanted tissues but also to virus-infected cells and tumor cells. Class Ib proteins are structurally similar to the class Ia molecules, and their functions are now being revealed. The numbers of both class Ia and class Ib genes vary between different individuals.

Three class Ia loci have been defined; K, D, and L in mice and A, B, and C in humans. The precise number of genes at each locus varies (e.g., some mouse strains lack an L gene locus; some strains have four D genes, whereas others have a single D gene), which is consistent with the occurrence of gene duplication/deletion events. Sequence homology occurs among these genes both within and between species, suggesting evolution from a common ancestor. The interspecies sequence similarities extend to the functional level because a human HLA gene expressed in a transgenic mouse appears to function similarly to the endogenous murine proteins. All share a genomic layout of eight exons that undergo splicing to generate a mature messenger RNA encoding the class I molecule, a glycoprotein of 350 amino acids. This molecule has five domains: three extracellular (a1, a2, and a3), one transmembrane, and one cytoplasmic. The class I molecules are expressed on the cell membrane in association with b_2M, a 12-kd protein encoded outside the MHC. The crystallographic structure of the MHC class I molecule has been solved. The a3 domain and the b_2M molecule, both of which are evolutionarily related to the constant region of Ig, are in intimate contact with the cell membrane, whereas the a1 and a2 domains interact at the top of the molecule to form the groove that presents the short peptides, most often nonameric, to $CD8^+$ T cells. Class I molecules are found in the membrane of almost all nucleated cells, which are therefore presumably able to present antigen. Neurons, an exception to this rule, transcribe b_2m but fail to transcribe the MHC heavy chain (88).

The peptides presented by class I MHC can originate from almost any protein synthesized within the cell, including self, tumor, and microbial proteins. It has been estimated that a single cell can display from 1,000 to 10,000 different peptides at any one time (84) and that $CD8^+$ T cell induction most often requires a threshold presentation of at least 100 MHC/peptide complexes per cell (31), although one report suggests that T cells can be activated by a single MHC/peptide complex (238). Essentially all viral proteins (including nucleoproteins, polymerases, and glycoproteins) can provide sequences to be presented to the cellular immune system. This provides a marked advantage to the host by allowing the entire coding region of the virus genome to be exposed to immune surveillance. Furthermore, the system allows presentation of virus antigens expressed early in infection. These often are non-membrane proteins, “invisible” to antibodies, and the MHC system thus provides a selective advantage to the host by permitting detection and removal of infected cells before virion particles have been produced. Analyses of MHC structure/function relationships provide a molecular explanation for the variability of T-cell responses in different mouse strains and in different individuals in a random-bred (e.g., human) population. The MHC class I proteins are highly polymorphic (i.e., there are many functionally distinct alleles at each locus). Sequencing studies of many different alleles show that the variable residues cluster around sites close to the antigen-presenting groove. Thus the functional polymorphism is caused by alterations affecting

the nature of antigen binding and presentation by individual MHC molecules; different MHC molecules will bind and present peptides of different sequence. Hence, one variable in the immune response is the ability of the host class I molecule to bind microbial sequences and present them to the T-cell repertoire. If a host, when challenged with a particular virus, lacks the class I sequences necessary to bind and present viral peptides, then that host will be unable to mount class I–restricted CTL responses, and therefore will probably be more susceptible to disease caused by the agent in question. In contrast, a host whose genotype encodes MHC molecules capable of binding and presenting a peptide from this particular virus is more likely to be able to mount a CTL response that will limit viral replication, dissemination, and virus-induced disease. Given the risk of failure to present, one might ask why we have not retained more than the six or so MHC class I alleles in each diploid host cell; if an individual could instead express, for example, 20 class I alleles, the risk of being unable to present epitopes from any microbe would be greatly reduced. The reason is straightforward. Some 98% of lymphocytes entering the thymus fail to develop into mature T cells, and a major cause of this attrition is negative selection, in which T cells with excessive affinity for self molecules (i.e., for MHC molecules associated with self peptides) are deleted. Thus the more MHC alleles we carry, the more self peptides we can present; and as more self peptides are presented, so we clonally delete more T cells in the thymus. With our present complement of MHC alleles (about six per person), we already delete some 98% of all of our T-cell specificities; increasing our MHC representation would most probably reduce our T-cell repertoire below effective levels. We are, therefore, in a state of equilibrium: our T-cell repertoire is balanced against our ability to present antigen for the perusal of T cells. We must present just the correct number of antigens; too many (too many MHC alleles) would decimate our population of mature T cells, whereas too few (too few MHC alleles) would increase the risk that we would be unable to present antigen to our T cells.

Early attempts to predict viral (or other) sequences that might bind class I MHC molecules were not consistent and thus were unsuccessful, probably because they attempted to predict the viral epitopes without taking into consideration the specific polymorphism (i.e., allelism) of the MHC molecules. However, two factors allowed more consistently correct predictive motifs to emerge for specific MHC alleles. First, the increasing database of CTL epitopes for the individual MHC alleles allowed comparison of the peptide sequences they presented, and allele-specific consensus sequences could be deduced. Second, work by the Rammensee and Nathenson groups showed that peptides can be eluted from MHC molecules and their sequences inferred or directly determined (84,207,208,251). By a combination of these two approaches, specific motifs have been identified for several murine and human MHC class I alleles. Most, but not all, peptide epitopes are nonameric, with two highly conserved anchor residues critical for binding of the peptide to the MHC molecule. The MHC class I/peptide complex has been studied at high resolution, allowing the detailed analysis of the interaction between MHC class I molecular grooves and the peptides found therein (18,19,51,132,277). The MHC groove contains several binding pockets, the most critical of which is that which binds the C-terminal residue of the peptide. The MHC residues that form this binding pocket have been identified, and their nature allows fairly confident prediction of which C-terminal residues will be favorable for a particular MHC pocket. Because the polymorphism of the amino acids forming this MHC pocket is fairly restricted, so too are the C-terminal amino acids of the binding peptides. Thus the great majority of peptides have as their C-terminal residue one of seven amino acids (I, L, V, R, K, Y, or F). Another pocket, located in the middle of the groove, appears important in determining allele-specific binding (i.e., in conferring fine specificity for specific peptide sequences) (132).

The “non-classical” class Ib MHC molecules are less well studied, but their functions are being slowly uncovered. In humans, four genes were identified and named HLA-E, -F, -G, and -H. In mice, three class Ib gene clusters, named Q, T, and M, have been found, but remain incompletely characterized. HLA-H is now known to be the gene related to 90% of cases of hereditary hemochromatosis (HH), and the HLA-H designation has therefore been replaced by HLE, the original name given to the gene conferring HH susceptibility; the protein is topologically similar to class I MHC, but its groove is too narrow to bind peptide. Thus it appears that this class Ib gene subserves no immunological function. The tissue distribution of HLA-E is similar to that of the class Ia proteins—that is, almost ubiquitous. Intriguingly, expression of this protein on the cell surface is greatly enhanced when its groove is loaded with a nonameric peptide derived from the leader sequence of class Ia molecules, ensuring that its surface expression is coordinately regulated with that of the classical molecules. In contrast to the groove of class Ia proteins, the HLA-E groove is relatively non-polymorphic, consistent with its role in binding a nonameric sequence which varies only slightly among the different class I leader sequences. HLA-E has been implicated in regulation of NK-cell activity (23), and it is possible that the very limited polymorphism allows it to interact with subtly different receptors on the NK cells (NK receptors are discussed later in this chapter). HLA-G has been implicated in antigen presentation, but it differs from other class I molecules in its distribution, being found mainly at the maternal/fetal interface; it has been implicated in protecting the fetus from rejection (113,116). The function of HLA-F remains obscure.

MHC Class II Genes

The existence in the mouse MHC of genes that regulate the humoral responses to antigen was clearly demonstrated more than 30 years ago. They were initially termed immune response (Ir) genes, subsequently termed Ia genes (easily confused with class Ia), and both terms have now been replaced by the term class II MHC glycoproteins. The class II locus of humans is termed HLA-D; and in mice, H-2I. Like their class I counterparts, the overall structure and function of class II genes appear similar in both humans and mice. Mature class II molecules are membrane-bound heterodimers resulting from the association of two MHC-encoded class II proteins, an a chain and a b chain (Fig. 3). Six a and seven b genes have been identified in the HLA-D region, divided into three clusters termed DR, DQ, and DP. Murine equivalents are present in the H-2 I region: two a and five b genes divided into two clusters termed A and E. The genes encoding the a and b chains are similar, although some variation between exon numbers is seen, even within a chain type. The resultant a or b proteins have two extracellular domains, a transmembrane region and a cytoplasmic domain. The top (membrane-distal) domain is named a1 (b1), whereas the lower one, adjacent to the membrane, is termed a2 (b2). The membrane-proximal paired a2 and b2 domains share significant homology with the class I a3/b $_2$M pairing, indicating once again common ancestry and possible functional relatedness. Indeed, there are many similarities in the overall topology of the two cell-surface MHC classes. Both classes have four extracellular domains; both the membrane-proximal domains are similar to Ig constant region, and the two distal domains interact to provide a groove for antigen presentation. The location of disulfide bridges is similar. The crystallographic structure of the class II molecule has been solved and appears similar to that of the class I heterodimer (25). Peptides bound by class II molecules are derived from a different processing pathway (see earlier) and are longer, usually between 15 and 20 amino acids. Unlike the class I peptides, in

which the C-terminal residue is firmly anchored, class II–bound peptides protrude at both ends of the groove. Some motifs have begun to emerge for peptide binding to specific class II dimers, but this work lags behind the class I studies (210).

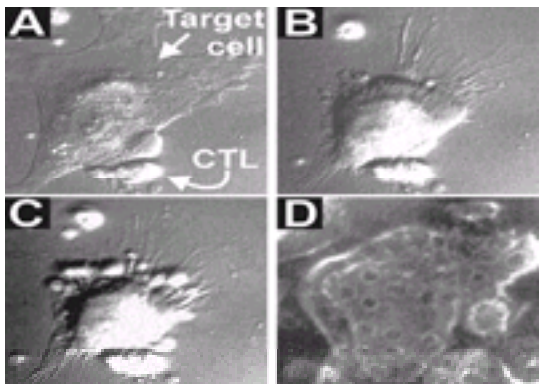

FIG. 3. Cytotoxic T lymphocytes (CTLs) kill target cells and insert perforin pores into the target cell membrane. Video-capture photographs (**A–C**) and an electron micrograph (**D**) are shown. **A:** A CTL encounters a target cell expressing an appropriate peptide/class I major histocompatibility complex (MHC), and within minutes the target cell begins to contract (**B**), and to show extensive blebbing of the cytoplasmic membrane (**C**). The CTL releases perforin, which self-assembles within the target cell cytoplasmic membrane, generating pores (visible in **D**), which permit entry of effector molecules, and also cause the cell to become hypoosmolar.

The polymorphism of class II molecules varies from gene to gene; for example, DQ a1 is highly polymorphic, DQ a2 much less so. However, the low polymorphism at certain loci is offset by the ability of class II genes to reassort. Thus in theory, the haploid genome encoding six a and seven b genes might make 42 different class II molecules, although in practice, reassortment is not entirely unrestricted. Reassortment cannot, of course, operate for the class I molecules because the heterodimer includes one monomorphic molecule (b_2M). The tissue distribution of class II molecules is markedly more restricted than that of class I. They are most abundant on B and T lymphocytes and cells of the macrophage/monocyte lineage. The class II/peptide complex is recognized by T cells bearing the CD4 surface marker; these cells are most frequently helper T cells rather than cytotoxic T cells.

CD1 Genes

The CD1 family encodes five proteins in humans (CD1a–CD1e), and the human CD1d gene has two homologs in mice (mCD1d1 and mCD1d2) (184). The intron/exon organization of these genes is similar to the MHC class I genes, and crystallographic analyses of the mouse CD1d1 protein revealed a strong similarity to the classical MHC structures, although the binding groove is deeper, presumably to accommodate the non-peptide (usually lipid/glycolipid) antigen described earlier (282). The tissue distribution of the CD1 proteins is severely restricted; like MHC class II genes, most CD1 proteins are expressed in professional APCs including B lymphocytes, macrophages, and dendritic cells.

TcR Genes

TcR genes belong to the Ig superfamily. Antigen specificity of T lymphocytes is bestowed by a heterodimeric surface-bound glycoprotein, the TcR. Four distinct genomic regions encode different TcR chains, which have been named a, b, g, and d. The great majority of circulating T cells, regardless of their helper/cytotoxic phenotype or MHC restrictions, bear ab receptors; gd receptor–bearing T cells have been identified quite recently, are far less well characterized, and are not discussed further in this chapter. The TcR genes are, like the Ig genes, products of somatic rearrangements that appose noncontiguous germline gene segments. The mature gene usually results from joining of V, D, J, and C regions; each chain type draws from its own pool of germline segments. The germline copy number of each of these regions varies among the chains but appears sufficient, when both combinatorial rearrangement and other V-region diversification mechanisms are invoked, to provide a TcR repertoire at least as large as that of antibodies. The mature TcR chains are glycoproteins of 260 to 300 residues in length, comprising a short cytoplasmic tail, a transmembrane region, and two extracellular domains, one constant and one variable. The antigen-recognition site, which recognizes the complex of antigenic peptide and MHC, thus consists of the Va and Vb regions. The crystallographic structures of several TcR/MHC class I/peptide complexes have been solved, and show that the TcR lies diagonally across the MHC/peptide complex; there is a degree of plasticity in the interaction, in which the TcR can to some extent conform to the peptide/MHC with which it interacts (55,56). In overall structure, the TcR molecule is similar to the distal (Fab) portion of an antibody molecule. However, the TcR differs from antibodies in several aspects. First, the effector function of an antibody is imposed on it by the constant region used in its generation, and there are multiple nonallelic constant-region genes to which the antibody-variable regions can be linked. In contrast, the copy number of TcR constant-region genes is very low, and the same C regions are represented in both CTLs and T helper (Th) cells. Thus the TcR C region does not dictate the T cell's effector function. The same Va and Vb gene pools are used in both class I and class II restricted T cells, indicating that the TcR does not fundamentally distinguish between the two classes of MHC molecule. Rather, the TcR recognizes the antigen/MHC complex, and other T-cell components determine the MHC class specificity (CD4/class II and CD8/class I) and the effector function (helper/cytotoxic) of the cell.

How restricted at the molecular level are T-cell responses to virus infection? This has been studied at the level of *in vitro* specificity of T-cell lines and clones, but much more rigorous analysis can be achieved by studying the TcR sequences of individual clones. This allows us to ask if responses to immunological stimuli are monoclonal or polyclonal, which is interesting not only at an academic level, but also at a practical one. If responses are monoclonal (i.e., if all TcRs are identical), then it may be possible to ablate specifically cells bearing that TcR, thus potentially diminishing

immunopathological effects related to the antiviral immune response. In contrast, if such responses are polyclonal, such intervention is likely to be difficult and unrewarding. The VDJ gene use in TcRs of cells responding to a variety of stimuli have been determined. In general, response to certain model antigens, such as cytochrome c, have shown quite restricted VDJ uses, but responses to virus infection have shown much broader use (79,279). Thus it appears unlikely that TcR-specific ablation will be a valuable therapeutic approach in control of virus-induced pathology. The catholic use of Va and Vb chains during acute infection extends also to the memory response. Certain T cells in an acute response will act as progenitors of the memory T-cell pool (175), and the selection process appears to be stochastic (228).

T-Cell Accessory Molecules

At the T-cell surface, both ab and gd receptors are intimately associated with several other proteins, collectively referred to as the CD3 complex. The CD3 complex comprises at least four transmembrane proteins that relay a signal across the T-cell membrane when the TcR is appropriately stimulated. Also on the T-cell surface, although not stably linked to the CD3/TcR complex, are the CD4 and CD8 molecules, which play critical roles in T-cell function. These nonpolymorphic glycoproteins, again members of the Ig superfamily, are differentially expressed during ontogeny, but mature circulating T cells generally express only one of the two molecules. There is a very strong correlation between the CD4/CD8 characteristic of a T cell and the MHC class that presents the antigen to that T cell. Thus almost all $CD4^+$ T cells are class II MHC restricted, whereas $CD8^+$ cells are class I restricted. There also is a correlation, although less absolute than the earlier one, between the T cell's CD4/CD8 characteristic and its effector function; most $CD4^+$ (class II–restricted) T cells are of the helper phenotype, whereas most $CD8^+$ (class I–restricted) T cells are of the cytotoxic phenotype. The CD4/CD8 molecules recognize specific regions of the MHC glycoproteins and may play a role in activating the transmembrane signal relayed by the CD3 complex (57). However, the outcome of signal transduction (help or cytotoxicity) is independent of the nature of the CD4/CD8 molecule or TcR; thus there are examples of $CD4^+$, MHC class II–restricted cytotoxic T cells. Interestingly, in animal model systems, these cells appear to modulate the outcome of virus infection, despite their presumed inability to recognize the great majority of infected cells (which will be class II negative) (39,80,146).

Immunoglobulin Genes

The humoral response to virus infection plays an important role not only in promoting clearance of primary infection but also in preventing the establishment of widespread infection on secondary exposure to the agent. All antibodies have a similar overall monomeric structure, comprising two disulfide-linked identical heavy (H) chains, each of which is flanked by a light (L) chain. The centrally located H chains usually have four domains: three constant and one N-terminal variable. The L chains contain one constant domain and one variable domain. The variable regions of each L+H pair combine to form a single antigen-binding site: an antibody monomer is thus divalent in terms of antigen recognition. The L proteins are segmentally encoded in the germline, as variable (V), joining (J), and constant (C) regions, and are linked by somatic recombination. The H proteins are similarly arrayed, although they also have diversity (D) regions. Variability at the antigen-binding site is brought about by a variety of mechanisms during somatic recombination (as for TcR). The rearranged VDJ region is then apposed to one of the constant regions; the nature of the constant region determines the class (and therefore the function) of antibody that results. There are five general classes of antibody, differing in function, time of appearance, and distribution in the body. Structurally, the classes differ in the constant region of their H chains. The classes are IgM (H chain is Cμ), IgG (Cg), IgD (Cd), IgE (Ce), and IgA (Ca). First exposure to a virus induces a rapid IgM response, which is usually independent of Th cells, and a slightly delayed IgG response, usually Th dependent and thus requiring antigen to be presented via MHC class II molecules.

In contrast, secondary exposure results in a similar IgM response but a much more rapid and massive IgG response. In many instances, the precise specificity of the secondary IgG antibodies is identical to that of the initial IgM molecules: that is, the antigen-binding sites have been retained, but the remainder of the molecule (which determines its effector functions) has been altered. This “class switching” occurs when, for example, an H chain VDJ region is moved from the Cμ gene to the Cg region; the new H chain has the same V region but a different C region. Two of these chains, in association with two unchanged L chains, generate an antibody identical to the original in specificity but different in effector function; it is now an IgG rather than an IgM. Initially, all B cells express membrane-bound IgM, but on exposure to specific antigen, they develop into plasma cells, usually undergoing class switching. The five classes of antibody subserve distinct effector functions, but only three are thought to play a role in opposing virus infection. IgM molecules form pentameric masses, each with 10 antigen-binding sites. These molecules are effective mediators of virus aggregation; they also activate both macrophages and the complement system. IgG, the main serum antibody, is monomeric, activates macrophages and complement, and can cross the placental barrier. IgA can exist as monomer, dimer, or trimer; it is transmitted to luminal surfaces (in saliva, tears, and intestinal and other secretions) to provide an initial defense against infection. IgE may play a role in immunity to parasite infection, but its major recognized task is the initiation of many allergic reactions, acting through histamine release from mast cells. The IgE-mediated release of histamine and other factors probably contributes to some of the respiratory manifestations of virus infection. The function of IgD remains obscure.

Genes Involved in Antigen Degradation in the MHC Class I and Class II Pathways

Many (but not all) of the peptides that eventually encounter MHC class I are generated by the proteasome, a multiprotein cytoplasmic organelle, the constitution of which is regulated by interferon g (IFN-g); this cytokine induces expression of three proteins, LMP (low-molecular-weight polypeptide)-2, LMP-7, and MECL-1 (multicatalytic endopeptidase complex-like), which replace three constitutive catalytic components of the “normal” proteasome, generating the “immunoproteasome” (52). Whereas MECL-1 is encoded outwith the MHC, both LMP-2 and LMP-7 are encoded next to the TAP genes, and should be considered MHC class III genes. Although several genes related to class I antigen presentation are MHC encoded, this is not true for some important genes related to the class II pathway; in both man and mouse, several genes encoding cathepsins [proteases vital to antigen degradation in the class II pathway (252)] are scattered throughout the genome (38,217).

Genes Involved in Peptide Transport and in Assembly of the Peptide/MHC Complex

Two TAP transporter genes are encoded in the MHC, called TAP-1 and -2; one copy of each combines to form a functional heterodimeric transporter. The role of these proteins in antigen presentation has been clearly demonstrated (247). Cell lines defective in transport are unable to present endogenously synthesized antigen in a normal fashion, but when the defective TAP is restored, antigen presentation proceeds (8,189). *In vivo* studies confirm these observations: TAP knock-out mice exhibit the expected phenotypes, having little or no cell-surface MHC and no mature $CD8^+$ T cells (253). There is some selectivity in the peptides transported by these molecules, as demonstrated in the rat; the class I modifier (cim) locus contains (at least) two genes (named mtp-1 and mtp-2), the latter of which has (at least) two alleles that result in presentation of different peptides by identical MHC molecules. The cim locus encodes a transporter, and the two alleles permit the passage of different peptides, thus providing the MHC class I molecules in the ER with different peptide populations from which to select (124,125,187,188). Direct evidence has been obtained showing that different transporter proteins display preferences for specific peptide sequences (153); one cim allelic product could transport peptides terminating in H, K, or R, whereas the other could not (70). Allelism has been identified in humans by sequencing the human TAP equivalents (186). Thus although the major contributor to epitope selection probably remains the class I molecule, the transporter proteins may play some role in limiting the range of peptides to which the class I molecules are exposed. However, this role is likely to be minor, for several reasons. First, many cell lines have been transfected with non-homologous MHC alleles, but these MHC molecules continue to present the same CTL epitopes as they did in their own background, suggesting that there is no major difference in the peptide pools generated in the differing backgrounds. Second, transporter heterodimers in which one component is murine and the other human appear to function the same as either of the natural heterodimers (280). Within the ER, several proteins are important to assembly of the class I/peptide complex (48). Four are quite well characterized: tapasin, which ensures association between the TAP transporter complex and the empty MHC-b_2M heterodimer, is encoded close to the MHC locus (75), whereas the genes encoding three chaperones (BiP, calnexin, and calreticulin) are not MHC linked.

CELL-MEDIATED EFFECTOR MECHANISMS

We have outlined our current understanding of immune responsiveness at the molecular level. In the following section, we discuss immune responsiveness at the organismal level, describing the biological roles of several different cell types and the effector mechanisms through which they act.

Virus-Specific $CD8^+$ T Cells

These cells have been extensively characterized in the mouse, and the results equate well with those obtained with human cells. First, the vast majority of these cells are restricted by class I MHC proteins, although a few have been identified that are restricted by class II or by CD1. The importance of these cells in the control and eradication of virus has been documented for several experimental infections. In the mouse, they are critical in combating infection with LCMV (272). In this case (105,106) and in murine cytomegalovirus (MCMV) infection (92), it is possible to fully protect a naive animal from virus challenge by immunization with a recombinant vaccinia virus (VV) expressing a single viral internal (i.e., nonmembrane) protein; the protection is mediated solely by $CD8^+$ T cells. Recombinant vaccines containing minigenes encoding isolated CTL epitopes as short as 11 residues can confer protection against normally lethal doses of challenge virus, and different epitopes can be linked on a “string of beads” to protect on several MHC backgrounds (5,6). No LCMV-specific antibody responses are induced by these vaccines, proving that protective effects can be mediated by cellular immune responses. In humans, it is clear that CTL responses can be generated against similar proteins. For example, the major CTL response to human cytomegalovirus (HCMV) is to a protein expressed immediately upon infection; a similar situation exists for varicella-zoster virus (VZV) and herpes simplex virus (HSV). Epstein–Barr virus (EBV) infects and transforms human B lymphocytes, and the control of this cell population appears to be managed in large part by class I MHC–restricted CTLs. Indeed some immunosuppressed individuals may develop EBV^+ lymphomata (198). Influenza virus infection induces CTLs directed against most viral components, but the major group is specific for the virus nucleoprotein. These CTLs, unlike anti-influenza antibodies, are cross-reactive; that is, they lyse HLA-matched target cells infected with a serologically distinct strain of influenza virus. However, their presence fails to confer absolute immunity to infection and disease caused by a serotypically distinct influenza virus. These results are sometimes used to challenge the hypothesis that the presence of virus-specific $CD8^+$ cells confers protection against virus-induced disease. It is important to understand that $CD8^+$ T cells cannot prevent infection; on the contrary, recognition by these cells requires that the target cell be infected. Only antibodies can prevent infection, whereas $CD8^+$ T cells limit virus production and dissemination; together they protect against disease. Thus the preexisting antiinfluenza CTLs, although failing to prevent infection, may diminish the ensuing morbidity (disease) and mortality (death). CTLs also have been found against measles virus, mumps, respiratory syncytial virus (RSV), and other agents. In patients with AIDS-related complex and AIDS, CTLs are detectable against gag, pol, and env proteins. CTLs to env correlate with clearance of initial viral load, and their absence heralds a return to high viral titers and AIDS. Antigen-specific recognition mediated by the TcR initiates a series of events including signal transduction across the cell membrane, which activates the $CD8^+$ T cell's effector functions. Several mechanisms have been proposed to explain the *in vivo* antiviral effects of virus-specific $CD8^+$ T cells.

CTLs Usually Release Perforin, a Pore-Forming Protein

CTLs contain granules, which align with the target cell upon recognition, and whose contents are released in a calcium-dependent manner onto the target cell membrane. These granules include a protein called perforin (183), which undergoes assembly into trans-membrane pores and thus punches holes in the cytoplasmic membrane of the target cell. Perforin shares immunological cross-reactivity with the C9 component of complement, and cloning of the murine perforin gene has allowed identification of a short stretch of amino acid homology between the two proteins. The membrane lesions caused by perforin are similar to those induced by the complement C9 complex. Thus CTLs and complement-mediated lysis seem to share one common mechanism of action. The importance of perforin in CTL activity *in vivo* has been demonstrated. Transgenic mice with a dysfunctional perforin gene are much less effective in controlling

infection by some (though not all) viruses (94,95,262).

CD8+ T Cells Can Induce Apoptosis in Target Cells

Apoptosis, or programmed cell death, is a well-recognized phenomenon responsible for several developmental processes, including the clonal deletion of T cells in the thymus. Its most characteristic features are nuclear blebbing and disintegration, resulting in a nucleosome stepladder of fragmented DNA. $CD8^+$ (and sometimes $CD4^+$) T cells can induce apoptosis when the fasL protein, expressed on the T-cell membrane (237), interacts with Fas protein on the target cell, initiating a signaling cascade which ends in target cell apoptosis. Virus-specific T cells may induce this process in infected target cells (220,269,287). A graphic representation of CTL–target cell interactions is provided in Fig. 3. In panel A, the CTL (small cell, bottom of panel) has just encountered a larger target cell expressing the appropriate peptide/MHC class I complex. Within minutes, the target cell contracts (B), and extensive membrane blebbing occurs (C). An electron micrograph shows the target cell membrane studded with pores, after CTL contact and perforin release (Fig. 3D).

CD8+ T Cells Release Antiviral Cytokines

Many $CD8^+$ T cells are capable of releasing high levels of cytokines, for example, interferon-g (IFN-g) and tumor necrosis factor-a (TNF-a). Mice lacking the IFN-g receptor have increased susceptibility to several infections, despite apparently normal CTL and Th responses (83). It has been cogently argued that a major role of the TcR/MHC/peptide interaction is simply to hold $CD8^+$ T cells in the immediate proximity of virus-infected cells, thus focusing cytokines on the infected cell (193,209), and convincing data from mice persistently infected with LCMV (168,242), from hepatitis B virus (HBV) transgenic mice (66,67), and from HBV-infected primates (68) has shown that viral materials can be eradicated *in vivo* from neurons (168,242) and hepatocytes (66,67) in the absence of cytolysis. We have recently shown that, even at the peak of the immune response, the great majority of virus-specific $CD8^+$ T cells are not producing cytokines; however, these virus-specific cells are exquisitely sensitive to antigen contact, initiating cytokine synthesis immediately upon encountering an infected cell, and terminating production the instant the contact is broken (225). This tight regulation is important because cytokines can be toxic to the host, and indeed, systemic cytokine release is responsible for many of the symptoms of microbial infection, and for the extensive weight loss seen in certain cancers.

Natural Killer Cells

Although our understanding of NK cells is in its infancy compared with that of T cells, significant advances have been made in the last 2 years (195). NK cells play an important role in the control of certain virus infections, as established by clinical studies in humans and experimental manipulations of mice. NK cell development normally occurs extrathymically; these cells are not antigen specific and therefore do not exhibit immunological memory. NK cells carry several non–antigen-specific receptors, through which they can be activated by a variety of mechanisms (112), and they kill susceptible target cells by perforin-mediated lysis. In addition to receptors through which they are activated, NK cells carry inhibitory receptors whose engagement with a specific molecule on a non-susceptible target cell *prevents* the NK cell's effector function. What might this inhibitory ligand be? Early work showed that NK cells had somewhat varied specificities (270) and could very effectively lyse target cells lacking MHC class I (265). Incubation of target cells with IFN-g increased their sensitivity to CTL, but diminished their sensitivity to NK cells (268). In 1990, the “missing self” hypothesis was proposed, which argued that NK cells are inhibited by the presence of self MHC class I, and that the absence of such molecules permits target cell lysis by NK cells (126). This is an intrinsically attractive idea, for the following reason. As stated earlier, MHC class I is vital to the control of most virus infections (and also for eradication of many tumors), and one might imagine that viruses (or tumors) that could down-regulate MHC expression would be at a selective advantage. However, such down-regulation, while protecting the cells from $CD8^+$ T cells, would make the cells more sensitive to NK-mediated lysis. Thus NK cells and antigen-specific $CD8^+$ cells together span the MHC expression spectrum.

Early studies of NK receptors and ligands were somewhat confusing, showing that only certain class I MHC molecules could inhibit recognition by certain NK cells (i.e., there was some form of MHC restriction, albeit different from that involved in T-cell recognition). These studies have been borne out, and greatly extended, by the identification of three families of inhibitory receptor proteins on NK cells, and of the target molecules with which they interact. The three NK inhibitory receptor families are (a) the CD94-NKG2 family (common to humans and rodents), (b) the Ly49 family (only in rodents), and (c) the KIR family (killer inhibitory receptor, only in humans) (112).

The first two families are members of the C-lectin superfamily and lie in an “NK complex” on mouse chromosome 6 or human chromosome 12. Despite being in the same superfamily, these two families are quite distinct. The CD94-NKG2 proteins are expressed on the NK cell surface as heterodimers of the monomorphic CD94 molecule (encoded by a single gene in the NK complex) along with one of the products of the four NKG2 genes.

In contrast, the mouse Ly49 locus contains at least nine genes, and the protein products are expressed on the cell surface as homodimers. As yet there is no evidence to suggest that different Ly49 alleles can interact to form a heterodimer, but the polymorphism of the Ly49 homodimers is increased by alternative splicing.

The KIR family is a member of the Ig superfamily, and probably includes about 12 genes; mouse equivalents have not been found. The target cell ligands with which these receptors interact are varied.

1. CD94-NKG2 receptors in humans interact mainly with the MHC class Ib molecule HLA-E (23) which, as described earlier, carries in its groove a peptide from the leader sequence of classical class I MHC proteins. Because the leader peptides vary slightly, it is possible that each different peptide/HLA-E combination is recognized by only one of the four NKG2 gene products (in association with CD94). In the majority of cases, these interactions inhibit NK cell activity (although, to complicate matters, there are reports that certain interactions can stimulate NK effector functions).

2. At least some of the Ly49 proteins interact with sites on the a1 or a2 domains of classical MHC class I molecules in mice. For example, Ly49A recognizes H-2D^d and H-2D^k, whereas Ly49C may recognize several ligands on the H-2b and H-2d backgrounds (24). The importance of MHC-bound peptide in these interactions is somewhat unclear.

3. Most of the KIR proteins interact with the a1 domain of the classical HLA class I molecules HLA-A, -B or -C, and some reports suggest that the MHC-bound peptide may alter NK recognition. Recent data (191) suggest that one of the KIR proteins is the NK receptor that interacts with HLA-G, which, as stated earlier, is expressed extensively at the fetal/maternal interface.

Thus the target cell ligands for all three NK receptors families appear to be MHC class I molecules of one sort or another, consistent with the missing self hypothesis. However, individual NK cells express on average five of the possible receptors (250), and different NK cells in an individual can express different receptor subsets. The regulation of NK receptor expression is poorly understood, and several conflicting hypotheses exist, comparison of which is beyond the scope of this chapter. Perhaps the most popular idea is the “at least one” hypothesis, which dictates that NK cells will (perhaps randomly) express one receptor, then a second, and so on, until at least one receptor has been expressed which can interact with a self MHC molecule, thus ensuring that the NK cell will not attack the host. In this model, the expression of an NK receptor able to interact with self MHC will then prevent the subsequent expression of still more NK receptor types. Consistent with this, NK cells in MHC-deficient mice expressed a wider variety of NK receptors.

Definitive studies on the role of NK cells in viral infections have been performed in the mouse model. Depletion of NK cells *in vivo* by administration of anti-NK cell antibodies leads to enhanced synthesis of some viruses, such as MCMV and VV, but not of other viruses, such as LCMV (266). NK cell–mediated resistance to MCMV is particularly profound. An age-dependent resistance to MCMV occurs in mice at about the third week of age, and coincides with the maturing NK cell response. Furthermore, adoptive transfers of NK cells into suckling mice protect them from severe MCMV infection. Lymphokine-activated killer (LAK) cells, which are NK cells highly activated by growth in culture in the presence of interleukin-2 (IL-2) and which have been used in a variety of clinical and animal model tumor therapy protocols, protect suckling mice against MCMV. The presence of prolonged NK cell activity during persistent infections may select for NK cell–resistant viruses; establishment of a persistent infection in severe combined immunodeficient (SCID) mice with an NK cell–sensitive variant of Pichinde virus resulted in the conversion of the virus into an NK cell–resistant genotype(s). The related arenavirus, LCMV, normally causes a persistent infection in mice in nature and is also NK cell resistant.

In general, NK cell activity peaks earlier than that of T cells (2–3 days) and diminishes rapidly thereafter. Clinical correlations have been observed between decreased NK cell activity as a consequence of disease or drug therapy and susceptibility to infections with HCMV and HSV-1. Examples of individuals with complete NK cell defects are rare, perhaps attesting to the importance of NK cells, but one adolescent with a complete and selective deficiency in NK cells presented with unusually severe infections with VZV, HCMV, and HSV-1 (17). Although the VZV and HCMV infections were life threatening, each infection resolved completely, probably because T-cell and B-cell immunity was normal in this individual.

We have now reviewed the most relevant aspects of cellular immunity, including T cells and NK cells. At this point, it is appropriate to present a tabular summary of the antigen-presenting molecules, the elements presented, the responding cells, and the nature of the responding cells' receptor (Table 2).

Presenting Molecule	Antigen Presented	To (Cell Type)	Responding Cell's Receptor
MHC class Ia	Peptide	CD8$^+$ T cells	Normal TcR
	Glycopeptide	CD8$^+$ T cells	Normal TcR
	Peptide (?)	NK cells	Ly49 and KIR families
MHC class Ib			
HMG	Formylated peptide	CD8$^+$ T cells	Normal TcR
HLA-E	Peptide from class Ia leader	NK cells	CD94/NKG2
HLA-F	Unknown		
HLA-G	? Fetal/maternal peptides	? NK cells	? Specific KIR
HLA-H	No immune function		
MHC class II	Peptides	CD4$^+$ T cells	Normal TcR
CD1	Microbial lipid & glycolipid	CD8$^+$ T cells	Normal TcR
	Glycosylceramide (self)	NK T cells	TcR of limited polymorphism

TABLE 2. *Summary of antigen-presenting molecules, the materials presented, the cells that recognize them, and the cells' receptors*

Antibody-Dependent Cell-Mediated Cytotoxicity

Antibody-dependent cell-mediated cytotoxicity (ADCC) may be mediated either by T lymphocytes or by NK cells. In the latter case, the antibody, usually IgG, is specific for a structure on the target-cell membrane. The antibody “coats” the target cell, and the NK cell then attaches by its Fc receptors, triggering release of perforin, and target cell death. ADCC can be brought about in a different way if the target cell bears Fc receptor. Incubation of uninfected cells with antibody to either the CD3 complex or the TcR idiotype itself causes a cytotoxic lymphocyte to attach to the target cell and lyse it. Note that in both cases, the specificity is conferred on the reaction by antibody; in one instance, the antibody is target cell specific, whereas in the other, it is effector cell specific. Although ADCC can commonly be identified *in vitro,* its contribution *in vivo* is still not known.

Macrophages

Mononuclear phagocytes (blood monocytes, tissue macrophages) are principal cellular players in the clearance and inactivation of most microbial pathogens. Interestingly, these same cells also represent a major target cell and infectious reservoir for many viruses that persist [e.g., measles, lentiviruses, and cytomegaloviruses (45,93,227), and see relevant chapters in these volumes]. There are at least four complications of infection of these cells. First, infectious virus can be transported by these cells to distant parts of the body where infection can continue.

Second, activated monocytes/macrophages are APCs, taking up, processing, and delivering viral antigens to T cells in regional lymph nodes (62,211). *In vitro* studies indicate that activated macrophages are 10- to 100-fold less efficient in antigen presentation compared with the major professional APC, the dendritic cell (see later), but are 10 to 100 times more efficient than antigen-presenting B cells. During certain virus infections, their ability to present antigens, or to initiate immune responses, can be compromised.

Third, viruses can interfere with the macrophage's capacity to degrade other microbial agents. For example, the microbe *Histoplasma capsulatum* normally replicates in quiescent macrophages, and is degraded upon macrophage activation. Activation depends, in part, on IFN-g. In a model system, persistent infection of macrophages by lymphocytic choriomeningitis virus clone 13 prevented IFN-g activation of macrophages; as a result, the mice succumbed to a normally sublethal dose of *Histoplasma* (257). This may be one reason that a number of virus infections are associated with secondary infections by microbes that are usually phagocytosed and inactivated by macrophages.

Fourth, the infection of macrophages or microglia (brain cells of macrophage lineage) can lead to pathology. For example, HIV primarily infects microglia in the central nervous system (CNS) (275) and indirectly, presumably by release of cytokines and perhaps other toxic molecules, leads to neuronal dysfunction mirrored clinically as AIDS dementia (123). Macrophages also can restrict virus infection or replication by acting as nonspecific effector cells for ADCC. The outcome of a macrophage–virus interaction is determined by a complex series of reactions that may dramatically affect the ability of macrophages to act as scavenger cell, immune effector cell, or susceptible target cell for virus replication. Mononuclear phagocytes have a vigorous phagocytic ability enabling them to eliminate virus from the circulation after a blood-borne infection. Their scavenger function constitutes an early line of defense for reducing the virus load either initially or in concert with the specific immune response. Monocytes and macrophages also can initiate a series of immune reactions through the cytokine network (see later) that assists or injures the host. Macrophages may be activated for enhanced antiviral activity by cytokines produced by virus-specific T cells.

Dendritic Cells

Recent attention in virology and immunology has focused on the dendritic cell. These are bone marrow–derived cells that are located in blood and lymph as well as in most tissues. They include Langerhans cells in the skin and mucous membranes, dermal dendritic cells, tissue-resident dendritic cells, and interdigitating dendritic cells in the thymic medulla, splenic white pulp, and lymph nodes (9,62,107). Activated dendritic cells express high levels of MHC molecules (classes I and II) together with accessory (co-stimulatory) molecules (e.g., CD80, CD86, CDIA, CD83) and, by producing a variety of chemokines, attract naive T cells. Infection or inflammation leads to the maturation of immature dendritic cells. For example, after a respiratory virus infection, immature dendritic cells lining the respiratory mucosa are infected, become activated, and carry viral antigens to T cell–dependent areas. These cells now appear as interdigitating dendritic cells and efficiently stimulate naive T cells, producing antigen-specific responses. *In vitro* studies show that only a few dendritic cells are required to provoke strong T-cell responses; for example, one dendritic cell can stimulate 10^2 to 3×10^3 T cells in a mixed lymphocyte reaction. Recent *in vivo* evidence revealed that adoptive transfer of only 10^2 to 10^3 activated dendritic cells to an immunologically naive animal could generate $CD8^+$ CTL-mediated protective immunity. In this study, transferred dendritic cells expressing an LCMV CTL epitope transgene were able to induce $CD8^+$ CTLs that were protective as early as 2 days after immunization, and lasted for at least 60 days (128).

Because dendritic cells play a primary role in the initiation of antigen presentation leading to immunity, a number of viruses can be expected to have evolved mechanisms to exploit these cells. The two best examples are HIV and LCMV. In the case of HIV, two conflicting events occur. First, it is likely that dendritic cells in the mucosal membrane are infected initially by HIV and migrate to lymphoid organs where effective presentation of HIV antigens triggers both $CD8^+$ and $CD4^+$ T-cell responses for the host's benefit. However, concurrently the dendritic cell has transported live HIV into lymph nodes, where it is believed to contribute to the spread of virus to $CD4^+$ T cells (107). LCMV has a different strategy. LCMV Armstrong clone 13 causes immunosuppression in adult mice (20) by preferentially replicating in the interdigitating dendritic cells in the T cell–dependent (white pulp) area. This leads to an early host antiviral $CD8^+$ T-cell response, which then destroys these infected professional APCs (an example of immunopathology). The result is generalized host immunosuppression. Evidence is accumulating that other viruses also can infect dendritic cells and alter their function, with resulting immunosuppression. Currently these reports are restricted primarily to *in vitro* studies, and *in vivo* confirmation is needed (for example, in measles virus/dendritic cell interactions) (104). However, owing to the olympian properties and role played by dendritic cells in acquired immunity, it is reasonable to predict that more examples of virus manipulation of dendritic cells will emerge. Furthermore, it seems likely that dendritic cells (or viruses that home to them) will prove useful for immunization.

CYTOKINES

Cytokines (for the purpose of this chapter, lymphokines and monokines) are proteins made by cells of the immune system, usually during cell activation. As soluble factors, they assist in regulation of the immune response and in elimination of viruses and other pathogens. Their activities and structure are reviewed elsewhere in this volume. Immune responses to particular antigens or microbes are often distinguished by a relatively distinct pattern of cytokines production by $CD4^+$ Th cell subsets. This in turn drives the immune system toward a predominant cell-mediated or humoral immune response (144). The subsets have been described in humans and mice. Th1 cells secrete IL-2, IFN-g, and lymphotoxin. They promote CTL-type and other delayed-type hypersensitivity reactions frequently required for clearance of intracellular organisms. Th2 cells produce IL-4, IL-5, IL-6, IL-10, and IL-13. They promote allergic-type

responses and stimulate the differentiation of B lymphocytes, mast cells, and eosinophils. The role and balance of Th1 and Th2 cells and their cytokines in viral infections is believed to be important in pathogenesis and immunity. Viruses themselves can use cytokines, or cytokine homologs, to their own advantage. Certain viruses contain genes encoding cytokine homologs, the expression of which potentially enables the viruses to modify immune responses to their own advantage. Some examples are provided later in this chapter. In addition, cytokines released from T cells, especially IFN-g, have been implicated in clearance of virus from infected cells in the absence of $CD8^+$ T cell–mediated lysis (30,67,168,242).

HUMORAL EFFECTOR MECHANISMS

Generation of B-Cell Responses and Antibody

The B-cell receptor for antigen is Ig, which is initially expressed as a membrane protein. The structure and genetics of Ig are well defined and were described earlier. During their development, B cells initially express IgM on their surface, followed by expression of different H chain classes IgG(g), IgA(a), or IgD(d). IgM and IgD may be expressed in all B cells, but once a cell has expressed an H chain class other than IgM, it remains committed to secrete that class of Ig. When a B cell encounters antigen, it usually differentiates into a mature plasma cell, secreting specific antibody as a single Ig class; memory B cells also are generated. This differentiation of B cells is controlled by specific B-cell growth and differentiation factors produced by T cells, analogous to the factors involved in the differentiation of T cells themselves. It has been suggested that B-cell responses to some viral antigens are more brisk than are those to isolated antigens because of the densely packed, paracrystalline nature of certain viral structures (284). Antibodies of all classes are usually generated in response to virus infection.

How Do the Various Antibody Classes Exert Their Antiviral Effects?

Antibodies neutralize viruses in a number of ways.

1. They may bind to the part of the virus that interacts with its cell-surface receptor, preventing virus attachment to the cell.
2. They may agglutinate many infectious particles into a single “clump,” thus reducing the number of cells that will become infected.
3. They may bind to virus glycoproteins on the cell surface, lysing the infected cell in association with complement, or modulating the intracellular viral replication.
4. Viruses may activate complement, releasing chemotactic factors such as C5a or C3q.

Recent animal experiments studying the effects of antiviral monoclonal antibodies on a wide variety of DNA and RNA infections have led to a new and important conceptual distinction between *in vitro* neutralization and *in vivo* protection. First, monoclonal antibodies that do not neutralize *in vitro* may nevertheless afford protection on *in vivo* transfer. Second and conversely, antibodies that have high neutralizing activity *in vitro* may not provide protection when adoptively transferred *in vivo.* Nevertheless, *in vitro* studies have provided much information regarding the probable mechanisms underlying the antiviral efficacy of each antibody class, and there often is a correlation between *in vitro* and *in vivo* efficacy. Note that intact antibodies are not a prerequisite for antiviral effectiveness; Fab fragments specific for RSV F glycoprotein, when instilled into the lungs of infected mice, were therapeutically effective (34). Such approaches hold promise, particularly in light of recent advances in technologies that allow the rapid production of antibodies of any desired specificity (10,97).

Immunoglobulin A

During natural infection, most viruses gain entry via mucosal surfaces, so it is not surprising that mucosal immunity plays an important role in control of viral infections (158). Development of mucosal immunity and secretory IgA are important in preventing mucosally restricted viral respiratory and enteric infections, and virus-specific circulating antibody plays an important role in limiting viremia and thereby aborting spread of virus to systemic sites. For successful induction of a secretory IgA response in the gut, virus must come into contact with a Peyer's patch, the gut-associated lymphoid tissue. Once a local response has been generated, IgA-producing memory B cells recirculate to other areas in the gut. This has been demonstrated in humans by using oral polio vaccine. The protective effects of IgA are usually attributable to antibody directly preventing virus binding to the mucosa. The approaches and concepts are discussed in detail elsewhere in this volume.

Immunoglobulin G

There are several IgG subclasses in humans and mice. For example, human IgG falls into four subclasses (IgG1, IgG2, IgG3, and IgG4), which normally constitute 70%, 20%, 8%, and 2% of the total IgG, respectively. However, IgG subclass–specific antiviral antibodies are generated in different amounts in acute or persistent infections. For example, IgG1 is the dominant IgG isotype found in HIV-infected individuals during the quiescent stage of disease, but it declines in those with progressive illness, perhaps because of a decrease in $CD4^+$ T-helper cells. IgG1 is the major complement-binding and opsonizing antibody in humans (231), and in patients recovering from acute hepatitis B virus infection, IgG1 and IgG3 dominate, with a switch from IgG3 to IgG4 (a non–complement-binding antibody) during chronic infection. IgG1 in humans corresponds to mouse IgG2a (major complement-binding and opsonizing antibody). In experimental mouse infection with LCMV, IgG2a antibody is the predominant isotype raised in acute infection with subsequent viral clearance (>90% of IgG isotype). However, during persistent LCMV infection, there is a switch to more than 90% of IgG1, a non–complement-binding antibody (243). This switch may be related to activation of different Th subsets and the cytokines they produce: in the mouse, IgG2a is associated with the Th1 subset (IFN-g, IL-2, and lymphotoxin production), whereas IgG1 is associated with the Th2 subset and low levels of IFN-g. Complexing of viruses with IgG antibody also will facilitate their phagocytosis by mononuclear cells and polymorphonuclear leukocytes via Fc receptors on these phagocytic cells. However, this latter process may actually enhance the infectivity of some viruses

(185). Sindbis virus infection of mice provides an interesting animal model demonstrating the antiviral effect of IgG. Sindbis virus, an alphavirus, can establish persistent infection of mouse neurons. Because neurons express little or no class I MHC (88,89), the virus is effectively hidden from $CD8^+$ T cells, but infusion of antiviral antibodies, or even of antiviral Fab'_2 fragments, clears virus infection almost entirely (118). The mechanism is unclear, but may involve modulation of viral replication, as demonstrated for measles virus in tissue culture (171).

Immunoglobulin M

This pentameric, decavalent molecule is produced early after virus infection, is usually independent of T-cell help, and acts as the initial antibody-mediated antiviral response. Later in infection, and on secondary exposure, most IgM-producing cells switch classes to IgG but retain antigen specificity. There is good *in vitro* evidence that IgM is effective in neutralizing the infectivity of virus in the fluid phase. This may result from the antibody preventing virus attachment to specific cellular receptors and from the aggregation of virus particles, thereby reducing the number of infectious particles.

Immunoglobulin E

IgE antibody is present at low concentration in serum; its principal biological activity appears to be mediated by binding to mast cells via specific receptors. When antigen reacts with the cell-bound IgE, inflammatory mediators are released from the mast cell. This IgE-dependent mechanism accounts for several of the clinical features of allergy, including wheezing with respiratory infections and pharmacologic relief with antihistamines. There has been little study of the antiviral IgE response or of the molecular mechanism(s) underlying its role in clinical disease.

Role of Complement

The human complement system comprises more than 20 plasma proteins, and most aspects of their interaction are now well understood (27,145). The classical pathway of complement is activated by the C1 macromolecule binding to IgG or IgM complexed to antigen. Attachment occurs via the Fc portion of Ig interacting with the C1q subunit of C1. C1r and C1s, two highly specific serine proteases, become activated in this process. C1s sequentially cleaves C4 and C2 with resultant assembly of C4b2a, the C3-cleaving enzyme or convertase of the classical pathway. C4b binds covalently to the target, whereas C2a carries the catalytic domain. The activity of C4b2a is restrained by the lability of C2b and to a lesser extent by specific regulatory proteins that inactivate C4b (these are the C4-binding protein and factor I). The more recently discovered mannan binding or lectin pathway of complement activation is similar to the classical pathway, except that it is triggered by the interaction of mannan- or lectin-binding proteins with sugars on microorganisms. A distinct plasma protease, analogous to C1s, is used to cleave C4 and C2.

The major difference involved in the initiation of the alternative, or amplification, pathway of complement activation is that it does not depend on antibody. The internal thioester linkage of C3 undergoes slow spontaneous hydrolysis in plasma, producing a C3b-like molecule. Although most of these C3b-like molecules are rapidly inactivated by factor H and factor I, some may bind directly to a target, and a proportion interact with factors B and D to form a C3-cleaving enzyme. The C3b generated by this transient fluid phase C3 convertase can then bind to surfaces. Most so-called “activators” of the alternative pathway, such as microbes and foreign surfaces, lack the membrane regulatory proteins expressed by host tissue. Therefore deposition of C3b on their membranes can trigger the alternative pathway. One property shared by activators of the alternative pathway, which are mostly particulate, is that when C3b is deposited on their surface from the fluid phase, it is relatively protected from inactivation by factors H and I. This protected C3b covalently attached to a target can then bind factor B, which is cleaved by factor D (a 25-kd serine protease in plasma) to give C3bBb, the C3-cleaving enzyme of the alternative pathway. This convertase has its catalytic site on the Bb fragment and decays rapidly, but the half-life of the enzyme is prolonged by properdin, which binds to the enzyme complex and retards this decay. An important point about the alternative pathway is that C3b, however generated (whether by the classical pathway, spontaneous hydrolysis, or cleavage of C3 by other proteolytic enzymes), serves to initiate the amplification of C3 cleavage. Thus the presence of an activator surface, lacking membrane regulators, unleashes a powerful biological positive-feedback loop. This emphasizes the importance of the regulatory proteins in the fluid phase and on cell surfaces, without which trivial amounts of C3b would result in unrestrained activation of the pathway, even in the absence of an activator surface. After cleavage of C3, additional C3b molecules may be incorporated into either C3bBb or C4b2a, giving C3bnBb or C4b2a3b; these complexes have C5-cleaving activity and are called C5 convertases. After C5 cleavage, sequential nonenzymatic binding of C5b to C6, C7, C8, and five to 10 C9 molecules results in assembly of the C5b-9 terminal complex. This membrane attack unit inserts into the lipid bilayer of cell membranes, where it can lead to osmotic lysis. By electron microscopy, the familiar lesion is visualized as a doughnut-shaped pore, and is similar to the lesion caused by CTL and NK cells when they release perforin, a protein with homology to C9.

In addition to the proteins of the classical and alternative pathways and the C5b-9 membrane attack complex, the complement system also includes cell-surface molecules that specifically bind activated or cleaved complement molecules in a receptor-mediated manner. These include receptors for C3b (CR1, CD35), iC3b (CR3, Mac-1, CD11b/CD18), and C3dg (CR2, CD21). The complement system is regulated by a number of plasma proteins (factors H and I, C1 inhibitor, C4-binding protein, S protein) and cell-membrane proteins [CD46 or membrane cofactor protein (MCP), CD55 or decay accelerating factor (DAF), and CD59, the membrane inhibitor of the membrane attack complex]. These regulatory molecules restrict inappropriate complement activation by sequestering activation fragments and/or facilitating their inactivation. The regulatory molecules do not block appropriate complement activation. They do, however, prevent chronic activation and restrict inappropriate activation (e.g., in plasma or on autologous tissue).

The major function of the complement system is to modify foreign membranes by opsonization or, less commonly, by lysis. Its second function is to promote the inflammatory response. Low-molecular-weight cleavage fragments (e.g., the C3a and C5a anaphylatoxins) induce chemokinesis and chemotaxis, mast cell degranulation, and other types of cell activation.

Complement is involved in innate as well as adaptive immunity. Complement enhances the neutralization of viruses by antibody. This is achieved by at least four different mechanisms. First, complement can blanket the sensitized (antibody-coated) virion, thus rendering it incapable of binding to its receptor. Second, complement can aggregate or clump sensitized virions, thereby reducing the number of infectious units. Third, by incorporating C3b into virus/antibody complexes, such sensitized virions can bind to C3b receptors on phagocytic cells, leading to their ingestion and degradation by these cells. Fourth, antibody and complement can directly lyse viruses possessing a lipid envelope. Complement likely plays an important role early during infection when small amounts of low-avidity antibody are made. [For a comprehensive review of the area, the reader should consult reference (78)]. Complement alone can inactivate certain viruses directly in the absence of antibody. Retroviruses from several species are lysed by human complement, in association with an anti-a-galactosyl natural antibody (206,267). This occurs because a viral protein alone (rather than an Ab/Ag complex) can act as a receptor for C1q. The alternative pathway of complement can be activated on the surface of a variety of microorganisms, including bacteria and yeasts. Thus an additional principal function of the complement pathway is as a nonspecific mechanism of host defense, capable of opsonizing or lysing a variety of microorganisms before the generation of specific immune responses. Hence it is not surprising that some viruses, notably the pox and herpes families, contain genes that interfere with selected complement molecules (36,127,226). Finally, at least three of the complement proteins are used as receptors by viruses. These include the C3d receptor (CR2 or CD21) used by EBV (47,90,154), the CD46 protein or MCP used by measles virus (41,131,151), and DAF, which appears to be used (probably as a co-receptor) by coxsackieviruses (15,215) and echoviruses (14). It is not known whether using complement proteins as a receptor confers any selective advantage to the virus. Possibilities include cell signaling via the complement protein, or protecting itself from complement attack.

Lysis of Virus-Infected Cells by Antibody and Complement

Antibody and complement can kill virus-infected cells *in vitro.* A variety of human cell lines (epithelioid, neural, and lymphoid) infected with a wide variety of human DNA and RNA viruses and expressing viral antigens on their surfaces are lysed by human serum when it contains both specific antibody to the virus and functional complement. Here, lysis appears primarily dependent on an intact alternative pathway of complement (33,223). A detailed analysis of this system using measles virus–infected cells as a model showed that the infected cell activates the alternative pathway in the absence of antibody, but that antibody is needed for lysis to occur. Despite the activation of the alternative pathway being independent of antibody, IgG enhances C3 deposition and thus facilitates lysis. The involvement of multiple cell types and viruses in similar events indicates the generality of the phenomenon. Although it is difficult to quantitate this mechanism *in vivo,* it is likely that cellular injury can be produced by antibody and complement *in vivo.*

Idiotypes and the Network Theory

The antigen-combining site of each Ig molecule itself represents a novel antigenic determinant known as an idiotype. Idiotypes can be recognized by other antibodies, and these latter “anti-idiotypic” antibodies often represent an “internal image” of the antigen to which the original antibody (bearing the idiotype) was directed. The “network theory,” formulated by Jerne (86), and for which he received the Nobel prize, stated this concept and extended it to view the whole immune system as a network of interacting idiotypes and anti-idiotypes. Simply put, all antibodies are anti-idiotypes, which also react with specific external antigens. When external antigen enters the system and produces expansion of specific clones of cells being particular idiotypes, the system is perturbed, and expansion of anti-idiotypic, anti–anti-idiotypic antibodies, and so on ensues. These were postulated to play a regulatory role in modulating the immune response. The same principles would apply to TcRs, which also bear idiotypes. These ideas have stimulated much current research. Anti-idiotypic antibodies have been demonstrated in some human autoimmune diseases and infections.

Does the network theory have any specific relevance for virus infections? Apart from their regulatory role in the immune response, anti-idiotypic antibodies that have the “image” of antigen may compete with that antigen for specific cellular receptors. For example, anti-idiotypic antibodies raised against antibody to reovirus glycoprotein can prevent the adsorption of the reovirus to its specific receptor on the cell surface, presumably because the anti-idiotype can itself bind to the receptor. In addition, anti-idiotype antibodies (which look like the original antigen) have been used as vaccines, because they induce anti–anti-idiotype antibodies, which can cross-react with the microbe, thus avoiding inoculation with the microorganism.

VIRUS STRATEGIES TO EVADE THE HOST IMMUNE RESPONSE

Viruses are relatively unstable outside a competent host, and for a virus to remain in circulation, it must be able to move efficiently among different hosts and/or to persist in an individual host. The virus' ability to evade host immunity is critical to the virus' survival both in the population and in the individual. For example, if there is widespread immunity in a population, a virus able to evade that pre-existing immunity will have an increased pool of susceptible targets. In the individual host, evasion of immunity is necessary to permit virus persistence, and immune evasion also may allow the virus to replicate to higher titers in the host, perhaps increasing its chance of transmission to a susceptible recipient.

Virus-induced immunosuppression is often directed against that component of the immune response which is most important in limiting the virus infection. For example, influenza viruses are highly sensitive to neutralizing antibodies, and this virus provides one of the best examples of antibody evasion; HSVs are controlled in large part by T cells, and HSV can inhibit antigen presentation by MHC class I; and NK cells combat primary cytomegalovirus infections, and this virus encodes an MHC homolog which may inhibit NK activity. These observations underline our contention that the intimate relationships between viruses and host immunity has been central to the evolution of both protagonists.

As with our overview of the immune response (Fig. 1), the array of viral evasion mechanisms could be categorized in a number of ways. We have chosen to classify the viral strategies as either “avoidance” (in which the virus “hides” from host immunity but does not actively interrupt it) or “inhibition” (in which the virus actively disrupts host immunity). This classification (the various underlying mechanisms of which are summarized in Table 3 and are discussed later) may have

some clinical significance, because the active inhibition of immunity may have serious consequences for the host.

Evasion Technique	Mechanism
Avoidance: Virus hides from the immune system but does not actively disturb host immune responses	Infection of immune privileged sites Down-regulation of viral gene expression Virus protein structure limits its immunogenicity Mutation or reassortment of genetic material
Inhibition: Virus inhibits either the induction or the effector phase of the immune response	Infection of thymus, leading to deletion of high-affinity T cells (negative selection), inducing partial tolerance Destruction of specialized APCs Inhibition of antigen presentation Expression of proteins that inhibit effector-cell function Expression of proteins that delay target-cell death Infection of T or B lymphocytes

TABLE 3. *Overview of how viruses can evade the host immune response*

Evasion by Avoiding the Immune Response

Infection of Immune-Privileged Sites

Certain regions of the body appear to be immune privileged, that is, not subject to the same rigorous immune surveillance as most somatic sites. Examples include the anterior chamber of the eye, perhaps because immune infiltration would be catastrophic to vision. Of all somatic cell types, perhaps we can least afford the wholesale destruction of neurons, which not only subserve critical functions, but also have very limited regenerative capacity. The CNS is protected by the blood–brain barrier, and has long been considered relatively immune privileged, but it is clear that it is accessible to activated T cells and cellular immunity and, if injured, to the passage of antibodies. Perhaps in part to ensure that neurons are less susceptible to immune-mediated destruction, they are one of the few somatic cell types that do not express class I MHC (88,89). Viruses have been quick to take advantage of this, and many viruses can infect neuronal cells (for example, polioviruses, coxsackieviruses, mengoviruses, LCMV, measles, alphaviruses). Several virus families have further exploited the loophole by using neurons as their site of persistent infection or latency. The best-characterized example, albeit still incompletely understood, is the herpesvirus family, several members of which (HSV, VZV) establish latent infection in sensory nerve ganglia. The costs and benefits of low neuronal class I expression have been recently reviewed (192).

Down-Regulation of Viral Gene Expression

How might viruses hide within cells that express class I MHC? Viruses may selectively reduce the level of expression of cell-surface glycoproteins, sometimes down-regulating them manifold in comparison with other, non-surface, viral products (169). This may help evade antibody-mediated detection, but, as has been stressed throughout this chapter, the detection of intracellular viral proteins is the major remit of T cells, in concert with the MHC. Thus the major problem facing intracellular virus is the evasion of this aspect of the host's detection systems. T-cell recognition requires as few as 1 to 100 MHC/peptide complexes to be displayed on the target cell, and this can be achieved with low levels of protein expression; it may therefore be difficult for viruses to down-regulate expression to a level preventing MHC presentation. Some viruses, such as herpesviruses, may be able to shut down protein synthesis during latency in neurons, but this luxury is available to only a few virus families. The inability to terminate protein synthesis completely is, presumably, one of the selective pressures which has driven many viruses (including even HSV) to inhibit actively MHC class I antigen presentation, as discussed later.

Virus proteins may be refractory to immune recognition. Virus proteins have been honed by constant interactions with the immune system, and over the millennia, many immunogenic sequences doubtless have been hidden or lost; those sites still recognized may reasonably be considered the residue. The intrinsic structure of a virus protein can render the protein less digestible to some part of the MHC class I processing pathway; the Gly-Ala repeats of the EBV antigen EBNA-1 act in cis to render the protein relatively resistant to proteasomal degradation (119). Furthermore, post-translational modifications can play a role. Many potential antibody epitopes on viral glycoproteins are masked by the glycosylation event (although the virus pays the price of inducing carbohydrate-specific antibodies). Although some modified peptides [e.g., glycopeptides (230)] can be presented by class I MHC molecules, it is plausible to suggest that certain modifications might prevent the protein's presentation, even if they do not actively inhibit the cells' ability to process other antigens. Consistent with this, an HCMV matrix protein appears to selectively inhibit processing of HCMV IE proteins, perhaps by phosphorylation of the IE products (59).

Mutation of Viral Gene Sequences

RNA viruses may be at a particular advantage, because the high mutation rate of RNA polymerases provides a rich diversity of virus mutants from which variants can be selected.

Evasion of Antibody Responses by Gene Mutation

This strategy is best exemplified by influenza virus, which contains a segmented RNA genome encased in a viral envelope studded with influenza proteins [hemagglutinin (HA) and neuraminidase (N)]. These proteins are critical for binding to, and entry into, target cells, and are obvious targets for the host antibody response; the host takes full advantage of the opportunity by producing neutralizing antibodies targeted to both proteins. The virus responds by generating mutant HA and N sequences, thus providing itself with the opportunity to evolve. This evolution is driven by

two requirements: the mutant proteins must retain their function (i.e., the virus must be viable), but they should be able to escape detection by the pre-existing antibodies. The resultant virus often has fairly subtle immunological changes, but these are sufficient to give it a temporary edge in the battle; it may have an expanded pool of susceptible hosts, because it should be able to infect individuals carrying the original set of antibodies. This phenomenon has been termed antigenic drift and plays a role in influenza epidemics, which occur sporadically. The process of antibody escape through mutation of viral target proteins is not restricted to influenza virus. Examples have been found in almost all viruses in which they have been sought, and indeed the need to escape antibodies doubtless has driven the diversification of many viruses into the different strains or serotypes that we recognize today. When we categorize viruses by the antibodies that do or do not recognize them, we are simply using the results of this ongoing evolutionary interplay.

Evasion of T-Cell Responses by Gene Mutation

In a manner analogous to evasion of antibody responses, viruses can mutate those sequences presented to the immune system as T-cell epitopes. The process of viral mutation toward CTL escape has been less widely studied than the antibody equivalent. Mice immunized with a minigene vaccine encoding a single LCMV CTL epitope, and later infected with LCMV, rapidly generate LCMV variants in which the vaccine epitope has been mutated (263), and CTL escape variants of LCMV replicate to higher levels in the host, although the viruses are eventually cleared (120). Studies using HIV have shown that viruses can mutate *in vivo,* in a natural infection, into CTL escape variants (181), and that this escape has important biological consequences (21). Finally, evidence of CTL escape variants comes from studies of a specific CTL epitope in EBV, presented by the HLA-A11 allele. This epitope is present in EBV strains circulating in a population in which the HLA-A11 allele is infrequent, but is mutated in virus circulating in an A11-rich population, and cannot bind to the A11 molecule (37).

Wholesale Replacement of Viral Proteins

In addition to gene mutation, which many viruses exploit, influenza virus escapes host immunity by using a second method, which is available only to virus families with a segmental genome structure. The influenza virus genome has eight RNA segments, and during co-infection of a single cell by two viruses (1 and 2), the segments from both infecting viruses can mix (reassort). The majority of RNA segments in the resulting virus may come from virus 1, but (for example) its HA-encoding segment may come from virus 2. In nature, such molecular reassortment appears to take place in non-human hosts, most probably porcine or avian, when there is co-infection of a human virus (which will infect pigs or birds only rarely) and an avian virus. The biological consequences of this event are difficult to predict, but if one of the resulting reassortants carries the avian HA but is able to infect humans, this virus may be immunologically unknown to, and highly pathogenic in, humans. It appears likely that such antigenic shift is the cause of severe influenza epidemics and pandemics.

Evasion by Active Inhibition of Immunity

Viruses have a developed a plethora of approaches to actively inhibit the development of host immunity, as outlined in Table 3.

Thymic Infection May Induce Tolerance

If viruses infect the thymus, the viral antigen presented by MHC in the thymic stroma may cause clonal deletion of virus-specific T cells (i.e., the virus is viewed as self). Thus no virus-specific cells will exit the thymus, and neither antibody nor T-cell responses will be induced by viral challenge; there is no generalized immune suppression, and the inhibition of immunity will be specific to the infecting virus. Congenital infection of mice with LCMV results in thymic infection, which begins in the fetus and is maintained for the life of the mouse. The mouse is persistently infected and cannot mount an LCMV-specific $CD8^+$ T-cell response. If the virus is eradicated (e.g., by adoptive transfer of LCMV-specific $CD8^+$ T cells), then the ability of the host to mount LCMV-specific responses is restored (103), because the transferred CTLs remove thymic cells expressing viral peptide/MHC complexes, and naive host T-cell precursors bearing TcR with specificity for the virus/MHC complex will no longer be deleted, and instead mature and exit the thymus. Recent studies indicate that, despite viral replication in the thymus, negative selection may be incomplete, with virus-specific T cells escaping to the peripheral lymphoid organs, and antiviral antibodies being generated. This occurs experimentally in LCMV infection and may similarly occur in HBV carriers. In both instances, antiviral antibodies are generated, and CTL can be induced by vaccination (162,170,180).

Destruction of Specialized Antigen-Presenting Cells

Many viruses can infect specialized APCs (dendritic cells, macrophages), the destruction of which may lead to generalized immunosuppression. Infection of, or absorption to, follicular dendritic cells by HIV-1 has been demonstrated and was proposed as the underlying cause of the late immune failure that characterizes AIDS (179,264). Certain variants of LCMV rapidly infect interdigitating dendritic cells, the major APCs, and the resulting antiviral CTL response destroys APCs and causes a transient global immunosuppression (20,283) affecting both antibodies (143) and T cells.

Inhibition of Antigen Presentation

Rather than destroying the infected professional APCs, viruses may instead inhibit antigen processing, thus providing themselves with a safe haven in which to produce progeny. This would apply not only to virus-infected specialized APCs (which express MHC classes I and II) but also to somatic cells, which can present antigen only via the class I pathway. Given that the vast majority of virus-infected cells are somatic cells, not specialized APCs, one might predict that viruses would have developed more tricks to inhibit MHC class I presentation than to disrupt the MHC class II pathway; this is the case, as recently reviewed (139). Herpesviruses are particularly skillful, blocking the class I pathway at almost every stage (87). The HSV IE protein ICP47 binds to the cytosolic surface of TAP and inhibits transport of peptides into the ER (77), whereas the HCMV US6 protein binds to the luminal aspect of TAP, to similar effect (3). The HCMV US3 gene

product retains peptide/MHC complexes in the ER (91), and the US2 and US11 proteins cause rapid degradation of class I heavy chains by the proteasome (274). Finally, IFN-g–mediated stimulation of MHC expression is inhibited by MCMV (71) and HCMV (138). Similar strategies are found in other viruses. Adenoviruses encode a 19-kd protein that binds to class I MHC and prevents its transport to the cell surface (111,229), rendering the adenovirus-infected cell relatively refractory to T-cell recognition. Although most examples of antigen presentation blockade involve class I MHC, some viruses do appear to inhibit MHC class II; for example, the EBV BZLF2 gene product binds to the HLA-DR molecule, inhibiting MHC class II antigen presentation (234) [and aiding entry of the virus into B cells (121)], as does the CMV-induced defect in IFN-g signaling mentioned earlier.

Expression of Proteins That Inhibit Effector Cell Function

Many years ago, it was shown that the pathogenicity of vaccinia virus could be modulated by addition of a host cytokine gene such as IL-2 (194). It now transpires that several poxviruses needed no help from us; they encode their own cytokine homologs, which can modulate the immune response (32,233,235). In addition, EBV encodes a potent equivalent to IL-10 (82). Finally, the HCMV UL18 protein is an MHC class I homolog expressed on the cell surface in association with b_2M; some studies have suggested that this molecule can engage inhibitory NK receptors, protecting the cell from NK lysis (197), although this is controversial (117).

Expression of Proteins Which Delay Target Cell Death

Antiviral $CD8^+$ T cells [and sometimes antiviral $CD4^+$ T cells (281)] can eradicate infected cells by induction of apoptosis. It would benefit the virus to retain cellular integrity for as long as possible, and many viruses have developed means to delay the apoptotic process. The EBV BHRF1 gene product encodes a bcl-2 homolog which prevents cell death (74), as does the adenovirus E1B/19k protein (271). The p53 and Rb proteins, important cell-cycle regulators that can drive cells into apoptosis, can be bound by SV40 T antigen (129,239), with resulting survival and proliferation of the infected cell, and the product of the poxvirus molluscum contagiosum gene MC159 protects cells from Fas-mediated apoptosis (16). Finally, reactive oxygen metabolites (free radicals) can be harmful to both viruses and cells; molluscum contagiosum virus gene MC066L encodes a selenoprotein with antioxidant functions (218).

Infection of T or B Lymphocytes

The simplest and most direct means by which viruses can disrupt immune effector mechanisms is by infecting the immune cells. As shown in Table 4, many acute virus infections, and the vast majority of persistent virus infections, involve cells of the immune system. HIV-1 infects $CD4^+$ T cells, causing their destruction directly, or through immunopathological attack. EBV infects B lymphocytes, as does coxsackievirus B3, perhaps another example of a virus “attempting” to counter that aspect of host immunity which most threatens it (136). An interesting mode of B-cell disruption is practiced by LCMV. The virus infects B cells which express surface IgM capable of binding to neutralizing epitopes on the viral glycoprotein, and these B cells present the viral epitopes on their class I MHC and are promptly killed by virus-specific CTLs; as a result, infected animals make very little neutralizing antibody (182). The specificity of this interaction is nicely shown by the fact that anti-NP antibody is produced in abundance, because B cells bearing NP-specific IgM are not selectively infected by the virus (because NP is not present on the surface of the infectious particle). LCMV also infects $CD4^+$ and $CD8^+$ T cells. During persistent infection, viral sequences and infectious virus (detected by infectious center assay) are found mainly in the $CD4^+$ population (2,22,244). Measles virus infects T cells, B lymphocytes, and APCs. Acute measles virus infection is a well-recognized cause of immunosuppression (indeed in the earlier part of this century, measles infection was used to treat certain immune-mediated diseases), and although the precise cause of the suppression remains unclear, the virus ability to infect T and B cells (and APCs) may be in some way involved.

Virus	Host	Infected Cells
Double-stranded DNA viruses		
Hepatitis B virus	Human, monkey	PBMCs, T and B lymphocytes
Woodchuck hepatitis virus	Woodchuck	PBMCs, bone marrow
Papovavirus	Human, monkey	PBMCs
Group C adenoviruses	Human	T, B, and null lymphocytes
Herpes simplex virus	Human	T lymphocytes
Epstein-Barr virus	Human	B lymphocytes
Cytomegalovirus	Human	Lymphocytes, monocytes
Pox virus	Rabbit	Spleen cells
Single-stranded DNA viruses		
Porcine parvovirus	Pig	Spleen cells
Minute virus of mice	Mouse	Lymphocytes
Positive-strand RNA viruses		
Poliovirus	Human	Lymphocytes, monocytes
Rubella	Human	T and B lymphocytes
Negative-strand RNA viruses		
Measles	Human, monkey	T and B lymphocytes, monocytes
Mumps	Human	T and B lymphocytes
Respiratory syncytial virus	Human	Lymphocytes, monocytes
Vesicular stomatitis virus	Human, mouse	T lymphocytes
Influenza A	Human	Lymphocytes, monocytes
Parainfluenza	Human	Lymphocytes, monocytes
Arbovirus RNA viruses		
Lymphocytic choriomeningitis virus	Mouse	T and B lymphocytes, monocytes
Junin virus	Human	Peripheral blood mononuclear cells
Retroviruses		
Murine leukemia virus	Mouse	B lymphocytes
Feline leukemia virus	Cat	T and B lymphocytes, monocytes
HTLV-I, -II	Human	T, B, and null lymphocytes
HIV-1	Human	T and B lymphocytes, monocytes
SIV	Monkey	PBMCs, macrophages
Ernstegamyxa (L-type virus	Mouse	Spleen cells
Bovine leukemia virus	Cow	PBMCs

TABLE 4. *Viruses that infect lymphocytes and monocytes*

IMMUNOPATHOLOGY OF VIRAL INFECTIONS: VIRUS-INDUCED IMMUNE COMPLEX DISEASE

One of the most common immunopathologic manifestations associated with viral infection (acute or persistent) is virus-induced immune complex formation. In many virus infections, antiviral antibody interacts with virus in the fluid phase, or with viral antigens on cells forming complexes that are shed into the fluid phase, resulting in the formation of virus/antibody (V-Ab) immune complexes. Such V-Ab complexes occur in most infections. Circulating complexes carry infectious virus, which can enter cells that express Fc or complement receptors (e.g., macrophages, activated lymphocytes). However, the production of circulating complexes does not inevitably lead to disease, as long as mesangial cells and macrophages can remove them sufficiently rapidly to prevent their deposition. When the infection is chronic, the continuous trapping of the complexes in renal glomeruli, arteries, and choroid plexus leads to arteritis,

glomerulonephritis, and choroiditis. V-Ab immune complex disease is the common pathogenic mechanism of animal nephritides, vasculitis associated with HBV infection, and glomerulonephritis seen in several chronic human infections, all presenting an immunopathologic picture similar to that seen in chronic human nephritis of unknown cause.

However, even in the face of persistent immune complex formation, some individuals escape disease. Why is that? It is a matter of genetics. Genetic studies of different mouse strains that displayed high or low levels of immune complexes revealed that high levels of complexes was a dominant trait. Backcrossing of F_1 mice to their parents mapped the effect to a single gene in the MHC locus (174). Additional studies using recombinant inbred mice mapped the effect to the MHC class II locus (IA in mouse, equivalent HLA-DR in humans). Thus the immune-response gene(s) control the antiviral antibody response and, at a certain antibody–antigen ratio, immune complexes can result.

The presence of immune complex disease is best documented by identification of viral antigen, host Ig, and complement in a granular pattern along the basement membrane of glomeruli or capillaries, or in the intima or media of arteries. Identification and quantitation of specific antiviral antibodies are accomplished by elution of Ig by low-ionic, high-ionic, or low-pH buffers to disassociate the V-Ab complex, recovery, and quantitation of the eluted Ig and determining the specific antiviral activity of the total Ig eluted (usually >75% compared with <1% specific viral Ig in circulation). Such immune complex depositions are constant companions of persistent viral infection of humans and animals (160,161).

VIRUS-INDUCED AUTOIMMUNE DISEASE

Viruses have been implicated in autoimmunity by three findings. First, human autoimmune responses are made *de novo* in, or are enhanced during, infection by a wide variety of DNA and RNA viruses (164,165). Second, in experimental animal models, both acute and persistent virus infections can induce, accelerate, or enhance autoimmune disease in high-responder mice (246). Third, investigation of molecular mimicry (one potential mechanism whereby microbes may cause autoimmunity) indicates that a number of etiologic agents may cause autoimmune disease (163,166).

How can viruses induce autoimmune responses? Certain viruses [or their protein(s)] have a mitogenic effect on unique lymphocyte subsets and hence act as polyclonal activators. However, because agents such as mycoplasma also can activate lymphocytes and may contaminate viral stocks, stringent evidence must be presented that the activation is due to the virus, and not to mycoplasma contamination. Viruses also can infect lymphocytes and macrophages, causing release of cytokines and chemokines. These molecules can modulate immune responses in a variety of ways (e.g., as growth or differentiation factors, or by regulating MHC class I and/or class II expression on cells). Finally, microbial agents share determinants with host (self) proteins (molecular mimicry). In this instance, an immune response mounted by the host against a specific determinant of the infecting agent may cross-react with the shared host sequence, leading to autoimmunity and, in some cases, tissue injury and disease. Many viral proteins share epitopes with host proteins, as determined by computer sequence analysis, or by analysis of monoclonal antibodies raised against a large panel of DNA and RNA viruses in which nearly 4% of more than 800 tested showed cross-reaction with host determinants expressed on uninfected tissues (236). The evidence that mimicry could cause disease came from experiments in which inoculation of a viral peptide homologous to the encephalitogenic site of myelin basic protein was able to cause the autoimmune disease, allergic encephalomyelitis (53). These observations suggest that the human viral encephalopathies which occur after measles, mumps, vaccinia, or herpes zoster viral infection, in which recovery of a virus is unusual, may have a similar pathogenic mechanism. In these instances, autoimmunity would occur only when the microbial and host determinants are similar enough to cross-react, yet different enough to break immunological tolerance. The induction and breaking of tolerance at both the B- and T-cell levels have been established in heterologous serum protein models, and the same kinetics would likely govern the establishment and the break of tolerance to microbial agents cross-reacting with host proteins. Such cross-reactions could occur either through antibody- or cell-mediated immune responses. Another process implicated in the pathogenesis of autoimmune disease is “epitope spreading,” initiated by an appropriate immune response to an infectious agent, but spreading to epitopes encoded on host proteins (140,254). For example, during the demyelination seen in Theiler's murine encephalomyelitis virus infection, the original virus-specific T-cell response extends to include T cells specific for self epitopes on myelin basic protein and proteolipid protein, and these autoreactive cells contribute to disease. It will be interesting to see if this process applies also to human autoimmune disease associated with virus infections.

VACCINE CONSIDERATIONS

At a time when new vaccines are being sought to counter new challenges (HIV, and even tumor vaccines) and because new methods of subunit vaccination are being suggested as replacements for older vaccines that have performed extremely well (e.g., live attenuated viruses), it is critical that we carefully determine the role of each aspect of the immune response in combating primary virus infection, and in preventing and/or limiting subsequent infection in an immune host. This can now be viewed in light of the foregoing discussion. Historically, two approaches have been taken to developing antiviral vaccines. First, live vaccines have been developed, usually by attenuating the pathogenic virus by tissue-culture passage in a variety of cell lines. Attenuation was then tested in animal models. When pathogenicity was sufficiently reduced, the attenuated virus was considered a candidate. Second, the pathogenic virus could be inactivated (e.g., by formalin treatment), and the killed vaccine tested for immunogenicity. A major difference is evident between these two groups: the antigens of live vaccines will be presented by both class I and class II MHCs, whereas those in killed vaccines will be presented mainly by the class II pathway. In Table 5 are listed the major vaccines in current use and their nature [live or dead (we have avoided the adjective “killed” because the hepatitis B vaccine is a recombinant protein, and thus killing of the hepatitis virus itself was not required for this vaccine)]. Included also are some of the immunological findings associated with each type of vaccine. Each vaccine has costs and benefits. Live vaccines give longer-lasting immune responses against most or all viral antigens (partly because the vaccine replicates to give a large antigenic dose), and they induce a balanced response that includes $CD8^+$ T-cell responses as well as $CD4^+$ T-cell and antibody responses. The induction of $CD8^+$ T cells may be in part responsible for the astonishing efficacy of the live vaccines listed earlier. Live vaccines carry the risk of vaccine-associated viral disease, caused by reversion to pathogenicity of the vaccine strain during its replication in the vaccinee. For example, several cases of adult polio occur annually in developed countries and are almost invariably vaccine associated; that is, the victims have been in recent

close contact with vaccinees. Molecular analyses of the viruses shed in the feces of recently vaccinated infants demonstrated that the vaccine virus had rapidly reverted toward neurovirulence (28,44). In contrast, killed vaccines should carry no real risk of revertant-associated disease, although if inactivation is inadequate, as in an early batch of the Salk polio vaccine (152), then vaccinees may receive virulent virus. However, dead vaccines do not induce good $CD8^+$ T-cell responses; the immunity is short-lasting and often not detectable against all of the viral antigens (in part because inactivation may selectively modify or destroy the immunogenicity of the various virus proteins). Furthermore, there is a risk of disease potentiation. Vaccinees who are subsequently exposed to the wild virus may get much more severe disease than do unvaccinated individuals. This has been well documented for killed measles (54) and RSV vaccines (98), which were withdrawn in large part for this reason. Such disease potentiation has rarely been shown for live vaccines, although recent experience with high-dose measles vaccine in Africa has indicated that occasional unpredictable problems might arise, thus counseling caution in use of new vaccines and mandating the continual monitoring of vaccinee status. The “ideal” vaccine therefore would combine the best features of both, while omitting the problems. It should deliver antigen into both class I and class II pathways to induce a balanced, long-lasting, and cross-reactive immunity without significant vaccine-associated risks. Since the last version of this chapter was written, many articles have been published on DNA immunization, which combines some of the advantages of both live and dead vaccines; several reviews are available (40,69,241). To date the approach has shown itself to be wonderfully adaptable and moderately successful. However, enhancements are needed, as the immunity induced by a DNA vaccine alone is usually less than that induced by a conventional vaccine.

	Live	Dead
	Polio (Sabin) Measles Mumps Rubella Yellow fever Vaccinia Varicella	Influenza Rabies Hepatitis B Polio (Salk)
Antibody induction	+++	+++
CD4 T cell	+++	+++
CD8 T cell	+++	–
All viral antigens?	Usually	Often not
Longevity of immunity	Mo/yr	Mo
Cross-reactivity among viral strains	+++	+
Disease potentiation	? High-dose measles	RSV, measles
Risk of viral disease	+	–

RSV, respiratory syncytial virus.

TABLE 5. *Summary of currently available antiviral vaccines*

TRANSGENIC MODELS OF VIRUS–IMMUNE SYSTEM INTERACTIONS

The ability to generate transgenic mice and mice with targeted gene deletions introduced by homologous recombination (known as knock-out mice) has already provided, and will continue to provide, unique opportunities to understand both the biology of virus infections and the molecular basis of virus-immune interactions. For several important human viruses (e.g., poliovirus, measles virus, and hepatitis B virus), small animal models of disease did not exist because of restricted host tropism of the virus. Transgenic mouse lines were created that express the human cellular receptor for either poliovirus [poliovirus receptor molecule (PVR) (108,196)] or measles virus [CD46 molecule (172)]. Transgenic mice expressing PVR are susceptible to poliovirus infection, and replication of virus in neurons gives rise to poliomyelitis. Measles virus infects transgenic mice expressing the measles virus receptor and gives rise to suppression of both cellular and humoral immune responses. Both models now allow probing of the generation and effect of the antiviral immune response in a well-defined genetic host (the mouse) in which various T and B lymphocytes, MHC class I and II molecules, activation and co-stimulatory molecules, and cytokine genes can be knocked out. However, neither the poliomyelitis nor the measles virus model totally recapitulates the natural human infection because, when the respective virus is provided by the natural route, little or no infection occurs. A third valuable transgenic model is that for HBV. Unlike the polio or measles virus approach, transgenic mice that express HBV sequences have been established by use of liver-associated promoters (metallothionein and/or albumin promoters) (30). Using these models, Chisari et al. (49,67) have shown the role played by both $CD8^+$ and $CD4^+$ T cells in control of HBV, and have demonstrated that IFN-g and TNF-a secreted by CTLs play a prominent role in clearing HBV sequences from the vast majority of hepatic cells. IFN-g and TNF-a triggered the down-regulation of HBV gene expression by a post-transcriptional mechanism, in which HBV RNA levels in hepatocytes are controlled by stabilizing (or destabilizing) influences of several HBV RNA-binding host proteins, whose activity is regulated through cytokine-induced signal pathways (72).

By a different approach, focal expression of a viral gene in a selected cell with a cell-specific promoter can be used as a means to evaluate virus-induced autoimmune disease (43,173,259). The viral transgene is integrated into the host's genome and passed on to progeny, essentially becoming a “self” antigen. This approach has been used to express a viral gene in the beta cells of the islets of Langerhans [a model for diabetes (159,173,259)] or in oligodendrocytes [a demyelination model for multiple sclerosis (43)]. Subsequent infection of the transgenic mouse with the appropriate virus allows one to determine whether related or unrelated viruses induce responses to the transgene, and how cytokines, or co-stimulatory molecules, might initiate or enhance autoimmune disease of the pancreas or CNS.

Gene dysfunction (knock-out) studies allow the generation of mice lacking specific immune cell populations (e.g., B cells, $CD4^+$ or $CD8^+$ T lymphocytes), T-cell effector molecules (e.g., perforin, IFN-g, TNF-a), and a litany of other molecules including specific MHC class I or class II molecules, cytokines, chemokines, activation and co-stimulatory, and adhesion molecules. Using such mice, the role of specific genes and their products can be defined during a virus infection; however, the redundancy of the genome, and the resultant capacity for compensatory changes, indicate that the results obtained with knock-out mice should be interpreted with some caution. For instance, there are several examples in which deletion of the gene has given results very different from prior studies that used monoclonal antibodies to inactivate the gene product in a developmentally normal mouse. It is not clear which of the two approaches has given us the “correct”

result. With this caveat, the various knock-out and expresser models presently available should help us to identify the roles played by various components in immunization and immunopathology, which in turn may lead to therapeutic strategies to optimize vaccination and to limit immunopathologic injury and disease.

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CHAPTER 12 Interferons and Other Cytokines

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GENERAL FEATURES OF CYTOKINES

Cytokines are protein factors made by cells to act on cells. The family of these includes a large and growing number of molecules acting on nonimmune and immune cells to mediate a variety of functions. Although their properties cannot be described briefly, certain characteristics are shared by most cytokines (Table 1). In general, cytokines are soluble, but there are examples of membrane-bound forms. They are structurally and biochemically similar to polypeptide hormones and growth factors (1,297). In particular, specific receptors for cytokines are expressed at cell surfaces, and cytokine binding to these activates intracellular signaling pathways. In addition, cytokines typically mediate autocrine and paracrine effects in a short radius, but they can also have endocrine effects under extreme conditions. Finally, some cytokines can themselves act as growth factors. One major difference between cytokines and their hormone and growth factor relatives, however, is that cytokines are tightly regulated for limited expression under basal conditions and are induced in response to host challenges, particularly microbial infections.

Most cytokines are simple polypeptides or glycoproteins ≤ 30 kd in size. Some cytokines form homoterms or homotrimers. One cytokine is a known heterodimer.
Cytokines are tightly regulated for limited expression under basal conditions. Various stimuli induce cytokine expression at the level of transcription, translation, or posttranslational protein processing.
Cytokine production is transient. The action radius is usually short, reaching or autocrine or paracrine functions. After profound conditions of stimulation or during systemic infections, however, some cytokines are found at circulating levels with endocrine functions.
Cytokines mediate their actions by binding to specific cell surface receptors (Kd in the range of 10^{-9}–10^{-12} M). Target cell responsiveness to a particular cytokine is dependent upon expression of receptors for the factor.
Most cytokine actions can be attributed to altered patterns of gene expression in target cells. Phenotypically, cytokine stimulation leads to an increase (or decrease) in the rate of cell proliferation, change in cell differentiation state, or change in the expression of some differentiated function.
Cytokines often act by regulating expression of other cytokines through the mechanism of inducing other cytokines in cascade responses or of decreasing certain other cytokines. Alternatively or additionally, cytokines can regulate the functions of other cytokines by positively or negatively modulating their receptors.
Structurally dissimilar cytokines sometimes mediate overlapping actions (redundancy), but structurally similar cytokines sometimes mediate different actions.
A single cytokine often has multiple target cells and multiple actions (pleiotropy).
Cytokines can have synergistic or antagonistic interactions. Because cells and tissues in the body are rarely at rest, responses to a single cytokine as a time, unperturbed biologic functions are likely to be the result of such interactions.

TABLE 1. *General characteristics of cytokines*

In terms of functions, cytokines are generally pleiotropic, activating multiple distinct consequences, but different cytokines can mediate overlapping functions. They are frequently observed in cascade responses with simultaneous or sequential expression of several kinds. This is because clusters of cytokines are sometimes induced by the same signals and because certain cytokines induce downstream activation of other cytokines. Because they can have synergistic or antagonistic interactions, the interactive biologic effects of the multiple cytokines elicited during infections may be more important than effects mediated by any single factor. After challenges with particular classes of infectious agents, such as viruses, bacteria, or parasites, unique or dominant cytokine responses can be induced and linked to help drive a cascade of initial to innate to adaptive subset cytokine and immune responses that are most effective in defense against the particular agent (Fig. 1). The earliest events appear to be controlled by stimulation of host receptors recognizing unique structures expressed by classes of infectious organisms but distinct from host determinants (23,24,181). Thus, there are many different kinds of cytokines, but expression of particular subsets appears to be regulated by host responses to unique conditions of challenge.

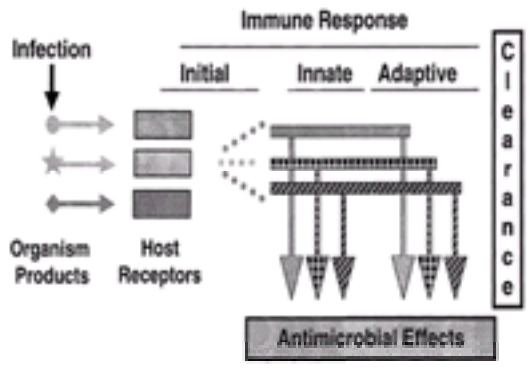

FIG. 1. General pathways of initial to innate to adaptive immune responses during infections. The picture emerging is that initial host responses to infectious agents are induced as a result of recognition, by specific receptors for unique microbial structures that are expressed in or on host cells. As a result of sensing structures expressed by classes of agents, these receptors activate a cascade of events, inducing subsets of innate and adaptive immune responses most effective in defense against the organism being encountered. Particular initial/innate cytokine responses (represented as broken lines) are major mediators in shaping the downstream immune responses.

Cytokine nomenclature reflects the evolution of knowledge from varied areas of investigations. Many of the factors have derived names from the biologic functions originally assigned to them. Examples are the interferons (IFNs), first identified by their ability to interfere with virus replication (222,297), and the tumor necrosis factors (TNF), which were demonstrated to induce necrosis of tumors (134,304). The first demonstrated function, however, is not always the most or only important one. The name interleukin (IL) was adapted for a number of cytokines originally thought to be leukocyte produced to act on other leukocytes. This has led to a long list of numbered factors (currently IL-1 through IL-18). Many of these, however, are now known to be made by, and act on, cells other than leukocytes.

Cytokines can be divided into four major groups based on general induction and function characteristics: (a) initial and innate cytokines, (b) adaptive cytokines, (c) chemokines, and (d) hematopoietic growth factors (1). The first three groups have well-characterized functions associated with abilities to mediate defense against infections by activating biochemical antimicrobial mechanisms or by regulating endogenous immune responses. These are reviewed in Table 2. Generally, initial and innate cytokines are associated during primary infections with early events; depend on nonimmune cells or cells of the innate immune system, such as polymorphonuclear leukocytes (PMNs), monocyte-macrophages (monocytic cells), natural killer (NK) cells, or dendritic cells (DCs), for expression; and contribute to innate immunity. In contrast, adaptive cytokines are associated with later events, depend on T and B cells of the adaptive immune system for expression, and contribute to adaptive immunity. Chemokines are associated with inflammation occurring during either innate or adaptive immune responses. These cytokines and their functions are discussed in more detail below. The fourth group is composed of mediators of leukocyte growth and differentiation. Although some of these factors may be induced during infections, they have been characterized primarily for function in normal leukocyte development, and such information is beyond the scope of this chapter.

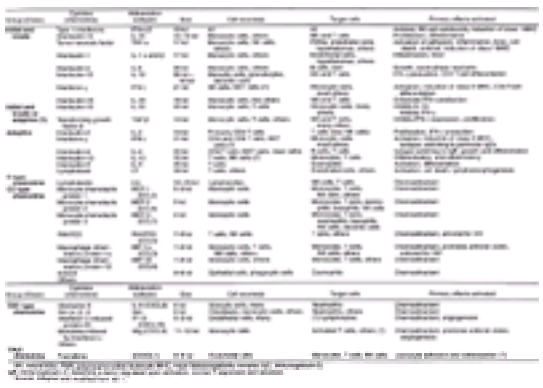

TABLE 2. *List of important cytokines and chemokines*

The focus of the material presented is current understanding of cytokine expression and function in the context of viral challenge. First, an overview is presented of important biologic functions mediated by, and kinetics of induction for, cytokine responses. This is a “low-power” view because events are considered in the context of the intact host and because the existing understanding at this level is still primarily extracellular. Known induction mechanisms and pathways accessed for mediating direct antiviral or direct and indirect immunoregulatory effects are discussed. Because the IFN cytokines induced by viral infections, such as the type I IFN family, appear to be expressed at particular high levels as well as to have unique or uniquely dominant roles in mediating direct antiviral functions and regulating immune responses, these factors are described in extensive detail. Review of type I IFN is at a “higher power” because the intracellular biochemical pathways inducing expression of these factors and delivering their antiviral functions are being precisely defined at the molecular level. The chapter concludes with general discussions of strategies evolved by viruses to avoid or utilize cytokine functions and of cytokine contributions to virus-induced disease.

SPECIFIC CYTOKINE CHARACTERISTICS, IMMUNOREGULATORY FUNCTIONS, AND FACTOR INDUCTION DURING VIRAL INFECTIONS

The immune system traditionally has been divided into innate and adaptive components based on kinetics of responses following primary challenges with infectious agents and on specificity for antigenic determinants associated with protection against secondary challenges. Innate cells, including monocytic cells, NK cells, PMNs, and DCs, express receptors with limited diversity, and tend to respond rapidly as large populations of cells. In contrast, adaptive T- and B-lymphocyte populations express an extensive range of antigen-specific receptors. Primary responses of T and B lymphocytes are delayed because specific subsets of low-frequency cells must be expanded. Innate immunity mediates defense, at times preceding activation of adaptive defense mechanisms. Innate events also make important contributions to shaping downstream immune responses. Such immunoregulatory functions are significant because only subsets of the various component immune responses activate mechanisms most effective in defense against particular challenges. Adaptive immunity is required to clear infections and mediate long-term memory defense. Thus, innate to adaptive responses are cascades in place both to mediate defense during each of the phases and to activate protective downstream immune responses (see Fig. 1). Cytokine constituent responses are known to be of major importance in delivery of both functions. Cell targets for cytokine actions express specific receptors for the factors.

To induce the chain of events activating immune responses most beneficial in defense against a particular infectious organism, the host links information available during the earliest times of infection to subset cascades. Unique microbial structures, distinct from host determinants, provide the early information. The known structures of this type are shared, and tightly conserved, by groups of pathogens sensitive to the same or similar defense mechanisms (181,319). Examples of unique microbial structures of this class are the lipopolysaccharide (LPS) structures on gram-negative bacteria, unmethylated CpG DNA motifs synthesized by invertebrate organisms, and other unique nucleic acid structures, such as double-stranded RNA (dsRNA), not detectable in normal cells but expressed in virus-infected host cells. These structures are sensed through receptors expressed by nonimmune or immune cells and result in activation of particular initial and innate cytokine cascade responses with shaping of downstream innate and adaptive immune responses.

It is difficult to assign responses to either initial or innate response phases. Initial events can be defined as those directly elicited by products of infectious agents, and innate components as those activated by initial response products. The adaptive phase depends on T or B cells. By these definitions, a particular cytokine may be part of all three phases if its expression can be stimulated directly by microbial products, and it can be produced by downstream innate and adaptive responses. Certain cytokines classified as initial or innate (e.g., IFN-a/b, TNF-a, IL-1, and IL-6) are induced in response to microbial products in both nonimmune cells and cells of the innate immune system. Other cytokines can be products of both innate and adaptive immune cells, such as IFN-g produced by NK cells as well as T cells. A few cytokines, such as IL-2, are exclusively assigned to one phase. These tend to be factors made only or predominantly by cells of the adaptive immune system. Cytokines can be related in terms of genetic homology, structural similarities, and overlapping use of receptor components.

Although much remains to be learned, initial and innate induction of high levels of the type I IFN family of cytokines (e.g., IFN-a/b) appears to be a unique or uniquely dominant characteristic of many viral infections (23,24). Other cytokine cascades often associated with responses to nonviral challenges, however, can sometimes be detected in the context of viral infections (Fig. 2). The cytokines known to be induced and functioning in the different phases of responses to primary viral infections are reviewed here (Table 3). The reader should keep in mind that the material is presented in idealized patterns based on results from infections studied in depth. Not all responses fit into these patterns because the host adapts to different conditions of viral infections, subtle or extreme, to access more appropriate defense mechanisms. Thus, many variations can arise.

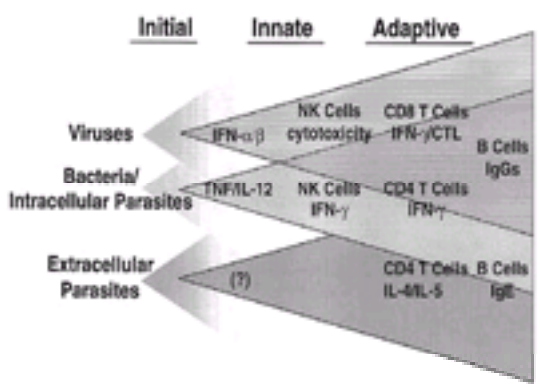

FIG. 2. Key known and proposed cytokine-regulated pathways shaping endogenous immune responses during infection with viruses, bacteria, and intracellular and extracellular parasites. Immune responses to bacteria or intracellular protozoan parasitic infections have dominant innate responses composed of interleukin-12 (IL-12) and natural killer (NK) cell interferon-g (IFN-g) production for shaping of downstream T_H1 type responses with CD4 T-cell IFN-g production. Challenges with certain organisms of these classes also are know to induce an innate cascade of proinflammatory cytokines, including tumor necrosis factor (TNF), IL-1, and IL-6. Responses to extracellular parasites of the helminth group fail to elicit an early IL-12 production and are linked to T_H2 responses with CD4 T-cell IL-4 and IL-5 production. Although responses to viruses have certain characteristics in common with T_H1 responses, they can also have dominant initial IFN-a/b production induced by unique viral products. This response is associated with NK cell cytotoxicity but not IFN-g production and is frequently accompanied by prominent downstream CD8 T-cell responses, including IFN-g production and cytotoxic T-lymphocyte (CTL) function. The patterns are idealized, and there can be overlap (represented by triangle overlap). This is clearly demonstrated during certain viral infections by induction of proinflammatory innate cytokine responses with IL-12 and NK cell IFN-g production and with mixed T-cell cytokine responses.

Virus	Cytokine expression	
	Intial and innate	Adaptive
Lymphocytic choriomeningitis virus	IFN-α/β	IFN-γ, IL-2 IL-4, TGF-β
Murine cytomegalovirus	IFN-α/β TNF, IL-1α, IL-6 IL-12, IFN-γ	IFN-γ, IL-2
Herpes simplex virus type 1	IFN-α/β TNF, IL-1α, IL-6 IL-12, IFN-γ	IFN-γ, IL-2 IL-4, IL-5
Influenza	IFN-α/β TNF, IL-18 IL-12, IFN-γ	IFN-γ, IL-2 IL-4, IL-5, IL-10
Human immunodeficiency virus	?	IFN-γ, IL-2 IL-4, IL-5, IL-10
Respiratory syncytial virus	IFN-γ IL-1, IL-6	IFN-γ, IL-2 IL-4, IL-5, IL-10

IFN, interferon; IL, interleukin; TGF, transforming growth factor; TNF, tumor necrosis factor.

TABLE 3. *Cytokines induced during viral infections*

Initial and Innate Cytokines

At least two clear pathways induce initial to innate cytokine responses. The first leads to IFN-a/b production and is elicited during many viral infections. The second leads to TNF, IL-6, IL-12, and downstream innate IFN-g and is elicited during many nonviral and certain viral infections. Numbers of other cytokines have been reported to be produced at the same time as, or to facilitate, these pathways. They include IL-1, IL-15, and IL-18. Still others, including IL-10 and transforming growth factor-b (TGF-b), have the potential to regulate particular innate cytokine responses negatively.

Interferon-a/b Cascade and a Potential Interleukin-15 Branch

Interferon-a/b

The type I IFNs are a superfamily with four subfamilies: IFN-a, IFN-b, IFN-w, and IFN-t. The IFN-a/b members are the best characterized and are known to be induced in response to viral infections. The factors are products of a single IFN-b and multiple IFN-a genes (222,297). They can be made by virtually any cell type in response to appropriate stimulation, and intracellular infections with viruses can be potent stimulators of IFN-a/b. These cytokines were first identified by their antiviral functions.

The transcriptional activation of the IFN-a and IFN-b genes is being extensively defined in regard to various mechanisms underlying stimulation during infections. Regulation of IFN-a/b genes is complex with potential for many pathways to expression. A well-characterized pathway is from one microbial product, that is, dsRNA, expressed in certain virus-infected, but not in uninfected, cells. Others, however, including interactions between certain viruses and their receptors and intracellular expression of particular viral proteins, exists (100,306). Several positive and negative regulatory gene elements participate in the transcriptional regulation process by binding specific transcription factors activated upon virus infections. The transcription factors are present in inactive forms in the cytoplasm of all cells. Their activation often requires phosphorylation of specific serine and threonine residues. The protein kinases involved in this process remain elusive, although participation of the IkB kinase complex and protein kinase R (PKR) in activating the NFkB transcription factor has been demonstrated (172). NFkB is one of the factors required for inducing transcription of the IFN-b gene and certain IFN-a genes. The other activated factors needed for induction of all IFN genes constitute the family of interferon regulatory factors (IRFs). There are at least 10 members of the IRF family (198). These molecules contain DNA-binding domains recognizing specific DNA elements (the interferon response element) in the IFN genes and in many IFN-inducible genes. The IRFs also contain transcriptional regulatory domains to activate or repress transcription, depending on the cell and gene context. Some IRFs are constitutively present in cells, whereas others are induced by IFNs. A few IRFs have cell type–dependent expression. The two IRFs most relevant for induction of the IFN genes are IRF-3 and IRF-7. A virus-activated factor that also mediates this process contains IRF-3, IRF-7, and the transcriptional coactivators p300 and the creb-binding protein (CBP) (306). This complex, in conjunction with NFkB and Atf-2/c-Jun, is recruited to the promoter region of the IFN-b gene to activate its transcription.

An interesting cascade operating in the IFN gene induction process has been recently discovered (173). The first members of this cascade are the IFN-b and specific IFN-a genes. They are activated by virus infection through the use of ubiquitously present IRF-3. These IFNs are then secreted and act through the IFN-a/b receptor to induce synthesis of IRF-7, not usually present in untreated cells. IRF-7, in turn, is used for the induction of other IFN-a members whose genes cannot be induced by IRF-3. This observation provides a molecular explanation for the phenomenon of priming for viral infection–induced IFN production by pretreating cells with a low IFN dose. It also couples, in a physiologic sense, the signal transduction pathways and the transcriptional induction mechanisms used by IFN and IFN-inducible genes. Thus, the direct intracellular signaling pathways for initial IFN-a/b induction, in response to infections, are being delineated clearly, and secondary type I IFN responses are induced in innate response to expression of the initial IFN-a/b gene products.

There are likely to be many additional mechanisms for IFN induction. The existence of the complex gene family may in fact be in place to respond to different stimulatory pathways. Several other biochemical pathways to IFN-a/b expression being identified include pathways induced during nonviral infections (66) or in response to the CpG DNA motifs synthesized by bacteria (15,277,288). Some of these mechanisms may preferentially stimulate IFN-a/b expression in particular cell types because they are preferred targets or are uniquely equipped to respond. In this regard, it is interesting to note that low-frequency populations of human DCs are potent producers of type I IFN in response to challenge with herpes simplex virus or influenza virus or to stimulation with CD40L (40,73,264). Much remains to be learned about IFN-a/b expression, but systemic levels of the factors appear to be uniquely induced to high and sustained levels during many viral infections, including those with lymphocytic choriomeningitis virus (LCMV) and murine cytomegalovirus (MCMV) (22,24). Production at this level is likely to be in part the result of recruiting large numbers of

cells, including nonimmune cell types such as fibroblasts and vascular endothelial cells, into IFN-a/b production. Major functions for such systemic and sustained IFN-a/b responses are to protect the many different host compartments and distal sites at risk for spreading or for secondary waves of infections. The conditions, however, are also likely to exert unique immunoregulatory effects on ongoing and developing immune responses (see later). A few viruses are reported to be poor inducers of IFN-a/b—most notably, respiratory syncytial virus (RSV) (45,232).

All nucleated cells express IFN-a/bRs. These are heterodimers comprised of IFNAR1 and IFNAR2c chains. A variety of generalized antiviral and cell-type specific immunoregulatory functions are activated as a result of binding the IFN-a/b forms to these receptors. The information available on the intracellular biochemical pathways activated through the IFN-a/bRs and on the complex potential and known immunoregulatory functions of IFN-a/b is reviewed later. Many IFN-a/b actions have been identified in vivo. Although some of these are direct, others are likely to be secondary effects. IFN-a/b cytokines can regulate expression of a number of other cytokines and cytokine receptors and, as a result, modify downstream consequences mediated by other factors.

Interleukin-15

In the context of cytokine cascades, IFN-a/b expression has a possible link to the induction of another innate cytokine, IL-15 (299). This factor was first identified as a stimulator of T-cell responses but is now known to activate both NK and T cells to proliferate, to be important for NK cell development, and to have a variety of other immunoregulatory functions. It is thought to be made by a number of different cell types and is known to be produced by monocytic cells in response to appropriate stimulation. The high-affinity receptor for IL-15 is a trimer (IL-15R) with a, b, and g chains (299). The IL-15Ra chain is specific for IL-15, but the b and g chains also bind the adaptive cytokine, IL-2, with a lower affinity and associate with an IL-2Ra chain to form the high-affinity IL-2R receptor. IL-15 can be made by a variety of cells, including monocytic cells. This cytokine may be induced with other proinflammatory cytokines (37). Its induction at the mRNA level, however, has also been observed after stimulation with type I IFNs, chemical inducers of type I IFNs, or viral infections (12,75,322). Expression of IL-15 is dependent on IRF-1, and this transcription factor can be activated by stimulation with IFN-a/b. Thus, there is a potential for IL-15 to be induced during viral infections either through direct stimulation or indirectly as a result of IFN-a/b–mediated effects.

***Other Immunoregulatory Functions of Interferon-* a/b**

IFN-a/b can positively or negatively regulate other cytokine functions by modulating expression of these factors or their receptors. Of particular relevance during innate responses, type I IFNs can inhibit expression of IL-12 (52,124,180). Other early changes induced by or associated with IFN-a/b expression are modifications in immune cell distribution *in vivo* (22,24,99,115), activation of NK cell cytotoxic activity (22), inhibition of NK cell responsiveness to IL-12 (200), *in vivo* induction of modest NK cell and memory CD8 T-cell proliferation (22,289), enhancement of pathways for presenting antigen on class I molecules of the major histocompatibility complex (MHC) (see later), and facilitation of T-cell IFN-g responses (31,53,234,235,251). Thus, the products of the initial and innate induction of the IFN-a/b cascade have the potential to regulate a variety of downstream innate and adaptive immune responses. Moreover, at extremely high concentrations, IFN-a/b inhibits the proliferation of a variety of cell types.

The Tumor Necrosis Factor-* a, *Interleukin-1, Interleukin-6 Cascade and an Interleukin-12, Interferon-* g *Branch

Other initial and innate cytokine responses may include a cascade of TNF-a, IL-1, and IL-6. This clustered response has been best characterized after stimulation with LPS components from gram-negative bacteria (1). TNF-a expression appears to be a major regulator of the cascade because treatment with factor alone induces the other two cytokines, but LPS can induce IL-1 in the absence of TNF-a. Often called the proinflammatory cytokine cascade, this response is generally detectable during inflammation. IFN-a can sometimes be seen under these conditions (86), but it is not clear that the factor is induced to the levels elicited by viral infections. Additional cytokines, such as IL-12, IL-15, IL-18, and NK cell–produced IFN-g, have also been reported to be associated with the innate TNF-a, IL-1, and IL-6 cascade under particular conditions, most notably stimulation with bacteria and bacterial products. An understanding of pathways to induction of this cytokine cluster is advancing. For example, CD14 and Toll-related receptors act in concert to bind LPS to induce signal transduction cascades, ultimately activating expression of the TNF-a, IL-1, and IL-6 genes (135,319).

Although the list of conditions under which certain of these proinflammatory cytokines are produced during viral infections is growing, less is known about how viruses elicit them. Expression of messenger RNA (mRNA) or protein is elevated for TNF-a, two major forms of IL-1, IL-6, and the two chains of heterodimeric IL-12 during infections with a variety of viruses, particularly the herpesviruses, including cytomegalovirus (CMV) (see Table 3) (2,121,122,192,207,208,226,243,244). TNF-a, IL-6, and IL-12 proteins are all expressed in the circulation during some viral infections and IL-18 has been detected during MCMV infection (223). The IL-1 cytokines are not readily detectable at the protein level. Induction of biologically active IL-12 during initial and innate immune responses elicits downstream induction of IFN-g production by NK cells, and NK cell–produced IFN-g can also be detected in the circulation during MCMV infections (208,209,243). The systemic TNF-a, IL-1, and IL-6, as well as the IL-12–to–IFN-g branch, cascade responses are likely to be in place to induce antimicrobial functions, promote inflammation at distal sites, and communicate with other organs, such as the brain and liver, to elicit secondary responses. As is the case with most cytokines, these responses must be tightly controlled. Under conditions of profound stimulation, including bacterial sepsis and certain viral infections, constituents of the proinflammatory cascade can be produced systemically, reach high levels in serum, and induce toxic shock conditions with coagulation, vascular collapse, and death.

*TNF-*a

Many of the important biologic functions mediated by TNF-a are related to its inflammation-promoting activity, that is, induction of enhanced adhesive properties of vascular endothelial cells and PMNs as well as vascular permeability (1,134,300). TNF-a mediates a variety of additional immunoregulatory and antiviral functions. Cell death (320), activation of monocytic cell functions (1), DC migration (231,302), enhancement of IFN-g responses (1), up-regulation of class I

MHC expression (1,296), and activation of certain antiviral pathways (91,159,197,314) are among the responses known to be induced by or associated with TNF-a expression. Under toxic shock conditions, TNF-a is required for disease induction and the detrimental conditions resulting in death (105,134). During systemic responses, the cytokine can also function as an “endogenous pyrogen” to communicate with hypothalamic regions of the brain and induce fever (1). TNF-a is made by a number of cell types, but activated monocytic cells are often the major producers. It is a member of a cytokine family including a related form, called lymphotoxin (LT), made primarily as an adaptive cytokine (41). TNF-a is synthesized as a type II membrane protein. The released form of the molecule is produced by proteolytic cleavage at an extracellular, membrane-proximal site. Both membrane-bound and released forms can mediate biologic functions. A number of different receptors for TNF-a, including TNF receptors type 1 (TNFR1) and 2 (TNFR2), are expressed on diverse cell types (283).

Interleukin-1 and Interleukin-6

The other members of this cascade, IL-1 and IL-6, are also made by both nonimmune cells and cells of the immune system. In comparison to TNF-a, however, they appear to be induced more readily in particular nonimmune cells (1). There are three gene products in the IL-1 family: IL-1a and IL-1b, functioning as stimulatory factors, and an IL-1 receptor antagonist, IL-1ra. Both IL-1a and IL-1b are synthesized as precursor molecules requiring proteolytic cleavage to generate mature forms. The IL-1a precursor may have some biologic activity, but the IL-1b form has to be cleaved to acquire function. An IL-1 protease, called the IL-1b-converting enzyme (ICE), or caspase 1, is responsible for most of the IL-1b processing (71,150). All three products of the IL-1 gene family can bind to two different characterized receptors. A type 1 receptor, however, may have higher affinity for IL-1b and is primarily responsible for IL-1–mediated responses. The type 1 receptor is expressed on virtually all cell types. A type 2 receptor can competitively inhibit IL-1 binding to the functional type 1 receptor and may be expressed on a more limited range of cell types. The functions mediated by IL-1 overlap with those mediated by TNF, in that the factor has been reported to enhance inflammatory and IFN-g responses and to be an endogenous pyrogen. In contrast to TNF-a, however, it does not appear to be required for many of the detrimental effects induced during cascade responses but can be a major contributor to eliciting IL-6. The two best-characterized functions of IL-6 are to promote B-cell growth and activate hepatocytes (1). Thus, prominent features of the proinflammatory cascade in total are (a) toxic potential of TNF-a, (b) overlapping functions for TNF-a and IL-1 in promoting inflammation and communication with the brain, (c) IL-1 induction of IL-6, and (d) unique IL-6–dependent links to other immune and hepatic responses.

Interleukin-12

Expression of IL-12, IFN-g, IL-15, and IL-18 can also be induced under conditions of LPS stimulation (37,279,317). In contrast to the expression of TNF-a, IL-1, and IL-6 by a variety of cell types, IL-12 appears to be primarily produced by monocytic cells, certain DCs, and PMNs (290). IL-12 is unusual among cytokines because it is a heterodimeric molecule composed of 35-kd (p35) and 40-kd (p40) subunits. Generally, the p35 subunit is constitutively expressed, and the p40 subunit is induced in response to challenge. Biologic activity is dependent on the presence of both chains. The receptor for IL-12 is composed of two subunits, the b1 and b2 chains, and is primarily expressed by NK and T cells (290). IL-12 was originally identified as a potent inducer of cytotoxicity and IFN-g production by NK cells and activated T cells. It is now clear that a major function for the factor is induction of NK cell IFN-g production under conditions of challenge with a variety of agents (21). It also appears to promote a particular subset of CD4 T-cell responses associated with IL-2 and IFN-g production (21,170,290).

Interferon-g

IFN-g was originally called type II because it can induce antiviral states. It was called immune IFN because, in contrast to the type I IFNs, it is made exclusively by cells of the immune system (29). IFN-g is primarily an NK cell product during innate responses, but it can be made by T cells during adaptive responses. A heterodimeric IFN-g receptor, distinct from the IFN-a/b receptor with IFNGR1 and IFNGR2 molecules, is expressed on virtually all nucleated cells. Despite the use of different receptors, certain IFN-a/b and IFN-g functions are shared because the signal transduction pathways activated through these receptors partly overlap (see later). However, IFN-g can mediate a number of unique immunoregulatory functions. Activation of monocytic cells to deliver antimicrobial defense mechanisms is an extremely important IFN-g function. After exposure to this cytokine, mononuclear phagocytes are induced to synthesize enzymes required for reactive oxygen intermediates contributing to the killing of phagocytosed organisms, particularly bacteria. IFN-g can act alone to induce nitric oxide synthase 2 (NOS2 or iNOS) (165). This enzyme contributes to the production of NO radicals for antimicrobial defense against a variety of agents, including viruses. TNF can enhance iNOS induction by either IFN-g or IFN-a/b, but the IFN-g effects are more potent. Thus, IFN-g is an important intermediary in induction of known effector mechanisms contributing to resistance against infections. Because there is a growing list of other enzyme responses to IFN-g, including the induction of a series of molecules having homologies to enzymes dephosphorylating guanosine triphosphate (GTP), that is, GTPases (28,36,286,287), there are likely to be additional or alternative mechanisms for IFN-g–induced antimicrobial defense. IFN-g can promote and amplify inflammatory responses by up-regulating expression of TNF receptors to potentiate the effects of this cytokine and by activating vascular endothelial cells. Finally, IFN-g enhances expression of a particular subset of chemokines. All of the effector mechanisms and inflammatory pathways are important consequences of IFN-g induction during either innate or adaptive responses.

A number of other immunoregulatory functions can be mediated or induced by IFN-g. IFN-g can activate NK cell cytotoxicity, but this may be of less importance than IFN-a/b activation during viral infections (22,92,208,211). Many other immunoregulatory functions appear to be in place to shape the development of adaptive immune responses. In particular, IFN-g induces increased class I and class II MHC expression on a wide variety of cell types (164), enhances CD4 T-cell differentiation to responses associated with IL-2 and IFN-g production by promoting expression of the IL-12 receptor (92,235,278), and acts on B cells to facilitate immunoglobulin class switching to particular subclasses, most notably immunoglobulin G2a (IgG2a) in the mouse (21). Taken together with the enhancement of mononuclear phagocytic cell functions, these immunoregulatory effects of IFN-g are likely to be most important in promoting antigen processing and presentation to CD4 T cells and in helping particular antibody responses against extracellular pathogens.

Interleukin-18

Less is known about the induction and function of IL-15 and IL-18. The IL-15 characteristics and proposed IFN-a/b induction were reviewed earlier. IL-18 was identified by its ability to promote IFN-g expression, and as a result, it was first named IFN-g–inducing factor (IGIF) (204). It is also sometimes called IL-1g because it has structural and biochemical similarities to the other IL-1 molecules. In particular, it is synthesized as an inactive precursor molecule and requires proteolytic cleavage by ICE (or other caspases) for release of the active form (71,87,101). Although only limited information is available concerning the IL-18 receptor, it is known to be different from those binding IL-1. It appears to be expressed on NK, T, and B cells. IFN-g expression can be induced by IL-18 alone or in synergy with IL-12. Most of the work characterizing induction of IL-18 expression has been done following stimulation with Propionibacterium acnes and LPS challenges. The cytokine appears to be readily elicited in response to this stimulation protocol and is found at high levels in liver (176,279). A natural soluble binding factor exists, IL-18–binding protein (203). IL-18 may not be required for driving T-cell IL-2 and IFN-g responses, but it can enhance them by facilitating IFN-g production and T-cell IL-12 receptor expression (233). In addition, there are intriguing data that indicate that IL-18 can act with IFN-a/b in promoting T-cell IFN-g responses (251).

Other Innate Cytokines

A number of other cytokines made by nonimmune or innate immune cells have the potential to mediate immunoregulatory functions during infections, including IL-10 and TGF-b, factors known to be potent inhibitors of IFN-g expression (163,239). These cytokines are induced under particular conditions of viral infections (250,276). Nonadaptive immune cell populations can be sources of the factors, but IL-10 and TGF-b have been more frequently observed at times of adaptive immune responses to viral infections.

Two other cytokines, IL-4 and IL-13, have structural similarities, share receptor components for intracellular signaling, induce overlapping biologic responses, and have potential to inhibit IFN-g expression and promote a subset of CD4 T-cell responses associated with IL-4, IL-13, and IL-5 production (44). These cytokines can be produced during adaptive immune responses and are induced at times of T-cell responses to certain viral infections. Little is known, however, about their expression during innate responses. Populations of mast cells and NK T cells, with similarities to classic NK cells but also expressing T-cell receptors for antigens, can be induced to make IL-4 under particular conditions of stimulation (18). It is not yet clear, however, whether these responses are activated during innate immune responses to challenge, and there is no definitive evidence for IL-4 stimulation during innate responses to viral infections. Likewise, although NK cell subsets making IL-5 or IL-13, but not IFN-g (22,110,220), have been identified, infection conditions under which such populations would be induced have not yet been defined.

Adaptive Cytokines

In comparison with initial and innate cytokine responses, adaptive cytokine responses are delayed and are associated with T-cell activation. A number of cytokines are produced during adaptive immune responses. Some of these are made exclusively by T cells. Some, however, are also made by other cell types but are produced at moderate or high levels by activated T cells. T-cell subsets can develop to produce preferentially particular adaptive cytokines, and the subset responses have been shown to be associated with defense against particular types of agents (see Fig. 2) or types of cytokine-mediated disease. For example, helper T-cell subtype 1 (T_H1) responses result in high production of IL-2 and IFN-g, are major contributors to delayed type hypersensitivity, and contribute to defense against bacterial or intracellular protozoan parasite infections (21,130,218). In contrast, helper T-cell subtype 2 (T_H2) responses result in IL-4, IL-13, and IL-5 production; are associated with antigen-induced airway hyperresponsiveness (asthma); and contribute to defense against certain parasitic infections (218,313). In general, polarization toward T_H1 responses is beneficial in defense against nonviral intracellular pathogens because IFN-g activates macrophage-dependent antimicrobial functions against such organisms. Development of T_H2 responses is beneficial in defense against extracellular parasitic pathogens, such as helminths, in part because IL-5 elicits production and recruitment of eosinophils and defense mechanisms delivered by these cells. Both cytokine profiles can promote B-cell production of antibody but are associated with different classes of antibody isotype production. High-level production of IgE is a characteristic of T_H2 responses.

The picture emerging from *in vitro* experiments and studies of nonviral infections is that development of a particular adaptive cytokine response is dependent on activation of T cells through their receptors for antigen (TCRs), the magnitude of stimulation, and the cytokine milieu in which the T cells are stimulated (2,25,92,235,260,278). For the most part, “polarized” subset responses have been characterized with CD4 T cells. Antigens for their activation are processed from extracellular forms and presented by class II MHC molecules. They are called helper responses because they promote T- or B-cell responses. As stated earlier, induction of IL-12 and IFN-g during innate immune responses preferentially promotes T_H1 CD4 T cells. In contrast, T_H2 CD4 T cells are promoted by the absence of IL-12 and IFN-g and the presence of IL-4 and IL-10 (218). A variety of infections with nonviral agents have been shown to result in polarized T_H1 or T_H2 profiles, but the T-cell cytokine responses characterized during a number of viral infections appear to be of a more mixed phenotype (see Fig. 2 and Table 3). In addition, some infections, particularly viral, induce CD8 T-cell responses. Antigens for activation of these cells are usually processed from intracellular forms and presented by class I MHC molecules. CD8 T-cell responses are generally associated with IFN-g expression and MHC class I–restricted, cytotoxic T-lymphocyte (CTL) function. Our understanding of the innate response conditions promoting particular CD8 T-cell functions is still limited.

Type 1 T-Cell Cytokine Responses

Interleukin-2

T cells produce IL-2, and a major function for this factor is promoting T-cell proliferation. Activated T cells express the

high-affinity trimeric form of IL-2R, comprised of the g and b chains shared with IL-15R and a specific IL-2Ra chain (299). NK cells generally express the g and b chains but are infrequently found with the high-affinity form of IL-2R. As a result, NK cells can be activated in response to IL-2 but are much less efficient than activated T cells in binding and utilizing the factor at the major times of production. In addition to acting as an autocrine growth factor for T cells, IL-2 is a potent inducer or facilitator of T-cell IFN-g production (51,271). B cells can respond to IL-2, and the factor promotes antibody synthesis. Viral infections have been shown to induce IL-2 expression (see Table 3). CD4 T cells are major producers, but CD8 T cells can express IL-2 under conditions of profound activation and during certain viral infections (2,51,128,129,275).

Interferon-g

Given its roles in supporting T-cell expansion and enhancing IFN-g expression, it is not a coincidence that IL-2 production is associated with T-cell IFN-g responses. T-cell IFN-g production is an important contributor to antiviral defense, and the pathways accessed for this are similar to those used by NK cell–produced IFN-g. The same cytokine is simply being made by two different cell types. The immunoregulatory functions, including facilitating switching of immunoglobulin classes and enhancement of antigen processing and presentation on MHC molecules, also predictably overlap. A major difference between NK cell and T-cell responses, however, is that T-cell responses are sustained for longer periods of time. As a result, there is potential for continued access to T-cell IFN-g production. Another apparent difference is that in contrast to the systemic NK cell IFN-g responses sometimes observed early during infections, T-cell IFN-g responses appear to be more often restricted to particular compartments. A likely contributing factor is the profound stimulatory effect for IFN-g expression mediated through TCR engagement (2,38). This signaling pathway needs T-cell exposure to antigen presented on MHC molecules. As a result, it provides an intrinsic regulation for need; that is, if viral antigen is cleared in vivo, T cells will not be stimulated through their TCRs.

A chain of events with innate IL-12 and NK cell IFN-g responses facilitating adaptive CD4 T-cell IFN-g responses is required to achieve optimal protection against nonviral, intracellular bacterial infections such as those with Listeria monocytogenes (130). Through this pathway, an NK cell IFN-g response can “pass off” a major function to a longer lasting-lymphocyte subset with different conditions for responding. It is not clear that such a pathway is needed during viral infections. CD4 T cells are induced to produce IFN-g, but T-cell IFN-g responses have been observed in the absence of endogenous biologically active IL-12, during a number of viral infection (53,188,207,213,258). This may be in part because many viruses elicit specific CD8 T-cell responses primed to produce high levels of IFN-g upon engagement of their TCRs. Most notable among these are infections of humans with Epstein-Barr virus (EBV) (141), influenza virus (55), and human immunodeficiency virus (HIV) (55,116) and infections of mice with LCMV (34,53,194), influenza virus (78), polyomavirus (160), and murine gamma-herpesvirus 68 (MHV-68) (274). Studies in culture suggest that the requirements for IFN-g expression in response to TCR stimulation are different for CD8 than for CD4 T cells, or that CD8 T cells may be more readily induced to produce IFN-g (38). Another pathway with IFN-a/b promoting CD8 T-cell IFN-g responses is being defined in the context of viral infections (see later).

Type 2 T-Cell Cytokine Responses

Interleukin-4 and Interleukin-13

As stated earlier, IL-4 and IL-13 have structural and functional similarities. T cells are major producers of both cytokines. IL-4 was first identified by its function in promoting B-cell responses. It is now know to be important in regulating IgE production and mast cell and eosinophil functions (1). IL-13 mediates overlapping functions, and both cytokines appear to contribute to recruitment of cells into tissues during allergic reactions (1,313). The cytokines use a common dimeric receptor expressed largely on nonlymphoid cells, including macrophages and vascular endothelial cells. One of these chains, however, can pair with the g chain common to the IL-15 and IL-2 receptors to form a specific IL-4 receptor on lymphocytes. Although polarized T_H1 and T_H2 responses are induced during certain nonviral infections, a number of different viral infections have been reported to express some level of IL-4 along with IL-2 and IFN-g (see Table 3). For the most part, IL-4 appears to be expressed at very low levels. Little information is available concerning the induction and function of IL-13 during viral challenges.

Interleukin-5

The major known function of IL-5 is induction and activation of eosinophils. These populations have not been observed frequently, or reported to mediate defense, during viral infections. They have been detected during respiratory viral infections, in particular those with RSV, and a predisposition for IL-5 expression after infectious challenge is induced by vaccination with the heavily glycosylated RSV G protein (see Table 3) (107,112,206,269).

Other Cytokines Made by T Cells

Lymphotoxin

T cells make a variety of other cytokines, including TGF-b and IL-10. They can make TNF-a but do not appear to be a major source of the factor under most conditions of in vivo challenge. They are important producers of a related cytokine, LT. LT has been called TNF-b because of its homology to TNF-a. It can compete for binding to TNF receptors. As a result of binding to these, it can induce functions overlapping with TNF. However, LT can also form a unique complex recognized by a different receptor identified as the LT b-receptor. This cytokine is often produced along with IFN-g by activated T cells and is generally considered part of T_H1 responses. A major unique function of LT is promotion of lymphomorphogenesis (41). Induction of LT expression at the mRNA level has been reported during a number of infections, but protective functions specifically mediated by LT under these conditions have not been defined.

UNFOLDING OF ENDOGENOUS IMMUNE RESPONSES DURING VIRAL INFECTIONS

The kinetics of virus-induced immune responses, as revealed during primary purposeful infections of mice and natural human infections, is schematically presented in Figure 3. Certain viruses activate component responses overlapping with those characterized as T_H1, but they also appear to elicit unique components. In particular, during initial and innate immune responses, uniquely dominant characteristics include prominent initial and innate IFN-a/b release and NK cell cytotoxicity (22,208). In LCMV infections of immunocompetent mice, IFN-a/b responses are observed without induction of detectable IL-12 (52,207). Moreover, although there are examples of both IFN-a/b and IL-12 initial and innate responses after infections with MCMV or influenza virus (188,207), they have dichotomous functions. During MCMV infections, IFN-a/b is necessary for induction of NK cell–mediated cytotoxicity but not IFN-g production. Conversely, and in contrast to the IL-12 role in activation of both NK cell responses during certain nonviral infections (23,256), IL-12 is required for MCMV infection–induced NK cell IFN-g production but not cytotoxic activity (207,208). Many of the NK cell–mediated antiviral effects depend on their being induced to make IFN-g (22,207,210), but the contribution of NK cell–mediated cytotoxicity to defense is less well documented (22,284). Thus, there are examples of initial and innate cytokine responses to viral infections sharing characteristics with those setting up for T_H1 responses during nonviral infections. These are associated with induction of NK cell–mediated antiviral effects, but there is also induction of IFN-a/b under these conditions. Moreover, certain viral infections induce IFN-a/b but fail to induce detectable IL-12.

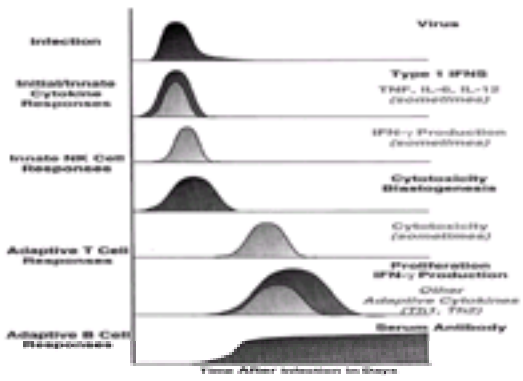

FIG. 3. Kinetics of known endogenous cytokine and cellular immune responses to viral infections during primary challenges. Initial events can include induction of prominent interferon (IFN)-a/b responses. Some viral infections also induce detectable tumor necrosis factor (TNF), interleukin-6 (IL-6), and IL-12 responses with an associated natural killer (NK) cell IFN-g production. NK cell cytotoxicity and blastogenesis are elicited as a result IFN-a/b responses. Adaptive responses include CD4 and CD8 T-cell responses with high-level expression of IFN-g, moderate expression of the T_H1 cytokine IL-2, and low-level production of the T_H2 cytokine IL-4. Dramatic CD8 T-cell proliferation with IFN-g production can be induced in a wide range of infections. Class I–restricted and virus-specific cytotoxicity mediated by CD8 T cells can be detected during some viral infections. B-cell antibody responses are extended for long periods of time.

In addition to the differences observed during initial and innate events, adaptive immune responses to viral infections can be contrasted with those to nonviral infections in terms of CD4 T_H1 and T_H2 and magnitudes of CD8 T-cell responses. Studies examining expression of the T_H1 cytokines, IL-2 and IFN-g, and the T_H2 cytokines, IL-4 and IL-5, indicate that T-cell responses to viral infections can be of mixed phenotypes with high IFN-g, moderate IL-2, and low IL-4 levels (35,97,112,166,167,187,201,237,250,275,310) (see Table 3). Moreover, CD8 T-cell responses can be induced to uniquely high levels with profound expansion, along with priming and activation of IFN-g production and cytotoxicity (2,6,34,53,78,194,274). Because these CD8 T-cell responses contribute to antiviral defense, their induction is important to the host. Remarkably, IL-12–independent T-cell IFN-g responses have been demonstrated during LCMV (53,207,213), influenza virus (188), and MHV (258) infections. Moreover, patients with deficiencies in IL-12 responsiveness have notably increased susceptibility to bacterial, but not viral, infections (5,62). Thus, the paradigm emerging from the studies of nonviral agents—that IL-12 is a required innate cytokine for promoting critical T-cell IFN-g responses—cannot be applied to the viral infections studied to date.

Given the direct pathways to IFN-a/b induction and the many immunoregulatory functions of cytokines, these factors are good-candidate molecules for dominant regulators of endogenous adaptive immune responses to viral infections; that is, they provide the key link between information available at early times during the infection and downstream immune responses. As stated previously, their expression results in profound early changes in cell distributions and modifies expression and function of other cytokines. By recruiting T- and B-cell repertoires into specialized areas for antigen presentation, the IFN-a/b–induced trafficking changes may increase the likelihood of activation of low-frequency antigen specific cells. Alternatively or additionally, they may localize adaptive cell populations into specific cytokine milieus elicited during innate responses to viral infections, and these could in turn mediate immunoregulatory functions directing downstream immune responses.

They may also uniquely regulate the endogenous cytokine milieu to promote the particular T-cell subset responses needed during viral infections. IFN-a/b set up conditions actively inhibiting IL-12–regulated pathways because they block IL-12 induction (52,53,124,180). This may be important to protect against viral infection–enhanced sensitivity to IL-12–induced toxicities (210,212) but would also limit access to a pathway promoting CD4 T-cell IFN-g responses. On the other hand, IFN-a/b may drive preferential stimulation of CD8 T-cell responses by enhancing class I MHC presentation, or IFN-a/b–induced NK cell-mediated cytotoxicity may negatively regulate T-cell responses (22,273). Finally, recent studies of a limited number of viral infections—influenza virus (83), herpes simplex virus (HSV) (144), and Sindbis virus (245)—in mice deficient in IFN-a/b functions show that these cytokines modulate viral cell tropism. If so, the characteristics of the primary host immune responses might be altered because of differences in antigen presentation or cytokine production resulting from infection of different target cells. Thus, many of the effects mediated by IFN-a/b have

the potential to promote particular T-cell subset responses during viral infections.

Nevertheless, understanding of exactly how CD8 T cells are regulated *in vivo* is still limited. IFN-a/b can enhance human T-cell IFN-g production under certain specific conditions in culture. However, experiments to date have examined only IFN-g roles for modest effects in association with IL-12 (169), for CD4 T-cell subset IFN-g responses (31,234,235), and for dramatic effects in association with IL-18 (251). Studies of the effects of blocking endogenous IFN-a/b functions during infections of mice with LCMV indicate that two divergent supporting pathways can act to promote CD8 T-cell IFN-g responses (53). Under normal conditions of infections, the CD8 T-cell IFN-g response is dependent on endogenous IFN-a/b effects but is IL-12 independent. In contrast, in the absence of IFN-a/b functions, an IL-12 response is revealed to substitute an alternative pathway to IFN-g. Thus, there appear to be important IFN-a/b–controlled pathways uniquely shaping T-cell IFN-g responses during viral infections, but the immune system has plasticity for accessing divergent innate mechanisms to achieve the same goal.

Although there are mechanisms by which IFN-a/b may promote activation of CD8 T cells and CD8 T-cell IFN-g responses, the cytokines are not essential for induction of CD8 T-cell cytotoxic function. Deficiencies in IFN-a/b functions are associated with poor detectable CTL responses during LCMV, but not vaccinia virus (VV), infections (193,294). CD8 T-cell cytotoxicity is essential for antiviral defense during certain viral infections (118), but the requirements for eliciting CTLs *in vivo* are poorly understood. IL-2 promotes CD8 T-cell expansion *in vivo* and enhances IFN-g production by these cells (51,275). As a result of supporting cell expansion, the factor also enhances the magnitude of CTL responses (51). It is not, however, absolutely required for *in vivo* induction of CD8 T-cell–mediated cytotoxicity (51,137). This may be in part because mixed cytokine responses can be observed during viral infections and because IL-4 can support certain CD8 T-cell responses (14). Alternatively or additionally, IL-15 made by non–T-cell populations may help promote CD8 T-cell responses (299). CD4 T-cell help for induction of acute CD8 T-cell responses is observed in some but not all viral infections (68,191,227), but CD4 T cells or IFN-g responses may be important for the general maintenance of CD8 T-cell memory (2,298). IFN-g does not appear to be required for CTL induction (96).

Acute and vaccine-induced changes in the conditions of RSV infections merit special consideration. Responses to respiratory infections with this virus appear to stand out from those characterized during many other viruses because RSV is a poor IFN-a/b inducer (45,232), eosinophils can sometimes be observed during infections with this agent (85), and vaccination with the highly glycosylated RSV G protein can predispose host responses upon challenge with the infectious agent to include IL-5 production and an accompanying lung pathology (7,94,206,269,304). Surprisingly, vaccination with one RSV antigen epitope presented to CD8 T cells as well as the G protein can enhance IFN-g production and protect against priming for the detrimental IL-5 response and disease (268). These observations suggest that there may be a particular requirement for inducing CD8 T-cell IFN-g responses at times of secondary CD4 T-cell responses to drive T_H1 over T_H2 responses preferentially to RSV. Although highly speculative, it is possible that the requirement is a result of the fact that alternative IFN-a/b–facilitated CD8 T-cell IFN-g responses are missing during infections with this virus.

CHEMOKINES

A group of low-molecular-weight cytokines, about 8 to 12 kd in size, with chemotactic functions are being described (see Table 2) (236,240,255). Because they are chemotactic cytokines, the mediators are called chemokines. This area of research is in a dramatic expansion period, and many newly identified factors are likely to be added to this group. The factors have important proinflammatory functions and can be induced in response to challenge with a number of infectious agents and their products and in response to particular cytokines. Although characterization of production has largely focused on immune cells, particular chemokines can be made by a variety of immune and nonimmune cells. The factors are broadly distributed into four families, C, C-C, C-X-C, and C-X3-C, based on their amino acid sequences. The C family has only one known member, lymphotactin. This factor targets lymphocytes for chemotactic responses. Many of the members of the C-C chemokine family are known to be produced by activated T cells and monocytes, but at least two members—the macrophage inflammatory protein-1a (MIP-1a) and a factor *r*egulated upon *a*ctivation, *n*ormal *T* *e*xpressed and *s*ecreted (RANTES)—can also be made by NK cells (26,72,205). C-C chemokine cell targets include monocytes, lymphocytes, and NK cells. The C-X-C chemokines are largely produced by activated monocytes, but certain family members can be produced by other cell types. A C-X-C chemokine, with a particular requirement for IFN-g in induction, is the monokine induced by IFN-g (Mig) (8,32,315,316). This factor may be uniquely restricted for, and especially potent at, induction of activated T-cell migration. Major target cells of the C-X-C chemokines include neutrophils, monocytes, and lymphocytes. The C-X3-C family has one known member, fractalkine. Monocytes and lymphocytes are major target cells of this factor.

Identification of specific roles for particular chemokines is complicated by the fact that they bind to and utilize different, but overlapping, receptors, and that the expression of multiple cytokines is generally induced simultaneously. As an example, the ligands MIP-1a, RANTES, and the monocyte chemotactic protein-1 (MCP-1) can all bind to the CC chemokine receptor CCR5 in culture systems (236). Moreover, conditions associated with MIP-1a induction are reported to induce other CC chemokines, including RANTES, during infections with VV (8), LCMV (11), mouse hepatitis virus (MHV) (142), HSV, and HIV (26,72). Nevertheless, there is good evidence supporting a specific requirement for a single chemokine (MIP-1a) during inflammatory responses *in vivo*. MIP-1a is crucial to migration of protective NK cells into infected livers during innate responses to MCMV infection (249). This results in local IFN-g driven Mig production (248). In addition, the chemokine is required for tissue inflammation at times associated with adaptive immune responses to influenza virus, coxsackievirus (50), and HSV infections (291).

Under the specific conditions of HIV infection, particular chemokines can also mediate direct antiviral functions (48). This is because HIV strains use the chemokine receptors CXCR4 and CCR5 as coreceptors with CD4 molecules for virus infection and because the natural chemokine ligands for these specific receptors can block HIV infection (4,19). The ligands include RANTES and MIP-1a. Both T and NK cells are capable of delivering this chemokine-mediated mechanism of protection.

CYTOKINE RECEPTORS AND SIGNALING PATHWAYS

Most cytokines use similar mechanisms to signal from the extracellular milieu to specific genes in the nucleus (113,281). The cellular receptors to which cytokines bind are generally type I ectoproteins with a single transmembrane domain (Fig. 4). Functional receptors may be composed of one, two, or three distinct protein subunits, each of which is a transmembrane protein. Particular subunits may be common to receptors of several cytokines. In some cases, one subunit binds the cytokine with high affinity, whereas in other cases, only composite multisubunit receptors do so. Structurally, the extracellular regions of these receptors contain fibronectin type III–like domains, cysteine-rich immunoglobulin-like domains, or combinations thereof. The carboxyl-terminal region of at least one of the receptor proteins forms a cytoplasmic tail used to recruit particular and crucial signaling proteins to provide specificity of intracellular signaling. Unlike many growth factor and hormone receptors, the cytoplasmic domains of cytokine receptors do not have intrinsic enzyme activity. Instead, they recruit specific tyrosine kinases, which are activated through events at the extracellular domains, resulting in ligand binding and receptor dimerization. A common family of such tyrosine kinases is the Janus kinase (Jak) group. One or more members of the Jaks are bound to specific sites on the cytoplasmic domain of different cytokine receptors. Ligand-bound receptors activate the Jaks, causing phosphorylation of specific tyrosine residues on the receptor subunits and on the Jaks themselves. Other tyrosine kinases, such as members of the sarcoma (Src) family, are also associated with particular cytokine receptors. Tyrosine phosphorylation of receptors and receptor-associated tyrosine kinases triggers a chain of events with cytoplasmic proteins containing Src-homology 2 (SH2) domains, to recognize phosphorylated tyrosines, recruited to the receptor and subsequently tyrosine phosphorylated. The most important signal transducing proteins for gene induction by cytokines are the signal transducers and activators of transcription (STATs). (Particular cytokine–cytokine receptor interactions associated with major specific STAT molecules are listed in Table 4.) Tyrosine phosphorylated STATs form homodimers or heterodimers and translocate to the nucleus, where they, by themselves or in association with other specific proteins, bind to specific DNA elements in cytokine-inducible genes to activate transcription. Thus, STATs are the true messengers carrying activation signals from specific receptors on the cell surface to specific genes in the nucleus.

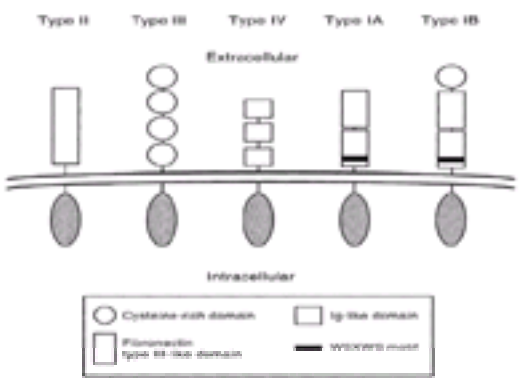

FIG. 4. Shared structural features among various cytokine receptors. The receptors are divided in groups by the nature of structural motifs present in their ligand-binding extracellular domains. Four such motifs are shown here. Type II receptors include those for type I and type II interferons (IFNs); these receptors are discussed in more detail in Figure 5. IL-10R is also a type II receptor. Type III receptors include TNF-RI and TNF-RII. Type IV receptors include IL-1RI and IL-1RII. Type IA receptors include IL-2Rbc, IL-2Rbc, IL-3Ra, IL-3Rbc, IL-4Ra, IL-5Ra, IL-7Ra, IL-9Ra. Type IB receptors include IL-6Ra, IL-6Rb, and IL-12Rb.

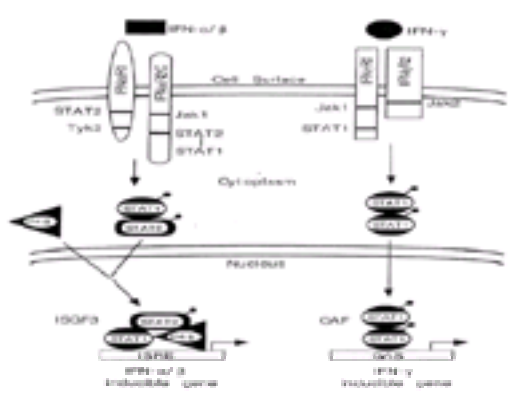

FIG. 5. Interferon (IFN)-signaling pathways. Binding of the IFN-a/b ligands to the extracellular domain of their receptor causes activation of Jak1 and Tyk2 and resultant Tyr-phosphorylation of STAT1 and STAT2. The phosphorylated STATs form a heterodimer and translocate to the nucleus, where they form a trimeric complex (ISGF3) with P48. ISGF3 binds to the ISRE elements of the promoters in IFN-a– and -b–inducible genes to activate their transcription. IFN-g binds to a different heterodimeric receptor, the intracellular domains of which bind to Jak1, Jak2, and STAT1. Ligand-induced activation of the tyrosine kinase causes STAT1 Tyr-phosphorylation, its dimerization, and nuclear translocation. The STAT1 homodimer (GAF) binds to the GAS elements of IFN-g–inducible genes and activates their transcription.

Cytokine	Jak	STAT
IFN-α/β	Tyk2, Jak1	1, 2, 3, (4)
IFN-γ	Jak1, Jak2	1
IL-2	Jak1, Jak3	(1), 3, 5
IL-3	Jak1, Jak2	5, 6
IL-4	Jak1, Jak3	6
IL-5	Jak2	
IL-6	Tyk2, Jak1, Jak2	1, 3
IL-7	Jak1, Jak3	
IL-9	Jak1, Jak3	3
IL-10	Tyk2, Jak1	1, 3
IL-11	Jak1	3
IL-12	Tyk2, Jak2	3, 4
IL-13	Jak1	6
IL-15	Jak1, Jak3	(1), 3, 5

IFN, interferon; IL, interleukin.
Parentheses denote cell-type specific activation.

TABLE 4. *Activation of JAK and STAT by various cytokines*

Because the Jak–STAT pathways were discovered through analysis of the mechanisms used by IFNs (271), signaling by these cytokines is described in detail. The pathways used by TNF also are discussed in brief because of their distinct nature. Little is known about the pathways used by cytokines for activating many of the immunoregulatory functions described earlier. Moreover, as most of the work characterizing these pathways has been done in culture, much remains to be learned about how they function in the context of the mixed cytokine responses elicited during viral infections in the host.

Interferon Receptors and Cell Signaling

IFN-specific intracellular signaling processes culminate in transcriptional activation of a set of IFN-inducible genes. Many of these genes are also induced in virus-infected cells by dsRNA or other viral products. The genes encoding the Jaks and the STATs themselves are often transcriptionally regulated by cytokines, thus providing positive feedback loops to the signaling systems. The intricate and partially overlapping signaling pathways used by IFNs and other cytokines provide opportunities for “cross-talking” among them, causing either synergistic or antagonistic outcomes. The cross-talking is physiologically relevant because a single cytokine is never induced in isolation in a virus-infected organism. Instead, cells are exposed *in vivo* to mixtures of cytokines simultaneously, and resultant changes in their gene expression reflect equilibrium between the multiple activated signaling pathways.

Interferon Receptors

Cells of all lineages, except possibly mature erythrocytes, express cell surface receptors for both type I IFNs (IFN-a, IFN-b, IFN-w) and type II IFN (IFN-g). Receptor numbers vary from 100 to 2000 per cell, and ligand-binding constants vary between 10^{-11} and 10^{-9} M. The receptors are multimeric transmembrane glycoproteins whose extracellular domains bind IFNs and whose cytoplasmic domains bind Jaks, STATs, and other signaling proteins. The type I IFNs bind to a receptor that has two major subunits, IFNAR1, a protein with 530 residues, and IFNAR2c, a protein with 315 residues (162). Neither subunit alone can bind IFN-a/b with high affinity, but the receptor complex can (49). The two IFNAR subunits associate with the tyrosine kinases Tyk2 and Jak1, respectively. Fully functional type I IFN receptors may contain additional transmembrane protein components because several other proteins coprecipitate with the ligand–receptor complex and because certain biochemical events cannot be reconstituted with the IFNAR1 and IFNAR2 molecules alone. The IFN-g receptor has two different subunits, IFNGR1 and IFNGR2, whose intracellular domains respectively bind the Jak1 and Jak2 kinases. IFNGR1 has 472 residues, whereas IFNGR2 has 315 residues (58). Functional IFN-g is a dimer binding to two IFNGR1 subunits to generate binding sites for two IFNGR2 subunits. Thus, the occupied, active receptor complex consists of two molecules each of IFN-g, IFNGR1, and IFNGR2. The crucial events in both type I IFN and IFN-g signaling are IFN-driven dimerizations of receptors. This leads to a cascade of tyrosine phosphorylation of proteins bound to the cytoplasmic domains of the receptor subunits.

Intracellular Signaling by Interferons

Both type I and II IFNs use similar principles in cellular signaling (Fig. 5). Specific protein tyrosine kinases of the Jak family are associated with cytoplasmic domains of the receptors, and ligand binding to receptor extracellular domains causes their activation to initiate a cascade of protein tyrosine phosphorylation events, with the STATs among the phosphorylated proteins (271). Once phosphorylated, STATs can form specific multimeric complexes that translocate to the nucleus and bind to specific cis-acting DNA elements present in the 5' regulatory regions of IFN-inducible genes. Thus, strong and specific transcriptional signals are transmitted from the outside of cells to specific genes in the nucleus. These signals are generated within minutes of ligand binding to the receptors. The effects are transient, decaying within hours of initiation. As a result, target gene transcription is activated in responding cells for only short periods of time.

At least seven proteins are essential for IFN-a/b signaling, including the two subunits of the receptor, two members of the Jak family of protein tyrosine kinases (Jak1 and Tyk2), two members of the STAT family (most notably STAT1 and STAT2), and a specific member of the IRF family, P48 (117,136,151,247,271). The interferon-stimulated response element (ISRE), cis-acting DNA sequences present in the regulatory regions of the IFN-a/b inducible genes to receive the stimulatory signal, has been defined (58). The relevant transcription factor binding to ISRE is ISGF3, a complex composed of three proteins, STAT1, STAT2, and P48. All of these proteins preexist in untreated cells, but the ISGF3 complex is formed only in IFN-treated cells after tyrosine phosphorylation of STAT1 and STAT2. P48 is a member of the IRF family of proteins, all capable of specifically binding to ISRE (198). Some IRF members stimulate transcription, whereas others repress it. Although the initial phase of positive signaling is transmitted to the ISRE by ISGF3, transcription of the IFN-stimulated genes (ISGs) may be sustained by specific IRF members, such as IRF-1. In addition to these proteins, the protein tyrosine phosphatase SHP-2 may also be involved in IFN-a/b signaling (69,117).

The previously described Jak–STAT pathway is thought to be the major signaling cascade used by IFN-a/b, but there is experimental evidence to implicate other STAT molecules, such as STAT4 (43,58). Moreover, cytosolic phospholipase A2 may be activated under particular conditions of IFN-a/b stimulation (76). An additional subtype-specific signaling pathway may be in place because a mutant cell line lacking Tyk2 cannot respond to IFN-a1 or a2 but retains a partial response to IFN-b and IFN-a8 (80). The possible existence of an IFN-b–specific pathway is supported by identification of a gene preferentially induced by IFN-b, that is, bR1 (228). IFN-a/b also activate the mitogen-activated protein (MAP) kinase pathway. This could be initiated by tyrosine phosphorylation and activation of an IFNAR1-associated MAP kinase identified as ERK2 (59).

The major IFN-g signaling pathway requires at least five cellular proteins: the two subunits of the receptor, the protein tyrosine kinases Jak1 and Jak2, and STAT1 (13,301). These receptor-bound STATs are also phosphorylated by the Jaks to cause their dissociation from the receptor complex (259,262,263). The dissociated Tyr-phosphorylated STAT1 proteins form a reciprocal homodimer, called the g-activated factor (GAF), and GAF translocates to the nucleus. The most commonly used cis element in IFN-g–responsive genes is called g-activated sequence (GAS) (61). GAF binds to GAS to promote gene transcription (see Fig. 5). Optimal transcriptional activation, however, requires further phosphorylation of STAT1 homodimers at Ser727 (59,309,316). The Pyk2 kinase with a MAP kinase–like specificity has been implicated in this process. IFN-g can also signal to ISRE and other elements. The compositions of the corresponding trans-acting factors remain to be determined. The IFN-g signal to ISRE may not be as strong as that mediated by IFN-a/b. Conversely, although ISGF3 is the major effector of IFN-a/b signaling, STAT1 homodimers are also formed under these conditions and can bind to GAS elements to drive transcription of some ISGs, such as the IRF-1 gene. Because only a subset of ISGs are induced, this pathway appears to be less prominent that the one induced by IFN-g. The STATs present in ISGF3 or GAF can interact with the basic transcriptional machinery by binding to the universal coactivator with its CBP/p300 components (323). Because induction of many genes by IFN-g, such as 9-27, P48, and class II MHC (see later), is not mediated by GAS elements, additional signaling pathways must exist. Although some features of these alternative pathways have been reported, their full descriptions await further investigation (27,157,230).

The mechanisms used by cells to down-regulate and terminate IFN signaling are not well understood. It is clear, however, that many components of the signaling pathway are inactivated in the process. Receptors are dephosphorylated (98) and down-regulated from the cell surface (221), and activated Jaks are inhibited by the suppressors of cytokine signaling (SOCS) family of proteins whose expression is induced by IFN (69,195,272). In addition, activated STAT1 is dephosphorylated by SHP-1 or similar tyrosine phosphatases (60) and possibly degraded by a proteasome-mediated process (131).

Interferon-Independent Signaling for Gene Expression in Virus-Infected Cells

Many IFN-a/b–stimulated genes are activated in virus-infected cells through signaling pathways independent of IFN. The IFN-independent pathways can be activated by dsRNA produced in cells infected with many viruses. These pathways are most convincingly demonstrated in cells devoid of type I IFN genes or incapable of responding to IFNs (16). The dsRNA-elicited signal is also received by the ISRE element. However, dsRNA-signaling does not require STAT1, STAT2, Jak1, Tyk2, or P48 used by IFN-a/b. Instead, dsRNA or virus-signaling is mediated by the dsRNA-activated factor (DRAF) (57) or virus-activated factor (VAF) (56) complexes binding to ISRE. The protein constituents of these factors are not as well defined as those of ISGF3 but contain IRF-3 and CBP/p300 (152,175,307,321). Regulation of these factors is poorly understood. IRF-3 phosphorylation is required, but the cognate protein kinase has not yet been identified. The dsRNA-dependent protein kinase PKR is likely to be involved in a cascade leading to activation. The pathway used by dsRNA to signal to ISRE is not well defined, but it and the ISGF3 pathway used by IFNs are distinct (143). Viruses may use yet another pathway to activate transcription from the same cis element because binding of virions or the relevant viral glycoproteins to the cell surface receptors can be sufficient to trigger transcription of specific ISGs (30,196,326).

Signaling by Tumor Necrosis Factor

There are two distinct TNF receptors to which both TNF-a and LT (also called TNF-b or LT-a) bind: TNFR1 or P55 and TNFR2 or P75. Their extracellular domains have homologies to many other cell surface receptors, such as Fas, nerve growth factor (NGF) receptors, and two poxvirus decoy TNF receptors, T2 and A53R. The cytoplasmic domains of the two TNF receptors are quite distinct, indicating that they elicit different intracellular signals. In general, TNFR1-generated signals cause cytotoxicity, increased cell proliferation, and antiviral activity, whereas TNFR2-generated signals are important in hematopoietic cells for specific functions (282,283). TNF binding to cell surface receptors causes pleiotropic intracellular responses, including activation and induced synthesis of transcription factors such as NF-kB and AP-1. As a result, transcription of many cellular genes is induced or repressed (296). The specific pathways used by TNF for signaling are not well characterized. MAP kinase is activated (295). Under most of the conditions studied to date, the Jak–STAT pathway does not appear to be activated by TNF. There are reports that TNF causes sphingomyelin hydrolysis generating lipid ceramide that activates a specific protein kinase to transmit the signal (133). The supporting evidence is, however, incomplete.

Because signaling by TNFR1 can cause apoptosis, it has been classified as a death receptor and has a characterized death domain in its cytoplasmic tail. Binding of TNF to the receptor causes its aggregation and the recruitment of intracellular signal transducers. One class of such proteins contains death domains as well (111). The TNFR1-associated death domain (TRADD) molecule is an example of a protein binding to the death domain of TNFR1. The second class of proteins, including those identified as TNF-receptor associated factors (TRAFs), do not contain death domains (114,242). They interact with each other and the receptor via a conserved C-terminal domain. A general picture of the signaling processes suggest that they lead to either apoptosis primarily through TRADD or activation of NFkB and cell survival through TRAFs (241,320). The balance between the two opposite outcomes depends on cell type. Thus, most normal cells are not killed by TNF, probably because strong NF-kB activation protects the cells from apoptotic effects. TRAF2 mediates both NF-kB activation and C-Jun N-terminus kinase (JNK) activation by TNF.

ACTIONS OF CYTOKINES ON VIRAL REPLICATION AND IMMUNE RESPONSES

Many cellular actions of cytokines are mediated by differentially induced proteins. These molecules are not expressed, or are expressed at a low level, in untreated cells. Most are products of genes whose transcription is directly induced, but some are products of genes requiring ongoing protein synthesis for expression. The latter proteins are likely to represent a second stage of a gene induction cascade. A variety of approaches, including gene array technology (63), have revealed that IFNs induce synthesis of a surprisingly large number of cellular proteins, counting in the hundreds. Type I and II IFNs induce two partially overlapping sets of proteins. Many of the type I IFN-inducible proteins can also be induced by host cell responses to dsRNA or virus infection (326). Although IFN-a and IFN-b primarily induce the same proteins, some proteins may be induced by one better than the other.

The characteristics of a few extensively studied IFN-induced proteins are described later. Their biochemical and cellular regulatory functions are well understood, and many of them play major roles in the antiviral functions of IFNs (Fig. 6). Particularly active in antiviral defense are certain enzymes with binding activity for dsRNA and molecules called Mx proteins. Progress is also being made toward understanding the induction and function of MHC molecules in response to IFNs. By promoting antigen processing and presentation, these effects can facilitate the development of adaptive T-cell responses. (TNF-a is also known to induce certain of these, but the pathways for induction are not as thoroughly characterized.) Exciting new information is becoming available about the biochemical pathways of IFN-mediated regulation of cell proliferation. These effects are likely to be important in shaping endogenous immune responses and in therapeutic effects resulting from IFN-a/b treatments of cancers. Finally, although less well characterized, IFN or TNF-a activates pathways inducing a nitric oxide synthase important in antiviral defense.

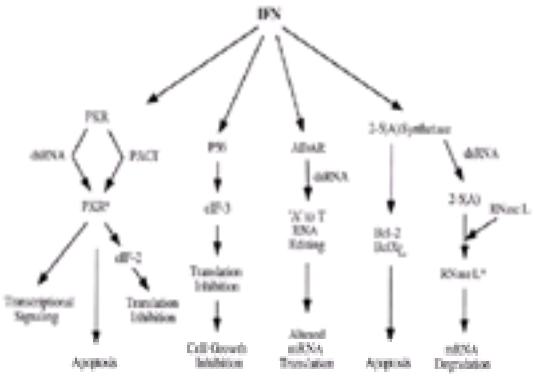

FIG. 6. Interferon (IFN)-induced biochemical pathways affecting cell growth and metabolism. Among the many proteins induced by IFNs are PKR, P56, ADAR, and 2-5(A) synthetase. IFN-induced PKR is inactive as such and requires either dsRNA or another cellular protein, PACT, for its activation by autophosphorylation. Activated PKR (PKR*) is a protein kinase with stringent substrate specificity. It can phosphorylate eIF-2a to inhibit protein synthesis. It can also cause cellular apoptosis and participate in specific transcriptional signaling pathways. The IFN-induced protein, P56, binds to the P48 subunit of eIF-3 and inhibits its function, causing translation inhibition and cell growth inhibition. The IFN-induced ADAR adenosine diaminase edits (A to I) dsRNA structures in viral and cellular RNAs, thereby changing their coding properties. The IFN-induced 2-5(A) synthetases are a family of enzymes, all of which are activated by dsRNA to synthesize 2'-5'–linked oligoadenylates [2-5(A)]. 2-5(A) binds and dimerizes inactive RNase L, causing its activation (RNase L*), and activated RNase L degrades mRNA. A specific isozyme, 9-2, of 2-5(A) synthetase has an additional cellular activity. It binds to the Bcl-2 family of proteins and causes cellular apoptosis.

Enzymes Dependent on Double-Stranded RNA

Studies of the IFN-induced proteins have led to the discovery of three interesting classes of enzymes, all of which bind to and require dsRNA for their functions: (a) the family of 2-5(A) synthetases (229) whose products, the 2'-5'-oligoadenylates, activate a latent ribonuclease, RNase L, to cleave cellular and viral RNAs (265); (b) the PKR, affecting many cellular processes and resulting in inhibition of viral replication (312); and (c) an isoform of dsRNA-specific adenosine deaminase, which converts adenosines to inosines (217). The functions of these enzymes are known to be important for induction of an antiviral state by IFNs, and the mechanisms for induction of the antiviral effects have been extensively studied.

As a result of binding dsRNA, the 2'-5'-oligoadenylate [2-5(A)] synthetases are activated to polymerize adenosine triphosphate (ATP) into 2'-5'–linked oligoadenylates. All 2-5(A) synthetase proteins are IFN induced, although basal levels are present in many cell lines. There are three size classes of these enzymes: small, in the 40-kd range; medium, in the 70-kd range; and large, in the 100-kd range (229,254). Each class is encoded by separate genes, which are all clustered in one genetic locus. Within each class, multiple isozymes are produced by alternative splicing of primary transcripts. Different isozymes have different posttranslational modifications and different subcellular locations. Studies with natural and recombinant proteins have shown that the three classes have different dsRNA optima and synthesize 2-5(A) of different lengths (90,252). Many small viral RNAs, such as adenoviral VAI RNA, EBV EBER RNA, and HIVI TAR RNA, can also activate these enzymes (64,168,261). Oligomerization of the small and medium proteins is essential for their enzyme activity (90,253). Recent studies with a medium isozyme, P69, have revealed that the catalytic domains of these proteins have an abbabbb structure similar to the catalytic domain of DNA polymerase b. Three Asp residues in this domain form the catalytic triad, and their mutation inactivates the enzyme without affecting ATP and dsRNA binding (253). The residues responsible for dimerization of P69 have also been identified.

The 2-5(A) product molecules activate RNase L by causing dimerization of inactive monomeric RNase L (265). The

N-terminal half of the protein containing P-loop motifs and ankyrin repeats binds 2-5(A) and serves as a repressor of enzyme function. The C-terminal half contains a region of protein kinase homology, a cysteine-rich domain, and the ribonuclease domain. Trimeric 2-5(A) is required for the activation of RNase L, dimers are inactive, and much longer oligomers of 2-5(A) may have inhibitory activity. The 2-5(A) synthetase–RNase L system has been shown to inhibit replication of specific families of viruses, especially picornaviruses. Constitutive expression of either the small or the medium isozyme causes inhibition of replication of encephalomyocarditis virus (EMCV) but not vesicular stomatitis virus (VSV) (42,89). Cells expressing a dominant negative mutant of RNase L and RNase L–null mice are deficient in the anti-EMCV effect of IFN-a/b (324). The constituents of the 2-5(A) synthetase–RNase L system can also affect cell growth. RNase L has been implicated in apoptotic pathways (324); and a small isozyme of 2-5(A) synthetase, 9-2, causes cellular apoptosis (88). The 9-2 activity is unique to this form of the 2-5(A) synthetase family and unrelated to 2-5(A) synthesis and RNase L binding. The function has been traced to a unique C terminus of 9-2 acquired by alternative splicing. This region contains a Bcl-2 homology 3 (BH3) domain, which enables it to interact with the antiapoptotic proteins Bcl2 and BclxL. Much remains to be learned about the effects on cell growth and death, however, because constitutive overexpression of P69 can cause a contrasting dose-dependent increase in cell doubling time and the formation of multinucleated cells (89).

Another dsRNA-activated enzyme is PKR (312). This enzyme is expressed in all cells at a low level but is induced to a higher level upon IFN treatment. The protein is inactive, but dsRNA binding activates it to cause its autophosphorylation in serine-threonine residues. Phosphorylated PKR can phosphorylate other proteins. The PKR dsRNA-binding domain is at the N-terminal half and the catalytic domain is in the C-terminal half of the molecule. The dsRNA-binding domain contains two dsRNA-binding motifs of about 70 residues. These motifs are present in other members of this family of dsRNA-binding proteins but not in 2-5(A) synthetases. The domain also mediates direct protein–protein interaction, causing PKR homodimerization (215) or allowing heterodimerization among different members of this family of proteins. The kinase domain contains the usual kinase subdomains and an insert domain shared with other eIF-2a kinases. PKR can be activated by other cellular and viral RNAs with partial double-stranded structures and heparin or other polyanionic compounds as well as by authentic dsRNA. Recently, a protein activator of PKR, PACT, has been identified (214). PACT also has the dsRNA-binding motifs and dimerizes with PKR. Activation of PKR by PACT is independent of dsRNA. Low concentration of dsRNA can activate PKR, whereas higher concentrations inhibit it. Many viruses, such as adenovirus, exploit this property by producing a large quantity of VAI RNA that inhibits PKR (280). Binding of dsRNA causes a conformational change in PKR, exposing the ATP-binding site and autophosphorylation. The most well-characterized PKR substrate is the translation initiation factor eIF-2a. Its phosphorylation inhibits the function of eIF-2 and causes an inhibition of translation. Thus, activation or overexpression of PKR causes an inhibition of cellular protein synthesis, and this is one mechanism for IFN-induced inhibition of viral replication. PKR can also phosphorylate histones, HIV Tat protein and IkB *in vitro* (312). It interacts with STAT1, STAT3, and P53 as well but does not phosphorylate them. A new nuclear dsRNA-binding protein substrate of PKR, DRBP76, has recently been identified (216).

Overexpression of PKR leads to inhibition of EMCV replication, and a dominant-negative PKR mutant or an antisense PKR cDNA suppresses the anti-EMCV action of IFN-a and IFN-b. To circumvent the actions of PKR, many viruses have evolved different mechanisms to inhibit its function. These viral evasion strategies are discussed in a separate section later. There are other cellular functions of PKR in addition to its antiviral effects. PKR has been shown to be required in transcriptional signaling by dsRNA (312). Through an as yet undefined pathway, dsRNA-activated PKR activates the IkK complex and causes degradation of IkB. PKR is also involved in cellular signaling by TNF-a, LPS, and IFN-g. There are suggestions that PKR may act upstream of the MAP kinase P38. Because PKR is a cell growth inhibitor, its transdominant inhibitors can promote unregulated cell growth and transformation of NIH/3T3 cells. In virus-infected cells, PKR promotes cellular apoptosis. Viruses encode or induce many antiapoptotic proteins to keep the cells alive during replication. One such inhibitor, P58IPK, is a cellular protein induced by influenza virus (280).

The IFN-inducible dsRNA-specific adenosine deaminase, ADAR, differs from 2-5(A) synthetases and PKR in the role dsRNA plays. The dsRNA structures are substrates of ADAR, whereas they are regulatory factors activating or antagonizing enzymatic functions of the other molecules. ADAR catalyzes C6 deamination of adenosine to inosine in viral and cellular RNAs (156,217). Consequences of this IFN-induced RNA editing are changes in target RNA coding capacities. Such site-specific editing can cause amino acid substitution and, as a result, synthesis of proteins with altered functions (155,156).

Mx and Guanylate-Binding Proteins

The Mx proteins and other guanylate-binding proteins are abundantly induced by IFNs in a number of species (270). The human MxA and MxB proteins are induced primarily by type I IFNs. These proteins belong to the dynamin superfamily of GTPase involved in endocytosis and vesicle transport (109). The Mx proteins were originally discovered as IFN-induced proteins with the ability to block replication of influenza viruses. Later, they were shown to be capable of inhibiting many other RNA viruses, including members of the Orthomyxoviridae, Paramyxoviridae, Rhabdoviridae, Bunyaviridae, and Togaviridae families (106). The Mx proteins are thought to interfere with virus replication through a dynamin-like force–generating mechanism by wrapping around viral nucleocapsids. The MxA protein associates, in the GTP-bound form, with the nucleoprotein component of Thogoto virus to cause an inhibition in nuclear transport of the viral nucleocapsids and viral genome transcription (132). The existence of considerable residual effects of IFN-a/b against EMCV in mice lacking functional RNase L, PKR, and Mx suggest the presence of additional pathways (325). Another family of IFN-induced proteins includes the guanylate-binding proteins, GBP-1 and GBP-2. They bind and hydrolyze GTP; that is, they have GTPase functions and may have mild antiviral activities (9).

Major Histocompatibility Complex Expression

Among the IFN-induced proteins playing major roles in recognition of virus antigens by cells of the immune system are the MHC proteins (29,153). The class I MHC proteins are induced by both types of IFNs, but the class II proteins are induced by IFN-g only. Enhanced surface expression of class I MHC proteins facilitates activation of CD8 T cells. Along with the class I proteins, IFNs promote the expression of other proteins that help constitute the class I MHC, including

b_2-microglobulin, three subunits of the proteasome LMP-2, LMP-8 and LMP-10, PA28, TAP, tapsin, and gp96 (185). The IFN-g–induced class II MHC response promotes CD4 T-cell activation. IFN-g up-regulates the class II MHC heterodimer, the invariant chain protein, two DM proteins that assist in assembly of the class II complex, and the transcription factor CIITA that is required for transcription of these genes (164). IFN-g regulates the expression of three cathepsins believed to be responsible for cleavage-processing of exogenous proteins into peptides for loading onto the class II MHC complex. IFN-g also induces the high-affinity Fcg receptor and the components of the complement system (54). PML and SP11, two human proteins localized in nuclear bodies within the nucleus, are IFN inducible (303).

P200 Gene Family

In mice, type I IFNs strongly induce synthesis of various members of the *P200* gene family (145). The cluster contains at least six IFN-regulated linked genes. The protein products of these genes are related in that they all contain at least one unit of a conserved domain of about 200 amino acids. The p202 protein inhibits cell proliferation by binding to and inhibiting the functions of a number of transcription factors, including NFkB, E2F, P53, c-fos, c-jun, and the retinoblastoma protein Rb (47). The p204 protein, on the other hand, inhibits rRNA transcription by binding to the UBF1 transcription factor that is required for ribosomal RNA synthesis (154). Genes from the homologous human locus are also induced by IFNs. Their products, IFI16 and MNDA, are DNA-binding nuclear proteins.

ISG56 Gene Family

In humans, a genetic locus encodes several proteins that are strongly induced by type I IFNs. They include P56, P54, P60, and P58 of the *ISG56* gene family (305). The corresponding mouse proteins also are known. These proteins are structurally related. They contain multiple tetratricopeptide domains thought to mediate protein–protein interactions. P56 is the product of gene *561* or *ISG56* (139). This gene is not expressed in untreated cells but induced very rapidly upon IFNa/b treatment. It is also induced by dsRNA or by infection with viruses. Both the mRNA and the protein are relatively short lived, and the protein, P56, is primarily cytoplasmic (103). The P56 cellular function is still poorly understood, but the molecule interacts with Int-6. The Int-6 protein has cytoplasmic and nuclear isoforms, arising from alternative translation initiation sites. The full-length cytoplasmic protein has a nuclear export signal at the N terminus and a nuclear localization signal near the C terminus (102). An internally initiated protein lacks the nuclear export signal and hence stays in the nucleus. P56 interacts with both proteins and keeps the nuclear isoform sequestered in the cytoplasm. Int-6 is identical to P48, a subunit of the translation initiation factor eIF-3 (10). As expected, P56 binds eIF-3, and purified recombinant P56 inhibits *in vitro* translation by blocking eIF-3 function (104). *In vivo* P56 and P48 are colocalized in the cytoplasm. When P56 is overexpressed in cells, the rate of protein synthesis is decreased, and the rate of cell growth is affected as a consequence.

Inducible Nitric Oxide Synthase

The inducible nitric oxide synthase (iNOS), alternatively identified as nitric oxide synthase 2 (NOS2), can be expressed in activated macrophages to mediate a variety of antimicrobial functions. Much of the work characterizing iNOS induction has been done in mice and in response to IFN-g or LPS stimulation. Inflammatory macrophages from humans, however, also express iNOS (165). Signaling for iNOS induction is incompletely characterized. The promoter regions of both mouse and human genes are complex, and there are likely to be multiple pathways to iNOS induction. The transcription factor, NFkB, can play a role in induction elicited by LPS. Moreover, the absence of IRF-1 results in significant iNOS deficiencies, and the absence of IFN-g induction of IRF-1 is likely to be a pathway to this effect (120). Although IFN-g is a potent inducer, TNF-a and TNF-a in synergy with IFN-a/b can also act as inducers of the enzyme (165). Through biochemical pathways with NADPH to oxidize and consume L-arginine, NOS enzymes catalyze production of NO, with L-citruline as a second product (165). NO is a lipid- and water-soluble gas that reacts with oxygen and its intermediates to yield other reactive molecules. The iNOS-dependent pathways have been shown to contribute to defense during a range of infections, including those with the RNA virus, VSV, and the DNA viruses, VV, EBV, HSV, and MCMV (2,126,127,165,182,284). Because of the variety of reactive intermediates and products, NO has the potential to mediate antiviral activity by modifying downstream target molecules. It has been shown to promote maintenance of EBV latency by down-regulating an intermediate-early transactivator protein (171) and to interrupt VV replication and protein synthesis by blocking required enzymes and intermediates (126,182).

VIRAL STRATEGIES FOR COUNTERACTING OR TAKING ADVANTAGE OF CYTOKINE AND CHEMOKINE ACTION

Viruses have evolved many strategies either to inhibit cytokine- or chemokine-mediated effects detrimental to their replication or to take advantage of cytokine and chemokine functions to promote viral propagation and spread in the host. In particular, viruses constantly try to evade antiviral IFN action. A virus that is a potent inducer of IFN and also highly sensitive to its antiviral effects will reproduce poorly and hence be at an evolutionary disadvantage. Thus, in nature, equilibrium is maintained between viruses and the IFN system. In the laboratory, this equilibrium can be shifted either way by increasing IFN concentration or viral multiplicity of infection: at high virus load, IFN is ineffective, and at high IFN dose, virus replication is totally shut off. A global strategy used by many virulent viruses is to inhibit cellular RNA and protein synthesis. As a result, both IFN synthesis and synthesis of IFN-induced proteins are impaired. Other viruses have developed more targeted and sophisticated strategies to interfere with different steps of the IFN and other cytokine systems (Table 5). These strategies are discussed subsequently.

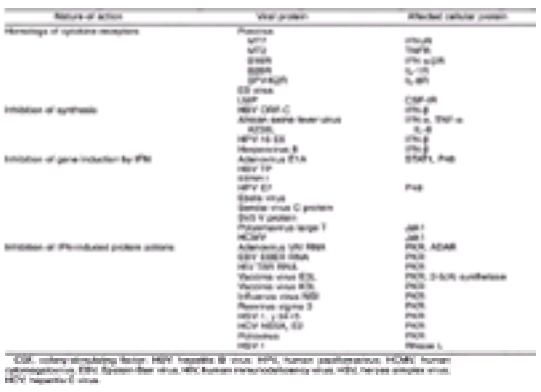

TABLE 5. *Viral interference with cytokines*

Inhibition of Interferon Synthesis

IFN synthesis is inhibited by some viral gene products. Hepatitis B virus ORF-C product inhibits IFN-b gene induction, as does the viral terminal protein (307). For IFN induction by VSV, mutations in the M protein may dictate this capacity of the virus (74). African swine fever virus that infects macrophages inhibits phorbol myristil acetate (PMA)-induced synthesis of IFN-a and TNF-a. Here, the product of the viral A238L gene encodes a homolog of IkB to inhibit NFkB actions in cytokine induction (224). Other viruses produce cytokine homologs. The myxoma virus MT-7 protein can bind IFN-g and serve as its decoy receptor. This protein can also bind several chemokines (252). VV encodes a homolog of IFN-a/b receptor that, unlike the cellular receptor, can bind type I IFN of many species (3,179). Tanapox virus–infected cells secrete a protein that can bind to and inactivate IFN-g, IL-2, and IL-5 (70).

Inhibition of Interferon Signaling

Another common step of interference by different viruses is at the level of signal transduction by IFNs. The Jak–STAT pathway loses its regulation and is constitutively activated by human T-lymphotrophic virus (HTLV) tax proteins (183) and by spleen focus-forming viral envelope protein gp55 that binds to the erythropoietin receptor (318). Herpesvirus saimiri activates the Jak–STAT pathway using lck, a src-related tyrosine kinase (161). In the opposite scenario, many viral proteins inhibit the Jak–STAT pathway. Infection with HCMV appears to decrease Jak1 levels in infected cells by enhancing degradation of the protein, and a consequence of this effect is disruption of induction of class II MHC expression on the surface of infected cells (184). Murine polyomavirus large T antigen binds to Jak1 and inhibits IFN signaling. This activity requires the pRB binding sites of the large T molecule. As a result, ISGF3 is not activated in response to IFN-a/b, and consequently, other viruses such as VSV are also not inhibited by IFN in cells expressing large T (308).

P>The most thoroughly studied example of interference with IFN signaling is that mediated by the adenoviral E1A proteins. Adenoviruses are insensitive to IFNs because several adenoproteins counteract the IFN system at multiple steps, with the earliest step at an IFN-signaling block by E1A. As a result of the E1A-mediated effect, IFN also cannot inhibit the replication of other viruses. E1A expression does not allow ISGF3 formation. The defect can be ascribed to a lowered cellular level of one or more of the signaling proteins, including STAT1 and P48 (146,147). E1A also disrupts the interactions between the STAT proteins and CBP/P300 required for ISG transcription by interacting with both STAT1 and STAT2 (20). Other DNA viruses can interfere with IFN-signaling. The hepatitis B virus (HBV) polymerase protein or its subdomain TP blocks ISGF3 activation (79). The EBV EBNA1 oncogene blocks gene induction and cell growth inhibition by IFN without interfering with the activation of ISGF3, indicating that the block may be at the level of ISGF3–coactivator interactions (123). The human papillomavirus E7 protein abrogates IFN- a signaling and blocks ISGF3 activation. In this case, E7 directly binds to P48 and inhibits its translocation to the nucleus (17). Numerous RNA viruses have also been shown to interfere with IFN signaling. Ebola virus inhibits both IFN- a and IFN-g signaling in endothelial cells (108). Sendai virus C proteins prevent the antiviral action of IFN, possibly by interfering with signaling (84). One specific C protein appears to be responsible. A similar virus, SV5, has a viral V protein blocking the activation of ISGF3 in response to IFN-a/b and GAF in response to IFN-g (65). This dual block is achieved by targeting STAT1 for proteasome-mediated degradation. Several viruses have strategies to block other steps. The HPV16 E6 oncoprotein binds to IRF-3 and blocks its transactivation functions (238). IRF-3 is activated in response to dsRNA and virus infection and promotes transcription of IFN genes and many ISGs. In the presence of E6, IFN- b is not induced by Sendai virus infection. Human herpesvirus type 8 (HHV-8) encodes a transcription factor similar to the IRF proteins (189). The viral IRF interferes with the actions of cellular IRF proteins and thereby blocks IFN and ISG expression. Thus, a variety of viruses have evolved to overcome potent antiviral effects induced by IFNs by blocking IFN-signaling pathways.

Interference with Functions of Interferon-Induced Proteins

Many viruses have developed mechanisms to counteract specific functions of various IFN-induced proteins. There are known viral inhibitors of the two well-studied IFN-induced antiviral pathways requiring dsRNA for activation. EMCV infection causes an inhibition of binding of 2-5(A) to RNase L, although IFN pretreatment overcomes this inhibition. Analogs of 2-5(A), which block activation of RNase L, are synthesized in herpesvirus- or SV40-infected cells (39). The most frequent IFN-induced antiviral mechanisms targeted, however, are those mediated through activation and function of PKR (119). Adenovirus produces abundant amounts of spoiler VAI RNA that bind to the dsRNA-binding domain of PKR but do not activate it. Similarly, EBV produces EBER RNA, and HIV-1 produces TAR RNA; at high concentrations, these viral products block PKR activation. The VV E3L protein, influenza virus NS1 protein, and reovirus sigma 3 protein, on the other hand, prevent PKR activation by binding dsRNA. VV also encodes K3L, which functions as a decoy of eIF-2 and an alternative substrate of PKR. A phosphoprotein of HSV-1 neutralizes PKR action by inhibiting phosphorylation of eIF-2 (46). The HSV-1 g 34 · 5 protein complexes with protein phosphatase 1a (PP1a) to promote dephosphorylation of

eIF-2a. PP1a may also be responsible for inactivating the enzyme itself by causing PKR dephosphorylation. Influenza virus activates a novel cellular chaperone protein, P58IPK, which inhibits PKR, possibly by interfering with its dimerization. Poliovirus and HIV promote degradation of PKR through unknown mechanisms. Hepatitis C virus encodes two proteins interfering with PKR action. The NS5A protein binds to the dimerization domain of PKR to block its activation (81), and differences in natural resistance to IFNs between different strains of HCV can be attributed to mutations in the NS5A protein. Another protein of HCV, the E2 glycoprotein, has also been shown to inhibit PKR (285). E2 contains a sequence identical to the phosphorylation sites of PKR and eIF-2 and binds to PKR to inhibit its action. The previous examples of many viruses evolving a variety of mechanisms to inhibit PKR support the notion that PKR functions as a major antiviral enzyme in the IFN system.

Other Mechanisms

In addition to those directed at IFN functions, viruses have evolved a number of other far-reaching mechanisms to counteract or take advantage of host cytokines or their actions (93,266,267). Although it is not possible to review all of these in detail, a few examples are given here. The large poxviruses are packed with genes having potential to inhibit or modify host immune responses. One of these is the T2 protein, which can protect virus-infected cells from the antiviral effects of TNF actions (93,158). The myxoma virus MT-2 molecule is a homolog of the cellular receptor for TNF and is released from infected cells to serve as a binding decoy and block signaling through functional host TNF receptors. Myxoma virus also produces a homolog of EGF/TGF-a (3). HHV-8 encodes a cytokine similar to IL-6, which promotes the growth of transformed B cells (33). The EBV BCRF-1 protein is highly homologous to mammalian IL-10 (186). The viral protein is biologically active and is thought to aid viral pathogenicity by suppressing production of immunostimulatory cytokines and by promoting B-cell growth and transformation. This gene function is essential for the transforming capacity of EBV. A VV gene for an enzyme promoting steroid production may act to inhibit endogenous immune responses by accessing the immunoregulatory pathways induced by these hormones (190). A different mechanism is used by the cow poxvirus to block IL-1b. It encodes an inhibitor of IL-1b converting enzyme that is required for the production of IL-1b by proteolytic cleavage from a larger precursor. Finally, there is an example of one virus, measles, using as its receptor a cell surface molecule delivering a signal to regulate IL-12 expression negatively (125).

Certain viruses also have genes with homology to host chemokines or chemokine receptors and other gene products with chemokine-binding activities (82,95,140,177,219,266,293). It is not yet clear whether all of these function to help virus escape from immune defense or whether they might also act to promote viral replication. In soluble forms, the chemokine receptor homologs could act as natural chemokine receptor antagonists or blockers. This appears to be the case during myxoma virus infections, in which certain viral products with chemokine-binding activity are virulence factor for infections and interfere with recruitment of leukocytes into virus-infected tissues. In contrast, chemokine homologs might promote virus spread by calling in cell for infection, and this appears to be the case with a chemokine homolog expressed by MCMV (77,246).

CYTOKINE-MEDIATED PATHOLOGY

Although characterization of immunopathology during viral infections has traditionally focused on adaptive immune responses resulting in particular antibody-dependent immune complex– or CTL-mediated diseases, cytokines can also contribute to immunopathology. This has been long appreciated in the case of bacterial infections. Cytokine-dependent consequences detrimental to the host have been clearly delineated during gram-negative bacterial sepsis and after exposure to LPS (105) as well as in response to gram-positive bacterial toxins such as the staphylococcal enterotoxins (174). High-level exposure to the first class of agents induces proinflammatory cytokines by innate immune response mechanisms. These conditions are associated with high systemic levels of TNF-a, IL-1, IL-6, IL-12, and IFN-g and with TNF-a essential for the detrimental effects and most of the other factors acting to increase sensitivity to TNF-a. Exposure to the bacterial endotoxins activates large proportions of T-cell subsets to produce cytokines amplifying the proinflammatory innate cytokine cascades. If uncontrolled, the conditions can result in coagulation, vascular collapse, and multiple-organ failure. Remarkably, the functions of certain of the proinflammatory cytokines, such as TNF-a, IL-1, and IL-6, include alerting the neuroendocrine system to the problem and activating the hypothalamic–pituitary–adrenal axis to induce the release of glucocorticoid (GC) hormones from the adrenal glands (178). The GC hormones can mediate a number of immunoregulatory effects to limit detrimental consequences of the endogenous cytokine responses. In particular, they can inhibit the expression of certain of the cytokines (2,178,202,257).

Little attention has been paid to potential cytokine-mediated diseases during viral infections. Recently, MCMV infections have been shown to induce systemic proinflammatory cytokines overlapping with, but not exactly identical to, those known to be induced under conditions of LPS exposure, that is, significant serum levels of TNF-a, IL-6, IL-12, and IFN-g but low or undetectable IL-1 responses (243,244). Under these conditions, an adaptive immune response–independent, but TNF-dependent, liver necrosis is induced and results in significant organ damage (209). Such a pathway may contribute to liver disease during other viral infections. In particular, infections with mouse hepatitis virus type 3 (MHV-3) also induce liver necrosis and disease associated with macrophage activation and coagulation (67,148,149). The conditions of systemic cytokine induction during MCMV infection result in an IL-6–dependent induction of endogenous GC production (243), and the GC response acts to protect the host from a TNF-dependent lethality during the infection (244).

Results of treatments with IL-12 (210,212) or challenge with LPS (199) suggest that there may be conditions under which T-cell responses to viral infections promote cytokine-mediated or cytokine-dependent disease during viral infections. Such pathways are likely to contribute to pathology during infections with Dengue virus (138) and perhaps Hantaan viruses, but this remains to be thoroughly investigated. Recent evidence suggests that effects mediated through the LT b-receptor also may contribute to viral pathogenesis (225).

SUMMARY

This chapter has reviewed current understanding of cytokine responses and functions during viral infections. We have

attempted to present the broad picture, but emphasis has been on unique pathways activated by and during viral infections. A focus has been on IFN-a/b. Key aspects of the integrated responses are schematically represented in Figure 7. Many viruses have evolved specific mechanisms to exert control over the responses and their functions. The overall balance is crucial to the host. As the material reviewed shows, our understanding is becoming sophisticated, but much remains to be learned. This is an active period of investigation. The authors look forward to the next few years of study and the knowledge to be derived from the results of this work, particularly in the areas of understanding how the systems work at the biochemical level to regulate immune responses *in vivo.*

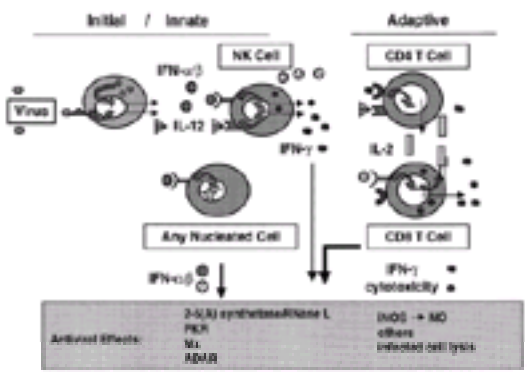

FIG. 7. Summary of known specific responses and their effects during viral infections. Initial events during infections include induction of interferon (IFN)-a/b ( ) in response to viral products. These cytokines then activate a variety of biochemical events through receptor stimulation of a Jak/STAT signaling pathway with activation of the ISGF transcriptional complex ( ) and lead to expression of proteins contributing to antiviral defense mechansims, including, but not limited to, 2-5 (A) synthetase/RNase L, PKR, and Mx. The IFN-a/b cytokines also promote the induction of innate natural killer (NK) cell cytotoxicity ( ) and can enhance adaptive T-cell IFN-g ( ) responses. Certain viral infections also induce an initial or innate interleukin-12 (IL-12) response ( ) response. This factor is associated with NK cell IFN-g production. Many viral infections induce profound CD8 T-cell expansion with activation of CD8 T-cell IFN-g production and cytotoxic T-lymphocyte (CTL) functions. T-cell IFN-g responses can be IL-12 independent during viral infections. IL-2 ( ) can promote CD8 T-cell expansion and facilitate optimal responses mediated by these populations.

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CHAPTER 13 Virus Evolution

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DEFINING EVOLUTION

Evolutionary change is a temporal process that is derived from the differential reproductive success of individuals and their inherited phenotypes that alters corresponding genotypes between generations. Evolution is therefore an inevitability in populations of entities that (a) self-replicate and (b) exhibit heritable variation in phenotypes that affect reproduction. Charles Darwin described evolutionary change as “descent with modification,” implying that characters exhibited by an ancestral population differ from those exhibited by its descendant population. Furthermore, this primarily statistical difference is brought about by the simple fact that not every member of the ancestral population is represented by progeny in the descendant population. The assortment of phenotypes inherited by the descendants of the chosen replicators is rarely identical to that of the previous generation, thus resulting in a changing phenotypic makeup through time. Evolutionary change does not need to be driven by reproductive success that is influenced by phenotype in any way, however. Genetic drift results from stochastic changes in population size that are independent of reproductive traits. This often results in a “sampling error,” bringing about a new mixture of genotypes that differs from that in the previous generation.

Viruses, being autonomous replicators that can exhibit profound degrees of intrapopulation genetically based phenotypic variation, are of course subject to the laws of evolution. Viruses do, in fact, use evolutionary change as both short- and long-term survival strategies that can allow the immediate escape of host antiviral responses as well as the transmission to new hosts, often through unpredictable environments. This is not to say, however, that viruses must evolve rapidly to exist. Many viruses change very little between generations, and this is often due to low mutation rates or unchanging, consistent host environments.

The Process of Evolution

Natural Variation

Naturally occurring variation in the form of genetically derived phenotypic diversity is an evolutionarily requisite characteristic of populations. Such diversity is the “raw material” on which both selection and drift act to influence characteristics of subsequent generations. This variation has many bases that will be discussed in more detail later, but the two primary causes of viral genetic diversity are mutation (including genetic exchange) and population size. As both increase, so too does the spectrum of characteristics available for inheritance by viral descendants.

The extent of this variation differs tremendously among viral types. Many RNA viruses, for instance, exist as *quasispecies* that are often broad distributions of mutant (and recombinant) viral genomes existing as a population within a single host. For example, a great deal of research has examined the quasispecies population structure that exists in foot and mouth disease virus (FMDV). FMDV has a single-stranded RNA genome about 8,500 nucleotides in length. It has been estimated that in natural populations of FMDV, nucleotide substitution rates range from 0.9×10^{-2} to 7.4×10^{-2} replacements per nucleotide per year (37). This translates into enormous genetic heterogeneity observable in natural isolates of the virus. As reported by Domingo and colleagues (37), during an outbreak of FMDV infection, 0.7% to 2.2% of nucleotides differed among typical sampled viral isolates. Within an infected individual, it was estimated that 0.6 to 2 nucleotide substitutions were occurring per round of genome replication.

In contrast to the highly variable RNA viruses are many DNA viruses that experience much lower mutation rates and thus vastly lower genetic variability. Included among these are the small DNA viruses such as papillomaviruses and polyomaviruses. These viruses use the highly accurate DNA replication machinery of their mammalian hosts and therefore exhibit substitution rates on the order of 10^{-7} to 10^{-8} per nucleotide per year (13,123). Not surprisingly these rates are close to those of their hosts. Perhaps as a result, these viruses often exist as silent, apathogenic infections and in addition are known to cospeciate with their hosts (116). This is not to say, however, that such viruses must display low genetic or phenotypic variation. Because of the almost incomprehensibly large population sizes (both intrahost and

interhost) that can approach 10^{12} particles within a single organism, the amount of total genetic variation is seemingly always adequate for adaptive survival and evolution no matter the environmental obstacle.

Natural Selection

Natural selection is a discriminating force that allows the production and survival of progeny from a subset of a parental population. This selectivity is driven by the specific abiotic and biotic conditions under which the population exists, and the heritable phenotypes that are best suited for reproduction under these conditions are the ones “allowed” to be transmitted to a subsequent generation. When natural selection is in operation, the result is a descendant population made up of genetic successors that exhibit phenotypes chosen from the previous generation based on their suitability to specific (yet variable) environmental characteristics.

It is commonly accepted that natural selection therefore leads to a certain type of evolutionary change that is termed *adaptive. Adaptation* refers to both the process by which a population becomes better suited for survival and reproduction in a given environment and the actual *phenotypic character* that arises by way of natural selection that bestows this ability. The closely affiliated and frequently employed word *fitness* is best understood as a relative term that describes the genetic contribution an individual makes to a subsequent generation compared with that made by other individuals in the same population. Fitness is therefore based on natural selection and increased by adaptation.

Different types and degrees of natural selection exist. The magnitude of natural selection is primarily determined by (a) the amount of change in the reproductive environment through time and (b) the width of the range of phenotypes allowed to reproduce in a certain environment. Along these lines there are three main types of natural selection that act on populations: (a) directional selection; (b) stabilizing selection; and (c) diversifying (disruptive) selection. These are best distinguished using an example. Figure 1 represents a hypothetical frequency distribution of some polymorphic phenotype in a viral population (e.g., rate of replication). Under directional selection, replication rates that fall within either area A or C might be selected for, and the result would be a correspondingly faster or slower mean rate of replication in the following generation. This type of selection might be more common in a changing environment. Under stabilizing selection, replication rates that fall within area B would be selected for, and consequently replication rates in areas A and C would be selected against. The result would be a similar mean replication rate in the descendant population but with less overall variation in that rate. This type of selection is common in stable environments that often have limited reproductive resources. Under diversifying selection, replication rates within both areas A and C would be selected for, resulting in an increase in the diversity of this phenotype in the next generation. This type of selection may occur when either replication environment or phenotypic diversity is highly variable.

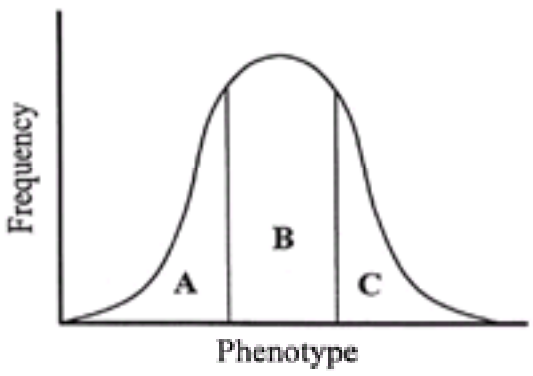

FIG. 1. Hypothetical phenotype frequency distribution.

Genetic Drift

Changes in genetic makeup through time (i.e., evolutionary change) need not be adaptive or driven by natural selection. Random and even predictable fluctuation in population size that translates into differential reproductive success can obviously influence future gene frequencies. Viruses can be particularly susceptible to the effects of genetic drift because host–host transmission frequently involves population size “bottlenecks” that occur independently of viral fitness. In other words, a specific viral type may be supremely adapted to replication in a particular host environment but, due to events of pure chance, is prevented from survival or reproduction. The outcome may be that a virus type of lesser reproductive capability is allowed to survive despite the decrease in mean population fitness this will cause. It is in this way that populations may actually become less adapted to their environments.

This phenomenon is well exemplified by viruses that rely on transmission routes, such as aerosol droplets, that involve extremely small inocula despite huge intrahost viremic population sizes. For example, human respiratory droplets are typically less than 1 to 10 μM in diameter. Therefore, on average, a given droplet is likely to carry few if any viral particles (6,58). Although there are few complete investigations of naturally occurring genetic drift imposed by host–host viral transmission, a number of studies have looked at the effects of experimental bottlenecks on population fitness. Vesicular stomatitis virus (VSV) is a negative-stranded RNA virus that frequently infects humans and other mammals. Duarte and colleagues examined the effects on viral fitness of experimental bottlenecks produced between clonal plaque–plaque transfers (45). Using direct replication competition assays, they found that after as few as 20 passages, profound decreases in viral fitness were observed in the VSV clones.

Why does this occur? The answer is an elementary statistical phenomenon known as *sampling error.* For an extremely large, highly diverse population (common among viruses), the ratio of highly fit to less fit genotypes may actually be a very small number. During a transmission event, only a few particles might be selected. The genotypic makeup of these

particles does not accurately represent the full genetic or fitness distribution of the original population and therefore often does not include members of the highly fit class because of their comparatively small numbers. The outcome is a new population established in a similar environment but with members of a small, less fit (on average) class of viruses. Mechanisms of escape from this form of evolutionary down-spiraling are discussed later (see Generation of Genetic Diversity).

Natural History

Evolutionary biology is a science that studies not only the mechanisms and processes by which evolutionary change takes place but also the historical record of that change. By applying knowledge about episodes of biological change and stasis in the past to our current understanding of the evolutionary process, it may be possible to predict the pattern and direction of change in the future. But why does one want to predict such change? Viruses and viral change have a profound impact on human health, agriculture, and indeed overall quality of life. The harmlessly silent viruses of today can undergo evolutionary change (via adaptation or otherwise) and seemingly overnight give rise to the nightmarish pandemic of tomorrow. What were the natural events that led to the global spread of lethal human immunodeficiency virus (HIV)? Why does influenza virus periodically cause widespread epidemics of dramatically high virulence? Questions such as these will probably never be answered completely, but significant progress is being made in epidemiology of viral and other infectious diseases with the help of evolutionary concepts and historical reconstruction.

Microbiologists and virologists in particular are at a severe disadvantage when investigating the past (geological) history of their target organisms. This is for two main reasons. First, evolutionary virology is a relatively new science that has only been made possible by modern advances in computing technology, microscopy, and molecular biology. Therefore, the amount of accumulated knowledge from past research is extremely small compared with other biological fields. Second, viruses leave no fossil record, in contrast to many multicellular organisms. Evolutionary virologists are therefore constrained to retracing past events mainly through mathematical analysis of protein and nucleic acid sequences from viruses that have been isolated almost exclusively during the 20th century. This by no means has a crippling effect on evolutionary investigation, however, because techniques of both sequence data production and sequence analysis are currently quite sophisticated and rapidly growing with advances in microprocessing, molecular biology, and information technologies (67). For instance, automated sequencing now allows determination of entire viral genome sequences in mere days. Multiple phylogenetic algorithms can reconstruct with great accuracy the evolutionary relationships of genes, proteins, and even genomes. In addition, population genetics theory can elucidate parameters such as population size fluctuations, evolutionary rate, and time to common ancestry.

Viral taxonomy is a principal benefactor of modern evolutionary analysis. Taxonomy is a science based on the hierarchical classification of organisms into separate groups based on similarity. This similarity is most ideally reflected in phylogenetic relationships. Taxonomy of cellular, especially sexually reproducing, organisms has its basis in the fundamental (lowest) unit of species. Many definitions of species exist, and all have evolutionary relevance. The commonly cited “biological species,” however, is described as a group of actually or potentially interbreeding organisms that are reproductively isolated from other such groups. This immediately presents an impasse to virologists because sex is not a required step in viral reproduction. Nevertheless, although viral classification schemes do include taxonomic groupings with names identical to those found in cellular taxonomy (i.e., species, genus, family, order), their functional definitions are mostly distinct.

The International Committee on Taxonomy of Viruses defines a *viral species* as a polythetic class of viruses that constitutes a replicating lineage and occupies a particular ecological niche (100,132). This is a necessarily broad definition that provides tremendous room for interpretation. In general, characters such as serologic cross-reactivity, host range, tissue tropism, gene sequence divergence, and transmission properties are used to denote viral species (132). Taxonomic ranks such as genus and family are assigned according to more slowly evolving characters like morphology, replication strategy, and genome characteristics (e.g., gene order) (100). The evolutionary relationships of viral groups are often assumed to mimic their taxonomic assignments, and although this may typically be assumed as accurate, it is not an inherent law of the science. Classifying viruses according to their true phylogenetic relationships, especially on the lower levels, may be a lofty goal given the practically exclusive reliance on molecular data analysis for determination of viral evolutionary history. In addition, gene sequences may actually transmit conflicting phylogenetic signals as a result of intergenomic genetic exchange. Phylogenetic information should be used as an influential consultant to taxonomic decision making and must not be an edict accepted as biological truth.

The limitations of molecular phylogenetics are conspicuous when considering viral taxa. This is most clearly demonstrated by the effects of viral recombination (discussed in more detail under Recombination). Phylogenies reconstructed using information from short, single, contiguous regions of viral genomes can be misleading. For example, in a report by Khimani and colleagues, phylogenetic analysis of complete murine leukemia virus (MuLV) genomes uncovered a likely recombination event involving only the *env* genes of Hortulanus MuLV and Cas-Br-E MuLV, two retroviruses isolated from wild mice (77). Nucleotide diversity analysis of other genomic regions (including *gag*) phylogenetically pairs Cas-Br-E with Moloney MuLV, whereas data from *env* nucleotide sequences indicate that the closest virus to Cas-Br-E is actually Hortulanus MuLV. This can be explained by a recombinational event that took place between the viruses, making their *env* sequences more similar than other genomic regions and thereby manifesting as disparate phylogenetic results. A taxonomist assuming that the closest relative to Cas-Br-E is Moloney MuLV based on *gag* sequence data is therefore likely deceived by not seeing the bigger picture.

VIRAL ORIGINS

General Considerations

Regarding viral origins, clearly two distinct issues emerge as important. First is the issue of original virus ancestors. This refers to the ultimate, ancient, and earliest appearance of particular viral groups. Second is the origin of modern viruses that have arisen long after the first viral progenitors and continually arise even today. The latter subject is discussed in

subsequent sections. Most researchers would agree that the available evidence points to multiple origins for viruses of different types. The unique reproductive strategy shared by all viruses (i.e., obligate molecular parasitism accompanied by genome encapsidation and intercellular transport) has most likely arisen more than once in biological history.

Viruses have presumably existed for many millions, and probably billions, of years on our planet, yet, as already emphasized, they have left no known fossil record. This predictably complicates investigation into their origins. We are left with their genomes, their genome products, their replicative behavior, and their hosts to formulate hypotheses about how viruses were evolutionarily conceived. This can be difficult, especially when considering rapidly evolving viral genomes (e.g., RNA-based genomes) whose informational content is periodically nearly replaced or "turned over." There are three primary theories used to explain the ultimate origin of viruses. These need not be mutually exclusive, however, and as discussed later, it is very possible that more than one evolutionary process was involved in the appearance of some viral groups.

The first theory is perhaps the most intriguing from the standpoint of Earth's biological history. It involves the possibility that viruses are actually remnants of a primordial precellular world. This was originally based on their structural simplicity but has since been adapted to incorporate genome biochemistry. The theory encountered a fair amount of scorn from researchers on the grounds that virus replication is so dependent on host cellular machinery that it is unlikely viruses could arise in the absence of such hosts (4,18). Discovery of and research into the enzymatic capabilities of RNA (22) have endowed this theory with a resurgence in attention because most known viruses use RNA as their genomic nucleic acid.

RNA-based primordial life is now strongly considered as possible, even likely (59,75). Therefore, even though modern RNA viruses may not possess characters detectable as being shared with precellular relatives, it is not inconceivable that they are ultimately derived from such a source. In fact, viroids (small, naked RNA plasmids) have been hypothesized to represent direct descendants of precellular molecular ancestors on account of their genomic material and self-splicing capabilities (19,34,35). Like viruses, viroids are infectious obligate molecular parasites, many of which replicate using a rolling, circular mechanism. Unfortunately, a lack of significant identifiable genomic homology between viroids and any known viruses prevents useful phylogenetic estimation of the evolutionary relationship between these two groups. It has been suggested, however, that RNA-based agents (perhaps related to viroid-like molecular entities) encoding reverse transcriptase facilitated, or even caused, the origination of DNA as genomic material (30,43,85,94).

Another theory suggests that viruses are descended from cellular pathogenic microorganisms such as bacteria that have gone through a process of "retrogression" (or, more accurately, an evolutionary decrease in complexity). This idea was constructed before the advent of modern molecular biology and therefore was based on crude viral characteristics such as size and pathogenic attributes (64,83). Modern advances in molecular techniques have allowed for more detailed examination of viruses, their genomes, and the effects they have on their hosts. Consequently, huge disparities between viruses and other pathogenic microorganisms have been elucidated. This has resulted in an all but total rejection of the retrograde hypothesis, but, as discussed later, it may still have some legitimacy, especially in combination with other theories of virus origins.

The third theory explaining the origin of viruses proposes that viral ancestors are cellular genetic elements that have "escaped." This currently has a great amount of empirical support and is therefore a widely accepted theory explaining the origins of some virus types. This "endogenous theory" was first alluded to by Lwoff in 1957, who discussed it in relation to bacteriophage latency (92), and was further developed along those lines by Waterson in 1960 (138). The idea was strengthened by Temin in his discussions about the origins of retroviruses (126,127). Evidence in support of this theory is most convincing when one considers the retroviruses and DNA viruses. The data exist mostly in the form of homologous coding sequences found in both viral and cellular genomes. These include DNA- and RNA-dependent DNA polymerases and oncogenes. In addition, the overall sequence similarities between some endogenous retroid agents and retroviruses cannot be ignored. The endogenous theory is more difficult to extend to riboviruses, which rely on RNA-dependent RNA synthesis—an activity that does not take place in DNA-based cellular hosts, the supposed viral progenitors.

Origins of RNA Viruses

Viruses are the only forms of life known to possess RNA genomes. In fact, RNA is the most common genomic nucleic acid among known viruses. Riboviruses are viruses that have RNA genomes that do not replicate via a DNA intermediate. They may have positive (+) sense (messenger sense), negative (–) sense (antisense), or double-stranded (ds) RNA. Of all known viral taxa, positive-sense RNA riboviruses are by far the most common (62) and include the picornaviruses and togaviruses among many others. Negative-sense RNA riboviruses include the orthomyxoviruses and arenaviruses. Double-stranded RNA viruses include the reoviruses. It is not known whether riboviruses and retroviruses are a monophyletic assemblage (i.e., descended from a single common ancestor). It is interesting to note, however, that all riboviruses use a virally encoded RNA-dependent RNA polymerase (RdRp) for genome replication, whereas all retroviruses use an RNA-dependent DNA polymerase (reverse transcriptase)—thus implying that the two groups have separate origins. In addition, a great deal of evidence exists indicating that since their appearance, both ribovirus and retrovirus genomes have obtained heterologous sequences from other viruses as well as their hosts.

A good starting point for discussion about the origins of riboviruses is evolutionary analysis of RdRp. Amino acid sequences of RdRp proteins are extremely important for two main reasons. First, sequence analysis of RdRp points to a single evolutionary origin of this protein for all riboviruses (62). It has been hypothesized that this ancestor arose before the first eukaryotic cells and is certainly one of the first enzymes to appear in biological history (76,109). Second, analysis of extant viral RdRp proteins from diverse groups has yielded extraordinary and unexpected phylogenetic information about the relationships therein. This has contributed strongly to the division of riboviruses into distinct taxonomic units.

Riboviruses are classified into eight main supergroups based on morphologic and genomic characteristics as well as phylogenetic analysis of their more highly conserved proteins. Phylogenetic analysis of RdRp (as well as other proteins,

such as virally encoded helicase) has indicated that the three main ribovirus genomic classes (+, –, and dsRNA) represent distinct monophyletic groups (17,61,80). Interestingly, within these groups, viruses do not always cluster phylogenetically according to host taxa, tissue, or replication ecology. For instance, based on RdRp sequence analysis, the flavivirus-like viruses (hepatitis C, West Nile, Japanese encephalitis, Dengue fever, and yellow fever viruses), which replicate in mammalian cells, are closely related to (and even derived from) the luteovirus, potyvirus, and tobamovirus groups that infect plant hosts exclusively (17). Perhaps even more remarkable has been the phylogenetic placement (based on RdRp sequences) of +RNA bacteriophages Qb, MS2, GA, and SP with such eukaryotic viruses as Dengue 4, yellow fever, and hepatitis C (80). These examples demonstrate the evolutionary fluidity exhibited by the RNA viruses with respect to the conditions under which they can replicate and persist.

Determining which RNA virus group is the most ancestral using molecular data exclusively is a complicated endeavor. If one assumes that evolutionary diversification increases with time, the +RNA viruses have been around the longest because they far exceed the other two groups in number of recognized taxa. It has been suggested, however, that dsRNA viruses gave rise to +RNA viruses, which gave rise to –RNA viruses (17,61). This might be consistent with the concept of dsRNA viruses as ribovirus ancestors because some phylogenetic analyses have placed them both within and even outside the +RNA evolutionary tree (61,81). In addition, it is known that dsRNA viruses are frequently pathogens of the more primitive bacterial and fungal species and less so of the “newer” eukaryotic species—perhaps another indication of their age. It is also possible that dsRNA viral genomes have evolved more than once.

These results tend to generate more questions regarding ultimate riboviral origins, however. It has been hypothesized that riboviruses (as well as other viruses) are derived from escaped host genetic material such as mRNA, perhaps even coding for RdRp. There are many solid examples of homologous cellular coding regions within riboviral genomes. For instance, bovine viral diarrhea virus (BVDV) is a +RNA flavivirus. A particular strain of this virus was found to have an approximately 270 base pair insert present in its genome that was nearly identical to the host mRNA coding for ubiquitin (27). Such insertions represent recent evolutionary events and are qualitatively much unlike the hypothetical escape of previral host RNA but do illustrate the blurry line between host endogenous and mobile exogenous genetic material.

The theory that riboviruses are living relics of the RNA world is frequently neglected because hard evidence is seriously lacking and it is also difficult to imagine their replication cycle, as we now know it, existing without a cellular host genome to be parasitized. The most likely group of extant life forms to resemble inhabitants of precellular Earth is the riboviruses. We argue that this theory should not be completely ignored for all riboviruses, and two important facts clearly stand out. First, riboviruses do not rely on DNA directly for any stage in their replication. This characteristic was certainly exhibited by all self-replicating entities in a precellular or pre-DNA world but is currently exhibited by no other organisms known. Second, RNA-dependent RNA polymerization would be required in an RNA biosphere, and it is probable, if not mandatory, that a protein or RNA–protein complex evolved to accomplish this efficiently. The only RdRp that is known to exist as a protein functionally related to reproduction is a component of every ribovirus genome.

One theory of riboviral origins that may be rejected with a high degree of confidence is evolutionary retrogression. This is because it is nearly impossible to imagine either (a) a previously autonomous RNA-based organism because none are known today or (b) the characteristic of genome nucleic acid actually changing from DNA to RNA during evolutionary retrogression.

Origins of DNA Viruses

More than 26 families of viruses use DNA, either single or double stranded, as genomic material. These viruses are common as parasites of prokaryotes, archaea, and eukaryotes. Although fewer in taxon numbers than the riboviruses, the DNA viruses as a group are in many ways more diverse. DNA viral genomes vary tremendously in size from as small as 1.8 kilobases (kb), as in the single-stranded porcine circulovirus, to as large as 670 kb, as in bacteriophage G. DNA viruses may be enveloped or nonenveloped, cytoplasmic or nuclear, polymerase encoding or non–polymerase encoding. Unlike the riboviruses, however, there exists no evidence that evolutionarily links the major divisions of DNA viruses. It is therefore likely that some DNA virus groups have had separate, independent origins.

It is perhaps easiest to begin by distinguishing two major lineages of DNA viruses. The most logical separation is between those that encode a DNA-dependent DNA polymerase (DdDp) and those that do not. As with RdRp discussed previously, the DdDp protein serves a function indispensable with respect to reproduction and is accordingly well conserved through evolution. DdDp proteins are much more divergent and varied than RdRp and are classified into four families (labeled A to D) based on amino acid similarity or disparity. All replicative viral DdDps belong to either family A or B, and all exhibit 3'5'-exonuclease activity. All DdDps, whether viral or cellular, are thought to have evolved from a single common ancestor (16,79,136). Viruses that encode family A polymerases include bacteriophages of the genera *Siphoviridae* and *Podoviridae* as well as the mycobacteriophages of fungi. Family B polymerases are contained within the genomes of phycodnaviruses of algae, herpesviruses, poxviruses, adenoviruses, and ascoviruses of vertebrates and other eukaryotic taxa, and baculoviruses of invertebrates.

Polymerase-encoding DNA viruses are a highly divergent group that may or may not have arisen from a single common viral ancestor. Sequence analysis of DdDp has resulted in some interesting, sometimes unexpected, results. It should be noted here that viral DNA polymerases are thought to be at least as old as any of their corresponding cellular homologs, prompting one to speculate about the ultimate origin of this protein (134). Viruses that use protein-primed DNA replication, such as the mammalian adenoviruses and bacteriophages f29 and PRD1, are closely related despite the distant relationship of their hosts (79). In addition, cytoplasmic eukaryotic viruses, such as vaccinia and entomopoxvirus, are paired with the T-even phages of bacteria (79). Other revealing information regarding the deeper relationships between DNA viruses include certain capsid morphologic and assembly characteristics that are shared between herpesviruses and bacteriophages, which may point to shared ancestry among these two groups (21,56,120).

An evolutionary link between the large DNA viruses and the small DNA viruses of eukaryotes (i.e., the papillomaviruses, polyomaviruses, and parvoviruses) cannot be established based on genomic analysis. This is because of an apparent

lack of homologous sequences, the existence of which may be driven by the high frequency with which DNA viruses are known to undergo recombination (see Recombination). Recombination between co-infecting viruses and between host and virus acts to create genetic homogeneity within virus types and heterogeneity between virus types. Because of their small genome size and their very strong dependence on host replication machinery, the small DNA viruses are perhaps the most likely candidate viruses to have arisen from endogenous cellular genetic material. Unfortunately, there currently exists no direct evidence in support of this method of origination.

Origins of Retroviruses

Retroviruses include both exogenous (horizontally transmitted) and endogenous (vertically transmitted) enveloped agents that contain RNA genomes but use reverse transcription (RNA ® DNA) as part of their replication process. To accomplish this, the viruses require the use of an RNA-dependent DNA polymerase (reverse transcriptase). Unlike most DNA and riboviruses, the retroviruses share obvious similarities with many nonviral, usually mobile cellular genetic elements. It is widely believed that retroviruses are evolutionarily derived from host genomic elements such as the LTR retrotransposons (126,127). The basic difference between the two groups is the presence of a genomic region coding for an envelope (packaging) structure in the former group. The acquisition of a genomic encapsidation strategy subsequently allowed for cell–cell and eventually host–host transmission. It is also accepted that all retroviruses, retrotransposons, hepadnaviruses, and caulimoviruses share a distant common ancestor (42,95,146).

The distribution of all retroid agents (defined as any genetic element that encodes reverse transcriptase) within the Earth's tree of life is quite skewed, with most being found in eukaryotes, much fewer being found in eubacteria, and none known to use genomes of the Archaea. The retrotransposons are found within the genomes of animals, plants, fungi, and protozoa, but because of their abundance and variety as well as the diversity of their replicative strategies, it is believed that they are possibly the oldest class of retroid agents (47). When considering just the retroviruses, only mammals, birds, and insects are known to act as hosts. This relatively small host phylogenetic distribution, along with similarity between different retroviral genome sequences and organization, suggests that the retroviruses have arisen recently in evolutionary time, especially when compared with most DNA and riboviruses (41).

Retroid agents, including retroviruses, have undoubtedly played many important roles in the evolution of life. The ubiquity and apparent ancientness of reverse transcription has led to speculation that this process was integrally involved in the transition from RNA to DNA as genomic material (30,43,85,94). In addition, the overwhelming presence of retrotransposons and endogenous retroviruses in the genomes of most eukaryotes provides abundant opportunity for emergence of new, exogenous retroviral agents (e.g., HIV).

VIRAL DIVERSIFICATION

As exemplified by the immense variety we observe in their morphology, replication strategies, host ranges, environmental tolerances, and transmission strategies, extant viruses have been shaped by a fantastic sequence of evolutionary change and divergence. As we shall see, the amount of viral evolution is related to both random and directional forces. Extremely mutable viruses need not change phenotypically between generations, and viruses with presumably stable genomes can change quickly depending on stochastic and environmental conditions. Although a broad generalization is often inadequate, two primary characteristics allow viruses to exhibit such great evolutionary plasticity: access to genetic variability and exposure to selective pressure.

Generation of Genetic Diversity

Genetic diversity within a virus population is ultimately the result of mutation or recombination. In addition, reassortment may allow the spread of beneficial genes after cellular co-infection. Without heritable genetic and corresponding phenotypic variability, a population, viral or otherwise, will not evolve. It has been theorized that the adaptive ability of a population is proportional to the amount of genetic variation therein. The average genetic variation exhibited by a specific virus type is dependent on diverse characteristics of its replication biology. For example, the fidelity of its genome replication, frequency of co-infection, population size and structure (both host and virus), mode of transmission, and replication rate all influence the generation of genetic variation.

Mutation

Mutation refers to the molecular synthesis or modification of a descendant gene that differs from the parental or template gene. This may include a deletion, insertion, or mismatch. The mutation or error rate therefore refers to the frequency of such errors per nucleotide per round of synthesis and is a good quantity with which to compare the mutability of virus types. Mutation differs from the process of base substitution in that the latter refers to the actual fixation or significant increase in frequency of a mutant gene in a population and is actually the result of evolutionary, not molecular, processes. As a group, viruses exhibit the broadest range of mutation rates among known genetic entities (97). For instance, natural mismatch mutation rates for genomic regions of some RNA viruses, such as phage Qb (12,38,40), VSV (69,115,143), and FMDV (36) have been estimated to be as high as 10^{-4} to 10^{-3}. Even more extraordinary, sporadic G ® A and A ® G error rates on the order of 10^{-2} to 10^{-1} (a process termed *hypermutation*) have been observed in some RNA virus and retrovirus types (97). The concept of *error threshold* refers to the highest possible mutation rate for a specific quantity of genetic information beyond which sustainable transmission of the information is not possible (48,125). Many viruses, such as poliovirus and VSV, have been hypothesized to exist very close to this value (70,71).

The number of viral mutations occurring per unit of time is largely determined by two main factors: polymerase fidelity and generation time. Polymerase fidelity differs greatly among nucleic acids. RNA polymerases and reverse transcriptases lack proofreading mechanisms that result in much higher mismatches per copying event (26,69,118). DNA viruses that rely on host replication apparatuses for genome duplication (e.g., papovaviruses) should not exhibit mutation rates that exceed those of their hosts. DNA viruses that encode their own polymerase exhibit varying mutation rates depending on template characteristics, proofreading capabilities, and intracellular conditions. In fact, little research has

been undertaken examining mutation rates of DNA viruses or their polymerases, but it is known that these rates do not approach those of riboviruses or retroviruses (44,97).

The number of mutations that occur per unit of time is also influenced by the number of replication events during that period, that is, generation time. Virus *generation time* is a measure that is difficult to estimate with accuracy in nature, and it is likely that it varies considerably among types and among hosts. Generation time may be affected by such characteristics as genome size, host tissue differentiation, cell cycle, availability of infectible host cells, and presence of antiviral agents. For instance, the generation time of HIV *in vivo* has been estimated at 2.6 days (107) and can translate into up to 200 sequential replications per year inside one host (114). Generation times of strictly vertically transmitted viruses, however, such as human endogenous retroviruses, match those of their hosts and thus can average many years. Shorter generation times result in more genetic variation per time unit; this is commonly observed in viruses that exhibit life strategies that involve acute infection.

Recombination

The importance of recombination in the evolution of viruses cannot be understated and is the focus of a great deal of research effort. Recombination is defined here as a process whereby genetic material is exchanged, resulting in the formation of a descendant nucleic acid composed of material from two or more parental nucleic acids. Recombination involving viruses may be between different viral genomes or between a viral genome and some other genetic entity (e.g., host genome, co-infecting parasite genome, plasmid). As is mutation, recombination is an important source of genetic variation. Recombination, however, can often be associated with a more immediate evolutionary reward (i.e., rapid divergence). In other words, a beneficial gene that may take mutation millions of generations to produce can, in theory, be accessed in one recombinational event between the gene's possessor and the virus “in need” of the gene. The evolutionary response to such genetic variation is discussed in more detail later (see Response to Selection).

Recombination between viruses can only occur during simultaneous co-occupation of a single host cell by more than one viral genome or its parts. The “horizontal” introduction of foreign genetic material into a viral genome will increase a population's genetic variation and allow novel combinations of genes on which natural selection can act. Much evidence exists indicating that viral recombination occurs within as well as between types. This evidence has given rise to a well-supported “modular” theory of virus evolution, which says that many viral genomes represent mosaics of genetic material obtained through recombination between types (15). For instance, the genomes of potyviruses are known to contain coding regions that closely resemble genes from flaviviruses, pestiviruses, picornaviruses, caulimoviruses, and tobamoviruses (60,84,137). Phages are also thought to undergo promiscuous recombination, especially as indicated by their tail fiber regions. As discussed by Kutter and colleagues (82) and Haggard-Ljungquist and associates (66), the makeup of tail fiber genes of many phages, including Mu, P1, P2, and T4, is clearly derived from genetic exchange between viral types.

As already briefly mentioned for BVDV, recombination is frequently noted between virus and host genomes. Baldo and McClure have demonstrated multiple instances of horizontal transmission of deoxyuridine triphosphatase (dUTPase) genes between many DNA and retroviruses and their eukaryotic, eubacterial, and archaeal hosts (8). For instance, using sophisticated sequence alignment and phylogenetic techniques, it was shown how dUTPase sequences from several mammalian poxviruses strongly cluster evolutionarily with dUTPase sequences from mammalian genomes rather than with those from other poxviruses. In addition, the authors demonstrated that it is highly likely that such dUTPase genes are of eukaryotic origin and were acquired by the viral genomes via horizontal transmission. Such events underscore the importance of recombination as a tool for generation of phenotypic diversity.

Reassortment

Interviral reassortment can occur when two or more viruses with segmented (or diploid, such as lentiviruses) genomes simultaneously co-infect a host cell. The genome partitions are (often randomly) packaged into progeny virions. These resultant progeny may therefore house genomic segments from more than one parent. This increases genetic variability because the offspring may contain novel combinations of genomic segments. This process is an important progenitor to virus survival and diversification, as illustrated by the antigenic shift commonly observed among the influenza viruses. As pointed out by Cann, two co-infecting influenza viruses, each with eight original genomic segments, may give rise to 2^8 (256) potential progeny genomes (20). Virus families that possess segmented genomes include the Orthomyxoviridae (7 to 8 –RNA segments), Reoviridae (10 to 12 dsRNA segments), Arenaviridae (2 –RNA segments), Birnaviridae (2 dsRNA segments), Bunyaviridae (3 –RNA and ambisense RNA segments), Nodaviridae (2 +RNA segments), and some members of Geminiviridae (2 single-stranded DNA segments). Segmented genomes are much more common in riboviruses. It has been suggested that this type of genome packaging, which allows for reassortment as a method of genetic exchange, has arisen and persisted evolutionarily because recombination in this nucleic acid is uncommon in many virus families (9,24,110,142).

Reassortment has been most thoroughly studied in the influenza A viruses that frequently infect fowl, swine, and humans often in agricultural settings. The pig has been considered a “reassortment vessel” for influenza viruses because it is known to be susceptible to both human and avian strains of the virus (141). Until recently, humans have not appeared to be directly susceptible to avian influenza strains, or vice versa (122). Therefore, it is theorized that swine, which harbor their own strains of the virus, are often co-infected with strains from more than one species. This co-infection can give rise to reassortants that can and do cause periodic influenza pandemics when successfully passed to human hosts. The process of reassortment in itself is obviously not enough to cause such a zoonosis. Its importance in this case is the production of genetic and phenotypic variants that can, under the appropriate conditions, become established in a new species or ecological niche. The potential variability of influenza attests to the importance of reassortment in viral diversification and has led some to suggest that segmentation and reassortment are an adaptation by which RNA viruses can maintain genetic variation to facilitate evolutionary survival (24,110).

Response to Selection

It is imperative to point out that the mere presence of genetic and phenotypic variation is not sufficient to direct or predict the evolutionary trajectory of any organism. Such variation, however, is a prerequisite for evolutionary change. Viral populations may be observed to change genetically through time. This change occurs because (a) there exist genetic differences between the successfully reproducing individuals in an ancestral population and (b) their genes are passed on to descendants disproportionately. When such disparities occur in response to forces imposed by the reproductive environment (i.e., nonrandomly), we say that evolution is driven by selection. This is not to say that selection, either natural or artificial, will have some directional or diversifying effect on viral characteristics. In fact, the opposite, a genetic homogenization of the population, can frequently be observed.

Selection is manifest as differential survival and reproduction of individuals competing for some limited reproductive resource. Unfortunately, the word *individual* can be a complicated term in the evolutionary sense, especially when considering viruses and other microparasites. This is because selection is capable of acting on many different types of characters—those that are exhibited by populations, individual genomes, gene combinations, even individual genes. Nevertheless, individuals that are capable of producing descendants at a higher overall rate than other simultaneously replicating individuals are considered to be of higher fitness. A detailed discussion of fitness and its components is beyond the scope of this chapter, and the reader is directed elsewhere for more thorough treatments of viral fitness (31,39). The overall result of differences in fitness is evolutionary change by way of natural selection acting on those differences.

Adaptation

Adaptation refers to a character that arises via natural selection and thus confers on its bearer the ability to succeed genetically in a specific environment. The word may also refer to the actual process of acquiring such traits. Adaptations are therefore an evolutionary response to selective pressure presented in the form of environmental conditions that affect reproductive output. Viruses are of course exposed to myriad such conditions. Selective pressures are directly related to survival and replication within a host and transmission between hosts (discussed in more detail under Ecology). Does selective pressure alone create adaptation? The answer is no. Replicative barriers and unexploited reproductive opportunities provide a mechanism by which certain mutants or novel genetic combinations can survive and ultimately outcompete other members of their population. These mutants exist in proportion to the amount of genetic variation that exists. The phenotypes that bestow higher relative fitness (adaptations) may eventually become fixed in the population as a result of the reproductive success they provide to the individuals that bear them.

Selection arising from direct competition between virus strains for access to reproductive resources has been observed in the laboratory to give rise to ongoing adaptation. Clarke and colleagues examined fitness changes in distinct, competing clonal populations of VSV (25). They used relative fitness tests to measure changes in the competitive ability of VSV populations that initially had identical fitness. When cultured together through time, one of the virus populations was inevitably totally displaced by the other. “Winner” and “loser” strains were then mixed with their ancestral wild-type strains to examine the absolute changes in their fitness (adaptive evolution). In all cases, the derived virus populations were able to outcompete and displace their ancestral populations. Adaptive change proceeded in such a way that overall competitive ability of present generations surpassed that of previous ones and was lower than that of the future generations. This exact outcome is predicted by an evolutionary concept termed the *red queen's hypothesis* (133), which states that when two or more populations exist in direct competition for some resource, the processes of adaptation and counteradaptation must be continually occurring for both populations to survive. Such adaptation within natural host populations is more difficult to confirm and has even been discounted for some mammalian RNA viruses (114).

Certain adaptations may not allow a virus to outcompete other individuals directly but instead may permit entry into an unusual reproductive environment. This may initially release a virus from competition because it will inevitably exist at a very low population density in its new surroundings. The result will be a new population (and eventually many populations), perhaps exhibiting a completely different reproductive lifestyle. Such is often the case when viruses cross tissue, host, or vector species barriers. For example, canine parvovirus (CPV) did not exist before the late 1970s, and it spread worldwide in less than 2 years (104). CPV is derived from feline parvovirus (FPV), and the two viruses differ genetically by only three amino acids of their VP2 capsid protein (103,105). Parrish was able to demonstrate that CPV viruses that were otherwise normal but contained lysine at VP2 position 93 were able to replicate in feline cells but lost their ability to replicate in canine cells (103). Furthermore, otherwise normal FPV that contained asparagine at positions 93 and 323 were able to replicate in both canine and feline cells. These latter two changes represent a recently acquired adaptation that allowed replication in a new, susceptible host species and subsequently enabled the rapid (and extensive) spread of parvovirus into the world population of canines.

Evolutionary Stasis

The presence of genetic variation does not ensure evolutionary change. Such change is dependent on the existence of some degree of either directional or diversifying selective pressure. Stabilizing selection, on the other hand, may result in long periods of little to no evolutionary change and can, when strong enough, cause tremendous genetic homogeneity among individuals in a population despite high rates of mutation or genetic exchange. This type of selection may be acting in situations where successful viral replication is contingent on (a) numerous factors that must converge precisely; (b) conditions that must reach extreme thresholds of optimality; and/or (c) environmental circumstances that are predictable but rare. Given such strict requirements, any phenotypic deviation from some selected ideal may translate into an infinitesimal probability of reproduction. The observed result would be very slow, if any, evolutionary change.

An excellent example of this is the arthropod-transmitted equine encephalomyelitis viruses (EEV) in the alphavirus genus. These viruses belong in the family Togaviridae and have +RNA genomes. Investigation of the evolutionary rates of EEVs has revealed that despite exhibiting the high mutation rates typical of riboviruses, they actually evolve very

slowly (111,128,140; reviewed in 139). For instance, sequential isolates of Eastern EEV from North America covering a period of 52 years revealed only 0.7% sequence divergence during this time (140). It has been estimated that the alphaviruses evolve at a rate that is on average ten times slower than other riboviruses (140). Weaver and colleagues have postulated that these viruses reproduce under stringent conditions that involve obligatory replication within both mosquito and mammalian hosts (139). The superimposition of an additional (and phylogenetically distant) species onto the virus replication cycle likely equals a proportional increase in the degree of selective constraints in operation. This theory is supported by the observation that other arthropod-borne RNA viruses (e.g., flaviviruses such as dengue 2 virus and yellow fever virus) also exhibit unusually slow rates of evolution (32,129).

Ecology

Ecology is a science that investigates the interactions between an organism and its environment. Environment refers to the physical, biological, and stochastic elements that constitute the habitat that allows an organism to reproduce at a given time. The environment thus uniquely determines the type and degree of natural selective pressure exerted on a population. Viruses have both general and specific requirements for replication and existence. All viruses require the use of a host cell's adenosine triphosphate (ATP), ribosome, nucleotide, transfer RNA (tRNA), and amino acid resources. Many viruses also require use of other cell functions and components, such as replication and splicing machinery. Often, viruses use more advanced host characteristics, especially when dealing with the demands of transmission. Here, they may take advantage of host ecology, feeding habits, sexual behavior, social structure, and so forth to facilitate host–host transfer.

Although viruses as a group are presumably capable of using any cellular organism for replication, individual virus types are usually specialized in the host species they parasitize. The specific set and degree of environmental conditions within which an individual (e.g., virus type, species) is adapted for survival and reproduction is referred to as an *ecological niche* (31,73). The ecological niche is unique to a particular life form and thus should be considered as a characteristic of that life form. For example, the ecological niches of bacteriophage lambda and HIV-1 differ dramatically, whereas the ecological niche of HIV-1 shares similarities with that of human T-cell leukemia virus and shares even more similarities with HIV-2. As discussed later, however, the ecological niche of a virus is not fixed and evolves just as any other viral trait or phenotype.

Most viruses exist with two main reproductive objectives: survival and replication within host cells and successful transmission of progeny to new host cells for subsequent survival and reproduction. This translates into distinct environments in which viruses must exist, that is, the intra- (within) host and inter- (between) host (57).

The Intrahost Environment

The intrahost environment consists of the replicative resources and conditions to which a virus is exposed both within the host organism (extracellular) and within the host cell (intracellular). This environment contains two primary forces that exert evolutionary pressure on infecting viruses: first, competition between individual viruses for intrahost resources, and second, the antiviral defense mechanisms exhibited by the infected host.

Intrahost resources required for replication (i.e., “reproductive substrate”) fundamentally include living cells of a necessary tissue type. Once an intrahost virus population has become established in, for instance, a multicellular eukaryote, the relative abundance of infectible cells is usually extremely high and not a limited resource for which viral genotypes “compete”; it has been estimated that less than 1% of susceptible host tissue is actually infected in most human viral infections (65). Within cells, competition for resources such as replication and translation machinery is dependent on the multiplicity of infection (moi) and thus the frequency of co-infection by multiple genotypes (23,131).

Investigation into intrahost competition is rare, and information about its overall influence on viral evolution is scarce. Turner and Chao have conducted an elegant series of laboratory experiments on the segmented dsRNA virus f6, which has provided evidence that intracellular competition between co-infecting viruses can be intense (130,131). In their studies, f6 was grown at both high and low moi for the purposes of examining the effects of genetic exchange (sex) between genotypes on rate of viral adaptation. What they discovered was that intrahost competition was often so intense that instead of evolving strategies to exploit the host species more effectively, viral adaptations arose that were aimed at influencing virus–virus competition. When multiple individuals of a virus type infect a single cell, genes are translated, genomes are copied, genomes are packaged, and virions are released. Genomes that are replicated faster accumulate higher copy numbers. Therefore, the probability of a “fast” genome being packaged into a given set of capsid proteins is proportionally higher regardless of whose genes were translated to produce these proteins. As a result, intracellular competition between viral genotypes can give rise to characters whose influence is focused on individual success in the presence of competitors rather than on optimal exploitation of the host species. This is also exemplified by the existence of defective interfering particles that arise and are capable of persisting during high moi infections. These particles are derived as mutants of the wild-type virus and are only capable of replication in the presence of high levels of the fully competent parental viruses whose enzymes and structural proteins they parasitize (10,78,112).

A larger (mostly theoretical) research effort has focused on the interaction between viruses and host antiviral defenses and the evolutionary effects of this interaction. Antiviral responses are theorized to create directional or disruptive selective pressures that drive viral diversification of traits that are susceptible to such responses (5,11). In many ways, the coevolution of viruses and the antiviral capabilities of their hosts should mimic classic predator–prey dynamics commonly cited in ecological research. The effect of a host's antiviral response on the evolution of a virus is selection for viral types that can evade this response. This outcome feeds back as selective pressure on hosts to evolve fresh antiviral responses against the new viral types and so on. Unfortunately, strong empirical evidence in support of these types of cause-and-effect viral–antiviral coevolutionary dynamics is severely lacking. The topic is discussed in greater detail under Antiviral Evasion.

The Interhost Environment

To persist evolutionarily, a virus must give rise to progeny that successfully infect at least one new host organism of the same or different species. To infect a new host, two principal conditions must be met. First, new, susceptible hosts must exist, usually within a limited spatial scale. Second, viruses must have access to a vehicle or pathway of safe transport to a new host. The interhost environment thus includes all the conditions to which a virus is exposed when it is not occupying a host organism. These conditions are mostly determined by host species biology (e.g., airborne, vector-borne, food-borne, water-borne). Viruses evolve transmission strategies that take advantage of their host species physiology, habitat use, population structure, and behavior. Viruses may also evolve ways to alter specific host characteristics such that they facilitate host–host transmission.

Viruses may be transmitted between hosts vertically (i.e., from parent to offspring through the germline), horizontally (i.e., from host to host via an extracellular stage), or through some combination of the two. The different pathways are advantageous under different ecological circumstances. For instance, vertical transmission may be selected for when direct host–host contact is rare, such as cases in which host populations are sparsely distributed, host organisms are sessile, or host populations are small. Viruses may also use mobile vectors (e.g., arthropods) for transmission to overcome these effects. Horizontal transmission may succeed when potential new hosts are abundant or when host–host contact is frequent.

It is difficult to define general rules of transmission that are followed by all viruses. Horizontal transmission, for instance, may take place by way of the external environment (e.g., aerosol) or by direct contact between hosts (e.g., tactile or sexual). Viruses have evolved diverse adaptations to allow for transmission through these routes. Exposure of some viruses to harsh conditions in the external environment (e.g., ionizing radiation, temperature extremes, pH extremes, aridity) necessitates characteristics that maintain the virion's viability. Many insect viruses (e.g., nuclear polyhedrosis and granulosis viruses) use multiparticle occlusion within proteinaceous crystalline structures to enhance environmental stability (1,14). An occlusion body may include only a few or as many as tens of thousands of viral particles each and can remain viable in the external environment for long periods of time (28). Particles have been observed to remain infectious after 21 years of laboratory storage and after exposure to pH values between 2 and 9 (28).

Bottlenecks and Founder Events

As mentioned under Genetic Drift, evolutionary diversification need not involve natural selection. Stochastic events often result in survival of individuals on the basis of pure chance rather than fitness. Such incidents can lead to immediate fixation of phenotypes and genotypes. A bottleneck refers to a large decrease in population size resulting in the survival of a small percentage of the original population (such as is common during host–host transmission of many viruses). A founder event refers to a situation in which a small number of individuals quickly become isolated from their original population, often in an unusual habitat. Both situations can exert a powerful influence over the genetic makeup and future evolution of the group. This is due to the general statistical phenomenon known as sampling error that can cause a small founding population to have a genetic makeup that is significantly dissimilar from the parental population. Under these circumstances, the probability that rare mutations (either beneficial or deleterious) are selected for future replication is much higher than if natural selection were allowed to operate on the population as a whole. If poorly adapted genotypes (with respect to the specific environment) are passed on, extinction is likely. This has been referred to as *Muller's ratchet* (99). Simply stated, this means that because any mutation is most likely deleterious (and as such, restorative back-mutations rare), its fixation via genetic drift will result in a decrease in population fitness. If, however, well-adapted genotypes are passed on, future generations will closely resemble members of the founding population instead of the parental population, and rapid evolutionary change will be observed.

Bottlenecks in natural virus populations are not frequently investigated but are most certainly a common occurrence in the life cycle of many viruses. The most obvious viral bottleneck can happen during host–host transmission. In HIV-1, for instance, transmission between hosts may involve infected cells or individual viral particles. In either case, the number of individual genomes (i.e., one to hundreds) that give rise to the virus population in the new host is extremely small relative to the original population size (i.e., more than 10^{10}). The effects of HIV transmission bottlenecks are illustrated by comparison of virus genetic variability between the infectious (donor) and infected (recipient) hosts. Heterogeneity in HIV populations generally increases with time after infection (96,102). Therefore, comparison of populations within recently infected hosts with those in the presumed donor hosts reveals great disparity in the amount of genetic variation between the two, indicating that the more homogeneous new population underwent a recent bottleneck event.

Founder events, as traditionally defined by evolutionary biology, have not been extensively examined in nature. This is not to say that they are rare or unusual, however, and they are known to be often responsible for adaptive radiation. In viruses, founder events may involve transmission to new or novel tissue types or, more importantly, new host species. The well-adapted founding virus may survive, reproduce, and evolve in this new environment. This will be discussed more extensively under Emergence and Evolution of Viral Disease.

VIRUS–HOST COEVOLUTION

Adaptation of viruses to abiotic conditions (e.g., temperature, salinity) obviously brings about no reciprocal change in those conditions. Adaptations that overcome or circumvent host characteristics, however, especially when they result in a decrease in host fitness, can generate selective pressure on the host. This selective pressure will make counteradaptations in the host advantageous. Thus, viruses and their hosts are often engaged in a coevolutionary process in which adaptive responses in one group impose selection on the other, which gives rise to more adaptation, and so forth (93). It is in this way that virus evolution in the present can indirectly influence virus evolution in the future. Although virus–host coevolution has not been extensively reviewed, many specific examples have been investigated (13,51,63,116)

Coevolution need not necessarily involve an “arms race” between host and virus. Situations may exist in which harm to the host is so strongly selected against that traits that cause host “overconsumption” cannot arise and persist. There exist numerous instances of both benign and mutualistic virus–host relationships. When the relationship between virus and host is so intimately weaved that neither can survive without the other, the evolutionary histories of the two groups may appear identical. In fact, it can even be questionable as to whether a particular virus is an autonomous entity. It is therefore insufficient to consider virus or host evolution outside the context of the effect that each has on the other's existence.

Virulence and Avirulence

Virulence, a relative measure of the amount of host damage (i.e., decrease in host fitness) caused by a specific parasite infection, is a complicated and perpetually evolving trait. Viruses are obligate parasites that cannot live outside their hosts. Because of this, any premature death to the host results in death to (i.e., nontransmission of) at least some infecting viruses. Therefore, it might seem paradoxical that viral infection would bring about any decrease in host life span. Indeed, it is still a common misconception among some scientists that all parasites evolve to become simply innocuous esidents of their host species and that the evolutionary age of a host–parasite relationship can be estimated by observing the level of virulence exhibited. The evidence against this idea is now overwhelming, which leaves researchers with numerous questions regarding why levels of virulence vary between parasite groups and strains.

Virulence is often (although not always) positively associated with the replication rate of the virus within the host. Intrahost virus concentration often increases at the expense of the cells being infected. This increase, however, although endangering the health or life of the host, also increases the probability that at least some of the viral progeny will be transmitted to a new, susceptible host. Therefore, the evolutionarily acceptable level of virulence is set by its relationship to transmission. An extremely virulent (lethal) virus that infects a host whose ecology prevents rapid host–host transmission no matter the intrahost viral titer will become extinct quickly.

Virulence as a trait is determined by the effects (both direct and indirect) of virus replication within the host. Therefore, characteristics that increase or decrease relative pathogenicity of virus strains often evolve under selective pressure that is based on competition for intrahost replication resources (54,55). In other words, virus strains within a host will exhibit varying rates of replication. Strains that manufacture viable progeny (reproduce) more rapidly will accumulate a larger relative population size. Assuming the size of the intrahost population is positively related to transmission probability, the larger population will have correspondingly higher fitness. The virulence level ultimately tolerated by a virus–host system is specific to the biology of both the virus and the host.

Is the level of virulence exhibited by a virus actually an adaptation that has arisen with the help of natural selection? It is probable that many specific symptoms of viral infection that have negative effects on host fitness may persist because they are associated with an increase in viral transmission. For instance, symptoms such as coughing and sneezing caused by viruses infecting respiratory regions (Adenoviridae, rhinoviruses, Coronaviridae, Paramyxoviridae, Orthomyxoviridae), diarrhea caused by enteric viruses (rotaviruses, Caliciviridae, astroviruses), and lesions caused by many sexually transmitted viruses as well as some poxviruses all have clear adaptive significance when considering interhost transmission routes because they place (shed) infectious particles into a suitable environmental position for entry into a new host. It has also been hypothesized that at least for some arboviruses, virulence represents a way to facilitate vector-mediated transmission by disabling a normally active and mobile host, thereby allowing easier access to feeding vectors (49,50). Dengue virus and yellow fever virus rely on hematophagous insect vectors for host–host transmission and induce fever, muscular aches, and/or encephalitis, which typically result in host inactivity. The host's sedentary state may allow for easier feeding by mosquitoes and therefore increase interhost transmission.

Conversely, many viruses exist as inapparent (silent) infections or even as symbionts that actually increase host fitness. These viruses obviously have a very different evolutionary relationship with their hosts. For instance, some human endogenous retroviruses exist as fully functional, chromosomally integrated, vertically transmitted agents that cause little to no apparent harm to their host (88,91,106). Their success is apparent in the fact that many have existed within the various primate lineages for tens of millions of years (119,124).

Polydnaviruses (PDVs) have actually evolved to the point of providing an essential role in the reproduction of their hosts. These viruses are found in the chromosomes of two families of endoparasitic wasps and are thus transmitted vertically (53,121). During oviposition within a susceptible lepidopteran larva, the eggs are coated with viral particles and transferred into the host body cavity. Once inside the host, PDV proteins assist development of wasp larvae by impairing host immune attack against the eggs. In addition, viral products actually alter the developmental rate of the larval host to allow for optimal tissue consumption by the young wasps (7,46,74,117,135). The important point is that viruses need not be pathogenic to exist, but only to increase the likelihood of being investigated by scientists.

Antiviral Evasion

Pathogenic viruses and their hosts appear to exist in a perpetual arms race of accumulating adaptation and counteradaptation. If a host were totally unable to develop and maintain any defense against containment or elimination of a viral infection that consistently caused a decrease in fitness, given enough time, the virus would have no habitat because of a corresponding reduction in host population. If a virus were likewise unable to penetrate the antiviral or innate defenses of a particular host species, it could also never exist therein. What we are able to observe in nature is the middle ground between these extremes. When considering the highly complex antiviral immune response of mammals, a correspondingly and equally complex array of evasion strategies has also evolved in the affected viral groups.

Escaping destruction by an intact immune system may be accomplished by escaping direct interaction with its components. This may involve replication within immunologically privileged tissue that is inaccessible to the host antiviral

response. The classic example of this is the herpesviruses (e.g., varicella zoster virus, herpes simplex virus [HSV]) that exist latently in nonreplicating sensory neurons that are not surveilled by the host immune system. In addition, the viruses turn off nearly all gene expression during latency, which further decreases their susceptibility because of the absence of viral encoded antigens. The viruses periodically move through axons to epithelial or opthalmic sites for replication and transmission (where they may eventually be suppressed by the immune response) but never actually leave the host completely. Other viruses replicate within tissues that experience limited antiviral activity. Human polyomaviruses BK and JC replicate within nephritic epithelial tissue and therefore not only benefit from limited T-cell penetration but also gain access to urination as a mechanism of dispersal (2).

A more typical way of escaping the host antiviral defenses is to be invisible to them. This is accomplished in a number of ways. Immunosuppression, for instance, results in the inability of the host to detect and destroy viruses effectively. This can involve the use of proteins, either viral or cellular, to disrupt the host antiviral response. Many viruses have the ability to sabotage production or function of host cytokines using their own gene products. Host molecules, such as interferons, interleukins, and other cytokines, are susceptible to the action of immunosuppressive viruses. Viral encoded molecules may prevent host gene transcription (e.g., hepatitis B virus terminal protein) or translation (e.g., adenovirus E1A), inhibit cytokine activated enzymes (e.g., RNAse L inactivation in HSV infection), or sequester molecules via complement binding (e.g., interferon-g–receptor decoy produced by myxoma virus). Viral gene products may also interfere with other aspects of the host antiviral response, such as antigen presentation (vaccinia virus, HSV) and down-regulation of host cell genes required for proper antiviral function (e.g., class I and II major histocompatibility complex, intercellular adhesion molecule, and lymphocyte function associated antigen 3 [LFA-3]) (see Chapter 11).

Another way for a virus to remain (at least partially) invisible to an adaptive host immune response such as exists in mammals is to change its appearance continually. This applies not to individual virions but rather to an infecting viral population. Many riboviruses and retroviruses, for instance, exhibit mutation rates that promote constant modification of the population's viral (especially surface) proteins through time. These proteins are markers that the immune response uses to separate self from nonself. Viruses such as HIV may persist in the host for long periods because their rate of and tolerance for protein modification exceeds that which can be successfully identified and cleared by the adaptive immune response (101,113). In this case, the phenotype that allows host occupation is mutability. In addition, HIV further diminishes the host's abilities to find and clear the virus by infecting and eventually destroying the very cells responsible for carrying out these activities (CD4+ cells, macrophages). Perhaps a classic demonstration of contemporary virus–host coevolution is the relative ability of some human hosts to exhibit increased resistance to HIV infection because of possession of a homozygous deletion in the CCR5 cell surface coreceptor. This deletion results in the inhibition of HIV cell entry (72). Many other viruses also infect cells of the immune system, including monocytes (cytomegalovirus, influenza virus) and B lymphocytes (Epstein-Barr virus, MuLV).

Pathogenic viruses that infect mammalian hosts are under continual selective pressure to reproduce in the presence of sophisticated and highly specific antiviral responses that have arisen through millions of years of evolution. Although it may seem that such hosts would have the upper hand in this ongoing battle, viruses continue to develop strategies of host occupation. Viruses are capable of much more rapid evolutionary responses to the antiviral threats they encounter because of their short generation times and huge population sizes (relative to their hosts), which translate into tremendous phenotypic variation from which natural selection can choose. More than 50 virus genes have been identified as influencing immune functions on numerous levels, and this number will most certainly grow (108). Such facts represent a strong demonstration of the impact that host antiviral immune responses have on the evolution of viruses.

Emergence and Evolution of Viral Disease

The overall importance of a given virus to society at large is related to its ability to cause disease in humans or economically or otherwise important species. This characteristic is only exhibited by a very small subset of all virus types on our planet. As already mentioned, however, viruses are capable of extraordinarily rapid modification and diversification (both genetic and phenotypic). Consequently, the benign, even unknown virus that today exists silently may represent tomorrow's crisis. Evolutionary theory has tremendous relevance to the investigation of viral disease emergence and spread. It is by way of the evolutionary mechanisms we have discussed that viruses (a) occupy novel host species and tissues and (b) change their symptomatology and pathogenicity for the purposes of (or as a by-product of) maintaining host–host transmission within a population. In addition, many of the ecological opportunities presented to viruses that lead to unfortunate (disease-related) consequences arise directly from intentional human activity; it is imperative to understand this as a catalyst of virus diversification to estimate the risks associated with such activity (33).

Individual virus types originate and are often considered new when they begin replication in atypical host species or tissue or when different symptoms are associated with infection. In fact, many viruses that are avirulent in the host species to which they evolved are highly virulent in other (often closely related) species upon exposure and infection. This is well exemplified by the myxomavirus, which infects New World rabbit species of the genus *Sylvilagus*. In this natural host, the virus exhibits little, if any disease. On introduction by humans into European rabbit pests (*Oryctolagus cuniculus*) occupying Australia, however, the virus initially had a higher than 99% mortality rate (3,52).

Viruses can only emerge into human populations when they are both adapted to do so and provided with the appropriate host exposure (68). We can do little to control the phenotypes that arise in natural virus populations, yet we do have the power to change the type and frequency of virus–host contact. The ability of a virus to reproduce sustainably in atypical host species generally appears to decrease with decreasing phylogenetic (evolutionary) relation between the species. In other words, a human lentivirus has a better chance of replication within a chimpanzee than within a salamander. Because of this, we can expect human viral diseases to emerge more frequently from mammal species than from other vertebrate or invertebrate groups.

Hantavirus is an excellent example of viral disease emergence through ecological opportunity. Hantaviruses are RNA viruses that naturally occur as persistent, nonfatal, genetically stable infections of rodents. The virus is shed in feces, saliva, and urine, and human contact with the virus can lead to infection and an often fatal respiratory illness (87). A hantaviral outbreak occurred in the western United States in the summer of 1993. Strains of the virus retrieved from

autopsy tissue matched those found in local rodent populations (genus *Peromyscus*), in which the virus exists as a persistent, asymptomatic infection (98). It was further discovered that rodent populations during that period were 10-fold higher than in previous years, and this was attributed to abnormally large amounts of rodent food brought on by increased rain (98). The explosion of local rodent populations most likely resulted in abnormally frequent human–rodent contact as well as an increase in viral load within individual rodent hosts. This contact was an ecological opening that increased opportunities for the resident hantavirus to infect a novel host species (it should be noted that in this case, human infection is an evolutionary dead end for the virus because sustained transmission between human hosts has failed to occur; this is obviously not true for every host species jump).

Ecological opportunity may also arise from certain changes in host behavior or population structure that directly affect competition between viral strains for host–host transmission. For instance, evidence suggests that HIV-1 existed in the human population for many years before the AIDS epidemic of the late 20th century (29,144,145,147). It has been suggested that its previously avirulent residence was altered in response to changes in human social conditions (e.g., population density, urbanization, transportation, sexual promiscuity, intravenous drug use), which allowed for greater transmission opportunities and thus permitted, even promoted, changes in virulence (49,50,89,90,147). Such a change in life cycle may have resulted in a modern adaptive radiation that is today manifest as the HIV and AIDS epidemic.

CONCLUSION

The study of viral evolution is a science unique within biology. Viruses are an astoundingly diverse assemblage of entities that is at least as varied as any higher-level cellular taxonomic group. Yet viruses mutate, adapt, and evolve at a diversity of rates that simply has no comparison in the cellular world. In addition, viruses are capable of influencing host evolution in ways we are just now beginning to uncover. The explosion of genomic sequence data (in humans as well as numerous other species) is bringing forth evidence of the enormous transformative power viral agents have had on their host organisms. This influence can be expected to endure for perhaps the remainder of Earth's biological future. Viruses and viral disease are certain to affect human health and quality of life for the foreseeable future both for the positive and negative. Joshua Lederberg made this point more eloquently in 1993: “The survival of the human species is not a preordained evolutionary program.... Our only real competitors remain the viruses....” (86).

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CHAPTER 14 Epidemiology

Neal Nathanson

DEFINITIONS AND METHODS

Epidemiology deals with the occurrence of diseases in populations. Historically, epidemics of viral disease were recognized long before their causal agents were discovered, and viral epidemiology was one of the first aspects of the science of virology to be developed. Evolving insights into the pathogenesis and molecular aspects of virology have provided an increasingly rational basis for understanding the epidemiology of viruses, and in this chapter, the biology of virus infections is used to explain the essentials of viral epidemiology. Readers interested in epidemiologic methods should consult several excellent introductory texts (27,45).

Rates: Incidence and Prevalence

The quantification of disease occurrence is the cardinal feature of epidemiology. To accomplish this, the concept of rates was introduced, and these have become the basic coinage of epidemiology. Rates are fractions in which the numerator is the number of cases of disease and the denominator is a measure of the population. Incidence (also called *attack rate*) is mainly used for acute diseases of short duration. A population and a time frame are defined, such as the population of Philadelphia for the year 1995, and the number of cases incident in that population during that interval of time is counted. Note that the denominator includes both size of population and time frame; that is, the denominator may be expressed as “million person-years” or “hundred-thousand person-weeks.” The incidence rate is then expressed as “cases per million person-years,” or a similar term. Also note that the time element is often omitted in expressing incidence; the reader must then determine by context the time frame used.

Prevalence is the alternate way of expressing rates and is most frequently used for chronic diseases, particularly when onset is insidious and not readily dated. When point prevalence is computed, a particular date is selected as “prevalence day,” and the population recorded for that day constitutes the denominator; all cases “prevalent” on that date constitute the numerator. Prevalence is expressed as a ratio, such as “cases per million”; note that there is no time parameter in this ratio.

In epidemiology, consistency is a virtue. When cases and populations are counted before computing rates, care must be taken to use the same definition for numerator and denominator. For instance, if incidence is computed for the population of Philadelphia for 1995, a case hospitalized in the city but resident outside the city would not be counted; likewise, a case with onset in December 1994 but still ill in 1995 would not be counted. Figure 1 illustrates the computation of incidence and prevalence rates.

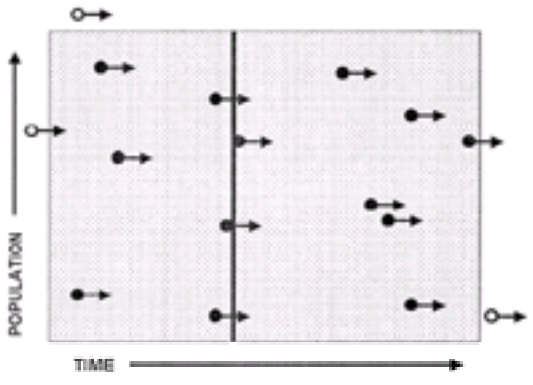

FIG. 1. Computation of incidence rate and prevalence ratio. The *shaded area* defines a population and a time frame that can be expressed as person-time units. Cases of disease are indicated by *circles* (placed according to the date of their onset) and *arrows* for duration of illness. *Solid circles* would be counted for computation of incidence, whereas *open circles* would be excluded because they had onset outside the designated time frame or were resident outside the population boundaries. Incidence equals the number of cases divided by population-time units. Prevalence would be determined by a *single vertical line* across the *hatched area:* cases active at that time point would be divided by the size of the population to compute the prevalence ratio.

Sources of Data

In practice, the accurate collection of data for the computation of rates is often a major undertaking, whereas the computations are relatively simple. Furthermore, the practicing epidemiologist must frequently work with incomplete and inaccurate information. In most developed countries, denominator (population) information is usually good, and numerator (case) information is the major problem. For infectious (viral) diseases, there are several alternate sources of case data.

Surveillance denotes the continuous reporting of disease; by law, many viral diseases are designated as “reportable” and, in theory, the reporting of all cases is required. Cases are reported by practicing physicians or hospitals to local health jurisdictions, which in turn relay reports to a central state office, from where they are transmitted to a national center (the Centers for Disease Control and Prevention in the United States). The weakest link is the initial report; in practice, only a small fraction of cases of many common viral diseases (such as 10% to 15% of measles or hepatitis B) are reported to the national center. If the proportion of cases reported is consistent among geographic locales and among years, however, these reports may be used to monitor trends. Consistency is an important caveat because the proportion of cases reported may increase if the absolute number decreases, such as during a campaign to reduce or eradicate poliomyelitis or measles. Reporting of certain rare or serious diseases, such as poliomyelitis or acquired immunodeficiency syndrome (AIDS), is often close to 100%; in such instances, reported cases can be used with much greater confidence for calculation of rates.

Epidemic investigation is the traditional approach to collecting information on outbreaks of disease. Such investigations are usually initiated by public health authorities. The investigation is tailored to the situation and to the resources at hand, but experience has set certain guidelines, particularly for recurring outbreaks such as food-borne diseases. The purposes of such studies are several: to classify the illness and determine the causative organism; to assess the extent of the outbreak and its economic and health impact; to abort the outbreak or prevent recurrent episodes; and to reassure the public. On occasion, the first recognition of a new disease or isolation of a previously unknown virus has been accomplished as a result of an epidemic investigation. Recent examples are the identification of Lassa virus, Marburg virus, and pulmonary syndrome hantavirus.

Serologic surveys may be used to detect the footprints that a virus leaves in a population. Surveys are particularly useful for viruses because most viral infections leave an imprint on all infected individuals, that is, the presence of antibody, which often is lifelong. Because many viruses cause asymptomatic infections or nondescript illnesses in addition to diagnosable diseases, serologic surveys may identify inapparent as well as apparent infections. In some instances, it is possible to infer the interval between detection of antibody and prior occurrence of infection by assays for immunoglobulin M (IgM) as well as IgG.

Prospective and Retrospective Studies

Modern epidemiology has made one outstanding contribution to the discipline: the extension of hypothesis testing from the laboratory to populations. In general, the epidemiologist attempts to test the hypothesis that one (or several) population variables influence the occurrence of a specified disease and, if so, to quantify this effect and estimate confidence limits. Two general designs are used for such studies: *prospective* (also called *cohort* or *longitudinal*), and *retrospective* (also called *case-control*) protocols. The essence of these designs is summarized next, and the reader is referred to appropriate texts for methodologic exposition (45) (Table 1).

Group	Total cases	Population	Rate per 100,000 person-years	Relative risk
Prospective study[a]				
Vaccinated	100	1,000,000	10	0.33
Unvaccinated	900	3,000,000	30	
Group	**Cases**	**Controls**	**Ratio**	**Relative risk**
Retrospective study[b]				
Vaccinated	10	25	10/25 (0.4)	0.33
Unvaccinated	90	75	90/75 (1.2)	

[a]Prospective study: two populations, vaccinated and not vaccinated, are followed for 1 year, and cases occurring in each are recorded. Rates are calculated, and the ratio of rates gives the relative risk for those who were vaccinated. In this instance, relative risk is lower for those with than without the attribute (immunization). The validity of this design depends on the assumption that vaccinated and not vaccinated groups would be at equal risk except for the attribute under study.

[b]Retrospective study: 100 cases and 100 controls are randomly picked to be representative of the groups from which they are drawn. Subjects in each group are classified as vaccinated or not vaccinated, and two ratios are computed: vaccinated cases/vaccinated controls and unvaccinated cases/unvaccinated controls. The ratio of these ratios yields the relative risk associated with vaccination. The validity of the retrospective design depends on two assumptions: (1) the cases and control groups are truly representative of the larger groups from which they are drawn, and (2) the number of cases is very small (less than 1/100) relative to the total population.

TABLE 1. *Hypothetical data to illustrate computations for a prospective and a retrospective study of vaccine efficacy*

Prospective Studies

The population is divided into two groups, one with and one without a specified attribute. Both groups are followed prospectively for the incidence of the disease under study, and incidence rates for both groups are then computed. A hypothetical example is shown in Table 1; in this example, the population attribute is immunization against a specified viral disease, and the attack rate in the immunized population is one third the rate in the nonimmunized control group. The relative risk (RR) associated with immunization is 0.33, and vaccine efficacy is 67% [(1 – RR)100]. There is one important assumption underlying the validity of such a study: the immunized and nonimmunized groups are at equal risk for the disease under study. For a variety of reasons, this assumption is often not correct; differences between the two study groups may be minimized by dividing each according to parameters, such as age, race, socioeconomic status, and so on, and then comparing rates for each subgroup. Even better, if possible, is to divide the study population into treated and placebo groups, which are then followed under a single- or double-blind protocol. Table 2 summarizes one such study and documents the problems of comparing groups (in this instance, vaccinated and unvaccinated) that are not at the same risk for disease (in this instance, poliomyelitis).

Study area	Vaccination group	Population	Paralytic cases	Rate per 100,000	Estimated efficacy
Placebo areas	Vaccinated	201,229	33	16	72%
	Placebo	201,087	115	57	
	Not inoculated	339,030	121	36	
Observed areas	Vaccinated	222,300	38	17	63%
	Controls	725,173	330	46	
	2nd grade, not inoculated	123,605	43	35	

[a]Vaccine was administered in the spring of 1954, and children were followed prospectively through the summer poliomyelitis season. Placebo areas were divided into volunteers who were vaccinated, volunteers who received placebo inoculations, and nonvolunteers (not inoculated). Observed areas were divided into second-grade volunteers (vaccinated), second-grade nonvolunteers (not inoculated), and first and third graders (controls).

Source: Data from ref. 28, with permission.

TABLE 2. *The 1954 field trial of poliomyelitis vaccine: comparison of attack rates for vaccinated and unvaccinated children*[a]

Retrospective Studies

Prospective studies are usually cumbersome because they require the enrollment of large numbers of subjects who must be followed for a period of months or years; the less frequent the expectation of disease, the larger the population or longer the follow-up needed. Needless to say, costs of prospective studies can be great, limiting them to special situations, such as the introduction of a new vaccine illustrated in Table 2. Retropective studies are much more cost-effective because they involve small numbers of subjects and do not require longitudinal follow-up. Table 1 shows a simplified version of such a study. In this instance, 100 cases of the study disease and 100 unaffected control subjects are identified and classified according to the attribute (vaccination) under study. For vaccinated and unvaccinated individuals, a cases/controls ratio is computed. Because these ratios have the same relationship as the rates computed in the prospective study shown in the upper half of Table 1, the relative risk can be computed as the fraction of the two ratios (0.4 / 1.2 = 0.33). Two assumptions underlie the validity of the case-control design: the two samples (cases and controls) must be representative of the groups from which they are drawn, and the incidence must be low so that cases make up less than 1% of the total population. Because retrospective studies are particularly useful to study risk factors for rare diseases, the second assumption is usually correct. The main flaws in retrospective studies are the choice of cases and particularly the selection of representative controls, a subject considered in depth in epidemiologic texts (45,72).

BASIC BIOLOGIC CONCEPTS

Immunity

Most acute viral infections confer lifelong immunity. Upon reexposure at any interval after initial infection, there is often a reinfection with minimal virus replication and an anamnestic immune response. Such reinfections are usually covert and almost never severe, and they result in minimal shedding of infectious virus. For certain viruses, such as poliovirus or rhinovirus, immunity is type specific and confers little protection against exposure to a different serotype. These simple

facts have profound implications for viral epidemiology. For epidemiologic purposes, a population may be divided into two groups: susceptible and immune. All people who have been infected at any time in the past may be considered immune and are exempt from disease and unlikely to act as links in a transmission chain, whereas susceptibles can both spread the agent and experience disease.

Persistent viral infections differ from acute infections because the infected individual may be capable of transmitting infection over many years and may develop disease at any time during virus persistence. In the instance of persistent viruses, the population may be divided into uninfected susceptibles and “immune” people who carry serologic markers of prior infection but who are still capable of acting as links in the chain of infection. Important examples are hepatitis B virus, human immunodeficiency virus (HIV), and several herpes viruses (herpes simplex, varicella zoster, and cytomegalovirus).

Parameters That Determine Incidence

For acute infections, the following three parameters together determine incidence: the proportion of the population susceptible, the proportion of susceptibles infected, and the proportion of infected people who become ill (case/infection ratio). If these three parameters are written as fractions and then multiplied, the product is incidence (cases/population), as shown in Figure 2.

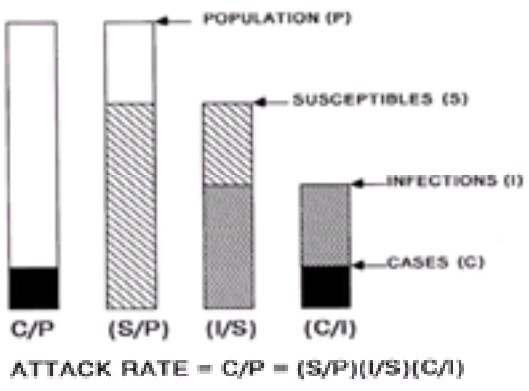

FIG. 2. The attack rate factored. A diagrammatic representation of the fractional parameters that, when multiplied and reduced, yield the fraction c/p (cases/population) or attack rate.

Proportion Susceptible

When a population is exposed to a specific virus, the susceptible part of the population will determine the spread of the agent and will account for all the cases of overt disease, and the proportion susceptible will reflect the past history of infection with the specific virus in the population under consideration.

Proportion Infected

The proportion of susceptibles infected during a fixed period of time (e.g., year or season) can vary widely, depending on the dynamics of transmission, which is influenced by factors such as the density of the susceptible population and season of year.

Case/Infection Ratio

The proportion of infections resulting in overt disease is characteristic for each virus and is strikingly different for different agents. One of the most important contributions of modern laboratory methods to viral epidemiology was the revelation of inapparent infections and the insight that most infections caused by some viruses were inapparent (67). The relative frequency of inapparent infections is expressed as the *case/infection ratio*, that is, the number of cases per 100 infections. The lethality of disease is a different parameter and is designated as the *case/fatality ratio*. Table 3 lists selected common viral infections and shows estimated case/infection ratios and case/fatality ratios for each. It is noteworthy that there is no regular relationship between case/infection ratio and severity of illness. For instance, measles has a very high case/infection ratio (above 95:100) but a low case/fatality ratio (less than 0.1:100 in the United States). Conversely, poliomyelitis has a low case/infection ratio (less than 1:100) but a much higher case/fatality ratio (about 5:100).

Virus	Cases per 100 infections	Fatalities per 100 cases	Comment
Smallpox	>95	<1	Variola minor
	>95	25	Variola major
Measles	>95	0.1	USA, 1970
	>95	2	Africa, 1988
Poliomyelitis	<1	5	USA, 1955
Hepatitis B	50	3	USA, adults
	<1	<0.1	Taiwan, infants

TABLE 3. *Estimated case: infection and case fatality ratios for selected viral infections*

Each of these three parameters can, under certain circumstances, be an important determinant of epidemics or other differences in incidence. The factors that account for variation in each parameter are discussed in subsequent sections of this chapter.

Incubation Period and Generation Time

The course of infection in a single individual can be conveniently divided into several periods. The interval from acquisition of infection to onset of illness is the incubation period. Note that onset of illness must be explicitly defined for each disease; it is often best measured by the first day on which pathognomic signs or symptoms are reported. For most viral diseases, the infected individual begins to shed virus before onset of illness and continues to shed during and sometimes after recovery from the acute illness. Therefore, the infected person is infectious for others over a period of time, and transmission may occur at any time during this period of infectiousness. The average period between infection and transmission is the generation time. From studies of selected outbreaks, it has been possible to determine the average interval between onsets of successive waves of cases. The generation time is often assumed to be equal to this interval, as the best approximation available. Generation time is usually slightly less than incubation period; that is, infections are transmitted slightly before onset of pathognomonic symptoms. These relationships are shown in Figure 3 for a typical acute viral infection; note that these definitions do not readily apply to certain persistent viral infections.

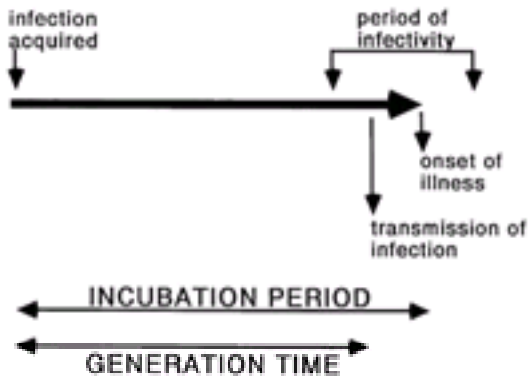

FIG. 3. Incubation period and generation time. Incubation period is the interval between acquisition of infection and onset of illness, whereas generation time is the interval between acquisition of infection and transmission to another person. This diagram shows the mean for each parameter; in practice, there is a spread around this mean.

Incubation periods for any viral disease vary around the mean, as illustrated in Figure 4. When a frequency distribution of individual incubation periods is plotted, it usually has a longer tail at the high end of the distribution (right-hand skew). If the frequency is plotted against the logarithm of time, the data often approximate a normal distribution. This log-normal distribution is characteristic of the incubation periods of many viral diseases and permits the comparison of the distributions of different incubation periods (70). The term *dispersion factor* has been introduced to quantitate the distribution of incubation periods; the dispersion factor is defined as the ratio of two incubation periods, as illustrated in Figure 4. The dispersion factor tends to be constant regardless of the absolute value of the incubation period; for most viral infections, it falls in the range 1.15 to 1.70.

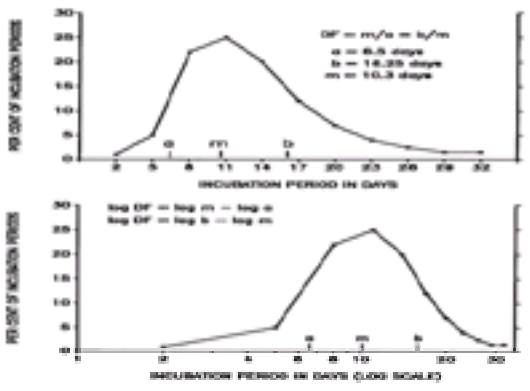

FIG. 4. Log normal distribution of incubation periods. **Upper panel:** incubation period distribution is shown for poliomyelitis; there is a right-hand tail. **Lower panel:** the appearance of incubation periods when plotted on a logarithmic scale. Logarithmic incubation period approximates a normal distribution, and the standard deviation may be computed (on the logarithmic scale). The dispersion factor (DF), a measure of the variation of incubation periods, is the antilogarithm of the standard deviation. The mean (m) is shown, together with one standard deviation (points a and b on the graph). The log DF equals the interval (log m – log a) or the interval (log b – log m). In this instance, the mean is about 10.3 days, a is 6.5 days, b is 16.25 days, and the dispersion factor is ± 1.58; that is, about two thirds of the incubation periods fall into the range (1.58 × mean) to (mean /1.58). (Data from refs. 8,13,38,56, and 69, with permission.)

TRANSMISSION OF VIRUSES

There are two major patterns of transmission into which most viruses may be classified: viruses maintained in a single species and arboviruses that alternately infect insect and vertebrate hosts. There are a few apparent exceptions, such as rabies and influenza viruses, which spread across species boundaries, but even in these instances, cycles of transmission within different species may be self-contained. For agents that infect humans, a second distinction may be made, namely, between those viruses that rely on human transmission for their perpetuation (the majority) and those viruses maintained in an extrahuman cycle for which humans are a deadend host. Examples of these patterns are set forth in Table 4.

Transmission pattern	Maintenance cycle	Example
Human to human	Human to human	Measles
		Hepatitis A
Animal to human	Animal to animal	Rabies
		Hantavirus pulmonary syndrome
Vector to human	Vector to human	Dengue
		Urban yellow fever
Vector to human	Vector to vertebrate	St. Louis encephalitis
		Western encephalitis

TABLE 4. *Major transmission patterns of viral infections of humans*

Viruses Maintained Within a Single Host Population

Each virus has a characteristic mode of host–host transmission. Viruses that cause acute short-term infections require efficient transmission to subsequent hosts and are usually spread by the aerosol respiratory route or by the gastrointestinal oral route. Such infections are characterized by the excretion of high titers of infectious agent over a limited period of time, as in the instance of influenza or smallpox. Viruses that cause persistent infections do not require such highly efficient modes of transmission because they are excreted continuously or intermittently for many years. Horizontal transmission is often used to designate all of these modes of transmission.

Vertical transmission is used to denote transmission between parent and offspring. Such transmission may occur during gestation, by passage of virus across the placenta (as in rubella and AIDS); perinatally during birth (herpes simplex or hepatitis B); or from the mother through colostrum or milk (AIDS). Germline transmission, another mode of vertical spread, is seen with certain animal retroviruses, such as murine leukemia virus, which are transmitted as an integrated provirus but which may be transcribed into infectious virus. Although retroviral sequences are carried in the human genome, there are presently no proven instances in which these have also been shown to function as infectious virions. Selected examples of horizontal and vertical transmission are listed in Table 5.

General category	Transmission mechanism	Example (virus)
Horizontal	Aerosol–respiratory	Influenza
	Gastrointestinal–oral	Rotavirus
Vertical	Placental–fetal	Rubella
	Maternal–parturition	Herpes simplex
	Maternal–neonatal	Human immunodeficiency virus
	Germline	?

TABLE 5. *Transmission mechanism of human viruses maintained by the person-to-person route*

Arboviruses

Arthropod-borne viruses (arboviruses) are usually maintained by continuously cycling between an insect host and a vertebrate host. In most instances, the virus replicates optimally in a single species or in a few closely related species of insects, which constitute the major, if not exclusive, vectors. Because most blood-feeding insects have a clear feeding preference for a few vertebrate species, this determines and limits the vertebrate species involved in the maintenance cycle. For some viruses, there are alternate maintenance cycles limited to the insect host, with transovarial or sexual transmission from generation to generation of insects. These insect cycles may be critical to perpetuation of the virus during adverse conditions, such as overwintering, but otherwise play a minor role in viral dissemination.

There are only two arboviruses, dengue and urban yellow fever, for which humans are the major vertebrate host. In other instances, such as St. Louis encephalitis and La Crosse encephalitis, humans are accidentally infected when the vector mosquito happens to feed on humans as an alternate to the preferred vertebrate blood source, such as birds or

woodland rodents.

Deadend Hosts

A *deadend* or *terminal host* is one that is not involved in maintaining a virus; such infections rarely result in transmission to other human or nonhuman hosts. Humans are occasional deadend hosts for a number of viruses that are maintained in extrahuman cycles. These include all arbovirus infections, with the special exceptions noted previously. In addition, there are a number of relatively exotic infections, most notably rabies, in which humans are deadend hosts. Transmission of rabies is often by bite, but for most other deadend infections in this group (such as Korean hemorrhagic fever and Bolivian hemorrhagic fever), environmental exposure to fomites is responsible. In such instances, the exact mode of transmission is obscure, although aerosolization of virus is often postulated. Table 4 lists examples of viral infections in which humans are deadend hosts.

Transmission of Persistent Infections

The transmission of persistent viral infections differs in many ways from that of classic acute infections. Persistent infections that are latent, such as herpes simplex or varicella zoster, are transmissible intermittently, during periods of activation. Thus, one individual may be infected in childhood and transmit infection 50 years later during a recrudescence; the ability for a single link in the infection chain to extend over such an interval has important implications for perpetuation and eradication of infection, as described later. Infections, such as those caused by hepatitis B and HIV, which are constantly transmissible over many years, are often transmitted inefficiently. Thus, hepatitis B is transmitted to susceptible household contacts at a frequency of less than 1 per 100 person-years of exposure, and HIV is transmitted to sexual contacts at a rate of less than 1 transmission per 100 contact episodes. For such infections, transmission may require passage of body fluids or of viable infected cells from infected to recipient host. Despite inefficient transmission, the number of new infections initiated by each persistent infection may be high. An epidemic curve caused by such a virus may stretch over years rather than weeks, and its mathematic modeling is profoundly different from that of acute virus infections because infectious individuals are not rapidly converted to noninfectious immune individuals but rather continue to accumulate over a long period.

Quantitation of Transmission

The transmission of acute viral infections may be quantified by the term *transmissibility,* or *T,* which is defined as the number of new infections initiated by each existing infection. Note that there is no time frame in this definition; instead, the focus is on the number of new infections initiated per generation. When T is greater than 1, incidence is increasing; when T is less than 1, it is waning. If the generation time has been estimated, as has been done for most acute viral infections, it is possible to use computer simulation to estimate T (46,86,87). T is determined by (a) the number of contacts between an infected individual and others, (b) the proportion of those contacts that are susceptible, and (c) the probability of transmission per susceptible contact.

DESCRIPTIVE EPIDEMIOLOGY AND ITS INTERPRETATION

A hallowed maxim in epidemiology is that a complete description of epidemic or endemic disease must include the parameters person, place, and time. The collection and display of this descriptive information is a necessary first step in understanding the epidemiologic mechanisms leading to occurrence, distribution, and course of an epidemic. This systematic approach is a surprisingly powerful tool in analysis. A few selected examples are presented in this section to demonstrate the application of descriptive epidemiology.

Person

In tabulating cases of a viral disease, the epidemiologist instinctively looks for features that distinguish affected individuals from the general population. Clues may exist in any feature, such as age, sex, race, occupation, residence, or any aspect of personal conduct, and none should be overlooked. Such variables are frequently the most important initial step in dissecting an outbreak.

The first recognized outbreak of St. Louis encephalitis in 1933 is a classic exercise in “shoe-leather” epidemiology (48); this led to the proposal that the disease was transmitted by an arthropod vector rather than by person-to-person contact. When this outbreak occurred neither the disease nor the causal agent were known. Because it was an acute neurologic disease, occurring in the summer, it was first thought to be a form of poliomyelitis or to be transmitted in the same manner (i.e., by interpersonal contact). The characteristic encephalitic manifestations made it relatively easy to identify many of the cases on a purely clinical basis. When these cases were assembled and rates estimated, it became apparent that the incidence was greater in the suburbs of St. Louis than in the city proper. Furthermore, there was a striking concentration of cases among the inhabitants of one institution located in the suburbs, but a comparison of several adjacent institutions revealed even more dramatic discrepancies, illustrated in Table 6. First, there were no cases in the personnel in an infectious disease hospital caring for many acute encephalitis cases; this was strong evidence against person-to-person transmission. Second, there were high rates in an almshouse but very low rates in two insane asylums; investigation revealed that the almshouse lacked screens, whereas the other three institutions were screened. These observations were strongly reminiscent of the classic findings of the Reed commission investigating yellow fever in Havana (68) and suggested mosquito transmission. Finally, it was observed that the suburbs of St. Louis, although home to many affluent residents, lacked the system of storm sewers present in the city; there were many more sites with standing ground water in the suburbs, and in the hot, dry summer of 1933, these were prime mosquito breeding sites. Although primitive, these observations suggested the mechanisms of transmission to the investigating epidemiologist.

Institution	Population	Cases	Rate per 100,000
Mental hospital	400	0	–
Hospital for the insane	4000	0	–
Isolation hospital	500	0	–
Almshouse (infirmary)	1200	13	10,800

[a]Of these four institutions, three had adequate screening whereas one (infirmary) did not. The isolation hospital cared for 300 patients with St. Louis encephalitis, but no cases occurred among the staff, estimated at 500.

Source: Data from ref. 48, with permission.

TABLE 6. *Attack rates for St. Louis encephalitis in four institutions in the suburbs of St. Louis during the epidemic of 1933*[a]

The “Cutter incident” offers another example in which descriptive epidemiology defined an unusual distribution of disease in a population, leading directly to the cause (56). In mid-April 1955, newly approved, inactivated poliomyelitis vaccine was distributed throughout the United States for immunization of children 5 to 9 years of age, who were considered the highest risk age group and therefore given preference in the use of limited vaccine supplies. About 2 weeks later, at the end of April, reports were received of cases of acute paralytic poliomyelitis, occurring in a small number of recently immunized children. Because this was close to the seasonal trough in poliomyelitis incidence, there were relatively few cases of poliomyelitis occurring in the general population at that time, so that a small number of vaccine-associated cases were particularly striking. Furthermore, vaccine-associated cases were confined to a few of the western states, and it quickly became apparent that this geographic distribution related to the manufacturer of vaccine. Vaccine had been produced by five manufacturers, and most cases were associated with vaccine produced by Cutter Laboratories, which was mainly used in western states. When rates were tabulated for different production pools of Cutter vaccine, the association focused on two high-rate pools, as indicated in Table 7. Subsequent investigation showed that there were inadequacies in the inactivation and safety testing protocols recommended by the government, permitting the release of vaccine lots containing residual infectious virulent virus.

Production pool	Number of inoculations	Paralytic poliomyelitis cases	Rates per 100,000
468	73,700	34	46.1
746	48,000	17	35.4
460	34,100	0	—
463	63,400	1	1.5
467	56,700	1	1.8
762	54,400	1	1.8
766	57,200	0	—
767	3,100	0	—

[a]Cutter vaccine was administered April 18–27, and this table is limited to cases with onsets between April 18 and May 14.

Source: Data from ref. 56, with permission.

TABLE 7. *The cutter incident: attack rates among children receiving cutter vaccine, according to production pool, United States, spring, 1955*[a]

Age Distribution

Age distribution of viral disease reflects differences in risk, which have important implications for the biology of virus infection. For many endemic human viruses, infection reaches 100% of the population at some time in life. In such instances, disease is confined to children or to children and young adults because older people are immune as a result of prior inapparent or apparent infection. Poliomyelitis is such an example, and the differences in age distribution of disease in different regions or in the same population in different eras reflects differing rates in the transmission of infection (21,22). Where this enterovirus is transmitted readily, all cases are confined to very young children; contrariwise, in countries with more rigorous personal hygiene, infection may be delayed so that poliomyelitis occurs up to 30 years of age or older (65,67). Figure 5 compares these age distributions and shows the parallelism with the acquisition of immunity.

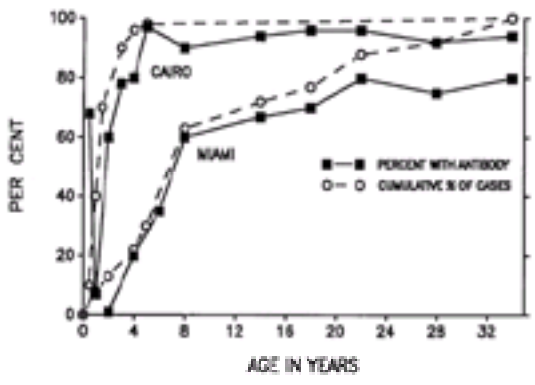

FIG. 5. Age distribution of poliomyelitis cases and immunity. A comparison of cumulative distribution of cases of

poliomyelitis and prevalence of type 2 (Lansing) antibodies in two populations, Cairo and Miami, to show that the age distribution of cases reflects the ages at which infection is acquired. There are few cases in individuals older than 3 years of age in Cairo and in those older than 30 years of age in Miami, the ages at which cumulative infection prevalence reaches 100%. (Data from ref. 65, with permission.)

For viruses that never infect more than a small proportion of a population during a lifetime, age distribution reflects differences in exposure or in the case/infection ratio rather than immunity. An example is St. Louis encephalitis (SLE), which produces unpredictable outbreaks in different areas of the central United States and is infrequent enough so that most age groups are susceptible. It has been repeatedly observed that SLE attack rates are lower in children and much higher in the elderly (10-fold differences). A classic investigation (47) of an epidemic in Houston showed that, surprisingly, the frequency of infection was similar for all ages, indicating that age-specific differences in the case/infection ratio accounted for age-specific attack rates.

Age-specific differences in case/infection ratios can be a key determinant of epidemiologic patterns. In some instances, infants experience much more severe disease than do adults. A classic example is the outbreak of measles that occurred in the Faroe Islands in 1846 (63). Because measles had not occurred in this isolated site for more than 50 years, women of childbearing age were seronegative, and their infants lacked maternal antibody. During the epidemic, the case fatality rate was about 20% in infants and below 1% in children and young adults (Table 8). This episode illustrates the biologic role of passively acquired maternal antibody. On the other hand, hepatitis B causes inapparent but persistent infection in infants, in contrast to acute liver disease followed by immunity in adults. In developing countries, infections are frequently transmitted from persistently infected mothers to their neonatal infants, whereas infection in developed countries is mainly transmitted to older children and adults (81). These differences account for the paradox that the attack rates for hepatitis B are higher in developed countries, whereas cumulative infection rates are much higher and the virus carrier state is more frequent in developing countries, as illustrated in Table 9.

Age group (y)	Annual mortality per 100		
	1835–1845	1846	Excess in 1846
<1	10.8	30.0	19.2
1–9	0.5	0.5	—
10–19	0.5	0.4	—
20–29	0.5	0.7	0.2
30–39	0.8	2.1	1.3
40–49	1.1	2.7	1.6
50–59	0.9	4.4	3.5
60–69	2.0	7.7	5.7
70–79	6.5	13.1	6.6
80–100	16.8	26.0	9.2

[a]The mortality for 1846 is for January to August, the period of the epidemic. The excess mortality provides a crude estimate of measles-specific mortality during the epidemic. At least 80% of the population had measles during the epidemic, which was the first to occur since 1781.
Source: Data from ref. 63, with permission.

TABLE 8. *Age-specific mortality rates on the Faroe Islands during the measles epidemic of 1846 compared with years 1835–1845*[a]

Population	Age group (y)	Anti-HBs (%)	HBs (%)
New York City blood donors (white)	<25	2	<0.1
	25-34	4	0.2
	35-44	3	0.2
	>44	8	<0.1
Taiwan (Chinese)	<10	28	23.0
	10-19	53	26.0
	20-29	48	18.0
	30-39	52	16.0
	40-49	48	15.0
	50-59	48	15.0
	60-69	48	10.0
	>69	48	7.0

[a]The prevalence of antibody and antigen is expressed as a percentage of those tested.
Source: Data from ref. 78, with permission.

TABLE 9. *Prevalence of hepatitis B in the United States and in Taiwan, measured by anti-HBS and HBS antigenemia*[a]

Place

Historically, mapping the distribution of disease preceded the development of epidemiology and became more significant with the introduction of place-specific rates. It remains one of the most powerful and intriguing aspects of descriptive epidemiology.

Two outbreaks of St. Louis encephalitis in two cities of Texas illustrate the complexities of geographic epidemiology. A large outbreak in Houston in 1964 was concentrated in the center of the city with decreasing rates toward the outskirts (Table 10). Subsequent serologic surveys showed that infection rates paralleled cases and also showed that high rates were associated with the lowest economic strata, open foundations, unscreened windows, and lack of air conditioning as well as with areas of standing water close to the banks of the river that flowed through center city. Conversely, in Corpus Christi, attack rates were highest in the suburbs, as shown in Table 11. In Corpus Christi, the determining factor was mosquito breeding in pools of standing water associated with lack of a storm sewer system rather than with reduced

protection from mosquito attack.

Circle	White upper class	White middle class	White lower class	Nonwhite	Totals
1 (innermost)	–	70	102	77	77
2	54	73	51	50	50
3	14	20	30	20	20
4	2	10	6	7	7
5 (outermost)	3	8	–	6	6

[a]The city was divided into concentric circles and into socioeconomically and racially stratified census tracts within each circle before computation of rates.

Source: Data from ref. 42, with permission.

TABLE 10. *Regional and socioeconomic differences in attack rates (per 100,000) for St. Louis encephalitis, Houston, Texas, 1964*[a]

Circle	Socioeconomic stratum	Population	Cases	Rates per 100,000
4 (innermost)	Lower	46,000	5	11
3	Lower middle	46,000	19	39
2	Upper middle	45,000	20	69
1 (outermost)	Upper	46,000	24	52

[a]The city was divided into four zones; zones 4 and 3 were concentric circles, whereas zones 2 and 1 were extensions to the South. Each zone contained a single socioeconomic stratum.

Source: Data from ref. 94, with permission.

TABLE 11. *Regional and socioeconomic differences in attack rates for St. Louis encephalitis, Corpus Christi, Texas, 1966*[a]

A striking example of geographic variation over a large area is the regional difference in attack rates for AIDS, shown in Figure 6. High rates are associated with two relatively small, high-risk groups in the population, namely, male homosexuals and intravenous drug abusers; both groups are concentrated in a few cities. AIDS associated with drug abuse is mainly an East Coast phenomenon, whereas AIDS in the homosexual population is seen on both coasts. Because HIV was only recently introduced into the population, its pattern is still evolving, with an increase in rates in areas initially little affected.

FIG. 6. Geographic distribution of cases of AIDS, United States, for cases reported for 1993. Figure shows annual incidence per 100,000 by State. (Courtesy of Harold Jaffe, AIDS Program, Centers for Disease Control and Prevention.)

Time: Seasonality

Many acute viral infections exhibit striking seasonal patterns in incidence that are very consistent. These differences reflect seasonal differences in transmission of infection because case/infection ratios remain stable at different times of year. Some viral infections peak in the winter, whereas others peak in the summer. In general, respiratory infections spread more readily in the winter, although they may peak at different times, such as January for influenza (23) and March for measles (86,87). Conversely, enteroviruses peak in the summer, although rotaviruses are a striking exception (85). Arbovirus infections are confined to the summer months when their vectors are active.

The underlying biologic explanation for seasonal differences has remained elusive. It is clear, however, that these variations correlate with changes in climate. An example is the seasonality of poliomyelitis in New England, which contrasts with the relative absence of seasonality for poliomyelitis in Hawaii, where humidity remains relatively constant throughout the year (Figure 7). This observation has led to the proposal, for which there is some evidence, that viruses

differ in their sensitivity to humidity, with poliovirus rapidly inactivated during winter months of low humidity, whereas influenza virus remains viable under the same conditions (57,87).

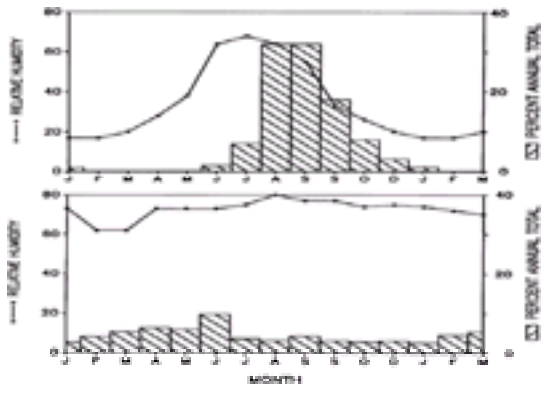

FIG. 7. Seasonal distribution of poliomyelitis in New England and in Hawaii, and the seasonal variation in relative humidity. **Upper panel:** cases for 1942 to 1951 in New England. **Lower panel:** cases for 1938 to 1952 for Hawaii. (Data from refs. 24,57, and 75, with permission.)

Time: Secular Trends and the Emergence of New Diseases

Secular trends in incidence of viral diseases fall into two categories: natural occurrences and those associated with programs of control or eradication. The latter are dealt with in a subsequent section.

One of the most dramatic aspects of historical epidemiology is the appearance of a new viral disease. This may reflect (a) the true appearance of a new virus in the population, a rare but real possibility; (b) an increase in the case/infection ratio so that an endemic infection is associated with a marked increase in disease incidence; or (c) the recognition of an existing but previously unidentified disease that can now be clearly diagnosed as a result of new laboratory tests accompanied by heightened practitioner awareness.

Emergence of a Virus New to the Population

AIDS and other diseases associated with HIV are an only too familiar example of the emergence of a virus new to the population. AIDS first appeared in the United States and Europe in about 1979, and serologic studies have demonstrated that infection first occurred in the male homosexual population of San Francisco 1 to 2 years before the recognition of the disease (16,17). The emergence of AIDS was reinacted in India and southeast Asia in the mid-1980s when newly developed serologic and virologic methods permitted the infection to be followed from its earliest entry into the population. A strong web of circumstantial evidence suggests that HIV-2 may have been derived from viruses indigenous to sooty mangabeys and HIV-1 may have originated in simian immunodeficiency virus strains circulating in chimpanzees, both in subsaharan Africa (30,59).

Canine parvoviruses appeared in the dog populations of the world in the late 1970s and spread round the world rapidly, causing a major pandemic. Circumstantial evidence strongly suggests that canine parvovirus was derived from feline panleukopenia virus, a long-established virus of cats (64). Wild-type feline panleukopenia virus will not replicate in canine cells, but a few point mutations will confer the ability to replicate in canine cells and cause disease in dogs.

Bovine spongiform encephalopathy (BSE) was first detected in the United Kingdom in the mid-1980s and was quickly identified as a spongiform encephalopathy similar to scrapie, an endemic disease of sheep. Detailed epidemiologic investigation (83) strongly suggested that BSE represents a common source epidemic because of the contamination of meat and bonemeal nutritional supplements routinely fed to dairy cattle. It is likely that scrapie-infected sheep tissues were included in the raw material used to produce the feed supplement and that a change (introduced in the late 1970s) in the production methods permitted scrapie infectivity to survive the production process at a residual level. The rapidly evolving epidemic (Fig. 8) led to the exclusion (in 1988) of ruminant tissues from feed supplements, which should result in the termination of the epidemic. However, the long incubation period (average, about 4 to 5 years) of BSE indicated that the epidemic might continue to evolve until about 1993 and begin to decline thereafter (59).

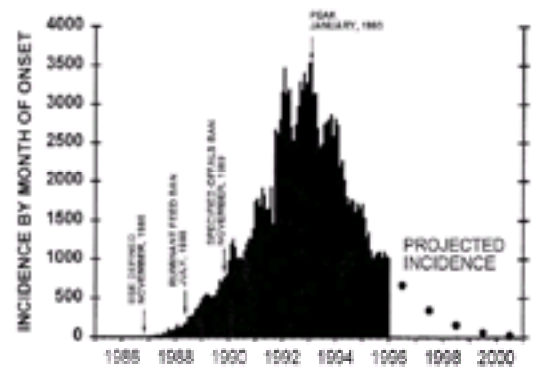

FIG. 8. The course of the epidemic of bovine spongiform encephalopathy (BSE) in the United Kingdom, shown as cases by month of onset, 1987 through April 1995. (Data taken from refs. 3,6,7, and 83, with permission.)

Increase in the Case/Infection Ratio

The dramatic appearance of epidemic poliomyelitis (known as *infantile paralysis*) in Europe and the United States in the 19th century (57,66) is illustrated in Figure 9. In retrospect, it is clear that polioviruses had been endemic for millenia but that cases of paralysis were few and sporadic. The likely explanation is that an improvement in standards of personal hygiene reduced the transmission of this enterovirus, delaying infection of young children beyond the age during which they were protected by maternal antibody (see Fig. 5) and resulting in the first epidemics of infantile paralysis. Support for this view (66) has been provided by the reenactment of the appearance of poliomyelitis in other countries (in the Middle East and Africa) (29) during the 20th century. In such instances, it was possible to conduct serologic studies that demonstrated the high prevalence of antibodies even before the appearance of clinical epidemics (66). Furthermore, the secular trend continued in the United States, with a gradual stepwise increase in the age distribution of paralytic poliomyelitis (Table 12), so that by the time of vaccine introduction in 1955, the term infantile paralysis had become outmoded.

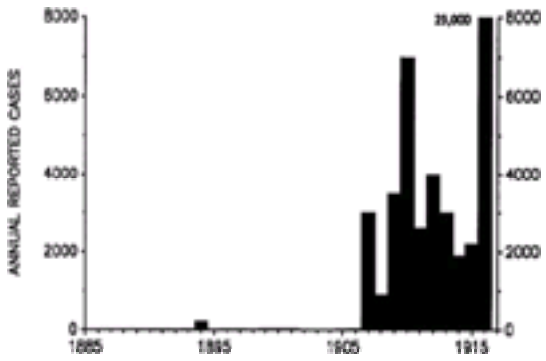

FIG. 9. The appearance of epidemic poliomyelitis in the United States, 1885 to 1916. The graph is based on reported cases (mainly paralytic) during an era when reporting was estimated at about 50%. (Data from refs. 44 and 57, with permission.)

Years	Percentage 0–4 y	Percentage 5–9 y	Percentage 10+ y
1912–1916	70	18	12
1920–1924	48	24	28
1925–1929	40	30	30
1930–1934	28	38	34
1935–1939	28	28	44
1940–1944	22	28	50
1948–1952	18	27	55

[a]Includes paralytic and nonparalytic cases.
Source: Data from refs. 22 and 57, with permission.

TABLE 12. *Age distribution of poliomyelitis in Massachusetts, 1912–1952*[a]

New Recognition of an Existing Virus

La Crosse virus is a mosquito-transmitted bunyavirus that causes La Crosse encephalitis. The causal agent was first isolated from a fatal case in 1964 (80) and led to the ability to distinguish this disease from the rubric of “arbovirus encephalitis, etiology unknown.” Beginning in 1964, about 100 cases have been reported annually mainly from the midwestern United States. It is likely that the emergence of this “new” disease reflects only the ability to make the specific diagnosis because the incidence has not risen since the 1970s (37) and because serologic studies (35) indicate that infections were occurring at a similar frequency long before isolation of the virus was reported in 1965.

A striking new acute pulmonary syndrome with high mortality was first reported in the southwestern United States in 1993, and combined epidemiologic and laboratory investigation quickly indicated that the agent (Sin Nombre virus [SNV]) was a previously unknown bunyavirus belonging to the hantavirus genus (39,60). Since the original reports, isolated cases due to related viruses have been detected in the southern and western United States, and several virus isolates have been made. SNV is an indigenous virus of deer mice (*Peromyscus maniculatus*), in which it causes a persistent infection accompanied by virus excretion leading to production of virus-infected aerosols and exposure of humans. Almost certainly, the emergence of SNV is the result of the recognition of a long-existing agent and disease that was brought to attention because of an unusual cluster of cases associated with a transient increase in the deer mouse population.

Possible Increased Frequency of Viral Disease Emergence

It appears that new virus diseases may be emerging at an increasing frequency, and the subject has captured considerable attention recently (53,54 and 55,59). A number of secular trends have enhanced the probability of emergence of new virus diseases (Fig. 10). First, the population of the world has inexorably continued to increase, and

urbanization has concentrated a higher proportion of people in densely populated areas, facilitating the transmission of any new infection. Second, modern transportation permits the carriage of infections around the globe within a single incubation period. Third, manmade perturbations of the environment are occurring at an increasing pace, which increases the likelihood of transmission of zoonotic and arboviral infections to humans. Finally, advances in fundamental virology have made available a new armamentarium of methods for the detection of previously unknown pathogenic viruses.

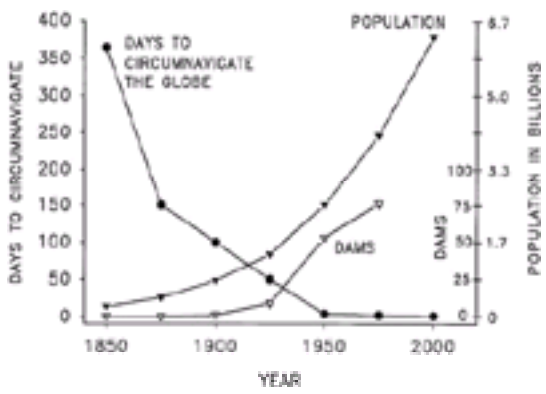

FIG. 10. Since the middle of the 19th century, many global changes have enhanced the probability of the emergence of new virus diseases of humans and animals. This chart depicts three examples of such trends. The rise in the human population has been accompanied by a massive increase in large urban populations, markedly increasing the risk for epidemic spread of infection. Dramatic reductions in the time to travel over long distances have increased the possibility of global transport of infectious agents over short periods of time. The number of large dams (dams more than 75 meters high built in the United States, 1890 to 1975) exemplifies increases in manmade manipulation of the environment. (Data from refs. 4,5, and 49, with permission.)

EPIDEMICS AND THEIR CAUSES

No medical phenomenon is more dramatic than the occurrence of an epidemic. The immediate cause of any viral epidemic is heightened transmission of the causal agent, and epidemics can be classified according to their principal mode of transmission as either common source or propagated.

Common Source Epidemics

Common source outbreaks are, as the term implies, due to exposure to the virus, usually either in food, water, aerosol, or injected product. Common source outbreaks have the potential to be explosive because of the simultaneous exposure of many individuals; because the exposure is frequently limited in time, such outbreaks may be of relatively short duration. Finally, common source outbreaks challenge the epidemiologist because unraveling the source may lead to termination of an ongoing outbreak or prevention of future recurrences.

Two examples illustrate common source epidemics. At the beginning of World War II, the military decided to immunize a large number of troops against yellow fever because it was clear that there would be action in several tropical theaters where jungle yellow fever might be encountered. The attenuated 17D strain of yellow fever virus was a newly developed vaccine considered to be a safe and effective immunogen. Because the vaccine efficacy depended on the infectivity of 17D virus, it was decided to enhance stability by including serum in the final formulation. Human serum was used to avoid serum sickness, and almost 1,000 donors were used, most of whom were medical students at Johns Hopkins University. Unfortunately, at least one individual was a carrier of hepatitis B virus. As a result, more than 400,000 troops received contaminated vaccine, causing a massive epidemic of hepatitis B (about 20,000 cases) in the spring of 1942 (71). Although the onsets of cases were spread over time, when the common source was recognized and cases were plotted from the time of initial immunization with 17D vaccine, they formed a classic log-normal distribution (Fig. 11) that provided a definitive estimate of the distribution of incubation periods for hepatitis B. Although this epidemic was originally considered to be one of infective hepatitis (hepatitis A), the great length of the incubation period (14-week mean) and the absence of secondary spread to contacts provided strong epidemiologic evidence that this disease was a distinct entity.

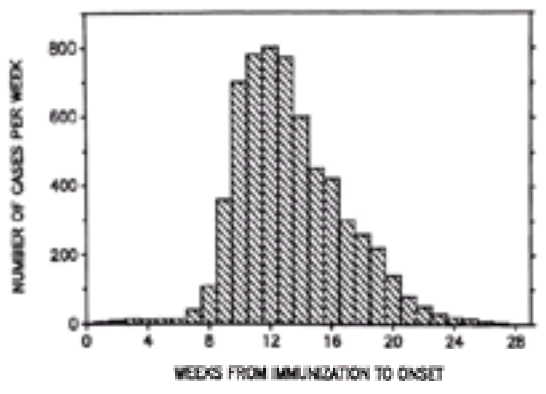

FIG. 11. Distribution of incubation periods for a common source epidemic of vaccine-associated hepatitis B. The graph shows cases of jaundice tabulated by weeks from immunization with 17D yellow fever vaccine to onset, for military units in California. (Data from ref. 71, with permission.)

An outbreak of hepatitis A occurred in 1961 in Pascagoula, Mississippi (51). In some cases, detailed food histories were taken that showed, by comparison with controls, that there was an association with raw oysters. Subsequent investigation disclosed that certain oyster beds in Pascagoula Bay were located near the discharge point for inadequately treated sewage from the city, and cases were associated with oysters supplied from these beds. This was one of the first instances of shellfish-associated hepatitis A, and it was later demonstrated that oysters, through their siphoning system, are capable of concentrating virus up to 1000-fold.

Propagated Epidemics

Propagated epidemics, as the term implies, involve host–host spread of virus. The occurrence of an epidemic is therefore due to the action of the three parameters that determine disease incidence, namely, the proportion of the population susceptible, the proportion of the susceptible population infected, and the case/infection ratio. To produce the unusually high incidence that defines an epidemic, at least one of these parameters must be operating above its usual level. In some outbreaks, it is possible to implicate a specific parameter.

Measles incidence in Iceland during a 50-year period is shown in Figure 12. There are intermittent epidemics alternating with periods of complete absence of measles. In this extreme situation, the virus invades the population, spreads widely and quickly, and fades out when the concentration of susceptibles drops below the level needed to perpetuate the virus. The virus disappears entirely, allowing susceptibles to accumulate as cohorts of infants are born. After enough susceptibles have accumulated, a single importation of measles can initiate a new epidemic. In this situation, the parameter required for epidemic initiation is the proportion of susceptibles in the population.

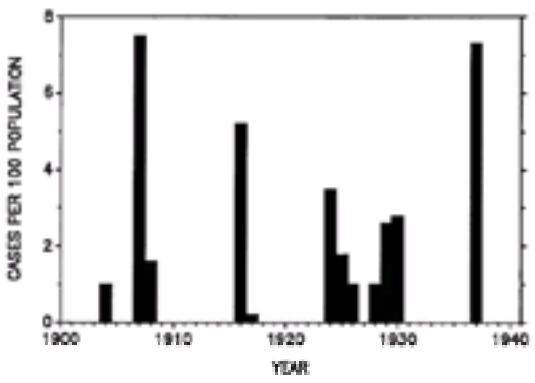

FIG. 12. Measles incidence in Iceland, 1900 to 1940. (Data from ref. 79, with permission.)

By contrast, outbreaks of arbovirus disease are unrelated to the proportion of susceptibles because infection is infrequent and a high proportion of the population is always susceptible. (Areas with hyperendemic yellow fever or dengue may represent a rare exception.) Epidemics are a reflection of the proportion of susceptibles infected; because humans are deadend hosts who do not act as links in the infection chain, the key determinants are ecologic factors, which result in a high concentration of vectors and a high infection rate in the vector population. Epidemics are most commonly preceded by those climatic conditions (rainfall, temperature, and the like) that maximize the vector population.

In some cases, an outbreak is clearly associated with an increase in the ratio of cases to infections, often associated with a virus strain of high virulence. Such occurrences are probably frequent but are rarely well documented. One well-studied example is the pandemic of avian influenza that overwhelmed the poultry industry of Pennsylvania in 1983. In this instance, there was a sudden increase in the virulence of an influenza virus, which was already enzootic in the poultry population, resulting in a pandemic that led to major commercial losses and that was controlled only by a widespread slaughter program. Unusually, isolates of the virus from before and after the epidemic were available for analysis, and a classic series of studies (82) showed that a point mutation in the viral hemagglutinin enhanced the replication ability and virulence of the virus.

Molecular Epidemiology

Expanding knowledge of basic virology has begun to provide molecular insights into the determinants of viral epidemics. In certain instances, it is possible to identify some of the molecular determinants of each of the three parameters (proportion immune, proportion infected, and case/infection ratio) that are responsible for propagated epidemics.

The proportion of the population immune is a relatively inflexible parameter for most viruses; however, influenza is a special instance. This virus is transmitted so effectively that it exhausts the supply of susceptible hosts. The major antigenic protein of influenza virus is the viral hemagglutinin, which is the viral attachment protein and the target for neutralizing antibodies. Influenza virus has a genome composed of eight segments, and these segments may reassort when two type A influenza viruses co-infect the same cells, with the creation of viruses containing a mixture of segments from the two parents. It has been proposed that, on rare occasions, a human strain of virus reassorts with an animal strain and acquires the hemagglutinin of the animal virus (42). This leads to major shifts in the antigenicity of the virus, such as occurred in 1957 with the appearance of the Asian strain, which had an H2 hemagglutinin, whereas previous influenza viruses had an H1 hemagglutinin. Such a radical shift in antigenicity provides the newly created virus with a global population that is relatively susceptible; as a result, a worldwide influenza pandemic may occur.

The proportion of susceptibles infected during a set time period will be determined by two characteristics of a given virus, namely, the generation time and the transmissibility. A virus with a relatively short generation time and high transmissibility, such as influenza will spread much more rapidly than a virus such as poliovirus. Generation time and transmissibility reflect the kinetics of the infection and the titers of virus excreted, and these parameters may be influenced by specific viral genes. Analysis of the genetic determinants of reovirus transmission have demonstrated that the l2 spike protein encoded by the L2 gene segment is a major determinant of transmissibility (41). Likewise, the transmission of California serogroup bunyaviruses, by their mosquito vector, *Aedes triseriatus,* is determined by the middle RNA segment that encodes the viral glycoproteins (31,36).

A major determinant of the case/infection ratio is viral virulence. Most viruses exhibit great natural variation in virulence. One classic example is variola virus (26), which caused virulent forms of smallpox (variola major) in India and Africa and much milder disease (alastrim or variola minor) in South America (Table 13). It has been known for many years that natural isolates of poliovirus varied greatly in their neurovirulence. Attenuated isolates were the starting point for the development of avirulent vaccine strains. Recent molecular analysis has begun to delineate the genomic determinants of virulence, which are located both in the structural genes and in the noncoding 5' region of the genome (1). This natural variation might have been a factor in the severity of poliomyelitis epidemics of the past. Another example is the 1983 pandemic of avian influenza cited previously (82), which was traced to a point mutation that increased the cleavability of the HA molecule, thereby enhancing viral maturation and infectiousness, with dramatic epidemiologic consequences.

Disease	Place and years	Cases	Deaths	Case fatality ratio (per 100)
Variola major	Minneapolis, 1924–1925	1,410	355	25.5
Variola minor	London, 1928–1934	13,395	34	0.2

Source: Data from refs. 35 and 77, with permission.

TABLE 13. *Comparison of case fatality rates for severe (variola major) and mild (variola minor) forms of smallpox*

PERPETUATION AND ERADICATION OF VIRUSES

Persistence of a virus in a population is a distinct phenomenon compared with persistence in a cell culture or individual host, and the term *perpetuation* may be used to emphasize that distinction. All viruses, whether they cause short-duration acute or long-term persistent infections, are capable of perpetuating in populations because perpetuation is a requirement for survival. Eradication is the converse of perpetuation and represents the ultimate method for control of an infectious disease. To determine the potential for eradication, it is necessary first to understand the requirements for perpetuation.

The principal ecologic patterns of viruses in host populations were discussed previously (Table 3 and Table 4), with emphasis on human viruses. In most cases, a virus is perpetuated in a single species, and discussion is limited to this dominant ecologic pattern.

Parameters that determine perpetuation include population variables (2,52) and viral variables. The following are population determinants: (a) the size of the population; (b) the turnover rate (rate at which susceptibles are introduced); (c) the density of the population; and (d) the proportion of the population susceptible. Viral determinants include the following: (a) transmissibility; (b) generation time; and (c) duration of infectiousness (acute or persistent). These three viral variables determine the rate of spread of a virus through a population (for a given set of population parameters). Paradoxically, an agent that spreads rapidly may exhaust susceptibles and disappear more rapidly from a small population than a virus that moves indolently through the same population. Perpetuation is discussed separately for large and small populations because population size is such an important determinant.

Small Populations

For small populations, a crude prediction of perpetuation may be made by determining the number of susceptibles entering the population per generation period. If this number is less than one, persistence will almost certainly not occur (87).

Isolated Human Populations

Small primitive human populations that have minimal contact with the outside world are occasionally available for serologic study. These populations provide a unique opportunity to determine the ability of viruses to perpetuate in their natural hosts. There are two principal patterns (10). Viruses that cause acute short-term infections produce abrupt outbreaks after introduction into human populations and then die out until reintroduced. Viruses that are capable of persisting in individual hosts are able to perpetuate in small, isolated groups.

Figure 13 presents the age distribution of antibodies to poliovirus types 1, 2, and 3 in an isolated Eskimo village. The profiles show that type 1 virus had infected essentially the entire population 25 years before the study, type 2 virus had

caused a similar wave of infections 15 years before the study, and type 3 virus had been present shortly before the collection of sera. Also, it is clear that after its intrusion, each virus had disappeared from the population, because there were few people younger than 25 years of age with type 1 antibody (the few individuals probably represent cross-reacting antibody after type 2 infection) and few younger than 15 years of age with type 2 antibody.

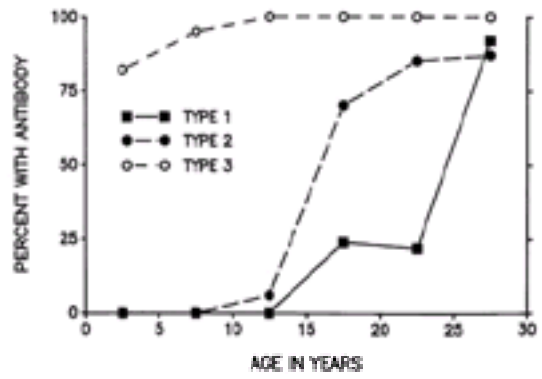

FIG. 13. Age-specific prevalence of antibodies to poliovirus types 1, 2, and 3 in an isolated Eskimo village in Greenland. (Data from ref. 62, with permission.)

In contrast, hepatitis B produces a considerable proportion of persistent infections, particularly in primitive populations, where infection occurs early in life. Figure 14, from another study of Eskimos, shows that infections occurred at all ages, with a gradual rise in cumulative incidence to 60% by 60 years of age; there was no evidence of disappearance of virus from the population or of exhaustion of susceptibles. The curve for hepatitis B surface antigen indicates that there were many persistently infected carriers in the population accounting for perpetuation in this small community.

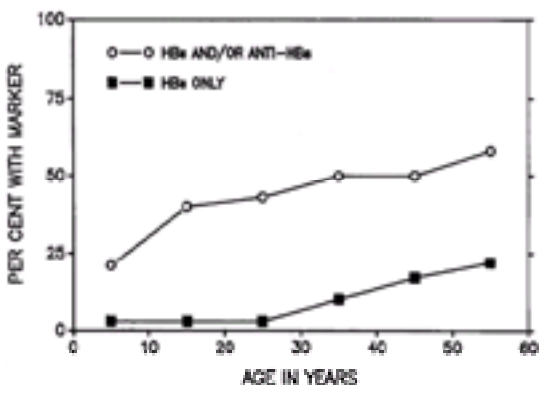

FIG. 14. Age-specific prevalence of hepatitis B antibodies (anti-HBs) and antigen (HBs) in Eskimos in southwest Greenland. (Data from ref. 76, with permission.)

Small Animal Populations

Animal populations differ radically from human populations in their relatively rapid rate of turnover, and this is a critical variable in virus perpetuation. Mean life expectancy in many animal populations ranges from 6 to 12 months, in contrast to a range of 30 to 70 years for humans. However, the kinetics of infection are similar in an individual human or animal host. For instance, the median incubation period (to rash) is about 14 days in smallpox and 10 days in mousepox. The consequence is that perpetuation may occur in animal populations (with rapid turnover) that are much smaller than the minimal human population required to sustain a virus that causes acute infections.

Laboratory animal colonies have provided an opportunity to document the quantitative impact of population turnover. Perpetuation in such colonies is of more than academic interest because enzootic infections present an important practical problem that frequently confounds biomedical research. One documented example is ectromelia (mousepox), which closely resembles variola in humans (25). Infection is initiated either by entry through the skin or by inhalation, and there is a generalized poxlike rash of the skin from which aerosolized virus is transmitted. As in smallpox, infections can be fatal, but recovered mice are solidly immune. Ectromelia is a relatively infectious agent and a notorious cause of acute and devastating epizootic results. When a few infected mice are placed in a larger group of susceptible animals, the virus spreads rapidly, all mice are infected within two incubation periods, and many mice die.

In these circumstances, ectromelia would not be expected to perpetuate in a small mouse population. A series of classic studies (33), however, demonstrated that this virus was readily perpetuated in a population of 100 to 200 animals. In one representative experiment, a mouse colony was established with 25 normal and 20 infected mice, and 3 uninfected mice were added daily; animals were removed only if dead. During a 30-month observation period, the total colony size gradually rose to a constant level of about 230 animals. The infection was maintained throughout the observation period; at equilibrium, about one fifth of the mice were uninfected, one fifth were actively infected (mortality rate, 50% to 60%), and three fifths were recovered immune subjects. It was possible to maintain this acute highly lethal virus over long periods of time in mouse populations with as few as 70 animals, demonstrating that high turnover markedly reduces the population size required for perpetuation.

Large Populations

To follow the pattern of virus infection in large populations, it is necessary to rely on reportable diseases that can be identified from clinical observations alone and that exhibit relatively high case/infection ratios. Measles is one of the few virus diseases that meets these criteria; however, even measles has its limitations because only a fraction (usually, 10% to 50%) of cases are reported. Although measles is a ubiquitous and highly infectious virus, its ability to perpetuate in human populations is surprisingly fragile (9). Table 14 shows that in cities of 200,000 to 300,000, measles faded out for at least 1 month in 56 of the 100 years recorded. Because a population of 300,000 might be expected to experience about 5,000 cases of measles in an average year, it is initially surprising that this would be insufficient to maintain the infection. The explanation becomes more apparent when the seasonal cycle of measles is examined (46,86,87). Data for the city of Baltimore (population 900,000) for 32 years of observation showed that annual reports averaged about 5,000 cases representing about 35% of cases; incidence rates ranged more than 100-fold from the peak in March to the seasonal low in August, when about 10 cases were reported. Because there were about three generation periods per month, this represents no more than 3 cases per generation period, barely sufficient to perpetuate the virus. A similar city of 300,000 would experience only 1 case per generation period; in such an instance, it is plausible that periodic fadeouts would occur.

Size of city (millions)	Number of cities	Average annual measles reports (thousands)	Years of study	Years with fadeout
1.0–7.5	6	3.5–91	120	0
0.5–0.9	9	2.9–4.0	180	1
0.2–0.3	5	0.9–2.1	100	56

[a]Estimate of reporting ranged from 14%–85% of measles incidence. Cities were as follows: large—New York, Chicago, Philadelphia, Detroit, Los Angeles, Montreal; medium—Cleveland, Baltimore, Boston, Toronto, Washington, Pittsburgh, Milwaukee, Buffalo, Minneapolis; small—Vancouver, Rochester, Dallas, Akron, Winnipeg.

Source: Data from refs. 9 and 86, with permission.

TABLE 14. *Fade-outs of measles in North American cities, 1921–1940*[a]

This analysis suggests that persistence of a short-cycle infection in a large population is dependent on the relationship between incidence during the seasonal trough and the generation time. Influenza, with a generation time of 1 to 3 days, has difficulty persisting in human populations through the seasonal trough. It probably circumvents the problem by shuttling between northern and southern hemispheres each year.

Requirements for Eradication

The recent history of smallpox (26) has demonstrated that eradication is an attainable objective for selected human viral infections. The salient epidemiologic features that made smallpox eradication possible were its relatively long incubation period (about 14 days) and low infectiousness; its marked seasonality; a case/infection ratio approaching 1; and the lack of an extrahuman reservoir. These features made it possible to abandon mass vaccination in favor of a search-and-containment strategy in which local outbreaks were identified and aborted by intensive immunization around each focus. The small number of local outbreaks during the seasonal trough was essential to the success of this strategy.

Several other human viral infections are now candidates for eradication, particularly poliomyelitis and measles (58). There are copious data available from the United States that provide an instructive comparison of these two viruses. Poliomyelitis in the United States exhibited marked seasonality, as shown in Figure 7. This means that it required a considerable population to maintain the virus through the seasonal trough. Computation suggests that in the northern United States, such as New England, before the introduction of vaccine, a population of 100,000 was just sufficient to perpetuate poliovirus. At the seasonal trough, it is estimated that there were about two infections per generation. In the United States in the prevaccine era, the poliovirus-susceptible pool was slightly more than 20% of the population. After the regular use of oral poliovirus vaccine, the susceptible pool of children was markedly reduced, perhaps to one fourth of its previous level (about 5% of the population). In the hypothetical city of 100,000 population, there would have been less than one infection per generation period at the seasonal trough, below the level required for perpetuation (58). In the United States, the stepwise drop in natural poliomyelitis was well documented (Fig. 15) and indicated that disease due to wild poliovirus totally disappeared after the summer of 1972 and has not reappeared since, with the exception of a localized introduction in 1979. During the period of fadeout, 1967 to 1972, poliomyelitis maintained its seasonality; furthermore, there was an annual stepwise reduction in the number of states reporting any natural poliomyelitis (58). These observations suggest that each year, after the seasonal disappearance of infection, fewer and fewer areas experienced reintroduction of virus during the summer upswing.

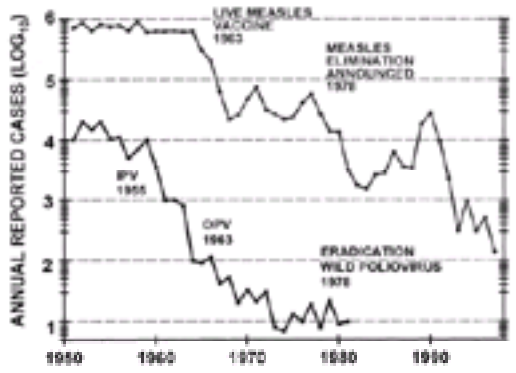

FIG. 15. Comparison of the decline in poliomyelitis and in measles in response to immunization programs, United States, 1951 to 1986. **Lower panel:** annual reported cases of paralytic poliomyelitis in the United States, 1951 to 1981. For the years 1973 to 1982, residual cases are either vaccine associated or imported, with the exception of an outbreak in 1979 in the unvaccinated Amish population. (Data from refs. 14 and 58, with permission.) **Upper panel:** reported cases of measles in the United States, 1960 to 1993. (Data from refs. 14,15,17,18 and 19, and 58, with permission.)

Measles has also been the target of elimination efforts in the United States, but with less success than for poliomyelitis, as shown in Figure 15. Surveillance data indicate that mass immunization has gradually reduced the number of states with endemic measles. Nevertheless, measles has stubbornly resisted eradication. Although measles fadeouts have periodically occurred in almost all states, during the period 1972 to 1987, no more than half (26 of 52), the states were measles free in any 1 year (58). This suggests that a critical epidemiologic difference between measles and poliomyelitis is the frequency of reintroduction of virus after a fadeout. In turn, this may be due to the greater transmissibility of measles, which is reflected in the fact that the mean age of infection with measles was 5 years of age, whereas that for poliomyelitis was 11 years of age, before the introduction of vaccines.

Eradication will continue to be a major public health goal in the future. Experience with smallpox, poliomyelitis, and measles indicates that a thorough understanding of the dynamics of perpetuation is essential to selecting virus diseases that are realistic targets for eradication.

APPLICATIONS OF EPIDEMIOLOGY

Identification of Etiologic Agents

Acute infections, which are readily transmitted and cause intense outbreaks, are often first identified as distinct clinical entities during epidemiologic studies. Furthermore, their epidemiologic behavior offers strong circumstantial evidence that they are caused by transmission of an infectious agent. Recent examples include rotavirus disease, fifth disease, AIDS, and hantavirus pulmonary syndrome. Epidemiologically, it is usually impossible to infer whether the cause is a virus or other infectious agent. Epidemiologic observations, however, may suggest the portal of virus shedding and the period of infectiousness, which is important for the collection of specimens for isolation.

Epidemiologic observations may also contribute insights into more obscure persistent infections associated with atypical chronic diseases. The spongiform encephalopathies or prion diseases are an example. This group of agents includes the causes of scrapie of sheep, kuru and Creutzfeldt-Jakob disease of humans, and BSE. The initial definitive evidence that scrapie, a chronic degenerative neurologic disease, could be transmitted as an infectious agent, occurred as an unwanted complication of the use of louping ill vaccine in Scotland in 1935 (32,34). Table 15 shows that three lots of vaccine against louping ill, a flavivirus, were injected into sheep in Scotland; unexpectedly, there was a very high incidence of scrapie in recipients of one of the three lots. Subsequent epidemiologic investigation showed that louping ill vaccine was prepared by formalin treatment of virus grown in sheep brain and that several of the sheep used were probably incubating scrapie because their parents subsequently came down with the disease. Because the scrapie agent is extremely resistant to inactivation by formalin, contamination of the vaccine was readily explained. Before this episode, it was not generally accepted that scrapie was due to a transmissible agent with an incubation period of several years.

Vaccine batch	Number of sheep vaccinated	Percentage with scrapie
1	>22,000	<0.1
2	18,000	5
3	>4,000	<0.1

[a]Batch 2 was prepared from 122 sheep infected with louping ill; of these 8 were offspring of scrapie-affected ewes. Interval between subcutaneous injection of vaccine and onset was 20 months or longer.

Source: Data from ref. 34, with permission.

TABLE 15. *Occurrence of scrapie in sheep immunized with louping ill vaccine, Scotland, 1935*[a]

A more recent episode in the United States has documented the potential transmissibility from human to human of the agent that causes Creutzfeldt-Jakob disease. Between 1964 and 1985, about 7,000 pituitary dwarfs were treated with

injections of growth hormone prepared from autopsy-collected human pituitary glands. From 1970 to 2000, more than 30 cases of Creutzfeldt-Jakob disease have occurred in these growth hormone recipients, and epidemiologic investigation (11,12) has provided strong evidence of the association with pituitary injections based on the fact that this disease has been virtually unknown in young adults and that the cases were associated with a few lots of hormone.

Evaluation of Vaccines

The ultimate test of viral vaccines is their efficacy and safety in humans. Epidemiology provides well-established methods for the design and analysis of prospective studies of vaccine efficacy (61). These are illustrated by the 1954 field trial of poliomyelitis vaccine (28). In this trial, a large number of children in the first three grades of elementary school were enrolled. As shown in Table 2, the trial comprised two different study designs: placebo and observed. In the placebo design, children fell into three categories: vaccinated, placebo inoculated, and nonvolunteers (uninoculated). In the observed design, the groups were vaccinated second graders, nonvolunteer second graders (uninoculated), and controls (all children in the first and third grades). The most valid comparison was vaccinated and placebo inoculated children in the placebo areas; this group yielded an estimated efficacy of 72% for paralytic poliomyelitis, whereas the observed control area yielded an efficacy of 63%. The discrepancy was explained by the fact that the nonvolunteer children in both areas experienced an attack rate that was considerably lower than that of the placebo controls or the observed controls. Apparently, volunteer children were at higher risk (presumably because of a higher percentage of susceptibles) than were nonvolunteer children. Thus, the strict placebo control was the best comparison group. When the placebo study was further refined by limiting analysis to laboratory-confirmed cases, efficacy was estimated even higher (85%), underlining the importance of etiology-proven cases in vaccine evaluation. Curiously, the 1954 field trial was the only properly designed large-scale trial of any viral vaccine now in regular use in the United States.

Epidemiology has also played a vital role in documenting vaccine complications. The occurrence of Guillain-Barré syndrome associated with the use of swine influenza virus vaccine in the fall of 1976 provides a good illustration (73). About 40 million people received influenza vaccine in a mass campaign. Cases of a relatively rare neurologic disease, Guillain-Barré syndrome, were reported in vaccinees at a frequency considered excessive, and the vaccine was withdrawn. When vaccine-associated cases were compiled by interval after immunization (Fig. 16), they exhibited a log-normal distribution typical of many incubation periods. This pathognomonic epidemiologic feature, along with the excessive rates seen during the early weeks after immunization, provided strong evidence of an association and justified termination of the vaccine program. Epidemiologic evidence was the key in this episode because the association (less than 1 case per 100,000 vaccinees) was only detectable in a massive field study. Because Guillain-Barré syndrome occurs at a baseline rate in the population and because those cases associated with the vaccine were not clinically distinguishable from the baseline cases, this episode presented a special epidemiologic challenge. The occurrence of Guillain-Barré syndrome was not predictable by any small-scale human trial or laboratory test, and the biologic explanation is still unclear. In fact, epidemiologic studies conducted with influenza vaccines used after 1976 have demonstrated that these vaccines (produced from nonswine strains of virus) apparently carry no risk of Guillain-Barré syndrome (74).

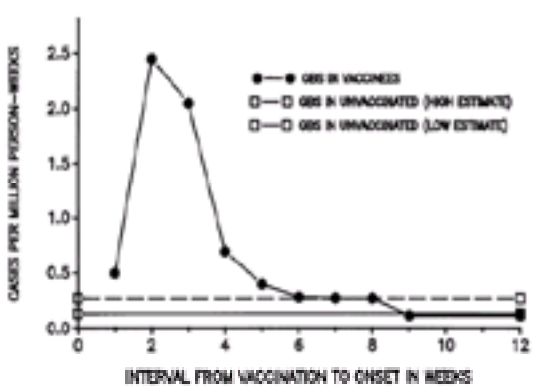

FIG. 16. Guillain Barré syndrome (limited to cases with extensive involvement) associated with swine influenza vaccine, according to interval between immunization and onset, United States, 1976. Two baseline rates are shown (upper and lower limits of *hatched area*) to define the expected rate of spontaneous Guillain Barré syndrome. The period of the epidemic is readily defined (1 to 6 weeks after immunization) and the intervals during this period of excess incidence form a classic log-normal curve. (Data from ref. 43, with permission.)

Development and Assessment of Control Measures

In addition to vaccines, other control measures are sometimes useful against virus infections. Such approaches seek to reduce the risk for exposure and are usually suggested by epidemiologic studies of transmission mechanisms. Examples include reduction of risk associated with blood or blood products for viruses such as hepatitis B and hepatitis C viruses, HIV, and human T-cell leukemia viruses types I and II. Another classic example is the control of urban yellow fever and dengue through reduction of *Aedes aegypti* mosquitoes. The use of epidemiologic control of virus infection is illustrated by a study of Bolivian hemorrhagic fever (40), an arenavirus that was endemic in certain rural villages. Ecologic studies suggested that this virus was enzootic in one species of peridomestic house-dwelling mouse, *Callomys callosus,* and was transmitted by aerosolized fomites. Table 16 shows a study in which rodent control was initiated in one half of a village and then extended to the other half. The dramatic effect of rodenticides confirmed the hypothesis of the virus reservoir and its transmission mechanism and provided a practical approach to control of a serious viral infection. A similar approach is being applied to the recently discovered pulmonary syndrome hantavirus (39,60).

Ten-day period beginning	New cases, East sector	New cases, West sector
April 1	12	9
11	8	17
21	12	8
May 1	7*	18
11	4	14
21	2	9
31	3	12
June 10	0	10*
20	5	7
30	0	0
July 10	0	0

Calomys callosus is the rodent reservoir of Bolivian hemorrhagic fever. Rodents were controlled by trapping and poisoning, beginning in the East sector on May 1(*) and in the West sector on June 15(*).

Source: Data from ref. 40, with permission.

TABLE 16. *Control of Bolivian hemorrhagic fever by reduction of the rodent reservoir, San Joaquin, Bolivia, 1964*[a]

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CHAPTER 15 Antiviral Therapy

Clyde Crumpacker

One of the remarkable scientific achievements of the past quarter century has been the growth of the infant science of antiviral therapy. There has been a steady stream of effective drugs being developed against an increasing number of viral pathogens. As recently as 1975, it appeared that antiviral therapy would not be possible because animal viruses were such complete obligate intracellular pathogens that inhibition of viral functions would necessarily mean cell death as well. The breakthrough occurred because of the identification of virally encoded enzymes that are essential for virus replication and that differ enough from cellular enzymes to permit selective chemical interactions to occur with viral enzymes but not cellular enzymes. The structural differences between viral and cellular enzymes that have similar functions have permitted chemicals to target viral enzymes and to spare cellular processes. The success of this approach is indeed a surprise. Resistance to antiviral therapy has been a most powerful tool for understanding the mechanism of drug action and the reasons for clinical treatment failure.

In this chapter, the virus groups in which antiviral therapy has succeeded will be described, and the mechanisms of drug action will be outlined. Progress in new antiviral therapies is developing rapidly, and there will be new drugs to inhibit new classes of viruses; these will undoubtedly be developed using principles described here.

HERPESVIRUSES

Historical Background

The first demonstration that antiviral therapy would be successful for treating herpesvirus infections was the use of 5'-iodo-2'-deoxyuridine (IDU) to treat herpetic keratitis. Iododeoxyuridine has significant toxicity in systemic use but was a real advance in the treatment of keratitis caused by herpes simplex virus (HSV), a significant cause of blindness (203). The successful use of IDU proved that herpes infections could respond to antiviral therapy. However, IDU was not a successful therapy in the treatment of HSV encephalitis (122), and its systemic use was associated with significant toxicity. Trifluorothymidine (TFT) is a nucleoside analog that is converted by cellular enzymes to the triphosphate, which inhibits herpes DNA polymerase. It is also used as a topical treatment for herpes keratitis and corneal ulcers (67) and for acyclovir-resistant mucocutaneous human immunodeficiency virus (HIV) lesions (284).

The first antiviral therapy that improved survival in a life-threatening viral disease was adenine arabinoside (Ara-A, or vidarabine), a nucleoside analog of adenine activated by cellular enzymes to form the triphosphate, which is the active inhibitor of the HSV DNA polymerase. Ara-A was administered intravenously to treat patients with biopsy-proven HSV encephalitis in 1977 (383). Because many other neurologic diseases might present with symptoms similar to those of herpes encephalitis, it was important to use a rigorous diagnostic test to establish a diagnosis. (The ability to culture HSV from temporal lobe brain biopsy tissue was required for enrollment in this study.)

Ara-A also provided effective therapy for other HSV infections, such as neonatal herpes and varicella zoster virus (VZV) infection in the immunocompromised host (380,382). An ointment containing a related compound, Ara-A monophosphate (Ara-Amp), was the first topical ointment to demonstrate an antiviral effect against recurrent herpes simplex labialis (cold sores) (338).

Another nucleoside analog, which possesses strong *in vitro* activity, is bromovinyl deoxyuridine (BVDU), which requires activation by the viral thymidine kinase (TK) to inhibit viral DNA polymerase (84). BVDU is metabolized in humans to bromovinyluridine (BVU), which possesses significant hepatic toxicity, but this drug was never approved for human use. A close relative, bromovinyluridine arabinoside (BVaraU; sorivudine), is much safer for humans; *in vitro* results showed that this nucleoside analog was the most effective inhibitor of the nucleoside analogs tested against human VZV (243). A multicenter clinical trial comparing sorivudine with acyclovir in patients with HIV and herpes zoster showed that the

former was more effective for cessation of new vesicle formation and total lesion crusting (147). It was not approved for use in the United States, however, because it enhances the severe toxicity of 5-fluorouracil (5-FU), and the U.S. Food and Drug Administration (FDA) was concerned that the drug would be used in patients receiving 5-FU for cancer treatment.

Acyclovir

Acyclovir [9-(2-hydroxyethoxymethyl)guanine; Zovirax] is a guanosine analog with an acyclic side chain at the nine position and a broken sugar ring. The chemical structure of acyclovir and related drugs for herpesviruses are shown in Figure 1. The structure of deoxyguanosine is included for comparison, to show the position of the broken sugar ring in drugs such as acyclovir, ganciclovir, and penciclovir. Acyclovir is the first truly specific antiviral drug, as it requires activation by a viral enzyme (TK) and has remarkably little toxicity for uninfected cells. Acyclovir is a specific inhibitor of HSV-1, HSV-2, and VZV replication (20,76,109). It is transported into cells either by passive diffusion or by a nucleoside transporter, which also transports guanine in erythrocytes (244). When in the cell, it is readily phosphorylated by the viral TK (see Fig. 1). The viral-encoded TK enzymes of HSV-1, HSV-2, VZV, and Epstein-Barr virus (EBV) catalyze the formation of acyclovir monophosphate (ACVMT), whereas acyclovir is a poor substrate for cellular TK enzymes (114,141,271,272). This accounts for the remarkable selectivity of acyclovir and the lack of toxicity in uninfected cells (35,271).

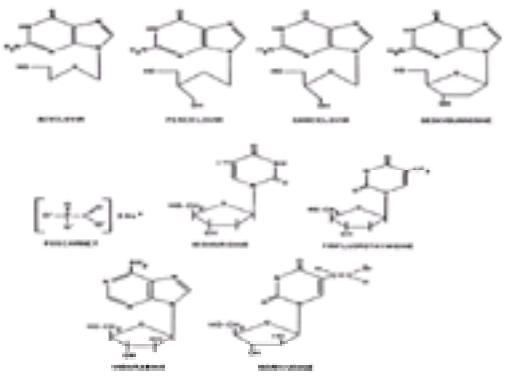

FIG. 1. Chemical structure of antiherpes, antiviral agents, acyclovir (*ACV*), penciclovir (*PCV*), ganciclovir (*GCV*), foscarnet (*PFA*), iodoxuridine (*IDU*), trifluorothymidine (*TFT*), vidarabine (*Ara-A*), and sorivudine (*SVD*). (Reprinted from ref. 172, with permission.)

In uninfected cells, the cytoplasmic 5' nucleotidase catalyzes the formation of very low levels of ACVMP (207), but in HSV-infected cells, the level of acyclovir triphosphate (ACVTP) may be 100-fold higher than in uninfected cells, because acyclovir is a much better substrate for the HSV-specific TK and thymidylate synthetase phosphorylating activities (113,138,141,206). A third important area of specificity derives from the fact that acyclovir triphosphate is a highly polar compound that does not easily pass through biologic membranes to exit cells, whereas acyclovir easily enters infected cells and is trapped there in high concentrations. Following its initial phosphorylation, ACVMP is converted by cellular kinases to the acyclovir di- and triphosphates. ACVTP actively inhibits HSV DNA polymerase (139,271,272) and may bind to it 30 times more effectively than to cellular alpha polymerase (97,143). ACVTP competes effectively with deoxyguanosine triphosphate (dGTP) for the viral DNA polymerase and is preferentially incorporated into elongating DNA chains as ACVMP (114,139). The 3'-terminal ACVMP results in chain termination because ACVMP lacks a 3' hydroxyl group on the 2' deoxyribose sugar, which is required for a 3'-to-5' reaction with the next incoming nucleoside triphosphate. A DNA chain that has been terminated with ACVMP is not a substrate for the 3', 5' exonuclease activity of the herpesvirus DNA polymerase and thus ACVMP is not excised (92). ACVTP has been described as an *obligate* chain terminator because viral DNA elongation stops completely when ACVMP is incorporated into the elongating viral DNA chain (305). In addition to inhibiting viral chain elongation, ACVTP has also been called a suicide inhibitor, because it is an example of an induced substrate inhibitor forming an inactive “dead-end” complex involving viral polymerase, ACVMP-terminated primer template, and the next specific deoxynucleoside triphosphate (dNTP) encoded by the template (305). Cellular DNA polymerases have poor affinity for ACVTP, and very little ACVMP is incorporated into the host cell DNA.

All this accounts for the very low genetic toxicity for host cells associated with acyclovir use (113). The role of ACVMP in chain termination of elongating viral DNA is illustrated in Figure 2. The binding of the next dNTP, which is incorporated immediately following ACVMP, prevents the viral polymerase from disassociating and moving to an alternative extendible primer template. This results in a time-dependent inactivation of HSV-1 DNA polymerase activity (305,306). This mechanism may be selective for the HSV-1 polymerase, as ACTVP does not inhibit the HeLa cell a polymerase (139,304,305). The HSV-1 polymerase is a single polypeptide of 1,235 amino acids. It has been expressed *in vitro*, in yeast, and in insect cells (101,159,249). The cloned protein possesses polymerase activity and can be inhibited by ACVTP in a manner very similar to purified HSV-1 polymerase (101). The functional enzyme consists of a heterodimer involving the HSV-1 DNA polymerase peptide and a smaller polymerase accessory viral protein designated UL42, which enhances polymerase activity and increases processivity of the HSV-1 polymerase (142,152). All herpesviruses probably utilize a similar mechanism—a functionally active polymerase peptide that exhibits greatly enhanced DNA polymerase activity in the presence of a virally encoded polymerase accessory protein. The inhibition of HSV-1 DNA polymerase activity by ACVTP occurs in the absence of the UL42 protein, indicating that ACVTP is able to bind to the polymerase peptide by itself (101).

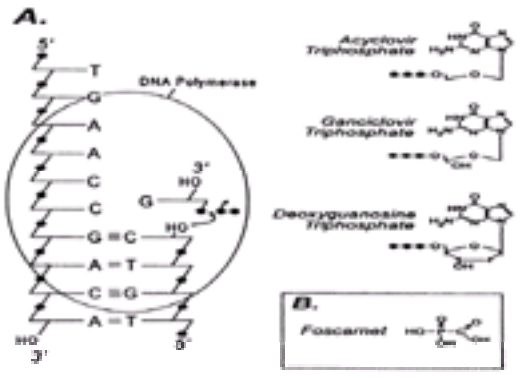

FIG. 2. Inhibitors of the polymerization reaction of herpesvirus DNA polymerase. (Reprinted from Fields BN, Knipe DM, Howley PM, et al., eds. *Fields Virology*, 3rd ed. Philadelphia: Lippincott-Raven, 1996, with permission.)

In summary, the selective action of acyclovir against HSV-1, HSV-2, and VZV is the result of several virus-specific factors: (a) specific phosphorylation by a virus-encoded TK enzyme; (b) specific inhibition of viral DNA polymerase polypeptide by ACVTP acting as a competitive inhibitor with the natural substrate dGTP; (c) chain termination of viral DNA by incorporation of ACVMP, and inactivation of viral DNA polymerase after the incorporation of ACVMP in the presence of dNTPS; and (d) failure of ACVMP and ACVTP to be exported out of cells, with build-up of high levels of intracellular ACVTP. In isolates of HSV-1, HSV-2, VZV, and cytomegalovirus (CMV) from human infections, the relative potencies of inhibition of virus replication in tissue culture are HSV-1 > HSV-2 > VZV, and there is a relative lack of inhibition of CMV replication (Fig. 3) (76). The reason that acyclovir is less active than HSV against VZV is probably related to the short half-life (t½) of ACVTP in VZV-infected cells, and to VZV's slower replication cycle. The slow replication cycle of VZV would require that a high level of ACVTP be present for a prolonged period, and this is not achieved with the short half-life (2.5 hours) of ACVTP in VZV-infected cell cultures. For comparison, penciclovir triphosphate has a half-life in VZV-infected cells of close to 12 hours. The main reason acyclovir is relatively inactive against CMV is that, because CMV does not possess a virus-encoded TK enzyme analogous to that of HSV, the intracellular concentration of ACVTP achieved in CMV-infected cells is very low. In contrast, ganciclovir triphosphate is specifically phosphorylated through the action of the viral phosphokinase UL97 gene product, and its levels have a prolonged half-life of 16.5 hours in CMV-infected cells (29).

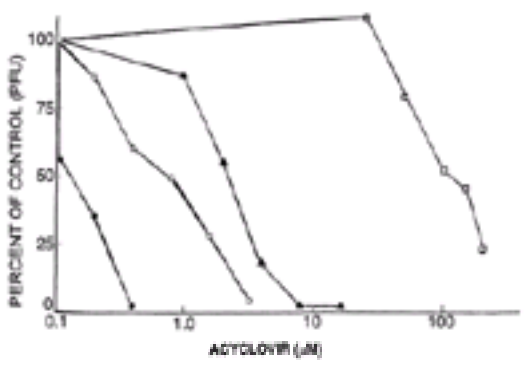

FIG. 3. Plaque reduction assay of herpesvirus clinical isolates from patients by acyclovir (acycloguanosine in Vero cells). HSV-1 (·); HSV-2 (○); VZV (▲); CMV (□). (Reprinted from ref. 76, with permission.)

Resistance

Resistance of HSV to acyclovir develops rapidly *in vitro* when HSV is grown in the presence of the drug, and this occurs also in animals and patients receiving acyclovir (75,128,329). The detection of drug-resistance mutations in a specific viral gene has been important to understanding the functions of these genes. Resistance to acyclovir is conferred by mutations in two viral genes, the viral TK and the DNA polymerase genes (55,129,329). The viral TK gene is important for HSV replication in resting or neuronal cells, and TK-deficient mutants of HSV reactivate inefficiently from latency (128,355,356). In many tissues, however, cellular TK enzymes appear to be able to substitute for the viral TK and to support replication of HSV TK mutants.

The most common form of acyclovir-resistant HSV isolate is the TK mutant (35). This may be the result of a single point mutation or a deletion mutant (35,72,212). It has been estimated that about 50% of mutations associated with acyclovir resistance in clinical isolates occur by deletion or addition of bases in strings of guanosine residues (G-strings) or cords of cytosine residues (C-cords) within the TK gene (144,324). These mutations usually result in stop codons producing premature shortening or truncation of the viral TK protein (34). The selection of mutant viruses with truncated TK enzymes also occurs in patients treated with acyclovir (34). The other 50% of point mutations can occur anywhere in the TK gene (144). A loss of almost all TK activity has also been associated with a single point mutation, producing an alanine-to-proline change in a region of the TK protein forming an alpha helix and still forming a full-length peptide (34). A single nucleotide deletion in a region with no G-string or C-cord residues, resulting in a truncated TK peptide, has also been associated with acyclovir resistance in a newborn infant (291). The three discrete classes of TK mutations that confer the acyclovir-resistant phenotype are as follows:

1. TK-negative mutants: The enzyme activity has been abolished, usually by truncation of the peptide and

phosphorylation of acyclovir does not occur.
2. TK-low producers: Partial TK activity is present but TK is not abolished. These are still able to produce disease in animals and reactivate from latency (80,121,354,355).
3. TK altered mutants: These can still phosphorylate thymidine, but they fail to phosphorylate acyclovir (80).

An early report that a TK virus could cause latency but not reactivate from ganglia referred to only a single mutant of HSV and should not be generalized to many TK-deficient viruses (54). Furthermore, in a neuronal culture with an HSV TK-negative mutant, the replication, latent infection, and successful reactivation of this mutant has been described (384). A study of 13 clinical acyclovir-resistant HSV-2 isolates from patients with acquired immunodeficiency syndrome (AIDS) revealed that the majority of these isolates establish ganglionic infection and reactivate in tissue culture, even though most of the strains contained truncated TK proteins (34,121). Another study showed that the human TK can effectively substitute for the viral TK, allowing the reactivation of a TK-mutant HSV-1 from murine ganglion (38). The HSV TK-low-producer and TK-negative mutants still retain an ability to reactivate, and they possess pathogenic potential in patients, particularly those who are immunocompromised (121,291).

Resistance to acyclovir is also mediated by mutations in the viral DNA polymerase gene (55,329). The acyclovir-resistant mutations result in an altered viral DNA polymerase, which functions in the synthesis of viral DNA in the presence of a high concentration of ACVTP (214). These mutations may also confer cross-resistance to other drugs that act directly on the viral DNA polymerases, such as phosphonoacetic acid (33,55,72). The herpesvirus DNA polymerases all contain regions of highly conserved amino acid sequences, which have strong homology to each other and to the human alpha polymerase (146,213,392). These form the family of alpha DNA polymerases. All the acyclovir-resistant mutations are found in conserved regions of the enzymes, designated I, II, III, V, VII, and A (230,392).

Drug-resistant polymerase mutations that are found to lie outside these regions may represent natural polymorphisms in the DNA polymerase and not actual resistant mutations (44,50). The conserved regions are probably involved in important enzymatic functions such as dNTP substrate recognition and binding, and pyrophosphate binding (145,146,214,230). In the absence of a crystal structure for the alpha DNA polymerase, the exact relationship among the most highly conserved regions of the herpesvirus polymerases is not clear. The HSV polymerase contains a consensus sequence, YMDTDS, which is remarkably similar to the sequence YMDD found in the active center of HIV reverse transcriptase (RT), hepatitis B virus polymerase, and many RNA polymerases (1). Site-specific mutagenesis of this highly conserved region of HSV-1 DNA polymerase revealed that a single amino acid change from serine (S) to alanzine (A) results in a highly acyclovir-resistant DNA polymerase that still maintains active catalytic functions (102,103). The acyclovir-resistant mutants in the DNA polymerase are relatively infrequent and have been identified only in immunocompromised patients (59,319).

Patterns of cross-resistance to other antiviral drugs occur with both TK mutants and DNA polymerase mutants. Acyclovir-resistant mutants caused by TK deficiency usually exhibit cross-resistance to all drugs that require the viral TK for activation, such as ganciclovir and penciclovir, which are phosphorylated by the viral TK (131,363). TK mutants retain susceptibility to drugs that do not require phosphorylation and that act directly on the viral DNA polymerase, such as phosphonoacetic acid or phosphonoformic acid (foscarnet, or PFA) (33,55,70).

Acyclovir resistance in humans was first documented in 1982 in a child with a severe combined immunodeficiency syndrome (75); in 1991, it was shown to be clinically significant in a study of patients who had TK-deficient mutants of HSV, all of whom responded to foscarnet therapy (320). The polymerase mutants selected with acyclovir therapy may also cause cross-resistance to other drugs that act on viral DNA polymerase, such as ganciclovir, vidarabine (52,53,59,73,74,319), and foscarnet. Double mutants in both the TK and polymerase genes exhibit a broad degree of cross-resistance to most antiviral nucleoside drugs with activity for HSV. The replication of both wild-type and acyclovir-resistant viruses can be inhibited in cells co-infected with a mixture of TK-deficient and wild-type HSV, because the wild-type TK is able to produce sufficient ACVTP to inhibit both viruses, but polymerase mutants are not inhibited in cells co-infected with wild-type and pol mutant HSV (55).

Clinical Trials

The clinical usefulness of therapy for HSV infection was established through a series of randomized placebo-controlled trials, which are models for clinical investigation. As mentioned, the first successful antiviral therapy to improve survival in a life-threatening viral infection was intravenous Ara-A (vidarabine) in biopsy-proven herpes simplex encephalitis (383). In a controlled trial, acyclovir was found superior to vidarabine for improving survival and reducing morbidity in herpes encephalitis (378).

The efficacy and safety of acyclovir for the prevention and treatment of infection with HSV-1, HSV-2, and VZV has been established through numerous multicenter, placebo-controlled, randomized clinical trials. An important result of these studies is that acyclovir is more effective in preventing infection when used early in the disease than when used for fully established viral disease, a finding also demonstrated with other antiviral drugs. Once a viral disease has become clinically evident, the effectiveness of acyclovir is less pronounced. Also, acyclovir is most beneficial when used to treat infections in the immunocompromised patient. This is because the replication of viruses is greater in immunocompromised patients, and the infections are more severe and cover a larger surface area, providing a greater opportunity to observe the effectiveness of the treatment.

The first study documenting the efficacy of acyclovir in prophylaxis involved patients receiving bone marrow transplantation (323). Bone marrow transplantation is frequently followed by reactivation of HSV. This infection may disseminate, producing systemic disease and compromising the regeneration of the marrow graft. In a study of 10 patients in the acyclovir treatment group and 10 patients in the placebo group, intravenous acyclovir was shown to prevent 90% of mucocutaneous herpes simplex (323). This result was highly statistically significant and established the usefulness of acyclovir therapy after transplantation to prevent herpes simplex recurrences. Other studies involving larger number of immunocompromised transplant recipients, patients receiving cancer chemotherapy, or patients with

HIV infection have confirmed and extended the usefulness of acyclovir as a safe and effective treatment and prophylaxis (105,321,322,368,379,382).

In the immunocompromised patients with large areas of mucocutaneous HSV infection, intravenous acyclovir markedly accelerates healing (75,100,379). Intravenous acyclovir is the drug of choice for treating serious infections of HSV or VZV (10,75,368).

Intravenous and oral acyclovir are effective for treating primary genital HSV infection, shortening the disease by about 50% (273,284). However, oral acyclovir has minimal clinical effect on recurrent disease (311). Oral acyclovir is primarily used for chronic suppression, to decrease the frequency of symptomatic recurrences, and to decrease asymptomatic shedding of HSV (104,274,343,370,371). Patients with frequent episodes of recurrent genital herpes infections (more than 12 episodes per year) will show a marked decline to less than one episode per year receiving while receiving oral acyclovir at a dose of 400 mg twice daily (343). Oral acyclovir is also effective for chicken pox in children (110) and herpes zoster (191).

The benefits persist even when patients take oral acyclovir for many years. Yet suppressive acyclovir is not a cure. When oral acyclovir that had produced prolonged suppression of genital herpes for 6 years was discontinued, infection recurred in 1 to 3 months. Acyclovir must be taken daily to prevent recurrence of genital herpes. When breakthrough episodes occur on chronic suppressive therapy, the episodes are usually not correlated with the emergence of acyclovir-resistant virus. Recurrences caused by resistant virus in a normal host have been reported but are rare (217,351). Acyclovir-resistant HSV has been reported in 0.1% of cultures from normal adults attending a sexually transmitted disease (STD) clinic in Northwest England, and in 5.9% of cultures from HIV-infected patients taking acyclovir (47). In the United States, a study by the Herpes-Resistance Task Force and the Centers for Disease Control has shown a similar prevalence, and resistance has been associated with the use of topical acyclovir ointment. This study enrolled a large number of normal patients attending STD clinics in many cities and over 1,000 patients who were infected with HIV.

In serious and life-threatening HSV infections, acyclovir has been successful in adults and newborn infants (100,379). Ara-A (vidarabine) has also been successful in the treatment of neonatal HSV infection (382). When acyclovir and Ara-A were compared directly in a randomized controlled clinical trial of patients with biopsy-confirmed herpes simplex encephalitis, acyclovir therapy was superior to Ara-A for improving survival and morbidity. After 6 months of treatment with acyclovir, 67% of survivors were back at work or school (378). Acyclovir is the therapy of choice in this infection. A controlled trial of vidarabine versus acyclovir in neonatal HSV infection showed that the two drugs were similar but acyclovir was easier to administer, had fewer undesirable side effects, and was the treatment of choice (377).

Valaciclovir is a valine ester of acyclovir. The valine group is cleaved by esterase enzymes in the intestinal mucosa and then free acyclovir is found in the portal vein (302). The presence of the valine group greatly enhances the bioavailability of valaciclovir and 68% of the drug is absorbed. This results in plasma levels of acyclovir that are 8 to 10 times higher than the level obtained by oral absorption of acyclovir, which is only 12%. This high degree of bioavailability makes it an attractive therapy for several herpesvirus infections. Valaciclovir may be taken at one 500-mg pill per day to provide effective suppression of genital herpes (312). It also provides effective therapy for herpes zoster infections in the immunologically normal host (19).

The use of valaciclovir to treat infections in the immunocompromised patient has been controversial because of an unexpected mortality and vasculitis with features of hemolytic uremic renal syndrome observed in patients infected with HIV who received 8 gm of valaciclovir per day to suppress cytomegalovirus retinitis. The direct association between this syndrome and high-dose valaciclovir remains unclear. When compared with acyclovir in the treatment of herpes zoster in immunocompetent adults, oral valciclovir at 1 gm, three times daily for 7 days, speeds resolution of pain and decreases the frequency of persistent pain (19).

Penciclovir is similar to acyclovir, with two CH_2OH groups instead of one (see Fig. 1). Following intravenous dosage, penciclovir is taken up by HSV-infected cells. It is then phosphorylated by the viral TK and by cellular enzymes to form the active inhibitor, penciclovir triphosphate, which has a longer intracellular half-life than ACVTP, persisting in cells for 12 hours (363).

A double-valine ester of penciclovir (famciclovir) has been developed. Following oral administration of famciclovir, of which approximately 68% is absorbed, one valine ester is removed by esterase enzymes in the gastrointestinal tract, and the second valine ester is cleaved by an enzyme in the liver. The plasma then contains high concentrations of penciclovir (363). Penciclovir is converted to the triphosphate and is effective in treating and preventing HSV and VZV infection (318,361). When HSV becomes resistant to acyclovir due to a mutation in the viral TK gene, the mutant virus is also resistant to penciclovir or famciclovir because viral TK is required to phosphorylate penciclovir to the monophosphate.

Penciclovir ointment (Denavir) shortens the duration of labial herpes (cold sores) caused by HSV-1 (339). With daily use, famciclovir has also been effective in preventing episodes of recurrent genital herpes (318). If begun within 24 hours of the onset of rash, oral famciclovir will decrease the severity of chicken pox in children (300) and is also effective to treat localized, ophthalmic, or disseminated herpes zoster infection (300,361).

Intravenous acyclovir is most effective in preventing visceral complications of varicella zoster in immunocompromised patients with disseminated cutaneous zoster. Acyclovir prevents the development of encephalitis, pneumonia, and hepatitis due to VZV dissemination (380). It is likely that penciclovir will also be found to be effective at preventing visceral complications, but this is has not been extensively studied. Acyclovir and the related compounds valcyclovir, penciclovir, and famciclovir inhibit viral DNA polymerase as the final antiviral target. Only acyclovir and the prodrug valcyclovir are clearly chain terminators for viral DNA elongation. Penciclovir greatly slows growth of replicating viral DNA by incorporation into the elongating viral DNA strand, but chain termination does not strictly occur. These drugs are the main therapies employed to treat infections with HSV and VZV (19,191,300,361,380).

CYTOMEGALOVIRUS

The b herpesvirus, CMV is the largest virus known to cause infection in humans. It produces acute illness with a mononucleosis syndrome in young adults, severe congenital infection in newborn infants, and primary infection or reactivation producing retinitis, gastrointestinal disease, or pneumonia in patients with AIDS or following organ transplantation (71). The first-line therapy for many CMV infections is ganciclovir (71,252), an analog of guanosine, containing an acyclic sugar ring and two CH_2OH groups at positions 3 and 5. The addition of the single CH_2OH group makes ganciclovir about 100 times more active at inhibiting CMV replication *in vitro* than acyclovir, another guanosine analog (71).

The three drugs approved for use against CMV are ganciclovir, foscarnet, and cidofovir (71). The structures of these drugs and others for herpesvirus infections are shown in Figure 1.

Ganciclovir

Mechanisms of Action

Like acyclovir, ganciclovir must be phosphorylated by a viral enzyme and cellular enzymes to form ganciclovir triphosphate, an active inhibitor of CMV DNA polymerase. CMV, however, does not possess a TK homolog. The CMV enzyme that phosphorylates ganciclovir is putatively a protein kinase, the product of the UL97 gene of CMV (27,36,238,348).

The best evidence that UL97 alone is necessary for phosphorylation of ganciclovir was obtained by insertion of the gene for CMV UL97 into a ganciclovir-insensitive vaccinia virus, which renders the recombinant virus fully susceptible to ganciclovir (266). The exposure of CMV-infected MRC-5 cells to the same concentrations of ganciclovir and acyclovir results in relatively large amounts of ganciclovir triphosphate being formed, but the formation of ACVTP is minimal because acyclovir is a poor substrate for UL97 enzyme (22). In CMV replication, the role of UL97 is still being defined, but the protein is able to phosphorylate serine residues (182). Its main function may be to phosphorylate CMV proteins, which are important in genome-packaging steps late in infection. After UL97 converts ganciclovir to ganciclovir monophosphate, cellular enzymes convert this to ganciclovir triphosphate, which is a potent inhibitor of CMV DNA polymerase and competitively inhibits the incorporation of dGTP into elongating viral DNA. On a molar basis, ganciclovir triphosphate is not as effective an inhibitor of CMV DNA polymerase as ACVTP, but the concentration of ganciclovir triphosphate in CMV infected cells is 10 times that of ACVTP (22,40,127). The very high and persistent levels of ganciclovir triphosphate within the infected cell effectively inhibits CMV DNA replication.

The half-life of ganciclovir triphosphate in CMV infected cells is 16.5 hours, compared to 2.5 hours for ACVTP; the prolonged intracellular half-life makes ganciclovir far superior to acyclovir for inhibiting CMV replication (22). Unlike ACVTP, ganciclovir triphosphate is not an absolute chain terminator. Ganciclovir monophosphate is incorporated into elongating viral DNA, but short subgenomic-length fragments of CMV DNA continue to be synthesized, especially in the region of the origin of CMV replication (166,165). Ganciclovir was employed in a series of creative experiments to map the origin of CMV DNA replication in CMV (166).

Ganciclovir monophosphate greatly slows the rate of CMV DNA elongation, and all of the drug's antiviral effects result from inhibition of CMV DNA elongation (40,71). In the presence of ganciclovir, CMV immediate-early and early proteins continue to be made, as these are produced before CMV DNA replication begins in infected cells. If one were to measure production of these CMV early gene products in the presence of ganciclovir, it might be mistakenly concluded that ganciclovir is not an effective antiviral drug for CMV (336).

Ganciclovir is also an effective antiviral drug for HSV because the HSV TK effectively phosphorylates ganciclovir to its monophosphate (71). Cellular enzymes in HSV-infected cells convert this to ganciclovir triphosphate, which is an effective inhibitor of HSV DNA polymerase (40,41,127). Some mutations within HSV DNA polymerase that confer resistance to ganciclovir do not confer cross-resistance to acyclovir (73). TK-deficient mutants of HSV, which are resistant to acyclovir, are also cross-resistant to ganciclovir (71,121).

Cytotoxicity

Uninfected mammalian cells do not phosphorylate ganciclovir and it has little toxicity for these cells. If murine cells are transfected with the gene for HSV TK, the transformed cells that express the viral TK are easily killed by ganciclovir (288). This fact has been exploited in gene therapy. When genetically altered cells are given to patients to correct genetic defects or treat diseases such as cancer, the inclusion of the HSV TK gene in these cells can serve as a safety feature or “suicide gene” to allow ganciclovir to kill the altered cells in case they proliferate too aggressively or otherwise harm the patient.

Ganciclovir also prevents proliferation of murine melanoma cells transfected with the HSV TK gene after they are inoculated into syngeneic mice (364). There is thus a potential utility for ganciclovir as an antitumor drug if tumor cells can be effectively transformed with a herpes simplex gene so that they are able to phosphorylate ganciclovir. Furthermore, when ganciclovir is used to treat tumors, not only are the cells that contain the HSV TK gene killed but surrounding tumor cells are also killed. This “bystander effect” may be the result of the diffusion of ganciclovir triphosphate into surrounding tumor tissue.

In addition to direct cell-killing effects, ganciclovir has significant mutagenic, carcinogenic, and teratogenic properties. In dogs receiving anciclovir, interference with spermatogenesis has been observed (71).

Mechanisms of Resistance

Ganciclovir-resistant mutants of CMV contain mutations in the UL97 gene, in the CMV DNA polymerase (UL54) gene, or in both (9,21,42,43,71,241,242,347). The selection of viral mutants that are unable to phosphorylate ganciclovir is the main mechanism of resistance (21,341). Most mutations that confer resistance to ganciclovir are located in two main regions of the UL97 gene (9,241). The position at codon 460 is important for ATP binding (241); the other region, located from codons 590 to 596 (9), is important for nucleoside binding. For efficient phosphorylation to occur, the phosphate group on ATP needs to be transferred to ganciclovir. It is reasonable then that mutations in both regions affecting this process would confer resistance to ganciclovir. A third mutation in the UL97 gene at codon 520 has also been found to confer resistance to ganciclovir in clinical isolates of CMV (118).

The initial ganciclovir-resistant mutant of CMV was selected in the laboratory by passage of the AD 169 strain of CMV in the presence of increasing concentrations of ganciclovir (21). A mutant was selected that was unable to phosphorylate ganciclovir, and it exhibited a decreased sensitivity to ganciclovir (21). This mutant virus contained a deletion in the UL97 gene from codons 590 to 593 (348). On further analysis, it also contained a mutation in the viral DNA polymerase gene (347). This mutant remained sensitive to inhibition by phosphonoacetic acid (PAA) and phosphonoformic acid (PFA), two pyrophosphate analogs, which act directly on the CMV DNA polymerase (21). Since the majority of clinical isolates that exhibit resistance to ganciclovir have mutations or deletions in a few discrete regions of the UL97 gene, it has been possible to develop a rapid diagnostic test for resistance based on restriction enzyme analysis of the UL97 gene (42). This assay detects about 80% of ganciclovir-resistant clinical isolates (42,43).

The CMV DNA polymerase gene is the other site for mutations that confer resistance to ganciclovir, as well as to foscarnet and cidofovir (50). Mutations that confer resistance to ganciclovir in the CMV DNA polymerase are found almost exclusively in the highly conserved regions of the enzyme (50). There is some natural polymorphism in the DNA polymerase gene of CMV clinical isolates, but a nucleotide sequence analysis of 40 clinical isolates revealed that with one exception all the variability in the ganciclovir- and foscarnet-sensitive CMV isolates occurred in regions other than the highly conserved regions of the DNA polymerase (Fig. 4) (44). A marker transfer experiment analyzed drug-resistance mutations within the CMV DNA polymerase of recombinant viruses. These viruses were created in a Cos cell line containing DNA fragments of a CMV genome into which the DNA polymerase from drug-resistant mutants had been inserted. The results showed that only mutations in the highly conserved regions of DNA polymerase would successfully transfer the resistant phenotype to the recombinant virus (50). Some ganciclovir-resistant mutants that exhibit a high level of antiviral resistance also exhibit cross-resistance to cidofovir, a nucleotide analog of cytosine that has been approved for treatment of CMV retinitis. The specific point mutations in the CMV DNA polymerase gene that produce resistance to ganciclovir were first identified in 1992 (242). Some of these mutations, such as those in region IV (N408D, F412V, F412C, D413E), in d region-C (L501F, L501I, K513N), and in region V (G987A), have been reported to produce cross-resistance between ganciclovir and cidofovir (HPMPC) (50). The L802M mutation has been reported to produce cross-resistance between ganciclovir and foscarnet without changing the sensitivity to cidofovir (45).

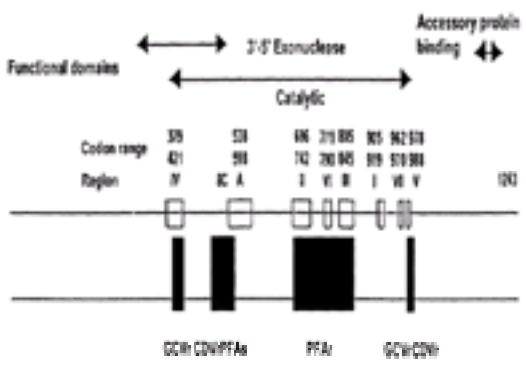

FIG. 4. Map of the cytomegalovirus (CMV) DNA polymerase. The CMV DNA polymerase showing functional domains and highly conserved regions of DNA nucleotide sequences (I–VII). *Shaded areas* are regions associated with drug resistance phenotypes. Codons, mapped to drug resistance in clinical isolates, are shown as *bars.* CDV, cidofovir; GCV, gancyclovir; PFA, foscarnet. (Reprinted from ref. 44, with permission.)

The V781I mutation in CMV polymerase region VI has been shown to induce resistance to PFA when the mutation was transferred to the Towne strain by cosmid recombination (9). A fivefold change in the susceptibility to PFA was noted, and the recombinant remained sensitive to ganciclovir. The same technique of cosmid recombination revealed that the L802M mutation conferred resistance to foscarnet, and the recombinant remained sensitive to ganciclovir (50). The difference between this report (50) and the one described in the preceding paragraph (45) may be due to different techniques, as the generation of recombinants in the Towne virus uses cosmids with many CMV DNA fragments, which may result in incorrect recombinations between some of the DNA fragments (45,50,70). Mutations at codons 700 and 715 have been reported to produce resistance to foscarnet and a slow-growth phenotype when these mutations were transferred to strain AD 169 (9A). A point mutation at K513N selected *in vitro* by cidofovir also has been reported to cause a slow-replication phenotype (50).

These two examples provide biologic support that drug-resistant mutations may also confer a loss of replication fitness because of mutations in the CMV DNA polymerase gene. In one case, a clinical isolate of CMV has been shown to be cross-resistant to ganciclovir, cidofovir, and foscarnet, and to possess a single nucleotide deletion in the DNA polymerase gene (46). To be sure that drug-resistant mutations in the CMV DNA polymerase really confer a

drug-resistant phenotype, marker transfer experiments through the generation of recombinant viruses are essential.

Clinical Trials

Multiple placebo-controlled trials have documented the efficacy of ganciclovir, foscarnet, and cidofovir for the treatment of selected CMV diseases in immunocompromised patients, mainly HIV-infected patients and transplant recipients (71). The most common CMV infection in HIV-infected patients is CMV retinitis. Before effective antiretroviral therapy was developed, 40% of all HIV-infected patients manifested CMV retinitis at some point during the course of the disease (193). This usually developed in patients with CD4 T-cell counts of less than 100 cells/mm ^{3}, and if untreated it progressed to blindness. Intravenous ganciclovir was shown to stabilize the retinitis in 2 weeks in over 91% of cases, whereas 61% had complete healing with intravenous ganciclovir therapy at 5 mg/kg twice daily (193). Another study in 108 patients treated with a similar regimen resulted in healing in 85% (30). Ganciclovir treatment of CMV retinitis was associated with clearing of viremia in 7 to 8 days (30) and clearing of CMV DNA from the plasma of infected blood in 8 days (69).

Foscarnet is also effective at treating CMV retinitis, and a direct comparison of foscarnet and ganciclovir in a multicenter randomized open-label trial in AIDS patients revealed no difference in the rate of progression of retinitis (286). The mortality rate, however, was higher in patients treated with ganciclovir, with a median survival of 8.5 months compared with 12.6 months in the foscarnet group (286). This important difference in survival was not fully explained, but it might result from the antiretroviral activity of foscarnet. A follow-up study showed no difference in survival between the two treatment groups, and a combination of foscarnet and ganciclovir was superior to either drug alone for treatment of retinitis (285). The conclusion of both studies was that ganciclovir and foscarnet were equally efficacious for CMV retinitis treatment, and that the combination of both drugs was superior, but that neither drug provided an advantage over the other for survival (71,285).

It was noted that essentially all patients had a relapse of retinitis after 20 to 30 days (median time) after the initial intravenous ganciclovir or foscarnet treatment was terminated. Maintenance therapy with daily intravenous ganciclovir at a dose of 6 mg/kg per day may delay time to relapse up to 72 to 75 days. Maintenance regimens of daily drugs are important to prevent relapse (30,106,285).

Oral ganciclovir was found to be almost as effective as intravenous ganciclovir as a maintenance treatment in patients treated initially with ganciclovir intravenously for 3 weeks (106). When progression of CMV retinitis was assessed by retinal photographs, the mean time to progression was 62 days for patients receiving intravenous maintenance therapy, and 57 days for those receiving oral maintenance. The oral ganciclovir group had fewer serious side effects, such as neutropenia and sepsis, related to the intravenous therapy (106,290). The study also showed that there was a marked decrease in the number of patients with positive urine cultures for CMV, indicating that both preparations decreased the replication of CMV in patients (106). For patients with sight-threatening retinitis, maintenance treatment with intravenous ganciclovir would still be preferred to preserve vision.

For the treatment of the gastrointestinal manifestations of CMV in patients with AIDS (e.g., esophagitis, cholecystitis, and colitis), ganciclovir has been the main therapy (57,97). Successful treatment of CMV colitis was first reported in 1986 (57), and a follow-up study of intravenous ganciclovir at a dose of 5 mg/kg given twice daily or placebo given for 14 days showed a greater reduction in the frequency of CMV infected colonic and urinary cultures in the ganciclovir group (97). Colonoscopy scores improved in patients who completed 14 days of ganciclovir therapy, but the frequency of diarrhea did not differ in the two groups (97). New, extracolonic CMV disease developed in 23% of patients in the placebo group, but in only 9% of patients in the ganciclovir group (p = .03). These results may indicate that treatment for 14 days is not adequate for the colon to heal and diarrhea to resolve. In bone marrow transplant recipients who had fecal CMV cultures on a regular basis, the cultures became negative in patients given ganciclovir, but gastrointestinal symptoms improved in both the ganciclovir and placebo groups, so there was no apparent clinical benefit of ganciclovir therapy (308). There is no evidence that maintenance therapy is useful in preventing a relapse of CMV colitis.

Treatment of primary CMV pneumonia with ganciclovir has been most successful in renal transplant recipients (184). In recipients of bone marrow transplants, however, attempts to treat CMV pneumonia with ganciclovir, acyclovir, vidaradine, human leukocyte interferon, or interferon plus acyclovir have been unsuccessful or of only limited success (71,334). For example, of the first seven patients treated with ganciclovir, four died of respiratory failure before completing a 14-day course of therapy (57). The three patients who survived for at least 14 days to complete therapy, died of pneumonia within 7 weeks. None of the other drugs that are active against CMV (foscarnet and cidofovir) have been successful at treating CMV pneumonia. In a group of 10 bone marrow transplant patients treated with ganciclovir for CMV pneumonia, only one patient survived, even though CMV was promptly cleared from pulmonary secretions (334).

Three uncontrolled studies showed that patients with CMV pneumonia who were treated with a combination of ganciclovir and high-dose intravenous CMV immune globulin had an improved survival of between 52% and 69%. As a result, this is the treatment of choice for CMV pneumonia (115,307,328).

Oral ganciclovir at a dose of 3 gm per day prevents CMV disease in patients with AIDS and fewer than 100 CD4 cells per cubic millimeter (337). A study in 725 patients comparing oral ganciclovir at 100 mg every 8 hours with placebo showed that 26% of patients in the placebo group acquired CMV disease, compared with only 14% of the ganciclovir recipients (337). In another study, in which retinal examinations were not routinely performed, however, and endpoints of disease progression may have been missed, oral ganciclovir could not prevent progression (28). Oral ganciclovir has proven to be effective in preventing CMV disease in recipients of kidney and liver transplants as well (26,129,143). Because it is the only oral agent for CMV, ganciclovir has been the only drug used to prevent disease. Prevention of pneumonia or other CMV disease with ganciclovir in recipients of bone marrow, heart, and liver transplants was clearly proven in several independent studies and was a major accomplishment (149,265,327,386). Intravenous ganciclovir was initially employed in prevention, but oral ganciclovir is also proving successful (26,143,185,335).

In some of these studies, a preemptive strategy was employed. It used measurement of CMV antigen in blood to determine when to start treatment with ganciclovir in renal transplant patients (185) and liver transplant patients (335). The presence of CMV antigen in bronchiolar lavage samples was used to deterine when to initiate ganciclovir treatment in bone marrow transplant patients (149). Preemptive strategies are effective and may avoid the expense of providing prophylaxis for all patients including those who are not at high risk of CMV disease.

Infection of the Nervous System

Progressive polyradiculopathy in patients with AIDS was the first peripheral nerve syndrome associated with CMV to be documented (112). This syndrome usually begins as low back pain with radicular or perianal radiation, followed in 1 to 6 weeks by progressive flaccid paraparesis.

Marked pathologic changes are found in the cauda equina and lumbosacral roots, with mononuclear-cell infiltrates, the destruction of axons, and CMV inclusions in Schwann cells and epithelial cells (112). This polyradiculopathy improved in a few patients who were treated early in the disease with ganciclovir alone or with ganciclovir and foscarnet, but treatment is more usually disappointing (117,136,153,196,269). Prospective clinical trials are needed to assess the possible benefits of combined ganciclovir and foscarnet therapy for this condition. Mononeuritis multiplex and painful peripheral neuropathy have also been described in patients with AIDS and have been attributed to CMV (135). Some of these patients were given ganciclovir with uncertain benefit. Anecdotal evidence suggests that CMV meningoencephalitis may respond to ganciclovir and foscarnet (117).

RETROVIRUSES

The human retroviruses associated with significant diseases are HIV-1 and HIV-2, the cause of AIDS (345), and human lymphotropic virus 1 (HTLV-1), the cause of T-cell leukemia and tropical spastic paralysis (393). Because AIDS is a devastating worldwide epidemic, it has been the subject of intense investigation, and a great deal is known regarding its molecular biology and the key events in HIV-1 replication.

Three classes of drugs are useful in treatment of serious HIV-1 infection in humans: nucleoside RT inhibitors, nonnucleoside RT inhibitors, and protease inhibitors. A massive program involving cooperation between the U.S. government, the pharmaceutical industry, and academic medical centers resulted in the development of over 15 antiviral agents that have been approved for therapy and prevention of HIV-1 disease. These drugs prolong life, decrease overall mortality, and prevent opportunistic infections, the hallmark of immune system decline caused by HIV-1 (293). One of the most remarkable consequences of effective antiviral therapy for HIV-1 is the resultant considerable immune reconstitution (4).

Successful antiviral therapy for HIV-1 to date has been directed at two viral enzymes that are essential for virus replication and maturation. These include the viral RT and the viral protease enzymes. Entry of HIV into cells involves the interaction of the envelope protein gp120 and the membrane-spanning protein gp41 with the cellular viral receptor CD4 and the chemokine receptors CCCR5 or CXCR4. This process of viral entry is itself a potential target for experimental therapies. Drugs that prevent viral entry by binding to the viral envelope gp120 protein (such as a bis-azo compound, FP21399) have been described (403). A potent peptide inhibitor of the conformational change in gp41 needed for fusion to occur between the viral envelope and the cell membranes has also been developed. This 36-amino-acid peptide (known as T-20) corresponds to amino acids 638 to 673 of the HIV-1 transmembrane protein gp41. When administered intravenously for 14 days to 16 HIV-infected adults, T-20 exhibited a dose-response effect; a dose of 100 mg twice daily was associated with a median decline in plasma viral RNA of 1.96 $\log_{10}$ (210). Another viral enzyme, viral integrase, is also a potential target of HIV replication inhibition. Compounds that inhibit integrase have been identified but not yet developed into proven drugs (181). Following receptor binding, the virion of HIV is internalized and uncoated, and reverse transcription takes place early in the cytoplasm. Components of RT are packaged and ready to function at once. There is evidence to indicate that reverse transcription begins within the virion (402). In the cytoplasm, in a preintegration complex of viral and probably cellular proteins, the conversion of single-stranded genomic RNA to a double-stranded DNA occurs. The HIV-1 RT catalyzes DNA synthesis and polymerization and also possesses RNase H activity to cleave the single-stranded RNA following initial single-strand DNA formation. The HIV RT, however, lacks an editing function, and errors that arise in the formation of double-stranded DNA are the source of the extensive genetic heterogeneity of HIV.

It is estimated that the mutation rate of this process is 10^{-4}, so an average of one nucleotide base error occurs for every round of reverse transcription (246). The viral preintegration complex is then transported into the nucleus, and the HIV-1 transcribed from proviral DNA is formed after the process of HIV integration. Replication of HIV can proceed following integration into the cellular chromosomal DNA of nondividing terminally differentiated monocyte macrophages and of CD4 T lymphocytes, which are arrested in stages G_2 and S (234,375).

Reverse Transcriptase Inhibitors

The first clinically useful drugs developed for HIV-1 were the nucleoside RT inhibitors (39,139). These drugs first require phosphorylation for the triphosphate to inhibit the HIV-1 RT in a manner analogous to that of most antiherpes drugs.

Phosphorylation is accomplished completely through cellular enzymes, as HIV does not encode any enzyme with similarities to the TK of the HSV. The mechanisms of action have strong similarities to acyclovir, in that the triphosphate is the active inhibitor of HIV-1 RT. Zidovudine (also known as azidothymidine, Retrovir, 3-azido-3-deoxythymidine, or AZT) is a synthetic pyrimidine analog that differs from thymidine in having an azido group at the 3' position of its deoxyribose ring instead of a hydroxyl group (188) (Fig. 5B). AZT was initially developed as an anticancer drug and was then found to inhibit RT of Friend leukemia virus in 1974 (188,292). In 1985, AZT was also shown to inhibit the RT of

HIV-1 *in vitro* (275). The active inhibitor is AZT triphosphate, which is a competitive inhibitor of RT. AZT-TP binds to the HIV-1 RT better than to the natural substrate deoxythymidine triphosphate (dTTP), and it functions as an alternative substrate for the enzyme (137,139,340). AZT-TP possesses a 100-fold greater affinity for the HIV-1 RT than for cellular DNA polymerases alpha or beta (137,139).

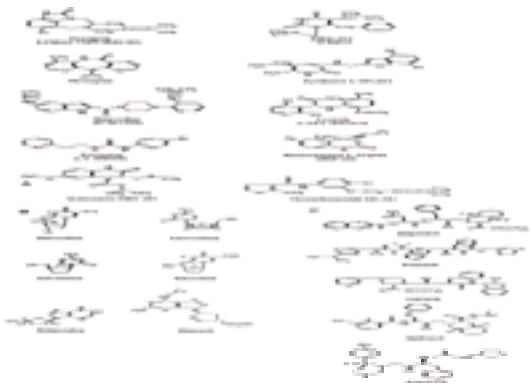

FIG. 5. A: Structures of nonnucleotide reverse transcriptase inhibitors. (Reprinted from ref. 86, with permission.) **B:** Nucleotide reverse transcriptase inhibitors. **C:** HIV-1 protease inhibitors. (Reprinted from ref. 167, with permission.)

This high affinity for HIV-1 RT makes the intracellular concentration of AZT-TP greater than the K_i value for HIV-1 RT but less than the K_i value for cellular DNA polymerase alpha and beta (139). The claim has also been made that AZT-MP is incorporated into growing DNA, and that this leads to premature chain termination (139). HIV-1 RT lacks an exonuclease, so the incorporated nucleoside analog cannot be excised and an incorporated AZT-MP does not posses a 3'-hydroxyl group to form a phosphodiester bond with an incoming nucleotide, so chain elongation is prevented and termination occurs at thymidine residues. The interaction of AZT-TP with the RT produces the selectivity of AZT for inhibiting HIV-1 replication (139,340). The phosphorylation is accomplished by cellular enzymes, and zidovudine is an efficient substrate for cellular TK. Cells exposed to AZT, whether infected or uninfected, accumulate high concentrations of AZT-MP because formation of AZT-DP (the rate-limiting step in the formation of AZT-TP) by host-cell thymidylate kinase is slow. AZT-MP is actually a competitive inhibitor of thymidylate kinase and reduces formation of the natural substrate dTTP. The high intracellular levels of AZT-MP may also impair the RNase H activity of HIV-1 RT (352). The formation of AZT-TP from AZT-DP is catalyzed by the cellular nucleoside diphosphate kinase (139).

As indicated, the phosphorylation of AZT is more efficient in rapidly replicating CD4+ T lymphocytes than in resting monocyte/macrophages. Other drugs may inhibit the phosphorylation of AZT. Ribavirin, a nucleoside analog, is also actively phosphorylated by cellular enzymes. In HIV-infected cells exposed to both zidovudine and ribavirin, the concentration of AZT-TP is diminished and the anti-HIV-1 activity of AZT is reduced (365). Ribavirin, however, increases the antiviral activity of purine analogs such as dideoxyinosine (ddI) by increasing the intracellular concentration of the inhibitor dideoxyadenosine triphosphate (ddATP) and decreasing the intracellular concentration of the natural substrate dATP. These effects of ribavirin are achieved by its inhibition of cellular inosine monophosphate dehydrogenase, which provides a competitive advantage for incorporation to ddATP over the natural substrate dATP (5).

Other nucleoside analogs, including didanosine (2',3'-dideoxyinosine, ddI), zalcitabine (2',3'-dideoxycytidine, ddC), lamivudine (3'-thiacytidine, 3TC), stavudine (didehydrodeoxy thymidine, d4T), and abacavir, also have activity against HIV-1 and have been approved for clinical use (167) (Fig. 5B). Didanosine is a prodrug of dideoxyadenosine (ddA) and avoids the reproductive toxicity of orally administered ddA. In the cell, ddI is converted to ddA and the active inhibitor of HIV-1 RT is, in fact, ddA-TP. The mechanism of action for all these nucleoside analogs is similar to that of AZT. Each triphosphate drug functions as a competitive inhibitor of the natural nucleoside triphosphate, and chain termination occurs following incorporation. The ddA, ddC, d4T, and 3TC are phosphorylated by cellular enzymes to the triphosphate and compete with dATP, deoxycytidine triphosphate (dCTP), dTTP, and dCTP (again), respectively, for binding to HIV RT. Abacavir is a guanosine analog and competes with dGTP for incorporation (167).

Resistance

Our knowledge regarding mechanisms of resistance of HIV-1 to RT inhibitors was greatly enhanced by solving the high-resolution crystal structure of this enzyme. The HIV RT is a heterodimer consisting of p51 and p66 subunits. The crystal structure has been determined at a resolution of 3.5 Å. The initial crystal structure was determined on HIV-1 RT complexed to the nonnucleoside RT inhibitor nevirapine, which enabled the enzyme to be stabilized in a conformation that would permit a structure to be determined (216). This was quickly followed by the determination of the HIV-1 RT crystal structure in a complex with a Fab antibody fragment (194). This same research team also determined the crystal structure of many mutant RT molecules. The structure of the HIV-1 RT at 3.2 Å in a complex with the double-stranded DNA template primer and a dNTP has also been described (190).

All of the RT crystal structures show a great deal of agreement, but there are also some contrasts. The structure of the covalently trapped catalytic complex of HIV-1 RT obtained at 3.2 Å, which shows the structure of HIV-1 RT complexed with the DNA template-primer and dNTP, provides a great deal of information regarding mechanisms of resistance and the key role of resistance mutations in RT function (190). The structure of RT containing the Try181Cys mutation and bound to the nonnucleoside RT inhibitor tetrahydrobenzodiazepine (TIBO) has also provided direct evidence of drug binding to an altered RT (82). The p66 subunit of RT contains the active center for dNTP binding, composed of a 183-tyrosine-methionine-aspartate-aspartate (183Y-M-D-D186) region that is absolutely conserved among all RT enzymes in retroviruses and hepatitis B virus (1). It is also conserved in the RNA-dependent RNA polymerase of poliovirus. The p66 subunit has been described as resembling the right hand, whose fingers interact with the position of

the template primer, with a palm region and a highly flexible thumb (216). The binding site for the nononucleoside RT inhibitor nevirapine in the first crystal structure of HIV-1 RT was found in the palm region (216). One of the surprises about the p51 subunit is that although it has the same amino acid sequences as the p66 subunit, its three-dimensional structure is completely different. The p51 subunit appears to function as a scaffold to hold the p66 subunit in place. The catalytic domains of RT are only within the p66 subunit (190,194,216).

The first report of HIV-1 clinical isolates exhibiting resistance to AZT involved patients who had received treatment for at least 6 months. Although the clinical significance of resistance could not be assessed initially (228), HIV-1 also develops resistance to AZT *in vitro* after serial passage of the virus in the presence of the drug (227). The mutations in the HIV-1 RT that confer resistance to AZT were initially reported at codons 67, 70, 215, and 219 (229), and later, mutations at codons 41 and 210 were added (187,205). Mutations at codons 215 and 41 are associated with a high level of phenotypic resistance to AZT and clinical progression of disease (79,200). The AZT-resistance mutations at codons 67 and 70 are associated with an RT enzyme that possesses increased processivity, whereas the mutations at codons 215 and 219 are associated with mutant enzymes possessing increased rates of pyrophosphorolysis, a reaction that is the reverse of nucleotide incorporation (2). This suggests that chain termination with the incorporated AZT-MP is reversible, and that the AZT-MP can be removed and viral DNA synthesis can be rescued. This appears to be a mechanism for AZT resistance.

Numerous studies to find altered binding of AZT-TP to the mutant enzymes have not conclusively shown it to occur. The rescue of chain-terminated DNA synthesis mediated by ATP or pyrophosphate (PPi) is considered to be an important mechanism that would explain HIV resistance to AZT (2,267). The finger region of the HIV-1 RT is flexible and able to position the incoming RNA template, and many drug-resistant mutations occur as point mutations affecting this domain of the HIV-1 RT. These include the change at K70R and D67N for AZT resistance, the change at K74V for ddI resistance, the change at codon 69 effecting ddC resistance, and the suggestion *in vitro* of codon 75 for d4T resistance (220,253).

Using wild-type RT, the sensitivity to dideoxynucleotides (25) was shown to depend on template length. When the template extension was more than three nucleotides, the wild-type RT began to incorporate dideoxynucleotides. If the RT contained the K74V mutation associated with ddI, then RT did not incorporate dideoxynucleotides, even with a long template extension (25). This clearly suggests a mechanism of resistance involving an altered response to the RNA template. In addition, a series of insertion substitutions, such as a run of serine-serine-serine at codon 69, has been associated with high-level AZT resistance and confers resistance to almost all of the nucleoside analogs (226,387).

An antiviral susceptibility assay that could measure phenotypic resistance to AZT in all the clinical viruses that grow in peripheral blood mononuclear cells was needed to assess the significance of AZT resistance in patients enrolled in clinical trials (199). Using such an assay, a high level of resistance [i.e., a 50% inhibiting concentration (IC_{50}) > 1.0 μM] was established in 1995, clearly showing that AZT resistance was strongly correlated with enhanced disease progression and death in patients with advanced HIV disease, even when other factors such as CD4 cell counts were controlled for (79). The association of clinical progression with AZT-resistance mutations was observed both in patients with extensive AZT treatment (66) and in those with little or no prior treatment (376). In the case of AZT, resistance and high levels of HIV RNA both strongly correlate with clinical disease progression (66,200,376). The presence of two AZT mutations at codons 41 and 215 in patients who were heavily pretreated with AZT is associated with high HIV RNA levels and enhanced clinical progression (200). Treatment of patients with another nucleoside chain-terminating compound, 3TC, is also associated with rapid selection of a single mutation in the active center of HIV-1 RT, known as the M184V mutation (24,303). The M184V mutation in RT confers high-level resistance to 3TC; it is caused by a change in the residue located in the YMDD motif that contributes to a portion of the polymerase active site of RT and other polymerases (303). The mutant RT containing the M184V mutation can discriminate between 3TC triphosphate (3TC-TP) and dCTP, resulting in an incorporation of 20 to 100 times less 3TC monophosphate (3TC-MP), than the wild-type enzyme (151,282,303). The mechanism of AZT resistance is quite different from 3TC resistance, as RT enzymes containing a combination of mutations conferring AZT resistance (M41L, D67N, K70R, T215Y, and K219Q) incorporate AZT-MP at rates that are almost equal to that of the wild-type enzyme. It has been shown that these AZT resistant enzymes have an increased rate of pyrophosphorolysis, a reaction that is the reverse of nucleotide incorporation (2). Thus, chain termination may be reversible and DNA synthesis can be rescued through removal of the incorporated AZT-MP. Unblocking of chain-terminated primers can also occur with nucleoside triphosphates in the case of AZT-MP (267,268).

The term *processivity* is defined as the property of an enzyme that determines the length of time that the viral RT remains in contact with the RNA template, permitting generation of long nucleotide polymers. The term *fitness* is defined as the capacity of the virus for efficient replication capacity. In comparison with wild-type RT, the HIV-1 RT M184V enzyme possessed diminished processivity (8,333), an increased fidelity (369), and diminished nucleotide misincorporation in the RNA-dependent and DNA-dependent DNA polymerase steps of reverse transcription as well as a diminished incorporation of 3TC-MP (313,369). However, a decreased pyrophosphorolytic cleavage and virtual inability to remove 3'-terminated 3TC-MP may be the strongest effect of the M184V mutation on RT function (151). The decreased pyrophosphorolysis may be an important factor, as it contributes to the reduced replication fitness of M184V-containing variants of HIV-1. The diminished rate of 3TC-MP incorporation by RT-M184V ensures that a low level of viral replication can continue in the presence of the drug. The RT enzymes associated with resistance to AZT possess properties that are opposite to those of the RT associated with 3TC resistance. AZT resistance is associated with increased pyrophosphorolysis in the presence of pyrophosphate (PPi) or ATP, and increased rates of rescue of DNA synthesis (2,267,268). AZT resistance and 3TC resistance appear to occur by opposite mechanisms, providing an explanation for data from clinical trials that shows that AZT-resistant viruses are not cross-resistant to 3TC (231). The fact that AZT and 3TC combination therapy is effective may be due to the diminished rescue capacity for AZT-terminated DNA synthesis in the presence of the RT M184V mutant virus (151,231) and the maintenance of AZT-sensitive virus. It has been claimed that several amino acid changes in addition to M184V (283) or polymorphisms at codon 333 (208) can facilitate coresistance to 3TC and AZT.

Resistance to other nucleoside analogs such as ddI and the active intracellular metabolite ddA is mediated by a mutation in the finger domain of the RT, specifically a mutation at L74V, and to a lesser extent at RT M184V (155,253). The L74V mutation confers only two- to fourfold resistance to ddI (332,385). This is very different from the 100- to 1000-fold

resistance to 3TC associated with the M184V mutation. The didanosine resistance mutation results in a decrease in inhibition of RT enzymatic activity by ddATP (332), which is very different from the case of AZT-associated mutations and AZT-TP. The AZT-associated mutation at K70A does result in a decrease in inhibition by ddATP (332), suggesting a possible mechanism for cross-resistance between AZT and ddI. The RT L74V mutation associated with ddI resistance results in a mutant virus with decreased fitness and diminished replication capacity (333). The decrease in fitness is associated with a diminished processivity of the RT L74V enzyme, which is similar to a decrease in processivity associated with the RT M184V enzyme (333). In a study comparing development of resistance in 34 paired isolates obtained at baseline and at follow-up of 6 to 12 months from patients treated with didanosine (ddI) and stavudine (d4T), only a 1.6-fold increase in resistance was observed to ddI and a 1.9-fold resistance to d4T (51). This study also showed that the combination of d4T and ddI apparently allows the selection of AZT-associated mutations, an observation also reported by others (89,388).

The thymidine analog d4T is also an effective inhibitor of HIV RT and is associated with much less anemia and neutropenia in patients than AZT. There are reports that resistance to d4T in tissue culture is associated with the RT V75T mutation (220). In clinical studies, the use of d4T has rarely been associated with the selection of V75T mutants, and the role of this mutation in virologic failure of d4T therapy is controversial, as isolates containing V75T remain susceptible to d4T (237). Resistance to almost all nucleoside agents for HIV has been associated with two distinct mutational patterns in the viral RT. The multinucleoside resistance complex has been characterized as a combination of A62V, V75I, F77L, F116Y, and Q151M mutations in the RT (192). A series of serine insertions at codon 69 has also been associated with multinucleoside resistance (230,387).

The mechanism of multidrug resistance associated with these mutations has not been characterized. The negative impact associated with AZT-resistance mutations on subsequent treatment with RT inhibitors has been frequently noted (258), and these mutations may make the virus less susceptible to other antiviral drugs.

Nonnucleoside RT Inhibitors

The p66 subunit of the HIV-1 RT has a hydrophobic pocket that is a binding site for drugs belonging to a family of compounds called nonnucleoside RT inhibitors (NNRTIs) (Fig. 5). These drugs do not need to be metabolized intracellularly to exert antiviral activity. They do not compete with a natural substrate for binding to HIV-1 RT but are able to directly inactivate the enzyme. They do not present the risks of toxic side effects due to interference with nucleotide metabolism and nucleic acid biosynthesis that are associated with nucleoside RT inhibitors. They may act very early in viral replication and may inactivate RT within the virion itself (402).

The NNRTIs were historically important in enabling the crystal structure of the HIV-1 RT to be determined (216). More than 25 different subclasses of NNRTIs have been described, and they show a great variety of chemical structures. Tivirapine is the candidate drug derived from the TIBO class of compounds, the first HIV-1-specific inhibitors targeted to the nonnucleoside binding site of HIV-1 RT (296,297). Hydroxyethoxymethyl phenothiothymine (HEPT) was the first NNRTI compound to be identified as a specific HIV-1 inhibitor (7), and MKC-442 is its clinically active derivative (6).

Nevirapine was the first NNRTI to be approved for clinical use after multicenter clinical trials demonstrated the benefit of adding it to regimens with AZT and ddI (78,278). Addition of nevirapine further improved virologic and immunologic parameters of HIV disease. In antiretroviral naive patients, this combination has been dramatically effective (278). Two other NNRTIs have also been approved for clinical use, delavirdine and efavirenz (DMP-266). Efavirenz is currently the most potent NNRTI (395). The IC_{50} to inhibit viral replication in tissue culture is 0.001µM, and the dose required to achieve a 50% reduction in the viability of noninfected cells (CC_{50}) is 80µM. This provides a selectivity index, the ratio of CC_{50} to IC_{50}, of 80,000 (395). Another NNRTI, pyridinone C-697,661, was shown to be markedly effective as monotherapy in reducing the HIV-1 viral RNA level in plasma, but this was a temporary effect because there was a rapid emergence of resistance to the drug (317). The rapid emergence of resistance has strongly limited the use of NNRTIs as monotherapy: Each must be used in conjunction with another antiretroviral drug to have substantial benefit.

Delavirdine is a highly active bisheteroarylpiperazine (BHAP) NNRTI that decreases viral load and increases CD4+ T-lymphocyte counts when used in combination with other antiretroviral drugs (90). Delavirdine selectively inhibits HIV-1, but it has no activity against HIV-2 or other retroviruses. It is synergistic with each of the five approved RT inhibitors: AZT, ddI, ddC, d4T, and 3TC (see Fig. 5). It binds to a hydrophobic pocket in the p66 subunit of HIV-RT near the catalytic site of the enzyme (86). Residues important for NNRTI binding include those at codons 181, 188, and 103 (108). Binding of all of the NNRTIs is thought to induce a conformational change in the RT that inhibits polymerization (353).

The NNRTIs share several features: They are active against HIV-1 but not against HIV-2 or other retroviruses. They inhibit HIV-1 replication at very low concentrations, a factor of 10^5 below the cytotoxicity threshold. They specifically bind to an allosteric nonsubstrate binding site of the HIV-1 RT. The mutations conferring resistance to these drugs are found surrounding this binding pocket (353). Dr. Edward Arnold and colleagues at Rutgers University employed x-ray crystallography to determine the structure of the first NNRTI, TIBO bound to the wild-type enzyme and to the mutant RT containing a Tyr181Cys substitution (Fig. 6). The amino acids whose mutations confer resistance to NNRTIs are Glu-138, Leu-100, Lys-101, Lys-103, Pro-236, Tyr-181, Tyr-188, Val-106, Val-108, and Val-179; the relatively conserved amino acids in the binding pocket are Leu-234, Phe-227, Pro-95, Tyr-229, and Tyr-318 (82).

FIG. 6. Computer graphics representation of the structure of HIV-1 reverse transcriptase (RT) showing the pocket region where nonnucleoside RT inhibitors (NNRTIs) bind. Shown is the inhibitor 8-C1 TIBO (tetrahydrobenzodiazepine). Edward Arnold and colleagues have used x-ray crystallography to determine structures of TIBO bound to both wild-type and Tyr181Cys HIV RT. Amino acids whose mutations confer resistance to NNRTIs are Glu-138, Leu-100, Lys-101, Lys-103, Pro-236, Tyr-181, Tyr-188, Val-106, Val-108, and Val-179; relatively conserved amino acids in the binding pocket are Leu-234, Phe-227, Pro-95, Trp-229, and Tyr-318. HIV-1 RT secondary structural elements (b6, b9, b10, b12, b13, and b14) are represented by ribbons. (Courtesy of Kaylan Das and Edward Arnold, Center for Advanced Biotechnology and Medicine, Piscataway, NJ, and Rutgers University, NJ.)

In contrast to the resistance mutations arising from *in vitro* cultures with the other NNRTIs such as nevirapine and efavirenz (Y181C or K103N), delavirdine induces a proline-to-leucine change at codon 236 of the HIV-RT (108). This p236 mutation is in the “roof” of the nonnucleoside binding pocket and confers a higher level of resistance to delavirdine than does K103N or Y181C (123). Delavirdine maintains some activity against the Y181C or K103N mutants *in vitro*, which is different from nevirapine or the pyridinones (108,124). The P236L mutant exhibits a hypersensitivity to other NNRTIs *in vitro* (123).

In patients receiving delavirdine as monotherapy, the most common mutations observed are at codons Y181C and K103N, even though P236L is the most common resistance mutation associated with delavirdine drug exposure *in vitro* (Fig. 7). In a trial of delavirdine as monotherapy, resistance to delavirdine developed within 8 weeks of therapy, but only 10% of resistance isolates had the P236L mutation. The majority of patients had isolates with the Y181C and the K103N mutations, even though these double mutants were considered to be unlikely (90). In patients taking AZT and delavirdine, the Y181C mutation was not detected, but when patients received delavirdine monotherapy, 50% of the isolates had the Y181C mutation (88,90). This is similar to what has been observed with nevirapine and AZT (89,315).

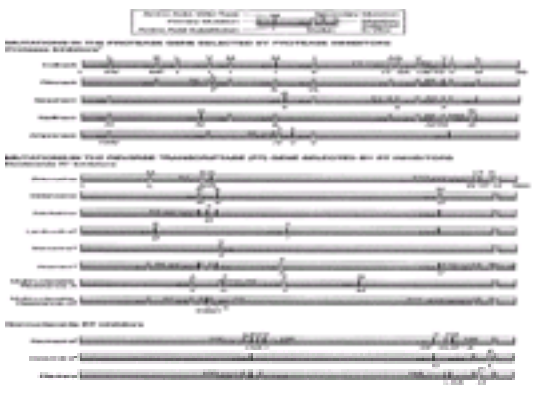

FIG. 7. Shown are the most common mutations in HIV-1 genes that confer drug resistance. For each residue, the *letter above* indicates the amino acid associated with the wild-type virus. The *italicized letters below* are substitutions that confer viral resistance. *Black bars* are primary mutations and cause decreased inhibitor binding. *White bars* contribute to drug resistance and may have less direct effect on inhibitor binding *in vitro*. *Black and white bars* are mutations selected *in vitro* and rarely seen in patients with treatment failure. (Reprinted from Hirsch MS, Brun-Vezinet F, D'Aquila RT, et al. Antiretroviral drug resistance testing in adult HIV-1 infection: recommendations of an International AIDS Society-USA Panel. *JAMA* 2000;283:2417–2426, with permission.)

NNRTIs inhibit RT noncompetitively with respect to its normal substrates (dATP, dGTP, dCTP, dTTP), commonly referred to as the deoxynucleoside triphosphates (dNTPs). The specific site (the pocket) of the HIV-1 RT where NNRTIs interact is functionally related to the substrate (dNTP) binding site. This pocket is located 10 Å from the substrate-binding polymerase (pol) active site within the palm domain of the p66 subunit of HIV RT. The binding of the NNRTIs to this site might indirectly affect the conformation of aspartate residues (Asp-185, Asp-186, and Asp-110) in the nearby pol active site (219). The binding of NNRTI to this pocket may lead to a repositioning of these aspartate residues; thus, the NNRTI may inhibit HIV-1 RT by locking the pol active site in an inactive conformation, reminiscent of the conformation of the p51 subunit of HIV RT (86,353).

Most of the NNRTIs assume a butterfly-like structure, with two hydrophobic wings connected by a polar central body consisting of urea (e.g., MKC-442), thiourea (e.g., tivirapine and trovirdine), carboxamide (e.g., loviride), or thiocarboxamide (e.g., thiocarboxanilide UC-781) (86). The NNRTIs appear to function as p-electron donors to aromatic side chain residues surrounding the NNRTI binding pocket. The side chain residues adapt to each bound NNRTI in a

highly specific manner, closing around the surface of the drug to make tight van der Waals contacts (219).

The contact points between the NNRTI butterfly and its surroundings in the HIV-RT pocket represent hot spots, where the HIV-1 RT preferentially undergoes mutations conferring resistance to the NNRTIs (i.e., L110I, K101E/I/Q, K103N/Q/R/T, V106A/I, E138K, V179D/E, Y181C/I, Y188C/L/H, G190A/E/Q/T, and P236) (86) (Table 1, and see Fig. 7). Amino acid substitutions at these sites give rise to resistance to the individual compound to which the HIV-1 RT has been exposed, often leading to cross-resistance to other NNRTIs.

Drug class	Mutations
Nucleoside reverse transcriptase inhibitor	Reverse transcriptase gene
Zidovudine (AZT)	41,67,70,210,215,219
Didanosine (ddI)	65,74,184
Zalcitabine (ddC)	65,69,74 184
Lamivudine (3TC)	44 118 184
Stavudine (d4T)	75
Abacavir (ABC)	41,65,67,70,74,115,184,210,215
Multinucleoside resistance A	62,77,116,151
Multinucleoside resistance B	41,67,69 (insert), 70,210,215,219
Nonnucleoside RT inhibitor	Reverse transcriptase gene
Nevirapine	100,103,190,108 181,188,190
Delavirdine	103 181,236
Efavirenz	100,103,190 [illegible] 188,190,225
Protease inhibitor	Protease gene
Indinavir (IDV)	10,20,24,32,36,46,54,71,77,82,84,90
Ritonavir	20,32,33,36,46,54,71,77,82,84,90
Saquinavir	10,48,54,71,73,77,82,84,90
Nelfinavir	10,30,36,46,71,77,82,84,88,90
Amprenavir	10,32,46,47,50,54,84

TABLE 1. *Mutations associated with resistance of HIV-1 to antiretroviral drugs*

Exposure to an NNRTI does not necessarily lead to cross-resistance to other NNRTIs, and switching compounds or combining drugs that have different resistance patterns is possible. To prevent the emergence of HIV-1 resistance to NNRTIs, the compounds should be administered in adequate doses. Several NNRTIs (e.g., tivirapine, MKC-442, nevirapine, pyridinone, delavirdine, quinoxalines, and thiocarboxanilides), when added to cells *in vitro* at high concentrations, can completely suppress viral replication so that repeated passage of the virus-infected cells in the presence of the compounds clears (“cures”) the cells of the virus (86). Failure to detect the presence of proviral DNA in the cells by using the polymerase chain reaction (PCR) was reported. The concentration at which viral clearance is achieved varies from one compound to another; the most potent compounds among the NNRTIs (i.e., HBY097 and thiocarboxanilide UC-781) achieve viral clearance at concentrations as low as 0.1 µg/mL, a concentration that is readily attainable *in vivo* through systemic (oral) administration of the compounds. This apparent “knock out” of virus *in vitro* can be achieved at lower drug concentrations when drugs are used in combination (12). Combinations of 3TC and any of the NNRTIs (e.g., MKC-442 and delaviradine) completely suppress viral replication under conditions and at concentrations where the individual compounds, when used as single-agent therapy, fail to do so (11). To achieve a synergistic effect on viral replication, compounds must be administered together, not sequentially. Only when 3TC is added to cells concomitantly with another NNRTI did the compounds act synergistically in preventing virus breakthrough (11).

Protease Inhibitors

The HIV-1 protease enzyme is a homodimer of two identical subunits composed of 99 amino acids. It is a member of the aspartate protease family, because the active center of the enzyme consists of two aspartate molecules. Other members of the family include cathepsin D, and angiotensin-converting enzyme. The HIV-1 protease was the first viral enzyme for which a crystal structure was determined, and atomic details are well understood (299,389). The homodimer has all the enzymatic activity and the monomer is enzymatically inactive (299).

Solution of the three-dimensional crystal structure of the protease enzyme allowed design of antiviral drugs and an understanding of HIV protease resistance and cross-resistance (Fig. 8) All of the HIV-1 protease-inhibiting drugs fit into an active center pocket that is large enough to accommodate seven amino acids. This binding pocket consists of several smaller subpockets designated S_1, S_2, and S_3. Each protease inhibitor binds preferentially to one or more of these subpockets. The seven amino acids define the recognition sequence required for HIV-1 protease to carry out cleavage in the viral replication cycle. The HIV-1 protease cleaves itself from the gag-pol precursor peptide. The protease acts at 11 distinct cleavage sites on the HIV-1 polyprotein precursor. All of the sites are located in the *gag* and *pol* genes except for one cleavage site within the nef protein (133). It is not clear how a single viral enzyme is able to effectively recognize and cleave at 11 distinct regions of amino acid sequence, but three of the HIV cleavage sites involve phenylalanine-proline or tyrosine-proline bonds, which are unusual sites of attack for mammalian proteases (119). This activity, however, is essential for HIV replication and maturation (133,215).

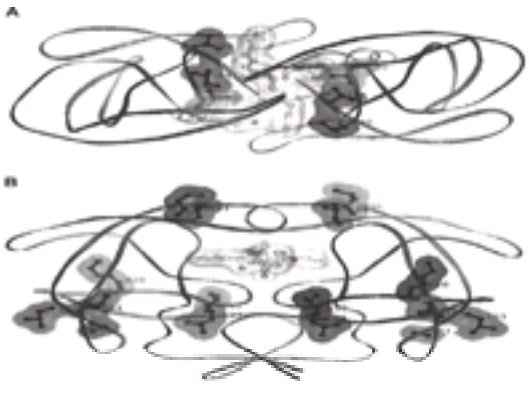

FIG. 8. Crystal structure of the HIV-1 protease showing locations of amino acid residues that induce drug resistance to indinavir. **A:** Top-down view showing residues known to affect inhibitor binding (V82, I84, L90). **B:** End-on view showing

eight residues away from the drug binding site. The structure of indinavir is shown; the catalytic D25 residue is on the loop below the drug. (Data courtesy of J. H. Condra et al. Structure by Z. Chen et al. Graphics by M. K. Holloway of Merck Research Laboratories.)

The work that established that HIV-1 protease is essential for viral replication was published as a landmark article by Dr. Irwin Siegel and his colleagues at Merck Research Laboratories (215). They described molecular approaches to construct viruses containing mutations within the HIV-1 protease gene. A single mutation involving the aspartate in the active center of the protease enzyme was shown to result in an inactive protein and the absence of viral infectivity. When viruses containing the mutant protease gene are transfected into cells, the gag p55 precursor polyprotein can be detected in cells, and RT precursors are still present. The RT, however, is not processed to its p66 and p51 functional subunits. Virion particles are produced by these cells but the virions are not infectious and do not contain mature core proteins or the processed RT components p66 and p51 (215).

The assay employed to measure processing of precursor proteins was effectively used to screen for potent antiviral compounds, which could inhibit the HIV-1 protease function, leading to the discovery of the protease inhibitor indinavir (133). The first protease inhibitor approved for human use was saquinavir in 1995. In 1996, ritonavir and indinavir were approved. There are now six licensed HIV-1 protease inhibitors in use, including nelfinavir, amprenavir, and ABT378 $_{R}$ (Kaletra, Lopinavir/Retonavir). All of these protease inhibitors fit in the active center. The protease inhibitors bind in this region and interact with several amino acids of this binding pocket depending on the size of the protease inhibitor molecule. Indinavir is the largest protease inhibitor and interacts with the largest number of amino acids in the protease dimer. The most frequent mutations associated with indinavir resistance occur at codons 48, 82, and 84 (63) (see Fig. 7 and Table 1).

Other protease inhibitors that, like ritonavir and saquinavir, are also large give almost complete cross-resistance profiles with indinavir (65). With saquinavir use, the first mutations selected are located at codons 48 and 90 (195). With prolonged use, many more mutations throughout the entire protease gene are selected. Retonavir selects for a broad cross-resistance among all the protease inhibitors (276). The initial mutation conferring resistance to retonavir is usually a change at codon 82, but with prolonged use, multiple mutations occur, producing high-level cross-resistance to all of the protease inhibitors (276).

The protease inhibitor with the smallest known molecular structure is amprenavir (133). The most common mutation selected by amprenavir occurs at codon 50, and it confers resistance in approximately half of the cases (294). The other half of the resistance cases to amprenavir are conferred by multiple mutations other than at codon 50. In addition to mutations occurring at codons for amino acids that directly touch the protease inhibitor, other amino acids associated with resistance are present in positions distant from the binding site of the drug to the protease enzyme (63,70). These altered amino acids are primarily considered compensatory mutations that increase the biologic activity of the drug-resistant enzyme (63). Some of the latter mutations, such as substitutions at L10, M46, and L63, were observed as initial events in the development of resistance to indinavir, leading to increased fitness of the viral protease. Specifically, studies of recombinant enzymes containing the M46I and L63P substitutions showed that the mutant proteins have improved catalytic efficacy (330).

Some mutations occur very commonly *in vitro* and were not expected to be associated with cross-resistance. For example, the drug nelfinavir, a very small molecule, touches the protease enzyme most closely at the position presented by codon 30. The D30N mutation predominates when resistance occurs *in vitro*. In clinical use of nelfinavir, however, other mutations are readily selected, particularly at position L90M. Virologic failure with nelfinavir is associated primarily with the mutation L90M (295). When HIV is grown in tissue culture, or the virus is isolated from patients receiving protease inhibitors, multiple amino acid mutations are observed in the protease enzyme and in the gag and gag-pol polyproteins that are the cleavage sites (404) for the protease. These mutations in the protease and in the gag-pol polyprotein cleavage sites result in processing defects and a loss of viral fitness (245,400). These less fit protease variants replicate more slowly in patients and are associated with a decrease in pathogenicity.

The inhibitors of the HIV-1 protease enzyme have had a greater impact on HIV disease than any other class of drug (186,293,374). For patients with a high level of HIV-1 RNA (100,000 copies of HIV-1 RNA per milliliter of plasma), protease inhibitors are dramatically effective at lowering viral load and improving patient outcome (157,164). It is not unusual for protease inhibitors alone to decrease the level of HIV RNA by a factor of three logs (133). A decrease of greater than two logs was essentially not seen with RT inhibitors. In some cases, the use of a protease inhibitor results in prolonged suppression of HIV replication (157,158). Thus, protease inhibitors are main components of antiretroviral combination therapies (137,157,164). This will be discussed later in the Clinical Trials section.

A major limitation of protease inhibitor effectiveness, however, is the high degree of protein binding by human sera that all of these drugs exhibit (277). Only the free drug penetrates cells and inhibits HIV. The most potent protease inhibitor, indinavir, is also the least protein bound. Penetration of indinavir into tissue compartments where viral replication is ongoing, such as the central nervous system, and into seminal fluid is higher than that of other protease inhibitors, and this may contribute to its considerable clinical effectiveness (133). An important observation for all of the protease inhibitors is that the presence of human serum attenuates the activity of the inhibitors, both for wild-type and utant HIV-1 strains (277). A careful study comparing the effect of human serum on the IC_{50} of retonavir, saquinavir, and nelfinavir when used against wild-type and protease mutant viruses found that their activities were markedly attenuated in the presence of human serum, whereas indinavir, amprenavir, and the compound ABT378 were only modestly affected (277). All of the HIV protease inhibitors also inhibit the cytochrome P450 enzymes, which metabolize these drugs, but ritonavir is the most potent inhibitor of these enzymes (91). When ritonavir is coadministered with other protease inhibitors, a powerful pharmacokinetic enhancement is observed, which greatly increases the plasma level of other protease inhibitors (209). This concept has been exploited to show that the phenotypic susceptibility of a clinical isolate (as an IC $_{95}$

corrected for human serum) could be greatly exceeded by a minimal concentration of drug (C_{min}) obtained when a protease inhibitor is coadministered with ritonavir. The ratio of C_{min}/IC_{95} (corrected) can give the value of the enhancement factor (called an inhibition quotient) that could be expected when small doses of ritonavir are used to increase the C_{min} of another protease inhibitor. This pharmacokinetic boost can be employed to achieve high plasma concentration and overcome virologic failure due to resistance to protease inhibitors. For example, a dose of indinavir at 800 mg and ritonavir at 200 mg given twice a day could achieve a C_{min}/IC_{95} ratio of 78.6-fold above the concentration achieved with indinavir alone (64). This provides a rationale for drug intensification with a twice-daily regimen of two protease inhibitors to overcome the virologic failure of a single protease inhibitor (64).

HIV-1 Drugs

Clinical Trials

Many randomized, controlled, double-blind clinical trials of antiretroviral therapies have examined the use of antiretroviral drugs in combination, as this is remarkably effective at decreasing viral replication and increasing the CD4 cell count in HIV-infected patients.

Assessment of the effectiveness of antiretroviral therapy has been closely linked to measurement of HIV-1 RNA levels in plasma (263). Plasma HIV-1 RNA, well established as an accurate measure of viral replication in patients (263), can be measured precisely by two commercially approved assays that show remarkably similar values when compared directly (66,376). One method employs RT-PCR to amplify the copies of RNA in plasma. The standard commercial assay has a limit of detection of 400 copies of HIV-1 RNA per milliliter of plasma and a dynamic range of up to 2 million copies per milliliter of plasma (66,376). An ultrasensitive PCR-based HIV-1 RNA assay detects HIV-1 RNA down to 50 copies per milliliter of plasma. Other experimental assays can detect as little as two to five copies of HIV-1 RNA per milliliter of plasma. Another method of detecting HIV-1 RNA is based on a signal amplification of RNA in plasma. This is called the branched DNA method for detection, and the latest version can detect 100 copies of HIV-1 RNA per milliliter of plasma (263). These two methods for detecting HIV-1 RNA in plasma and fluids in other body compartments are widely used to follow the response to antiviral therapy and to assess virologic failure.

The first effective antiviral drug for treating HIV disease was AZT, which was licensed in the United States in 1987 (132). In a study of 4 months' duration, AZT yielded an improvement in survival, and fewer opportunistic infections, which led to its approval by the FDA. In this study, however, the placebo recipients had an unusually high mortality, rarely seen in other trials. The short-term benefits of AZT were transient and not supported in longer-term studies (162). In a trial comparing two doses of AZT, the lower-dose regimen was found to be associated with improved clinical outcome, but patients receiving the lower dose also received more prophylaxis to prevent *Pneumocystis carinii* pneumonia (PCP), the main infection to which patients with HIV were subject. The favorable response of the lower-dose AZT treatment regimen was attributed to a decrease in the toxic side effects of AZT. This interpretation, however, is confounded, because more patients also received prophylactic treatment against opportunistic infections in the low-dose AZT arm (131). AZT does have significant side effects, including anemia, neutropenia, and lymphocyte toxicity (314). There were claims initially that disease markers such as the CD4 cell count could be improved in patients who were treated at an early stage of disease with AZT, and disease progression was decreased in patients treated for 1 year with AZT (366).

The Concorde Study, conducted by the British and the French, evaluated treatment early in disease with AZT for 3 years. It concluded that AZT provided no survival benefit even though CD4 cell counts were slightly improved after 1 year of treatment (61). In time, it became clear that treatment of HIV-1 disease with a single RT inhibitor produces little long-term benefit, and that resistance rapidly emerges. With AZT therapy alone, HIV RNA levels decreased transiently and then returned to baseline levels. One of the few exceptions to the limitations of monotherapy was with didanosine therapy. In the first study of the AIDS Clinical Trials Group (ACTG 175) to show an improvement in survival with the use of two RT inhibitors, ddI monotherapy performed as well as the use of two drugs in providing clinical benefits and improving mortality (163). Therapy with ddI provided a greater decrease of HIV RNA than did AZT monotherapy, but over the short duration of treatment, two nucleoside RT inhibitors had a more significant effect on lowering HIV RNA than did ddI alone. After 1 year of treatment, however, there was no statistical difference between therapy with ddI alone and therapy with a combination of two RT inhibitors (163,202). The first ACTG study to show a statistically significant decrease in viral RNA levels was ACTG 116/117, a study that enrolled patients heavily pretreated with AZT (66,201). This study showed that if patients switched to ddI there was a significant decrease in viral RNA levels compared to those who stayed on AZT alone (191). The drop in RNA was small, only about 0.5 log, and no survival benefit could be documented with ddI therapy (66,201).

The data from ACTG 116/117 also provided the first evidence to show that high-level resistance to AZT resulted in a more rapid progression of AIDS and an enhanced rate of death (79). This association of clinical failure with high-level AZT resistance is the best example of the clinical significance of resistance to any antiretroviral therapy. For other antiretroviral agents, virologic failure with an increasing HIV RNA level in plasma has been noted, but the clinical consequences have not rigorously been determined.

Although the ACTG 175 trial using two RT nucleoside inhibitors was the first study to clearly show a persistent survival benefit associated with treatment, it was not until a protease inhibitor was included in the treatment regimen for patients with advanced HIV disease that a dramatic improvement in survival was shown. In the ACTG 320 study, patients treated with indinavir, AZT, and 3TC had a 50% decrease in mortality at a mean follow-up of 36 weeks, and this was superior to results in patients treated with AZT and 3TC alone (164). The study was terminated early because of the statistically significant increase in survival benefit noted. There was a major drop in deaths caused by the major opportunistic infections of CMV, *Pneumocystis carinii*, and *Mycobacterium avian* complex in patients receiving triple therapy. This clinical improvement was associated with a greater drop in viral RNA level in those receiving triple therapy compared to two-nucleoside therapy (164). The Merck 035 study looked at the same regimen of indinavir, AZT, and 3TC; it showed that markers of HIV-1 RNA and CD4 cells improved in a group of heavily treated patients with HIV-1 infection (157). The Merck study showed that even in a group of patients in whom 81% had AZT drug-resistance mutations, after 2 years of

follow-up 84% of those still taking their medication had an HIV-1 RNA level below the limit of detection of 400 copies per milliliter of plasma (157). After 3 years of follow-up, 67% of these patients had an HIV-1 RNA level of less than 400 copies per milliliter of plasma (158). Although the number of patients who were still on this regimen was small, 21 of 31 (65%) had viral RNA levels of less than 50 copies per milliliter. There was a steady increase in CD4 cells in the patients in this study, with a mean increase of 230 cells noted after 3 years (158). It was shown in a study by Autran et al. that patients on highly active antiretroviral therapy (HAART) initially have an increase in CD4 memory T-cells in the peripheral blood, largely from mobilization from lymph nodes (4). With continued time on HAART, naive CD4 lymphocytes also contribute to the increase in CD4 cell numbers.

In addition to antiviral drug regimens containing protease inhibitors, regimens containing the potent NNRTIs efavirenz, AZT, and 3TC are also highly effective at suppressing HIV-1 replication for a prolonged period of time. In the 48-week Dupont 006 trial, the efavirenz, AZT, and 3TC combination was superior to indinavir, AZT, and 3TC in the number of patients who were suppressed below the limit of detection of 50 copies per milliliter of plasma (342). It is important to keep in mind that this was an open trial, where patients knew the regimens that they were receiving and the group taking indinavir, AZT, and 3TC needed to take a very high number of pills every day, and this was associated with a very high drop-out rate (342).

Long-term suppression of HIV-1 RNA levels has been most convincingly demonstrated for patients receiving the protease inhibitor indinavir and two nucleoside RT inhibitors, such as AZT and 3TC, or d4T and 3TC. It is clear that other regimens containing NNRTIs, such as efavirenz, AZT, and 3TC (Dupont 006 Study) (342), or nevirapine and d4T and ddI (INCAS Study) (278), can also suppress HIV-1 RNA for considerable periods. Whether the duration of suppression will be as long as that seen in patients on protease-inhibitor-containing regimens is the subject of ongoing clinical trials. Regimens containing three nucleoside RT inhibitors such as AZT, 3TC, and abacavir are also able to suppress HIV-RNA effectively, but the results of longer-term suppression studies are awaited. A clear finding emerging from all of these studies is that replication-competent HIV-1 remains in resting memory CD4 lymphocytes in spite of plasma suppression by highly active antiretroviral therapy (48,130,391). The amount of virus detected in plasma may be undetectable (less than 50 copies of HIV-1 RNA per milliliter of plasma), but replication-competent virus is still present in the patient.

HIV-1 is likely to accrue drug-resistant mutations as long as it is replicating. With prolonged time, increased replication is likely to result in virologic failure in spite of antiretroviral therapy. When viral rebound does occur, the virus is usually not resistant to all of the components of active antiretroviral treatment, but primarily to the RT inhibitors (93,168). Since the rebounding HIV remains sensitive to the protease inhibitors, there is hope that treatment strategies based on intensifying the protease part of the combination regimen will be successful at lowering viral RNA (64).

A major success of antiretroviral therapy has been achieved in the ability of Zidovudine to decrease the rate of transmission of HIV-1 from mother to infant by almost 70% (65A,337A). A double-blind, placebo-controlled trial enrolled HIV-1–infected women with more than 200 CD4 T-cells/mm^3, who were between 14 to 34 weeks of gestation, and most were Zidovudine-naïve. The mothers took Zidovudine at 100 mg orally 5 times per day through pregnancy and received intravenous Zidovudine by continuous infusion of 1 mg/kg/hour during labor until delivery. The newborn took Zidovudine at 2 mg/kg orally every 6 hours for the first 6 weeks of life. This regimen decreased perinatal transmission of HIV-1 from 22.6% to 7.6%. The benefits of Zidovudine could not be fully explained by reductions in maternal viral load (337A). Another regimen, studied in African women, of one capsule of nevirapine to a mother during labor and one pill of nevirapine to the newborn in the first 72 hours of life was successful in reducing transmission by 50%. The effects of combination antiretroviral therapy on decreasing perinatal transmission are still under study.

In many regimens that show the capacity to decrease HIV-1 RNA levels in plasma to very low levels, replication-competent virus remains. When antiretroviral therapy is stopped, viral rebound will occur quickly. The source of rebounding virus may not be predicted by virus present in latent reservoirs—it may emerge from unknown sources. The current goal of antiretroviral therapy is to find new therapies that act on other virologic targets, such as viral entry or the integration of viral provirus by integrase, to suppress viral replication for many years.

HEPATITIS B

The human hepatitis B (HBV) is a small DNA virus that is hepatotropic and infects only humans. Infected humans are the only known reservoir of the virus (250). Similar viruses are found in American woodchucks, ground squirrels in California, Peking ducks in China, and some other rodent hosts (250,256,350). The HBV genome, one of the smallest of all known or animal viruses, consists of a 3,200-base-pair, double-stranded, circular DNA molecule that contains a single-stranded region of different lengths of viral DNA molecules (189,225). The virion DNA molecules are packaged into virions before viral DNA replication is complete in the synthesis of the plus strand. Viral DNA replication by RT is a unique mechanism for a DNA virus, and it is shared by the hepadnaviruses and the cauliflower mosaic virus (357). The similarity in replication mechanisms and the nucleotide sequence homology between HBV, retroviruses, and cauliflower mosaic virus indicate a phylogenetic relationship between these virus families (270,357). When HBV infects cells, the relaxed circular virion DNA genome is converted to a closed circular DNA, which serves as a template for viral transcription. This is accomplished by the product of the polymerase gene (which makes up 75% of the viral genome), a 90-kd polypeptide that possesses RT DNA polymerase and ribonuclease H activities. The polymerase protein also serves as a primer for DNA minus-strand synthesis (23,349).

The long RNA transcript and the RT product of the *pol* gene are encapsidated in immature core particles, and viral DNA synthesis takes place by reverse transcription of this long transcript in the cytoplasm of infected cells (349). Unlike HIV, HBV does not possess an integrase enzyme. HBV DNA is found in infected liver as an episomal form involved in virus replication, and it is also integrated in cellular DNA. It is not known whether viral integration occurs in all or some virus-infected cells. Without an integrase for orderly integration of the viral genome, integrated viral DNA is found at many random cellular DNA sites. Moreover, integrated HBV sequences are not preserved and small deletions or rearrangements are present in all integrants (316). The process of integration has been called illegitimate recombination (316), because HBV integration interrupts the viral genome so that replication is not possible from integrated sequences

in naturally infected cells.

Because of the strong similarities between the HBV DNA polymerase and the HIV RT, drugs that inhibit HIV RT also inhibit HBV RT-like functions and HBV replication. Lamivudine (3TC) is an effective inhibitor of HBV replication *in vitro* and it proved to inhibit HBV effectively in humans as well (96,222) (Fig. 9). In a 1-year trial of 358 patients with chronic HBV hepatitis in China, 56% of patients taking 100 mg of 3TC daily improved histologically, and 72% had a sustained normalization of aminotransferase activity (222). Sero conversion to the presence of antibody to HBe antigen (HBeAG), indicating an improvement in the immune protection against severe hepatitis progression, occurred in 16% of patients after 1 year and increased to 27% after 2 years of treatment (154,222). For patients who undergo a liver transplant for HBV-induced cirrhosis and liver failure, 3TC at 100 mg daily for 90 weeks reduces the risk of HBV reinfection (154).

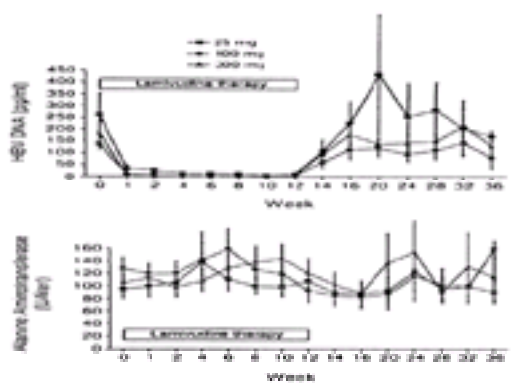

FIG. 9. Changes in mean (±SE) serum levels of HBV DNA (*upper panel*) and alanine aminotransferase (*lower panel*) in patients with chronic hepatitis B undergoing 12 weeks of therapy with 25, 100, or 300 mg of lamivudine per day. (Reprinted from ref. 96, with permission.)

The HBV RT pol enzyme contains the conserved YMDD motif, which is common to all of the avian and mammalian RT enzymes. When using 3TC for patients with chronic HBV infection, drug resistance can emerge (17). In a study of patients with chronic HBV infection and active hepatitis, 14% developed resistance after taking 3TC for 1 year and 38% developed resistance after 2 years (17). Although 3TC-resistant variants of HBV replicate less well *in vitro* (262), they have been associated with progressive liver disease in transplant recipients (17). Resistance is most commonly associated with an M552V change in the YMDD motif in the highly conserved catalytic center of the HBV RT where resistance to 3TC in the HIV RT occurs, the M184V change (17). For those HBV mutant viruses that possess the M552V mutation, a loss of fitness and a slower viral replication cycle have been reported (262). When an additional mutation at codon 526 of the polymerase occurs, replication fitness is restored to wild-type levels.

Another HIV RT inhibitor, adefovir, is also a potent inhibitor of HBV DNA replication. Adefovir is an experimental nucleotide analog that contains a phosphonate group; it is phosphorylated by cellular enzymes to adefovir triphosphate. Adefovir triphosphate is an active inhibitor of HBV DNA replication (Fig. 10) (87). HBV mutants that contain the M552V mutation and exhibit resistance to 3TC, retain susceptibility to adefovir (289). Thus, adefovir may provide an alternative treatment for those patients who have failed with lamivudine.

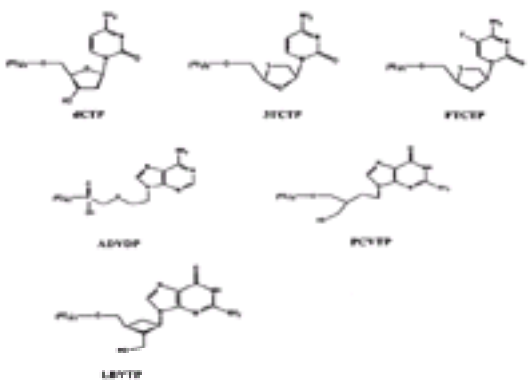

FIG. 10. Structure of the triphosphate of nucleosides and nucleotides that inhibit hepatitis B polymerase. dCTP, deoxycytosine triphosphate; 3TC-TP, lamivudine triphosphate; FTC-TP, emtricitabine triphosphate; ADV-TP, adefovir diphosphate; PCV-TP, penciclovir triphosphate; LBV-TP, lobucavir triphosphate. (Reprinted from Das K, et al. Molecular modeling and biochemical characterization reveal the mechanism of hepatitis B virus polymerase resistance to Lamivudine (3TC) and Emtrilitabine (FTC). *J Virol* 2001;75, with permission.)

Human a interferon or recombinant a interferon expressed from the human a interferon gene in *Escherichia coli* is a potent inhibitor of HBV replication and is useful as a treatment for chronic active hepatitis B infection. Until the development of 3TC, the use of human a interferon was the mainstay of treatment for chronic active hepatitis B infection in humans and provided one of the first examples of effective antiviral therapy for any disease. With interferon treatment, about 30% of patients exhibit a dramatic decrease in circulating HBV DNA, and improvement in liver function tests and histology on follow-up liver biopsies. In some patients, these favorable responses are maintained when treatment is discontinued. The difficulty is that the favorable outcome is observed in only a small percentage of patients, and the benefits of treatment are usually short-lived. The side effects of interferon therapy are frequently severe, with flu-like

illness characterized by fatigue, headache, muscle aches, chills, and fever being commonplace. Other problems such as neuropsychiatric problems and the need for continuous parenteral therapy make treatment difficult. These side effects limit the ability of patients to tolerate therapy for an extended duration (260,281).

Human a interferons are a group of potent glycoproteins that have many actions in addition to antiviral activity. One of the first demonstrations of antiviral efficacy of a interferon was in the treatment of chronic HBV infection (282). Of the three major classes of human interferon (interferon-a, interferon-b, and interferon-g), only interferon-a is approved for treating viral infection. The gene for human interferon-a, which can be expressed in a recombinant form in *E. coli* in a cost-effective manner, is produced by two companies for human use (Roche and Schering). These are designated interferon-a2a and interferon-a2b, respectively. Both of these recombinant interferons are approved for treatment of chronic HBV hepatitis (see Fig. 10).

Interferons do not act directly on the virus but induce cellular proteins to produce an antiviral state. These include proteins that inhibit the synthesis of RNA, enzymes that cleave viral and cellular DNA, and proteins that alter cell membranes so that release of replicated virions is blocked (204,282). The use of subcutaneous or intramuscular interferon-a2a (3 million units) three times per week for 6 months in chronic HBV hepatitis patients produced a normalization of abnormal alanine aminotransferase (ALT) levels in 40% of patients, and a virologic response of decreasing HBV DNA levels in 30%. Within 6 months of treatment, however, 50% of patients relapsed (204). In another study of interferon-a2b treatment of chronic hepatitis B, subcutaneous injections three times per week for 16 weeks resulted in clearance of hepatitis e antigens and HBV DNA in 50% of patients compared to 7% of control subjects (282). The normalization of liver function tests and a loss of hepatitis B surface antigens occurred in fewer patients. Although interferon-a and 3TC are effective in treating chronic HBV infection, there is no evidence that combining these agents results in a higher response rate.

HEPATITIS C

The hepatitis C virus (HCV) is a positive sense single-stranded RNA virus with a lipid envelope. The virus is classified as a genus of the *Flavivirus* family, which also contains the yellow fever virus (95,309). The viral particles are 50 to 75 mm in diameter. Two types of viral particles have been described, a low-density one with a specific gravity of 1.03 g/mL, which is most infectious, and a high-density particle (1.2 g/mL), which is less infectious (309). The HCV genome contains a little more than 9,000 nucleotides and consists of a single open reading frame with nontranslated regions at the 5' and 3' ends. The HCV genome (Fig. 11) encodes structural proteins that comprise the core and envelope proteins, as well as nonstructural proteins required for viral replication.

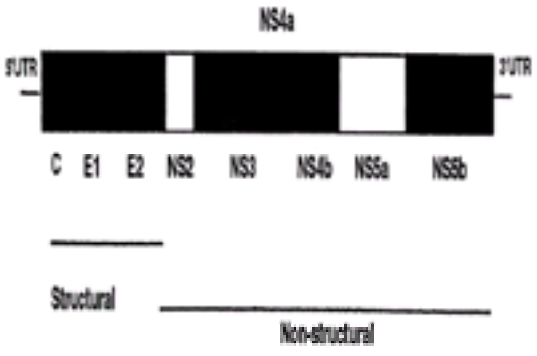

FIG. 11. Structure of HCV genome. C, core protein; E1 and E2, envelope proteins; NS2, cysteine protease; N3, serine protease, nucleotide triphosphatase, helicase; NS5A, putative interferon sensitivity–determining region; NS5B, RNA-dependent RNA polymerase. (Reprinted from ref. 100, with permission.)

In the middle of the viral genome, a single large HCV open reading frame encodes a large polyprotein that is processed into 10 polypeptides. The structural proteins consist of a capsid protein (C) and two envelope proteins, E1 and E2. The capsid protein binds to the 5' untranslated region of viral RNA and forms multimers, with several copies combining to form the capsid structure of the viral particle. The replication of HCV occurs mainly in the cytoplasm, but small forms of the capsid protein are transported to the nucleus. HCV core protein in the nucleus may interact with cell genes related to the cellular growth and contribute to HCV-mediated liver disease, such as hepatocellular carcinoma (95,309).

The HCV nonstructural proteins are designated NS2, NS3, NS4A, NS4B, NS5A, and NS5B. The NS2 protein is the cysteine protease, which is necessary to cleave NS2 and NS3 (239). The NS3 gene encodes three enzyme activities including serine protease, nucleotide triphosphatase, and helicase (211,239). The amino-terminal end of NS3 encodes the HCV serine protease, which is frequently found in a complex with NS4A and is necessary to cleave the polyprotein between NS8 and NS4A, and between NS4A and NS4B. The HCV protease is a serine protease, whereas the HIV-1 protease is an aspartate protease. The three-dimensional crystal structure of the HCV protease has been solved; it possesses a cleft in the active site where an inhibitor is likely to bind (239). Unfortunately, this cleft is very shallow, and it has proven very difficult to develop effective inhibitors to bind in this active site. The NSSA phosphoprotein is a replication component. The NS5B protein contains a GDD motif, which is characteristic of RNA-dependent RNA polymerases of other viruses such as influenza A; this enzyme may also prove an effective target for antiviral therapy (95,239).

Clinical Trials

The first active therapy for chronic hepatitis C infection was interferon-a. In studies of HCV treatment response to

interferon-a, treatment was defined as normalization of ALT levels and disappearance of HCV RNA from serum to levels below detection. A response was considered to be sustained if ALT levels remained normal and HCV RNA continued to be absent beyond 6 months after discontinuation of treatment. A nonresponder is a patient in whom HCV RNA never disappears from the serum. A patient who relapses is one in whom HCV RNA returns following an initial response.

Treatment with interferon-a at a dose of 3 million units subcutaneously three times a week for 6 months is effective in only a minority of patients. This regimen produced a biochemical response in 40% of patients (351), but it was usually short-lived, and only 15% to 20% of patients achieved a response that persisted for 6 months after treatment was completed. With a 6-month course of treatment, plasma HCV RNA levels fell to undetectable in 30% to 40% of patients. This was sustained in only 10% to 15% of patients at 1 year. By increasing the duration of therapy to 1 year, however, the sustained response rate could be increased to 20% to 25% (247). A meta-analysis of 20 randomized controlled clinical trials, which included only previously untreated patients who were randomized to at least 2 million units of interferon-a three times a week for 6 months, revealed an overall sustained response rate of only 14% (32). When the duration of therapy was extended for 12 to 24 months, a sustained response rate of 27% could be achieved ($p < .001$) (260). Thus, extended-duration interferon therapy is recommended as the standard treatment of chronic HCV in the United States (281). In those few patients who achieved a sustained response to interferon treatment, the long-term prognosis is very good. Most of them continued to have undetectable plasma HCV RNA levels and improving liver histology for the next 5 years (260).

Recently, it was shown that the covalent attachment of a 40-kd branched chain of polyethylene glycol (PEG) to interferon-a2a results in a compound (PEG-interferon-a2a) that has sustained absorption with a slower rate of clearance and a longer half-life than unmodified interferon-a2a. This form of PEG-interferon requires only a single dose of 180 mg subcutaneously once per week. In a study of 267 patients with chronic hepatitis, this favorable response at week 72 was more common in the PEG-interferon group (45%) than in the interferon group (25%) (401). The severity and frequency of adverse events were similar in the two groups and reflected the side effects typical of interferon-a. These findings were extended to a group of patients with chronic hepatitis C and cirrhosis, and again the main virologic end point was the proportion of patients with a sustained response of a hepatitis C RNA level of less than 100 copies per milliliter at week 72 (183). Those patients with cirrhosis or chronic hepatitis C received 180 mg of PEG-interferon subcutaneously once per week for 48 weeks, or 6 million units of interferon-a2a subcutaneously three times per week for 12 weeks, followed by 3 million units three times per week for 36 weeks. The PEG-interferon group was associated with a higher rate of virologic response, 69% compared to 28% at week 48, and 39% compared to 19% at week 72 (183).

The return to normal levels of serum ALT in patients with bridging fibrosis has been more difficult to achieve, but once-weekly administration of PEG-interferon-a2a was more efficacious in these patients than was a three-times-per-week regimen of unmodified interferon. The group of 271 patients with cirrhosis or bridging fibrosis received either 3 million units of interferon-a2a three times weekly (88 patients), 90 mg of PEG-interferon once weekly (91), or 180 mg of PEG-interferon (82) for 48 weeks, followed by an observation period of 24 weeks. HCV RNA was undetectable at week 72 in 8%, 15%, and 30% of patients treated with interferon, 90 mg of PEG-interferon, or 180 mg of PEG-interferon, respectively. Also at week 72, ALT had returned to normal in 15%, 20%, and 30%, respectively. There was also an improvement in liver biopsy histology in 184 patients with paired liver biopsies: 54% of patients receiving PEG-interferon showed improved liver histology by at least 2 points on a 22-point histology activity index. The histologic response correlated with a sustained virologic response at 72 weeks, as 80% of those receiving interferon-a2a had a histologic response, as did 100% of those receiving 90 mg of PEG-interferon-a2a and 88% of those receiving 180 mg PEG-interferon-a2a. HCV genotype influenced outcome: In patients with HCV genotype 1, the sustained virologic response was 2% for those receiving PEG-interferon (183). In patients with other than HCV 1 genotype, the sustained virologic response was 15% with interferon-a2a and 51% with 180 mg of PEG-interferon-a2a (183).

Interferon monotherapy, however, provides little hope of viral eradication in the majority of HCV-infected patients. The use of combination therapy for hepatitis C (as described for HIV-1) offers considerable promise. Ribavirin, a guanosine analog with an altered base, can be combined with interferon therapy to enhance biochemical and virologic benefit. Other combinations with interferon have been tried for HCV without benefit, including corticosteroids, nonsteroidal antiinflammatory drugs, quinoline antibiotics, amantadine, thymosin, granulocyte-macrophage colony-stimulating factor, and pentoxifylline (260).

Ribavirin monotherapy does not lower HCV RNA levels, but it yields improvements in liver histology and temporarily reduces levels of serum ALT in about 50% of patients with chronic hepatitis C infection (260). The effects of ribavirin on liver injury do not appear to be mediated by its antiviral properties (247). In fact, its mechanism of action against hepatitis C virus is not completely understood, but it inhibits replication of both RNA and DNA viruses, including several that are related to HCV. Ribavirin decreases mortality in patients infected with Lassa fever virus (259), and an aerosol formulation benefits infants with respiratory syncytial virus (RSV) pneumonia (161) (although it is no longer used to treat RSV routinely). Ribavirin inhibits inosine monophosphate dehydrogenase in Sindbis virus–infected cells, leading to a decrease in guanosine triphosphate concentrations, and it inhibits the RNA-dependent RNA polymerase of influenza A and influenza B viruses (120,344). Ribavirin also inhibits the 5' cap structure on vaccinia messenger RNA (155). Thus, ribavirin may have several antiviral mechanisms, and this may contribute to the lack of development of ribavirin-resistance mutants following passage in the presence of the drug (344). Ribavirin also has immunomodulatory actions, including macrophage inhibition, a decrease in CD4 cells in patients with AIDS, and alteration of the cytokine balance (223,310).

In three randomized trials, the combination of ribavirin plus interferon-a was compared to interferon monotherapy in treatment-naive patients. Each trial was small in sample size, but very consistent results were shown (37,223,310). In the largest of the three studies (100 patients), the sustained virologic response was 42% in the interferon–ribavirin combination group, compared to 20% in the interferon monotherapy group ($p = .03$). In a study of 40 patients treated for 48 weeks with interferon and ribavirin, a virologic response of 43% was observed, compared to 6% in those receiving interferon monotherapy. This response was maintained without additional therapy when observed at 96 weeks (223). In the third study of 30 patients, 43% of patients treated with the interferon–ribavirin combination had a sustained virologic

response, compared to 7% of the monotherapy patients (37).

These initial studies showed the promise of an interferon–ribavirin combination therapy regimen and led to a large multicenter trial in the United States. The aim of this trial was to compare safety and efficacy of interferon alone versus interferon plus ribavirin as initial treatment for chronic hepatitis C, and to determine the optimal duration of combination therapy (261). The study was carried out in 912 adult patients with chronic hepatitis C, elevated ALT values, detectable serum HCV RNA, and a liver biopsy showing changes of chronic hepatitis C infection within 12 months of study entry. Patients were randomized into four regimens: interferon plus placebo for 24 weeks (231 patients), interferon plus placebo for 48 weeks (225 patients), interferon plus ribavirin for 24 weeks (228 patients), and interferon plus ribavirin for 48 weeks (228 patients). Patients weighing less than 75 kg received ribavirin at 1,000 mg/day, and those weighing more than 75 kg received ribavirin at 1,200 mg/day (261). Interferon was given in the standard dose of 3 million units subcutaneously three times per week. The main endpoint of the study was a sustained virologic response, defined as the absence of serum HCV RNA at 24 weeks after treatment. Normalization of ALT levels and improvement in histology on liver biopsy were important secondary endpoints. Seventy percent of patients in the study were infected with HCV genotype 1.

The results showed that the sustained virologic response rate was higher in patients treated with interferon and ribavirin for 48 weeks (38%) or 24 weeks (31%) than in those receiving interferon-a alone for 48 weeks (13%) or 24 weeks (6%). The relapse after treatment was also less frequent in those receiving combination treatment, being 42% at 24 weeks and 28% at 48 weeks (260,261). Improvement was more common in both groups receiving interferon–ribavirin combination therapy than in the monotherapy group treated for 48 weeks ($p < .001$). Histologic improvement was the greatest in patients receiving combination therapy for 48 weeks. The biochemical responses, particularly reduction in ALT levels, were very similar to the virologic response rates. Histologic improvement was seen in all four treatment groups (260).

HCV genotype differences are of clinical importance, as they may reflect differences in response to therapy (83). In patients with genotype 1, sustained virologic response rates with combination therapy were fourfold higher than with interferon alone after 48 weeks of treatment (261). Moreover, the response in patients with genotype 1 treated with combination therapy for 48 weeks was significantly more gratifying than at 24 weeks ($p = .01$). The sustained virologic response rates for 24 and 48 weeks were similar for patients with genotype other than 1, indicating that 24 weeks of therapy may be adequate for such patients (261). The differences in responses among treatment-naive patients with genotypes 1 versus 2 or 3 is striking. In 70% of patients with genotype 2 or 3 they experienced a sustained virologic response to combination therapy with 24 weeks of treatment. In contrast, 12 months of combination therapy produced a sustained response in only 30% of patients infected with genotype 1 (260,261). Genotypic differences also have a significant effect on the response of relapsing patients to interferon-a2b and ribavirin: 75% of relapsing patients with genotype 2 or 3 achieve a sustained response, but only 30% of relapsing patients with genotype 1 achieve a response.

Like HIV-1, HCV isolates consist of groups of related genetic variants, which are designated quasi-species. These variants have only minor sequence differences, consisting of less than 5% of the viral genome (83). The response to therapy according to HCV genotype indicates that genotypic differences are clinically significant, but current evidence does not suggest that quasi-species are clinically important. Testing for quasi-species in an individual patient is not yet practical in clinical settings. In addition, since no specific therapies for genotype 1 exist and some patients with known genotype 1 do respond to therapy, genotyping is not recommended to predict eligibility for treatment. As shown previously, patients with genotypes other than HCV-1 respond better to PEG-interferon than do those with genotype 1. Studies of PEG-interferon and ribavirin combination are in progress.

A given inoculum of HCV may contain a large number of quasi-species, and not all quasi-species are infectious. The quasi-species possess a range of replicative efficiencies (83) and the diversity of quasi-species is related to the size of the original inoculum infecting that person (316). Some studies suggest that the greater diversity in HCV quasi-species correlates with more severe liver disease (298,358). The reasons for this observation have not been defined, but it has been suggested that the greater diversity results in an increased number of targets for cytotoxic T lymphocytes to attack the liver (358). Interferon therapy reduces the heterogeneity of quasi-species as viral replication decreases (358). Some quasi-species appear to be more responsive to interferon-a therapy than others, and after interferon therapy different quasi-species may become dominant. Resistance to interferon has not been clearly demonstrated and the change in quasi-species is usually interpreted as evidence that interferon therapy is effective (148,397). If patients have more than two major quasi-species, however, a response to interferon therapy may be poor (148). In the use of combination therapy to treat HCV infection, it is clear that the interferon part of the treatment is responsible for the antiviral effects, because ribavirin treatment alone has not been effective at decreasing HCV replication and decreasing plasma HCV RNA.

INFLUENZA A AND B

Influenza A and B viruses are members of the family *Orthomyxoviridae*, and they produce major worldwide epidemics. The influenza viruses contain single-stranded, negative-sense RNA genomes organized into eight individual segments (359). The RNA molecules are transcribed separately and are packaged into viral ribonucleoprotein particle (vRNPs) in association with a matrix protein (M1) and a nucleocapsid protein (NP). There is a nonglycosolated integral membrane (M2) protein that is expressed on the surface of infected cells and is present in limited amounts in virions (224,255,301). The transmembrane domain of the M2 protein is highly conserved for all human and avian strains of influenza A virus, and this protein functions as a channel for hydrogen ions (Fig. 12) (301). This channel allows hydrogen ions to flow from the endosome into the virion interior before fusion between the viral envelope and the endosome membrane can occur. A decreased pH within the virion may induce conformational changes in the nucleocapsid protein, allowing disruption of the vRNP-M1 complex and permitting transport of the vRNP into the nucleus during early infection (254,255). Influenza viruses also encode an RNA-dependent RNA polymerase, which is essential for viral RNA replication. This enzyme is another target for antiviral drugs.

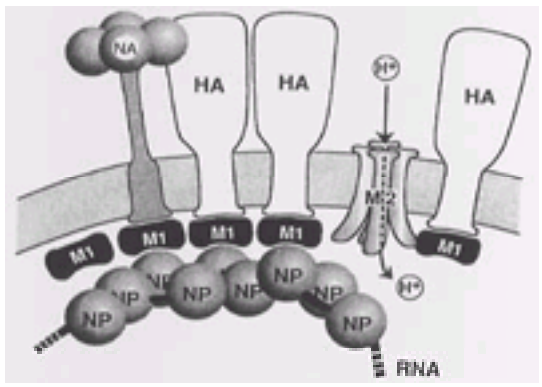

FIG. 12. Molecular interactions between structural components of influenza virus relevant to the mechanism of action of amantadine and rimantadine. (Reprinted from Fields BN, Knipe DM, Howley PM, et al., eds. *Fields Virology*, 3rd ed. Philadelphia: Lippincott-Raven, 1996:434, with permission.)

The influenza virus attaches by viral hemagglutinin (HA) to cells of the respiratory epithelium. Another viral surface protein, neuraminidase, cleaves terminal sialic acid residues and destroys receptors recognized by the HA (60). This action is essential for release of virus from infected cells, prevention of viral aggregation, and effective spread within the respiratory tract. Inhibition of the enzymatic function of the influenza neuraminidase by neuraminidase inhibitors has proven to provide a powerful approach to treatment of influenza and prevention of spread within families exposed to influenza (177,178,180,232).

Amantadine and Rimantadine

The first drug to be useful in preventing symptoms of influenza A infection was amantadine. It was joined later by a closely related drug that was better tolerated—rimantadine. Amantadine and rimantadine are symmetric tricyclic amines that specifically inhibit influenza A viruses at low concentration. By plaque reduction assay, the inhibitory concentrations of the drugs range from 0.1 to 0.4 μg/mL for many human influenza A viruses including H_1N_1, H_2N_2, and H_3N_2 subtypes (29,176). Both drugs work by similar mechanisms of action, but rimantadine is 4 to 10 times more active *in vitro* than amantadine (29). Both agents are active in prophylaxis and treatment of influenza A infection.

Amantadine and rimantadine exhibit two concentration-dependent mechanisms of action against influenza A viruses (171). The mechanism that appears to be primary and that occurs at a very low concentration is the inhibition of the ion channel formed by the influenza A matrix protein M2 (171,372). Inhibition of this ion channel involves inhibition of the M2-mediated reduction in pH, which is essential for viral uncoating and disassembly during the process of endocytosis (49). The other effect of amantadine occurs during the late steps of influenza replication, particularly for the H_7 subtype, in which assembly is mediated by a change in the pH of the trans-Golgi apparatus. This pH change induces an alteration in the HA during transport across the Golgi, which facilitates virion assembly (49,169,172). In the presence of amantadine, the intraluminal pH is decreased so that the HA undergoes a premature acid-induced conformational change, which decreases release of infectious viral particles (49). This has been shown especially for the fowl plague virus, a subtype of influenza A for which the M2 protein is known to regulate the pH of intracellular compartments and stabilize the HA into the native form that it assumes at neutral pH (287).

Resistance

The understanding of the mechanism of action of amantadine and rimantadine has been promoted by studies with amantadine-resistant influenza mutants. These have been selected both *in vitro* and *in vivo* and exhibit widespread cross-resistance to rimantadine (16,331). Resistance, as manifested by a 100-fold increase in the IC_{50}, is associated with single amino acid substitutions at critical points in the transmembrane region of the M2 protein (16,169).

Single nucleotide changes are observed at residues 26, 27, 30, 31, and 34, with mutations at codon 31 being the most common (16,169,331). The amino acid residues at these positions line the ion channel and thus may form the binding site for amantadine and rimantadine. The drugs appear to form “bird cage” structure that block the channel and prevent ion flow. Residues associated with drug-resistance residues prevent the drugs from binding in the channel. Usually, a single amino acid change is sufficient to cause resistance to amantadine, but mixed populations containing independent resistance mutations have been described (169,176,331). The same mutations in M2 that abolish drug susceptibility will abolish drug susceptibility to the early and late effects of amantadine. In avian strains of influenza A, the resistant viruses are virulent, genetically stable, and able to compete effectively with wild-type virus, and transmission of drug-resistant virus has been documented after therapy is stopped (13,179).

Amantadine- and rimantadine-resistant viruses emerge rapidly during virus passage *in vitro*, and in people within a few days of initiating therapy. Close to 30% of drug-treated children and adults shed resistant virus by day 5 of treatment (175,179). Immunocompetent persons shed resistant virus and still resolve their illness promptly, whereas immunocompromised patients can shed viruses for a prolonged period and remain ill (175,179). Transmission of resistant virus has occurred in household contacts of rimantadine-treated index cases and in nursing home residents who have received amantadine and rimantadine (175,257). These resistant variants can cause typical influenza illnesses. It is suggested that contact should be avoided between treated patients and susceptible high-risk individuals. For amantadine- and rimantadine-resistant viruses, ribavirin and the neuraminidase inhibitors zanamivir and oseltamivir are active *in vitro* and in patients.

Clinical Trials

The clinical usefulness of amantadine and rimantadine is limited primarily to prevention of influenza A virus infection (99,105). They are less useful in treatment of established influenza A infections (233,362). Amantadine and rimantadine are both used at a dose of 200 mg per day in adults, and they are between 70% and 90% protective against the development of clinical illness caused by strains of influenza A, including the worldwide pandemic strains (173). Prophylaxis has also been effective in preventing nosocomial influenza and in curtailing outbreaks in nursing homes (3), and protection against influenza A clinical illness adds to the protection afforded by influenza A subunit vaccine (236). Rimantadine at 5 mg/kg per day decreases the risk of influenza A in school-age children and possibly their household family contacts (68). Treating an index case before amantadine or rimantadine is given to other family members may select for resistant viruses, which spread to other members of the family and make their treatment ineffective. Therefore, all high-risk members of a family should probably receive treatment at the same time.

Amantadine and rimantadine are also effective for uncomplicated influenza A illnesses, especially if treatment is started early (18,170,362,396). It is uncertain whether treatment is effective at reducing complications in high-risk patients or whether it is useful in elderly patients with chronic pulmonary disease. In otherwise healthy individuals, when therapy is begun within 2 days of the onset of symptoms, a dose of 200 mg per day may reduce the duration of fever and systemic symptoms by 1 or 2 days. Some studies also report that duration of virus shedding is decreased by treatment, but this is not universally accepted. In elderly patients, therapeutic benefits were observed in treatment with rimantadine (18). In children with influenza A H_3N_2 subtype infection, rimantadine treatment reduced fever and virus titers during the first 2 days of treatment, but the rimantadine-treated patients were noted to have a longer total duration of virus shedding (160). In summary, the main use of rimantadine and amantadine has been in prophylaxis of influenza illness, and treatment benefits remain controversial.

Zanamivir and Oseltamivir

The viral neuraminidase enzyme is essential for pathogenicity of both influenza A and B virus. This enzyme cleaves the terminal sialic acid residues on cells, destroys the viral HA receptors, and permits the spread of virus in the respiratory tract. Zanamivir (4-guanidino-neu-5Ac2en, GG167) is a sialic acid analog that is a potent inhibitor of the influenza A and B virus neuraminidases (367). Zanamivir must be administered intravenously or by aerosol spray. A transition-state analog of sialic acid cleavage (GS 4071) was identified with antiviral potency and activity similar to that of zanamivir (232,264). Oseltamivir (GS 4104) is an orally bioavailable prodrug of this analog (264). Both zanamivir and oseltamivir are competitive, reversible inhibitors of the influenza viral neuraminidases and inhibit these enzymes at very low concentrations (232,394). The main differences between these drugs is that zanamivir is administered as an aerosol spray to upper airways, whereas oseltamivir is administered orally. Both drugs are nontoxic for cells and inhibit the neuraminidase from mammalian sources or other pathogens at million-fold higher concentration. The drugs inhibit the purified influenza neuraminidase at concentrations from 0.6 to 8μM, whereas a broader range of concentrations, from 0.002μM to 10μM, is required to inhibit influenza A and B replication in cell culture (394).

The active site of the influenza neuraminidase is designed to bind sialic acid, which is cleaved by the enzyme. Sialic acid, through its glycerol and carboxylate groups, binds to amino acids in the active site. Zanamivir replaces the hydroxyl group of sialic acid with a larger, positively charged guanidine, which forms strong attachments to two negatively charged amino acids in the cleft of the active site. Oseltamivir also binds to a hydrophobic pocket in the active site (Fig. 13). Because zanamivir and oseltamivir bind to different regions of the active site of neuraminidase, the active site that decreases binding to zanamivir may still be inhibited by oseltamivir (156,232). For example, the mutant Glu119Gly, with decreased binding of zanamivir, is still inhibited by oseltamivir (156,246). A mutation in part of a catalytic triad of neuraminidase at Arg292Lys may exhibit decreased inhibition by both drugs, so some mutations do confer cross-resistance (156). These altered neuraminidase enzymes may have reduced activity or stability and result in a less fit virus (156,246). Influenza viruses with these altered enzymes manifest decreased infectivity in animals (156,246).

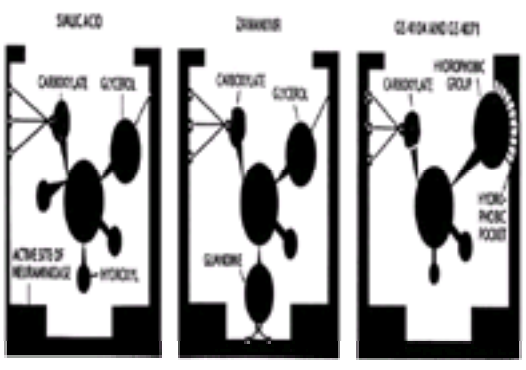

FIG. 13. The active site of influenza A neuraminidase, showing the binding of sialic acid, the normal substrate, and the binding of zanamivir and GS 4071 (oseltamivir), two drugs that clog the active site. (Reprinted from ref. 232, with permission.)

Clinical Trials

The use of zanamivir and oseltamivir for prevention of influenza A has proven highly effective. Zanamivir prevents experimentally induced influenza in humans when virus is administered intranasally, and against naturally occurring influenza when given by inhalation once daily (279). Inhaled zanamivir provided a 67% rate of protection against

influenza-associated illness and 32% protection against infection during a 4-week trial of prophylaxis in young adults (279). Oral oseltamivir also proved effective in preventing naturally occurring influenza in a large study of 1,559 healthy nonimmunized adults who received oseltamivir at 75 mg, either once or twice daily for 6 weeks during a peak period of influenza activity (174). In the patients assigned to once or twice daily oseltamivir, the risk of developing influenza was 1.2% or 1.3%, compared to 4.8% in subjects assigned to placebo (p = .001). Thus, the protective efficacy of oseltamivir was 74%. For culture-proven influenza, the average protective effect of oseltamivir in preventing influenza efficacy was 87%. Moreover, oseltamivir led to a 50% decrease in the proportion of subjects with symptomatic or asymptomatic laboratory-confirmed influenza (174).

Inhaled zanamivir was also studied for the prevention of influenza A in families where amantadine and rimantadine have been ineffective because of transmission of drug-resistant variants. A double-blind placebo-controlled trial of inhaled zanamivir for the treatment of the index case and prevention of influenza in families showed a 79% reduction in the proportion of families with at least one affected contact (177). Zanamivir protected against influenza A and influenza B. Among the laboratory-confirmed and treated influenza cases, the median duration of symptoms was 2.5 days shorter in the zanamivir group than in the placebo group. The study concluded that prophylactic treatment of family members with once-daily inhaled zanamivir was well tolerated and prevented the development of influenza when combined with treatment of index cases. The emergence of resistant influenza variants was not detected (177).

The neuraminidase inhibitors zanamivir and oseltamivir are effective in treating influenza and offer the possibility of treating severe disease. In a study of oseltamivir in treating acute influenza, the clinical benefits of treatment were statistically significant in both young and middle-aged adults (360). Oseltamivir was well tolerated, but in this one study 12% of subjects taking it daily experienced nausea compared to 7.1% in the placebo group (360). Oseltamivir was most effective if used within 2 days of symptoms. In the treatment, 95% of patients were infected with influenza A, 3% with influenza B, and 2% were indeterminate, and all benefited from treatment (360). In a random controlled trial of experimental human influenza, oseltamivir treatment improved the symptom score, reducing days of fever and malaise and decreasing viral shedding (180). Inhaled zanamivir treatment has also shown efficacy in studies involving adults, adolescents, and children 5 years of age or older (178). Zanamivir has been effective at treating influenza in the elderly. The main side effect of inhaled zanamivir has been bronchospasm in elderly patients.

To prevent influenza in families, treatment of index cases with inhaled zanamivir shortens the median duration of symptoms by 2.5 days (178,359). Zanamivir does not impair the humoral immune response to inactivated influenza vaccine (177), and it is likely that zanamivir and oseltamivir can be used in conjunction with vaccination. Further studies need to be carried out in immunocompromised patients to determine that these drugs can act synergistically with vaccination against influenza. These two neuraminidase inhibitors provide treatment possibilities against influenza disease and may provide protection against the influenza pandemic strains.

FUTURE DIRECTIONS

The future of antiviral therapy will consist of a series of step-by-step advances against each viral pathogen, beginning with drug design, and followed by knowledge of mechanisms, resistance, and pharmacokinetics of each drug. The great successes so far have focused on drugs that inhibit viral enzymes, and this is likely to continue as the crystal structures of the herpesvirus protease enzymes have been determined, indicating that effective drugs may soon follow. The future may also show progress in preventing viral protein–protein interactions, such as blocking the herpes polymerase enzyme from binding to its accessory protein, or preventing the conformational change in the gp41 of HIV-1 needed for viral entry. Small molecules that mimic the three-dimensional structure of viral peptides will also show progress. The use of antisense oligonucleotides is just at the beginning and will continue to be an active area of investigation and promise. A newly developed antisense phosphorothiolate oligonucleotide (fomivirsen), which is complimentary to RNA of the human CMV major immediate-early gene, has been approved for topical treatment of CMV retinitis. This represents the first antisense molecule with antiviral activity approved for clinical use, and others may soon follow.

Several important human viral pathogens will continue to provide active areas for development of antiviral therapy. The human papillomas virus, which causes cervical cancer, may respond to antiviral therapy if targets can be identified. The human rhinoviruses, which cause the common cold, require the action of a viral protease for maturation to an infectious virion, and the viral protease has long been a target for development of effective therapy.

Effective protease inhibitors for rhinoviruses and enteroviruses appear close to receiving approval for human use. Although the protease for hepatitis C virus has not been shown to be an effective target for antiretroviral therapy, the RNA-dependent polymerase of hepatitis C may lead to effective antiviral therapy against this virus. The ability to develop new forms of antiviral agents such as Kaletra, valganciclovir, and PEG-interferon, which possess enhanced pharmacokinetic plasma levels, establishes the role of medicinal chemists in providing new antiviral agents. The continued collaboration between chemists, structural biologists, virologists, and experts in clinical trials will open new paths in the development of antiviral therapies.

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CHAPTER 16 Immunization Against Viral Diseases

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VIRAL ANTIGENS RECOGNIZED BY THE IMMUNE SYSTEM

Resistance to virus infection depends on the development of an immune response to antigens present on the surface of virions or virus-infected cells. Immunologic responses to viral glycoproteins or protein antigens displayed on the surface of virions or virus-infected cells are needed to effectively eliminate those viruses or cells. Responses to the nonsurface viral antigens are thought to play only a minor role in resistance to infection. Important historical evidence supporting these conclusions was acquired in studies of the 1957 Asian influenza pandemic and the 1977 Russian influenza epidemic. The 1957 Asian influenza A (H2N2) virus contained surface glycoproteins (the hemagglutinin and the neuraminidase) that were novel—that is, the human population had not been exposed to strains containing these or related surface antigens. In contrast, the major nonsurface proteins of the new virus, the nucleoprotein and the matrix protein, were closely related to corresponding antigens of previous strains of subtype H1N1, to which most individuals had been exposed. Despite this prior and (in many instances) recent experience with related nonsurface antigens, the Asian influenza A virus spread rapidly without apparent immunologically mediated restriction and caused a major pandemic (207). In contrast to this experience, observations following the reemergence of the H1N1 virus in 1977 indicated that homotypic humoral immunity provided a high level of resistance that lasted over 20 years.

Additional evidence for the importance of the surface glycoproteins of influenza A virus in immunity is provided by passive and active immunization studies in mice. Monoclonal antibodies directed against the hemagglutinin (HA) or the neuraminidase (NA) protected mice against challenge with virulent wild-type virus, whereas monoclonal antibodies specific for the internal nucleoprotein or matrix protein did not alter the course of disease (207). Analysis of a set of recombinant vaccinia viruses, each expressing one of the 10 known viral gene products of the influenza A virus, showed that only recombinants expressing the HA or the NA glycoprotein induced resistance to virus challenge (81). These and other observations with paramyxoviruses (291) indicate that a successful strategy of immunoprophylaxis against many virus infections requires the generation of an immune response to the surface antigens displayed on virions and on virus-infected cells. To facilitate discussion of these important proteins or glycoproteins, we will refer to them as *protective* antigens. Identification of protective antigens in the manner just described represents an important first step in the development of effective viral vaccines, whether nonliving or live.

The surface glycoproteins of enveloped viruses and the capsid proteins of nonenveloped viruses are generally the major protective antigens, and their important role in resistance has been confirmed using monoclonal antibodies directed against these antigens. The following generalizations can be made about the nature of protective antigens and about the antibody response to them. First, and most important, antibodies predominantly recognize conformational epitopes on protective antigens. Such conformational epitopes are very difficult to mimic with peptides or other forms of an immunogen in which the protective antigenic sites are denatured. Thus, an immunogen that assumes the structure of the

native protein induces antibodies that best recognize the conformational epitopes and that mediate immunity most effectively.

Second, antibodies recognize many antigenic sites on viral proteins. Within each antigenic site, there are multiple epitopes. For example, the parainfluenza virus attachment glycoprotein (HN) has at least six antigenic sites, three of which are recognized by neutralizing antibodies (51). After infection, not all individuals develop antibodies to each antigenic site of the infecting virus (50). After one or more reinfections, the antibody response broadens to recognize most of the antigenic sites. These findings provide a partial explanation for the failure of immunization to induce complete immunity in certain circumstances, and they also provide a rationale for recommending reimmunization to achieve optimal protection.

Third, immunoglobulin (Ig) molecules generally do not have direct access to most of the functional sites on viral protective antigens, such as (a) the receptor-binding site; (b) sites with enzymatic activity; and (c) fusion domains, which are mostly buried within the protein structure of the protective antigen. As a consequence, these sites remain relatively inaccessible to the antiviral activities of antibodies. Therefore, antibodies usually exert their antiviral activities by mechanisms other than direct inactivation of functional sites on the protective antigens. Instead, antibodies most often act directly or indirectly to impede attachment of virus to the cell or to prevent uncoating of virus that has attached to or entered the cell. In addition to viral structural proteins, protective immune responses can be directed at nonstructural proteins displayed on the surface of infected cells such as the NS1 protein of dengue virus or the T antigen of simian virus 40 (SV40).

IMMUNE ECHANISMS THAT ELIMINATE VIRUS OR VIRUS-INFECTED CELLS

Immune mechanisms have evolved to protect the host against a diverse assortment of viral pathogens. The major purpose of the immune response is to inactivate free virus and to eliminate infected cells that have the potential to release infectious virus. It is important to emphasize at this juncture that the various cellular and humoral antiviral immune mechanisms function together to eliminate virus after its first encounter with the host, as well as to protect the host against reinfection (11,69,180,207). Thus, there is considerable redundancy inherent in immunity to viruses. For example, both CD8+ T cells and antibody plus complement can lyse virus-infected cells. Another well-studied example of this redundancy involves CD4+ and CD8+ T cells, which can separately effect clearance of virus, as shown in studies of influenza A virus in mice. Animals lacking CD8+ T cells readily clear influenza virus from the lungs, indicating that CD4+ T cells and antibody are sufficient for viral clearance (158,265). The therapeutic efficacy of antibodies acting in the absence of other immune functions has also been demonstrated in experimental influenza A virus infection of mice. Antibodies to the influenza A virus surface HA cleared virus from the lungs of persistently infected several combined immunodeficient (SCID) mice (265). CD4+ T cell–deficient animals also readily clear influenza virus from the lungs by a CD8+ T cell–dependent mechanism (158). The popular concept that CD8+ T cells are solely responsible for viral clearance has been replaced with the knowledge that CD4+ T cells (plus the antibodies secreted by B cells for which they provide help) and CD8+ T cells can mediate viral clearance separately and that the relative importance of these two arms of the immune response varies in the resolution of particular viral infections. Even in the case of lymphocytic choriomeningitis virus (LCMV), the prototype arenavirus that has long been considered the paradigm for CD8+ T cell–mediated resistance, antibodies and CD4+ T cells each make a significant contribution to the resolution of primary viral infection, and antibodies play a major role in resistance to reinfection (11). Although it is clear that CD4+ and CD8+ T cells do play an important role in the resolution of viral infections, the major mediator of resistance to reinfection is antibody.

Cellular Immunity to Viruses

Major histocompatibility complex (MHC) class I–restricted CD8+ cytotoxic T lymphocytes (CTLs) and MHC class II–restricted CD4+ helper T cells (T_H cells) each function independently as antigen-specific antiviral effector cells.

CTLs

Cytotoxic T lymphocytes are the major T-cell effectors of antiviral activity. The CTL receptor recognizes a short peptide derived from an endogenously produced viral protein in the context of the MHC class I b2-microglobulin heterodimer expressed on the surface of an infected cell. Because the MHC class I restriction elements are present in a relatively high concentration on almost all cells except neurons, CTLs can exert their antiviral effects against most infected cells. In most instances, antigen presentation by the MHC class I b2-microglobulin heterodimer is restricted to viral peptides that are produced and processed during infection. Hence, the function of CTLs is not to prevent infection (87) but to eliminate cells already infected or, alternatively, to inhibit virus replication by the elaboration of antiviral cytokines (91,197,306). The net effect of CTL activity is to prevent further spread of virus and to terminate infection in cells that are already infected. The importance of CTLs in recovery from viral infection is indicated by the diverse strategies that viruses use to escape from CTL immunity (279). While CTL antiviral activity is generally associated with the clearance of virus and the reduction of virus-associated pathology, disease enhancement can be observed under experimental conditions in which CTLs have been passively transferred to viral antigen–bearing recipients (37,78,192).

The time course of CTL activation during viral infection in the lungs is consistent with its important role in clearance of primary virus infection. Primary (also called direct) CTL cytotoxic activity of pulmonary lymphocytes peaks early (day 7) during acute virus infection, such as that produced by influenza A virus or respiratory syncytial virus (RSV), and it declines to barely detectable levels by day 12 (6). Thus, restriction of virus replication mediated by CTLs is active early during the acute stage of virus infection, but this activity can wane rapidly (53). Residual memory CTLs were not able to accelerate the clearance of RSV after viral challenge on day 45 (149). These and other (87,284) observations suggest that the CTL component of the immune response is active primarily during the early phase of infection from the time just before the peak titer of virus is reached to the time virus-infected cells are cleared, and later these cells persist in an inactive (or memory) state that does not provide a high level of resistance to reinfection (87). For viruses that replicate rapidly in the host, such as the influenza viruses and paramyxoviruses, the proliferation of memory CTLs does not appear

to occur with sufficient rapidity to alter the peak titer of virus attained in the target organ (87). Because disease usually develops by the time the peak titer of virus is attained, it is not surprising that immunization with antigens that predominantly induce CTL activity without an accompanying stimulation of neutralizing antibodies is less effective in restricting virus replication and preventing illness than immunization that induces a sustained neutralizing antibody response (77,103).

Because of the important role of CTLs in clearance of established viral infections, the incorporation of CTL epitopes into viral vaccines has been considered a requirement. The following characteristics of the CTL response *in vivo* decrease the attractiveness of including CTL epitopes as the sole or main protective component of a viral vaccine. First, the ability of a CTL epitope to induce an immune response is MHC dependent. Because human MHC restriction elements are highly polymorphic, many protective CTL epitopes would have to be incorporated into a single vaccine to protect a large proportion of the population. Second, the duration of immunity mediated by CTLs following primary infection can be very short (10,87). However, some resistance to virus replication following reinfection can be mediated by CTLs (90,106), indicating that this arm of the immune response can restrict replication of virus during reinfection, but the level of restriction is generally weak. Third, there appears to be a limited number of protective CTL epitopes in most viruses (81,149,150). Thus, the number of protective CTL epitopes per MHC haplotype is small for many viruses. Fourth, viruses can escape from protective CTL responses by mutations within the CTL epitopes (190,232). Because of this, vaccinees whose resistance was induced by immunization with only one protective CTL epitope would be fully susceptible to infection with a CTL epitope escape mutant. Fifth, the protective effect of a CTL response can differ dramatically among CTL epitopes, some of which induce strong protection against disease, whereas others are only weakly protective (115). Thus, only those CTL epitopes capable of inducing a strong protective immune response could serve as suitable immunogens for inclusion in vaccines. Sixth, CTLs themselves can cause disease by an immunopathologic reaction in the target organs in which virus is replicating, indicating that a fine balance exists between protective efficacy and disease-enhancing capacity (37,78,192). Seventh, the induction of CTL responses using minimal peptide epitopes is problematic because peptides do not induce CTL responses efficiently. Finally, CTLs represent a second line of defense against infection because CTLs are effective only against infected cells, whereas antibodies can neutralize the infectivity of the virus and decrease the number of cells initially infected (87).

For these reasons, it is unlikely that CTL epitopes by themselves will replace native capsid proteins or surface glycoproteins in the formulation of viral vaccines. However, their inclusion in vaccines is expected to be beneficial. The induction of a CTL memory response by immunization might be helpful against viruses that replicate relatively slowly in their host or that cause persistent infection. In such situations, memory CTLs could be activated and amplified to an antiviral state and participate in the control of infection. It should be noted that live virus vaccines induce both antibodies and CTLs efficiently, whereas peptide, inactivated, or subunit virus vaccines as currently formulated are poor immunogens with respect to induction of CTLs.

T_H Cells

Helper T cells provide help to B cells, thereby augmenting the antibody response. However, T_H cells can also provide direct antiviral activity *in vivo* (117,170,194,305). The contribution to immunity made by the direct antiviral activity of T_H cells appears to be less than that of CTLs. This is consistent with the more limited distribution of the restricting element of T_H cells, namely the MHC class II, a,b heterodimer, which is present predominantly on lymphocytes and antigen-presenting cells such as macrophages and dendritic cells. In addition to having antiviral activity, T_H cells also can mediate immunopathology (5,179,297).

The T_H cell response to certain internal viral proteins may play a cooperative role in the development of effective resistance by augmenting the antibody response to a major protective antigen (258). For example, immunization with the membrane (M) protein of influenza A virus can prime for a subsequent augmented antibody response to the HA glycoprotein on virus particles (258). In this manner, the entire T_H cell repertoire developed against the viral surface and nonsurface proteins can be called into play to amplify a B cell response to a protective antigen. Because T_H cell epitopes are far more numerous on viral proteins than CTL epitopes (150), the repertoire of T_H cells capable of augmenting the antibody response is indeed large. A viral antigen can induce a protective immune response mediated by T_H cells in the absence of B cells or CTLs (170), indicating that this arm of the immune response can make an independent contribution to resistance against both acute and chronic viral infections (117). For these reasons, it may be necessary to include virus structural protein antigens capable of inducing T_H cell responses in live and nonliving virus vaccines to achieve maximal immunogenicity.

Humoral Immunity to Viruses

Antibodies have the major responsibility for rendering free virus noninfectious, and the principal manner in which this is achieved is by neutralization of infectivity. The lifelong immunity mediated by antibodies stems from plasma cells, whose considerable longevity contrasts with that of the short-lived, activated CTL response (272). Passive transfer of antibodies can also exert antiviral activity against cells that contain viral glycoproteins inserted into the outer cell membrane. Specific human antibodies have been shown to prevent disease caused by a wide variety of viruses belonging to diverse RNA or DNA virus families (41). Antibodies acting alone, even at low concentrations, are effective at preventing or modifying human disease caused by hepatitis A virus (HAV), hepatitis B virus (HBV), measles virus, poliovirus, and varicella zoster virus (VZV). When given at birth, human IgG was shown to be effective in preventing chronic HBV infection in high-risk infants whose mothers were chronically infected with HBV and who had HBV e antigen as well as HBV surface antigen (HBsAg) in their blood (15). The results of these studies in humans implied that vaccines that induced such antibodies should protect against disease. Subsequent field trials of immunogenic HAV, HBV, poliovirus, measles, and VZV vaccines validated this view. Passive antibodies were shown to protect against mucosal viral infections, such as those caused by RSV (109), but higher quantities of passive antibodies are needed to restrict virus replication on mucosal surfaces than at systemic sites.

Mechanisms by Which Antibodies Decrease Viral Infectivity In Vitro

Antibodies can decrease the infectivity of a virus by several distinct mechanisms. First, antibodies directed against a surface antigen can aggregate the virus particles, thereby causing a decrease in the virus titer and an accelerated clearance of virus from the bloodstream (127). Second, antibodies acting alone or in the presence of complement can irreversibly alter the structural integrity of the virus and render it noninfectious (65,317). Third, the major mechanism by which antibodies inactivate free virus is by neutralization in which the virus is rendered noninfectious, although its structural integrity remains largely intact. Neutralization of infectivity is achieved either by inhibiting attachment of the virus to its cell receptor (214) or, more commonly, by preventing release of the nucleocapsid from virus bound to the receptor on the cell surface or from virus present in an endocytic vacuole (68). Antibodies can act synergistically to achieve these goals (97). Inhibition of attachment of virus to its cell receptor is thought to be predominantly steric in nature, because most receptor-binding sites on viral attachment proteins are inaccessible to antibodies. After binding to cell receptors, extensive modifications occur in the conformation of viral glycoproteins or the capsid proteins of icosahedral viruses during the events leading up to release of viral nucleic acid into the cytoplasm, and it is thought that most neutralizing antibodies act to prevent successful completion of this phase of virus entry.

The very important role that neutralizing antibodies play in immunity to viral infection is highlighted by the observation that viruses use several distinct mechanisms to escape from neutralization by antibodies. Certain viruses decorate their surface proteins with N- or O-linked carbohydrates to evade neutralization by antibodies (248). The V2 loop of the human immunodeficiency virus (HIV) envelope glycoprotein appears to shield an as yet unidentified region of the protein that is rich in neutralization epitopes (281). It might be necessary to modify such carbohydrate or protein regions of a live or nonliving virus vaccine candidate to increase its immunogenicity, especially for viruses that are weak inducers of neutralizing antibodies such as HIV. Viruses such as the influenza A virus develop missense mutations in the neutralization epitopes of the HA (i.e., the attachment protein) in an attempt to evade herd immunity of the human population conferred by previous infection with influenza A viruses of the same subtype. In addition, influenza A virus can acquire an antigenically unrelated HA from an avian influenza virus source via gene reassortment. Escape of influenza A viruses from neutralization by either mechanism allows the antigenic variant to become the predominant viral strain circulating in the human population, because it displaces other previously dominant strains; this successful escape from antibody is the basis for the unique need for annual immunization against this viral pathogen. Adenoviruses have been found to undergo a similar, but slower, form of antigenic change by illegitimate recombination in the gene encoding a major protective antigen (60). A persistent virus such as HIV evades the humoral immune response in a single host by generating waves of antigenic virus variants that resist neutralization by antibodies induced by their progenitors (218). Finally, the neutralizing antibody response to an acute viral infection can be severely blunted by CTL-mediated lysis of neutralizing antibody–producing B cells that have been infected by virus via the membrane-associated antibody receptor present on the B cell (236).

Antibodies Active Against Virus-Infected Cells

Viruses that mature at the cell surface by budding or that insert viral glycoprotein(s) into the cell surface membrane render the infected cell susceptible to lysis by humoral (antibody-dependent, complement-mediated lysis) and cellular [antibody-dependent, cell-mediated cytotoxicity (ADCC)] mechanisms. Antibodies plus complement can lyse virus-infected cells and thereby decrease the amount of infectious virus released by the cells. Cytophilic antibodies can arm leukocytes in the ADCC reaction, which has been shown to be important in the resistance of infant mice to herpes infection and for clearance of a retrovirus (52,146). Some herpesviruses encode an IgG Fc receptor homolog, which enables the virus to evade ADCC, underscoring the importance of the ADCC effector mechanism *in vivo* (72). These mechanisms in which antibody acts cooperatively with cells or complement to exert antiviral effects *in vivo* represent, like CTLs, a second line of defense against infection because infection of cells must occur for these immune mechanisms to act effectively.

Nonneutralizing Antibodies with Antiviral Activities

Nonneutralizing antibodies can also mediate resistance to viral infections but are less effective than neutralizing antibodies (196). Nonneutralizing antibodies can be directed against antigens on the surface of virus particles or against virion or nonstructural proteins displayed on the surface of infected cells. *In vivo*, nonneutralizing antibodies mediate antiviral activities by several different mechanisms. First, virus particles coated with nonneutralizing antibodies can undergo accelerated clearance from the bloodstream. Second, nonneutralizing antibodies directed at surface glycoproteins such as the NA of influenza A virus or the hemagglutinin–esterase protein of coronavirus can decrease the level of virus replication in the target organ, presumably by inhibiting the release of virus from infected cells (29,334). Third, nonneutralizing antibodies can lyse virus-infected cells via an IgG Fc-dependent mechanism (266).

Antiviral Activity of Antibodies in Vivo

In the past, conventional wisdom discounted the importance of antibodies in resolution of virus infections. Instead, clearance of virus was thought to be mediated primarily by CTLs. However, several recent observations cast doubt on this generalization and indicate that antibodies do indeed participate in the resolution of virus infections and, in some instances, can independently bring about resolution of infection, even one occurring at a mucosal surface (14,89,122,195,314). For example, in SCID mice, a persistent influenza virus infection of the lungs or an alphavirus infection of the neurons in the brain can be cleared rapidly by parenteral inoculation of glycoprotein-specific monoclonal antibodies (195,314). The mechanism responsible for this dramatic effect in the brain is IgG-subclass independent but requires that the antibody be bivalent (314).

Other studies in mice indicate that antibody directed against the sigma 1 outer capsid protein of reovirus (the viral attachment protein) can interrupt the spread of established reovirus infection within the central nervous system (CNS) as well as infection that moves from the brain to the eyes (313). Secretory IgA antibodies have been shown to function

within cells of the mucosal epithelium. Dimeric IgA secreted by plasma cells present in submucosal tissues is transported from the basal layer of mucosal epithelial cells to their apical surfaces in association with the polymeric Ig receptor to which dimeric IgA initially binds. During its transport through Sendai virus–infected cells, virus-specific IgA can reduce the level of replication of infectious virus within the cell (173). The mechanism responsible for this effect is not clear at this time, but it is likely that the endocytic pathway used for IgA transport and the exocytic pathway used for transport of viral glycoproteins intersect at some location within the cell (94). Thus, antibodies play diverse roles *in vivo* and can act not only to prevent virus infection but also to modify an infection that develops in a partially protected host.

In light of the great diversity and complexity of the *in vitro* and *in vivo* actions of antiviral antibodies, it is unlikely that simple immunogens such as peptides or antiidiotype antibodies could induce the antibody repertoire that is required for the control of viral infections *in vivo.* This failure may limit the usefulness of such immunogens. Immunization can best be achieved by including as many of the protective antigens of the virus in the immunogen as feasible (21,102).

Antibodies Active on Mucosal Surfaces

Antibodies present in the systemic circulation protect internal organs against viruses that are introduced directly into the bloodstream (e.g., HBV, HIV) or that spread via the bloodstream from primary sites of replication such as the respiratory or gastrointestinal mucosa. As mentioned previously, diseases produced by viruses that fall into the latter category (i.e., measles, polio, hepatitis A, rubella, smallpox, and varicella) can be prevented or modified by Ig prophylaxis, often with small amounts of antibody that are difficult to detect in the blood of recipients (41), and such antibodies are relatively easy to induce by parenteral immunization with live or nonliving virus vaccines. In contrast, antibodies present in the systemic circulation do not provide efficient protection against viral diseases that are limited to mucosal surfaces unless these antibodies are present in high titer. This is because only a small proportion of such Ig molecules traffic from the plasma to the lumenal surface of the mucosal epithelium by transudation (41,198). Therefore, to prevent viral diseases that are limited to mucosal tissue, two different strategies can be employed. The first, and the preferred strategy, is to induce secretory IgA antibodies at the mucosal site by virus replication at that site. The second, and less efficient, strategy is to stimulate a high titer of serum IgG antibodies that can protect mucosal surfaces following transudation.

When IgG antibodies present in the blood are passively transferred by transudation to mucosal surfaces, where they can exert antiviral activity, there is a gradient regarding the ability of serum IgG–derived antibodies to restrict virus replication on mucosal surfaces. This gradient is lung > nasopharynx > lower intestinal tract (198). IgG antibodies, if present in the serum in high enough titer, can provide almost complete resistance to pulmonary replication of RSV, but resistance in the upper respiratory tract is more difficult to achieve (41). Immunization of the female genital tract with a live virus vaccine can induce the local synthesis of IgG antiviral antibodies such that the specific activity of mucosal IgG antibodies significantly exceeds that of serum, indicating that IgG antibodies present on a mucosal surface can come either from serum via transudation or from IgG antibody–secreting cells present in the submucosal tissues (227).

Although passively transferred IgG antibodies can provide mucosal immunity in the lower respiratory tract, the major mediators of resistance to viral infection of the upper respiratory tract, the larger airways of the lungs, and the gastrointestinal tract are IgA antibodies, which are selectively transported across mucosal surfaces to exert antiviral effects on the lumenal surface (198). Both nonneutralizing (32) and neutralizing (257) IgA antibodies have antiviral effects on mucosal surfaces, with the former active intracellularly during transcytosis and the latter active both intracellularly and on the lumenal side of the epithelium. Antiviral IgA antibodies function to clear viral infections, to modify the severity of disease on reinfection, and to prevent infection on reexposure to virus. The level of antiviral IgA antibodies, as well as the number of virus-specific IgA secreting cells, often correlates with resistance to viral infection (198,336). Since IgA antibodies recognize the same protective antigens as IgG antibodies, the greater role of IgA versus IgG antibodies in mucosal immunity is largely the result of the higher levels of Ig antibodies present on the lumenal surfaces, which results from their selective secretion via the polymeric-Ig receptor-mediated transport system.

The primary mucosal IgA response peaks within the first 6 weeks after infection and usually decreases to a low, often barely detectable, level by 3 months. The transient nature of the primary mucosal antibody response is a factor in the ability of many viruses to reinfect mucosal surfaces, and it underlies the need for two or more doses of vaccine to efficiently immunize mucosal surfaces. The virus-specific IgA antibody response is greater at the site of virus replication or of antigen administration than at distal sites, suggesting that mucosal immunity is best induced by antigenic stimulation of sites directly involved in replication of the wild-type virus. The appearance of IgA antibodies in mucosal secretions coincides with the cessation of virus excretion during infection in both the respiratory and gastrointestinal tracts (198).

Thus, to achieve optimal resistance to virus infection in mucosal tissues, immunization should induce IgA antibodies efficiently. This can be accomplished most effectively by infection of the mucosa with a live virus vaccine. It has also been recognized that the upper respiratory tract is an effective inductive site for stimulating IgA responses in distal mucosal sites such as the genital tract (12,98). In studies in which the intranasal and parenteral routes of immunization with a herpes simplex virus (HSV) type 2 glycoprotein D vaccine candidate have been directly compared for their efficacy against a genital challenge with HSV-2, the parenteral route of immunization was superior to the intranasal route (219). This observation indicates that parenteral immunization can provide superior immunity to a mucosal infection than can immunization at a distal mucosal site.

Adverse Effects of Antibodies

Antiviral antibodies are not always helpful in the elimination of virus infection and can, in certain circumstances, augment viral infectivity and participate in the potentiation of disease. For example, it has been proposed that heterotypic antibodies to the envelope glycoprotein of dengue virus play a central role in the severe clinical entity known as dengue hemorrhagic fever/shock syndrome (148). Dengue viruses replicate primarily in cells of the mononuclear phagocyte lineage, and subneutralizing amounts of dengue virus antibodies enhance dengue virus infection of these cells *in vitro* by increasing uptake of virus via IgG Fc receptors present on these cells (113). However, the mechanism by which augmented disease results from enhanced access of dengue virus into mononuclear cells remains unclear (256). It has

been proposed that antibody enhancement of infectivity also plays a role in the enhanced disease seen in immunized cats challenged with infectious peritonitis virus, a coronavirus that also infects cells of mononuclear lineage (57). These observations suggest that special attention should be given to the functional activity of antibodies induced against viruses that replicate in cells bearing receptors for the Fc fragment of IgG. Development of antibodies that bind to viral surface antigens but fail to neutralize infectivity effectively could render vaccinees more susceptible to infection as well as to enhancement of disease once infection has occurred.

OBSTACLES TO IMMUNIZATION IN EARLY LIFE

Viruses that cause disease in early infancy present special problems for the development of effective vaccines. One example is provided by RSV, which produces severe lower respiratory tract disease with highest frequency during the second month of life, generally causing disease in those infants possessing lower levels of maternally derived antibodies. For a vaccine to be effective against this virus, it must be administered to infants by 1 month of age. Because RSV is a ubiquitous pathogen and reinfection occurs commonly, most young infants possess a relatively high serum titer of passively acquired, maternally derived RSV-neutralizing antibodies. Both immunologic immaturity and a high level of maternally derived antibodies act to suppress the immune response of the young infected infant to the protective antigens of RSV (i.e., the F and G glycoproteins) (143,199). In experimental animals, passively administered RSV antibodies had adverse effects, both quantitative and qualitative, on the immune response to RSV glycoproteins expressed by a vaccinia-F (vac-F) or vac-G recombinant virus (203). For example, the frequency and magnitude of the serum antibody response to the F and G protective antigens was markedly suppressed when RSV immune serum was administered to animals the day before they were infected by a parenteral route with vac-F and vac-G (203). Furthermore, antibodies to the RSV protective antigens that did develop had abnormally low neutralizing activity. Immunized animals that had their immune response suppressed by passively transferred antibodies were more susceptible to challenge infection with RSV than were animals that received control serum lacking RSV antibodies. This form of antibody-mediated immunosuppression has been observed with purified protein or whole virus antigens, vector-expressed antigens, and antigens encoded by DNA (73,233,268,320). Fortunately, this form of immunosuppression can be overcome, in part, by immunization with a vaccine virus that actively replicates in the respiratory tract (73). Although virus-specific antibody responses to vector- or DNA-encoded viral antigens can be suppressed by passively acquired viral antibodies, T_H cell and CTL responses can be induced in such circumstances, and accelerated immune responses can occur upon subsequent boosting (268).

A second important effect of maternally derived antibodies on immunization is their ability to restrict replication of a live attenuated virus, thereby decreasing its immunogenicity. This is a well-known phenomenon that adversely affects the efficacy of parenterally administered live attenuated measles virus vaccine during infancy. It constitutes the basis for delaying immunization until after 12 months of age, by which time a sufficient decay of maternally acquired neutralizing antibodies has occurred to permit efficient replication of the live attenuated vaccine virus. Since maternally derived antibodies are less efficient at restricting replication of vaccine viruses administered mucosally rather than systemically, it is possible to successfully immunize neonates who possess maternally derived antibodies if the viral vaccine can replicate on a mucosal surface. For example, live attenuated poliovirus vaccines are effective immunogens in young infants because virus introduced into the gastrointestinal tract is not neutralized by the maternally derived serum IgG antibodies, which allows replication to high titer at this site.

The ability of maternally derived antibodies to restrict replication of live attenuated vaccine virus can be beneficial in some cases. The licensed live rhesus rotavirus vaccine exhibits mild reactogenicity for fully seronegative infants over 3 months of age (230). This effect can be abrogated significantly, without losing immunogenicity, by immunizing younger infants who possess passively acquired serum antibodies. In this instance, the passively acquired serum antibodies further restrict replication of the partially attenuated vaccine virus, and this restriction of replication of vaccine virus contributes to the overall attenuation of the vaccine for the young infant (134). This partial contribution of maternal antibody to the attenuation of the rotavirus vaccine is the basis for the recommendation that the vaccine not be given to older infants and children in whom maternally acquired antibodies have decayed to a low level.

GOALS OF IMMUNIZATION AGAINST VIRAL DISEASES

Prevention

The major goal of immunization against viruses is the prevention or modification of disease. Most viral vaccines prevent or modify disease without necessarily preventing infection. The ways in which this is achieved are diverse. Whereas most viral vaccines are administered before infection occurs, prevention of CNS disease in individuals inoculated with rabies virus by an animal bite can be achieved by administration of vaccine shortly after infection has occurred. This is possible because the long incubation period of the infection permits the development of an effective immune response in time to modify infection and prevent disease. Similarly, individuals exposed to smallpox virus can be protected against a fatal outcome if vaccinia virus vaccine is administered up to 4 days after initiation of smallpox virus infection.

The major goal of immunization against rubella virus is prevention of fetal abnormalities caused by intrauterine infection. To achieve this goal, both males and females are immunized. Immunization of males has little direct benefit to the vaccinee other than prevention of the relatively mild illness caused by the rubella virus. The fetus of a vaccinated female is protected by immunization of the mother before pregnancy. Herd immunity resulting from extensive immunization of males and females provides some protection to the fetus of an unvaccinated female by decreasing the circulation of virus in the community.

Some vaccines can prevent infection in individuals who have not been vaccinated, not only by engendering herd immunity but also by the spread of vaccine from recipients to contacts. This has been observed with oral live poliovirus vaccine, which spread from vaccinees to unvaccinated contacts (262). This resulted in immunization of a significant proportion of the population that had not been administered vaccine, markedly reducing the incidence of poliomyelitis in both vaccinated and unvaccinated individuals. Spread of vaccine virus has been viewed by many as a desirable property

because it confers the benefits of immunization on many individuals who were not vaccinated. Of course, this has been a mixed blessing because of the rare occurrence of vaccine-associated paralytic disease among contacts of vaccinees. Although live poliovirus vaccine is no longer recommended for use in the United States, immunization of the unvaccinated still appears to be a positive feature of the live poliovirus vaccine especially when viewed in the context of interruption of virus spread in the community and, more importantly, for eradication of poliovirus worldwide.

Although most vaccines in use today are effective in preventing disease, they are inefficient at preventing infection. Prevention of infection is a highly desirable property for specific vaccines such as HBV and HIV. Infection with HBV during the neonatal period is associated with a greatly increased risk of progression to the chronic carrier state that is strongly associated with the development of chronic liver disease and hepatocellular carcinoma later in life. Therefore, a major goal of an HBV vaccine is to prevent transmission of infection from persistently infected mothers to their infants during the neonatal period. Considerable success in this effort has been achieved through the use of HBsAg vaccine or hyperimmune HBV antiserum plus vaccine immediately after delivery (124). Since HIV can progress to acquired immunodeficiency disease (AIDS) many years after initial infection, prevention of infection would be desirable.

In addition to preventing the acute effects of viral infection, two vaccines, one licensed (HBV) and one under development [human papillomavirus (HPV)], have as their goal the prevention of the most serious long-term sequelae of viral infection, namely cancer of the liver or cervix, respectively (124).

Immunotherapy

HSV-1 and HSV-2, as well as VZV, produce latent infection in sensory ganglia that is periodically activated, resulting in development of new lesions and increased opportunity for transmission of virus. Therefore, a goal of immunization of persons previously infected with these viruses is to prevent or ameliorate recurrent disease caused by reactivation of their latent infection (21,156). This form of immunization is referred to as immunotherapy. Other illnesses targeted for immunotherapeutic vaccines include warts and cancer caused by HPVs (156), and chronic hepatitis caused by HBV (169). Effective immunotherapeutic vaccines for recurrent HSV will be difficult to develop because the vaccinees have been infected by a wild-type virus and will be effectively reimmunized during each recurrence. It will be difficult to augment an immune response in such repeatedly immunized hosts.

Eradication

Appropriate use of a highly effective vaccine can help eradicate a major disease when humans are the only natural host of the virus. During the 1970s, this goal of eradication was achieved for smallpox largely through the use of the live vaccinia virus vaccine, and this ranks as one of the major public health achievements of this century. Two other viruses have been similarly targeted for eradication, namely poliovirus and measles, and significant progress toward this goal has been made for both viral pathogens. Live virus vaccines have played and continue to play a central role in these current eradication efforts.

CURRENTLY LICENSED VIRUS VACCINES

This chapter will focus solely on active immunization, and the reader is referred to Chapter 45 on RSV for a discussion on the only widely used form of passive immunization, a monoclonal antibody to the F glycoprotein used for immunoprophylaxis of high-risk children.

The first vaccine used in humans, live vaccinia virus, was developed by Jenner more than 200 years ago for the control of smallpox. During the ensuing two centuries, vaccinia virus had a noble record of success in controlling this disease and played an essential role in the final eradication of smallpox. It is ironic that the origin of this most important vaccine virus strain has remained obscure. Attempts to determine the origin of vaccinia virus using modern molecular biologic techniques have yielded equivocal results. Vaccinia virus appears to be a distinct species, different from both variola and cowpox viruses (167).

Since the introduction of vaccinia virus, licensed vaccines have been developed for 21 other viral pathogens (Table 1). Several of these vaccines already have helped to control the diseases against which they are directed—notably, poliomyelitis, yellow fever, mumps, measles, and rubella.

TABLE 1. *Viral vaccines licensed in the United States*[a]

CURRENTLY LICENSED NONLIVING VIRUS VACCINES

Nonliving virus vaccines are available in the United States for the prevention of disease caused by 10 separate viral agents (Table 1). These vaccines are prepared from virus that is grown in eggs (influenza types A and B), a continuous monkey kidney cell line (poliovirus types 1, 2, and 3), human diploid fibroblasts (rabies, hepatitis A), or mouse brain (Japanese encephalitis virus vaccine). Virus is then inactivated with a chemical such as formalin or disrupted with a detergent (influenza). The level of efficacy of these vaccines differs: Inactivated poliovirus vaccine is highly effective in preventing disease, whereas influenza virus vaccine is only partially protective.

Semipurified influenza virus subunit vaccines have been developed in an attempt to increase purity and decrease toxicity associated with whole virus vaccines. Such vaccines containing the HA and NA surface antigens of the virus are less toxic than are inactivated whole virus vaccines, but there is no increase in protective efficacy (226). In fact, in unprimed individuals, influenza virus subunit vaccine appears to be less immunogenic than inactivated whole virus vaccine, and two doses are needed for effective immunization in immunologically naive persons (226).

Finally, one of the latest nonliving virus vaccines to be developed is a highly purified HBV subunit preparation that contains the 22-nm HBsAg virus-like particle (VLP). The initial version of this vaccine, designed to protect against type B hepatitis, was unusual because surface antigen was purified from the blood of persons persistently infected with HBV. An effective second-generation HBsAg vaccine was developed using a recombinant plasmid to express the gene for HBsAg in yeast. Yeast-produced HBsAg self-assembles into typical HBsAg VLPs and is immunogenic in humans.

Advantages of Nonliving Virus Vaccines

Inactivated virus vaccines offer the advantage of immunization with little or no risk of infection. Only rarely do such vaccines contain a contaminating adventitious agent or residual infectious virus that has resisted inactivation. An example was paralytic disease produced by some of the early lots of inactivated poliovirus vaccine that contained residual infectious virus (213,231). Contamination by an infectious adventitious agent was also detected retrospectively in some early lots of inactivated poliovirus. This was of some concern because the contaminating SV40 was oncogenic in hamsters (123). Fortunately, long-term follow-up of individuals who received SV40-contaminated vaccine parenterally during early infancy failed to show evidence of an oncogenic effect (191,286). Live vaccinia virus was present in a preparation of inactivated vaccinia–HIV recombinant virus used to augment immunity in HIV-infected subjects. Unfortunately, an immunodeficient recipient of this vaccine developed progressive vaccinia initiated by residual live virus in the inoculum (337). The use of purified viral proteins or synthetic peptides should eliminate both types of contamination. In addition, the preparation of vaccines free of nucleic acid precludes the possibility of long-term adverse effects associated with infection or integration of viral nucleic acid into the host genome.

Disadvantages of Nonliving Virus Vaccines

Several nonliving virus vaccines have potentiated disease rather than prevented it (95,140). This was first observed with formalin-inactivated measles virus vaccine (95). Initially, this vaccine prevented measles, but after several years vaccinees lost their resistance to infection. When subsequently infected with naturally circulating measles virus, the vaccinees developed an atypical illness with accentuated systemic symptoms and pneumonia (95,209,244). Retrospective analysis showed that formalin inactivation destroyed the ability of the measles fusion (F) protein to induce hemolysis-inhibiting antibodies, but it did not destroy the ability of the H (hemagglutinin or attachment) protein to induce neutralizing antibodies (216,217). When the immunity induced by the H protein had waned sufficiently to permit extensive infection with measles virus, an altered and sometimes more severe disease was seen at the sites of measles virus replication (16,33). This atypical disease is believed to be mediated in part by an altered cell-mediated immune response in which T_H2 cells were preferentially induced, leading to heightened disease manifestations at the sites of viral replication (238). Chemical inactivation of poliovirus for use in a vaccine results in modification of a major neutralization epitope. This indicates that inactivation of the infectivity of nonenveloped as well as enveloped viruses can modify the antigenicity of the vaccine (83).

Potentiation of disease also was observed after parenteral administration of an experimental formalin-inactivated RSV (FI-RSV) vaccine (140). In clinical trials conducted in the mid 1960s, an FI-RSV vaccine induced a measurable serum neutralizing-antibody response but did not protect the young vaccinees. Unexpectedly, upon subsequent natural infection with RSV, the FI-RSV vaccinees developed severe RSV lower respiratory tract disease significantly more often than did infants and young children who had received an inactivated parainfluenza virus vaccine (140). Speculation on the mechanism of disease potentiation by the inactivated RSV vaccine has centered on several possible aberrations of the immune response to vaccine that involve an imbalance between or within various compartments of the immune system (43). First, it is likely that the vaccine failed to induce appreciable local respiratory tract RSV secretory IgA antibodies, thereby leaving vaccinees susceptible to infection under conditions in which the adverse effects of a series of other unbalanced responses might exacerbate disease.

Second, vaccinees immunized with FI-RSV developed a high titer of serum antibodies to the F glycoprotein of the virus, but these antibodies exhibited a low level of neutralizing activity that was not sufficient to provide effective resistance (205). This observation was confirmed later in an experimental setting; passive transfer of antibodies with such low neutralizing activity failed to protect rodents from RSV infection (54). The young infant vaccinees also developed a low level of antibodies to the other major RSV protective antigen, the G glycoprotein (205).

Third, shortly after the immunization phase of the vaccine trials was completed, it was observed that lymphocytes from the young RSV vaccinees exhibited an exaggerated proliferative response to RSV antigens *in vitro* that was significantly greater than that detected after RSV infection (141). It is thought that the vaccine most likely induced a high level of virus-specific memory T_H cells, probably of the CD4+ lineage. Recent studies have shown that CD4+ T cells predominate in the pulmonary infiltrate that develops after RSV challenge of FI-RSV-immunized mice (56). On the other hand, studies

in mice indicate that FI-RSV vaccine does not induce an RSV-specific CD8+ CTL response (56). These observations suggest that the vaccinees also experienced an imbalance in their cell-mediated immune response, in which the CD4+ component predominated and the CD8+ component was deficient (43,56). Finally, an additional imbalance attributable to immunization with FI-RSV was identified recently, namely an alteration in the T_H1 and T_H2 components of the CD4+ T-cell response to RSV infection. Ordinarily, T_H1 cells predominate in the lungs of RSV-infected mice, but in animals previously immunized with FI-RSV the ratio of T_H1 to T_H2 CD4+ T cells in the lungs of RSV-infected mice appeared to be reversed and sharply skewed to favor the latter (55,104). Since CTLs promote the development of a T_H1 response, their absence in the FI-RSV vaccinees partially contributed to the skewing of the T_H cell response toward the T_H2 subset (280). The absence of a protective immune response in the presence of heightened T_H2 cell immunity resulted in increased inflammation at the sites of RSV replication in the lung, which translated clinically into a more severe form of bronchiolitis and pneumonia in the FI-RSV vaccinees. In many ways, this adverse effect of immunization with an FI vaccine is reminiscent of the potentiation of measles that occurred following the use of the licensed FI measles virus vaccine.

Several lessons were learned from the potentiation of disease by FI-RSV. First, the chemical treatment and physical purification procedures used for the preparation of each new inactivated vaccine should be evaluated for their effect on the immunogenicity of the major viral protective antigen(s). The ability of a candidate vaccine to induce a high titer of serum antibodies with relevant functional activity (usually neutralization of virus infectivity) that is equivalent to that induced by infection, would constitute a sign that the protective antigen(s) had not been perturbed significantly. Retention of immunogenicity of the major viral protective antigen(s) present in the vaccine should preclude an unbalanced immune response deficient in protective antibodies.

Second, evidence for an imbalance involving the CD4+ T-cell response to the vaccine should be sought in experimental animals. An exaggerated CD4+ response, especially in the absence of an adequate protective antibody response, would represent a danger signal suggesting that other methods for inactivation of virus infectivity or for purification of virus particles or proteins should be investigated before proceeding further in vaccine development.

Third, the potential dangers inherent in pursuing a strategy for selective induction of T-cell responses in the absence of a protective antibody response should be evaluated in experimental animals, if possible. Failure to do so could result in the preparation of an inactivated vaccine that causes potentiation of disease rather than its prevention or amelioration. This caution applies not only to CD4+ T_H cells but also to CD8+ CTLs because adoptive transfer of the quantity of polyclonal or cloned CTLs required for rapid resolution of RSV infection can cause disease in recipient mice, and in some circumstances may kill the host (36).

Potentiation of disease by an inactivated virus vaccine is not limited to vaccines containing measles virus or RSV. For example, administration of an inactivated caprine lentivirus vaccine has also been associated with an accelerated, more severe disease in animals subsequently challenged with virus (176). Also, immunization with a subunit vaccine, but not an inactivated whole virus vaccine, of another lentivirus, equine infectious anemia virus (EIAV), enhanced disease caused by subsequent challenge with a heterologous EIAV strain (128). The immunologic mechanisms responsible for these two examples of enhanced lentiviral disease have not been identified.

Another disadvantage of nonliving virus vaccines is that parenteral immunization against viruses that infect and cause disease that is limited primarily to a mucosal surface may not be completely effective because of the failure of parenterally administered antigen to stimulate a satisfactory local IgA antibody response (198). This means that resistance in the upper respiratory tract or in the intestines may be less complete than that conferred by local immunization or infection.

Inactivated virus vaccines also may be at a disadvantage in relation to live attenuated–virus vaccines with respect to induction of a CTL response. This type of response is induced most efficiently by infected cells in which processed peptides derived from endogenously synthesized viral proteins are presented to the immune system in association with MHC class I proteins. Inactivated influenza virus is considerably less effective than virus infection in stimulating a primary CTL response in mice. However, inactivated influenza A virus or purified surface glycoproteins derived from it can stimulate a secondary CTL response in sensitized mice or humans (2,79,80,322). As described earlier in this chapter, methods to induce effective durable immunity mediated by CTL epitopes are not currently available.

The Guillain-Barré syndrome was associated with widespread use of inactivated influenza A H1N1 (swine) virus vaccine during 1976 and 1977 (153), indicating that unanticipated, delayed, or untoward side effects can be induced by nonliving virus vaccines. Surprisingly, this syndrome has been associated only rarely with subsequent nonliving influenza virus vaccines, although these products were prepared in the same manner as the 1976 vaccine (153).

It has been recognized that antigenic changes can occur during selection and amplification of influenza viruses in the allantoic cavity of embryonated eggs, the substrate used to produce virus for the inactivated vaccine (252). The genetic basis for these mutations involve host cell selection of mutants that replicate more efficiently in eggs. These variants acquire a mutation in or near the receptor-binding pocket of the HA that increases their efficiency of replication in eggs but also alters their antigenicity and immunogenicity. However, the influenza viruses used to prepare inactivated vaccine for humans are grown in eggs, and such vaccines have been repeatedly shown to be protective. A similar situation has been described for the inactivated poliovirus vaccine (255). Poliovirus grown in tissue culture contains an uncleaved outer capsid protein VP1, which is usually cleaved in a natural infection by intestinal proteases. Thus, tissue culture–grown poliovirus has limited ability to induce antibodies that neutralize viruses containing a cleaved VP1. This creates a potential problem because the uncleaved VP1 antigenic site (site 1) of poliovirus type 3 appears to be immunodominant over antigenic sites 2 and 3. Thus, the major immune response to inactivated type 3 poliovirus vaccine is directed against intact antigenic site 1, a site not present on type 3 poliovirus that has been cleaved. Infection by attenuated vaccine or wild-type poliovirus type 3 induces antibodies capable of efficiently neutralizing virus with both cleaved and intact VP1. Presumably, during virus replication in the gastrointestinal tract, viruses with either cleaved or

uncleaved VP1 are present and induce antibodies to both forms of virus.

In humans, each of two inactivated poliovirus vaccines, one containing a cleaved VP1 of poliovirus type 3 and a second containing an uncleaved VP1, induced antibodies able to neutralize virus with either a cleaved or an uncleaved VP1, and each of the two induced a comparable level of resistance to replication of subsequently administered live attenuated poliovirus vaccine (234). These findings indicate that immunization of humans with an inactivated poliovirus bearing an uncleaved VP1 is efficacious. These observations suggest that inactivated virus vaccines derived from tissue cultures or eggs may not mimic the full array of virus forms generated during an infection of the host. Thus, immunization with such antigens may induce antibodies with diminished ability to efficiently neutralize the total repertoire of viruses produced during infection.

Immunization of monkeys with FI simian immunodeficiency virus (SIV) produced in human cells induces antibodies directed at antigens originating from the cells used to produce the virus (275). These anticellular antibodies can neutralize the infectivity of SIV produced in human cells, but not of virus produced in monkey cells, by interacting with human antigens incorporated into the virion's outer membrane. This form of neutralization, which results from complement-mediated virolysis, can yield an overestimate of vaccine immunogenicity. Hence, an effort should be made to avoid immunization with virions into which foreign host-cell antigens have become incorporated (275).

POTENTIAL SOURCES OF ANTIGENS FOR FUTURE NONLIVING VIRUS VACCINES

Major progress has been made using monoclonal antibody and recombinant DNA technology to produce large quantities of purified protective viral antigens for use in immunoprophylaxis. Monoclonal antibody immunoaffinity chromatography or lectin chromatography facilitates purification of viral antigens from infected cells, and recombinant DNA technology makes it possible to express viral proteins in eukaryotic cells of yeast, insect, or mammalian origin. The use of these techniques has yielded candidate vaccines encompassing a large array of viral proteins that are capable of inducing protective antibodies in experimental animals. The production of individual viral proteins in prokaryotic cells has been less successful, but VLPs have been made in bacteria (157). Other approaches to immunization involve the production of synthetic peptides representing immunologically important domains of viral surface antigens and the use of antiidiotype antibodies as antigens to elicit an antiviral antibody response. Conventional techniques for producing FI whole virus vaccines are still being actively pursued for various flaviviruses (242) and alphaviruses (1).

Production of Viral Proteins in Eukaryotic Cells

Before expression of viral proteins in eukaryotic cells can be used as a method for vaccine production, several obstacles must be overcome. First, it is essential to identify the correct immunogen. Second, it necessary to produce protein in a highly efficient manner to make this approach economically feasible. Strategies that have been pursued to enhance expression of viral proteins focus primarily on identification of strong promoters of gene expression and development of techniques for efficient gene amplification. Third, viral proteins must be effectively separated from host cell protein and DNA. This latter requirement is of special concern because continuous mammalian cell lines constitute the most feasible substrate for production of viral proteins; therefore, potentially oncogenic cellular DNA must be greatly reduced or absent from the final vaccine. Recombinant DNA techniques to promote secretion of viral glycoproteins into the medium have been developed to facilitate effective separation of viral proteins from host cell proteins. Fourth, the procedures used to produce and purify viral protein must be gentle enough to maintain the protein in its native state, thereby preserving the conformationally dependent neutralization epitopes needed to induce protective antibodies. Finally, because the purified proteins are relatively poor immunogens, adjuvants need to be developed to enhance their immunogenicity. The following discussion of these approaches is not intended to be exhaustive in scope but illustrative of current strategies in use or in development.

Recombinant DNA techniques have been used to express viral proteins in yeast, insect, and mammalian cells. The usefulness of yeast was described previously in the discussion about the production of the HBsAg vaccine; yeast is currently being used to express other VLPs such as HPV (161). However, it has proven difficult to produce purified viral proteins such as the rabies G protective antigen in a native conformation in yeast (263), and other expression systems are currently being developed using insect or mammalian cells.

A major advance in viral antigen production has been the development of baculovirus vectors to express foreign genes at high levels in insect cells under control of a strong baculovirus promoter such as the polyhedrin promoter (164). Protein can be produced in large quantity (1 mg/10^6 cells) using this system. The baculovirus expression system has been used to express the influenza A virus HA protein, which has been found to be as safe and as immunogenic as the licensed nonliving virus vaccine in humans (239). Hepatitis E virus (HEV) protective antigen produced in a baculovirus expression system protected rhesus monkeys against HEV challenge (312). The baculovirus system can also be used to express more complicated viral immunogens that require polyprotein processing to generate native immunogenic protective antigens. An example of this is expression of the entire structural protein region (C-E3-E2-6K-E1) of an alphavirus to generate authentic, processed viral proteins capable of inducing an efficient neutralizing antibody response in experimental animals (120).

Mammalian cells also have been used for the production of viral proteins that can be incorporated in a subunit vaccine. Mammalian cell cultures represent an optimal system for the production of viral proteins, because protein folding, transport, and processing closely approximate those that occur in the infected host. Viral proteins have been produced from mammalian cells infected with the virus of interest or with a recombinant virus expression system engineered for a high level of expression of the foreign viral antigen. Two such expression systems are the vaccinia/T7 recombinant virus system (93) and an alphavirus replicon expression system (92,99,307). In the former, cell cultures are infected with two vaccinia recombinant viruses: One recombinant expresses the foreign gene under control of the T7 bacteriophage promoter, and the second recombinant expresses the T7 polymerase protein under control of a vaccinia promoter (93). Expression of the T7 polymerase (controlled by the vaccinia promoter) results in a high level of expression of the foreign viral gene (controlled by the T7 promoter). As a consequence, doubly infected cells produce large quantities of the

protein encoded by the foreign gene. Infectious vaccinia virus must be removed from the final product.

Alphavirus replicons have also been used to produce large quantities of proteins in mammalian or insect cells. Their success, like that of the vaccinia/T7 recombinant virus system, results from the selective amplification of the mRNA for the foreign protein (92,99,133,307). The expression system is based on a self-replicating RNA, termed a replicon. The replicon vector consists of an RNA molecule with authentic alphavirus 5' and 3' genomic ends; it contains (a) an alphavirus packaging signal located within the coding sequences for the nonstructural proteins located toward the 5' end of the replicon, and (b) an alphavirus subgenomic promoter sequence located downstream of the genes encoding the nonstructural proteins but upstream of a cloning site into which the foreign gene is inserted. The foreign gene replaces the genes encoding the alphavirus structural proteins. This replicon RNA is transcribed *in vitro* and then transfected into cells where the transcriptase/replicase encoded by the nonstructural genes amplifies the vector RNA and the mRNA encoding the foreign protein of interest. Co-transfection of cells with the replicon RNA and a defective helper RNA that lacks the packaging signal but expresses the alphavirus structural proteins results in the production of a high titer of defective virus particles that contain only replicon RNA. These replication-defective viral particles are able to infect cells at a high multiplicity and induce a high level of expression of the foreign protein. This type of system can be used for the expression of proteins of viruses that (a) cannot be grown efficiently in cell culture, such as HBV or the parvovirus B19, (b) are poorly infectious, or (c) represent a significant biohazard and, hence, cannot be considered for use in an inactivated whole virus vaccine (e.g., certain lentiviruses, filoviruses, hantaviruses, and arenaviruses).

In each of the eukaryotic systems described before, recombinant DNA technology can be used to introduce mutations into the expressed viral glycoproteins that facilitate subunit vaccine production. For example, the cell membrane anchor domain of viral glycoproteins can be deleted, with the result that the viral glycoprotein is secreted into the medium (282), thereby greatly facilitating purification of the protein. Chimeric proteins containing the antigenic portions of two protective antigens fused to form one immunogen have been produced, but the advantage of a chimeric protein over other forms of the immunogenic proteins has not been established (54,121). To induce a protective immune response using purified proteins that form heterodimers in virions or in virus-infected cells, it may be necessary to immunize with the protein complex rather than with the individual proteins, as has been done for the gH-gL complex of HSV (229). For larger viruses such as HSV, which encodes over 10 protective antigens, it has proven difficult to successfully immunize with single purified virion proteins, and it may be necessary to administer a mixture of proteins to achieve satisfactory protection (21,102).

Immunoaffinity chromatography, lectin chromatography, and physical separation techniques have been used to purify a variety of viral glycoproteins that have induced partial to complete resistance in animals. When directly compared in animals, resistance induced by immunization is greatest when the purified viral glycoprotein is in its native conformation (316). For RSV, HSV-1, and HSV-2, the neutralizing antibody response of animals immunized with purified proteins has been less than that observed after virus infection (177,206). In the case of RSV and Epstein-Barr virus (EBV), purified glycoprotein vaccine induced antibodies that were able to bind to glycoprotein in an enzyme-linked immunosorbent assay (ELISA), but these antibodies had a very low level of neutralizing activity (206,330). The titer of neutralizing antibodies stimulated by the RSV or EBV proteins was less than that induced by infection. In contrast to purified protein immunogens, RSV glycoproteins expressed by a vaccinia virus RSV F or G recombinant induced a titer of neutralizing antibodies comparable to that developed by animals infected with the virus (54). Observations of this type underscore the need to use purification techniques that yield glycoproteins that retain the capacity to induce antibodies with a high level of neutralizing activity or other desirable functional characteristics.

Novel strategies are being explored to increase the level of expression and immunogenicity of expressed antigens that have not yet induced a satisfactory humoral immune response following infection or immunization, such as the gp160 of HIV. One such strategy being evaluated for gp160 is to increase protein expression in cell culture by modifying codon usage to match that used by mammalian cells rather than that encoded in the virus genome (111). A second strategy involves immunization with formalin-fixed whole cells expressing HIV virions bound to receptor-bearing cells to induce neutralizing antibodies to antigens that are uniquely exposed during the process of initiation of the virus–cell membrane fusion reaction (151). It remains to be determined whether such strategies will be useful in the production of future vaccines.

Plants have been genetically modified to express virus proteins, and it is possible that some future viral vaccines will use proteins purified from plants (40). Alternatively, it might be possible to be immunized by simply eating a plant containing expressed virus proteins or VLPs (329).

Production of Viral Proteins in Prokaryotic Cells

In general, production of immunogenic viral protective antigens in prokaryotic cells has met with little success because it has been difficult to express conformationally authentic viral glycoproteins in such cells due to differences in protein processing and in microenvironment. Several experimental vaccines have been prepared using viral antigens that self-assemble to form VLPs (see later) (157,211) or viral proteins that have linear B-cell epitopes such as certain picornavirus capsid proteins and the HSV gD protein (145,323).

Virus-like Particles

The development of nonliving virus vaccines has been greatly facilitated by the observation that protective antigens of a large number of enveloped and nonenveloped viruses can self-assemble into VLPs. The capsid of most nonenveloped viruses is composed of two or more proteins that assemble into one or more shells that surround the viral genome. In contrast to virions, VLPs consist of capsid proteins assembled into a similar shell-like structure, but viral nucleic acid is not present within the shell. These empty shells can display conformational epitopes that are not present on individual purified capsid proteins. Some of these epitopes are formed by the juxtaposition of parts of two different capsid proteins. VLPs offer several advantages as immunogens. First, VLPs present conformational epitopes to the immune system in the same way that native infectious particles do, so that neutralizing antibodies and other protective immune responses are

induced efficiently. Second, many of the viruses for which immunogenic VLPs have been developed replicate poorly or not at all in tissue culture (e.g., the B19 parvovirus and the papillomaviruses), thus precluding use of purified virus as immunogen. Third, because VLPs are noninfectious, inactivation with formalin is not required. For this reason, VLPs might prove to be better immunogens than FI whole virus vaccines because the deleterious effects of formalin on immunogenicity can be avoided. Fourth, the immune system responds better to a viral antigen presented as an assembled multimeric particle than to one presented as an individual monomeric protein. VLPs have been engineered successfully for viruses belonging to a wide range of virus families. The success of the first VLP vaccine in humans, the yeast-grown HBV vaccine, is encouraging for the development of additional VLP vaccines against other pathogens.

Initially, it was observed that picornavirus capsid proteins could self-assemble during the *in vitro* translation of viral RNA, to yield 75S to 85S particles (222). Later studies indicated that VLPs could form *in vitro* following translation of picornavirus vRNA lacking most of the 5' noncoding region (46). Next it was observed that expression of the open reading frames of capsid proteins of nonenveloped viruses by a baculovirus or vaccinia vector in appropriate eukaryotic cells resulted in generation of VLPs (47,144,338). For picornaviruses, particle assembly requires expression of the entire open reading frame to permit the proteolytic processing of the structural proteins by nonstructural viral-encoded proteases.

Currently, VLPs are being actively pursued as experimental virus vaccines for two viruses, HPV and parvovirus B19, largely because these viruses replicate poorly in cell culture and alternative vaccines do not exist. The two major diseases caused by HPVs in humans for which vaccines are being prepared are cervical cancer, which is most frequently associated with HPV-16 or HPV-18 infection, and genital warts, caused by HPV-6 and HPV-11. VLPs for HPV-16 and HPV-11 that induce neutralizing antibodies following parenteral or mucosal administration have been produced in prokaryotic and eukaryotic systems (12,157,211,254). VLPs have also been prepared against a variety of animal papillomaviruses, and prophylactic efficacy against a variety of these viruses causing cutaneous or mucosal tumors has been demonstrated (12). VLPs containing either the L1 capsid protein alone or both L1 and L2 capsid proteins have been efficacious. This VLP-induced immunity was shown to be mediated by antibodies, as protection could be readily conferred to naive animals by passive transfer of antibodies from animals rendered immune by vaccination. It is likely that immunization of humans with an HPV-16 or HPV-18 vaccine will restrict replication of HPV in the cervix sufficiently to decrease the frequency with which infection progresses to malignancy. An L1/E7 chimeric protein containing CTL epitopes present in the HPV E7 oncoprotein is able to assemble into a VLP and to induce antitumor immunity in an animal model (107). It is possible that such chimeric VLPs might be more effective as prophylactic vaccines than the L1 VLPs, and that the chimeric VLPs might be active immunotherapeutically. Bacterial vectors transformed with HPV L1 capsid protein can assemble VLPs, and infection of mice with live recombinant bacteria has resulted in the production of antibodies in serum and mucosal secretions that were directed against conformationally dependent antigens, indicating that it might be possible in the future to deliver VLPs by bacterial vectors (211).

Human parvovirus B19 causes erythema infectiosum, acute and chronic red cell aplasia, and other illnesses. A vaccine consisting of the human parvovirus B19 VP1 and VP2 capsid proteins that form VLPs is being developed (13). Immunization of humans with this vaccine induces neutralizing antibodies that achieve levels that are equivalent in magnitude and neutralizing activity to those seen following natural infection, indicating that this VLP also represents a promising vaccine candidate (24).

Synthetic Peptides as Immunogens

During the early 1970s, the use of synthetic peptides as immunogens was studied using a synthetic icosapeptide representing the immunodominant domain of the outer coat protein of bacteriophage MS-2 (152). This peptide, conjugated to a carrier and emulsified in adjuvant, induced antibodies in rabbits that neutralized the infectivity of the bacteriophage when goat anti–rabbit Ig antibodies were added to the mixture. Although synthetic peptides were subsequently shown to induce neutralizing antibodies that did not depend on the aggregating effect of anti-Ig antibodies and to stimulate immunity to certain animal viruses *in vivo* (22), the immunity induced was less than that achieved when full-length protein, inactivated whole virus, or live virus was used as immunogen. The decreased immunogenicity of synthetic peptides is a consequence of their failure to mimic the conformational epitopes of protective antigens. Strategies to augment the immunogenicity of synthetic peptides, such as (a) inclusion of both B and T_H cell epitopes in the peptide, (b) presentation of peptide in a noninfectious viral particle, or (c) expression of peptide by infectious virus, have not overcome the inherent deficiencies of this approach. Until these are achieved, it appears unlikely that a synthetic peptide vaccine will prove protective (289).

The recent demonstration that epitopes seen by CTLs consist of short peptides of about eight to 10 amino acids in length presented in the context of the MHC class I heterodimer suggested that it might be possible to produce peptide vaccines to induce CTL-mediated immunity (75). Success with such CTL-mediated vaccines has been achieved in several experimental systems. For example, a peptide, in adjuvant, induced CTLs and protected mice against Sendai virus infection (137). In addition, immunization with a vaccinia recombinant virus expressing a CTL epitope either in the context of the entire viral protein (115,149) or as a minigene (CTL epitope only) induced transient resistance *in vivo* (328). Immunization with vaccinia virus expressing a series of LCMV CTL epitopes induced resistance to LCMV in mice of different MHC haplotypes (328). However, immunization with CTL epitopes is fraught with obstacles (see section on CTLs in this chapter). It has yet to be demonstrated that a peptide-CTL-epitope vaccine induces immunity that is more effective and more durable than that induced by either a protein antigen or a VLP or by infection with an attenuated virus.

Antiidiotypic Antibodies as Immunogens

Another approach to induction of viral neutralizing antibodies involves the use of antiidiotypic antibodies as immunogens. Infection or immunization with a virus induces a neutralizing antibody (so-called antibody 1), which bears on its Fab variable region combining site a reverse image of the virion protein that is the target of that particular antibody. This unique region of the Fab is called an idiotype. Immunization with antiviral antibody (antibody 1) induces antiidiotype antibody (antibody 2), which has a conformation at its combining site that mimics the structure of the original viral epitope. This antiidiotype antibody can then be used instead of the viral antigen to elicit an immune response (antibody

3) to the viral protein. Immunization with antiidiotype antibody has induced a neutralizing antibody response to reovirus, poliovirus, rabies virus, coxsackievirus, or HBsAg (100,175,246,315), and protection of experimental animals has been demonstrated for poliovirus (315) and HBV (139). However, the level of neutralizing antibodies induced by these antiidiotype antibodies has been less than that elicited by infection or by immunization with inactivated viral particles. Thus, this approach to vaccine development does not appear to have an advantage over that of other, more conventional approaches used to induce protective antibodies.

Immunization with DNA Encoding Protective Antigens of Viruses

A new and potentially useful strategy for immunization involves direct intramuscular or intradermal inoculation of plasmid DNA that encodes viral protective antigens (290,331). Expression of the protective antigen is driven by a strong promoter capable of efficient activity in a variety of mammalian cell types. The feasibility of immunization with DNA was demonstrated by the induction of antibodies to human growth hormone in mice immunized with a plasmid encoding this foreign protein (290). Subsequently, immunization with DNA that encoded viral antigens, including antigens that can form VLPs, from a wide range of viruses has induced functional (i.e., neutralizing or syncytium-inhibiting) antibodies and/or resistance to viral infection (70). Immunization with DNA has several advantages over immunization with purified viral antigens. The most important advantage is that viral antigens such as viral glycoproteins can be expressed on the surface of transfected cells and are presented to the immune system in a native state. During the process of purification of viral proteins, purification and assembly of VLPs, or inactivation of viruses with chemicals, the integrity of epitopes on protective proteins can be adversely affected, resulting in altered and weakened immunogenicity. DNA immunization, which circumvents these problems, more closely resembles immunization with a live virus vaccine than with a nonliving viral antigen. Viral antigens encoded by DNA, whether entire proteins or minimal CTL epitopes, are presented efficiently in the context of class I MHC antigens and are able to induce T_H cells and CTLs (335). In this way, it is possible to induce a balanced immune response more similar to infection-induced immunity than immunity induced by administration of preformed viral antigens. A second advantage of immunization with DNA is its ability to transfect cells without interference by viral antibodies. Whereas live attenuated vaccine virus, such as that present in the measles virus vaccine, is effectively rendered noninfectious by maternal antibodies, DNA immunization should not be affected in this manner. Finally, it has been observed that the foreign antigen can be expressed *in vivo* for several months following DNA immunization (333). If transfected cells can present antigens over a longer period of time than live or nonliving virus vaccines, more durable B- and T-cell responses might result, but the rate of rise of immune responses following DNA immunization can be slow. Immunization with DNA encoding rabies G glycoprotein by gene-gun delivery of plasmid into skin (a delivery method considered highly efficacious) protected monkeys against challenge with virulent rabies virus. Nevertheless, the conventional vaccine induced a much more rapid rise to peak antibody titer, indicating that it would still be the preferred immunogen for postexposure prophylaxis (160). To date, immunization with DNA has induced lower titers of serum antibodies and less protective immune responses than that resulting from virus infection or from other more conventional forms of immunization (171,212,335). The major reasons underlying the weak immunogenicity of DNA vaccines are the paucity of cells that are transfected *in vivo* and the low level of expression of viral antigen in the transfected cell. Both of these deficiencies will need to be improved considerably if this form of immunization is to be useful in the next generation of vaccines.

The technology to improve the immunogenicity of DNA vaccines is evolving rapidly, and it is clear that this form of immunization has not yet been optimized. The immunogenicity of DNA vaccines has been significantly increased by a variety of methods (7,19,27,142,264,269), and it remains unclear which of these methods or which combination might bring DNA-based vaccines into the clinic. Recent success in augmenting the immunogenicity of DNA vaccines has been achieved using plasmid vectors based on the alphavirus replicon system, in which the plasmid vector expresses both the nonstructural alphavirus proteins and the foreign gene. The latter is selectively amplified in the cells transfected by the vector, as it is under control of the alphavirus subgenomic promotor (116).

The safety of DNA vaccines will require continued evaluation, but the likelihood of mutagenesis by integration of the vaccine DNA into the human genome appears at this time to be very small (27).

Adjuvants

A characteristic feature of purified viral proteins produced by recombinant DNA technology or peptide antigens is that they are weakly immunogenic in an immunologically naive host. This is because the immune system has evolved, in part, to respond most efficiently to viral antigens that are present on virus particles or on virus-infected cells—that is, the strongest immune responses are to multimeric, particulate antigens. An adjuvant is needed to increase the immunogenicity of recombinant viral antigens that are predominantly individual monomers or oligomers, but the only adjuvant licensed for human use is alum. This is a relatively weak but safe adjuvant. It is not surprising to find that VLPs, because of their particulate nature, can be highly immunogenic without an adjuvant. There is a considerable effort being made to develop safe and effective adjuvants for human use (4,59,220). Adjuvants function as antigen depots, as immunostimulants or immunomodulators, or as organizers of antigens into particulate forms. Many adjuvants combine two or more of these functions.

The depot function of adjuvants is designed to release antigen for a long period that exceeds that of an aqueous antigen suspension and that mimics an acute viral infection in which there may be sustained release of antigen for 2 to 4 weeks (59). Alum, an insoluble precipitate of aluminum hydroxide and aluminum phosphate, functions as a short-term antigen depot in which antigen that is electrostatically bound to alum is slowly released at the site of inoculation. A second type of depot is a water-and-oil emulsion from which antigen is slowly released as the biodegradable oil is metabolized or removed by macrophages. A third type of depot is that in which viral antigen is complexed with a synthetic polymer to form microparticles that can be degraded by tissue enzymes, thereby slowly releasing the viral antigen. Microparticles can be designed to release antigen over a long period of time.

The immunostimulatory and immunomodulatory functions of adjuvants serve to augment the antibody, CTL, and/or T_H cell responses to viral antigens. In addition, adjuvants can modify the T_H cell balance between the T_H1 and T_H2 subsets

achieved by immunization. For example, alum stimulates a T_H2 response, whereas monophosphoryl lipid A, a detoxified bacterial endotoxin, stimulates predominantly a T_H1 response. If the goal of immunization is to achieve a CTL response to the viral antigen, then one might choose to include in the adjuvant a saponin, which can form immunostimulating complexes (ISCOMs) (319). Various cytokines are also being explored as adjuvants (159). Bacterial toxins such as the cholera toxin and the *Escherichia coli* labile toxin, which have been molecularly engineered to exhibit greatly reduced toxicity *in vivo*, are potent systemic and mucosal adjuvants, but they can elicit an immune response to themselves that might limit their usefulness as general adjuvants (221). Last, it has recently been recognized that mammals have the capacity to recognize specific sequences, such as CpG sequences, in bacterial DNA, and to respond by augmenting the immune response to a viral protective antigen (64). For this reason, oligonucleotides containing CpG sequences have been incorporated into certain experimental adjuvant formulations.

Particulate adjuvants include (a) liposomes formulated to contain lipophilic or hydrophilic viral antigens; (b) ISCOMs, which are cagelike structures formed by a complex of a defined mixture of lipids and saponins, containing viral antigens; and (c) microparticles made from biodegradable polymers complexed with viral antigens (59).

Currently, one adjuvant formulation, designated microfluidized emulsion 59 (MF59), has been extensively tested in monkeys and humans, and its safety and immunogenicity profiles appeared sufficiently promising that it has been licensed in Italy for use with an inactivated influenza virus vaccine (4,220). MF59 consists of the biodegradable oil squalene and the surfactants sorbitan trioleate and Tween 80. Each of these three components has been previously used in human medicines. Squalene serves the depot function, but it remains unclear which component(s) functions as an immunostimulant. MF59 was originally developed as a vehicle for the immunostimulant MTP, a derivative of muramyl dipeptide, the active component of the *Mycobacterium* cell wall fraction in Freund's complete adjuvant. Clinical studies in humans indicated that MF59 itself was as immunostimulatory as MF59/MTP, with less toxicity (105). MF59 appears to be a safe adjuvant in nonhuman primates (308,318) and humans (105,184) but increased immunogenicity is accompanied by an increase in reactogenicity. The ability of MF59 to augment the humoral immune response appears to be greater in an unprimed host (308) than in a previously primed host (184).

CURRENTLY LICENSED LIVE VIRUS VACCINES

Except for vaccinia virus, each of the 16 currently licensed live vaccine viruses (Table 1) has a clear lineage. Vaccinia virus and the simian (rhesus) rotavirus are not of human origin. The orally administered enteric-coated adenovirus vaccine strains (types 4 and 7) are wild-type human viruses that produce an asymptomatic infection that is limited to the lower intestinal tract, in which disease with these types of adenovirus does not occur (42). One of the most widely used vaccine strains is a naturally occurring attenuated human poliovirus that was identified by its lack of virulence for the brain and spinal cord of monkeys (type 2 poliovirus, strain 712) (261). The quadrivalent rotavirus vaccine is composed of a simian rotavirus (serotype 3 specificity) and three reassortant viruses in which the VP7 gene (the determinant of serotype specificity) of the simian virus has been replaced by a human rotavirus gene with serotype 1, 2, or 4 specificity. Finally, the remaining vaccine viruses were derived from wild-type human viruses by serial passage in cell cultures prepared from an unnatural host, leading to the emergence of mutants that were partially restricted in humans at the portal of entry and/or the target organ(s). In this manner, attenuated mutants of rubella virus and types 1 and 3 poliovirus were selected after passage in monkey kidney tissue culture. Vaccine strains of yellow fever virus (YFV; 17D strain) and measles virus were generated by multiple passages in chick embryo cell culture, whereas embryonated eggs were used for this purpose for attenuation of mumps virus.

For the YFV and poliovirus vaccines, it was possible to identify promising vaccine strains by their attenuation for experimental animals. In monkeys, the 17D strain of YFV exhibited decreased tropism for the liver, and this served as a sign that this mutant might be suitably attenuated for humans (300). This was confirmed during subsequent clinical trials. In a similar fashion, naturally occurring and tissue culture–passaged strains of poliovirus were evaluated for neurovirulence in monkeys by intraspinal inoculation (260,261). This system was selected because a number of observations suggested that the spinal cord of monkeys was more permissive than that of humans to the neuronolytic effect of poliovirus (260,261). Viruses least neurovirulent for monkeys were identified, and these strains were then cloned and recloned by the plaque technique to yield progeny with the lowest possible level of monkey neurovirulence. These candidate vaccine strains were then subjected to additional cycles of selection, which led to the identification of mutants that replicated with high efficiency in the intestinal tract without significant increase in neurovirulence for nonhuman primates. These viruses were then evaluated in clinical trials and shown to be satisfactorily attenuated and immunogenic.

Experimental systems for evaluating virulence were not available for the other vaccine viruses. Hence, these viruses were tested for attenuation directly in humans. Initial candidate measles and rubella vaccines were not suitably attenuated, but further passage of rubella virus in monkey kidney cell culture and selection of a cold-adapted, temperature-sensitive mutant of measles virus yielded satisfactory vaccine strains. Attenuation of mumps virus passaged in eggs was also monitored in humans. Interestingly, the Jeryl Lynn strain of mumps virus vaccine has recently been found to be a mixture of two closely related strains of mumps virus (3).

Poliovirus, adenovirus, and rotavirus vaccines are administered orally, whereas yellow fever, rubella, measles, and mumps virus vaccines are given parenterally. It should be noted that, except for adenovirus and rotavirus vaccines, each of the currently licensed live-virus vaccines is directed against a virus that has a complex pathogenesis of infection in which virus is introduced by inoculation (yellow fever) or by implantation on a mucosal surface (measles, mumps, rubella, and poliomyelitis), with subsequent spread systemically to the target organ(s).

Basis for Attenuation

Mutants selected by passage in an unnatural host accumulate many mutations, often making it difficult to define in a precise manner the genetic basis for their attenuation. In a sense, these satisfactorily attenuated mutants are the product of a process of genetic roulette, followed by selection of mutants with the desired properties of attenuation and immunogenicity. The unpredictability of this process is illustrated by the failure of Theiler (299) to produce additional

attenuated mutants of YFV using the protocol that yielded the satisfactorily attenuated 17D strain of virus. The genetic basis for attenuation of measles, mumps, rubella, yellow fever, rotavirus, and vaccinia viruses is unknown, whereas the genetic determinants of attenuation of two of the three live poliovirus vaccine strains are better characterized. The net result of most attenuating mutations is to reduce the level of replication in the host, at the portal of entry or at internal sites, to a level that does not cause significant disease. For some viruses, the specific level of replication in humans or nonhuman primates that is associated with attenuation is known, although it is not known for most viruses (200,202,298).

The poliovirus vaccine strains are temperature sensitive with respect to replication and exhibit greatly decreased neurovirulence in monkeys after intraspinal administration of virus (260). Because the poliovirus vaccine viruses replicate efficiently in the gut of vaccinees and can produce viremia, it is likely that restriction of replication in neurons represents the basis of attenuation of these viruses for the CNS of humans, monkeys, and rodents. The genetic basis of this neuroattenuation of each of the three poliovirus vaccine strains has been partially defined (108). The identification of mutations contributing to neuroattenuation of the three vaccine viruses derives from a large series of studies involving (a) sequence analysis of virulent parental type 1 and type 3 viruses and their attenuated vaccine strain derivatives; (b) sequence analysis of neurovirulent “revertant” virus isolated from vaccineassociated cases of poliomyelitis (101); (c) *in vitro* generation of chimeric recombinant viruses produced from cDNA clones of virulent and attenuated viruses or viruses bearing site-specific mutations thought to be associated with neuroattenuation on the basis of sequence analysis of revertant viruses (25); or (d) evaluation of the neurovirulence of these naturally occurring or *in vitro*–generated viruses in monkeys, normal mice, or mice transgenic for the human poliovirus receptor. These analyses have identified an attenuating nucleotide substitution in the same region in the 5' noncoding sequence of each of the three vaccine viruses (nucleotide 480 in type 1 virus, 481 in type 2 virus, and 472 in type 3 virus) and other attenuating or *ts* mutations in the 3' noncoding region or in the structural or nonstructural coding regions of type 1, type 2, or type 3 polioviruses (25,101,249). The mutation in the 5' noncoding region (a) alters the predicted secondary structure of this important regulatory region, (b) is responsible in part for the *ts* phenotype of the vaccine viruses, and (c) decreases virus replication *in vitro* in cells of neural origin, presumably by altering the function of a neural tissue specific internal ribosomal entry site (108). These mutations can now be monitored during manufacture (45,249) and following replication *in vivo*. Importantly, reversion in several of these attenuating mutations has been detected during the *in vitro* passage of virus that is required for the production of vaccines (45) or after replication *in vivo*, even as early as 47 hours after vaccine administration. Specifically, the rare reversion of the attenuating mutation at nucleotide 472 of the 5' noncoding region of the Sabin type 3 vaccine strain that occurs during vaccine production can now be detected using sensitive polymerase chain reaction techniques. Vaccine lots with an increased frequency of reversion at residue 472 exhibit increased neurovirulence, and such lots can be discarded as unacceptable for human use (45). Thus, it is now possible to use molecular virologic techniques to control the manufacture of vaccines by identifying the presence of the desired attenuating mutations as well as the absence, or low frequency, of unwanted reversions (45).

A different approach was taken to develop the live adenovirus vaccines that are currently licensed for use in military personnel, where adenoviruses are the major cause of epidemic respiratory tract disease (42,74). Attenuation is achieved by limiting infection to an area of the body in which disease does not develop. The current vaccine contains wild-type virus that is administered orally in an enteric coated tablet. Virus is released when the tablet enters the intestines. There it establishes an asymptomatic, immunizing infection that does not spread to the respiratory tract, the region in which disease develops during natural infection. Although vaccine virus is shed in feces, spread to susceptible contacts occurs with a very low frequency. Selective enteric infection stimulates a vigorous systemic immune response that confers complete or almost complete resistance to adenovirus disease (74). Resistance to reinfection is also impressive (70%) but not complete (74,274).

The Jennerian approach to the development of live attenuated viruses involves the use of a virus strain of mammalian or avian origin to immunize humans against a human virus that is related antigenically to the animal or avian strain. Mammalian and avian viruses that are well adapted to their natural host often do not replicate efficiently in humans and hence are attenuated. At present, we lack a thorough understanding of the genetic basis for this form of host-range restriction. However, those mammalian or avian viral genes that have been identified as being responsible for host-range restriction in humans exhibit significant divergence of nucleotide sequence from that of the corresponding human viral genes. The recently licensed quadrivalent rotavirus vaccine is an example of the Jennerian approach to vaccine development in which a nonhuman rotavirus strain, the rhesus rotavirus (RRV), was found to be attenuated in humans and protective against human serotype 3 to which it is antigenically highly related (135). Since there was a need for a multivalent vaccine that would induce resistance to each of the four major human rotavirus serotypes, the Jennerian approach was modified by constructing three reassortant viruses, each of which contained 10 RRV genes plus a single human rotavirus gene that coded for the major neutralization antigen (VP7) of serotype 1, 2, or 4. The intent was to prepare single gene substitution RRV reassortants with the attenuation characteristics of this simian virus and the neutralization specificity of human rotavirus serotype 1, 2, or 4. The quadrivalent vaccine based on the host-range restriction of the simian RRV in humans induced a high level of resistance to human rotavirus disease in infants and young children (230). However, the licensed vaccine retains mild reactogenicity in older seronegative infants lacking maternal antibody, and second-generation Jennerian vaccines, based on strains of bovine rotavirus, is being developed to replace the RRV vaccine (48).

Advantages of Live Virus Vaccines

The major advantage of live virus vaccines is the activation of all components of the immune system yielding both a balanced systemic and local immune response, and a balanced humoral and cell-mediated response. This is particularly important for infections in which cell-mediated immunity plays an important role, and for mucosal infections in which both local and systemic immunity are required for optimal resistance. Mucosal infection with a live virus vaccine is usually more effective in stimulating a local response in the unprimed host than is a nonliving virus vaccine administered parenterally (198). Live virus vaccines also stimulate an immune response to each of the protective antigens of a virus, and this obviates the difficulties that arise from selective destruction of one of the protective antigens that may occur during preparation of a nonliving virus vaccine, which can result in disease potentiation. This is especially important for complex viruses encoding many protective antigens, as it is difficult to immunize economically with multiple purified proteins or to construct multivalent vectors. Furthermore, immunity induced by live virus vaccines is generally more

durable and more effective (132). It should be noted that there are also practical advantages to many live virus vaccines, such as low cost of production and ease of administration.

Disadvantages of Live Virus Vaccines

The major concerns with live virus vaccines are that they (a) can contain adventitious agents, (b) can cause illness directly or lose their attenuation during manufacture or during replication in vaccinees by reverion or by second-site compensatory mutations, (3) can spread to contacts (see preceding), and (4) can lose infectivity during storage, transport, or use. The potential for contamination with live adventitious agents always exists. Fortunately, this has rarely been a problem. Some of the early lots of live poliovirus vaccine were contaminated with live SV40, and the live YFV vaccine was initially contaminated with an avian leukosis virus. Follow-up of individuals who were given live vaccine contaminated with SV40 or avian leukosis virus has failed to identify any long-term adverse effect, including cancer, associated with exposure to these adventitious viruses (191,286,321). Current vaccines grown in chicken cells contain a presumably noninfectious retrovirus derived from the cell cultures, and this contaminating particle does not appear to be infectious for vaccinees (311). Nonetheless, prudence dictates that live virus vaccines should be free of such contaminants.

Some live virus vaccines, such as the measles virus, rubella virus, and YFV vaccines, retain a low level of residual virulence. The reactions produced by these vaccines are minor and have not interfered with acceptance and widespread use of these products. A more serious problem is that of restoration of a varying degree of virulence during infection by vaccine virus. This occurs with the poliovirus vaccine at an extremely low frequency i.e., about one in 10^6 to 10^7 immunizations) (215). Most vaccine-associated cases of paralysis in vaccinees or their close contacts occur after the first dose and involve the type 3 vaccine strain (215). A significant proportion of the paralytic illnesses associated with poliovirus vaccine occur in individuals who are immunocompromised, but this may not represent a manifestation of genetic alteration of vaccine virus (215). In most instances, however, vaccine virus appears to have regained neurovirulence by mutation, allowing it to produce disease in the vaccinee or a close contact (101). In addition to mutation, polioviruses with recombinant genomes have been isolated from vaccinees with paralytic disease, suggesting that restoration of virulence might occur by the process of interserotypic recombination as well as by mutation (96).

The genetic basis for the very rare increase in virulence of the poliovirus vaccine strains during replication *in vivo* is relatively well understood. However, a paradox exists, in that mutation toward a higher level of virulence for the CNS occurs rapidly and frequently during replication of type 3 poliovirus in the intestinal tract of vaccinees, but vaccine-associated paralytic disease is extremely rare (183,188). It is now clear that mutations that increase neurovirulence of the virus for primates can occur in each of the three Sabin vaccine strains during infection of vaccinees (165,293,296), but, fortunately, poliomyelitis in vaccinees or contacts is very rare. During manufacture of vaccine, the mutations that are associated with restoration of virulence can be detected at variable low frequency by sequence analysis, and neurovirulence of the final product can be held to a low level by rejecting vaccine lots that exceed the accepted standard for frequency of these mutations (45). Genetic instability was also a problem with the 17D YFV vaccine during early field trials. Encephalitis was observed in 1% to 2% of young vaccinees administered virus that had been passaged 20 times beyond the original seed lot (88,300).

The problem of genetic instability of live attenuated virus vaccines following replication *in vivo* is an even larger concern for viruses that can cause latent or persistent infection *in vivo*, such as herpes viruses or lentiviruses (e.g., HIV). Despite this concern, a successful vaccine has been developed for VZV, a herpes virus that causes chickenpox and zoster, indicating that a successful live attenuated vaccine can be developed for a virus that causes a lifelong infection in humans.

During studies of attenuated lentivirus mutants, SIV in monkeys and HIV in humans, the time required for expression of residual virulence proved to be very long (9,155). These mutants, constructed in the laboratory (SIV) or detected initially in persons with long-term inapparent HIV infection, contained multiple mutations including deletion of the *nef* gene as well as other regions of the viral genome. Nevertheless, immunodeficiency did develop (9,155). The long incubation period to disease and the development of additional second-site mutations that suppressed the attenuation phenotype of an SIV deletion mutant (20) emphasize the daunting challenge of the live attenuated vaccine approach to prevention of AIDS.

Because live poliovirus vaccines can cause persistent infection and paralytic disease in immunodeficient children (332), there is an increasing concern that, as the numbers of immunodeficient humans increase, other live attenuated virus vaccines might need to be phenotypically more stable in the future. Rubella virus can be recovered from the lymphocytes of immunologically normal individuals with arthritis up to 6 years after immunization or natural infection (44), but the rubella virus vaccine does not appear to be associated with chronic arthritis (245). Interestingly, persistence of measles vaccine virus has not been detected, nor has the vaccine virus been implicated in subacute sclerosing panencephalitis (182). Instead, widespread use of live measles vaccine has almost eradicated this rare but serious sequela of persistent wild-type measles virus infection.

Several other concerns have been identified for live attenuated virus vaccines. First, although there is a theoretical risk that infection of the fetus with rubella vaccine virus during the first trimester of pregnancy might lead to development of the congenital rubella syndrome, the actual risk appears to be negligible (225). Second, naturally occurring wild-type viruses may interfere with infection by a live virus vaccine, resulting in a decrease in vaccine efficacy. Such interference has been observed primarily with live poliovirus vaccine strains and is caused by a wide variety of enteric viruses. This phenomenon has also been seen with experimental live attenuated alphavirus vaccines (174). To overcome this virus–virus interference, multiple doses of polyvalent live virus vaccines are required to ensure a protective immune response to each component. Third, defective interfering particles have been identified in one preparation of live measles virus vaccine (35). The presence of such particles could modify the clinical response to vaccine and possibly produce variation in immunogenicity and level of attenuation among successive lots of vaccine. Fourth, live attenuated measles virus vaccine has been associated with a generalized immunosuppression that was observed to be directly correlated

with the level of immune response to the vaccine (126).

Finally, it should be noted that stability is a serious problem with labile vaccine viruses such as measles virus. The need for storage and transport of measles vaccine at low temperature (4°C) has limited its usefulness in some tropical areas, where maintenance of a “cold chain” for transport and storage is difficult.

DIRECTIONS FOR FUTURE DEVELOPMENT OF LIVE ATTENUATED VIRUS VACCINES

Conventional Approaches and Naturally Occurring Attenuated Mutants

Conventional techniques for attenuation of viruses, such as passage of virus at low temperature, mutagenesis followed by selection of mutants with the desired phenotype(s), or passage of virus in heterologous tissues, will continue to have a role in the development of live virus vaccines (17,168). These techniques are being used to generate candidate vaccine strains for cytomegalovirus, ortho- and paramyxoviruses, alphaviruses, flaviviruses, HAV, and arenaviruses. In the case of RSV, a candidate vaccine strain that was partially attenuated for susceptible young infants by multiple *in vitro* passages at low temperature in embryonic bovine kidney cells was subsequently subjected to chemical mutagenesis, and further attenuated derivatives containing attenuating temperature-sensitive (*ts*) or small plaque mutations were identified (201). In this way, two conventional approaches were combined to introduce different classes of attenuating mutations (i.e., host-range mutations introduced by passage in a heterologous host, and *ts* mutations introduced by chemical mutagenesis). This process of combining different attenuating mutations is thought to be desirable because the different mutations can have synergistic effects on the stability of the attenuation phenotype *in vivo* (204).

Variation in virulence of naturally circulating human viruses has been documented, but this phenomenon has not been studied systematically, nor have its implications for immunoprophylaxis been adequately explored. Almost 45 years ago, Sabin (259,260) observed that strains of poliovirus recovered from healthy individuals exhibited a wide range of neurovirulence as measured by intracerebral or intraspinal inoculation of monkeys. Approximately one fifth of these strains were highly attenuated. This suggests that systematic study of viruses recovered from healthy persons might identify naturally occurring attenuated mutants of viruses other than poliovirus. As indicated previously, the type 2 poliovirus strain of the live oral vaccine was identified in this manner.

The use of conventional methods for achieving attenuation will diminish as the newer techniques for construction of viruses with defined attenuating mutations are applied to development of live virus vaccines. Viruses bearing stable, defined, identifiable attenuating mutations or attenuating gene constellations represent the vaccine strains of the future because the genetic basis for attenuation will be known and can be monitored directly during all phases of vaccine development, manufacture, and utilization in humans.

Attenuating Mutations Introduced into Coding Regions

Missense Mutations

A missense mutation that specifies an amino acid substitution in an encoded viral protein can decrease its function *in vivo*, thereby attenuating the virus for its host. Three classes of missense mutants with easily identifiable phenotypes have been evaluated extensively for their level of attenuation and their potential usefulness in live attenuated viral vaccines: (a) *ts* mutants, (b) protease activation (*pa*) mutants (also designated as cleavage mutants), and (c) mutants with altered interaction with host cell receptors.

The *ts* mutants are conditional lethal viruses that replicate efficiently at permissive temperatures (32° to 34°C) but that are restricted in replication at the high end of the normal temperature range (37° to 39°C). The *ts* mutants can be grown efficiently *in vitro* at the permissive temperature for vaccine production because the attenuating *ts* mutations generally restrict replication only at elevated temperature. Attenuating *ts* mutations have been identified in most, if not all, genes of the viruses that have been mutagenized and then examined systematically (267). Also, multiple sites within a given protein can sustain *ts* mutation (154). Thus, it is possible to introduce a *ts* mutation into almost any gene of a candidate vaccine virus. Alternatively, several *ts* mutations can be introduced into the same gene using recombinant cDNA technology (288,327), and two nucleotide changes per codon can often be engineered to specify the desired amino acid substitution, thereby increasing the stability of the missense mutation. It is also possible to export an attenuating missense mutation that is present in a virus to the corresponding site of a member of a distantly related virus genus or subfamily, and in this way the number of mutations that are available for use to attenuate candidate vaccines can be significantly expanded (270). Thus, recombinant DNA technology offers new flexibility to add or subtract mutations in vaccine candidates to achieve the desired delicate balance between attenuation and immunogenicity that is needed to produce a successful vaccine.

The use of *ts* mutations to attenuate respiratory viruses is especially attractive because the *ts* lesion should restrict virus replication in the warmer lower respiratory tract (37°C), the major site of disease; however, sufficient replication should occur in the cooler upper respiratory tract (32° to 34°C) to immunize the host (250). Importantly, virus replication in the latter site is usually sufficient to immunize the host against infection with virulent virus (17,250). However, genetic instability has presented a problem for *ts* mutants because there is strong selection *in vivo* for ts^+ viruses that have undergone reversion to wild-type sequence or have sustained suppressor mutations that abrogate the *ts* phenotype (304). The phenotypic stability of candidate vaccines bearing *ts* mutations can be enhanced by increasing the number of *ts* mutations per virus or by combining *ts* and non-*ts* attenuating mutations, which can act synergistically to augment stability of the attenuation phenotype (204,271,327).

The second approach to producing missense mutations that specify attenuation involves the isolation of *pa* mutants. Proteolytic cleavage of the F glycoprotein of paramyxoviruses is required for activation of infectivity, and this process is thought to play an important role in tissue tropism and pathogenicity. Trypsin-like enzymes that are present in host tissues are required for the cleavage of the fusion protein precursor F0 to its disulfide-linked F1 and F2 subunits. The *pa*

mutants of murine parainfluenza virus type 1 (PIV-1; Sendai virus) were selected by growing virus in the presence of another protease such as chymotrypsin or elastase (294,295). The *pa* mutants selected in this manner were resistant to activation by trypsin but formed plaques in the presence of the selecting protease. These *pa* mutants, which sustained amino acid substitutions that abolished the cleavage site for trypsin and created a new site for cleavage by chymotrypsin or elastase (129), were attenuated and protective in animals. However, revertants did develop during replication *in vivo* (186).

Viral surface proteins can also undergo mutation that results in attenuation. This is not surprising because missense mutations affecting surface proteins can alter tissue tropism and pathogenesis of infection (277). This was shown most convincingly in studies in which mutants were selected by growing virus in the presence of a monoclonal antibody that neutralized virus infectivity and by isolating antigenic mutants that were no longer neutralized by the selecting antibody. These variants, which are referred to as monoclonal antibody-resistant (*mar*) mutants, generally sustain only a single amino acid substitution. Some *mar* mutants have been described that have properties desirable for a candidate vaccine—namely, they have a decreased ability to enter and infect cells in the target organ in which the virus produces disease, but they are able to replicate at other sites *in vivo* and in tissue culture (277,278). It has been possible to increase the stability of *mar* mutations by the introduction of multiple mutations sequentially into the capsid proteins of coxsackievirus B4 or the G glycoprotein of rabies (58,240).

Because it appears that attenuation can be achieved with even a small number of mutations in certain circumstances, it should now be possible using modern molecular techniques to introduce the requisite number of attenuating missense mutations into a virulent or partially attenuated virus to produce a vaccine virus that has a stable attenuation phenotype (327). Missense mutations can be combined with attenuating deletion mutations to fine-tune the level of attenuation while maintaining a high level of stability of the attenuation phenotype that will allow use even in immunocompromised persons.

Deletions

Deletion mutations that represent either partial or complete deletion of a given protein sequence can attenuate a virus for its host. Deletion mutations can be used in several ways to construct candidate vaccine viruses. First, deletion mutations can be produced in one or more genes that are not required for replication *in vitro* to attenuate the virulence of the virus for its host (67,166,276). Second, a protein that mediates a specific function in the host that would be undesirable in a vaccine virus can be deleted—for example, a gene encoding neurovirulence for a herpes virus or a gene encoding a protein involved in immune evasion. The use of deletion mutations as the basis for the attenuation of a vaccine virus has appeal because this form of mutation cannot be corrected directly as this would require restoration of deleted sequence. Although phenotypes specified by a deletion mutation can be modified by a suppressor mutation or by another genetic mechanism such as a sequence duplication (20,38,310), attenuation specified by an appreciable deletion is more stable than that specified by a single missense mutation.

To be useful in construction of live vaccine viruses, deletion mutations should restrict virus replication *in vivo* while permitting virus to grow efficiently *in vitro* during vaccine production. This is most easily achieved for poxviruses, herpesviruses, and adenoviruses, whose large genomes encode a number of functions that are not essential for virus replication *in vitro*, but it also has been accomplished for RNA viruses that bear genes that encode accessory functions (138,325). Furthermore, deletions have been introduced in noncoding regions of a viral genome to achieve attenuation without greatly restricting replication (178).

Attenuating deletion mutations can be designed to abrogate specific undesirable functions of a virus. The function of immune evasion genes can be deleted via mutation, resulting in attenuation *in vivo* (163) or in an increased susceptibility to immune mediators of protection (163,210). Deletion of specific genes of HSV can (a) reduce the number of latently infected neurons, (b) decrease the frequency of reactivation from latency, (c) or decrease neurovirulence while maintaining efficient replication *in vitro* (66,130,301).

Insertions

A virulent virus can be attenuated by the insertion of genes that encode proteins with known antiviral activities or with known immunoregulatory functions, such as interferons, interleukins (ILs), or tumor necrosis factor (31,84,147). Such recombinant viruses encode foreign proteins that function to restrict replication of the virus, and this results in attenuation of the virus for its host. Vaccine candidates possessing insertions that provide antiviral activity must be produced in cells that are refractory to this activity. Some DNA or RNA viruses bearing such attenuating foreign immunomodulatory proteins have been found to be satisfactorily attenuated in animals, with some recombinants even being highly attenuated for immunodeficient animals (31,84,147). It is also possible to augment immunogenicity of a candidate vaccine by inclusion of desired ILs—for example, inclusion of IL-5 or IL-6 in a vaccinia virus increased mucosal IgA responses to vaccinia virus (243). Usually, the restriction of replication imposed by the cytokine insertion decreases immunogenicity; however, for RSV, insertion of the interferon-g gene attenuated the virus while retaining a vigorous humoral immune response (31). Rapid attenuation of many viruses can now be achieved in a single step by the simple insertion of one or more specific attenuating cytokines. However, the genetic stability of the insert following manufacture and use in humans remains an issue.

Attenuating Mutations Introduced into Noncoding Regions

It is possible to attenuate a virus for its host by introduction of mutations into the 5' or 3' noncoding regions of the genome as well as into other cis-acting noncoding regions of the genome. These noncoding regulatory regions contain cis-acting signals required for efficient replication, transcription, and translation that represent target sites for modification of these functions by mutation. Attenuating mutations in these cis-acting regulatory regions can result in (a) a tissue-specific attenuation (108,339), (b) increased sensitivity to interferon (339), (c) temperature sensitivity (326), (d) a general restriction in level of replication (178,208), and (e) host-specific restriction of replication [e.g., a dengue virus that replicates in mammals but not in mosquitoes (34)]. These attenuating mutations can be achieved by point mutations,

exchanges of sequences between related viruses, or deletions.

Host-Range Mutations: The Jennerian Approach

The Jennerian approach to the development of live attenuated viruses has been successfully employed to develop the recently licensed rotavirus vaccines (see preceding) and is being explored to develop vaccines for PIV types 1 and 3 and for HAV (76,125,136). In each case, the animal virus is antigenically related to the human virus, and infection of experimental animals with the animal strain induces resistance to the human virus. The Jennerian approach to the development of live attenuated vaccines for influenza A viruses was attempted, but it failed to produce a consistently attenuated vaccine for use in humans (283).

Attenuation by Gene Incompatibility

The proteins of the PB2, PB1, and PA influenza virus polymerase complex function as a unit, performing a variety of biochemical activities. These proteins must be compatible with each other to function efficiently. Disruption of this compatibility can bring about attenuation. An example of the use of this strategy to produce an attenuated vaccine candidate is an influenza A reassortant virus derived by mating an avian influenza virus with a human influenza virus (287). This reassortant, which possesses a mixed polymerase complex, with the PB1 and PA genes derived from the human influenza A virus parent and the PB2 gene derived from the avian influenza A virus parent, was attenuated in humans. It replicated efficiently in avian tissue but grew poorly in mammalian cell culture and in the respiratory tract of humans. Incompatibility of polymerase genes also contributed to the attenuation of influenza A/PR/8/34 reassortants for humans (86). The phenomenon of gene incompatibility resulting in attenuation is also seen with DNA viruses. Intertypic recombinants between HSV-1 and HSV-2 often exhibit decreased neurovirulence for experimental animals compared with either parent (112,131,302).

Antigenic Chimeric Viruses

Live attenuated antigenic chimeric virus vaccines possess the protective antigens of a virus against which the vaccine is directed, plus the remaining coding and noncoding regions of another related virus that bears attenuating mutations. Live attenuated antigenic chimeric viruses comprise three of the four components of the quadrivalent licensed rotavirus vaccine. Each of the three antigenic chimeric viruses contains the attenuating backbone of 10 genes of the rhesus rotavirus plus the gene for a human VP7 serotype 1, 2, or 4 protective antigen. Similarly, the live attenuated influenza virus vaccine candidates are antigenic chimeric viruses that are antigenically updated annually by replacement of the HA and NA genes of the attenuated donor virus with those of a new epidemic or pandemic virus.

Recombinant DNA technology is actively being used to construct live attenuated antigenic chimeric virus vaccines for flaviviruses and for paramyxoviruses. For flaviviruses, a live attenuated virus vaccine candidate for Japanese encephalitis virus (JEV) has been made by the replacement of the premembrane (prM) and envelope (E) regions of the attenuated YFV with those from an attenuated strain of JEV. The JEV-YFV antigenic chimeric recombinant vaccine candidate was attenuated and immunogenic *in vivo* (110). Both components of this chimeric virus have come from an attenuated virus. Other strategies for developing live attenuated antigenic chimeric flaviviruses are also being pursued. Antigenic chimeric recombinant candidate vaccines have been made between a naturally attenuated tick-borne flavivirus (Langat virus) and a wild-type mosquito-borne dengue 4 virus, and the resulting recombinant was found to be significantly more attenuated than its tick-borne parent virus for mice (237). This is an example of an attenuating effect that stems from partial incompatibility between the two components of the chimeric virus.

A third strategy is being pursued for the production of a quadrivalent dengue virus vaccine in which a dengue 4 backbone containing an attenuating deletion mutation in the 3' noncoding region is used to construct antigenic chimeric viruses containing the protective antigens of dengue 1, 2, or 3 viruses (28,178). An antigenic chimeric virus containing the HN and F protective antigens of PIV-1 substituted for those of an attenuated PIV-3 virus has been constructed and shown to be attenuated and protective in experimental animals (292). This strategy is currently being pursued to make vaccines effective against PIV-1, -2, and -3.

VIRAL VECTORS

Replication-Competent Viruses as Vectors

Stable attenuated viruses, some of which have been used as vaccines in humans, are being used to construct viable recombinants that express the protective antigens of other viruses. The use of a viral vector for immunization has the general advantage that the foreign viral antigens are expressed naturally in the context of an infected cell, thereby inducing cellular as well as humoral immune responses. To date, the most success has been achieved using vaccinia virus as the recombinant vector (193). This virus has a large genome into which a variety of foreign viral genes can be inserted and expressed without seriously compromising the capacity of vaccinia virus to replicate. Recombinant vaccinia viruses expressing the protective antigen (or antigens) of a large number of viruses have been constructed and have been shown to be protective in experimental animals. Polyvalent live vaccinia virus recombinants have been used to successfully immunize experimental animals, even in the presence of immunity to one of the foreign viral antigens (85), and vaccinia–rabies virus recombinants are being used to control rabies in foxes (228). Unfortunately, vaccinia virus recombinants are not sufficiently attenuated for use in an immunocompromised host such as persons with HIV infection (82,247), and hence more attenuated poxvirus vectors were sought. A modified vaccinia virus Ankara (MVA), which was derived by more than 500 passages in chick embryo cells (181), has been evaluated as a vaccine vector for a variety of viral antigens. MVA is a highly attenuated host-range mutant that replicates well in avian cells but not in most mammalian cells, including those of monkeys and humans (23,39,71). MVA is nonpathogenic in immunocompromised hosts and has been administered to large numbers of humans without incident (172,285,324). Attenuation of the MVA recombinant resulted from the extensive deletions that the viral genome sustained during its many passages in embryonated eggs (8), and in this regard it resembles other further attenuated derivatives of vaccinia virus that have been attenuated by

extensive targeted deletions (223). Use of avian poxviruses, referred to as avipox viruses, as vectors represents a second type of host-range restricted poxvirus vector. Avipox viruses are naturally occurring host-range restricted viruses that are deficient in replication in mammalian cells and mammal hosts (223). Each of these poxvirus vectors has the property of efficient replication *in vitro* in avian cell culture but restricted replication in primates.

Although each of these poxvirus vectors is safe for immunodeficient persons, they, and most other viral vectors, have two other additional obstacles to overcome. The first obstacle is that vaccinees eventually develop immunity to the vector backbone itself, which restricts use of the vector to a limited number of immunizations (224). It is possible in certain circumstances to overcome this immunity (induced by previous parenteral immunization with a poxvirus) by readministration of vaccine by a mucosal route rather than a parenteral route (18). The second obstacle is that the immunity induced in primates by poxvirus vectors is often less than optimal (61), and additional research is required to optimize immunogenicity by coexpressing various cytokines and immunostimulatory molecules. The role that these poxvirus vectors will have in virus vaccines of the future remains to be defined, but it will depend in part on the success of efforts to augment immunogenicity.

A variety of other viruses that are being considered for use as vectors include adenoviruses (30,185) and rhabdoviruses (251), each of which has one or more features that offer specific advantages for its use as a replication-competent vector. It is likely in the future that such vectors will find use in immunization against viruses to achieve defined goals in immunization against specific diseases.

Replication-Defective Viruses as Vectors

Replication-defective virus vectors have had genes deleted that are essential for replication *in vitro* or *in vivo*. Replication-defective viruses can replicate to high titer in cell lines engineered to express the protein encoded by the deleted gene. The viruses produced in such complementing cells can infect cells *in vivo* but are unable to support multicycle replication. Such vectors undergo an abortive infection *in vivo* but express the foreign protein during this abortive infection and can thereby present antigens efficiently to both the humoral and cellular arms of the immune response. Such nonreplicating vectors would be expected to be safe in the immunosuppressed host.

Adenoviruses that lack the EIA gene replicate to high titer in the 293 cell line that constitutively expresses the EIA-encoded proteins (185,303). This type of defective adenovirus recombinant is prepared by inserting a foreign gene encoding viral protective antigens into the EIA region and then growing the adenovirus recombinant in 293 cells. Immunization of experimental animals with replication-defective recombinant adenoviruses has protected the animals against viral challenge (185,303).

Replication-defective HSVs have been produced in a manner analogous to that of the EIA-deficient adenoviruses—that is, they have had one or more genes deleted that are essential for replication, but replication *in vitro* is supported in a cell line that constitutively expresses the essential protein (26,62,63,119,189). In contrast to the EIA-deficient adenoviruses, the replication-defective HSVs are primarily being developed as vaccines to protect against disease caused by HSV itself rather than as a vector of foreign viral proteins. An immune response is induced not only to the antigens present in the input virus but also to the viral gene products expressed during the abortive infection *in vivo*. Such a replication-defective HSV could also be used as a vector of foreign viral proteins.

Replication-deficient alphavirus particles that cannot undergo multicycle replication *in vivo* have been generated (see preceding section on eukaryotic expression systems). Importantly, the high efficiency of the replicon amplification system *in vivo* permits a high level of expression of foreign protein *in vivo*, which induces a strong and effective immune response. The *in vitro* expression system has been modified to include the replicon along with two helper RNAs engineered to minimize the possibility of generating replication-competent alphavirus through recombination (241,273). Cell lines are being engineered to express various components of this tripartite system to increase the quantity of replication-defective virus generated *in vitro*. Replication-defective alphavirus vectors have induced a significant immune response, both cell-mediated and humoral, to a variety of viral proteins (118,241). Importantly, successful immunization to multiple pathogens has been achieved by sequential immunization (241). These replication-deficient alphavirus vectors offer promise for the development of future vaccines.

COMBINED USE OF LIVE AND NONLIVING VIRUS VACCINES TO ACHIEVE OPTIMAL PROTECTION

Sequential immunization with inactivated poliovirus vaccine followed by live attenuated virus vaccine was previously recommended for use in the United States to replace the three doses of the live virus vaccine that were in use. Several factors have contributed to this change. The live poliovirus vaccine causes paralytic disease very rarely in vaccinees or contacts, but since polioviruses have been eradicated from the Americas (except for the sporadic occurrence of imported cases), the vaccine-associated cases have received much more scrutiny. Since the vaccine-associated cases are seen predominantly following the first dose of vaccine, it was hoped that the use of the enhanced-potency inactivated poliovirus vaccine for the first several doses would induce sufficient immunity to abrogate even the rare cases of vaccine-associated polio caused by the live virus vaccine. Thus, the inactivated vaccine is used to protect against the rare cases of CNS infection caused by the live virus vaccine. The live virus is used because even the enhanced potency vaccine, in contrast to the live virus vaccine, provides relatively weak protection in the gastrointestinal tract (187,234). The live-virus vaccine is needed to optimize immunogenicity in the individual and to provide herd immunity to the population by inducing sufficient mucosal immunity to prevent the spread of imported strains of poliovirus. Currently, only the inactivated poliovirus is recommended for use in the USA.

Simultaneous administration of two different influenza virus vaccines to elderly subjects, namely, an intranasally administered live attenuated influenza A virus candidate vaccine and a parenterally administered licensed nonliving virus vaccine, induced greater resistance to illness than did administration of the inactivated virus vaccine alone (309). In this situation, the greater immunogenicity of the live virus vaccine in the respiratory tract coupled with the greater efficiency of

the inactivated virus vaccine in stimulating systemic immunity appear to act in concert to optimize efficacy. Sequential immunization with a live attenuated virus alphavirus vaccine candidate followed with a boost with an inactivated virus vaccine induced higher levels of antibody than live virus alone (235). A replication-defective HSV vaccine was found to be most immunogenic when administered by both parenteral and mucosal routes, indicating that the same virus preparation can be given by a combination of routes to achieve optimal protection (189).

For viruses that are relatively weak immunogens, such as HIV, various strategies are being explored to optimize immunogenicity by employing different types of immunogens in a sequential immunization regimen referred to as a prime-boost immunization schedule. The components of this strategy include vectored antigens or DNA vaccines, followed, in one scenario, by a boost with a purified protein vaccine (49,114,162,253). This varied-component, sequential immunization schedule is designed to stimulate multiple components of the immune system using different forms of the immunogen to attain a protective immune response.

SUMMARY

Over the next decade, it is likely that a large number of new viral vaccines will be successfully introduced. This increase will stem largely from the contribution that recombinant DNA technology has made to the generation of many candidate live and nonliving virus vaccines currently being evaluated in experimental animals and in humans. Vaccinologists of the future will have a greatly expanded choice of vaccine strategies, including the use of various combinations of different vaccines to achieve optimal protection.

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CHAPTER 17 Virus Vectors and Their Applications

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Gene therapy is a novel form of molecular medicine that is likely to have a major impact on human health in the coming century. Rarely has a medical technology elicited so much excitement, although that excitement is tempered by unfulfilled expectations. Excitement originates from the simplicity of the concept—introduce the gene and its product should have the ability to correct a genetic disorder and slow the progression of a disease. Disappointment stems from the inability to execute this task. How does one deliver genes into a wide variety of cells, organs, and whole animals? How long is the foreign gene expressed in its new milieu? Are there adverse immunologic consequences of both the delivery vehicle and its cargo? The quest for the perfect delivery vehicle is still incomplete!

The currently available delivery vehicles fall into two categories: The first consists of viral vectors, which take advantage of a variety of naturally occurring viruses to shuttle the foreign cargo either to be integrated permanently into the host's chromosome or to masquerade as an autonomous unit. The second category of delivery vehicles is made up of the nonviral vectors, which rely on direct delivery of either naked DNA or a mixture of genes with cationic lipids (liposomes). In this chapter, the currently available and widely used viral vectors, and their advantages and limitations, will be discussed.

RETROVIRAL VECTORS

Retroviruses can be classified into two categories (160): Simple retroviruses, encompassing the murine leukemia–related viral group [e.g., murine leukemia virus (MuLV)], and a group of complex retroviruses such as lentiviruses [e.g., human immunodeficiency virus (HIV)]. Gene therapy vectors have been developed from both groups of retroviruses and will be covered in separate sections.

Retroviral vectors based on MuLV were the first viral vectors to be used in a gene therapy trial that started a decade ago, with the aim to treat two children suffering from severe combined immunodeficiency (SCID) (16). Although the efficacy of this treatment and whether the disease was indeed cured by gene therapy could not be clearly ascertained because the patients received supportive conventional therapy during the trial, the retroviral gene transfer into the target cells (T cells) was partially successful.

Further underlining the potential of this class of gene delivery vehicles is the success of a clinical trial that resulted in the full correction of a disease phenotype (24). In this trial, CD34+ bone marrow cells were isolated from SCID patients and infected *ex vivo* with a MuLV-based retroviral vector carrying the cytokine receptor gene, which is mutated in these SCID patients. Cavazzano-Calvo et al. (24) were able to demonstrate expression and function of the transgene over a 10-month period, and most important, they showed the development and sustained function of the immune system, resulting in clinical improvement of the patients receiving gene therapy.

In principal, gene therapy vectors are either replication competent or they are rendered replication defective by deletion of essential viral genes from the vector. Replication-defective vectors are able to perform only one initial infectious cycle within the target cell. Although replication-competent retroviral vectors have been designed—especially in the early days of vector development—that are able to productively infect the target cell (106), replication of the viral vectors is unwanted in most gene therapy approaches, to avoid spreading of the recombinant virus.

Replication-Defective Retroviral Vectors

Most of the retroviral vectors (106, 176) presently used in gene therapy approaches are derived from MuLV. To generate replication-defective MuLV, all of the protein-encoding sequences are removed from the virus and replaced by the transgene of interest (Fig. 1A, Fig. 1B). However, the sequences required for encapsidation of the vector RNA [i.e., a packaging signal (Y)] have to be included in the vector construct. The absolute minimal MuLV packaging signal has been narrowed down to a region encompassing bases 215 to 355 (96, 97). In addition, the 5' end of the *gag* gene has been shown to carry sequences that are required for efficient encapsidation of the vector RNA. Inclusion of this region

[nucleotides 215 to 1,039 (10)] resulted in so-called Y^+ or gag^+ vectors with a 50- to 200-fold increase of vector titers (7,10) compared to vectors containing only the minimal Y signal. To ensure reverse transcription and integration of the vector DNA into the host genome, the long terminal repeats (LTRs), the primer transfer RNA (tRNA) binding site (PBS), and the polypurine tract (PPT) have to be incorporated into the vector construct (see Fig. 1B). The proteins needed for production of infectious viral particles can be supplied in trans in packaging/producer cells (see next section, Packaging of Retroviral Vectors).

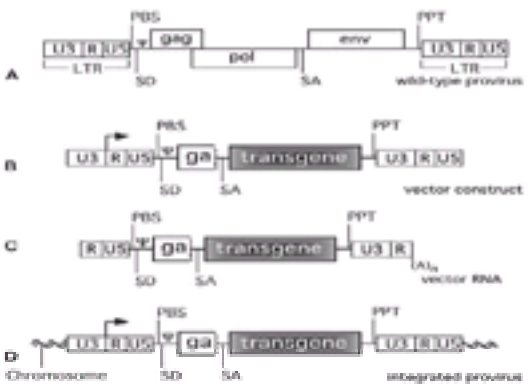

FIG. 1. Retroviral vectors. **A:** Schematic representation of the retroviral provirus. PBS, primer binding site; SD, splice donor; Y, packaging signal; SA, splice acceptor; PPT, poly purine tract; LTR, long terminal repeat. **B:** Retroviral vector. The transgene replaces the viral genes and is flanked by the retroviral LTRs. Apart from the minimal packaging signal (Y), portions of 5' *gag* sequences are included to increase packaging efficacy. **C:** Vector RNA packaged into virions in the producer/packaging cells. **D:** Integrated provirus. The transgene of interest is transcribed from the promoter/enhancer region within the U3 of the 5' LTR (*arrow*).

Although the retroviruses and their genomic organization is described in detail in Chapter 57, Chapter 58, Chapter 59, Chapter 60, Chapter 61 and Chapter 62 the life cycle of a retroviral vector is briefly summarized here (see Fig. 1B,Fig. 1C and Fig. 1D). The vector DNA is transcribed into RNA in the producer cells and packaged into viral particles. The promoter/enhancer regions of the 5' U3 region are not transcribed, and the U5 sequences of the 3' LTR are replaced by a polyadenylation [poly(A)] tail as a result of the presence of a poly(A) signal in the R region (see Fig. 1C). In the infected cell, the vector RNA genome is reverse transcribed and the provirus DNA integrates into the host genome. The U3 regions of the 5' and the 3' LTR of the provirus are derived from the U3 regions of the 3' LTR originally present in the vector RNA, and both U5 regions are derived from the 5' LTR originally present at 5' end of the vector RNA (see Fig. 1C,Fig. 1D). This has important practical implications for the vector design. The transgene should not contain a poly(A) signal sequence, as this would lead to the replacement of the 3' U3/R region with a poly(A) tail during transcription of the vector RNA (106). Furthermore, the duplication of the 3' U3 region during reverse transcription is also the basis for the development of self-inactivating vectors (see later).

Several changes of the 5' as well as the 3' LTR region have been introduced into the retroviral vectors to increase vector yield and to improve the biosafety of the vectors, respectively. Among the most important changes concerning the efficacy of virus production is the replacement of the U3 region of the 5' LTR with the immediate-early region of the human cytomegalovirus (CMV) enhancer-promoter (Fig. 2A), which results in a CMV/LTR hybrid that has a high transcriptional activity when introduced into the appropriate cell line (47), such as human embryonic kidney (HEK) 293 cells. This cell line expresses the adenoviral E1 gene products (59), which have been shown to superactivate the CMV promoter (55). After transient transfection of CMV/LTR vectors (47,120) together with CMV-driven packaging constructs (47,120) in 293 cells, this vector system yielded titers that were 100-fold higher than those obtained with retroviral vectors carrying the parental MuLV 5' LTR.

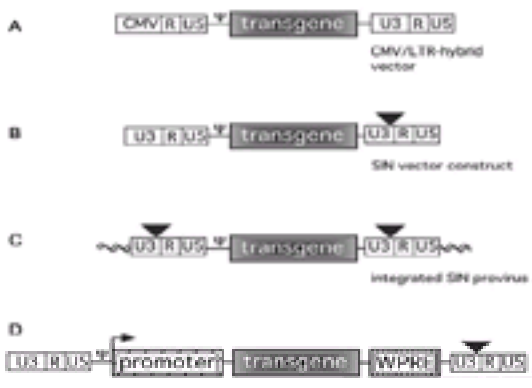

FIG. 2. Retroviral vector development. **A:** The CMV/LTR-hybrid vector system [e.g., r*kat* or pCL vectors (47,120)] allows a high-titer vector production in 293 cells. **B,C:** Retroviral SIN (self-inactivating) vectors with deletions (*black triangle*) of the promoter/enhancer regions of the 3' LTR of the vector construct, which replaces the 5' U3 region in the final integrated SIN provirus. **D:** An internal, heterologous promoter can be included in the vector construct to increase transgene expression in the target cell and/or to restrict transgene expression to certain cell types and tissues. To avoid interference with the promoter/enhancer of the viral LTRs, a SIN vector can be used. Incorporation of the WPRE into the vector enhances transgene expression.

To improve the biosafety of the retroviral vectors, deletion mutations were introduced into essential regions of the 3' LTR. Because the U3 promoter/ enhancer region of the 5' LTR of the integrated provirus is derived from the 3' LTR of the vector construct, deletion of promoter activity from the 3' LTR should be carried over to the 5' LTR during reverse transcription in the target cell (see Fig. 2B, Fig. 2C). This concept of transcriptional inactivation of the provirus is also termed self-inactivation, and these vectors are known as SIN (self-inactivating) vectors (178,182). Because the transcriptional activity of wild-type LTRs can affect the expression of host proto-oncogenes, the SIN strategy is a major improvement in the biosafety of the vectors. The drawback of the inactivation of the transcriptional regulatory elements of the proviral LTRs is a substantial loss in viral titer, which was at least 10- to 100-fold lower than the parental retroviral vector (178,182). On the other hand, partially disabled vectors (178) with an intact TATA box still exhibit a low level of LTR-driven transcription (0.1% to 1%).

The use of SIN vectors may be necessary to avoid interference of the viral promoter and enhancer regions with internal promoters of the vector in the target cells. In the most simple vector design, the 5' LTR of the integrated provirus drives the expression of the transgene (see Fig. 1D). To achieve a higher level of transgene expression and/or specific expression in certain cell types and tissues, heterologous promoters can be placed internally in the retroviral transcription unit to drive transgene transcription (see Fig. 2D). However, the transcriptional activity of the proviral 5' LTR can interfere with the activity of internal promoters (42). Thus, elimination of the promoter and enhancer functions of the proviral 5' LTR may be necessary, and this can be achieved by using SIN vectors (see Fig. 2D).

Another improvement of transgene expression can be achieved by inclusion of cis-acting posttranscriptional regulatory elements (PRE) (186). PREs are present in herpes simplex and hepatitis B virus, and the latter has been shown to enhance expression of a heterologous cDNA (e.g., b-globin cDNA). Incorporation of the PRE of the woodchuck hepatitis virus (WPRE) 3' of the transgene (see Fig. 2D) increased gene expression at least fivefold in MuLV-derived vectors (186). Interestingly, the enhancement of transgene expression by the WPRE was independent of the cell type or the cell cycle status of the target cells (186). The precise mechanism of WPRE function remains unknown.

Packaging of Retroviral Vectors

To package the replication-defective vector into virions, the viral proteins required are provided in trans in the packaging cell (Fig. 3 and Fig. 4). Retroviral packaging constructs are either transfected transiently into the packaging cells, or a cell line is established that stably expresses the viral proteins. The first-generation packaging cell lines (such as Y-2) carried a mutant MuLV virus with deletion mutations of the essential Y sequence (97) (see Fig. 3B). Deletion of Y results in a dramatic reduction of encapsidation of MuLV. In addition, the host range of vector particles produced by this packaging cell line was rather small. The tropism of MuLV is defined by Env and the species distribution of its receptor (see Chapter 57). Ecotropic viruses—such as MuLV—can infect only murine cells that express the receptor for Env [a sodium-independent cationic amino acid transporter (2)]. Since Y-2 cells carry the ecotropic *env* of MuLV, only murine cells could be infected with viral vectors produced by these cells. The host range of the vectors can be expanded by replacing the MuLV *env* gene with envelope sequences from amphotropic viruses (see Chapter 57; and refs. 32,108). These chimeric retroviruses contain *gag* and *pol* sequences from MuLV and the *env* gene from the amphotropic viruses (e.g., 4070A) (see Fig. 3B). Transfection of NIH3T3 with these chimeric packaging constructs, which lack the encapsidation signal, together with a retroviral vector, resulted in the production of high-titer [>10^5 transducing units (T.U.)/mL] stocks of helper-free recombinant retrovirus with amphotropic host range, able to infect murine and human cells (32).

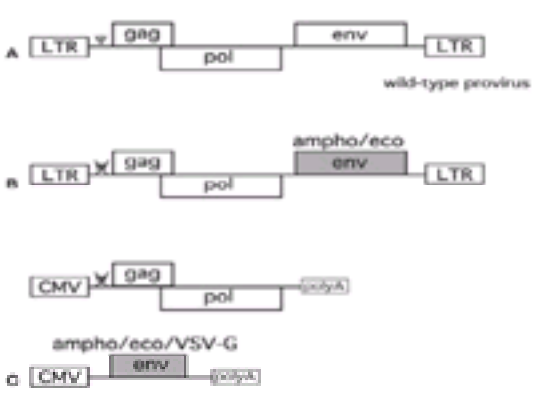

FIG. 3. Retroviral packaging system. **A:** Schematic representation of the wild-type retroviral provirus. **B:** First-generation packaging strategy with deletion mutations of the packaging signal (Y). Replacement of the wild-type *env* gene (*eco*) with, for example, amphotropic *env* (*amph*) results in a broadened host range. **C:** Split-genome packaging strategy: *gag*/*pol* and *env* are encoded on separate plasmids.

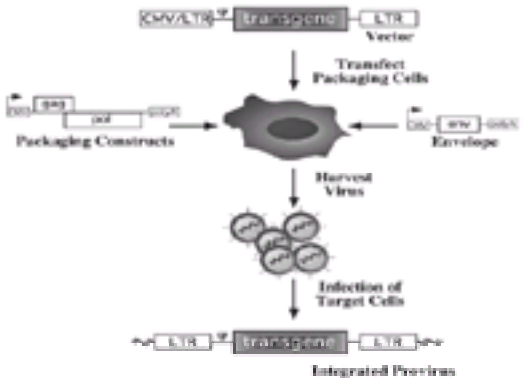

FIG. 4. Production of retroviral vector particles. Retroviral vectors can be produced by transient co-transfection of the vector plasmid (containing a hybrid CMV-LTR promoter/enhancer at the 5' LTR position) with packaging constructs (coding for *gag* and *pol*) together with the envelope plasmid (a total of three plasmids are transfected) into packaging cells (e.g., 293 cells). The CMV promoter drives the expression of the different expression cassettes in the packaging cells. After infection of the target cells, the integrated provirus containing the foreign gene is transcribed from the 5' LTR.

An important biosafety issue of these early generations of packaging systems was contamination with replication-competent recombinants (RCRs), also called helper viruses. This problem was addressed by introducing further deletions into the packaging construct: the 3' LTR and the PPT site (see Fig. 1A) were replaced with the simian virus 40 (SV40) poly(A) signal (107). Thus, in the rare case of encapsidation of the packaging construct into infectious virions, the packaging RNA will not be completely reverse-transcribed because it lacks the site for initiation of second-strand DNA synthesis. In addition, the 5' end of the 5' LTR that contains the cis-acting signal sequence required for integration (also called *att* element; see Chapter 57 for details) was removed. This deletion mutation should prevent integration of recombinant viruses that contain the 5' part of the packaging construct. However, there was still a considerable amount of homology between the packaging construct and the retroviral vector, which could lead to the production of helper virus after a single recombination event between the two plasmids. Therefore, a packaging strategy based on split packaging constructs, one containing the *gag* and *pol* genes and the other carrying the *env* gene, was introduced (see Fig. 3C) (36,98). Splitting the packaging genome into multiple units not only increases the safety of retroviral vectors, but also facilitates pseudotyping of retroviral vectors with the envelope of other viruses, because different envelope genes can be included in the packaging cells by a simple exchange of the envelope construct.

A major hurdle for the use of retroviral vectors in human gene therapy was that virions, which incorporated ecotropic or amphotropic envelope proteins, could be concentrated only with a very low efficiency and labor-intensive protocols. Importantly, these virions could not withstand the shear forces of ultracentrifugation. It was previously shown that MuLV could be pseudotyped with the G protein of the vesicular stomatitis virus (VSV-G) (43). VSV-G mediates viral entry by membrane fusion via the interaction with phospholipid components of the cell membrane, and therefore it has a broad host range. Additionally, VSV-G-pseudotyped vectors can be concentrated 100- to 300-fold by ultracentrifugation to titers above 10^9 infectious units (IU)/mL (21). The major disadvantage of using VSV-G as an envelope is its toxic effects in the packaging cells, especially as high levels of VSV-G expression are required for high-titer virus production (21). The packaging cells express the VSV-G protein also on their cell surface, which leads to the formation of syncytia and cell death after several days of transfection with the VSV-G plasmid (21). Because of the toxicity of VSV-G, attempts to produce retroviral vectors from cell lines stably expressing VSV-G failed until inducible expression strategies (26,124) were developed using the tetracycline-repressed regulatable system (TrRS) (57).

The TrRS is based on the tetracycline resistance operon of *Escherichia coli* and combines the specificity of the tet repressor (tetR) for the tet operator sequence (*tet*O) with the activity of the herpes simplex virus transactivator (VP16) in eukaryotic cells. A conditionally active tetracycline transactivator (tTA) was created by fusion of the C-terminus of VP16 to the C-terminus of the tetR protein. Alternatively, tetR can also be fused to the ligand-binding domain of the estrogen receptor, leading to hormone-dependent function of the fusion protein (26). In the absence of tetracycline, the tetR domain of tTA binds to a tetracycline-regulated promoter (tRP), consisting of seven *tet*O repeats placed upstream of a minimal CMV promoter, and activates transcription of the transgene. Binding of tetracycline to tTA leads to a conformational change and highly reduced affinity of tTA for tRP, and the transgene expression is switched off. This strategy is also known as tet-off system (see Fig. 5D). The tetracycline system exhibits an impressive degree of regulation and can be turned off and on within 24 hours (77). The two components of this regulatory system, the transactivator tTA and the regulated promoter tRP, together with the VSV-G expression cassette were transfected into 293 (HEK) cells that stably express *gag* and *pol* [293GP cells (21)], and stable clones were selected (26,124). Finally, the retroviral vector is introduced into the stable packaging cell clones, either by infection with a recombinant virus (SIN vectors cannot be used for this purpose) or by transfection of the vector construct. Withdrawal of tetracycline induces a VSV-G expression in the packaging cells (26,124), with maximal virus production between 48 and 96 hours after removal of tetracycline (124).

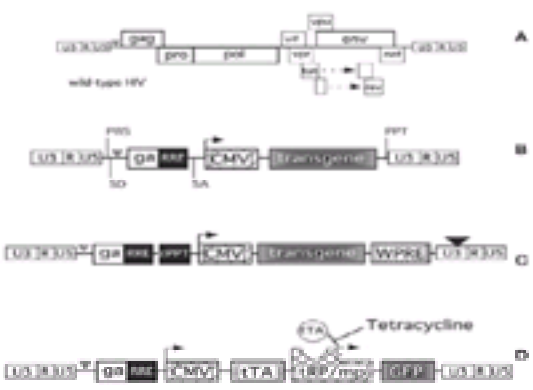

FIG. 5. Lentiviral vectors. **A:** Schematic representation of the wild-type HIV provirus. **B:** Lentiviral vector containing the lentiviral LTRs, a packaging signal (Y), the primer tRNA binding site (*PBS*), and a polypurine tract. 5' *gag* sequences and the Rev-responsive element (*RRE*) are included to increase packaging efficacy and to allow efficient transcription and cytoplasmic export of full-length vector transcripts. **C:** The most advanced lentiviral vector construct with improved performance is a result of the incorporation of a central polypurine tract (*cPPT*) to enhance nuclear translocation of the vector in the target cell. In addition, the WPRE is included to improve transgene expression in the target cell and deletions in the U3 region of the 3' LTR (*triangle*) to achieve self-inactivation of the vector in the target cell. **D:** Lentiviral vector incorporating the tetracycline-regulated system. The vector contains the tetracycline transactivator (*tTA*) (a fusion of tetR and VP16) and the tetracycline-regulated promoter (*tRP*), consisting of *tet*O repeats placed upstream of a minimal

CMV promoter (*mp*). Expression of the tetracycline responsive elements is driven by a CMV promoter. Binding of tetracycline to tTA inhibits tRP-dependent gene expression; the transgene (e.g., GFP) expression is switched off.

Taken together, the recent improvements of the retroviral vector and packaging system are important steps toward using retroviral vectors for gene therapy trials in humans. The stable packaging cell lines allow the standardized production of retroviral vectors that can be screened for the presence of helper virus contamination. The split-genome approach to packaging (see Fig. 4) is an important safety feature that reduces the risk of such contamination. Pseudotyping of the virions with VSV-G broadens the host range and allows concentration of the virus with easy ultracentrifugation protocols. The toxic effects of the VSV-G protein in the packaging cells can be controlled by inducible expression strategies (e.g., the tetracycline-regulated system). The development of human-derived packaging cell lines (based on 293 cells) allows the production of clinical-grade virus preparations and offers several advantages over the—so far—frequently used NIH3T3 cells: Human-derived cells do not harbor a large number of endogenous retroviral genomes that could participate in recombination events to generate replication-competent viral mutants (147). Retroviral vectors produced from murine cells have been shown to be inactivated by human serum (natural antibodies and complement). The use of vectors that carry a CMV–LTR hybrid (see Fig. 2A) and CMV-driven packaging constructs (see Fig. 3C and Fig. 4) in 293 cells makes use of the fact that 293 cells express the adenoviral E1A protein, which stimulates of CMV-dependent transcription.

Furthermore, transient transfection of the viral vector, the packaging construct (carrying *gag* and *pol*), and the envelope construct (e.g., VSV-G) into 293 cells is a suitable way to generate high-titer (>10 ^{9} IU/mL) preparations of many different viral vectors for laboratories that work on animal models for gene therapy (177).

LENTIVIRAL VECTORS

Lentiviruses belong to the family *Retroviridae*, whose characteristics—virion structure, genome, replication strategy, and host spectrum—are described in detail in Chapter 59, Chapter 60 and Chapter 61. An important genetic difference between simple retroviruses and lentiviruses is the presence of regulatory (*tat* and *rev*) and auxiliary genes (*vpr*, *vif*, *vpu*, and *nef*) that have important functions during the viral life cycle and are relevant for lentiviral pathogenesis.

In contrast to simple retroviruses, lentiviruses are able to infect nondividing, terminally differentiated mammalian cells. This feature of lentiviruses makes them a very attractive tool for gene delivery (119,159).

The development of lentiviral vectors is based on the techniques and strategies developed for vectors studied in simple retroviruses. Previous experiences with retroviral vectors proved to be very helpful in the development of lentiviral vectors.

Replication-Defective Lentiviral Vectors

Like murine retroviral vectors, replication-deficient lentiviral vectors were designed by splitting the viral genome into cis- and trans-acting components necessary for gene transfer into the target cells (Fig. 5 and Fig. 6). The viral proteins required for the infection of the target cells are provided in trans by producer/packaging cells, which package the transfer vector that contains the transgene into infectious viral particles. In the vector constructs, the transgene is flanked by viral LTRs that contain cis-acting sequences necessary for packaging, integration, transcription, and polyadenylation (see Fig. 5).

FIG. 6. HIV-derived packaging constructs. **A:** HIV-1 provirus. **B:** First-generation packaging plasmid. The reading frames of *env* and *vpu* are blocked, and the 5' and 3' LTRs are replaced with the CMV promoter and a polyadenylation [poly(A)] site, respectively [e.g., pCMVDR9 (118)]. **C:** Deletion of *env*, *vif*, *vpr*, *vpu*, and *nef* resulted in the second generation of packaging construct [e.g., pCMVDR8.91 (188)]. **D:** Tat-free, nonoverlapping split genome packaging system. In this third generation of an HIV-based packaging system, *gag* and *pol* are encoded on one plasmid [e.g., pMDLg/pRRE (40)], and *rev* is expressed in trans from another plasmid [e.g., RSV-REV (40)]. **E:** The envelope of the lentiviral vector is provided by a plasmid containing the VSV-G coding region flanked by the CMV promoter and a poly(A) signal [e.g., pMD.G (118)]. Y, packaging signal; RRE, Rev-responsive element; SD, splice donor.

As in the MuLV-based vectors, inclusion of sequences extending further into the *gag* gene (up to 722 nucleotides) significantly increased vector propagation and gene transfer efficiency (19,128). In addition, incorporation of the Rev-responsive element (RRE) of *env* (see Fig. 5B) increases encapsidation and transfer efficacy (128,137). Analysis of the ratio of unspliced to spliced cytoplasmic vector RNAs in packaging cells showed a significant reduction of unspliced

RNA in the HIV-based vectors lacking the RRE compared to vectors containing the RRE (128).

The ability of lentiviruses to infect nondividing and terminally differentiated cells has been extensively studied, but the precise mechanism by which lentiviruses transduce nondividing cells remains elusive. The preintegration nucleoprotein complex (PIC) is recognized by the nuclear import machinery of the target cell and actively transported through the nucleopore (see Chapter 59, Chapter 60, and Chapter 61). HIV-1 contains multiple nucleophilic determinants that allow the nuclear entry of the lentiviral PIC. Three karyophilic proteins have been identified in HIV-1: the *gag*-encoded matrix protein (MA) (20), integrase (IN) (52), and Vpr (69). The MA contains two nuclear localization signal (NLS) motifs (65), and inactivation of both of them renders the mutant virus defective in nuclear transport, even in the presence of Vpr and IN. IN also harbors sequence homologies to NLS (51,130), and mutation of these regions has caused nuclear exclusion of IN but not of the HIV PIC (130). Both MA and IN are included in the HIV-based lentiviral vectors, but the multiply attenuated lentiviral vectors produced with the second- (see Fig. 6C) (188) or third-generation packaging systems (40) (see Fig. 6D) lack Vpr (see later section, Development of Lentiviral Packaging Systems). At least for the gene transfer into the central nervous system, Vpr is completely dispensable, as Vpr-deficient (Vpr^-) vectors transduce a similar number of adult rat neurons as Vpr-positive vectors (75,188). In addition, quiescent human and murine hematopoietic stem cells are transduced by Vpr^- vectors (23,99). However, the requirement of lentiviral accessory genes for the transduction of other cells such as macrophages may differ, as Vpr can rescue the ability of MA NLS-mutant viruses to infect nondividing macrophages (69).

Apart from these karyophilic viral proteins, HIV-1 genomic cis-acting sequences have recently been found to enhance the nuclear DNA import (50,183). In addition to the 3' PPT, located just 5' of the U3 region of the LTR (see Fig. 5B), all lentiviruses contain a central copy of the PPT (cPPT), at which synthesis of the downstream plus strand is initiated (183). Inclusion of the upstream cPPT element (see Fig. 5C) enhances nuclear import of the HIV-1-derived vector genome (50,183). Because the cPPT element is situated in the *pol* gene, the HIV-based gene transfer vectors devoid of *pol* lack this sequence, and the nuclear import of these vectors should be retarded as compared to wild-type HIV-1. Interestingly, *pol*-deficient lentiviral vectors are able to transduce terminally differentiated, nondividing cells such as neurons (118), muscle (75), hepatocytes (75), retinal photoreceptors (112,156), and hematopoietic stem cells (23,111), showing that at least in these cell types the cPPT is not necessary for nuclear import. An important difference between the $cPPT^-$ and $cPPT^+$ vectors is the time course of the transgene expression *in vitro* and *in vivo* and the total amount of protein expression achieved (50): After intravenous injection of $cPPT^+$ vectors carrying a CMV-driven expression cassette encoding human factor IX into SCID mice, the plasma level was twice as high as that achieved after injection of the $cPPT^-$ vector. Interestingly, inclusion of a cPPT has also been shown to enhance two- to threefold the transduction efficacy of simian immunodeficiency (SIV)-based vectors (95). Thus, the inclusion of a cPPT-element into lentiviral vectors improves their performance *in vivo* (50,183).

Enhancement of Lentiviral Transgene Expression

Vector performance can also be improved by increasing transgene expression after integration of the recombinant lentivirus into the host genome using cis-acting transcriptional regulatory elements. Inclusion of the posttranscriptional regulatory element of WPRE in HIV-derived vectors increased the reporter gene (green fluorescent protein [GFP] and luciferase) expression five- to eightfold after transduction of both dividing and arrested 293T cells (186). The WPRE has to be present within the transgene transcript in sense orientation, and it is placed 3' of the transgene cDNA upstream of the 3' LTR (see Fig. 5C).

SIN Lentivectors

As in all retroviruses, the U3 and U5 LTR regions of the lentiviral provirus are derived by duplication of the 5' U5 and the 3' U3 regions of the viral RNA during reverse transcription. Therefore, the same principal used to generate retroviral SIN vectors (see earlier and Fig. 1 and Fig. 2) can be employed for the production of lentiviral SIN vectors. SIN lentivectors (see Fig. 5C) were designed by incorporating extensive deletions (133 to 400 bp) in the 3' U3 region of HIV-1 LTR, leaving intact the *att* element at the 5' end of the U3 that is required for integration into the host genome (110,187). The deleted U3 regions include the TATA box, the three SP1 binding sites, the NFkb sites (110) up to the NF-ATc sites located further upstream, and the so-called negative response element (187). Co-transfection of the SIN vectors with packaging constructs resulted in titers that were similar to those obtained with the original vector plasmid without U3 deletions (110,187). Analysis of the effect of the U3 deletions on SIN lentivirus–derived RNA production in the target cells revealed an almost complete loss of the LTR promoter activity (187). Importantly, using a sensitive virus rescue assay, it was shown that the amount of virus rescued from SIN vector–infected cells was 10,000-fold lower than from cells infected with the parental vector without SIN mutations (110). Thus, the use of SIN vectors reduces the risk of mobilization of integrated provirus by replication-competent virus, minimizing the risk of vector virus spread.

Furthermore, after one round of viral replication, the U3 regions in both LTRs are derived from the U3 region of the 3' LTR (see Fig. 1), thus allowing the replacement of the U3 region of the 5' LTR with the CMV promoter in the vector plasmid (110,187). This modification of the 5' LTR is important because SIN vectors combined with hybrid CMV-LTR contain only one U3 region (with deletions). Thus, recombination to generate a wild-type U3 region is not likely. In addition, replacement of the U3 region of the 5' LTR with the CMV promoter resulted in Tat-independent transcription (110,187). This allows the production of HIV-derived vectors in the absence of Tat (see later).

Regulated Gene Expression

Spatial and temporal regulation of transgene expression would be a highly desirable feature of gene therapy vectors. Among the different regulatory systems that have been developed for use in gene therapy vectors (1), the TrRS (57) offers many advantages and is one of the best characterized regulatable systems (see also Packaging of Retroviral Vectors).

Because lentiviral vectors can package and deliver large transgenes, such as human factor VIII cDNA, a

tetracycline-regulatable lentiviral vector system has been developed (77). In this system (see Fig. 5D), the two regulatory components of the TrRS [i.e., the tTA (tetR/VP16 fusion) and the tRP (*tet*O/minimal promoter)] are encoded on a single vector that also carries the transgene (77). After transduction of 293 cells with the regulatable lentiviral vector carrying a GFP transgene (pCL-CTIG), GFP expression was 500- to 1,000-fold induced by withdrawal of doxycycline from the culture medium (77). Maximal suppression of GFP transcription was achieved within 24 hours of addition of the tetracycline analog. In addition, after transduction of adult rat brain with pCL-CTIG, tetracycline-regulated GFP expression was achieved *in vivo*: Only a low level of GFP expression in a few cells could be detected in the brains of doxycycline-treated animals, whereas the nontreated or doxycycline-removal group demonstrated a very high level of GFP, as far as 2 to 3 mm from the site of virus injection.

So far, only the tetracycline-regulated system has been used in combination with lentiviral vectors, but studies are underway that employ the insect hormone ecdysone (121) or other nuclear hormone receptors as regulatory elements in the context of lentiviral vectors. Also, the rapamycin (FK506) system (138), which has been shown to function in an adeno-associated viruses (AAV)-inducible system (139,175), holds great promise, because it exhibits low baseline expression and a high induction ratio, as well as control by an orally bioavailable small-molecule drug.

Development of the Lentiviral Packaging System

Like retroviral packaging cells, all lentivirus proteins necessary for virus production are provided in trans by packaging plasmids, and virus particles are assembled by expressing viral proteins in producer/packaging cells (see Fig. 4). As a result of biosafety concerns, the packaging constructs lack cis-acting sequences required for the transfer of the viral genome to target cells (see Fig. 6). The aim is to identify the minimal number of viral genes required for efficient virus production and to minimize the number of lentiviral genes present in the packaging plasmids.

The first genuine lentiviral packaging constructs (19,128,133,149) carried a deletion mutation of Y, and/or lacked the 5' and 3' LTRs (which were replaced by the CMV promoter and a polyadenylation signal, respectively). Although deletion of LTR and Y eliminated the sequences crucial for packaging—reverse transcription and integration of transcripts derived from the packaging plasmids—these packaging constructs still contained more than 90% of the HIV genome. Because these initial packaging systems incorporated the endogenous HIV envelope, the host range of these vectors was restricted to CD4-positive cells, and there was a risk of generating wild-type HIV. In addition, only low infectious titers [in the range of 10^1 to 10^2 IU/mL (19,128)] were obtained, and the vector particles could not be efficiently concentrated.

A crucial step for the advancement of lentiviral gene transfer was the incorporation of envelope proteins of other viruses. Dual infection of cells with HIV and a murine amphotropic retrovirus led to the production of HIV virus that carried the amphotropic envelope, demonstrating that HIV-1 can be pseudotyped with heterologous envelopes (151). By using replication-defective HIV-1 mutants that carry deletions of gp160, it was shown that the HIV enevelope can be provided in trans (125). Expression of the envelope gene of an amphotropic virus or of the human T-cell leukemia virus in cells transfected with the gp160-deficient HIV mutant resulted in the production of infectious viral particles (88,125).

Replacement of the HIV envelope glycoprotein with VSV-G (see Fig. 6B,Fig. 6E) expanded the host range of the HIV vectors (117,136). After transient co-transfection of packaging and vector plasmids, the virus can be harvested by collection of the culture medium of the 293T producer cells and concentrated to titers of up to 10^8 IU/mL by using a facile ultracentrifugation procedure (117,136). First-generation, VSV-G pseudotyped lentiviral vectors (see Fig. 6B, Fig. 6E) were able to transduce nondividing, G1/S- and G2-arrested HeLa cells (118). Furthermore, comparison of the transduction efficiency of MuLV-based with HIV-based VSV-G pseudotyped vector in unstimulated human peripheral blood CD34+ cells, which are highly enriched for G0/G1 cells, revealed an eightfold higher number of transduced cells after infection with the lentiviral vector (136). More importantly, lentiviral vectors (10^8 IU/mL) were able to transduce terminally differentiated, nondividing neurons *in vivo* (118).

With an approach similar to that used for HIV-1-based vectors, packaging systems for vectors based on feline immunodeficiency (FIV) (132), SIV (95), HIV-2 (131), and equine infectious anemia virus (123) have been developed, achieving titers (after concentration by ultracentrifugation) in the range of 10^7, 10^7, 10^8, and 10^6 IU/mL, respectively. Because HIV-1-based vectors have been exploited more extensively, we have used them as a model system for discussion of lentiviral vectors.

The initial packaging plasmids contain most of the lentivirus genes except for *env* (see Fig. 6B). In an effort to improve safety, a second version of the packaging system was introduced with extensive deletions of the viral genome (see Fig. 6C). Because it was shown that accessory genes of HIV-1 (*vpr*, *vpu*, *vif*, and *nef*) are not required for efficient production of viral particles (188), a packaging system containing only the *gag*, *pol*, *tat*, and *rev* genes of HIV-1 was developed (75,188). Thus, these packaging constructs are devoid of all the accessory genes required for efficient replication in human cells. HIV-based vectors packaged with this system are able to deliver genes into dividing and nondividing cells *in vitro*, and to transduce adult neurons and muscle *in vivo* (75,188).

In the latest, third generation of packaging strategies, the *tat* gene is deleted (40) (see Fig. 6D). Tat is a strong transcriptional activator of the HIV-1 LTR promoter element and is essential for viral replication (34,41). Tat expression in the packaging cells is necessary for the production of high-titer HIV-vectors (40), because interaction of Tat with the LTR promoter region is essential for the transcription of the vector plasmids in the packaging cells. Transcription from the HIV LTR in the absence of Tat was 10- to 20-fold reduced compared to the levels obtained in the presence of Tat in the first and second generation of packaging systems. This limitation of the Tat-free system was overcome by the inclusion of strong constitutive promoters, such as CMV (40,110) or respiratory syncytial virus (RSV) promoters (40), in the U3 region of the 5' LTR of the vector constructs, resulting in CMV/RSV–HIV hybrid LTRs.

Another prominent feature of the third-generation packaging system is a split genome strategy (40). Initially, only the *gag* and *pol* genes (without the sequences upstream of the *gag* initiation codon) were cloned into one plasmid. However, transfection of this construct resulted in almost no detectable expression (40). Efficient expression was achieved after

Dull et al. (40) inserted the HIV RRE 3' of *pol* (see Fig. 6D) and included the *rev* gene—on a separate plasmid—in the packaging system. This minimized HIV-genome, split-genome packaging strategy is a significant improvement of the biosafety of the viral vectors.

Stable Packaging Cell Lines

Most of the studies using lentiviral vectors published so far rely on transient transfection of packaging and vector plasmids for vector production. Using the highly transfectable 293T cells, one routinely obtains titers of 10^{9} to 10^{10} IU/mL after transient transfection with the latest generations of packaging (containing VSV-G) and vector constructs followed by concentration of the virus particles by ultracentrifugation. However, standardization of the virus production is not easily achieved with transient transfections. Given the problem of possible contaminations of the lentivirus preparations with RCRs and cellular debris (e.g., proteins and lipids shed by apoptotic producer cells), stable producer cell lines may be necessary to address biosafety concerns before HIV-derived vectors are used in clinical trials.

The development of a packaging cell line for lentiviral vectors was curtailed by the fact that VSV-G and some lentiviral proteins inhibit cell proliferation (e.g., Vpr) and/or exhibit cytotoxic effects (Vpr, Gag, and Tat). Because a long-term, stable expression of these proteins is not feasible, cell lines that carry deletion mutations of most of the accessory genes and that do not contain the VSV-G envelope gene were developed (22,33,181). However, these cell lines produced only low-titer virus with a limited host range. A very promising approach to address this problem is the development of cell lines that express the toxic components from inducible plasmids (78,181). The first inducible packaging cell line for the production of HIV-based vectors was described by Yu et al. (181). In this cell line, the expression of HIV genes is regulated by a dual system: HIV *rev* and *env* are encoded on a plasmid that carries the tetracycline-regulated system (see preceding section, Packaging of Retroviral Vectors), whereas the HIV late transcripts Gag, Pol, Tat, and Vif are expressed from a second plasmid that lacks the 3' LTR, Y, and the other HIV proteins. Only in the absence of tetracycline, is Rev expressed, which in turn up-regulates the expression of the other HIV proteins (i.e., Gag, Pol, Tat, and Vif). In the presence of tetracycline, no infectious particles were detected (by measuring reverse transcriptase activity after stable transfection with the vector plasmid) (181), because of the regulatory function of Rev for the expression of late HIV transcripts.

In the packaging cell line developed by Kafri et al. (78), all HIV-1 genes (except the HIV envelope) are transcribed from a single expression cassette that is regulated by the tetracycline-regulated promoter. In addition, the VSV-G gene too is under the control of the tetracycline-responsive system. Withdrawal of tetracycline after transduction with the vector constructs results in the production of vector particles at titers greater than 10^{6} IU/mL for 3 to 4 days, after which cell viability declines dramatically (78). Recently, a third-generation lentiviral packaging cell line has been generated (82) (T. Kafri, personal communication, 2000).

Transduction of Nondividing Cells

VSV-G pseudotyped lentiviral vectors derived from HIV-1 have been shown to transduce a broad spectrum of terminally differentiated, nondividing cells *in vivo*, such as neurons (118) and retinal (110,156), hepatic (75), and muscle cells (75). The absence of a cellular immune response at the site of injection, as measured by staining for CD4+ T lymphocytes or macrophages, after injection of lentiviral vectors (1 to 3×10^{7} IU/mL) into the liver and muscle of the rat, indicates that lentiviral vectors might constitute immune-tolerated vectors. However, higher vector concentrations (>10^{9} vector genomes) and different tissues have to be tested, especially in higher mammals, and also the humoral immune response has to be assessed.

The fact that lentiviral vectors transduce human CD34+ hematopoietic stem cells *in vitro* without cytokine prestimulation is of high clinical relevance and a clear advantage in comparison to MuLV-based retroviral vectors (23,50,111). The transduced CD34+ cells provided long-term repopulation and were capable of engrafting and differentiation into multiple hematopoietic lineages after transplantation into nonobese diabetic (NOD)/SCID mice (111).

The correction of several human genetic diseases by gene therapy will require the delivery of large DNA fragments. For instance, sickle cell anemia and b-thalassemia, two genetic disorders of the b-globin gene, will require high-level expression of the b-globin gene in hematopoietic and erythroid (31) cells. To achieve this, introns of the b-globin gene and parts of the locus control region (LCR) of the human b-globin gene (122) will have to be included in the vector. Viral vectors based on simple retroviruses exhibit genetic instability if genomic fragments, such as introns, are included in the vector DNA. Therefore, retroviral vectors containing b-globin transcription units and LCR fragments are very unstable, with multiple rearrangements on transmission of the provirus (91,122). Genetic instability of retroviruses occurs by (a) inappropriate splicing or polyadenylation of the viral genome, and/or by (b) rearrangements during reverse transcription (91). In contrast, lentiviruses such as HIV-1 have the ability to package unspliced viral genomes, and Rev has been shown to permit the nuclear export of unspliced or incompletely spliced HIV-1 RNAs (94). Because RRE is included in HIV-based lentiviral vectors (118), it was speculated that lentiviral vectors might overcome the problem of retroviral instability.

It has recently been shown that the use of HIV-based viral vectors enables the efficient transfer and faithful integration of the human b-globin gene together with large parts of its LCR into hematopoietic stem cells (99). The high efficiency of the lentiviral gene transfer, together with an optimized b-globin-LCR configuration and the absence of vector rearrangements, yielded human b-globin expression in the therapeutic range in a mouse model of b-thalassemia after bone marrow transplantation of transduced stem cells. In summary, lentiviral vectors clearly fulfill many of the promises of a suitable gene delivery vehicle.

ADENO-ASSOCIATED VIRUS

Adeno-associated viruses are nonpathogenic, nonenveloped DNA viruses that belong to the parvovirus family. AAV virions contain a single-stranded DNA genome of only 4,680 bases, which is flanked by two inverted terminal repeats

(ITRs). Because productive (lytic) infection with AAV normally requires co-infection with an unrelated helper virus, either adenovirus or herpesvirus, these viruses were also termed dependoviruses (for a detailed description, see Chapter 69 and Chapter 70). In the absence of helper virus, AAV establishes a latent infection and integrates into a host chromosome via its ITRs. The ITR consists of 145 nucleotides, with the first 125 bases forming a palindromic sequence that forms a Y- or T-shaped structure. The ITR plays a crucial role in the AAV life cycle: It is involved in integration of the AAV genome and its rescue from the integration site. Latent infection in human cells with wild-type AAV results in site-specific integration into the AAV-S1 region of chromosome 19 (86,146). The integration sites are clustered, and they show microhomologies between the host DNA and the ITR (173). Because the integrated AAV provirus can be mobilized by subsequent superinfection of the host with a helper virus, latent infection seems to be a mechanism for ensuring the survival of AAV in the absence of helper virus. On the other hand, the helper-free life cycle of AAV is the basis for the development of AAV-based gene therapy vectors (12,61,113,114,145,152).

Adeno-Associated Viral Vectors

So far, six serotypes of AAV have been found in primates (AAV-1 to AAV-6). Type 2 is the best-characterized primate serotype, and it was the first AAV used for the development of vectors for gene transfer (103,144). Most of the vectors currently in use are derived from AAV-2, but vectors based on AAV-1 have been recently described (169).

In contrast to retro- and lentiviral vectors, in which vector development not only resulted in different versions but also achieved significant improvements of transduction efficacy and biosafety, the original design of AAV vectors has not changed over the last decade. The AAV-2-based vectors contain only the left and right ITRs, and 139 (103) or 45 (144) nucleotides of nonrepeated AAV sequences adjacent to the right terminal repeat, respectively. Samulski et al. (143) were able to show that all the cis-acting AAV functions required for replication and virion production are located within the ITR and the immediately adjacent 45 nucleotides. In the AAV-based vectors, the two viral open reading frames (ORFs), which code for the capsid proteins (VP1, 2, and 3) and the four nonstructural Rep proteins (Rep78/68 and Rep52/40) are replaced with the transgene of interest and its promoter (Fig. 7B,Fig. 7C) and then transfected into the producer cells, where the viral genes necessary for virus production and packaging of the vector genome are provided in trans by packaging plasmids and helper viruses/plasmids (see next section, AAV-Vector Packaging Strategies).

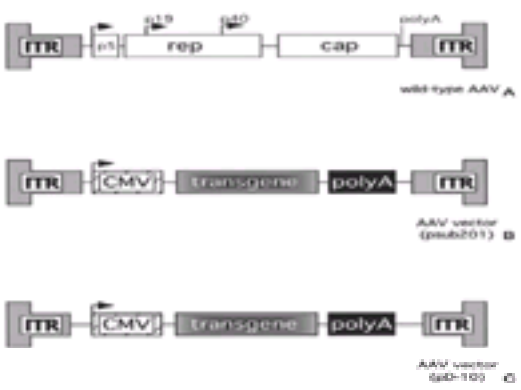

FIG. 7. Adeno-associated viral vectors. **A:** Scheme depicting wild-type AAV. The inverted terminal repeats (*ITRs*) and the promoters (*p5*, *p19*, and *p40*) of the *rep* gene are indicated. **B:** Recombinant AAV vector [e.g., p*sub*201 (143)]. The viral *rep* and *cap* genes are replaced with the transgene of interest, its promoter (e.g., CMV) and a polyadenylation signal sequence [*poly(A)*]. **C:** AAV vector carrying deletion mutations of the ITRs [e.g., pD-10 (164)] to minimize recombination between the vector and packaging/helper plasmids carrying adenoviral terminal repeats.

One disadvantage of AAV-based vectors is their size limitation: The optimal size for AAV vectors lies between 4.1 and 4.9 kb (37). Although larger genomes can be packaged, the packaging efficacy is sharply reduced and the maximal size of the vector genome (including the two ITRs) is 5.2 kb (37). Because of this size limitation, a dual vector, trans-splicing AAV vector system was developed (39,116,155,172), which allows the transduction of target cells and tissues with two AAV vectors that contain a split expression cassette. This promising approach to AAV-based gene transfer is based on the finding that episomal recombinant AAV (rAAV) genomes form circular multimers that concatamerize in a head-to-tail orientation (38). The dual-vector strategy functions as follows: After co-infection of the target cells, the two AAV vectors that carry the split genome form head-to-tail heterodimers through sequence homology of the ITRs, thereby rejoining the split gene into one continuous DNA molecule. Although such rejoining leads to the disruption of the gene-coding sequence by the AAV ITR, this problem can be overcome by inclusion of eukaryotic splicing signals to remove the ITR sequences during RNA maturation. With this novel dual-vector approach, the *E. coli lacZ* transgene (116,155), a luciferase reporter gene (39), and the human erythropoietin (172) gene were successfully delivered *in vitro* and *in vivo* (to muscle and liver of the mouse). Although the efficacy of gene delivery in the dual-vector system was consistently reported to be lower than in the unsplit, conventional AAV vector system, the individual efficacies vary between 16% (155) and 70% (116) as compared to unsplit vectors.

AAV-Vector Packaging Strategies

The basic principal of the AAV packaging system is the same as in other vectors: The vector plasmid contains the required cis-acting viral sequences necessary for packaging and integration and the transgene, while the packaging plasmids provide the viral components required for virus production in *trans.* However, because AAV is a dependovirus, its propagation depends on the functions provided by a helper virus (adenovirus or herpesvirus). Although replication has been described to occur in a helper-independent fashion if the host cells are stressed with genotoxic agents such as irradiation, adenovirus infection is still the most efficient way to induce AAV replication.

The initial studies with AAV vectors revealed that both the AAV *rep* and *cap* genes can be provided in trans in producer cells (103,144), and this resulted in the first generation of packaging systems (103,144). In this system, the packaging plasmid [pAAV/Ad (144)] encodes the *rep* and *cap* genes, and the essential helper functions are provided by the adenovirus (Fig. 8). Transfection of the AAV packaging construct together with the vector plasmid into adenovirus-infected packaging/helper cells results in excision of the ITR-flanked transgene from the vector plasmid, followed by its amplification and packaging into AAV capsids. This first generation of AAV packaging yielded up to 102 rAAV particles per cell, but there is a major disadvantage: the coproduction of wild-type adenovirus along with the AAV vector particles. Although these AAV preparations can be purified [e.g., by centrifugation in multiple cesium chloride (CsCl) gradients] and/or the adenovirus contamination can be inactivated (e.g., by heating the preparations for 30 minutes at 56°C), a complete removal of adenoviral capsid components is not always possible. In addition, this may lead to a loss in the activity of the AAV particles. Given the fact that viral proteins derived from inactivated adenovirus preparations elicit a cytotoxic T lymphocyte (CTL) immune response at the site of injection (76), it is clear that this packaging system is suboptimal, at least for clinical use.

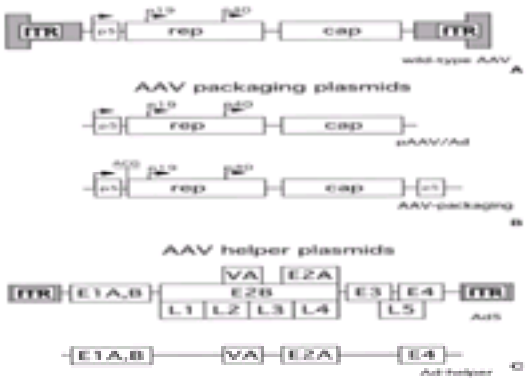

FIG. 8. AAV packaging/helper constructs. **A:** Wild-type AAV**. B:** AAV packaging constructs of the first [e.g., pAAV/Ad (144)] (*top*) and of the second generation [e.g., pXX2 (171)] (*bottom*); the latter contains a mutation of the start ATG to ACG, and two p5 promoter/enhancer elements to optimize *rep* and *cap* expression. **C:** Ad5 (*top*) can be used as an AAV helper virus. The use of adenovirus-derived helper plasmids [e.g., pXX6 (171)] that contain the required viral genes minimizes the risk of helper-virus contamination.

To overcome this problem, a packaging system was developed (171) that is free of adenovirus. The adenoviral genes that provide essential helper functions during the productive life cycle of AAV are the E1A, E1B, E2A, E4, and VA RNA genes (reviewed in Chapter 69 and ref. 114). E1A functions as a transactivator of AAV *rep* and *cap* transcription. Interaction of E1B with E4 stabilizes AAV mRNAs and/or facilitates the mRNA transport to the cytoplasm. A similar role has been described for E2A and VA RNA. By cloning of the E2A, E4, and VA RNA genes into a high-copy plasmid, Xiao et al. (171) generated a helper plasmid (pXX6) that contains all the essential helper genes but lacks the adenovirus structural and replication genes and the adenoviral terminal repeats (see Fig. 8C). The missing E1 gene products are provided by using 293 producer cells (59). Surprisingly, supplying the adenoviral helper functions from a plasmid that resulted from deleting most of the adenoviral genome significantly increased AAV vector production (171). This is presumably the result of the lack of competition between the AAV and adenovirus for adenoviral gene products in the packaging cells, in addition to the removal of the lytic action of the helper virus (145).

A further, major improvement of AAV vector production was the development of a second generation of packaging constructs (171): Because *rep* expression has been shown to cause a paradoxical inhibition of rAAV production if the Rep68/78 proteins are overexpressed in the producer cells (92), the ATG translation start codon of the *rep* gene was mutated into an inefficient ACG codon to achieve attenuation of Rep68/78 synthesis (see Fig. 8B). Because the p5 promoter exerts positive effects on p19 and p40 but negatively regulates its own transcription (100,129), Xiao et al. (171) reasoned that inclusion of a second copy of the p5 promoter/enhancer element 3' of the *cap* gene might have positive effects on AAV vector production (see Fig. 8B). Indeed, these changes increase vector yields approximately 15-fold over the conventional packaging plasmid pAAV/Ad. Thus, co-transfection of 293 cells with this second-generation packaging system, containing three plasmids (the vector, the pXX2 packaging plasmid, and the pXX6 helper construct) results in viral titers of up to 10^{10}/10-cm plate (171).

A problem with this packaging system is that the adenoviral terminal repeats present in pAAV/Ad and pXX2 have been shown to be involved in illegitimate recombination with the AAV ITRs. This recombination between the AAV vector and the packaging plasmids can lead to the production of wild-type AAV-2 particles in the range of 4% of the total physical titer (164). However, removal of the distal 10 nucleotides (in the D sequence of the ITR; see also Chapter 69) of the AAV vector ITRs [pD-10 vector construct (164)] reduced the amount of wild-type AAV contaminants to below the detection level, when a packaging plasmid was used that lacked adenoviral ITRs (164). The elimination of replication-competent wild-type AAV and helper virus (e.g., adenovirus) contaminations from the rAAV preparations is an important step toward the generation of clinical-grade vectors.

Packaging Cell Lines for AAV

Adeno-associated-virus vector production in stable cell lines has to overcome several problems associated with the toxicity of viral proteins and the viral life cycle. The Rep proteins exert an antiproliferative effect on mammalian cells (174), and they have a negative effect on heterologous gene expression (92). Therefore, it was not surprising that initial attempts to establish a stable cell line containing AAV-2 *rep* and *cap* (30,157) were not very successful. The initial AAV cell lines gave titers of 30 IU/cell (30), whereas transient transfection of the second generation of packaging/helper

system yields up to 1,000 IU/cell (171). Recently, a number of promising inducible cell lines have been developed, in which the expression of Rep and Cap proteins is regulated by the tetracycline-inducible system (73) (see earlier section, Packaging of Retroviral Vectors). Infection of the cell line with adenovirus after the addition of tetracycline or doxycycline to the medium resulted in AAV vector production with titers 10 times higher (i.e., 10,000 IU/cell) than those obtained by transient transfection. However, after 3 to 4 days in doxycycline, the number of particles per cell dropped dramatically (73).

A major drawback of the presently available packaging cell lines is the requirement for the infection with a helper virus, and an extensive purification procedure has to be applied to minimize the amount of helper virus contamination. In addition, an important problem with stable cell lines for AAV vector production is that expression of the helper viral genes leads to the excision of the vector genome from the integration site, and thus prolonged expression of helper virus genes might disturb the genomic stability of the packaging/producer cells.

Purification of Recombinant AAV and Titering

In contrast to retroviral vector particles that are secreted into the culture medium of the packaging/producer cells, rAAV accumulates intracellularly and has to be purified from the producer cells. Lysis of the producer cells (e.g., by freeze/thaw cycles) is necessary if a packaging/helper system is used that is devoid of lytic helper-viruses. The conventional purification protocol involves a step-wise precipitation of AAV particles with ammonium sulfate, followed by up to three rounds of CsCl density gradient centrifugation (170). After each round of CsCl centrifugation, the gradient is fractionated and the active fractions are identified by dot-blot hybridization or polymerase chain reaction analysis. Because this purification method is labor intensive and often results in substantial loss of viral particles, new methods have been developed to purify rAAV using gradient media other than CsCl or column chromatography (29,157,185). A major improvement of AAV purification is the iodixanol method developed by Zolotukhin et al. (185), which decreases toxicity from rAAV aggregates and avoids the toxicity of residual hyperosmotic CsCl. Iodixanol is an iso-osmotic, inert x-ray contrast reagent that has been extensively tested. Crude producer cell lysates can be purified at least 100-fold, and 70% to 80% of the starting infectious particles are recovered. With this method, lysates obtained from 2.5×10^{9} cells can generate 10^{14} virus particles or 10^{12} IU/mL (185).

An important further step toward clinical-grade virus preparations was the development of ligand affinity chromatography purification techniques. Two laboratories have developed heparin affinity chromatography procedures using commercially available heparin columns (29,185). This approach exploits the fact that heparan sulfate proteoglycan acts as a cellular receptor for AAV-2 (154). Furthermore, it appears to be superior to earlier attempts to purify AAV using ion exchange or size-exclusion columns (29,157). The combined iodixanol/heparin chromatography procedure results in 50% to 70% recovery of virus that is better than 99% pure and is more infectious than the CsCl method (particle-to-infectivity ratios are less than 100:1 for iodixanol/heparin, versus 1,000:1 for the traditional purification strategies) (185). Recently, an immunoaffinity column chromatography approach to AAV purification has also been explored (60).

Host Range and Infection of Nondividing Cells

AAV-2-derived viral vectors exhibit a broad host range and infect a wide variety of tissues and cells. Furthermore, AAV is a nonpathogenic virus and is able to transduce nondividing cells such as muscle cells (170), hepatocytes (150), and neurons (101). These cells are terminally differentiated, and progression through the cell cycle seems not to be a prerequisite for transduction, as indicated by analysis of 5'-bromo-2'-deoxyuridine (BrdU) labeling of livers infected with rAAV (104). However, it has been reported that transduction efficacy is influenced by the cell cycle phase of the target cells and that the transduction frequency is significantly higher in cells in S phase (142). In addition, there appears to be a preference of rAAV for certain cell types: For example, rAAV infects neurons, but no transgene expression was found in astrocytes or microglia after infusion of AAV vectors into the adult rat brain (9). Furthermore, neuronal AAV infection was reported to be restricted to certain areas of the brain (e.g., the hippocampus), whereas neurons in the cerebral cortex were not transduced (101). Possible explanations are that rAAV might require a certain metabolic state, or that the receptors needed for AAV attachment and entry are not expressed on cells refractory to AAV transduction. The latter notion received further attention when the cellular receptor and coreceptors for AAV were identified. Heparan sulfate proteoglycan has been identified as the primary receptor for AAV (154) that mediates attachment of AAV-2 to the cell membrane of the target cell. In addition, integrin a_vb_5 and the fibroblast growth factor receptor 1 (FGFR1) have been identified as coreceptors for AAV infection (134,153). Interestingly, coreceptors have been identified for both adenoviruses (Chapter 67 and Chapter 68) and herpes simplex viruses (Chapter 72 and Chapter 73). Thus, AAV may have acquired coreceptor dependency as a mechanism for co-infection with its helper viruses that allows the optimal production of progeny virions (153).

Apart from the cell cycle phase and the requirement for AAV receptor, expression of helper virus genes (46) and exposure to agents that induce genotoxic stress (e.g., irradiation) and DNA repair (46,140) can significantly increase transduction efficiency. Not only does adenovirus help wild-type AAV to initiate productive infection, it also enhances rAAV transduction (reviewed in ref. 145). It has been shown that the E4 protein is directly involved in this process, and that the E4- and genotoxic stress-induced increase in rAAV transduction is caused by enhanced second-strand synthesis (46). The latter is of importance for AAV-based gene delivery, because the conversion of the single-stranded AAV genome to duplex is the rate-limiting step for rAAV transduction in many cell types (145).

Integration Versus Episomal Persistence of rAAV

Site-specific integration would be a highly desirable feature of a gene therapy vector, as it would ensure long-term (theoretically even life-long) expression of the transgene and minimize the mutagenic and carcinogenic potential—major drawbacks of random integrating vectors based on retroviruses. Although the ITRs are the only genomic elements necessary for integration, efficient integration and site specificity require the presence of the viral Rep protein (165). Because AAV vectors lack the Rep expression cassette, it comes as no surprise that AAV vectors integrate with low efficacy and low specificity into the host genome. Integration of rAAV has been observed in dividing cells (e.g., HeLa

cells) (173), as well as in nondividing cells *in vitro* (human neuronal cell lines) (168). Nakai et al. (115) provide direct evidence for integration of rAAV into the mouse genome. Further analysis by pulse-field electrophoresis and fluorescence in-situ hybridization (FISH) showed integration of the rAAV in approximately 5% of liver cells (105).

Apart from integration into the host genome, the presence of episomal forms of rAAV has been demonstrated *in vivo* (38,49,115). Several studies demonstrated the presence of AAV vector DNA in an episomal circular form in muscle and brain tissue transduced with AAV vectors (38,115). Because this form of episomal AAV DNA persists for up to 9 months (38,115), the episomal AAV DNA might be a major contributor to the long-term expression of transgenes delivered with AAV vectors. The characteristic feature of these circular genomes is that they contain two viral ITRs in a head-to-tail orientation, which appears to confer an increased persistence of episomal DNA at least in certain cell types (e.g., HeLa cells) (38). Presently, not much is known about the exact mechanisms that lead to the formation of these concatamers, but it appears to involve recombination events between separate viral genomes. This inherent ability of AAV to form concatamers fusing the 5' and 3' ends of two genomes of two viruses is the basis for the development of trans-splicing, dual-vector systems (39,116,155,172).

In summary, completion of second-strand synthesis appears to be the rate-limiting step of rAAV infection (recently reviewed in refs. 113,141), and it is associated with an increase in transgene expression, which can take several weeks after administration *in vivo*. Long-term (i.e., several months) transgene expression could be achieved by integrated concatamers, or by stable concatameric episomes, or both.

Immune Response Against AAV in Humans

Single administration of rAAV into the muscle of immune-competent mice does not result in a detectable cellular immune response as determined by staining for CTLs (170). However, a detailed analysis of the immune response in mice that received rAAV via intramuscular (i.m.), intraperitoneal (i.p.), intravenous (i.v.), and subcutaneous (s.c.) injection revealed that all these different routes of administration elicited a humoral response (17). Interestingly, a potent transgene-specific CTL response was detected after i.p., i.v., and s.c. injection, whereas only a minimal CTL activity was observed after i.m. administration (17).

The preexisting humoral immunity against AAV in humans is likely to be a hurdle for many gene therapy approaches in humans. A recent study by Chirmule et al. (27) showed that virtually all patients had antibodies to AAV-2, and in 32% of the cases neutralizing antibodies were found. In addition, 5% of the subjects had peripheral blood lymphocytes that proliferated in response to AAV-2 antigens. All patients enrolled in the first clinical trial of intramuscular injection of an AAV vector carrying human factor IX in patients with hemophilia B (79) had detectable titers of neutralizing antibodies against AAV before treatment. However, factor IX expression was demonstrated in muscle biopsies even in the patient with the highest pretreatment antibody titer. Administration of the AAV vector elicited a 10- to 1,000-fold increase in the neutralizing antibody titer.

Therefore, especially if vectors are readministered, efforts to overcome the humoral immunity against AAV have to be considered. Temporary block of CD4 T cell function to inhibit memory B cells (28), and the selective modification of the AAV capsid (54) are additional approaches. The latter is also a way to alter AAV-2 tropism and may be employed for targeting of AAV vectors *in vivo*.

ADENOVIRAL VECTORS

Adenoviruses are medium-sized DNA viruses that have been isolated from avian and mammalian species. Their genomic organization and virion structure are described in detail in Chapter 67 and Chapter 68. The double-stranded linear DNA genome is 36 kb long and is flanked by two ITRs that contain the origin of replication (Fig. 9A). The Ad genome is packaged in a nonenveloped icosahedral protein capsid. On infection of permissive cells, the viral genome is replicated and transcribed in two major stages: an early and a late phase. The boundaries of the two phases are delineated by viral replication. The early genes lie in five noncontiguous regions designated as early region 1A (E1A), E1B, E2, E3, and E4. Most late transcripts are initiated from the major late promoter and are subsequently processed to generate five families of transcripts (L1 to L5) that share the same carboxyl terminus.

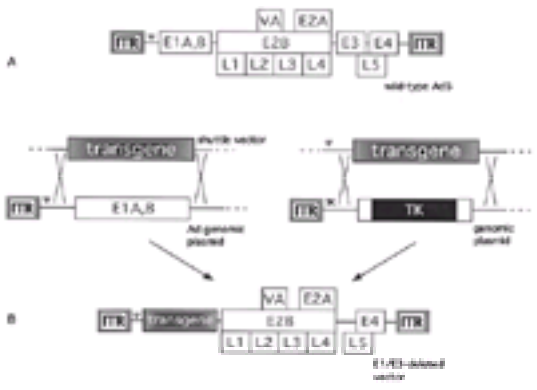

FIG. 9. Adenoviral vectors. **A:** Wild-type adenovirus (Ad5) **. B:** First generation of adenoviral vectors. *Left:* Homologous recombination of the shuttle vector that carries the transgene flanked by adenoviral genomic sequences with the genomic plasmid results in the replacement of the E1 region with the transgene in the recombinant Ad vector (*bottom*). *Right:* Deletion of the packaging signal and/or insertion of a selection marker such as thymidine kinase (TK) reduces the amount of contamination with unrecombined parental virus.

Development of Adenoviral Vectors

Of the approximately 50 known serotypes of human adenovirus, which are categorized into six subgroups, most adenoviral vectors (63,71,166) have been derived from serotype 5 (Ad5) of subgroup C. An important finding for the development of adenoviral vectors was that Ad5 carries a cis-acting DNA encapsidation signal at the end of the viral chromosome, and that recombinant viral genomes lacking these signal sequence cannot be packaged into viral particles, although normal levels of viral proteins were synthesized from these mutants (68). Two other important findings are (a) that Ad DNA can circularize in infected cells and that circular Ad genomes can be propagated as bacterial plasmids (reviewed in ref. 13), and (b) that co-transfection of two plasmids with overlapping sequences can generate infectious viral particles via homologous recombination *in vivo*.

Based on these findings, the first generation of Ad-derived vectors were designed by replacing adenoviral coding regions E1 and/or E3 with foreign genes. Because the E1A proteins are essential for transcription of other early viral genes and promote the expression of host cell proteins needed for DNA replication, deletion of the E1 region should render the vector replication-defective. The E1-deleted vectors can be propagated in 293 cells, which express 11% of the Ad5 genome and complement for E1 function (59). Thus, nearly all the E1 coding sequences (up to 3,181 nucleotides) can be removed (13), as long as the packaging signal and the coding sequence of protein IX, which is colinear with E1B but uses a different promoter (see Chapter 67), is not affected. Because the packaging capacity of Ad5 virions is 105% of the wild-type genome (14), transgenes of up to 4.7 to 4.9 kb can be accommodated in E1-deleted adenoviral vectors. The capacity of the viral vectors can be further increased by deletion of the E3 region (11,66), which is not essential for viral replication and growth in cell culture. However, for gene therapy vectors, the E3 protein is of relevance, as it has immunosuppressive functions (71): From the seven proteins encoded by the E3 region, the gp 19-kd protein binds to MHC class molecules and the 14.7/10.4-kd proteins inhibit apoptosis. The largest viable E3 deletion described so far is 3.1 kb (13). A combination of both E1 and E3 deletions in one mutant virus results in a cloning capacity of 8.3 kb.

Several strategies have been developed to insert the transgenes of interest into the Ad vectors. The most commonly used strategy relies on homologous recombination in the 293 producer cells (see Fig. 9). The transgene is inserted into a shuttle vector that contains adenoviral genomic sequences that flank the recombination target site (i.e., the E1 coding region) in the recipient Ad genomic plasmid. Through homologous recombination in the 293 cells, the transgene sequence is integrated into the Ad vector DNA, replacing the E1 gene. This E1-deleted Ad vector is able to replicate in the 293 cells because of their E1 helper function, and it is possible to produce up to 10^{11} viral particles per 10-cm cell culture dish.

A drawback of this first generation of Ad vectors is the contamination with wild-type virus. Two major changes have been incorporated into the Ad genomic plasmids to solve this problem (see Fig. 9): (a) removing the packaging signal (13), or increasing its size beyond the maximum that can be packaged (102), thereby inhibiting packaging of the parental genome, and (b) incorporating a selection marker (e.g., by replacing the E1 region with the thymidine kinase gene) that allows for the selection against wild-type virus (72). In addition, strategies have been designed for the production of recombinant viral DNA that do not rely on recombination with parental infectious viral genomes in 293 cells. Instead, Ad genomic DNA can be manipulated in yeast (80) and bacteria (25). The recombinant Ad genome can then be purified from these hosts and used to produce virus in 293 cells.

A recurrent problem with the first generation of Ad vectors is the generation of replication-competent adenovirus (RCA). To address this problem, further deletions of viral genes were introduced into the vector backbone, resulting in the second generation of Ad vectors. Reduction of the number of viral genes present in the Ad vector should also reduce the immune response against the vector and vector-infected cells. These second-generation vectors (71,161) are based on deletion of the E2 and/or E4 regions within the E1-deleted vectors. The E2 region encodes proteins required for the replication of the viral chromosome: the precursor terminal protein, DNA polymerase, and DNA-binding protein (DBP). The first attempts to delete the E2 region were hampered by the fact that the production of the E2-deleted vectors require expression of E2 in trans, which is not easily achieved because of the toxicity of the E2 protein (83). Therefore, Engelhardt et al. (44) constructed an E1-deleted adenoviral vector that harbors a temperature-sensitive mutation of DBP. This mutation reduces the ability of the virus to replicate at 39°C. Because of the “leakiness” of this system and the conflicting results regarding persistence of transgene expression [especially at a later time (beyond 7 days) (44,45)], vectors with deletions of DBP (56), of adenoviral DNA polymerase (5), and of the precursor terminal protein (148) were designed that can be produced in packaging cell lines that complement for the deleted protein (4,56,89,184).

E4 is the other genomic region that has been targeted for the development of second-generation vectors. Located at the right end of the Ad genome (see Fig. 9A), E4 encodes proteins that are required for viral DNA replication, mRNA splicing and accumulation, late protein synthesis, and inhibition of host cell protein synthesis. Deletion of the entire E4 region is theoretically possible; however, in practice such vectors have been shown to result in a significant drop in vector yield (18). The mechanism for this decrease in viral titer of E4-deleted vectors is not completely understood, but it was shown that total deletion of E4 causes a reduction in the expression of late region 5 (L5) mRNA. It was postulated that the spatial organization of E4, L5, and the right ITR itself might be of importance for the regulation of L5. The defect of E4-deleted vectors was overcome either by insertion of spacer sequences into the E4 locus (18) or by only partially deleting the E4 region (53,162,179). To propagate the E1/E4-deleted vectors, E4-complementing cell lines have been developed. An important aspect of E4 function is the discovery that the product of ORF6 can substitute for the entire E4 region. Because expression of E4 ORF6 together with E1B is detrimental to the packaging cells, inducible E4 expression systems have been developed (87,163). Although deletion of the majority of E4 should decrease the host immune response and increase the cloning capacity of the Ad vector, the E4 region may be required for long-term expression, especially if the CMV promoter is used as internal promoter in the vector construct (6,8).

High-Capacity, Helper-Dependent Vectors

The third-generation Ad vectors (63,71,84) have been termed, according to their characteristics, high-capacity,

helper-dependent, mini, and “gutless” adenoviral vectors. They were designed to minimize the amount of Ad viral genes present in the vector. This would increase the cloning capacity of the vector and should reduce the host immune responses against the Ad vector-transduced cells.

Earlier attempts (48,64,109) to divide the adenovirus genome into separate cis- and trans-acting functions to establish clearly separated vector and packaging/helper constructs were successful. However, two major problems were encountered (71): The vector preparations were highly contaminated with up to a 1,000-fold excess of helper virus (48). Vectors that are significantly below the size of the wild-type Ad genome proved to be unstable and undergo DNA rearrangements (48,64). Parks and Graham (127) analyzed the size constraints of Ad vectors: The optimal DNA packaging length ranges from 75% to 105% of the wild-type (36-kb) genome. Vectors smaller than 75% were packaged with a much lower efficacy, and were unstable (127).

An important step in the production of helper-dependent vectors was the introduction of the Cre/ *loxP*-strategy in the Ad system to remove viral DNA sequences (67,93,126). The Cre-recombinase of bacteriophage P1 excises DNA sequences that are flanked by *loxP* recognition sites and has been successfully used for gene targeting experiments in mice (62). The Cre/*loxP* systems allows even the manipulation of mammalian chromosomal regions (up to 3 to 4 cM) (135). Insertion of *loxP* sites so that they flank the genomic region to be deleted is a promising way to restrict the Ad genome (Fig. 10). Lieber et al. (93) deleted 25 kb of adenoviral genome encompassing the pIX, the E2, and the L1 to L4 regions by infection of 293 cells stably expressing the Cre recombinase (293-cre) with an Ad vector that contained *loxP* sites flanking the region to be deleted. These authors (93) introduced the *loxP* sites into an E1-deleted vector backbone that carried a transgene in the place of the E1 region, and the final result after Cre-mediated excision was a 9-kb vector that contained only the transgene, a *loxP* site, the packaging signal, and the E4 region flanked by ITRs (see Fig. 10). Unfortunately, this vector was unstable, presumably because of its size, and it provided only limited transgene expression *in vivo*.

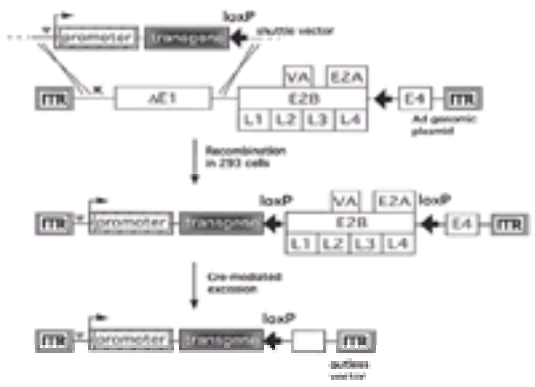

FIG. 10. High-capacity adenoviral vectors. High-capacity, adenovirus vectors can be obtained by introduction of two *loxP* sites (*black arrows*) into an Ad genomic plasmid that carries deletions in the packaging signal, in the E1 region, and in the E3 region (93) (*top*). Infection of 293-cre cells that express the Cre recombinase leads to the excision of the sequences flanked by the *loxP* sites (*middle*), thereby deleting almost all viral sequences. The resulting “gutless” vector contains only the ITRs, the packaging signal, the viral E4 region, and the transgene and the internal promoter.

An alternative Cre/*loxP* system was designed that targeted the sequences essential for packaging of the helper virus to prevent its packaging (67,126). Previous studies (85) had shown that using a helper virus that carried a deletion of 91 bp of the Ad packaging signal, together with a helper-dependent Ad vector, resulted in a considerable improvement of the helper virus contamination, reducing it to less than 1% after CsCl purification. The helper virus with the *loxP*-flanked packaging signal can be easily propagated in conventional 293 cells. After infection of 293-cre cells with this helper virus, the packaging signal is excised, and the helper virus retains its capability to provide the Ad functions required for replication and packaging of the helper-dependent virus. With this system, Parks et al. (126) obtained about 10^{10} IU, with less than 0.01% of total virus being helper virus.

The principal advantage of helper-dependent vectors over first- and second-generation Ad vectors and other gene therapy vectors is their enlarged cloning capacity: If all viral genes are deleted, up to about 35 kb of DNA could be packaged in the Ad5 virion. The cis-acting elements required for replication and packaging (i.e., the packaging signal and the ITRs) of the vector are contained within 500 bp at the end of the Ad genome (58). In addition, the removal of viral genes from the vector is a promising way to avoid the host immune response.

Replication-Competent Adenoviral Vectors

Although replication of Ad vectors is unwanted in most gene therapy approaches, viral replication, virus assembly, and release of the progeny virus by lysis of the infected cell can be exploited for cancer gene therapy. To use adenoviruses for oncolytic gene therapy, conditionally replicative adenoviruses have been developed that replicate exclusively in tumor cells (3). An example of such a virus is an Ad mutant that replicates selectively in p53-deficient cells (called dl1520 or ONYX-015) (15,70). The dl1520 is a human subgroup C Ad that carries an 827-bp deletion and a point mutation in the E1B region that prevents expression of the E1B-encoded 55-kd (E1B-55K) protein. E1B-55K normally binds and inactivates the tumor suppressor p53 (180) and contributes to oncogenic transformation by Ad. Because p53 function must be blocked to allow efficient virus replication, it was hypothesized that replication of the E1B-55K-deficient Ad virus would be suppressed in normal cells, whereas cancer cells that lack p53 would support viral replication. Indeed, the E1B-55K-deficient virus was subsequently shown to productively infect a broad spectrum of human tumors with p53 abnormalities (e.g., p53 mutations and/or deletions) and to exert a replication-dependent cytopathic effect in these tumors *in vitro* and *in vivo* (70). On the other hand, human cells with functional p53 were highly resistant to the

E1B-55K-deficient virus (70). Given this promising result, a phase II clinical trial was started with intratumoral administration of E1B-55K-deficient Ad mutant in combination with standard i.v. chemotherapy in patients with recurrent squamous cell carcinoma of the head and neck (81). The combined therapy caused a more than 50% decrease in tumor volume in 63% of the patients, and none of the responding tumors progressed for 6 months, whereas all tumors treated with chemotherapy alone did progress. Thus, the approach of using specially engineered replication-competent Ad vectors in the treatment of cancer is a promising direction for gene therapy.

Immunologic Impediments

The ability to generate high-titer (10^{14} particles/mL) recombinant Ad vectors (RAd) and their efficient transduction of both dividing and nondividing cells boded well for a very successful vector. Unfortunately, length of expression of the transgene was transient (7 to 10 days) (35). It soon emerged that transient expression was caused by an immune response, because in immunocompromised animals, expression in slow-turnover cells such as muscle and neurons is observed for long periods of time. It is now generally accepted that cells transduced by RAd vectors are eliminated by CTLs generated against viral proteins or the transgene (76,158). It was for this reason that a number of investigators generated second- and third-generation vectors in which chances of synthesis of viral proteins are minimized. Unfortunately, RAd vectors present a unique problem because even the inactivated RAd vector can elicit potent CTLs against viral proteins (76). Furthermore, the RAd vectors elicit a potent humoral response that will effectively eliminate all subsequent transduction. This is even more pertinent to adenoviral vectors because they do not integrate, and hence they suffer loss by cell division and by DNA degradation, necessitating a repeat infection with the vector. Because there are scores of adenoviral serotypes, one strategy to overcome this problem might be to use different serotypes.

The high-titer recombinant adenoviruses are potentially toxic, even though the precise mechanism associated with this toxicity remains uncertain. The death of a patient directly associated with recombinant adenoviruses is a tragic warning sign that signals the need for further improvement of viral gene therapy. The RAd vectors will, however, continue to be utilized in situations where a high-level but transient expression of the foreign gene is required—for example, restenosis and cancer.

OTHER VECTORS

Our emphasis in this chapter was on most commonly used viral vectors, but there are other viral vectors based on SV40, Sindbis virus, herpes viruses, and others. Among these, the herpes virus–based vectors have the best potential for clinical applications. The presently available herpes viral vectors, where viral immediate-early genes have been deleted, appear to be nontoxic and therefore likely to be useful for clinical applications. We refer the reader to excellent reviews on the subject of emerging vectors (74) and herpes viral vectors (90,167).

The last two decades have witnessed the birth of the field of gene therapy, which has generated great hopes and great hypes. The promise of influencing the outcome of a vast array of diseases, ranging from birth defects to neurological disorders and from cancer to infectious disease, although far-reaching, is not beyond reach. With the completion of the sequence of the human genome, over 50,000 genes will be available to the practitioners of gene therapy. The potential benefits for human health are vast. Gene therapy has many challenges and hurdles ahead, but they are surmountable. It will continue to remain an exciting field of research.

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CHAPTER 18 Diagnostic Virology

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PERSPECTIVE

Until recently, diagnostic virology was outside of the mainstream of clinical laboratory medicine. The reasons were clear: virology laboratories depended on tissue or cell culture techniques that were slow and expensive, commercial availability of reagents was very limited, and few treatments were available for viral infections. Consequently, most hospitals did not have clinical virology laboratories. Those that existed were usually associated with children's hospitals, having been established to assist in the diagnosis of poliomyelitis.

In contrast, diagnostic virology is presently integrated into routine medical practice. The expanded role of diagnostic virology has several explanations. First, the human immunodeficiency virus (HIV) and acquired immunodeficiency syndrome (AIDS) epidemic and the success of organ transplantation have greatly increased the pool of patients at risk for serious opportunistic viral infections. Second, an increasing number of antiviral agents are available, and their use often depends on establishing a laboratory-based diagnosis. Third, technologic developments such as monoclonal antibodies and the polymerase chain reaction (PCR) have made rapid viral diagnosis a reality. It is still true that diagnostic virology laboratories as distinct entities are by and large limited to large hospitals, especially tertiary referral centers that are often within academic medical centers.

An important characteristic of modern diagnostic virology is the use of multiple methods for detecting viral infections. Viral culture, detection of viral antigens and nucleic acids, and detection of viral antibodies are all used to diagnose current viral infection. Light microscopy is used to detect the effect of viral infection in tissue and to define the role of viral infection in producing disease. Serologic techniques are used to define the presence of specific antiviral immunity. Tests using these methods may be carried out in a number of different clinical laboratories, including serology, blood bank, surgical pathology, chemistry, molecular diagnosis, and retrovirology laboratories in addition to traditional diagnostic virology laboratories. Several books on diagnostic virology provide more detailed treatment than is permitted by the scope of this chapter (97,112,184).

Diagnostic virology is much more important in some areas of clinical medicine than in others. Patient groups whose care often involves the use of diagnostic virology tests include immunocompromised patients, especially recipients of solid organ or bone marrow transplants and those with HIV or AIDS; patients with sexually transmitted diseases, acute respiratory infections, gastrointestinal infections (especially children but increasingly adults as well), infectious mononucleosis-like illnesses, acute or chronic viral hepatitis, serious ocular infections; and infants (and fetuses) suspected of having congenital infections. The management of antiretroviral drug therapy in patients with HIV or AIDS is based on assays to measure plasma HIV RNA plus genotypic and phenotypic assays to detect HIV drug resistance. The approaches developed for HIV are establishing new paradigms for the care of patients with chronic viral infections in which viral diagnostic tests are used to direct and monitor ongoing antiviral therapy.

Diagnostic virology has also been important in public health. For example, viral culture surveillance of patients with acute respiratory infections is crucial for the recognition of new strains of influenza. Specific diagnosis of cases of measles or rubella may initiate aggressive emergency immunization programs. Recognition of cases of arboviral encephalitis may set in motion mosquito control programs. Detection of Norwalk agent or other caliciviruses may be important in

understanding and controlling food- and water-borne gastroenteritis.

HISTORY OF DIAGNOSTIC VIROLOGY

The modern era of medical virology began in 1898 with the discovery by Loeffler and Frosch that foot and mouth disease of cattle was caused by a filterable agent referred to as a virus. Viral inclusions had been noted previously in 1892 by Guarneri (81), who described intranuclear and intracytoplasmic inclusions at the base of smallpox lesions. Knowledge of viruses as causes of human disease increased rapidly in the first quarter of the 20th century. The earliest methods that were widely used for diagnosing these infections were serologic, directed at the detection of viral antibodies or antigens. The complement fixation test described by Bedson and Bland in 1929 was first used to detect antibodies to smallpox, vaccinia, and varicella zoster viruses (VZV) (18). Serologic testing for antiviral antibodies is still widely employed to diagnose acute viral infection as well as to define specific antiviral immunity.

Diagnostic virology leaped forward in 1948 with the report by Weller and Enders of the first growth of pathogenic human viruses in tissue culture (197). Because the newly developed techniques for maintaining tissue cultures were beyond the scope of existing clinical laboratories, virology laboratories were established as distinct entities. Cell culture, the growth of defined populations of cells not formed into discrete tissues, was a refinement of tissue culture that formed the basis of diagnostic virology for many years. It is now being gradually replaced by nonculture techniques based on immunologic or molecular methods.

The first application of fluorescent antibody (FA) staining to diagnostic virology was the detection of influenza virus antigen in nasal smears by Liu (114). The method was further pioneered by Gardner and McQuillan (72). The development of monoclonal antibodies in the 1970s (101) greatly increased the availability of immunologic reagents with well-defined specificity for individual viruses. Rapid viral diagnosis became a practical reality during the 1980s as diagnostic virology laboratories implemented monoclonal antibody–based assays to detect a wide variety of viral antigens directly in clinical specimens. The introduction of molecular techniques into the diagnostic virology laboratory accelerated with the development of PCR, described in 1985 (169), and led to a period, still ongoing, of rapid evolution of diagnostic virology laboratories.

SPECIMENS FOR VIRAL DIAGNOSIS

The quality of viral diagnosis can never exceed the quality of the specimen that is received in the laboratory. Important variables include the timing of the specimen in relation to the patient's illness, the type of specimen, the quality and amount of specimen material obtained, and the time and conditions of transport to the laboratory. Although this concept is so basic as to seem trivial, optimizing the variables listed requires knowledge on the part of the physician and attention to logistic considerations that can present substantial barriers.

For the diagnosis of acute viral infections, the best specimens are usually obtained from the site of disease. For example, in the patient with suspected viral meningitis, cerebrospinal fluid (CSF) is the best specimen. In infections involving skin or mucosal surfaces, specimens obtained from that surface are usually the only ones required. Viral titers are highest early in the illness, so that specimens obtained within the first few days after onset are most likely to be positive.

Viral culture requires more attention to conditions of transport than specimens submitted for detection of viral antigens or nucleic acids because the viability of the virus must be preserved. A number of clinically important viruses are labile and will not survive prolonged transport. General instructions for transporting specimens for viral culture are shown in Table 1. These conditions are usually also suitable for detection of viral antigens and nucleic acids, but consultation with the laboratory performing testing is advised. Specimens other than blood should usually be kept at 4°C during transport, especially if transport will require more than 1 hour. If a delay of more than 24 hours is anticipated, the specimen should be frozen at a temperature of –70°C or lower. The recovery of some viruses, such as respiratory syncytial virus (RSV) and human cytomegalovirus (CMV), is seriously compromised by freezing. Blood specimens can be transported at room temperature, but recovery of CMV by culture is unlikely if the time of transport is 24 hours or greater, regardless of whether the blood is at room temperature or 4°C (160).

Specimen	Instructions
Blood	Usual volume required is 3–10 ml. The appropriate tube is determined by the test to be performed, the blood component on which the test is performed (whole blood, leukocytes [buffy coat], plasma, or serum), and the preference of the laboratory. Whole blood, leukocytes, and plasma require collection into a tube containing an anticoagulant. The most commonly used anticoagulants are EDTA (purple-top tube), heparin (green-top tube), and acid-citrate-dextrose (ACD). Note that heparin is inhibitory for some polymerase chain reaction (PCR) procedures, especially those performed on plasma. Some nonculture tests performed on blood may have specific collection tube requirements that can be determined through communication with the laboratory. Most serologic procedures, some cultures (e.g., for enteroviruses), and some PCR assays (e.g., for parvovirus B19) are performed on serum. Serum specimens require collection into a tube that does not contain an anticoagulant (red-top tube). Blood specimens do not have to be placed on ice if transport requires less than 1 day. Note that prolonged transport can severely compromise the ability to recover cytomegalovirus in culture from blood specimens.
Swab	Swabs with Dacron or rayon tips are preferred. If available, swabs designed specifically for collection of viral specimens should be used. Swabs can also be place in viral transport media. In this case, the shaft of the swab may have to be cut to allow the top of the transport vial to be securely closed. The swab or tube of transport media should be placed on ice if transport will require more than 1 hour.
Fluid	Place in a sterile container.
Tissue	Place in a sterile container with a small amount of viral transport media, sterile saline, or phosphate-buffered saline to maintain moisture.
Stool	Obtain at least 4 g of stool. Place in a clean or sterile container.

[a]Specimens other than blood should be transported on ice if more than 1 hour is required for arrival at the laboratory. If a delay of more than 24 hours is anticipated, specimens should be frozen at –70°C and transported on dry ice. Note that some viruses, such as respiratory syncytial virus and cytomegalovirus, are unlikely to retain viability with freezing. Specimens should not be frozen at –20°C.

TABLE 1. *Instructions for obtaining and transporting specimens for viral culture*[a]

For serologic diagnosis, an acute-phase serum specimen should be obtained within the first few days of illness and a convalescent phase specimen 2 to 4 weeks later. If a virus-specific immunoglobulin M (IgM) assay is available, the acute-phase specimen may be sufficient by itself. Immunoglobulins are stable in serum or plasma. Proper handling of specimens for serologic diagnosis begins with separation of serum or plasma. If testing is performed within several days, the serum or plasma can be stored at 4°C. If a longer delay is involved, the specimen should be frozen at –20°C or

–70°C. For certain viral infections, serologic tests can be performed on saliva or urine.

METHODS USED IN DIAGNOSTIC VIROLOGY

Viral Culture

Viral culture is the procedure that sets diagnostic virology apart from other areas of diagnostic microbiology. Because viruses require cellular machinery for replication, living systems must be used. Historically, inoculation of animals and eggs was used to cultivate and propagate viruses, but these techniques have been largely supplanted by cell culture in virtually all diagnostic virology laboratories. Viral culture has important advantages and disadvantages compared with other diagnostic methods. By its very nature, culture is an amplification method that increases the amount of the putative pathogen, facilitating detection and characterization. Viral culture is unique among detection methods in that it provides an isolate of viable virus that can be further characterized as necessary and can be stored for future studies. Another important feature is that culture methods allow the detection of many different viruses, including some not suspected at the time the culture is established. Even viruses not previously known can be discovered. This is in contrast to immunologic or nucleic acid–based diagnostic tests, which ordinarily detect only the specific virus to which the diagnostic reagent is directed. Viral culture also has significant disadvantages as a diagnostic method, including requirement for specialized facilities and expertise, expense, relatively prolonged time to detection, and relatively limited range of viruses that can be detected.

Growth of Cell Cultures

Cells are grown in the laboratory using the appropriate nutrient media, temperature, and atmosphere of incubation. A number of defined basal media are commercially available and are often supplemented with additives such as glutamine, fetal bovine serum, and antibiotics to counteract bacterial or fungal contamination. Most cell cultures used in diagnostic virology laboratories grow as cell monolayers that are adherent to glass or plastic surfaces. Most commonly, cultures are planted in individual glass tubes, but other vessels, including plastic flasks and microtiter trays, can be used. Some cell culture types do not adhere and are grown as suspension cultures. No one type of cell culture can grow all medically important viruses, and therefore diagnostic virology laboratories must use several different types of cell culture. Cell cultures can be maintained and propagated within the laboratory, or alternatively, they can be purchased from commercial suppliers who maintain supplies of a wide variety of cell culture types.

Types of Cell Culture

Cell cultures can be divided into four distinct categories. Primary cell cultures are derived directly from the source animal. Examples include primary monkey kidney cells and primary rabbit kidney cells. Viruses endogenous to the source can occasionally be propagated in these cells, and care must be taken to distinguish these viruses from viruses derived from the clinical specimen. Diploid or semicontinuous cells are capable of a limited number of passages before undergoing senescence. The most widely used semicontinuous cells are human fibroblast cell cultures such as MRC-5 and WI-38. Continuous or transformed cell lines are immortalized cells that can be “passaged” without limit. Examples include HEp-2, HeLa, A549, and Madin-Darby canine kidney cells. Hematopoietic cells, such as peripheral blood mononuclear cells or umbilical cord mononuclear cells, are required for the growth of viruses such as Epstein-Barr virus (EBV), human herpesviruses types 6 to 8, and HIV. These cultures are grown in suspension and must be set up from the source material within a few days before inoculation of the specimen. Although necessary for growth of the viruses mentioned, hematopoietic cell culture systems are not used routinely in most diagnostic virology laboratories. Table 2 lists commonly used cell cultures and the medically important viruses that grow in those cultures.

Culture type	Examples	Viruses Supported
Primary	Monkey kidney	Influenza virus, parainfluenza virus, enteroviruses
	Rabbit kidney	Herpes simplex virus
	Human embryonic kidney	Adenovirus, enteroviruses
Diploid	Fibroblast	Cytomegalovirus, varicella zoster virus, herpes simplex virus (HSV), rhinovirus, enteroviruses (some), adenovirus, respiratory syncytial virus (RSV)
Continuous	HEp-2	RSV, adenovirus, HSV, parainfluenza virus (some), enteroviruses (some)
	A549	HSV, adenovirus, enterovirus
	MDCK	Influenza virus
	LLC-MK2	Parainfluenza virus
	Rhabdomyosarcoma (RD)	Echoviruses
	Buffalo green monkey	Coxsackieviruses

TABLE 2. *Cell culture types used for the detection of medically important viruses*

The determination of which cells to use for a given specimen is based on the viruses likely to be present in that specimen. Thus, communication between clinicians and the laboratory is essential to maximize yield. When specific communication does not take place, cell cultures should be selected based on the viruses likely to be present in a given specimen type.

Specimen Processing and Inoculation

Performing a viral culture requires the following steps: processing of the specimen and inoculation onto the cell culture, maintenance of the inoculated cell culture, and detection of viral growth. Normally sterile body fluids, such as CSF, may be inoculated directly onto cell cultures. Urine specimens should have the pH adjusted toward neutrality before inoculation. Specimens from body sites typically contaminated with bacteria, such as respiratory or genital specimens,

are treated with antibiotics before inoculation to prevent bacterial or fungal overgrowth. After inoculation, cultures are incubated at 35° to 37°C and inspected periodically, for example, once daily or every other day. Respiratory cultures directed at detection of influenza or rhinoviruses may be incubated at 33°C.

Detection of Viral Replication

Viral growth in cell cultures is most often detected based on the development of microscopically visible cytopathic effect (CPE). Some viruses, however, can grow to high titer without producing visible CPE and must be detected by other means.

Cytopathic Effect

When viruses grow in cell culture, they often produce changes in the infected cells, or CPE. A number of viruses reveal distinctive morphologic changes resulting from CPE that can be visualized microscopically and that allow their recognition in the culture with considerable confidence. Important characteristics of CPE include which cell culture types are affected, the timing and rate of progression, the distribution (focal, diffuse, limited to the margins of the cell culture), and the nature of the morphologic changes. Several examples of CPE caused by common viruses are shown in Figure 1. The CPE may be sufficiently distinctive to allow unequivocal identification of the virus, but in other cases, techniques such as FA staining performed on cells scraped from the cell culture are used to confirm the presence of the suspected virus.

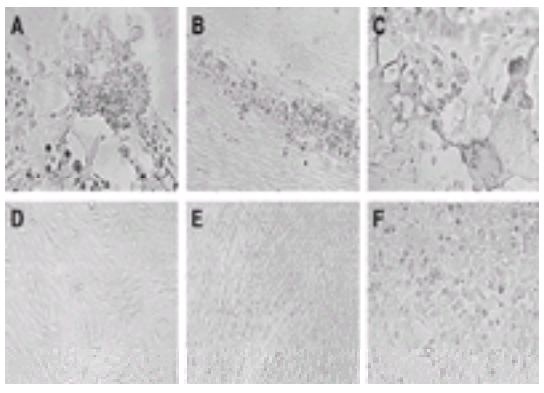

FIG. 1. Cytopathic effect caused by viruses growing in cell culture. Herpes simplex virus growing in primary rabbit kidney cells (**A**); uninfected primary rabbit kidney cells (**D**). Cytomegalovirus growing in human embryonic lung fibroblast cells (**B**); uninfected human embryonic lung fibroblast cells (**E**). Respiratory syncytial virus growing in HEp-2 cells (**C**); uninfected HEp-2 cells (**F**).

Hemadsorption

Paramyxoviruses, including influenza, parainfluenza, and mumps viruses, may produce only minimal visible CPE and instead are routinely detected by testing the inoculated cell culture for hemadsorption. Hemadsorption occurs because infection of cell cultures with certain viruses brings about the expression on the cell surface of viral proteins that bind to erythrocyte membranes. The binding is specific for the erythrocytes of certain species, such as guinea pig, rat, or monkey. To test for hemadsorption, a suspension of guinea pig erythrocytes is introduced into the cell culture previously inoculated with a clinical specimen. After a 30-minute incubation period, the culture is inspected microscopically to detect attachment of the erythrocytes onto the cells in culture. If hemadsorption is detected, the causative virus can be identified by FA staining of cells from the culture.

Interference

The interference assay is based on the phenomenon that a cell culture infected by one virus may be rendered resistant to infection by other viruses to which it was originally sensitive. To carry out this assay, a culture previously inoculated with a specimen is “challenged” with a test virus to which it is ordinarily susceptible. A companion culture of the same type that was not inoculated with a clinical specimen is challenged simultaneously to demonstrate viability of the challenge virus. Interference is present if the challenge virus grows in the companion tube but does not grow in the tube inoculated with the clinical specimen. When interference is demonstrated, the virus responsible can be identified by FA staining of cells from the culture.

Other Detection Methods

A variety of other methods can be used to detect viral growth in cell culture when CPE is not visible. A particularly important one is electron microscopy to detect viral particles. Electron microscopy is especially useful when there is uncertainly regarding the identity of the virus that might be present, precluding the use of immunologic or nucleic acid reagents. When a specific virus is suspected, its presence in the culture can be detected using virus-specific antibodies or nucleic acid assays.

Shell Vial Culture

Shell vial culture refers to a modified cell culture in which the specimen is centrifuged onto the cell monolayer and viral growth is detected by antigen detection procedures, whether or not CPE is visible. The method was developed originally

for CMV (77,79) but has subsequently been applied to many other viruses, including herpes simplex virus (HSV), VZV, respiratory viruses, and enteroviruses. The main advantage of the shell vial culture method is that it can decrease the time required to detect the presence of the virus being sought. For a slow-growing virus such as CMV, this difference can be dramatic. A diagram of the method is shown in Figure 2. For some viruses, either the centrifugation step or the antigen detection component of the full shell vial procedure can be omitted, although for CMV, inclusion of both components is required to maximize the sensitivity of the method.

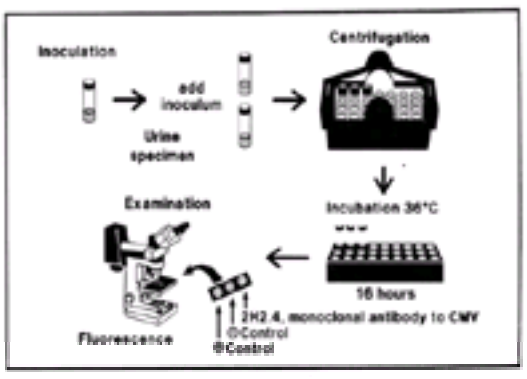

FIG. 2. Shell vial assay for cytomegalovirus. (From Shuster EA, Beneke JS, Tegtmeier GE, et al. Monoclonal antibody for rapid laboratory detection of cytomegalovirus infections: characterization and diagnostic application. *Mayo Clin Proc* 1985;60:577–585, with permission.)

In a common application of the shell vial method, human fibroblast cells are grown on a cover slip that is placed at the base of a 1-dram vial (these thin-walled vials are also known as shell vials). After inoculation of the specimen, the vial is centrifuged at low speed (i.e., 700 × g for 45 minutes) and then incubated for at least 16 hours. After the incubation period, the cover slip is removed and subjected to FA staining using a monoclonal antibody specific for an immediate early antigen of CMV. The presence of one or more fluorescent nuclei is evidence of the presence of CMV in the specimen. The sensitivity of the method varies among laboratories and is affected by a number of technical variables, including the age and condition of the fibroblasts, the timing of the staining, the monoclonal antibody used, and the quality of the fluorescent microscope (7). In some laboratories, the sensitivity of the method exceeds that of conventional viral culture (7,78).

Genetically Engineered Cell Lines

Techniques of genetic engineering have been used to modify cell lines to either make them susceptible to viruses that they are not otherwise susceptible to or create novel means of detecting viral growth. For example, HeLa CD4 cells are HeLa cells that have been transfected with the gene encoding the CD4 molecule. Expression of CD4 on the cell surface renders the cells susceptible to HIV (162). Another example is a cell line developed by Stabell and Olivo for the detection of HSV (181,182). This cell line, shown in Figure 3, consists of baby hamster kidney cells that have been transfected with the b-galactosidase gene from *Escherichia coli* under the control of a promoter from the HSV gene *UL39.* The promoter is activated by exposure to HSV proteins ICP0 and VP16. Exposure of the cells to a specimen containing HSV specifically activates the promoter, causing production of b-galactosidase, which can be detected by a simple histochemical stain of the culture, performed 16 to 24 hours after inoculation. A commercial version of this system called *ELVIS HSV* is marketed by Diagnostic Hybrids (Athens, OH) and has been shown to have sensitivity comparable to conventional viral culture for detection of HSV in clinical specimens (182). The system has also been adapted to the performance of HSV susceptibility assays (188).

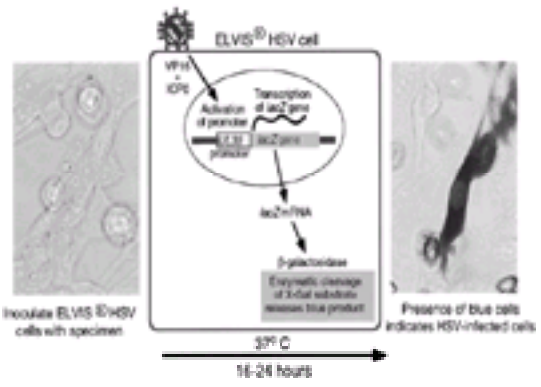

FIG. 3. ELVIS herpes simplex virus (HSV), a genetically engineered cell line for the detection of HSV in clinical samples. ELVIS cells are baby hamster kidney cells that contain a b-galactosidase gene under the control of the HSV promoter UL39. If HSV is present in the specimen, viral particles enter the cells, and the HSV proteins ICP0 and VP16 activate the UL39 promoter, leading to synthesis of b-galactosidase. The presence of b-galactosidase is detected by adding X-gal, a substrate for the enzyme. The activity of b-galactosidase on X-gal results in blue staining of ELVIS cells, visible microscopically.

Electron Microscopy

Viral infections can occasionally be diagnosed using electron microscopy for the direct visualization of viral particles in specimens. Advantages include speed, lack of requirement for viral viability, and the fact that many different kinds of viral particles can potentially be seen. Thus, like viral culture, electron microscopy is an “open” procedure. Disadvantages include the cost and complexity of maintaining an electron microscope, the need for a skilled operator, and relative lack of sensitivity related to the fact that a relatively high concentration of viral particles (10^5 to 10^6 per milliliter) is required for visualization (128).

Direct Specimen Examination

The main area in which electron microscopy has been used in diagnostic virology is the evaluation of stool specimens from patients with suspected viral gastroenteritis (93). None of the major viral causes, including rotaviruses, enteric strains of adenovirus, caliciviruses, and astroviruses, is readily cultivable. However, all can be seen by transmission electron microscopy of material that has been negatively stained using phosphotungstic acid or uranyl acetate. Rotaviruses and adenoviruses are easily seen, but most laboratories rely on immunoassays rather than electron microscopy for diagnosis of gastroenteritis caused by these agents. Detection of caliciviruses such as the Norwalk agent is enhanced by preincubation with immune serum (immune electron microscopy) to create viral aggregates that are more easily visualized than individual viral particles. Other applications of electron microscopy to the direct examination of specimens include examination of blister fluid to discriminate between herpesvirus and poxvirus particles and detection of filoviruses (e.g., Marburg and Ebola viruses) in clinical samples such as serum or urine.

Examination of Fixed Tissue

Electron microscopy can also be used to detect and classify viral particles in fixed tissue obtained by biopsy or at autopsy. Screening of tissue samples by electron microscopy is not useful because of the small area of tissue that can be examined. Thus, electron microscopy is best employed when it is directed by evidence of viral infection detected by routine histology.

Examination of Infected Cell Cultures

Finally, electron microscopy can be used to identify viruses growing in cell culture when they cannot be identified by other methods. Although identification to the species level is not possible, identification of a virus family based on morphology can provide a starting point for more detailed identification.

Light Microscopy

Although viral particles, with the exception of poxviruses, cannot be directly visualized by light microscopy, indirect evidence of viral infection may be detected. The most characteristic signs of viral infection are inclusion bodies (composed of masses of virions), multinucleated cells, and syncytial cells. These findings may be present in cytologic specimens or in tissue examined after histologic staining. Viral inclusions can be found in the nucleus or cytoplasm of infected cells. The location is characteristic of the responsible virus. The location of viral inclusions is summarized in Table 3. Microscopy for detection of the effects of viral infections in clinical specimens is generally carried out in surgical pathology, rather than diagnostic virology, laboratories.

Virus	Nuclear	Cytoplasmic
Herpes simplex virus	X	
Varicella zoster virus	X	
Cytomegalovirus	X	X
Adenovirus	X	
Polyomaviruses (JC and BK)	X	
Parvovirus B19	X	
Poxviruses		X
Measles	X	X
Parainfluenza virus		X
Rabies		X

TABLE 3. *Viral inclusions*

Cytology

Cytologic examination for evidence of viral infection can be performed on smears prepared by applying a specimen directly to a microscope slide, on slides prepared by cytocentrifugation of fluids such as bronchoalveolar lavage fluid, and on touch preps prepared from pieces of unfixed tissue. Viruses that may produce cytologic evidence of infection in respiratory specimens include HSV, CMV, adenovirus, and measles virus (Warthin-Finkeldey cells). Cytologic examination is usually not a sensitive method for detection, but it provides strong evidence for tissue involvement. Examples of cytologic findings suggestive of viral infection are shown in Figure 4.

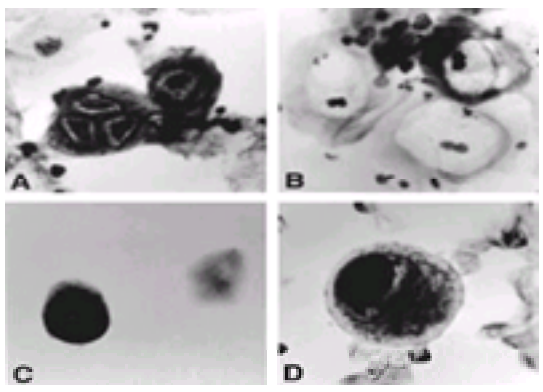

FIG. 4. Cytologic findings suggestive of viral infection. **A:** Cervical smear showing multinucleated cells and the Cowdry type A intranuclear inclusions of herpes simplex infection. **B:** Papanicolaou smear showing binucleate squamous epithelial cells with distinct perinuclear halos. These characteristics are described as *koilocytosis* and are the cellular features associated with human papillomavirus infection. **C:** Urinary epithelial cell containing an enlarged nucleus with smudgy chromatin and a small pale “glassy” intranuclear inclusion indicative of polyomavirus infection. **D:** Cell from bronchoalveolar lavage with a large intranuclear inclusion with a perinuclear clear space (“owl's eye” cell) indicative of cytomegalovirus infection. (Photographs provided by Dr. Leslie Boucher, Department of Pathology, Washington University, St. Louis.)

Tzanck Smear

A specific example of cytologic staining for viral infection is the Tzanck smear, first described in 1949 (193). The Tzanck smear has been used for the rapid detection of HSV or VZV infection in skin lesions. Tzanck smears are prepared by applying material from the base of a lesion on a microscope slide, followed by air drying, fixation in acetone, and staining with Wright stain, Giemsa stain, or a variety of other stains. The presence of multinucleated giant cells or intranuclear inclusions suggests the presence of HSV or VZV virus. The technique does not distinguish between the two viruses. For VZV, the Tzanck smear is more sensitive than culture but less sensitive than FA staining (174). The main advantages of the technique are that it is rapid and does not require sophisticated equipment.

Papanicolaou Staining

Another application of cytology for viral diagnosis is Papanicolaou staining of cells obtained from the uterine cervix. Human papillomavirus (HPV) infection produces characteristic changes in keratinocytes, including a condensed nucleus with a prominent perinuclear clear zone referred to as *koilocytosis.* More specific evidence of the presence of HPV can be provided by histochemical stains or molecular techniques.

Urine Cytology

Cytologic staining of cells present in urinary sediment may reveal intranuclear inclusions indicative of infection with either CMV or the polyoma viruses JC and BK. Culture for CMV and PCR for polyoma viruses are more sensitive techniques. Cytology does not distinguish between JC and BK viruses.

Histology

Histologic examination of stained tissue can provide unique information about the role of viral infection in producing tissue inflammation or injury and can be useful in distinguishing between asymptomatic viral shedding and clinically significant infection. This is particularly useful for CMV infections, in which prolonged viral shedding can occur without producing disease. For example, the presence of cytomegalic inclusion cells in tissue obtained by biopsy or at autopsy is often considered to be the gold standard for diagnosing clinically significant CMV infection. Histopathology can be enhanced by immunohistochemistry (IHC) to detect specific viral antigens and by *in situ* hybridization (ISH) to detect specific viral nucleic acids. These methods can enhance the sensitivity of detection and allow identification of the presence of a specific viral agent. IHC can often be performed on formalin-fixed tissue, but for some antigen–antibody combinations, sensitivity is better on fresh-frozen tissue. ISH can usually be performed on formalin-fixed tissue. The technique of *in situ* PCR has been developed to allow the PCR to be performed directly on tissue. *In situ* PCR can allow exquisitely sensitive detection of viral nucleic acid in tissues but is currently available only in a limited number of laboratories with a special interest in the technique.

Antigen Detection

The detection of viral antigens directly in clinical specimens has become an essential component of the methodologic repertoire of diagnostic virology. Antigen detection methods can provide diagnostic information within a few hours of the receipt of the specimen in the laboratory. The lack of requirement for virus viability is another important advantage over viral culture, especially when specimen transport time is prolonged or otherwise suboptimal.

Antigen detection methods can be applied when the following conditions are met: (a) viral antigen is expressed and is present in an accessible specimen, (b) an appropriate antibody (usually, but not necessarily, monoclonal) is available, (c) antigenic variability does not preclude recognition by immunologic reagents of different strains of the target virus, and (d) the antigen being detected is sufficiently stable that it does not degrade during transport and processing of the specimen. Methods used for viral antigen detection include FA staining, immunoperoxidase staining, and enzyme immunoassay (EIA). The latter can be subdivided into solid-phase and membrane immunoassays. The viruses for which antigen

detection assays are in widespread use are shown in Table 4.

Specimen	Viruses detected
Respiratory (nasopharyngeal swab or aspirate, nasopharyngeal wash, tracheal aspirate, bronchoalveolar lavage)	Respiratory syncytial virus Influenza virus A, B Parainfluenza virus 1-3 Adenovirus Measles
Skin or mucous membrane scraping	Herpes simplex virus Varicella zoster virus
Conjunctival or corneal scrapings	Herpes simplex virus Adenovirus
Stool	Rotavirus Adenovirus (enteric serotypes)
Blood	Cytomegalovirus (pp65) Hepatitis B (surface antigen) Human immunodeficiency virus (p24)

TABLE 4. *Viral antigen detection: specimens used and viruses detected*

Fluorescent Antibody Staining

FA staining is widely employed for detection of cell-associated viral antigens. In the direct format, a fluorescent label, usually fluoroscein isothiocyanate (FITC) is conjugated directly to the antibody that recognizes the viral antigen. In the indirect format, the antiviral antibody is unlabeled and is detected by a second antibody that recognizes immunoglobulins from the animal species of origin of the antiviral antibody. The second antibody carries the fluorescent label. After staining, the specimen is viewed with epiillumination using ultraviolet light of the wavelength needed to excite the fluorescent label. The direct method is simpler to use but requires conjugation of each antiviral antibody with the fluorescent label. The indirect method is slightly more sensitive and more versatile because only the antiimmunoglobulin antibody has to be conjugated with the fluorescent label.

The main applications of FA staining have been for the detection of respiratory, ocular, cutaneous, and bloodstream pathogens. Many different respiratory specimens can be used, including nasopharyngeal swabs or aspirates, nasal washes, tracheal aspirates, and bronchoalveolar lavage fluid. In each case, the specimen is processed in the laboratory to prepare a pellet of cells, which is spotted onto one or more microscope slides. The cells are air dried, fixed in acetone, and stained with monoclonal antibodies to one or more viruses. For ocular infections, material is scraped from the conjunctiva or cornea, placed on microscope slides, and processed as described for respiratory specimens. Staining is usually directed at detection of HSV and adenovirus antigens. For cutaneous infections, the lesion is scraped with a scalpel blade or swab, and cellular material obtained is placed directly on one or more microscope slides. Alternatively, the base of the lesion can be rubbed vigorously with a swab and the swab submitted to the laboratory, where cellular material is washed from the swab and spotted onto a microscope slide. The use of cytocentrifugation to prepare the slides has been shown to improve the results (110). Staining is directed at detection of HSV and VZV. In the CMV pp65 antigenemia assay, peripheral blood leukocytes are separated from anticoagulated blood, spotted onto a microscope slide, usually by cytocentrifugation, and stained using a monoclonal antibody specific for the CMV pp65 antigen.

Immunoperoxidase Staining

Immunoperoxidase (IP) staining is similar in principle to FA staining except that horseradish peroxidase is used in place of a fluorescent label. After the peroxidase-labeled antibody is incubated with the specimen, a substrate for peroxidase is added that changes color when acted on by the peroxidase. The advantage of this method is that the staining can be viewed by light microscopy, thus obviating the need for a fluorescent microscope. IP staining is particularly advantageous when performed on intact tissue because the tissue can also be stained by histochemical stains, allowing examination of the spatial relationship between viral antigens and cellular structures. The disadvantages of the IP method are that it is more cumbersome than FA staining, and endogenous peroxidases in some specimens can produce background staining.

Enzyme Immunoassay

EIA is a versatile and widely used method that can be applied to the detection of antigens regardless of whether they are cell associated or in fluid phase. Because intact cells in the specimen are not required, specimen integrity is less important than for FA or IP staining. This may be advantageous when specimen transport time is prolonged. A common assay format for antigen detection is the double antibody sandwich, shown in Figure 5. In this assay, a “capture” antibody specific for the viral antigen being sought is bound to a reaction surface; for example, the wells of a plastic microtiter tray or the surface of a plastic bead. When the specimen is added, viral antigen present in the specimen binds to the capture antibody. Bound antigen is detected using a second antiviral antibody, the “detector” antibody. The detector antibody can be labeled with an enzyme or can be detected by the addition of an enzyme-labeled third antibody with specificity for immunoglobulin of the species from which the second antibody was derived. The addition of an enzyme substrate produces a color change or light emission if the enzyme is present. Thus, color change or light emission indicates that viral antigen was present in the specimen being tested. Advantages of EIA include applicability to diverse specimens and potential for automation. Laboratory instruments are now available that can perform EIAs for the detection of either antigens or antibodies. Viruses for which antigen EIAs have been widely used are RSV, influenza, rotavirus, enteric adenoviruses, HSV, hepatitis B virus (HBV), and HIV.

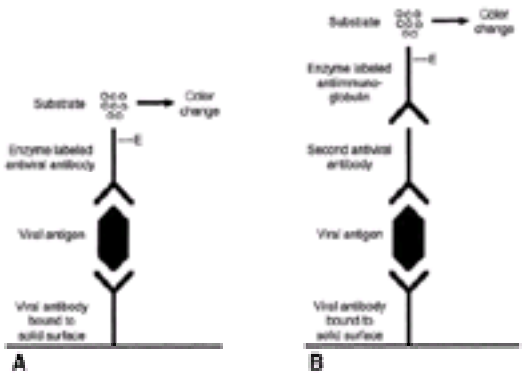

FIG. 5. Enzyme immunoassay (EIA) for antigen detection. **A:** Direct. **B:** Indirect.

Membrane Immunoassays

The membrane immunoassay is a variant of EIA in which antigens or antigen–antibody complexes are captured directly on membranes. An assay called the Directigen test (Becton-Dickinson, Cockeysville, MD) is commercially available, and versions exist for detection of influenza A and B and RSV. Membrane immunoassays are manufactured as self-contained cassettes that are convenient for testing single specimens. They are simple enough to be used in physician's offices or in other outpatient facilities.

Assay Performance

For certain applications, especially RSV and CMV, the sensitivity of antigen detection exceeds that of viral culture (148,190). For the other respiratory viruses and a number of other targets, however, the sensitivity is often less than culture and is certainly less than PCR and other nucleic acid amplification techniques. Overall performance of FA and IP staining techniques is affected by many variables, including the source, timing of acquisition, and transport of the specimen; the quality of the reagents and microscope in use; and the skill of the microscopist. EIA techniques tend to be less affected by the skill of the individual performing the assay and are more suitable for automation.

Nucleic Acid Detection

Diagnostic virology is being revolutionized by the application of nucleic acid detection techniques. These techniques detect specific nucleic acid sequences and can be applied to the detection of virtually any virus. Depending on the target sequence, the assays can be specific for a single virus species or for a group of related viruses. The latter characteristic is particularly advantageous because it allows nucleic acid detection techniques to be applied to groups of viruses, such as the enteroviruses, for which antigenic diversity has precluded successful application of antigen detection techniques. In the case of the enteroviruses, highly conserved sequences in the 5' nontranslated region of the genome have been exploited to develop reverse transcription–polymerase chain reaction (RT-PCR) assays that can simultaneously detect almost all enterovirus serotypes (31,164).

The earliest attempts at nucleic acid–based diagnosis involved direct hybridization of nucleic acid probes to viral nucleic acids present in clinical specimens. Direct hybridization was never widely adopted because it lacked adequate sensitivity, requiring the presence of 10^4 to 10^5 copies of the target nucleic acid. The development of PCR (169) and other nucleic acid amplification techniques overcame that sensitivity barrier and has led to the development of nucleic acid–based diagnostic tests for many viruses. Nucleic acid amplification assays are particularly attractive for viruses that are difficult or impossible to cultivate, viruses that grow slowly in culture, and viruses for which antigen detection cannot be applied because of antigenic diversity or because the level of viral antigen in clinical specimens is too low to permit successful detection. Because nucleic acid amplification techniques can often be performed on a small volume, they are also uniquely useful for specimens such as CSF or ocular fluid that may be available only in small amounts. Still another advantage of nucleic acid detection as a diagnostic method is the stability of DNA, so that detection of viral nucleic acids can be done even when conditions lead to loss of virus viability.

Nucleic Acid Amplification Assays

Nucleic acid amplification assays can be divided into those that amplify target nucleic acid, those that amplify the probe itself, and those that amplify the detection signal.

Target Amplification Assays

PCR is the prototype of target amplification assays. PCR employs short oligonucleotide primers and a thermostable DNA polymerase, such as Taq polymerase, to amplify a segment of target DNA that is typically 100 to 1000 base pairs in length. The PCR itself includes 25 to 40 cycles, each consisting of denaturation, primer annealing, and extension steps that take place at different temperatures. Progression through the steps of the cycle is controlled by an instrument called a *thermal cycler* that controls the temperature of the reaction. After PCR amplification, the PCR product, or *amplicon,* is detected by gel electrophoresis or one of several probe-hybridization techniques, such as Southern blot. The analytic sensitivity of PCR can be as low as 1 to 10 copies of target DNA. Because of its simplicity and broad applicability, PCR has been widely used in viral diagnosis.

Another important target amplification technique is called *transcription-based amplification* (82,105) (Fig. 6). These techniques use three enzymes: reverse transcriptase (RT), RNase H, and T7 RNA polymerase to amplify a target RNA sequence, employing a series of reactions that mimic the retrovirus replication scheme. An advantage of

transcription-based amplification assays is that they are isothermal and do not require complicated instrumentation. The assays begin with synthesis of a DNA strand that is complementary to the RNA target, using a primer that contains a T7 polymerase–binding site. The resulting DNA–RNA hybrid is converted to double-stranded DNA by the action of RNase H and a second primer that also contains a T7 polymerase–binding site. The double-stranded DNA product then serves as a template for transcription brought about by T7 RNA polymerase. The newly synthesized RNA transcripts serve as templates for additional cycles of the reaction. The assay is being developed commercially as the NucliSens assay (Organon-Teknika Corp., Durham, NC) for the detection of HIV and CMV RNA.

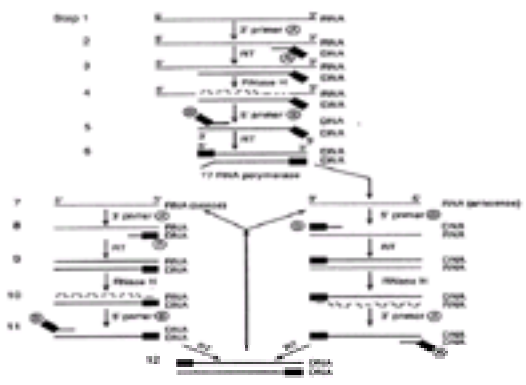

FIG. 6. Transcription-mediated assay, also called 3SR. The 3SR reaction depends on a continuous cycle of reverse transcription and transcription reactions to replicate an RNA target by means of cDNA intermediates. Oligonucleotides A and B prime DNA synthesis and encode the promoter sequence for the T7 RNA polymerase (*black boxes*). Steps 1 to 6 depict the synthesis of a double-stranded cDNA, which is a transcription template for T7 RNA polymerase. Complete cDNA synthesis is dependent on the digestion of the RNA in the intermediate RNA:DNA hybrid (step 4) by RNase H. Transcription-competent cDNAs yield antisense RNA copies of the original target (step 7, *right*). These transcripts are converted to cDNAs containing double-stranded promoters on both ends in an inverted repeat orientation (steps 7 to 12). These cDNAs can yield either sense or antisense RNAs, which can reenter the cycle. Thin lines, RNA; thick lines, DNA; RT, reverse transcription. (From ref. 82, with permission.)

Signal Amplification Assays

Examples of signal amplification assays include the branched-chain DNA (bDNA) assay (54) and the hybrid capture assay (88). The bDNA assay (Fig. 7) was originally developed by Chiron Corporation (Emeryville, CA) and is being applied to the detection of HIV and hepatitis C RNA. The assay uses short branched-chain oligonucleotides to capture the target nucleic acid sequence. Other branched-chain oligonucleotides link multiple molecules of detector enzyme to the captured target. A chemiluminescent substrate allows detection of the target, and measurement of the intensity of emitted light makes it possible to quantitate the input target accurately. Because the target itself is not amplified, this reaction is less susceptible than PCR to carryover contamination (see later).

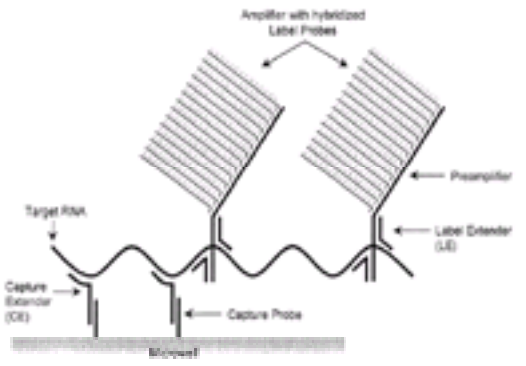

FIG. 7. Branched-chain DNA assay as used for ultrasensitive detection of human immunodeficiency virus (HIV). After liberation of viral nucleic acid from the clinical specimen by a lysis buffer, viral nucleic acid is hybridized with two sets of bifunctional oligonucleotide probes, each of which contains sequences complementary to the target. One set of probes also contains a generic sequence complementary to a capture probe, which is bound to the surface of a microtiter tray, and serves to bind the target to the solid surface. The other set of probes contains a sequence complementary to preamplifier molecules. Additional specificity is achieved because two of the target-specific probes must be juxtaposed in the correct orientation to stabilize the binding of the preamplifier molecule. Each preamplifier molecule binds numerous amplifier molecules, each of which bind many subsequently added alkaline phosphatase-labeled probes. A chemiluminescent substrate is added, and generated light is read by a luminometer. The cascade effect results in amplification of the signal generated from initial binding of probes to the target and allows for detection and quantitation of nucleic acid present in the specimen. (Courtesy of Bayer Diagnostics, Emeryville, CA.)

The hybrid capture assay (Fig. 8) is another signal amplification assay that is being developed commercially for viral diagnosis (Digene Corporation, Beltsville, MD) (116,124). The assay involves a liquid hybridization reaction between the denatured DNA target and RNA probes specific for the viral DNA sequence of interest. If the viral DNA is present, DNA–RNA hybrid molecules are formed and are captured and detected using an antibody specific for DNA–RNA hybrids. This assay can be used as either a qualitative or quantitative assay. The hybrid capture assay is being developed for

detection of the HPV, CMV, and HBV DNA.

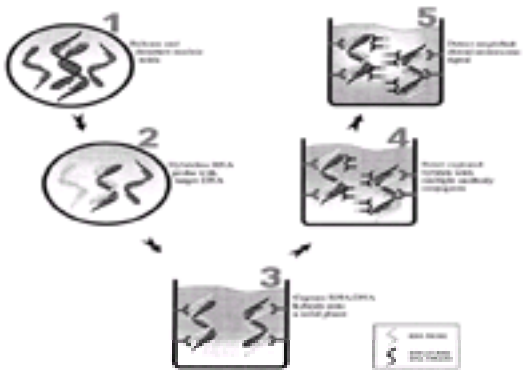

FIG. 8. Hybrid capture assay. In step 1, target DNA is released and denatured. In step 2, RNA probe hybridizes with target DNA. In step 3, RNA:DNA hybrids are captured by an antibody to RNA:DNA hybrids that is bound to the sides of the reaction vessel. In step 4, an enzyme-conjugated antibody that recognizes RNA:DNA hybrids binds to the hybrids. In step 5, a chemiluminescent substrate is added, and light is emitted if hybrids have been formed. (Courtesy of Digene Corporation, Beltsville, MD.)

Contamination

A general concern regarding highly sensitive nucleic acid amplification assays is the occurrence of false-positive results resulting from contamination of the reaction with exogenous nucleic acids. The source of contaminating nucleic acids can be either amplified product from previous reactions or viral nucleic acids present in a different specimen, especially one containing a high level of the intended target. Prevention of contamination requires fastidious attention to procedural detail within the laboratory. A number of specific preventive techniques are widely used to prevent carryover contamination after PCR, including the use of positive displacement pipettes or plugged pipette tips to block aerosol production, ultraviolet irradiation of reaction components (not including Taq polymerase and PCR primers that can be inactivated by ultraviolet irradiation), use of small working aliquots, and frequent cleaning of equipment and laboratory surfaces with 10% bleach. An important precaution is the physical separation of the laboratory into sections where (a) the specimen is prepared, (b) the PCR reaction is set up, (c) the PCR amplification reaction takes place, and (d) the detection of amplification products is carried out. After PCR has taken place, the reaction tube is only opened in the section of the laboratory devoted to product detection.

Two other specific methods have been developed to prevent PCR carryover contamination. The first is the use of uracil-*N*-glycosylase (145). In this method, the original amplification reaction is carried out with deoxyuracil triphosphate substituted for deoxythymidine triphosphate, leading to incorporation of deoxyuracil residues into any amplified nucleic acid. Subsequent PCR reactions are set up including uracil- *N*-glycosylase in the reaction mixture. The action of this enzyme cleaves deoxyuracil residues from DNA. The sites where deoxyuracil residues have been cleaved are susceptible to scission during any subsequent PCR reaction, rendering the DNA unsuitable for amplification and eliminating any risk related to carryover contamination. The second method involves the use of furocoumarin compounds such as psoralens and isopsoralens (145). These agents bind to DNA when exposed to ultraviolet light of the proper wavelength. DNA to which the furocoumarins have bound cannot be amplified by Taq polymerase. In this method, the furocoumarin compound is included in the PCR reaction mixture. After amplification but before the reaction tube is opened, the mixture is subjected to ultraviolet irradiation to allow binding of furocoumarin to DNA. The cross-linked DNA cannot serve as the target for additional PCR amplification, thus eliminating danger of carryover contamination.

RNA Detection

Because Taq polymerase uses only DNA as a template, the use of PCR to detect viral RNA sequences requires the inclusion of an RT step before PCR. This can be done in the same reaction tube in which PCR will take place, and the combined reaction is called RT-PCR. The RT reaction can be performed using a devoted enzyme such as Moloney murine leukemia virus RT or avian myeloblastosis virus RT. Alternatively, heat-stable multifunctional enzymes, such as recombinant *Thermus thermophilus* DNA polymerase, are available that can carry out RT as well as DNA polymerase reactions. Special care must be used in specimen processing because of the susceptibility of RNA to digestion by ribonucleases that may be present in clinical samples. RT-PCR has been applied to the detection of numerous RNA viruses, including HIV, hepatitis C, enteroviruses, RSV, influenza and parainfluenza viruses, rotavirus, and other RNA viruses that cause gastroenteritis. RT-PCR can also be applied to the detection of viral messenger RNA (mRNA). This may be particularly useful in the diagnosis of infection caused by viruses that have a latent phase in their life cycle. For these viruses, detection of viral DNA might not distinguish between latent and productive infection, whereas detection of an mRNA expressed only in productive infection would be evidence of active viral infection. Transcription-based amplification assays (see Fig. 6) are ideally suited for amplification of RNA.

Multiplex Polymerase Chain Reaction

Multiplex PCR refers to PCR reactions in which more than one primer set is included, allowing the detection of multiple targets. This can be an efficient means for detecting within the same reaction multiple agents that can produce the same clinical syndrome. For example, a multiplex assay has been described to allow the simultaneous detection of enteroviruses, HSV-1 and HSV-2, and VZV in specimens of CSF from patients with encephalitis or meningitis (149). The agents detected do not have to be only viruses; for example, a PCR reaction has been described for the simultaneous detection of EBV and *Toxoplasma gondii* in CSF from immunocompromised patients with mass lesions within the brain

(161).

Quantitative Polymerase Chain Reaction

A variety of methods can be used to allow PCR and other amplification methods to quantitate the amount of target nucleic acid present in a specimen. A widely used method is the quantitative-competitive PCR reaction, in which an alternative target (referred to as the *competitor*) which has the same primer binding sites as the legitimate target is included in the reaction mixture. The competitor must be different from the legitimate target in some way, such as size or partial sequence difference, that allows the amplified competitor to be distinguished from the amplified legitimate target. After amplification, the amount of legitimate target in the original specimen is determined from the ratio of amplified target to amplified competitor.

Real-Time Polymerase Chain Reaction

Several new PCR assays have been developed in which the synthesis of the PCR product is detected as it occurs (75,89). These assays have been termed *real-time PCR.* Two specialized instruments are available for carrying out real-time PCR assays, and more are under development. The two instruments currently available are the Prism 7700 manufactured by Perkin-Elmer (Foster City, CA) (also known as the *Taqman* instrument), and the Light Cycler, manufactured by Roche Diagnostics (Mannheim, Germany). The two instruments have important differences and are adapted to run different real-time PCR assays, including the 5'-exonuclease assay, the hybridization probe assay, and the SYBR Green DNA-binding assay. All three formats can be run on the Light Cycler, whereas the Prism 7700 is currently configured to run the 5'-exonuclease and SYBR Green assays.

The unique feature of the Light Cycler is very short PCR cycle times, achieved by using very small-volume capillary tubes whose surface area is large in proportion to the volume. This feature, combined with an air-based thermal system, achieves rapid heating and cooling of the PCR tubes, allowing amplification reactions to be completed in as little as 20 to 30 minutes. Reaction products are detected with a fluorescence detection system consisting of a light-emitting diode that delivers excitation light to each reaction tube and an optical unit with three detection channels to record emitted light of three different wavelengths. The instrument can accommodate 32 capillary tubes, all of which can be analyzed simultaneously.

The Prism 7700 is based on a Perkin-Elmer 9600 thermal cycler with a laser-based optical system that delivers excitation light to each of 96 wells of a microtiter tray and reads emitted fluorescent light from the same wells. The PCR cycle characteristics are similar to those used in conventional PCR. The Prism 7700 is particularly advantageous for applications requiring high throughput.

The simplest system for detection of real-time PCR products uses the DNA-binding dye SYBR Green, which fluoresces when it binds to double-stranded DNA. When SYBR Green is included in a PCR reaction, the intensity of fluorescence is proportional to the amount of PCR product. Because SYBR Green binds nonspecifically to any double-stranded DNA, signal is generated by undesired amplification products, such as primer-dimers, as well as by intended amplicons. Discrimination among different amplification products can be achieved through the use of dissociation curves (also referred to as *melting-point analysis*). Melting-point analysis is performed after completion of the amplification reaction by recording fluorescence as the temperature of the reaction mix is gradually increased. When the dissociation temperature (melting point) of the double-stranded DNA reaction product is reached, SYBR Green is released, and fluorescence decreases. Because the melting point is affected by both length and sequence of the PCR product, intended and unintended amplification products can usually be readily distinguished.

The other two detection systems are based on the use of oligonucleotide probes that are labeled with fluorescent dyes that interact with one another according to principles of fluorescence resonance energy transfer. The hybridization probe assay format requires two oligonucleotide probes that are homologous to adjacent portions of one of the strands of the amplified DNA. The probes are chosen so that the 5' end of one probe is within a few nucleotides of the 3' end of the other probe. These adjacent ends are each labeled with a fluorescent dye. The dye on the 3' end is termed the *donor dye* and the dye on the 5' end is termed the *accepter* dye. The required property of these dyes is that when they are in close proximity to one another (within a distance of several nucleotides), excitation of the donor dye leads to emission of light by the accepter dye. When both probes bind to their target sequences, the two dyes are within the proximity required for excitation of the accepter dye, and fluorescence occurs. The fluorescence intensity is proportional to the amount of PCR product.

The other detection system based on interacting fluorescent dyes is the fluorogenic 5'-exonuclease assay (also referred to as the *Taqman* assay). This assay uses a probe that is complementary to a segment of the intended PCR product located between the PCR primers. This probe is labeled with two fluorescent dyes, one called the *reporter,* which is linked to the 5' end of the probe, and the other called the *quencher,* which is linked to the 3' end. When they are in close proximity (i.e., bound to opposite ends of an oligonucleotide probe), the quencher prevents fluorescence by the reporter. During the annealing step of PCR, the probe labeled with both dyes binds to the PCR product. During extension, the 5'-exonuclease activity of Taq polymerase cleaves nucleotides from the bound probe, releasing the reporter dye, which allows it to emit light upon excitation. As in the hybridization probe assay, the intensity of fluorescence is proportional to the amount of PCR product.

Real-time PCR assays have several important advantages over conventional PCR. Because the accumulation of PCR product is monitored in the reaction tube, no separate detection method, such as gel electrophoresis, is required, thus shortening the effective assay time markedly. In addition, the chance of contamination by amplified PCR product is decreased because the systems are closed, with no handling of the reaction contents after completion of PCR. The use of multiple fluorescent dyes with different emission wavelengths makes it possible to perform multiplex reactions with simultaneous amplification of more than one product. Quantitation of PCR targets is readily achieved because the

generated fluorescence is proportional to the amount of PCR product.

Polymerase Chain Reaction and Nucleotide Sequencing

Sequencing of PCR amplification products can provide valuable information about the identity of a virus whose nucleic acid was amplified or the presence of viral mutants associated with resistance to antiviral drugs. The process of obtaining nucleotide sequence data from PCR reactions is achieved through the use of the cycle sequencing reaction, which uses Taq polymerase and dideoxynucleotides in the PCR reaction mixture (98). The resulting reaction products can be analyzed by polyacrylamide gel electrophoresis followed by automated laser scanning and sequence assembly. The main application in diagnostic virology is for the detection of antiviral resistance in HIV directly from patient plasma specimens (94). Similar assays have also been used to detect antiviral resistance in CMV (37).

Other uses of nucleotide sequencing in diagnostic virology are restricted to highly unusual circumstances. For example, rapid sequencing was used to determine the identity of the hantavirus, sin nombre virus, when it was discovered as the cause of hantavirus pulmonary syndrome (138). In this case, because of clinical and laboratory suspicion that a hantavirus was the cause of the syndrome, PCR reactions were carried out using primers that matched conserved sequences of previously discovered human hantaviruses. Nucleotide sequencing of the amplification product of PCR performed on clinical specimens from patients with hantavirus pulmonary syndrome revealed the presence of a new hantavirus.

Chip Technology

It is now possible to bind precisely hundreds or thousands of oligonucleotide probes to a small silicon chip and to carry out hybridization reactions directly on the chip. Confocal microscopy is used to scan the chip, detecting fluorescent signals that reveal hybridization at precise locations on the chip. Use of probes representing all possible nucleotide sequence variations within a target sequence allow rapid determination of nucleotide sequence (144). Chip technology has the potential to allow simultaneous detection of multiple infectious disease pathogens, viral and nonviral. The first application in diagnostic virology has been for rapid sequencing to detect HIV mutations associated with resistance to antiretroviral drugs (102).

Applications of Nucleic Acid Amplification Techniques

PCR has been used to detect numerous viruses and is increasingly used to assess prognosis, monitor response to treatment, and assess progression of infection. Some of the more widely used diagnostic applications are listed in Table 5. Widespread use of nucleic acid amplification techniques is limited because few assays are available as commercial kits. The only nucleic acid amplification assays currently licensed by the Food and Drug Administration are the Amplicor assay for HIV plasma RNA (Amplicor HIV-1 Monitor Test, Roche Molecular Systems, Mannheim, Germany) and the NucliSens assay for CMV pp65 messenger RNA (Organon-Teknika, Durham, NC). Other assays have been developed by individual laboratories as “home-brew” assays. This situation has resulted in a proliferation of different assays that are not uniformly standardized and thus are not directly comparable. Performance of home-brew assays varies widely among laboratories, and interlaboratory proficiency testing is still in its infancy. In addition to variation in the PCR assays themselves, varying approaches to the choice of specimen, technique used to process the specimen within the laboratory, and technique used to detect the products of the PCR reaction can have important effects on the performance of PCR. Thus, it is important for the user of PCR to be familiar with the laboratory performing the assay. It may be appropriate to request data that the laboratory used to validate a specific PCR reaction.

Target	Specimen(s)	Application(s)
Human immunodeficiency virus (HIV) (DNA)	Leukocytes from infant born to HIV-infected mother	Perinatal infection
HIV (RNA)	Plasma	Viral load (prognosis, response to treatment)
Herpes simplex virus	Cerebrospinal fluid (CSF), ocular fluid	Encephalitis, meningitis, retinitis
Varicella zoster virus	CSF, ocular fluid	Encephalitis, meningitis, retinitis
Cytomegalovirus	Leukocytes, plasma, whole blood, CSF, ocular fluid, amniotic fluid	Diagnosis of systemic infection after organ transplantation, encephalitis, radiculomyelitis, retinitis, congenital infection
Epstein-Barr virus	CSF, leukocytes	Primary central nervous system lymphoma in acquired immunodeficiency syndrome (AIDS), posttransplantation lymphoproliferative disorder
Parvovirus B19	Serum, amniotic fluid	Chronic parvovirus B19 infection, aplastic crisis, congenital infection
JC virus	CSF	Progressive multifocal leukoencephalopathy
BK virus	Urine	Hemorrhagic cystitis
Human papillomavirus	Genital secretions	Detection of viral types associated with cervical carcinoma
Enteroviruses	CSF	Meningitis
Hepatitis C virus (qualitative)	Plasma	Diagnosis of infection
Hepatitis C virus (quantitative)	Plasma	Prognosis, monitoring response to therapy
Hepatitis B virus	Serum	Monitoring response to therapy

TABLE 5. *Nucleic acid detection for viral diagnosis*

Serology

The measurement of antiviral antibodies was one of the first methods used for the specific diagnosis of viral infections and remains an important tool in the diagnostic virology laboratory. The role of serology may be for the diagnosis of acute or current infection or for the determination of immune status to specific viruses. Serologic diagnosis is important for viruses that cannot be readily cultured or for which culture is slow or otherwise impractical. Viruses for which serology is useful in diagnosing acute infection are shown in Table 6. Serology is uniquely useful for defining immunity with respect to specific viruses. It is important to use sensitive assays, such as EIA, for this purpose because antibody levels indicative of past infection may decline to low levels years after the infection. The specific assay used must also be selected with care to ensure that it measures antibodies that correlate with immunity. Viruses for which serologic testing is used to determine immune status are shown in Table 7.

Epstein-Barr virus[a]
Cytomegalovirus (mononucleosis syndrome)[a]
Hepatitis viruses (A–E)[b]
Measles[a]
Rubella[a]
Mumps[a]
Parvovirus B19[a]
Encephalitis viruses[a]
Rabies
Hemorrhagic fever viruses[a]
Dengue[a]
Human immunodeficiency virus
Human T-cell lymphotrophic virus types I and II

[a]Virus-specific immunglobulin M (IgM) assays are used.
[b]Virus-specific IgM assays are used for hepatitis A and B (IgM anti-HBc).

TABLE 6. *Serology for diagnosis of acute infection*

Varicella zoster virus
Herpes simplex virus
Epstein-Barr virus (immunoglobulin G antibodies to the viral capsid antigen)
Rubella
Measles
Parvovirus B19
Hepatitis A virus (total antibodies)
Hepatitis B virus (anti-HBs)

TABLE 7. *Serology to determine immune status*

Kinetics of the Antibody Response

Serologic diagnosis is based on the kinetics of the antibody response to viral infection. Virus-specific antibodies are absent in a susceptible individual but typically become detectable several weeks after the onset of infection. Virus-specific IgM antibodies are often detectable for a short period before virus-specific IgG antibodies. The IgM response tends to decline within about 1 to 2 months, although low levels may persist for 1 year or more in some viral infections. IgG antibodies are much more long-lasting and may persist for the life span of the individual. According to this model, the absence of virus-specific IgG and IgM antibodies signifies susceptibility to infection; the presence of virus-specific IgM antibodies, with or without virus-specific IgG antibodies, signifies current or very recent infection; and the presence of virus-specific IgG but not IgM antibodies signifies past infection. The latter pattern often indicates immunity to subsequent infection.

The relationship between the onset of clinical manifestations of infection and the presence of virus-specific antibodies depends on the incubation period of the infection. In infections with short incubation periods, such as acute respiratory infections, antibodies are absent at the onset of symptoms. In viruses with incubation periods of 1 month or longer, however, virus-specific IgG and IgM antibodies are typically present at the onset of symptoms.

Serology in Reinfection and Reactivation

The serologic diagnoses of reinfection and reactivated infection are more complicated than is the diagnosis of initial infection. In both of these cases, the infection occurs in an individual with preexisting virus-specific IgG antibodies. In reinfection, there may be an anamnestic response that results in an increase in the level of virus-specific IgG antibodies with continued absence of virus-specific IgM antibodies. Reactivation of latent infection, for example, in the case of herpesvirus infection, may result in the appearance of virus-specific IgM as well as IgG antibodies.

Serology in Chronic Infections

The interpretation of serology is unique for viruses that typically cause chronic infection, such as HIV and the related retroviruses human T-cell lymphotrophic virus (HTLV) types I and II. For these agents, the detection of antibodies of any isotype to these viruses (in the absence of artificial immunization) virtually always signifies current infection. In the case of hepatitis C virus, the confirmed presence of antibodies to the virus corresponds to active infection in about 85% of individuals.

Serologic Assays

Binding Assays

The most widely used assays are those that directly measure binding of antibodies to viral antigens. Examples of binding assays include EIA, radioimmunoassay (RIA), and the indirect immunofluorescent antibody assay (IFA). Binding assays can be used in many different formats. In one common format, a viral antigen (which may consist of virally infected cells, a purified viral preparation, or a recombinant viral protein) is attached to a solid surface such as the inner surface of the well of a microtiter tray, a plastic bead, or a microscope slide in the case of IFA. Serum is added, allowing the binding of

antiviral antibodies that might be present in the serum. After an appropriate incubation period, the serum is removed, and the well is extensively washed. The next step is the addition of a second antibody (also referred to as a *detector* antibody) with specificity for human immunoglobulins. The second antibody is typically a mouse monoclonal antibody or a polyclonal antibody from a nonhuman species. It is linked to an enzyme such as horseradish peroxidase. The second antibody binds to any human antibody present (ideally because of specific binding to the viral antigen). The well is again extensively washed, and the presence of the second antibody is detected based on a colorimetric, radiometric, or fluorescent signal. An important advantage of binding assays is that they can be modified to detect IgM- or IgA-specific antiviral antibodies through the use of isotype-specific detector antibodies.

Separated Antigen Assays

The Western blot and related recombinant immunobinding assays are binding assays that allow the identification of antibodies to specific viral proteins. In the Western blot assay, viral antigens present in infected cells are denatured, separated by gel electrophoresis, transferred to a membrane, and allowed to react with the serum specimen. Binding of antiviral antibodies is detected through the use of labeled detector antibodies. The Western blot is cumbersome but provides a high level of specificity because of the ability to identify binding to individual viral proteins and the production of a characteristic blot fingerprint. Thus, its main application in diagnostic laboratories is the confirmation of screening assays, such as EIA, that are less specific. For example, the Western blot is widely used to confirm a positive HIV EIA. Western blot is also considered the gold standard for determining the type specificity of HSV antibodies. The recombinant immunobinding assay differs from the Western blot in that specific viral antigens are produced in *in vitro* expression systems and are artificially attached to membranes in a predetermined configuration, often in dots or in bands. The membranes are then incubated with serum, and bound antibodies are detected as in the Western blot. Because the antigens used in the immunobinding assay are not denatured as they are in routine Western blots, these assays may have advantages in detection of antibodies to conformational epitopes. A recombinant immunobinding assay is widely used to confirm positive hepatitis C EIA results.

Functional Assays

Functional assays are based on detection of specific activities resulting from the binding of specific antibodies to viral antigens. An important example is the neutralization assay, which measures the ability of antibodies to block viral infectivity. In neutralization assays, serial dilutions of the serum to be tested are incubated with a standardized quantity of infectious virus. After a short incubation period, the serum–virus mixtures are added to cells that support the growth of the virus, in parallel with a similar inoculum of virus that has not been incubated with serum. The neutralizing antibody titer is the highest serum dilution that prevents infection of the cells. The neutralization assay correlates well with protection from infection and is sometimes considered the standard against which other serologic assays should be measured. Because it is cumbersome and expensive, the neutralization assay is now rarely used in routine diagnostic laboratories. Other examples of functional assays are the hemagglutination-inhibition assay, in which the presence of antiviral antibodies is detected by their ability to block virus-induced hemagglutination, and the complement fixation assay, which measures the ability of antiviral antibodies to fix complement, preventing the complement from lysing indicator erythrocytes. Although the hemagglutination-inhibition and complement fixation assays have been widely used in the past, they are increasingly being replaced by binding assays.

Agglutination Assays

Viral antigens can be bound to a variety of particles, including fixed erythrocytes and latex particles. Agglutination assays are performed by mixing dilutions of serum with a suspension of antigen-coated particles. The agglutination titer is the highest serum dilution that results in visible agglutination of particles. The advantage of agglutination assays is simplicity; they are rapid and do not require sophisticated equipment. Therefore, they are well suited to “stat” testing and for use under field conditions. For example, a rapid latex agglutination assay for varicella antibodies may be employed for rapid determination of the varicella immune status of health care personnel who are exposed to a patient with VZV.

Virus-Specific Immunoglobulin M Assays

Assays for virus-specific IgM antibodies are important because they often allow a specific diagnosis to be made based on analysis of a single serum specimen obtained during the acute phase of illness. These assays are most likely to be useful in diseases with incubation periods that are sufficiently long that virus-specific IgM levels are present at detectable levels at the onset of illness. Hepatitis A provides an excellent example; because of its incubation period of 1 to 6 months, hepatitis A IgM antibodies are virtually always detectable at the time patients seek medical care.

A number of methods are used to measure virus-specific IgM antibodies. Two older methods include (a) separating IgG and IgM fractions before performing the virus-specific antibody assay and (b) performing the virus-specific assay before and after procedures, such as treatment with b-mercaptoethanol, that selectively destroy IgM antibodies. Many modern virus-specific IgM antibody assays use IFA or EIA with a detector antibody that binds only to human IgM. The IgM capture assay is an alternative approach in which IgM antibodies present in the serum sample being tested are first “captured” using an antibody specific for human IgM. A preparation of viral antigen is added, followed by an enzyme-labeled antibody specific for the viral antigen. Binding is detected by the addition of the appropriate enzyme substrate.

Virus-specific IgM assays have several potential pitfalls and must be interpreted in conjunction with clinical findings. False-positive results can occur if the serum being tested contains rheumatoid factor (IgM antibodies to human IgG) and virus-specific IgG antibodies that form complexes that may be falsely detected by the assay as virus-specific IgM antibodies. This problem can be avoided by pretreatment of the serum to remove either rheumatoid factor or IgG antibodies. One or the other of these pretreatment methods is included routinely in many commercial virus-specific IgM antibody assays. False-negative results can occur if the serum being tested contains high levels of virus-specific IgG antibodies that may compete with the virus-specific IgM antibodies. The IgM-capture assay configuration avoids this

problem, as does serum pretreatment to remove IgG.

Complexities in the nature of the IgM antibody response must also be considered when interpreting virus-specific IgM antibody tests. In some patients, the IgM antibody response is transient or low level, leading to failure to detect virus-specific IgM antibodies, whereas in others, low levels of IgM antibodies may persist for 1 year or longer, decreasing the specificity of the assay as an indicator of recent infection. In herpesvirus infections, IgM antibodies can sometimes be detected in reactivations as well as in primary infection.

Saliva and Urine Assays

Virus-specific antibodies can often be detected in saliva and urine. These body substances are attractive for use in serologic assays because they avoid the need for phlebotomy. Assays for HIV antibodies in these fluids are commercially available and have performance characteristics similar to assays carried out on serum (71,194).

Cerebrospinal Fluid Serology

Serologic testing can be applied to CSF for the diagnosis of central nervous system (CNS) infection. For unusual viruses, such as rabies, the presence of any virus-specific antibodies within the CSF is diagnostic of active infection. For the diagnosis of encephalitis caused by the alphaviruses, bunyaviruses, or flaviviruses, the presence of virus-specific antibodies in CSF is highly suspicious, and the presence of virus-specific IgM antibodies in CSF is diagnostic. For more common viruses, such as the herpesviruses or the common respiratory viruses, the mere presence of virus-specific antibodies in CSF is not diagnostic of CNS infection because antibodies produced in the blood are present in the CSF even in the absence of CNS infection. The problem is further complicated by the fact that defects in the blood–brain barrier that may occur during many neurologic diseases can increase the passage of antibodies from blood to CSF. Therefore, for the common viruses, intrathecal synthesis of specific antiviral antibodies must be demonstrated to provide evidence of CNS infection.

Intrathecal synthesis of a specific antiviral antibody is evaluated by determining the quotient of two ratios: (a) the ratio of specific antiviral antibody level in CSF to the level in serum ($CSF_{\text{specific antibody}}/serum_{\text{specific antibody}}$ ratio), and (b) the ratio of total IgG in CSF to total IgG in serum ($CSF_{IgG}/serum_{IgG}$). A quotient greater than 1.5 is evidence of intrathecal antibody synthesis of the specific antibody. The antibody assay used to measure the specific antiviral antibody must have certain special characteristics. First, it must be very sensitive because CSF antibody levels are usually low (about 1000-fold less than serum levels). Second, it must be capable of providing a quantitative result that allows calculation of the CSF/serum ratio of specific antibody level. EIAs are usually used, but testing of a series of dilutions of CSF and serum is required to obtain linear quantitative estimates of antibody level suitable for calculating the necessary ratio. Because of the complexities involved, accurate determination of intrathecal antibody synthesis of specific antiviral antibodies is performed in only a limited number of reference laboratories.

Measurement of intrathecal antibody synthesis has been used in a variety of CNS infections. The application for which experience is most extensive is HSV encephalitis. In HSV encephalitis, the utility of determinations of intrathecal antibody synthesis is limited because intrathecal antibody synthesis may not be detectable until 7 to 10 days after the onset of illness. Interestingly, intrathecal antibody synthesis may persist for years after the acute infection (150). Therefore, the main utility is in cases in which specimens were not available early in the illness to allow a definitive diagnosis based on PCR. A caveat concerning diagnosis by demonstration of intrathecal antibody synthesis is that nonspecific polyclonal intrathecal antibody synthesis may occur in some diseases, such as multiple sclerosis (150). This pitfall can be avoided by evaluating intrathecal synthesis of antibodies to at least one other virus other than the one of primary interest.

Antiviral Susceptibility Testing

In recent years, antiviral drugs have been licensed for the treatment of infections caused by HSV, VZV, CMV, influenza A and B, RSV, and HIV. Not surprisingly, increasing use of these drugs has been accompanied by the appearance of antiviral drug resistance. Testing for resistance may be required for optimal patient management, particularly in infections caused by HSV, CMV, VZV, and HIV, for which resistance testing can guide the choice among several potentially effective drugs. Antiviral drug susceptibility testing is performed in some diagnostic virology laboratories.

Phenotypic and Genotypic Assays

Antiviral susceptibility testing is divided into phenotypic and genotypic methods. Phenotypic methods analyze the effect of antiviral drugs on the growth of virus, which can be measured by infectivity, viral antigen production, or viral nucleic acid production. Phenotypic assays are attractive because they directly measure the effects of antiviral drugs on viral growth. However, they tend to be labor intensive and slow. They have also been difficult to standardize, and results can vary based on the test system used. Table 8 shows recommended cutoffs that define resistance to several drugs used for HSV and CMV infections.

Virus	Drug	Susceptible	Intermediate	Resistant
Herpes simplex virus[195]				
	Acyclovir	<6 μM		≥6 μM
	Foscarnet	<200 μM		≥200 μM
Cytomegalovirus[9]				
	Acyclovir	≤6 μM	6–12 μM	>12 μM
	Foscarnet	≤400 μM		>400 mM
	Cidofovir	≤6 μM		>6 μM

Source: Data on herpes simplex virus from ref. 167, with permission; data on cytomegalovirus from ref. 58, with permission.

TABLE 8. *Cutoffs for antiviral susceptibility testing for herpes simplex virus and cytomegalovirus using the plaque reduction assay*

Genotypic assays test for the genetic basis for resistant phenotypes and can involve detection of restriction fragment length polymorphisms, differential hybridization assays, or nucleotide sequencing. Genotypic assays are informative only when the genetic basis for resistance is known, and they are most attractive when the resistance being evaluated is accounted for by a limited number of genetic changes.

Plaque Assay

The plaque assay is a traditional infectivity-based method that measures the inhibitory effect of an antiviral drug on the production of viral plaques in cell culture. In this assay, a standardized viral stock is inoculated into multiple identical cell cultures, usually grown in wells of a cluster plate. A solidifying agent such as agarose is added to the cultures to minimize spread of virus through the media. The viral titer of the stock is previously determined and is adjusted to yield a countable number of plaques in each well. Increasing concentrations of the antiviral drug being evaluated are incorporated into a series of wells. The effect of the drug is often expressed as the 50% inhibitory concentration (IC_{50}), defined as the lowest concentration of drug that results in a 50% decrease in the number of viral plaques compared with control wells with no antiviral drug. The IC_{90} can be similarly determined and is of additional interest because it better reflects the presence of minority resistant subpopulations. The plaque assay is considered the gold standard for antiviral susceptibility testing but is labor intensive and time-consuming. Completing the assay requires initial growth of the virus, titration of the viral stock, and the assay itself. For a slow-growing virus such as CMV, the time involved is often more than 6 weeks. For HSV, the assay can be completed in less than 2 weeks.

Other Phenotypic Assays

Assays measuring viral antigen or nucleic acid production are alternatives to infectivity-based assays. A variety of assays have been used in individual laboratories, but only two assays are commercially available. The Hybriwix assay (Diagnostic Hybrids, Athens, OH) uses ^{125}I-labeled probes to measure viral DNA that has been bound to nylon wicks placed in the culture wells. The IC_{50} is the minimal concentration of drug that results in a 50% decrease in viral DNA. The results of the Hybriwix assay for HSV and CMV correlate well with those measured by the plaque reduction assay (50,186). The ELVIS assay illustrated in Figure 3 has also been adapted for the performance of antiviral susceptibility testing (188).

Recombinant phenotypic assays have recently been developed for HIV. These assays involve insertion of the polymerase and protease genes of HIV from a patient being evaluated into a vector consisting of a rapidly replicating laboratory strain of HIV that also contains a reporter gene, such as luciferase, that is used to measure viral growth (142). These assays allow relatively rapid testing of the susceptibility of HIV to multiple drugs as well as to drug combinations.

Genotypic Assays

Genotypic assays have been most widely used for CMV and HIV, since phenotypic assays for involving these viruses are slow or expensive. A relatively limited number of mutations in the CMV *UL97* gene account for resistance to the antiviral drug ganciclovir in about 80% of resistant CMV isolates (35,36). The most important of these are mutations at *UL97* codons 460, 594, and 595, which can be detected using an assay based on PCR amplification followed by digestion of the PCR product with restriction endonucleases that differentiate between wild-type and mutant sequences. In many cases, this assay can be carried out directly on patient specimens, providing a result within 2 days. If a resistant pattern is not detected, nucleotide sequencing of the regions of the *UL97* and polymerase genes that have been implicated in resistance is required for complete detection of resistance (59). Resistance to the drugs foscarnet and cidofovir has been localized to mutations in the polymerase gene, and nucleotide sequencing is the only method available for detecting these mutations (59).

Genotyping testing for resistance is increasingly used for antiretroviral drug management of patients with HIV. This testing involves RT-PCR amplification of regions of the polymerase and protease genes, followed by nucleotide sequencing. Certain mutations are highly associated with phenotypic resistance and drug failure, whereas the significance of other mutations found in drug-resistant strains is less clear. Interpretation of HIV genotypic testing is complicated because of the diversity of mutations and the evolving knowledge base regarding the interaction of multiple mutations in determining the viral phenotype and response to therapy (94).

Genotypic testing for HSV is less useful than phenotypic testing because of the diverse genetic loci within the HSV thymidine kinase and polymerase genes that can account for resistance (73,168).

DIAGNOSTIC APPROACHES TO SELECTED CLINICAL PROBLEMS

Herpesviridae Infections

The diagnosis of infections caused by members of the family Herpesviridae is complex because of the diversity of clinical syndromes, frequently involving immunocompromised individuals. Additional complexities are related to the life cycle of the members of the family. After primary infection, herpesviruses become latent, with periodic reactivations. Reactivations are typically associated with the presence of infectious virus but may or may not be clinically significant. The following

discussion is subdivided according to the three Herpesviridae subfamilies.

Alphaherpesvirinae

The members of the alphaherpesvirinae subfamily that are human pathogens are HSV and VZV. B virus (also known as *Cercopithecine herpesvirus 1* or *Herpesvirus simiae*) is a virus that infects Old World macaques and can cause severe human disease when it is introduced by the bite of an infected monkey or through other exposures to infected secretions or animal products, including cell cultures. These viruses are characterized by rapid replication, efficient destruction of infected cells, and tendency to establish latency in sensory ganglia. Most clinical infections with HSV and VZV involve vesicular lesions of the skin and mucous membranes. Both viruses can also infect the CNS and produce encephalitis, meningitis, or myelitis. Likewise, B virus produces severe infection of the CNS. Visceral infection with HSV and VZV can also occur but is unusual.

Herpes Simplex Virus and Varicella Zoster Virus

Mucocutaneous Infections

The main diagnostic approach to mucocutaneous HSV and VZV infections involves direct detection of the virus, either by culture or antigen detection. Virus can be cultured during either primary or reactivated infection with either virus, although titers are higher with primary infection. Specimens are obtained by unroofing a vesicle and using a swab to soak up vesicular fluid as well as to scrape the base of the lesion. Calcium alginate swabs should not be used because this material inhibits the growth of HSV (48). HSV grows well in cell culture, usually producing CPE in 1 to 3 days (25). A variety of cell culture types can be used, but rabbit kidney, mink lung, and guinea pig embryo cells are particularly susceptible (111). Some laboratories use shell vial culture techniques or the ELVIS genetically engineered cell line (Diagnostic Hybrids, Athens, OH) (181,182). The sensitivity of cell culture techniques for HSV is strongly influenced by the stage of the lesion, with higher sensitivity for vesicular and pustular lesions than for lesions that are crusted (106). The overall sensitivity of culture for the diagnosis of genital HSV is about 80% (133). HSV-1 and HSV-2 grow equally well in culture. They can be differentiated by FA staining of the infected cell culture.

VZV is more difficult than HSV to grow in culture. This virus grows only in human fibroblast cells and monkey kidney cells and typically requires 4 to 8 days to become detectable. The sensitivity of culture detection is lower than for HSV but can be enhanced through the use of the shell vial technique (174).

Rapid Diagnosis

Rapid diagnosis of both HSV and VZV infections can be accomplished by FA staining. For this technique, cells from the base of a lesion are obtained by scraping the base with a scalpel blade or rubbing vigorously with a Dacron or rayon swab, and placing the material obtained on one or more microscope slides. This material is air dried, fixed with acetone, and then stained with fluorescent-labeled monoclonal antibodies to HSV and VZV. This technique is particularly advantageous for VZV because of the difficulty of culturing that virus (174). FA staining is preferable to a Tzanck smear because it allows specific viral identification and is more sensitive. PCR can be used to detect HSV or VZV DNA in cutaneous lesions with greater sensitivity than cell culture (87,165).

Serology

Serology has little role in the diagnosis of acute HSV infection because viral culture or antigen detection is usually successful, because serology requires acute and convalescent phase specimens, and because of difficulties in interpretation related to cross-reactions as well as asymptomatic reactivations. VZV serology is occasionally useful for diagnosis of acute VZV if appropriate specimens were not obtained for culture and antigen detection or if those tests were negative despite a clinical impression that VZV was the true diagnosis. Detection of seroconversion is the strongest serologic evidence of infection, although detection of VZV-specific IgM antibodies also supports the diagnosis of recent infection. Virus-specific IgM antibodies can be present with reactivation (zoster) as well as with primary infection (12,47).

Serology is widely used for determination of immune status, especially to VZV. The reference test for this purpose is the fluorescent antibody to membrane antigen test (199), but EIA, IFA, and latex agglutination assays are more widely used. None of these assays has optimal sensitivity for detection of VZV antibodies produced in response to varicella immunization. Latex agglutination is more sensitive than EIA or IFA for this purpose (183), although it is less sensitive than an EIA that uses purified glycoproteins as antigen (146). The latter assay is not commercially available at the current time.

Assays have become available that accurately distinguish antibodies made in response to HSV-1 or HSV-2 infection (13). These assays detect antibodies to type-specific glycoproteins, such as glycoprotein G. These assays may be useful in several settings. For example, in individuals with recurrent genital lesions who have never had a positive culture, detection of HSV-2 antibodies suggests that the genital lesions are probably herpetic. Type-specific antibody testing may also be useful for counseling couples. For example, if one partner is known to have HSV-2 infection, testing the other partner may reveal prior infection, suggesting no risk of transmission, or alternatively, absence of infection, indicating susceptibility and highlighting the need for precautions to prevent transmission. Testing of pregnant women may be helpful in identifying those who are seronegative but have a seropositive partner. These couples should take precautions to avoid transmission during the pregnancy. Pregnant women who are seropositive should be monitored with particular care taken to identify the presence of active lesions at the time of delivery. The issues involved in the use of type-specific HSV serology are complex and require much further evaluation.

B Virus

Most cases of human B virus infection have occurred after a bite or other exposure to infectious secretions or excretions

from a monkey infected with B virus. Symptomatic disease may not be apparent in the monkey. Typically, a vesicular lesion develops at the site of the bite, leading to lymphangitis and regional lymphadenopathy, followed by ascending encephalomyelitis. Recommendations for evaluation and treatment of suspected cases have been published by an expert working group (95). When B virus infection is suspected, specimens should be obtained from mucocutaneous lesions using a Dacron or cotton swab. CSF and conjunctival and pharyngeal swabs can also sometimes yield the virus, which grows in several types of primary and continuous monkey kidney cell cultures. Attempts to isolate the virus should be carried out only by appropriate reference laboratories with the necessary biologic safety capabilities (see later for listing of qualified laboratories). PCR may also be used to detect B virus. Acute and convalescent serum specimens should also be obtained, although cross-reactions with HSV can make interpretation difficult.

The following laboratories provide consultation as well as testing for B virus:

•Julia K. Hilliard, Ph.D
Southwest Foundation for Biomedical Research
Department of Virology and Immunology
7620 Northwest Loop 410
San Antonio, Texas 78228
Telephone (210) 674-1410 extension 280; or (210) 673-3269
Pager 1-800-443-7243; ID 011422
•Seymour S. Kalter, Ph.D.
Virus Reference Laboratory
7540 Louis Pasteur Drive
San Antonio, Texas 782229
•David Brown, M.R.C. Path.
Virus Reference Division
Central Public Health Laboratory
61 Colindale Avenue
London NW9 5HT, England
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Betaherpesvirinae

The betaherpesvirinae include CMV, HHV-6, and HHV-7. These viruses are characterized by restricted host range, slow growth in culture, enlargement of infected cells, and latency in secretory glands and lymphoreticular cells. These viruses are widespread in the population, usually causing asymptomatic infection, but sometimes causing serious disease, especially in immunocompromised patients. The diagnosis of infection with these viruses is complicated by the frequent presence of these viruses in the absence of clinically significant manifestations. Thus, the presence of the virus, particularly in saliva or urine, is not diagnostic of disease. General approaches to this problem involve detection of the virus in less usual sites, such as blood; detection of the viruses in tissue, along with histologic examination to provide evidence that the infection is associated with pathologic findings; and quantitation of the level of virus present. For CMV, a number of laboratory methods are well established, including culture, antigen detection, nucleic acid detection, and serology.

Cytomegalovirus

Culture

CMV can be readily cultured in the diagnostic virology laboratory, although the virus is labile, and rapid transport to the laboratory is important (160). CMV grows only in human fibroblast cells and typically requires 7 to 21 days to exhibit CPE. For this reason, the shell vial technique is important because it provides results within 24 to 48 hours. Its sensitivity compared with that of conventional culture varies widely among individual laboratories but can be comparable or greater (7,78). The interpretation of CMV cultures can be difficult because of frequent shedding of the virus in saliva and urine in symptom-free individuals. Cultures of blood are more helpful diagnostically because asymptomatic CMV viremia does not occur in nonimmunocompromised hosts. In the immunocompromised patient, positive blood cultures indicate active infection but may occur in the absence of symptoms. Culture of CMV from urine or saliva usually has little diagnostic significance.

pp65 Antigenemia Assay

The pp65 antigenemia assay (154,195) is commercially available in both FA and IP formats and is widely used for the rapid diagnosis of CMV infection. In this assay, leukocytes are separated from other blood elements, spotted onto a microscope slide, and stained using a monoclonal antibody to pp65, the CMV lower matrix phosphoprotein. The assay has a number of desirable attributes, including potential availability of results within a few hours of receipt of the blood sample in the laboratory, sensitivity exceeding that of culture, capability of providing quantitative data, and a strong although not perfect correlation with the presence of clinically significant infection (190). A concern in the use of the assay, particularly for quantitative purposes, is that the antigen is labile, and results may be inaccurate if transport to the laboratory takes more than 24 hours (109,139).

Nucleic Acid Detection Assays

Detection of CMV DNA can be accomplished using PCR or the Digene hybrid capture system (Digene Corporation, Beltsville, MD). An assay for CMV pp65 RNA is also commercially available (NucliSens, Organon-Teknika, Durham, NC). PCR assays can be performed on any body substance or tissue but are usually performed on blood for the diagnosis of active CMV infection. Detection of CMV DNA in blood of normal, CMV-seropositive individuals is unusual and strongly suggests clinically significant infection either currently or in the recent past (155). In immunocompromised patients,

however, detection of CMV in blood is much more common and is poorly correlated with the presence of clinically significant CMV disease. Increasing evidence suggests that quantitative tests that measure the amount of CMV DNA in peripheral blood will be more useful in clinical decision making about the need for antiviral therapy (21,43,159,179). Further studies are required to define whether the optimal specimen is plasma, leukocytes, or whole blood.

Serology

CMV serology is of less value for the diagnosis of infections in immunocompromised patients than are direct tests for the virus (121). It is useful, however, for the diagnosis of CMV infection in the normal host (see later) and for defining CMV immune status, for example, in blood donors or in transplant donors and recipients. EIA is the assay format most widely used for detection of CMV antibodies and is more sensitive than the complement fixation test, which was widely used in the past.

Cytomegalovirus in the Normal Host

The most common disease manifestation of CMV in the normal host is a mononucleosis-like illness. In these cases, the heterophile antibody test is negative, as are tests for EBV IgM antibodies. Because these are primary infections, detection of CMV IgM antibodies is a convenient approach that usually provides a diagnosis (96). Acute EBV infection can also produce a false-positive CMV IgM test; therefore, acute EBV infection should be excluded before a positive CMV IgM test is considered diagnostic (86,96). In patients with CMV mononucleosis, CMV is regularly detected in the urine by culture and often in the blood by culture, the pp65 antigenemia, or PCR (155). Of these, PCR is the most sensitive, reaching 100% sensitivity in one study (155).

Cytomegalovirus in the Immunocompromised Host

CMV is the most common cause of opportunistic infection in patients who have received either solid organ or bone marrow transplants. It is also the most common virus that causes opportunistic infection in patients with AIDS. A common manifestation of CMV infection, especially after transplantation, is a systemic syndrome without specific organ localization. Involvement of specific organs can also occur, either along with a systemic syndrome or without systemic manifestations. The availability of antiviral drugs, such as ganciclovir and foscarnet, that are effective both for prevention and treatment of CMV infections has created a variety of viral diagnostic needs that affect the choice of test used.

Diagnosis of Systemic Cytomegalovirus Infection

No available diagnostic test is perfect for the diagnosis of current systemic CMV syndrome after transplantation. Detection of CMV in the blood correlates better with the presence of systemic disease than does detection in urine or saliva. Detection by culture of peripheral blood leukocytes suggests that a systemic syndrome can be attributed to CMV but is not absolutely diagnostic because CMV viremia also occurs in asymptomatic transplant recipients (121,127). The sensitivity of blood cultures for the diagnosis of systemic CMV infection is also imperfect because not all patients with a systemic CMV syndrome after transplantation have positive blood cultures. The pp65 antigenemia assay is both more sensitive and more specific than blood culture for identification of current disease. High levels of antigenemia or increasing levels in sequential specimens increase the positive predictive value (190). PCR is even more sensitive, but less specific. Failure to detect CMV DNA in blood by PCR virtually excludes CMV as a cause of a systemic syndrome, but a positive test has a low predictive value. High levels of CMV DNA detected by quantitative PCR may have a higher predictive value.

Diagnosis of Localized Disease

Patients with localized CMV infection are not always viremic. The diagnosis of CMV pneumonitis or gastrointestinal tract disease is best made by histologic examination of tissue from the site of disease. The diagnosis of CMV CNS infection is best made through the use of PCR to detect CMV DNA in CSF (10). The diagnosis of CMV retinitis is usually a clinical diagnosis made by ophthalmologists, but in cases with atypical features, CMV PCR can be performed on vitreous or aqueous humors (125).

Diagnosis of Impending Disease

Detection of CMV in blood by culture, pp65 antigenemia assay, hybrid capture assay, or PCR can be used as a trigger to initiate antiviral therapy before symptoms have occurred. Transplant programs that use preemptive therapy regimens typically evaluate patients for evidence of CMV viremia every 1 to 2 weeks during the first 4 months after transplantation, when the risk for disease is highest. In general, PCR is the most sensitive test, detecting CMV DNA in the blood of virtually all transplant recipients before the onset of symptoms, but also detecting CMV DNA in recipients who never develop symptoms, thus having a low positive predictive value. The hybrid capture assay and the pp65 antigenemia assay become positive several days to 1 week after PCR and occasionally fail to detect CMV antigenemia before the onset of symptoms, but they have higher positive predictive values (20,175). Culture is the least sensitive and often the last test to become positive. The proper choice of test depends on the vulnerability of the patient population being monitored, with more sensitive tests generally recommended for more vulnerable patients. It is likely that quantitative testing of blood will improve the predictive value of CMV detection assays (43,159). The level of CMV DNA in plasma as measured by quantitative CMV PCR correlates with the likelihood of subsequent CMV infection in patients with AIDS (179).

Monitoring of Patients During Antiviral Therapy and Prediction of Relapse

After the initiation of antiviral therapy, cultures become negative within a few days, followed by the pp65 antigenemia assay, followed by PCR (74). Further study is required to define whether persistence of antigenemia or CMV DNA during therapy is associated with relapse. Quantitative assays may be useful in providing a measure of response to therapy,

with rising levels potentially providing an early warning of antiviral drug resistance (159).

Congenital Cytomegalovirus Infection

Congenital CMV infection can be readily diagnosed by culture or PCR performed on urine within the first week of life. It is important to perform testing during this period because later cultures may not distinguish congenital infection from infection acquired in the peripartum period (156). *In utero* CMV infection can be diagnosed by culture or PCR performed on amniotic fluid. False-negative results have occurred when amniotic fluid was obtained too early in gestation or too early after maternal infection (27,55,56). Repeated sampling is suggested for maximal sensitivity when the level of clinical suspicion is high.

Human Herpesvirus Types 6 and 7

HHV-6 is the cause of roseola in infants. It has also been suggested as the cause of cases of encephalitis, febrile seizures, hepatitis, and secondary bone marrow failure after bone marrow transplantation. One study has suggested a role for HHV-6 as a cause of multiple sclerosis (29). HHV-7 has also been implicated as a cause of some cases of roseola. The diagnosis of these infections is difficult because both are ubiquitous, and asymptomatic viral shedding is common. HHV-6 DNA has been detected in the CSF of infants with acute HHV-6 infection, but the clinical significance of this finding in individual cases is unclear (26). HHV-6 DNA has also been detected in brain in the absence of neurologic disease (118).

Culture of HHV-6 and HHV-7 presents practical difficulties because it requires the use of umbilical cord lymphocytes, which are not available in most diagnostic virology laboratories. Furthermore, no criteria exist for the interpretation of diagnostic tests for HHV-6 and HHV-7 in immunocompromised patients. In infants, seroconversion is diagnostic of acute infection. Other findings suggestive of acute infection are a positive PCR performed on peripheral blood mononuclear cells in conjunction with a negative antibody titer (33) and the presence of HHV-6 in peripheral blood mononuclear cells but not in saliva because salivary shedding has later onset than viremia (41). In unusual cases, tissue should be examined by IHC and ISH whenever possible to assist in understanding the relationship of HHV-6 to the patient's pathologic process.

Gammaherpesvirinae

The members of the gammaherpesvirinae subfamily that infect humans are EBV and HHV-8, also known as Kaposi's sarcoma virus. The characteristics of these viruses are replication in lymphoid and epithelial cells, latency in lymphoid cells, and a proliferative effect on latently infected cells. EBV is the cause of infectious mononucleosis. HHV-8 has been linked to Kaposi's sarcoma in patients with HIV-related immunosuppression.

Diagnostic Tests for Epstein-Barr Virus

The cultivation of EBV requires immortalization of umbilical cord lymphocytes with demonstration of the presence of EBV antigens in the immortalized cells. This procedure is not practical for routine diagnostic laboratories, and accordingly, most diagnosis of EBV infections is based on serologic testing. Most cases of infectious mononucleosis are diagnosed based on the presence of atypical lymphocytes in peripheral blood plus the presence of heterophile antibodies, which are IgM antibodies that agglutinate the erythrocytes of sheep, oxen, and horses. The classic Paul-Bunnell-Davidson test has been largely replaced by slide agglutination tests that use horse erythrocytes and do not require differential absorption with beef erythrocytes and guinea pig kidney. Failure to produce heterophile antibodies is common in children, especially those younger than 4 years of age (185), and occurs occasionally in adults as well. Specific EBV antibody tests are used for diagnosis in these patients.

Specific Epstein-Barr Virus Serology

EBV serology consists of tests to measure antibodies to the viral capsid antigen (VCA), early antigens (EA), and nuclear antigens (NA). In some laboratories, tests for anti-EA distinguish between antibodies to diffuse and restricted components, based on the location of fluorescent staining and the differential sensitivity of the two components to acetone and methanol fixation. Testing for IgM antibodies to VCA is useful in defining acute infection because IgM anti-VCA is usually detectable with the onset of clinical manifestations and remains detectable for only several months (60,91). Testing for IgG VCA is useful in defining EBV immune status because these antibodies are detectable for the life of the individual. Anti-EA arise within a few weeks of infection but are not detected in all patients with acute infectious mononucleosis (91,92). The presence of antibodies to the diffuse component is more specific for recent infection because these antibodies do not persist for more than a few months. In contrast, antibodies to the restricted component may persist for several years after infection. Antinuclear antibodies arise later after infection than anti-EA and persist for life (90,91). Thus, measurement of anti-EA (especially the diffuse component) and anti-NA is occasionally useful for determining the timing of infection. The use of EBV antibodies for the diagnosis of chronic fatigue syndrome is of no value because the link once thought to exist between EBV infection and this syndrome has been disproved.

Epstein-Barr Virus Infections in Immunocompromised Patients

Like other herpesviruses, EBV can produce serious disease in immunocompromised patients. In patients with AIDS, EBV is associated with primary CNS lymphoma. PCR to detect EBV DNA in CSF is useful for making this diagnosis (9,38,51). EBV is also associated with the syndrome of posttransplantation lymphoproliferative disorder (PTLD) seen in solid organ and bone marrow transplant recipients. EBV serology is not useful for making this diagnosis. PTLD is confirmed by detecting EBV antigens (201) or nucleic acid (115,147) in lymphoid tissue showing the appropriate histologic findings. Several reports suggest that using PCR to monitor EBV DNA levels in peripheral blood mononuclear cells may be useful for the early recognition and clinical management of this syndrome (99,117,158).

Respiratory Viral Infections

The viruses that are common causes of respiratory infection include influenza and parainfluenza viruses, RSV, adenovirus, rhinovirus, coronavirus, and enteroviruses. All but the coronaviruses are frequently identified in routine diagnostic virology laboratories. Testing involves viral culture and antigen detection techniques. The preferred specimens are nasopharyngeal aspirates, washes, and swabs. Tracheal aspirates and specimens obtained through a bronchoscope, especially bronchoalveolar lavage fluid, are also suitable specimens. Antigen detection techniques, such as FA staining, are widely used to achieve rapid diagnoses.

Culture for Respiratory Viruses

Culturing for respiratory viruses is complicated because of the diverse growth and detection requirements of the different viruses that might be present. The influenza and parainfluenza viruses replicate best in primary monkey kidney cells, although continuous cell lines can be used, such as Madin-Darby canine kidney for influenza viruses and LLC-MK2 for parainfluenza viruses. Serum-free medium is used for incubating these cells for recovery of influenza and parainfluenza viruses, and some strains of influenza virus replicate best at 33°C. Trypsin is included in the media when Madin-Darby canine kidney cells are used. The influenza and parainfluenza viruses may replicate without producing CPE, and hemadsorption with guinea pig erythrocytes is used for detection. Early detection of viral replication can be accomplished by hemadsorption as early as 24 hours after inoculation (130). RSV replicates optimally in HEp-2 cells. This virus is very labile, and delayed transport to the laboratory may result in failure to isolate RSV. Adenoviruses replicate optimally in human embryonic kidney cells (104). Rhinoviruses replicate only in human diploid fibroblast cells, and they replicate better in some fibroblast cell lines than in others. Incubation at 33° to 34°C is required for optimal rhinovirus recovery. Coronaviruses do not replicate in currently available cell culture systems, but require tracheal organ cultures for cultivation in the laboratory.

Antigen Detection for Respiratory Viruses

Antigen detection techniques can be used to achieve rapid diagnosis of RSV, influenza, parainfluenza, and adenovirus respiratory tract infection. Viral antigens are visualized by FA staining of respiratory epithelial cells obtained by nasopharyngeal aspirate, wash, or swab. Aspirates and washes provide a higher cellular yield and have higher viral titers than swabs (2,84,123), but nasopharyngeal swabs are convenient and are used successfully by many laboratories (2,66). Tracheal aspirate and bronchoalveolar lavage specimens are also suitable for examination. On arrival in the laboratory, a cell pellet is prepared, spotted on a microscope slide, air dried, fixed, and stained. The entire procedure takes about 2 to 3 hours. Staining can be performed using monospecific antibodies or pools of monoclonal antibodies that can detect multiple respiratory viruses. Convenient membrane immunoassays that can be performed by personnel with less training than is required for FA staining are now commercially available for RSV and influenza. These assays can be performed in an emergency unit or physician's office. Other new rapid assay formats for detection of influenza viruses include an optical immunoassay for influenza antigens (45) and an assay for direct detection of neuraminidase activity.

A consistent finding is that the sensitivity of antigen detection methods exceeds that of culture for RSV (148). For influenza, parainfluenza, and adenovirus, some laboratories have reported sensitivities comparable to culture (72), but many laboratories find lower sensitivity compared with culture for these viruses (148). Rhinoviruses and coronaviruses are not detected by antigen detection methods because of antigenic diversity and lack of availability of appropriate antibodies. In general, antigen detection methods have higher sensitivity in children than in adults. The sensitivity of antigen detection methods to detect RSV in adult patients is particularly poor (61).

Nucleic Acid Amplification for Respiratory Viruses

PCR and RT-PCR have been applied to the detection of respiratory viruses (32,40,62,63,67,76,132,137,143,196). These techniques are probably more sensitive than either antigen detection or culture, but whether the increased sensitivity justifies the increased cost and complexity requires further evaluation. An intriguing concept is the use of multiplex PCR to detect multiple respiratory viruses simultaneously (63). Nucleic acid amplification may be most useful for detection of viruses that are not optimally detected by current methods, such as coronaviruses, rhinoviruses, influenza C virus, and parainfluenza virus type 4. It may also be useful for special patient groups, including adult patients with low levels of virus, immunocompromised patients, and perhaps asthmatic patients.

Need for Specific Viral Diagnosis

There is ongoing debate about the need for specific viral diagnosis of patients with acute viral respiratory infection. Proponents argue that specific diagnosis is useful for establishing prognosis, limiting antibiotic usage, and cohorting hospitalized patients to prevent nosocomial transmission (1,103). Skeptics argue that in the absence of specific antiviral therapy, patient management is not changed. There is little doubt that making a specific diagnosis is helpful in patients with unusual clinical manifestations and in immunocompromised patients.

Gastrointestinal Viral Infections

Viruses that are important causes of gastrointestinal infection are rotaviruses, enteric adenoviruses (especially serotypes 40 and 41), caliciviruses, astroviruses, and possibly toroviruses. Interestingly, none of these viruses is readily cultivable, and laboratory diagnosis has depended on electron microscopy and antigen detection techniques. Nucleic acid amplification techniques have also been applied.

Electron Microscopy

Electron microscopy was instrumental in the discovery of each of these agents (6,64). Negative staining with uranyl

acetate or phosphotungstic acid can be performed directly on stool specimens with minimal preparation. Some of the viruses, especially the caliciviruses, are better visualized using immune electron microscopy, in which viral particles are agglutinated using a specific antiserum before negative staining. Electron microscopy is not in widespread use in diagnostic virology laboratories but is used with excellent yield in selected laboratories with a special interest in the technique.

Antigen Detection

Antigen detection methods are widely used in the laboratory diagnosis of viral gastroenteritis. Many commercial assays that use a variety of EIA and particle agglutination formats are available for rotavirus detection, and at least one commercial adenovirus assay is available. Because the amount of rotavirus antigen present in fecal specimens is as high as 10^{11} particles per gram of stool during rotavirus gastroenteritis, the sensitivity of rotavirus antigen detection tests tends to be high. EIAs tend to be somewhat more sensitive than agglutination tests (53), although the latter may have advantages in convenience. There is some variation in specificity among the various commercial assays (53). Antigen detection assays for astrovirus have been used in investigations (131) but are not commercially available.

Nucleic Acid Amplification

RT-PCR and PCR assays have been developed for the main viral agents of gastroenteritis, but their use is restricted to research laboratories. These assays are unlikely to replace antigen detection assays for rotavirus because of the adequate sensitivity and greater simplicity of antigen detection assays. Likewise, at least one investigation of astrovirus infections showed no advantage in sensitivity for RT-PCR compared with EIA (70). The diversity of the caliciviruses is so extensive that no single primer set is yet available to amplify all pathogenic caliciviruses. Nucleic acid amplification methods may be especially useful for environmental studies and studies of molecular epidemiology (14,140,198).

Viral Meningitis and Encephalitis

A diverse group of viruses cause meningitis and encephalitis. No single diagnostic test method detects all possible suspects, and clues from the patient's history of exposure and clinical findings must be used to guide the choice of tests. The likelihood of making a specific diagnosis has been greatly increased by the application of nucleic acid amplification methods. Different diagnostic approaches are required for patients with (a) meningitis, (b) encephalitis, and (c) opportunistic viral infection of the CNS. Multiplex PCR assays are potentially applicable to each of these clinical problems and are likely to be an important approach in the future (149,161).

Meningitis

Enteroviral Meningitis

The echoviruses and coxsackieviruses are the most common viral causes of meningitis. RT-PCR assays that amplify a segment of the 5' nontranslated region of the enterovirus genome that is conserved among most enteroviral types are rapidly becoming the standard for diagnosis (31,163,171). These assays improve on viral culture of CSF, which has a sensitivity of 70% or less, is not successful for some coxsackie A virus strains, and requires at least several days to detect viral growth. Several studies have suggested that RT-PCR assays can improve patient management and may be able to decrease length of hospital stay for children with enteroviral meningitis (3,85,176).

Herpes Simplex Virus Meningitis

Most HSV meningitis is associated with HSV-2, which may cause recurrent episodes of meningitis. Some episodes are accompanied by genital lesions. Laboratory diagnosis using techniques other than PCR can be difficult because viral cultures are uniformly negative in recurrent cases (19). PCR appears to be both sensitive and specific for HSV meningitis. Use of PCR has revealed that many cases of recurrent meningitis of unknown etiology, previously referred to as *Mollaret's meningitis,* are actually caused by HSV (189).

Varicella Zoster Virus Meningitis

VZV can cause meningitis as a complication of either primary or reactivation infection. PCR can be used to amplify VZV DNA from the CSF of these cases. Experience is not yet adequate to provide data regarding sensitivity and specificity. The results of VZV PCR performed on CSF must be interpreted with the knowledge that VZV DNA is present in some cases of zoster without neurologic complications (83). PCR may be uniquely useful in recognition of cases of VZV meningitis without cutaneous lesions (58). A recent study that used a multiplex RT-PCR and PCR assay to detect simultaneously the nucleic acids of enteroviruses, HSV, and VZV indicated that VZV meningitis in the absence of skin lesions may account for a substantial proportion of cases of viral meningitis, especially in adults (149).

Encephalitis

Herpes Simplex Virus Encephalitis

PCR performed on CSF has revolutionized the approach to the diagnosis of HSV encephalitis, which previously required a brain biopsy for laboratory diagnosis (15,107). The sensitivity and specificity of HSV PCR for the diagnosis of HSV encephalitis were found to be 96% and 99%, respectively, in a metaanalysis of published studies (187). HSV DNA is still detectable in CSF for several days after initiation of acyclovir therapy (107). Laboratory performance may vary widely because an HSV PCR assay is not currently available as a commercial kit.

Arbovirus Encephalitis

Diagnosis of arboviral encephalitis is best accomplished by serologic testing. Either EIA or IFA assays can be used to detect arboviral specific IgM antibodies, which are diagnostic of recent infection when detected in either CSF or serum. About 40% of patients have arboviral IgM antibodies detectable in serum or CSF by 4 days after onset, and this percentage increases by about 10% per day, so that by day 10, nearly all cases are positive. It is important to be aware that low levels of arboviral IgM antibodies can persist in serum for up to 1 year after infection (191).

Rabies

The diagnosis of rabies is accomplished by a variety of methods depending on the stage of the infection. In the earliest phase, diagnostic tests are directed at detection of the virus by culture, antigen detection using FA staining, or nucleic acid amplification. Important specimens include saliva and CSF for culture and RT-PCR, and skin (usually from the nape of the neck) for FA staining. A brain biopsy is not indicated for the diagnosis of rabies, but if brain tissue is available, it should be analyzed for rabies antigen by FA staining. After the 8th day of illness, rabies antibodies may be present and are diagnostic if the patient had not received rabies immunization. The presence of rabies antibodies in CSF is always diagnostic of rabies (178).

Other Causes of Meningitis or Encephalitis in Immunocompetent Hosts

A wide variety of other viruses can cause meningitis or encephalitis in immunocompetent hosts, including EBV, HHV-6, B virus, lymphocytic choriomeningitis virus, measles, mumps, rubella virus, influenza virus, and adenovirus. These cases can be diagnosed by a combination of culture, serology, and nucleic acid amplification. Viral diagnostic studies other than serology are negative in cases that represent postinfectious demyelination.

Opportunistic Viral Infections of the Central Nervous System

Several viruses are important causes of CNS infection in immunocompromised patients. CMV is an important cause of encephalitis and radiculomyelitis in patients with advanced AIDS. The virus can only rarely be cultured from CSF in these cases but is detectable by PCR (10). Quantitation of the level of CMV DNA is useful to distinguish between clinically significant CMV CNS infection and cases with asymptomatic or presymptomatic infection (8). EBV is strongly associated with primary CNS lymphoma in patients with AIDS. PCR performed on CSF can detect EBV DNA in most cases, with a reported sensitivity of 83% to 100% and reported specificity of 88% to 100% (9,38,39,51). PCR for JC virus is useful for diagnosing progressive multifocal leukoencephalopathy, although the sensitivity in most laboratories is less than 100% (39).

Viral Hepatitis

Six hepatitis viruses (A to E and G) are currently known. Of these, A to E are known to cause clinical hepatitis. Because these viruses are difficult or impossible to culture, diagnosis has been based primarily on serologic tests.

Hepatitis A

Acute hepatitis A is diagnosed using assays for hepatitis A IgM antibodies. This test is virtually always positive when symptoms develop and remains positive for several months. Assays for total antibodies to hepatitis A virus are used to define previous exposure and immunity.

Hepatitis B

HBV infection is diagnosed using several different serologic tests. The test for hepatitis B surface antigen (HBsAg) defines current active infection with HBV. This test is usually positive with the onset of symptoms of acute HBV. Persistence of a positive HBsAg test beyond 6 months defines the HBV carrier state. Antibodies to hepatitis B surface antigen (HBsAb) and core antigen (HBcAb) develop after HBV infection. Anti-HBc is the first antibody to appear and is always present during the symptomatic phase of illness. Anti-HBs develops later. Both antibodies may persist for many years. A test for HBc IgM antibodies is used in the diagnosis of acute HBV infection. It is regularly present at the onset of symptoms and is particularly useful because occasional patients with acute HBV may be in the window period when they are negative for both HBsAg and anti-HBs. Only anti-HBs is produced in response to hepatitis B vaccine, and its presence indicates HBV immunity. Hepatitis e antigen is a component of HBc. It is detectable in the serum during active infection, and its presence signifies active viral replication. Anti-HBe is produced in response to HBe, and its presence in HBsAg carriers, generally indicates a less active state of infection. A quantitative assay for HBV DNA polymerase is also used to define active infection. PCR for HBV DNA is the most sensitive test for HBV. HBV DNA is sometimes detectable in serum by PCR when all other HBV markers are negative (24).

Hepatitis C

The screening test for HCV is the hepatitis C EIA. This assay may not become positive for months after infection. Because false-positive tests are common, especially when the test is used to screen low-risk populations such as blood donors, a positive HCV EIA must be confirmed by an additional test. In blood donor testing, the additional test is usually the radioimmunobinding assay. This test detects antibodies to the same antigens as the EIA, but because antibodies to individual antigens are visible, it is helpful in the recognition of false-positive tests. In high-risk patients, the additional test is often a test for HCV nucleic acid. These tests, including RT-PCR and the bDNA assay performed on plasma, define the presence of active HCV infection. Quantitative HCV RNA tests are used to assess the likelihood of response to antiviral therapy and to monitor the response when treatment is given. Molecular techniques, including hybridization or

nucleotide sequencing, are used to define the HCV genotype, which may be important because it affects the likelihood of response to interferon therapy and is used to determine the duration of treatment.

Hepatitis D, E, and G

Infection with hepatitis D virus (HDV) (also known as the delta agent) is diagnosed by the detection of antiviral antibodies. HDV produces infection only in patients who are also infected with HBV. HDV RNA can be detected by RT-PCR and may also be useful for defining active infection (170). Hepatitis E is diagnosed using an EIA that measures antibodies to HEV. The presence of anti-HEV IgM antibodies indicates recent infection. Tests for hepatitis G virus are not used in diagnostic virology laboratories because of uncertainty regarding the pathogenicity of this virus.

Human Papillomaviruses

The HPVs are important because of their close association with cervical cancer. They also cause the common wart and several other conditions affecting the skin and mucous membranes. Multiple types exist and differ in their relationships to various disease manifestations. Some types, such as 16, 18, 31, and 45, are considered high-risk types in relation to cervical cancer. HPV cannot be grown in cell culture; therefore, laboratory diagnosis depends on other techniques. Characteristic cytologic findings on cervical Papanicolaou smear (i.e., koilocytosis) are indicative of HPV infection. PCR and the hybrid capture assay have been used to detect HPV DNA in patient specimens. The most widely used PCR assay detects a segment of the gene encoding the L1 major capsid protein that is common to all HPV types (173). Typing is accomplished by differential hybridization of the PCR product with DNAs from individual types. The hybrid capture assay is a commercial system (Digene Corporation, Beltsville, MD) usually applied to swabs obtained from the cervix. This assay is configured to detect pools of HPV types designated as high risk or low risk. It is less sensitive than PCR but more sensitive than Southern blot (44). It also can provide quantitative data on the amount of HPV DNA present.

The indications for HPV testing are not well defined. One situation in which it may be helpful is the case of cervical lesions that are mildly atypical or have low-grade dysplasia. In these cases, the presence of high-risk HPV may justify more aggressive management (46). The question of whether HPV testing should be part of routine cervical cancer screening is still under study (49,120).

Human Polyomaviruses

Two polyomaviruses, the JC and BK viruses, are linked to human disease. Both are common human viruses that cause disease in only a tiny minority of infected individuals. JC virus is the cause of progressive multifocal encephalopathy, most commonly seen in patients with AIDS. BK virus is the cause of hemorrhagic cystitis, which occurs most often in bone marrow transplant recipients. Neither virus is routinely cultured in diagnostic virology laboratories. BK virus grows in human embryonic kidney cells, and a shell vial culture method has been described (122). JC virus grows only in human fetal brain cultures and a human fetal cell line called SVG (119). PCR assays are available for both viruses and are usually used on CSF for the detection of JV virus and on urine for detection of BK virus (11).

Exanthematous Viral Diseases (Measles, Rubella, Mumps, Parvovirus B19, Enteroviral Infection)

Measles, mumps, and rubella have become rare because of the success of immunization programs. Curiously, this increases the need for laboratory diagnosis of individual cases because many physicians trained in the vaccine era are not skilled in recognizing the diseases. In addition, specific diagnosis is important because extensive public health control measures may be implemented based on the occurrence of even a single case. All of the viruses discussed in this section, with the exception of parvovirus B19, can be cultured, but other laboratory methods are more important because of their potential for rapid diagnosis.

Measles

Measles virus can be grown in a variety of human and simian cells. Specimens from which the virus can be recovered include nasopharyngeal and conjunctival secretions, blood, urine, and CSF. Cultivation of the virus is slow and not always successful. Rapid diagnosis can be accomplished by FA staining of nasopharyngeal secretions or urine for measles antigen (69,129). An alternative approach for rapid diagnosis is to test for measles-specific IgM antibodies, which are always detectable within several days of appearance of the rash (192). The diagnosis can also be made by comparing measles antibody titers in acute and convalescent phase specimens, with seroconversion or a four-fold rise taken as diagnostic of recent infection. The presence of measles virus IgG antibodies in serum is evidence of measles-specific immunity. Subacute sclerosing panencephalitis, a late sequela of measles virus infection, is diagnosed by the demonstration of high levels of measles antibodies in CSF (42). Other CNS complications of measles may be diagnosed by using RT-PCR to detect measles virus RNA in CSF (136).

Rubella

Rubella virus can be cultured from specimens of nasopharyngeal or throat secretions, urine, stool, blood, and joint fluid. African green monkey kidney, Vero, or RK-13 cells can be used to grow the virus. Culturing for rubella virus is complicated by the fact that the virus may grow without producing CPE and must be detected by using the interference assay, in which the culture is “challenged” with a virus to which it is ordinarily susceptible. Failure of the challenge virus to grow is evidence of the presence of an interfering virus, whose identity can be confirmed by specific FA staining or nucleic acid detection techniques. Because this technique is cumbersome and time-consuming, serology is the preferred approach for the diagnosis of rubella. A rapid diagnosis can be accomplished by testing for rubella virus–specific IgM antibodies, which are usually detectable within a few days of onset of the rash. Rubella virus antibody titers can also be compared in acute and convalescent specimens, with seroconversion or a four-fold rise taken as diagnostic of recent infection. The presence of rubella virus IgG antibodies in serum is evidence of rubella-specific immunity.

Mumps

Mumps virus can be grown from specimens of saliva, urine, and CSF. Rhesus monkey kidney cells and human embryonic kidney cells can be used. The virus grows slowly and often requires hemadsorption with guinea pig erythrocytes for detection. A rapid diagnosis can be made by detecting mumps virus–specific IgM antibodies, which are usually present in serum at the time of clinical illness. Mumps virus antibody titers can also be compared in acute and convalescent specimens, with seroconversion or a four-fold rise taken as diagnostic of recent infection. Cross-reactivity with parainfluenza viruses can complicate the interpretation of serologic tests but is not usually a major issue because of differences in the clinical presentation of disease caused by the mumps and parainfluenza viruses.

Parvovirus B19

Parvovirus B19 produces diverse clinical manifestations depending on the stage of infection and the immune status of the host. The diagnostic approach is different for the different clinical manifestations. The virus cannot presently be cultured in routine diagnostic virology laboratories; therefore, diagnosis is based on serologic and nucleic acid testing. Acute parvovirus B19 infection causes fifth disease, also known as *exanthem subitum.* The optimal approach for documenting acute parvovirus B19 infection is to test for parvovirus B19–specific IgM antibodies, which are present within several days of the onset of symptoms (5). Parvovirus B19–specific antibody titers can also be compared in acute and convalescent specimens, with seroconversion or a four-fold rise taken as diagnostic of recent infection. The presence of acute parvovirus B19 infection in an individual with chronic hemolytic anemia may produce hemolytic crisis (177). Hemolytic crisis occurs earlier after infection than fifth disease, and parvovirus B19 antibodies may not be detectable for about 1 week after the onset of crisis. However, parvovirus B19 DNA is regularly present in serum at this time and can be readily detected by PCR (166). Parvovirus B19 infection in immunocompromised patients may result in chronic infection of erythroid progenitor cells, resulting in chronic red blood cell aplasia (68,100). Patients with chronic parvovirus B19 infection may fail to develop parvovirus B19–specific antibodies, but the diagnosis can be established by detecting parvovirus B19 DNA in serum by PCR or other DNA detection assays (100). Finally, parvovirus B19–specific immunity can be demonstrated by testing for parvovirus B19–specific IgG antibodies.

Enteroviruses

The enteroviruses include coxsackie A and B viruses, echoviruses, polioviruses, and several viruses classified only as enteroviruses. This group of viruses can produce a wide variety of clinical syndromes, including meningitis, encephalitis, nonspecific febrile illness with or without a rash, pharyngitis, hand-foot-and-mouth disease, upper respiratory tract infection, hemorrhagic conjunctivitis, myocarditis, pericarditis, and pleuritis. CNS infections are best diagnosed using RT-PCR to detect enteroviral RNA in CSF.

Enteroviral infections of body sites other than the CNS are best diagnosed by culture. The best specimens are stool and nasopharyngeal or throat secretions. The coxsackie B viruses grow well in primary monkey or human embryonic kidney cells as well as in a number of continuous monkey and human cell lines. Many of the coxsackie A viruses require newborn mouse inoculation for growth. The echoviruses grow in human fibroblast cells as well as in primary monkey or human embryonic kidney cells. Cultures often become positive in 2 to 8 days. Viruses growing in culture can be classified to group by FA staining using group-specific monoclonal antibodies, but type-specific identification requires the laborious neutralization test. Enteroviruses can typically be cultured from respiratory tract specimens for 1 to 2 weeks after infection, and from stool specimens for 4 to 6 weeks. Because of the prolonged shedding, the significance of positive cultures is not always clear, and clinical judgment is required. Rapid recovery of enteroviruses from patients with compatible clinical syndromes is highly suggestive of a causal relationship. Serologic testing is not widely available because of the antigenic diversity of the enterovirus group.

Congenital and Neonatal Infection

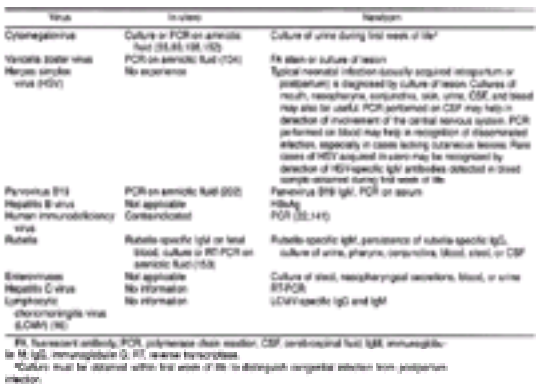
A variety of viruses can infect the fetus, resulting in congenital infection. Some of the same viruses, as well as others, may cause infection in the peripartum or immediate newborn period, resulting in neonatal infection. The possibilities for diagnosing congenital and neonatal viral infections have increased dramatically because of the convergence of several developments: (a) improvement and very widespread use of intrapartum ultrasound examination, which can detect morphologic evidence of *in utero* infection; (b) improved techniques for obtaining fetal samples; and (c) molecular techniques that can be used to analyze small sample volumes.

Diagnosis of infection *in utero* depends on acquisition of fetal samples. Techniques that are employed include chorionic villus sampling, amniocentesis to obtain amniotic fluid, and cordocentesis to sample fetal blood (200). Important considerations include the volume of specimen obtained and the timing during the pregnancy in relation to (a) acquisition of fetal infection and (b) the synthesis of antibodies by the fetus. Chorionic villus sampling provides a fetal sample from the first trimester of pregnancy, whereas amniocentesis and cordocentesis are usually performed during the second or third trimesters.

Viral diagnostic tests that are employed for the diagnosis of *in utero* or neonatal infection include viral culture, viral nucleic acid amplification assays, and tests for virus-specific IgM antibodies. Because IgM antibodies are not passively transferred across the placenta, the detection of virus-specific IgM antibodies in the fetus or newborn is presumptive evidence of congenital infection. The capability of synthesis of IgM antibodies is present starting at about 20 weeks of gestation (4). Assays for virus-specific IgM antibodies used to diagnose congenital infection must include procedures to prevent false-positive results from rheumatoid factor, which can be present either in the fetus or in the young infant and can cause nonspecific test results (151,180). The use of batteries of IgG serologic assays, such as TORCH titers (antibody titers to *Toxoplasma gondii, Treponema pallidum,* rubella virus, CMV, and HSV), are not useful for diagnosis of fetal or congenital infection because the presence of virus-specific IgG titers may represent passive transfer of maternal

antibodies rather than fetal infection.

The specific tests used depend on the virus and the period of gestation or age of the newborn. Table 9 provides a summary of approaches to the diagnosis of viral infections of the fetus and newborn. The reader is referred to chapters on the individual viruses and to other sources (200) for additional information.



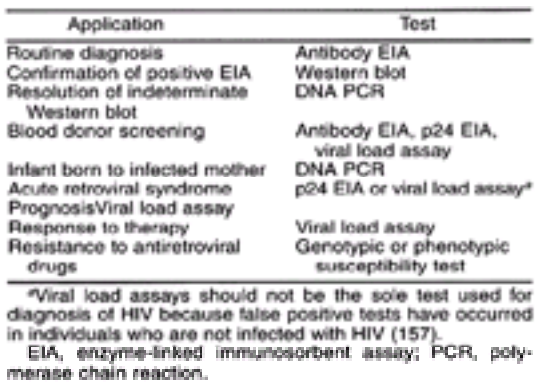
Virus	In utero	Newborn
Cytomegalovirus	Culture or PCR on amniotic fluid (68,69,108,152)	Culture of urine during first week of life[a]
Varicella (latent virus	PCR on amniotic fluid (124)	
Herpes simplex virus (HSV)	No experience	Typical neonatal infection (usually acquired intrapartum or postpartum) is diagnosed by culture of lesion. Cultures of mouth, nasopharynx, conjunctiva, skin, urine, CSF, and blood may also be useful. PCR performed on CSF may help in detection of involvement of the central nervous system. PCR performed on blood may help in recognition of disseminated infection, especially in cases lacking cutaneous lesions. Rare cases of HSV acquired in utero may be recognized by detection of HSV-specific IgM antibodies detected in blood sample obtained during first week of life
Parvovirus B19	PCR on amniotic fluid (202)	Parvovirus B19 IgM, PCR on serum
Hepatitis B virus	Not applicable	HBsAg
Human immunodeficiency virus	Contraindicated	PCR (32,141)
Rubella	Rubella-specific IgM or fetal blood, culture or RT-PCR on amniotic fluid (153)	Rubella-specific IgM, persistence of rubella-specific IgG, culture of urine, pharynx, conjunctiva, blood, stool, or CSF
Enteroviruses	Not applicable	Culture of stool, nasopharyngeal secretions, blood, or urine
Hepatitis C virus	No information	RT-PCR
Lymphocytic choriomeningitis virus (LCMV) (96)	No information	LCMV-specific IgG and IgM

RIA, fluorescent antibody; PCR, polymerase chain reaction; CSF, cerebrospinal fluid; IgM, immunoglobulin M; IgG, immunoglobulin G; RT, reverse transcriptase.
[a]Culture must be obtained within first week of life to distinguish congenital infection from postpartum infection.

TABLE 9. *Tests used for the diagnosis of viral infection of the fetus and newborn*

Human Retroviruses

Essentially all patients who are truly seropositive for HIV have active infection. This concept forms the basis for the use of the HIV antibody test as the standard approach to the diagnosis of HIV infection. Other assays, such as the p24 antigen assay, DNA PCR, plasma RNA, and genotypic and phenotypic susceptibility assays, are used for a variety of specialized purposes, described subsequently and summarized in Table 10.



Application	Test
Routine diagnosis	Antibody EIA
Confirmation of positive EIA	Western blot
Resolution of indeterminate Western blot	DNA PCR
Blood donor screening	Antibody EIA, p24 EIA, viral load assay
Infant born to infected mother	DNA PCR
Acute retroviral syndrome	p24 EIA or viral load assay[a]
PrognosisViral load assay	
Response to therapy	Viral load assay
Resistance to antiretroviral drugs	Genotypic or phenotypic susceptibility test

[a]Viral load assays should not be the sole test used for diagnosis of HIV because false positive tests have occurred in individuals who are not infected with HIV (157).
EIA, enzyme-linked immunosorbent assay; PCR, polymerase chain reaction.

TABLE 10. *Laboratory tests used for the diagnosis and management of human immunodeficiency virus (HIV) infections*

Human Immunodeficiency Virus Antibody Assays

Virtually all HIV antibody testing is performed using EIA. A number of assays are commercially available, with sensitivity and specificity often exceeding 99%. These assays detect all serotypes of HIV-1, with the exception of some group O viruses (172). Some commercially available assays, including all of those used for screening of blood donors, are designed to detect both HIV-1 and HIV-2. The remaining HIV-1–specific assays detect about two-thirds of cases of HIV-2 (52). Current HIV EIAs typically become positive about 22 days after infection (23).

Despite the high specificity of the HIV EIA, when it is used for screening purposes in low to moderate risk populations, a high proportion of all positive results detected are false-positive results. Therefore, Western blot testing is required to confirm positive EIA results. The two-step algorithm of EIA followed by Western blot if positive has extremely high sensitivity and specificity. The major difficulty with this approach is that occasional Western blot results are indeterminate. These troublesome situations can often be resolved by molecular tests (see later) or by repeating the EIA and Western blot after several months.

HIV antibody testing can be performed on oral transudate (71) or urine (194). Use of oral transudate and urine tests can facilitate screening in situations in which it is not practical to draw blood. Particle agglutination assays that can be performed on serum to provide results within minutes of collection are also commercially available, and the U.S. Public Health Service has recommended the use of such tests in selected situations for testing individuals who may not return to learn of test results (28).

p24 Antigen Assays

Assays for HIV p24 antigen in serum can detect HIV infection about 6 days earlier than the antibody EIA (23). These assays are used by blood banks for screening all collected blood units and are occasionally used for the diagnosis of the acute retroviral syndrome. Other uses have been replaced by plasma HIV viral load assays.

Nucleic Acid Amplification Assays

HIV DNA PCR assays that detect proviral DNA are used to detect infection in infants born to HIV-infected mothers. HIV antibodies are present in these infants as a result of transplacental transfer from the infected mother and do not indicate infection of the infant. Current estimates are that HIV DNA PCR is positive in about 40% of these infants within the first few days of life and in more than 90% by 2 weeks of age (22,141).

Assays for plasma HIV RNA, also referred to as *viral load assays,* have become an essential tool for the management of HIV. The level of HIV RNA provides information about the prognosis of HIV (126) and is used to evaluate the response to treatment. Methodologies that are employed include RT-PCR, branched-chain DNA, and transcription-mediated amplification. Each of these methods is able to provide accurate quantitation of HIV RNA in plasma over a very large range. Ultrasensitive modifications of these assays can detect as few as 40 copies of HIV RNA per milliliter of plasma. Because these assays provide the earliest detection of HIV after infection, they are now being evaluated for screening of blood donor units as well as for the diagnosis of individuals with the acute retroviral syndrome. Viral load assays should not be the sole test used for diagnosis of HIV because false-positive tests have occurred in individuals who are not infected with HIV (157).

Antiretroviral Susceptibility Testing

The availability of multiple therapeutic agents for HIV and the potential for the virus to become resistant to these agents has created a need for tests to measure the susceptibility of HIV to antiretroviral agents. These tests are classified as genotypic and phenotypic (94). Genotypic assays are based on nucleotide sequencing of HIV RNA amplified directly from plasma. The presence of specific mutations correlates with specific drug resistance. Use of these assays has been shown to be useful in minimizing HIV viral load (17). Phenotypic assays assess the effect of antiretroviral drugs on the growth of HIV in culture. Recombinant assays are commercially available in which the polymerase and protease genes of a patient's virus are inserted into a standardized molecular clone that is used to carry out viral growth assays. The role of genotypic and phenotypic assays in clinical practice is evolving rapidly.

Human Retroviruses Other Than Human Immunodeficiency Virus Type 1

HIV-2, HTLV-I, and HTLV-II share with HIV-1 the characteristic that essentially all infected individuals are chronically infected. This means that assays for antiviral antibodies can be used to diagnose active infection. Antibody assays are available for each of these viruses and are used to screen all blood donor units in the U.S. Western blot and radioimmunoprecipitation assays are also available to provide supplemental testing. PCR can also be used to confirm infection and to discriminate among the various human retroviruses.

When evaluating patients for HIV infection, the possibility of HIV-2 infection must be considered in individuals who may have acquired infection in West Africa or from an individual infected in that area. Commercially available HIV assays, including antibody EIA, p24, PCR, and viral load assays, vary in their ability detect HIV-2. In cases in which HIV-2 is a possibility, consultation with the laboratory performing the testing is recommended to determine whether the tests in use detect HIV-2.

Exotic Viral Infections

In the era of global travel, infections may become symptomatic far from where they were acquired. Therefore, it is important for virology laboratories to be able to provide assistance in the diagnosis of these infections. Some agents, such as the filoviruses and the arenaviruses, can be hazardous to laboratory personnel, and all testing must be performed in Biosafety Level 4 facilities. Consultation with State Health Departments and personnel at the Centers for Disease Control and Prevention is important when exotic viral infections, especially the hemorrhagic fevers, are diagnostic considerations. Table 11 provides a summary of exotic viral infections, geographic locations, and diagnostic tests.

Virus or syndrome	Location	Tests*
Sin nombre virus (hantavirus pulmonary syndrome)	Western U.S.	Virus-specific IgM, RT-PCR
Other hantaviruses	Asia, Europe	Virus-specific IgM
Colorado tick fever	Western U.S.	Culture of blood clot, IFA stain of erythrocytes, RT-PCR
Dengue	Asia, Central and South America	Virus-specific IgM, RT-PCR
Hemorrhagic fevers (filoviruses [Ebola and Marburg], arenaviruses [Lassa and South American hemorrhagic fevers], yellow fever, Congo-Crimean hemorrhagic fever)	Africa, South America, Asia	Virus-specific IgM, culture of serum, RT-PCR, EM of urine or blood (Ebola and Marburg), antigen detection assays
Rift valley fever	Africa	Virus-specific IgM, culture, RT-PCR
Alphaviruses (Chikungunya, Chyorg nyorg, sindbis, Mayaro, Ross River)	Africa, Europe, Asia, Central and South America, Australia	Virus-specific IgM, culture, RT-PCR

*Performed on blood unless otherwise specified.
IFA, fluorescent antibody; IgM, immunoglobulin M; RT-PCR, reverse transcriptase polymerase chain reaction.

TABLE 11. *Exotic viral infections*

DISCOVERY OF NEW VIRAL AGENTS

In recent years, numerous new viral agents have been discovered. In some instances, conventional viral culture techniques have yielded new viruses. For example, detection of Hendra virus, a new paramyxovirus that causes infection

of horses and humans, was based on growth of the virus in cell cultures in use in diagnostic virology laboratories (135). In other cases, powerful nucleic acid–based techniques have been applied to detect viruses that could not be cultivated. Application of these techniques is likely to yield additional new viruses in coming years. Short descriptions of the culture-independent discovery of three human pathogenic viruses follow.

The first outstanding example of culture-independent virus discovery is hepatitis C virus. This virus was discovered through molecular cloning of plasma known to be infectious for chimpanzees. Serum from a person with non-A, non-B hepatitis was used to screen an expression library of about 1 million candidate clones, resulting in discovery of one clone expressing an HCV gene product. Nucleic acid sequencing provided the identification of the new virus as a flavivirus and led to the development of a blood test used for screening of donated blood units (34). Cultivation of HCV has still not been achieved.

The discovery of sin nombre virus, the cause of hantavirus pulmonary syndrome, began with the detection of antibodies in the serum of affected individuals that cross-reacted with known human hantaviruses. Based on that clue, an RT-PCR assay was designed using primers based on sequences conserved in the four hantaviruses previously known to be human pathogens. Sequencing of a product amplified from specimens obtained from human cases revealed the presence of a new hantavirus (138). That discovery has led to the discovery of numerous additional hantaviruses (57). Laboratory cultivation of sin nombre virus and related viruses remains extremely difficult.

Kaposi's sarcoma virus (HHV-8) was discovered as the cause of Kaposi's sarcoma through use of the technique of representational difference analysis (113). This technique involved the use of DNA extracted from Kaposi's sarcoma tissue, which was used along with DNA extracted from normal tissue. Unique sequences were detected that were homologous to known gammaherpesviruses (30). The new virus was subsequently shown to be highly associated with Kaposi's sarcoma as well as with multicentric Castleman's disease and AIDS-related body cavity–based lymphoma. Cultivation of the virus in cell culture has subsequently been achieved (65).

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CHAPTER 19 Plant Viruses

Sondra G. Lazarowitz

HISTORY

The oft cited color variegation in tulip flowers, caused by tulip breaking virus, is probably the most splendid example of disease symptoms produced by a viral infection of plants. Tulips, with their naturally solid colored flowers, acquired this infection when introduced from Constantinople to the Netherlands in 1551. The spectacular floral patterns that resulted were so highly coveted that a tulip bulb could command an astonishingly high price: 5,200 guilders were reportedly paid for a tulip bulb in 1637, whereas Rembrandt's fee for his masterpiece “The Night Watch” in 1642 was a mere 1,600 guilders (59). Given the tulip mania that seized the Netherlands, the affected tulips were reproduced in great numbers by noted 17th-century Dutch painters and growers alike (Fig. 1). Although considered an example of the beneficial consequences of some virus infections, most plants do not fare as well in the face of virus infection. Indeed, viruses are currently responsible for severe losses in crop plants of every type worldwide (Table 1). It is difficult to place a precise dollar figure on the total losses annually, but a single virus, such as tomato spotted wilt virus (TSVW), is estimated to cause annual crop losses in excess of $1 billion.

FIG. 1. “Study of Tulips” by the Dutch botanical artist Jacob Marrel (1614–1681). Reprinted with permission of the Metropolitan Museum of Art, New York, Rogers Fund, 1968 (66,68).

Virus disease	Geographic distribution	Host crop
Sugarcane mosaic	Worldwide	Sugarcane, corn
Cassava mosaic	Africa	Cassava
Citrus quick decline (tristeza)	Africa, Americas	Trees[a]
Swollen shoot of cocoa	Africa	Cocoa (heavy losses)
Plum pox (sharka)[b]	Europe	Plums, peaches, apricots
Barley yellow dwarf	Worldwide	Grains
Tomato yellow dwarf	Mediterranean, Caribbean	Tomatoes, beans
Tomato spotted wilt	Worldwide	Tomatoes, tobacco, peanuts, ornamentals, others[c]

[a]Tens of millions of trees infected as of 1997.
[b]Severe, spreading epidemic.
[c]Extremely broad host range.

TABLE 1. *Viruses that cause severe crop losses worldwide*

The origins of the science of virology in general are rooted in research at the end of the 19th century on tobacco mosaic disease, caused by what came to be called tobacco mosaic virus (TMV). The interest in TMV at the time was economic: the diseased tobacco leaves were not suitable for making cigars. Adolf Mayer, Director of the Agricultural Research Station at Wageningen, was investigating this tobacco mosaic syndrome and in 1886 showed that he could transfer the disease by injecting healthy tobacco plants with leaf extracts from diseased plants (222). Thus, he had established the

infectious nature of this disease, but he failed to identify the pathogenic agent. In 1892, Dmitrii Ivanowski, working in the Crimea, reported that such extracts from mosaic-diseased tobacco, when passaged through candle filters that would exclude bacteria, were still infectious (157), thereby establishing the cause of this disease as a “filterable agent.” However, despite this and additional evidence from his own work, he wrongly concluded that the causal agent was a toxin or bacterial agent that could still pass through the filters. It was Martinus Beijerinck at the Technical School of Delft, repeating and extending these filtration studies, who made the conceptual leap to report in 1898 (22) that this mosaic disease of tobacco was caused by a “contagium vivum fluidum”—a contagious living fluid. Thus, TMV was the first of the new class of “filterable agents” of disease, which eventually would simply be called *viruses,* to be identified, in the same year that Loeffler and Frosch reached similar conclusions for the cause of foot-and-mouth disease in livestock.

During the first half of the 20th century, TMV remained a central player in the development of modern virology, in general, and plant virology in particular. This was due in part to the ease of infecting and propagating a virus in plants (as opposed to obtaining human volunteers as did Reed and Carroll in their studies of yellow fever) and largely to the stability of TMV and the large amounts of virus that could readily be purified from infected tobacco plants. TMV was the first virus to be purified in a crystalline state and shown to consist of ribonucleoprotein (RNP) particles (19,325), and the first for which the architecture of the particles was revealed by electron microscopy and x-ray diffraction (25,44,107,168). It was the first virus shown to reassemble into infectious particles *in vitro* from viral RNA and coat protein (105) and the first for which the intrinsic infectivity of the virion RNA was demonstrated (118). As studies became more molecular, TMV has continued to be in the forefront of the development of techniques and concepts in modern plant virology. Techniques to prepare plant protoplasts and infect these with TMV were a major technical breakthrough in plant virology (15,338). The concept that plant virus movement between cells was an active process facilitated by a viral-encoded “movement protein” was established based on studies of temperature-sensitive mutants of TMV (244). The first successful example of pathogen-derived resistance was the engineering of transgenic plants resistant to TMV infection based on the expression of the viral coat protein gene (270), and much of our current thinking about the function of virus movement proteins derives from the initial demonstration that the TMV movement protein could alter the gating properties of plasmodesmata (376). Just as for animal viruses, research on plant viruses has been both applied and basic, with very similar goals: to understand how plant viruses cause diseases so as to prevent these and their impact economically and in terms of human and animal nutrition, and to use them as models to investigate plant cell biology and gene expression. In contrast to animal viruses, this latter goal has taken longer to realize because of technical limitations in investigating plant viruses.

REVERSE GENETICS AND THE MODERN ERA OF PLANT VIROLOGY

From the mid-1950s on, the availability of animal cell tissue culture systems meant that clonal isolates of animal viruses, essential for genetic studies, could be readily obtained and that synchronous infections could be done to dissect viral multiplication cycles. Thus, investigations of animal viruses became more molecular, and our knowledge of animal virus–host interactions advanced. Lacking appropriate tissue culture systems, plant virus studies relied on the ability to infect plants by mechanical means—rubbing a suspension of virus particles onto a leaf with a finger or spatula, often in the presence of an abrasive agent such as fine-grade carborundum—or using appropriate vectors, usually insects. For a number of plant viruses, protoplasts (isolated plants cells from which the walls had been enzymatically removed) could be transfected with the viral genome or, in some cases, virions. However, this advance did not occur until 1968, at which point plant virus studies began to focus more on virus replication and on the synthesis and translation of viral messenger RNAs (mRNAs). In particular, advances were reported for positive-sense RNA plant viruses (including TMV) that could be readily propagated because the purified viral genome was infectious, often multipartite (i.e., segmented), and could be translated *in vitro* using available rabbit reticulocyte and wheat germ extracts, or when injected into oocytes from frogs or toads. Thus, from the 1970s to the early 1980s, knowledge about virus replication was advanced for those positive-sense RNA viruses that accumulated to high levels in infected plants and could be easily purified. The coat proteins of brome mosaic virus (BMV) and TMV were shown to be translated from a subgenomic mRNA (152,317). Protoplast studies began to identify nonstructural proteins encoded by these viruses and other viruses, and genetic studies began to identify specific viral genes through the study of temperature-sensitive mutants and genetic reassortment studies for viruses having segmented genomes.

Against this background of technical limitations, the impact of reverse genetics on the investigation of plant viruses in the early 1980s was immense, ushering in the current era of research on plant virus–host interactions at the cellular level. Ahlquist and colleagues (8) were the first to report the generation of infectious plant virus transcripts from cloned complementary DNA (cDNA) of the genome of BMV. Their success was facilitated by the fact that the BMV genome consists of three segments of positive-sense RNA, the largest being 3.2 kb. Their approach was to clone each of the three BMV cDNAs behind a modified bacteriophage lambda P_R promoter such that transcription would correctly initiate at the first nucleotide of the inserted sequence. Transcripts of each segment with correct 5' and 3' ends were generated *in vitro* using *Escherichia coli* RNA polymerase, and these proved to be infectious when mechanically inoculated onto susceptible host plants or transfected into plant protoplasts. Generating full-length cDNAs that could be transcribed into an infectious transcript for the larger (at least in terms of contiguous nucleotides) genomes of RNA plant viruses like TMV (6.4 kb) and the potyviruses tobacco vein mottling virus and plum pox virus (~10 kb) proved to be more difficult, but followed within a few years (83,288).

It was more straightforward to clone infectious DNA copies for plant viruses with DNA genomes like the geminiviruses with their single-stranded DNA (ssDNA) genomes, or the pararetrovirus cauliflower mosaic virus (CaMV) with its double-stranded DNA (dsDNA) genome. However, here the problem was how to efficiently infect plants with these cloned DNAs. The same issue in principle existed for positive-sense RNA viruses like the luteoviruses that could not be mechanically transmitted to plants, and for the negative-sense plant rhabdoviruses and tospoviruses (in the *Bunyaviridae*), in which the genome alone would not be infectious. The earliest solution was developed in studies of CaMV and the geminivirus tomato golden mosaic virus (TGMV) and took advantage of the ability of *Agrobacterium tumefaciens,* the bacteria that causes crown gall tumors on plants, to transfer its tumor-inducing (Ti) plasmid into plant cells by a mechanism that resembles bacterial conjugal DNA transfer (126). In crown gall tumor, bacterial Ti plasmid sequences are integrated into the plant chromosome, and studies to define the mechanism by which this occurs led to the development of vectors that are commonly used to generate transgenic plants. In the agroinfection procedure that

was developed, the CaMV or TGMV genomes were each cloned into vectors that would autonomously replicate in *A. tumefaciens.* These would be transferred into plant cells by the same mechanism as Ti plasmid transfer when the bacteria were either injected into the plants or introduced at wounded sites (Fig. 2). Once inside the plant cells, infection was initiated when inserted viral sequences would replicate or recombine out of the bacterial vector to reform the virus genome. This same general approach can be used to infect plants with the cloned cDNA copies of RNA virus genomes and viroids, as well as to generate transgenic plants that contain specific viral sequences (126). The introduction of biolistic bombardment in the early 1990s to "shoot" cloned DNAs into plant cells provided an alternative approach to using *A. tumefaciens* to transform plants, as well as to infect them with cloned plant virus genomes or introduce reporter gene constructs for transient expression assays.

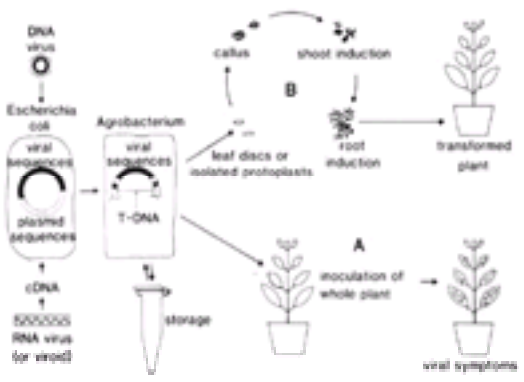


FIG. 2. Agroinfection: transfer of a viral genome or viroid RNA to plants. The gene or genome of interest is cloned into a plasmid that can replicate in *Agrobacterium tumefaciens*. It contains at least the Ti plasmid right border sequence; thus, it can be transferred into plant cells by the action of the bacterial *Vir* genes. The outcome of infection, or integration to create a transgenic plant, is determined by the Ti plasmid having a left as well as a right border sequence (integration) and on whether the introduced plasmid contains a viral sequence that can autonomously replicate when released from the plasmid in the plant. (From Grimsley N, Bisaro DM. Agroinfection. In: Hohn TH, Schell J, eds. *Plant DNA infectious agents.* New York: Springer-Verlag, 1987:87—107, with permission.)

The impact of reverse genetics on plant virology cannot be overstated. Having clonal isolates of plant virus genomes and the techniques in place to introduce these back into plants in the absence of a vector meant that defined mutations could be introduced and studied for their effects on the virus replication, transcription, and host interactions. Thus, individual viral genes could be readily identified and functions assigned to these. Viral genomes and genes were used for *in situ* hybridization to track virus multiplication and infection in the plant and were expressed in *E. coli* to generate antisera for identifying and localizing individual virus-encoded proteins in infected plants. Sequence analysis and hybridization studies established the relationships among viruses and, extended through the use of the polymerase chain reaction (PCR), have produced a veritable growth industry in the identification and cloning of new plant viruses, which has profoundly affected studies on plant virus epidemiology. b-Glucuronidase (GUS) and the jellyfish green fluorescent protein (GFP) expressed from plant virus genomes have provided visual reporters to trace the path and tissue specificity of virus infection in the plant and, fused to individual plant virus proteins, are identifying the cellular components and pathways used by plant viruses. Given the relative ease with which a number of plant species can be transformed, the functions of individual plant virus genes and their role in pathogenesis are being studied in transgenic plants. Indeed, plant virus genomes themselves have been termed a veritable "tool box" for plant genetic engineering and crop protection (374), being the source of strong promoters and translational enhancers that are routinely used to express foreign genes in plants, and of individual virus genes to confer resistance against virus infection. For example, it is almost impossible to read about transgenic plants without coming across reference to the strong CaMV 35S promoter (24). What we have learned is that plant viruses are not fundamentally different from animal viruses in their basic replication and gene expression strategies, which is logical because they are after all eukaryotic viruses. However, because of fundamental differences in the structure of plant and animal cells and in the physiology of plants and animals, plant viruses are fundamentally different in how they enter and exit from cells and systemically invade their hosts, and they have evolved some clever strategies to deal with these problems.

In the previous edition of this textbook, it was noted that interactions between plant viruses and their vectors or the earliest events in the infection process were poorly understood, that detailed information about plant virus replication and cell-to-cell movement was just beginning to accumulate, and that the mechanisms by which plant viruses induce disease responses had not been determined (313). Yet, John Shaw in writing this was optimistic that it seemed "certain that the increasingly powerful techniques of molecular and cellular biology should provide us with explanations of these phenomena within a relatively short period of time." His optimism was not misplaced. Since 1996, when the last edition of this text was published, we have made major advances in all of these areas. This chapter focuses on the molecular and cellular details of plant virus replication and pathogenesis, highlighting the studies of a number of representative model plant viruses that have advanced our understanding of how plant viruses interact with their insect vectors, how different plant viruses replicate and regulate their gene expression, how these viruses move from cell to cell to systemically infect the host, and the interplay between virus and plant that results in disease or resistance of the host. An excellent overview of the families and genera of plant viruses, with descriptions of representative viruses within each group, can be found in the third edition of this book (313). The *Seventh Report of the International Committee on Taxonomy of Viruses* (ICTV) (347) and the *Encyclopedia of Virology* (123) present more detailed descriptions of all known viruses and viroids and their current classification. Additional excellent texts and reviews that describe the diseases caused by and the epidemiology of different plant viruses, as well as subviral agents (viroids and satellites) that infect plants, are also available (5,73,294).

THE PLANT VIRUS FAMILIES

According to the latest ICTV report (347), the number of recognized viruses is 3,618, of which ~25% (957) are plant viruses. In addition, there are subviral agents (formerly called *unconventional agents*) that can infect plants, namely viroids and satellites. Satellites are analogous to the d-agent of hepatitis B virus, being encapsidated RNAs that are not essential for the infectivity of a particular plant virus “partner” but can modulate the pathogenicity of that virus (294). Viroids, now classified in seven different genera, are small (~250 to 370 nt), highly structured RNAs that are responsible for a number of important diseases in plants, including potato spindle tuber disease, coconut cadang-cadang, and avocado sunblotch.

As for animal viruses, a system based on the use of Latin names has been adopted to classify plant viruses into families and subfamilies. Genera within these are based on running together the first few letters of the name of the virus that is considered to be the “type member” for that group. For example, *Tobamovirus* comes from *tobacco mo*saic virus, *Potexvirus* from *pot*ato virus *X*, and *Tospovirus* from *to*mato *spo*tted wilt virus (Fig. 3). This approach to classification has at times led to vigorous debate because the genera designations are not necessarily intuitive for the nonexpert, and even those in the field can find them difficult to remember. As is the case for animal viruses, researchers generally use common names to refer to individual viruses.

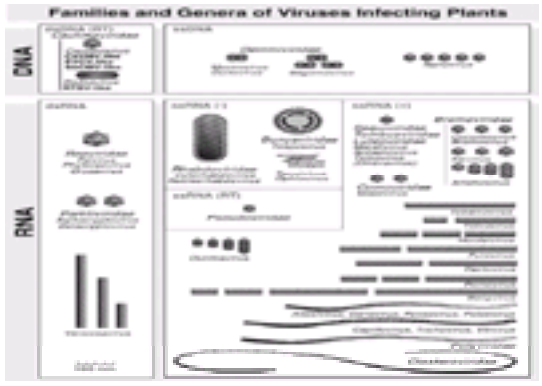


FIG. 3. Classification of plant viruses. (From ref. 347, with permission.)

Figure 3 illustrates several important points about plant viruses in relation to animal viruses. The vast majority of plant viruses are nonenveloped positive-sense ssRNA viruses. There are few enveloped plant viruses, and these are negative-sense (the rhabdoviruses) or ambisense (the tospoviruses and tenuiviruses) ssRNA viruses. There are few DNA plant viruses, and none of these are true dsDNA viruses. Thus, there are no simple dsDNA viruses corresponding to the papovaviruses, and there are no dsDNA viruses of the complexity of the adenoviruses, herpesviruses, or vaccinia viruses. The caulimoviruses and badnaviruses have dsDNA genomes of ~7.2 to 8.2 kb in size but employ a reverse-transcribing replication strategy through a positive-sense ssRNA intermediate, as do the hepadnaviruses (see Chapter 86). The geminiviruses and the nanoviruses have small multicomponent circular ssDNA genomes (one or two genome segments of ~2.6 to 2.9 kb for geminiviruses, and at least six genome segments of ~1.0 to 1.2 kb for nanoviruses) and, like the parvoviruses, replicate through dsDNA intermediates. In contrast to animal viruses, in which segmented genomes are encapsidated in a single particle, the majority of plant viruses (positive-sense ssRNA, and ssDNA) have segmented genomes (referred to as *multicomponent*), with each genome segment encapsidated in a distinct particle. Only a few plant viruses belong to recognized families of animal viruses: *Reoviridae, Rhabdoviridae,* and *Bunyaviridae.* The diversity of plant virus families and genera classified according to the scheme in Figure 3 is striking. Yet despite this diversity, sequence analyses have revealed striking similarities, both among the RNA plant viruses and between plant and animal RNA viruses, as well as among caulimoviruses, retroviruses, and hepadnaviruses. Such comparisons have led to the grouping of plant and animal RNA viruses into “superfamilies” that share common sequence arrangements in their RNA-dependent RNA polymerases (RdRp), helicases, methyltransferases, genome-linked viral-encoded protein (VPg) or encoded proteinases, and common strategies of gene expression (see Chapter 13). Several of these points are discussed further in the following sections in the context of functional studies on specific plant viruses and the differences between plants and animals.

CONSEQUENCES OF THE PLANT CELL WALL

The production of a rigid cell wall by plant cells is one of the two fundamental alterations that account for most of the differences between plants and animals (the other is carbon dioxide [CO_2] fixation through photosynthesis). Thus, perhaps it is not surprising that most of the basic differences between plant and animal viruses can be traced to plant cells being surrounded by this wall of long cellulose microfibrils (extensive polymers of b1®4–linked glucose residues) embedded in a matrix of branched polysaccharides (hemicellulose) and pectins, and the resultant rather sedentary lifestyle this imposes on plants. The plant cell wall is an effective barrier to virus entry and spread within the plant. External surface layers composed of cutin and waxes provide an additional protective barrier for the plant as a whole. Thus, to enter the plant initially and establish infection in a cell, a virus must mechanically breach these barriers. In addition, the virus will have to be delivered to the plant.

Plant-to-Plant Transmission

Although in nature a few plant viruses have been reported to be transmitted by direct plant-to-plant contact, and some can be introduced by mechanical abrasion with contaminated implements, most plant viruses are transmitted to their hosts through the action of a vector. In the vast majority of cases, this involves introduction into the aerial parts of the plant by the action of insects feeding on leaves and stems (124) (Table 2). The insect acquires the virus by feeding on infected plants and then transmits it when feeding on uninfected plants. These insect vectors include aphids, whiteflies,

leafhoppers, mealy bugs, thrips, mites, and beetles. The type of insect vector is specific for a particular genus of plant viruses. The insect-transmitted form is the virion itself, as suggested by genetic studies that show the plant virus coat or capsid protein (CP) is invariably required for insect transmission. The virus may simply become associated with the insect cuticle (noncirculative), or it may have to be altered by passing through tissues of the insect (circulative) before it is transmitted back to plants (Table 3). Because insects feed multiple times on a given plant, viruses are usually introduced at high multiplicities. This could explain the prevalence of multipartite plant viruses having separately encapsidated genome components.

	Aerosols (airborne) or ingestion (waterborne or foodborne)	Fluids (direct contact)	Parent to offspring	Vectors
Animal viruses	Most	Few	Few	Many
	Picorna	Hepadna	Retro	Toga
	Orthomyxo	Retro	Herpes	Flavi
	Corona	Herpes	Arena	Bunya
	Reo	Papilloma		Rhabdo
Plant viruses	None	Few	Many	Most
		Tobamo	Hordei	Poty
		Tombus	Ilar	Potex
		(water, mechanical)	Poty (seeds, pollen, bulbs, cuttings)	Gemini
				Luteo
				Tospo (Bunya) (insects, nematodes, fungi)

[a]Examples indicate that some, not necessarily all, viruses within the family are transmitted by the given mode. (Based on ref. 124.)

TABLE 2. *Transmission of viruses between hosts*[a]

	Noncirculative (cuticle associated)		Circulative (tissue associated)	
	Stylet borne (nonpersistent)	Foregut borne (semipersistent)	Nonpropagative (persistent)	Propagative (persistent)
Example	Potyviruses	Caulimoviruses	Luteoviruses	Tospoviruses
Acquire[a]	Seconds	Minutes	Hours	
Inoculate[b]	Seconds	Minutes	Hours	
Retain[c]	Hours	Days	Days	
Latent period[d]	No	No	Yes	

[a]Minimum feeding time required for an insect to acquire[a] or transmit[b] a virus.
[b]Period of time for which an insect retains the ability to transmit a virus.
[c]Lag between the time an insect acquires a virus and the time it becomes competent to transmit that virus.
Based on ref. 124.

TABLE 3. *Modes of plant virus insect transmission*

Viruses that are closely related are transmitted by the same type of vector and in a similar manner. For example, luteoviruses and potyviruses are both transmitted by aphids, but their relationships to the aphid differ. For potyviruses, the aphid is similar in principle to a flying syringe, although the interaction is more specific. The virus binds to a receptor(s) in the aphid stylet (the feeding probe), but the aphid is not a host for the virus: it simply serves to inject the virus into a plant during feeding, probably by a process of ingestion and salivation (124). This type of transmission mode has been termed *nonpersistent* and is now referred to as *stylet borne.* It is characterized by rapid insect acquisition and inoculation times (on the order of seconds), and the insect retains the ability to transmit the virus for only a few hours (see Table 3). In contrast, luteoviruses have to circulate through the aphid, passing from the insect midgut to the hindgut and crossing the hindgut wall to enter the hemocoel. From the hemocoel, the virus moves to the salivary glands and then enters the salivary canal, from which it will be inoculated into plants during aphid feeding. Thus, there is an obligatory biological relationship between the luteovirus and the aphid, one in which the acquired virus is altered in preparation for transmission. There is evidence to suggest that this alteration may involve proteolytic cleavage of a proline-rich extension in a subset of the viral CP subunits that make up the viral capsid. Although luteoviruses do not in fact multiply in the aphid, the insect is referred to as a “host,” but a nonpropagative one (see Table 3), and it is considered “infected” with the virus. This circulative mode of transmission is characterized by long acquisition and plant inoculation times, on the order of hours. It was formerly referred to as “persistent” because the insect can transmit the virus for many days, and potentially throughout its life, once it has acquired the virus.

A number of plant viruses that are transmitted by the circulative mode have been shown to multiply in their insect vector, thus infecting the insect in the more conventional sense. The insect in these cases is termed a *propagative host* (see Table 3). Perhaps not surprisingly, most of these plant viruses, which when viewed from their ability to multiply in the insect could be considered to be “animal viruses,” belong to well-recognized animal virus families— *Reoviridae, Rhabdoviridae,* and *Bunyaviridae*— although some apparently do not (marafiviruses) (see Table 3). The tospoviruses, named for the type member TSWV, are a genera within the *Bunyaviridae,* and TSWV illustrates at least some of the adaptations a plant virus may evolve to achieve this dual lifestyle.

TSWV, like the animal-infecting *Bunyaviridae,* has a tripartite ssRNA genome encapsidated in three nucleocapsids, which are surrounded by an envelope. The envelope contains heterodimeric spikes composed of the viral-encoded G1 and G2 glycoproteins. TSWV is transmitted by thrips in a circulative and propagative manner. The virus, once ingested, passes to the thrip midgut, where it may bind to a receptor(s) (17), and crosses the midgut wall, apparently by endocytosis. TSWV multiplies in the infected thrip, eventually reaching the salivary glands and salivary canal, from which it is transmitted to other plants. Comparison of the TSWV genome organization with that of the animal-infecting *Bunyaviridae* shows that its M segment, which encodes G1 and G2 in all *Bunyaviridae,* is uniquely ambisense, being transcribed into a subgenomic RNA that is translated into a plant virus-specific NSm protein (see Chapter 48). Both the glycoproteins and

NSm are needed for TSWV to exit from an infected cell, but in very different ways. G1 and G2, indeed the entire enveloped structure, are required for multiplication in and transmission by thrips, but are completely dispensable for infecting plants. This was graphically shown by the studies in which TSWV was serially passaged in tobacco plants by mechanical abrasion in the complete absence of the insect vector (285). After a number of passages, virus was obtained that was highly infectious when mechanically inoculated on plants but could not be transmitted by thrips. This mutated TSWV would still react with antinucleocapsid antisera but was no longer recognized by either anti-G1 or anti-G2 antisera, suggesting that the glycoproteins were absent. This was confirmed and extended in electron microscopic studies that showed these mutant TSWV preparations consisted of the typical viral RNPs, with no enveloped particles being present. Molecular studies in insect cells and gel overlay assays with tissue extracts from thrips suggest that the glycoprotein spikes are required for attachment and penetration in the thrips vector (17,171). NSm is not required for insect transmission or replication of the viral genome (272). Rather, it is a unique plant virus protein—a movement protein—that is needed for the virus to cross the cell wall and systemically infect the plant.

The specificity of the virus–insect interaction requires virus-encoded protein and presumably a receptor protein(s) in the insect, although few such candidate receptors have been identified. As shown for the geminiviruses, in nonenveloped viruses, the CP can be the only virus-encoded protein required for the specificity of interaction with the insect vector. There are different genera of geminiviruses, and the begomoviruses, such as African cassava mosaic virus (ACMV), are transmitted by whiteflies, whereas the curtoviruses, such as beet curly top virus (BCTV), and the mastreviruses, such as maize streak virus, are transmitted by different leafhopper species (see Fig. 3). When ACMV was engineered to express the BCTV CP in place of its own, the recombinant ACMV genome encapsidated in the BCTV CP was now transmitted by leafhoppers, and not by whiteflies, which demonstrated a direct role of the geminivirus CP in insect recognition (31). However, for some nonenveloped plant viruses, a virus-encoded transmission accessory or “helper” factor is required, in addition to the CP, for the specific interaction with receptors in the insect. This is the case for the potyviruses and their aphid vectors, where it has been suggested that a dimeric form of the viral-encoded helper factor (helper component, the HC domain of HC-Pro) acts as a bridging molecule between the virion capsid and receptor(s) in the cuticle lining the aphid stylet (264). The viral spikes in TSWV appear to bind directly to candidate receptor proteins in thrips (17,171), and it is reasonable to think that this may be generally true for all enveloped plant viruses that are transmitted by insects. Our understanding of plant virus attachment to and passage through, or replication in, the insect vector is currently rudimentary owing to the general lack of stable virus mutants that are defective in insect transmission, and of cultured insect cells in which to study plant virus attachment and replication. The functions in virus penetration or circulation of the few candidate receptor molecules that have been identified remains to be defined. Recent technical advances, such as the establishment of cultured thrips and leafhopper cell lines for replication studies, and genetic approaches to generate stable virus mutants (172,241,279) should aid in addressing these questions and increase our knowledge of how virions are altered as they pass through their insect vectors.

Elementary as our knowledge is about insect transmission of plant viruses, we understand even less about the transmission of plant viruses by fungi or nematodes, or their horizontal transmission through seed or pollen (see Table 2). In those instances in which it has been is studied, both virus-encoded and host-encoded components appear to be important in these modes of transmission. As for insect transmission, the evidence suggests that virus particles are transmitted by nematodes and fungi because, in the few cases in which it has been investigated, at least the viral CP is required for transmission by these vectors (125,212). About 18% of plant viruses can be transmitted through seeds in at least one host, a process that is more significant in terms of economic and epidemiologic impact than this number might imply once one considers the number of infected seeds that can be produced by a field of plants. In all cases, except for TMV, seed transmission requires that the embryo be infected either directly or indirectly through infection of the gametes prior to fertilization (221). Molecular studies of barley stripe mosaic virus (a hordeivirus), the potyvirus pea seed-borne mosaic virus, and the tobravirus pea early browning virus indicate that the process is complex. Nevertheless, in two of these cases, a viral-encoded protein that is a “pathogenicity determinant” has been identified as a determinant of seed transmission (96,162,362). This suggests that perhaps efficient systemic invasion of the host is a factor in the ability of some plant viruses to overcome tissue barriers and invade the developing embryo.

Adaptation to the Cell Wall: The Fundamental Difference Between Plant and Animal Viruses

Once mechanically introduced into a plant, by insect feeding or other means, plant viruses will initiate infection of the inoculated cells, most commonly within a leaf. However, the problem of the plant cell wall does not end here because it is an effective barrier to plant viruses exiting from as well as entering a cell. The virus must do this to spread from cell to cell within the inoculated leaf and then invade the growing plant, a process termed *systemic infection.* A fundamental distinction from bacteriophage, which also have to negotiate their way across cell walls, is that plant viruses infect and invade an integrated multicellular host, not a population of autonomously functioning walled cells. Plant cells, although surrounded by walls, are connected by transwall channels termed *plasmodesmata,* the formation and function of which can be developmentally regulated. Through these channels, all plant cells are potentially in communication with each other through the passage of solutes and small molecules, and recent studies suggest that even rather large proteins may pass through plasmodesmata at certain developmental stages (252,291). The term *symplasm* was coined to reflect this potential cytoplasmic continuity among plant cells. The spaces between plant cells constitute the *apoplasm.* If plant viruses were to adapt the solution of most phage (except f1/M13) of encoding the equivalent of a lysozyme function (a “cellulase”) to dissolve the wall of the infected cell, they would find themselves in the apoplasm facing the next cell wall. Worse yet, the “cellulase” could digest the walls of the surrounding cells, which, given their high internal hydrostatic pressure, would burst—not a useful outcome for the virus.

Although the cell wall presents a barrier, the plasmodesmata present an opportunity for the plant virus to move directly from cell to cell without having to go through an extracellular phase. This is similar in principle to cell-to-cell spread of vaccinia virus or human immunodeficiency virus (HIV), but in a manner that is potentially less destructive to the plant cells. All plant viruses encode a unique class of proteins termed movement proteins (MPs), which modify or alter plasmodesmata so that the virus genome can spread directly from cell to cell, effectively “tunneling” through the cell wall in a nondestructive way. The MP is essential for this process, as indicated by the name, which originates from the genetic studies that first identified the genes encoding such proteins. A plant virus that is mutated in the gene encoding this MP will still replicate in individual plant cell protoplasts and assemble virus particles, but this virus will be defective in

spreading within the inoculated leaf and systemically infecting the host. The classic studies on the TMV temperature-sensitive *Ls1* mutant (245) were among the first to show that such MPs exist. TMV *Ls1* can systemically infect tomato plants at 22°C but will not do so when the plants are grown at 32°C, although it replicates well at both temperatures. When tomato plant leaflets mechanically inoculated with this *Ls1* mutant were grown for 24 hours at 22°C or 32°C and stained with fluorescently tagged antivirus antibody that would detect TMV CP, fields of large numbers of adjoining infected cells that accumulated viral CP could be seen at the permissive temperature of 22°C. This was also true for leaflets at both temperatures inoculated with wild-type TMV. In contrast, only individual single cells were found to accumulate CP in the *Ls1*-inoculated leaflets grown at 32°C. This demonstrated that the *Ls1* mutant could replicate at the nonpermissive temperature because it was synthesizing viral CP. However, the fact that only individual single cells were seen to be infected, rather than fields of contiguous cells, showed that this *Ls1* mutant was defective at the high temperature in moving from the inoculated cell into neighboring cells within the leaf. As described in more detail later, we now know that this *Ls1* mutant has a missense mutation in the 30-kd MP of TMV (71).

Although the MP is essential for this cell-to-cell movement, the viral CP may or may not be required. TMV and several other positive-sense RNA viruses do not require CP to move locally from cell to cell within the infected leaf. Neither do the ssDNA bipartite geminiviruses (begomoviruses). As discussed later, although the mechanisms differ, the genomes of these viruses can move from cell to cell in a complex with a movement protein. Other plant viruses, such as TSWV, CaMV, and potato virus X (PVX), require CP in addition to movement proteins to move locally from cell to cell, moving as virions or subviral particles. Thus, although plant viruses are transmitted from plant to plant in the form of virus particles, virions are not necessarily the form in which the plant virus genome is transmitted directly from cell to cell, at least within an infected leaf (194). This is in stark contrast to animal viruses and phage. This does not mean that virus particles do not play a role in systemic infection of the plant. For most plant viruses, movement out of the inoculated leaf to distant parts of the plant to establish systemic infection occurs through the nutrient transport system of the plant, the phloem, by way of companion cells connected to the sieve elements or “tubes” that form the conduits for long distance transport of nutrients. Evidence suggests that most plant viruses require CP for this long-distance movement, although whether this occurs in the form of stable assembled virions in all cases remains to be shown.

As suggested from the preceding considerations, an additional distinct feature of plant viruses when compared to animal viruses and phage is that there is no evidence for the attachment of virus particles to specific receptors on the surface of plant cells to initiate infection. Such virus–receptor interactions do appear to be important for recognition of plant viruses by their insect vectors, and perhaps other agents, for transmission. It also seems likely that within the vector, receptor-mediated endocytosis or receptor binding, followed by cell surface fusion, will be used by plant viruses that infect their insect vectors such as the topsoviruses and plant rhabdoviruses. Whether this represents another plant virus adaptation to the presence of the plant cell wall or the physiology of the plant, such an interaction has not been demonstrated to play a role in virus infection of a plant host. Few studies have focused on the mechanism by which plant virus particles or the genome in some other packaged form moves out of phloem sieve elements to penetrate plant cells when invading a new leaf, or on the uncoating of the plant virus genome, and we understand little about these processes.

PLANT VIRUS GENE EXPRESSION AND REPLICATION STRATEGIES

Given the relatively compact nature of their genomes, plant viruses, like animal viruses, have devised a variety of strategies to expand their coding capacity and regulate their gene expression while adhering to the rules for the translation of mRNAs in their eukaryotic hosts. These strategies, and the viral replication schemes, are all found among the animal viruses. Plant viruses with ssRNA genomes each replicate through an antigenomic or complementary strand, which serves as template for the synthesis of progeny strand genome ssRNA: the antigenomic replication template is negative-sense for a positive-sense RNA virus and the same polarity as mRNA (positive) for a negative-sense ssRNA virus, but it is not translated. Thus, exactly like ssRNA animal viruses, plant ssRNA viruses have to reconcile their genome structure with the requirement for monocistronic mRNAs in eukaryotic cells. In doing so, positive-sense ssRNA plant viruses regulate their gene expression at the level of transcription, through the production of subgenomic RNA (sgRNA); and at the level of translation, through polyprotein synthesis, leaky ribosome scanning, suppression of termination (called *readthrough translation*), ribosomal frameshifting, or internal ribosome entry (see Chapter 5). The production of sgRNA is the most common strategy employed; however, most positive-sense ssRNA plant viruses use more than a single strategy to regulate and optimize their gene expression. The ambisense tospoviruses, in the *Bunyaviridae* (see Chapter 48), regulate their expression at the transcriptional level through cap snatching and the production of sgRNA, and by a polyprotein strategy at the translational level. The negative-sense plant rhabdoviruses probably employ a strictly monocistronic transcriptional strategy, as do the animal rhabdoviruses (see Chapter 38). Reinitiation of translation and RNA editing, strategies employed along with these others by different animal ssRNA viruses, have not been reported for ssRNA plant viruses.

In terms of plant DNA viruses, geminivirus circular ssDNA genomes are replicated through dsDNA intermediates by a rolling circle mechanism, as has been shown for the replication of the ssDNA genome of bacteriophage fX174 and proposed for the replication of herpesvirus dsDNA (see Chapter 22 and Chapter 72). The geminivirus dsDNA forms are also templates for the transcription of viral mRNAs, and gene expression is temporally regulated through transcriptional cascades, as it is in animal DNA viruses, such as the papovaviruses, adenoviruses, and herpesviruses, and in DNA bacteriophage. Alternative splicing of transcripts, as is found in many DNA animal viruses and the retroviruses, also regulates gene expression in some geminiviruses and in the caulimoviruses, such as CaMV and figwort mosaic virus (FMV). For these latter viruses, and the badnaviruses, the dsDNA genome is replicated by a reverse-transcribing mechanism through a positive-sense ssRNA in a manner similar to the hepadnaviruses (see Chapter 86). CaMV also regulates its gene expression through ribosome shunting (also used by adenovirus type 2) and the use of two promoters, and a virus-encoded transactivator acts to couple the translation of genes so that the viral 35S transcript is translated as a polycistronic mRNA even though it uses the eukaryotic translation machinery.

These strategies and principles are illustrated in the following sections by examining in detail the multiplication of plant viruses within several genera in an attempt to present the current state of our knowledge and highlight the experimental approaches being used. The particular viruses are not necessarily the most important in terms of disease potential and economic impact, but rather were chosen because they have been extensively characterized at the molecular,

biochemical, and/or cellular levels and each illustrates particular points about the regulation of gene expression and virus interactions with plant cells. All plant viruses that have been sequenced and characterized to date employ some combination of the strategies that are discussed in these sections. Unsegmented (monopartite) positive-sense ssRNA viruses are presented first, chosen to illustrate the spectrum of positive-sense RNA virus strategies in a variety of combinations. These are followed by an example of a positive-sense ssRNA plant virus with a segmented genome, enveloped plant viruses, and plant DNA viruses. All of the RNA viruses in the following sections, except for some of the rhabdoviruses, are cytoplasmic viruses. In addition, all of the viruses that are presented, save for the *Luteoviridae* and *Geminiviridae*, infect and replicate in mesophyll cells (the cells between the veins that make up the bulk of a leaf). Viruses in the family *Luteoviridae* are limited to infecting the phloem tissue within the veins, and this is also true for a number of the *Geminiviridae*.

Positive-Sense Single-Stranded RNA Viruses

Potyviruses: Polyprotein Strategy

The largest of the plant virus families, the *Potyviridae,* encompasses the potyviruses, rymoviruses, and bymoviruses, along with several other genera (347). The potyviruses themselves constitute the largest genus of known plant viruses (~30%) and as a group are responsible for the highest losses of crop plants worldwide (5). Although *pot*ato virus *Y* is the type member for this genus (hence “poty”), other potyviruses, such as tobacco etch virus (TEV), tobacco vein mottling virus (TVMV), and plum pox virus (PPV), have been better characterized at the molecular level. The virions are flexuous filamentous rods 11 to 15 nm in diameter and 680 to 900 nm in length (see Fig. 3) and are transmitted by aphids. The capsid is assembled from subunits of a single protein, the viral-encoded coat protein (CP). The potyviral genome is an unsegmented (monopartite) positive-sense ssRNA of ~10 kb that has a viral-encoded protein (VPg) covalently linked to its 5' end and is polyadenylated at its 3' end (reviewed in 290). With the initial complete sequencing of the genomes of TEV, TVMV, and PPV, it became clear that the potyvirus genomic RNA encodes a single open reading frame (ORF), which could be translated into one large polyprotein of 340 to 370 kd, similar to the animal picornaviruses. This, and similarities with picornaviruses and flaviviruses, as well as plant nepoviruses and comoviruses, in predicted amino acid sequence motifs, and the organization of proteins potentially involved in RNA replication, eventually led to the proposed superfamily of picorna-like plant and animal viruses (120) (see Chapter 13).

Prior to the availability of complete genome sequences and infectious clones, several of the potyvirus-encoded proteins were identified by a combination of biochemical and immunological studies of virions, insect (aphid) transmission, and infected plant tissue, and their names reflect these original associations. Thus, the viral CP was originally “Cap” for capsid protein, VPg is the genome-linked viral protein, and HC is the “helper component” N-terminal domain of HC-Pro (found in amorphous cytoplasmic inclusions) that is required as an accessory factor for insect transmission of virions (this has been extensively characterized, as reviewed in 264). Three additional viral proteins were associated with characteristic inclusions in infected cells: CI with cytoplasmic *cylindrical inclusions,* and NIa and NIb with *nuclear inclusions*.

The single polyprotein encoded by the potyvirus genomic RNA is cotranslationally and posttranslationally cleaved to produce all of the mature viral proteins by the action of three viral-encoded proteinases: P1, HC-Pro, and NIa (39,40,116,135,353) (Fig. 4). P1 and HC-Pro are responsible for two distinct autoproteolytic (*cis*) cleavages within the N-terminal region of the polyprotein. NIa, which resembles the picornavirus 3C proteinase, carries out the remaining cleavages (both in *cis* and in *trans*) within the C-terminal two-thirds of the polyprotein. NIa is composed of an N-terminal VPg domain and a C-terminal proteinase domain. This proteinase domain, and the C-terminal proteinase domain of P1, are both structurally related to the chymotrypsin class of serine proteinases. HC-Pro, also having a C-terminal proteinase domain, is a papain-like cysteine proteinase. P1, HC-Pro, and NIa are not only required for the correct proteolytic processing of the viral-encoded polyprotein: each proteinase is a multifunctional protein with additional roles in the virus life cycle. This multifunctionality appears to be true for most of the potyvirus proteins.

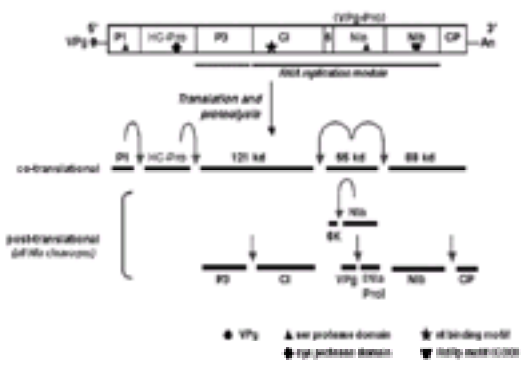


FIG. 4. Genome organization and polyprotein processing of tobacco etch virus (TEV). (Adapted from refs. 287 and 290, with permission.)

P1 autoproteolytically separates itself from HC-Pro (see Fig. 4). This proteolytic cleavage is essential for virus infectivity, but P1 proteolytic activity *per se* is not. This was shown in studies with TEV using mutants that were nonviable because each encoded a processing-defective P1 proteinase. Each mutant could be restored to infectivity by the insertion of an NIa cleavage site between P1 and HC-Pro (350). P1 can bind ssRNA *in vitro* (30,352), and based on this and sequence comparisons, it was long speculated that P1 might be an MP. To address this point, a modified strain of TEV was engineered to express the bacterial *GUS* gene (TEV-GUS) as a reporter. When protoplasts prepared from tobacco plants are transfected with TEV-GUS RNA, the level of virus replication can be determined based on a fluorometric assay to

detect GUS activity, produced from the viral genome, in cell extracts. When plants are inoculated with TEV-GUS, in addition to quantitating GUS activity in leaf extracts using this assay, intact leaves can be fixed and stained for GUS activity using a histochemical substrate (X-gluc) that will stain GUS-expressing cells blue (Fig. 5). Thus, using this GUS-expressing virus, mutations in P1, or in any other viral protein, can be directly assayed in plants for their effects on virus movement as well as virus replication. A mutant in which the P1 coding sequence was deleted from TEV-GUS replicated to low levels in protoplasts (~3% wild-type levels), suggesting that while P1 was not essential for replication, it did play an accessory role. Despite this poor replication, this mutant was capable of cell-to-cell and leaf-to-leaf (systemic) movement in inoculated plants (351). The level of replication in protoplasts could be increased if the protoplasts were prepared from transgenic plants engineered to express P1. Thus, P1 is not an MP but may be a *trans*-acting accessory factor that stimulates viral genome amplification. P1 could exert this effect directly by interacting with viral RNA or proteins in the replication complex, or more analogous to the 2A proteinase of picornaviruses, could indirectly affect genome amplification by stimulating viral RNA translation (351) (see Chapter 23).

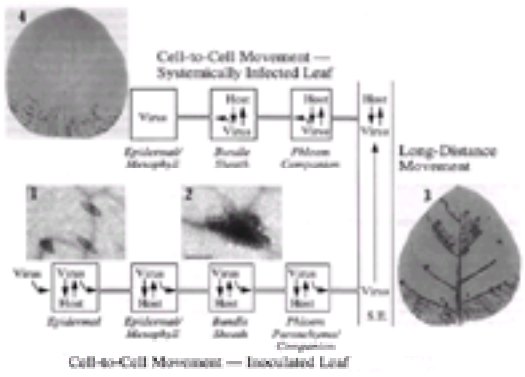


FIG. 5. Path of virus local and long distance movement as visualized in plants infected with tobacco etch virus (TEV) expressing a *GUS* reporter gene. Virus that is mechanically inoculated onto a leaf first moves from the surface epidermal cells to the underlying mesophyll cells. It then moves from cell to cell in the mesophyll to reach bundle sheath cells, from which it enters phloem parenchyma and companion cells. Long distance movement to enter a distal leaf occurs through the sieve elements of the phloem. At each stage, the virus is interacting with host factors (indicated by *boxes*). (From ref. 43, with permission.

The CI-6K-NIa-NIb region of the potyviral polyprotein is similar in sequence and organization to the 2C-3A-3B-3C-3D region of the picornavirus polyprotein, which led to its original designation as the RNA replication module (290) (see Fig. 4). Molecular genetic and biochemical studies have established that this similarity extends to function as well, with NIa (VPg-proteinase) playing an essential regulatory role similar to that of the picornavirus 3C proteinase (see Chapter 23). Potyvirus replication occurs in the cytoplasm, apparently in association with endoplasmic reticulum (ER)-derived membranes (Fig. 6). Several lines of evidence suggest that the 6K protein, probably in the form of the 6K-NIa polyprotein (see Fig. 4), may act to target and anchor replication complexes to these membrane sites. TEV infection causes a collapse of the ER network into aggregated structures, and 6K appears to be associated with these ER-derived membranes. This was shown by expressing a fusion of 6K and the jellyfish green fluorescent protein (GFP:6K) in living cells from plasmids or the TEV genome, which could be introduced into leaf cells by biolistic bombardment, transfected into protoplasts, or inoculated onto plants. Using confocal microscopy and immune staining for marker proteins known to be located in the ER or Golgi, the GFP:6K was shown to localize to ER-derived membrane vesicles, and subcellular fractionation studies showed that both replication complexes and the 6K protein were associated with ER-like membranes (304).

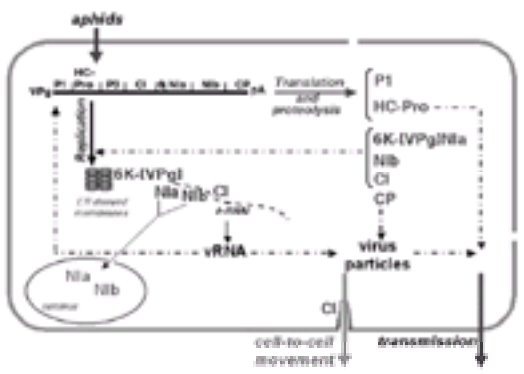


FIG. 6. Multiplication of tobacco etch virus (TEV). See Fig. 4 for the proteolytic events catalyzed by P1, HC-Pro, and NIa to process the TEV polyprotein into the individual encoded proteins.

Proteolytic processing by NIa, as well as NIa interacting with NIb, the viral RdRp (100,207), appear to be critical in orchestrating these events, with NIa acting in *cis* and NIb in *trans* (see Fig. 6). The latter conclusion is based on the finding that NIa mutants cannot be rescued for replication when transfected into protoplasts prepared from transgenic plants that express NIa, but NIb mutants can be rescued in transgenic protoplasts that express NIb (206,303). NIa can bind RNA *in vitro* (58), and TEV and TVMV NIa have also been shown to interact with NIb in yeast two-hybrid and *in vitro* binding assays (100,146,207). Genetic studies indicate that NIa–NIb interaction is essential for virus replication (57). TEV NIa cleaves sites that contain the consensus heptapeptide motif Glu-X-X-Tyr-X-Gln¯Gly/Ser at differing rates, and it

shows *cis*-preferential cleavage at a number of sites in the polyprotein, including those at CI/6K, 6K/NIa, and NIa/NIb. Whether NIa is retained in the cytoplasm or transported to the nucleus appears to be regulated by its association with 6K: the 6K-NIa polyprotein is retained in the cytoplasm, apparently because 6K masks the nuclear localization signal (NLS) which is present in NIa. However, after cleavage to produce the two separate proteins, NIa itself is efficiently transported to the nucleus mediated by its NLS, which is located in the VPg domain of the NIa (287). A suboptimal site that is slowly processed separates the VPg and proteinase domains of NIa itself. Mutants altered at this site to accelerate autocleavage by NIa are defective in amplifying the viral genome, as are mutants in the VPg linkage site (Tyr-62), the NIa (VPg) NLS, or the proteinase active site (303). Thus, nuclear targeting of NIa and slow internal processing between the VPg and proteinase domains, as well as VPg (or NIa) linkage to the viral genome and NIa proteinase activity, are required for viral RNA replication. The model that is consistent with these findings is that the 6K-NIa polyprotein bound to viral RNA by its NIa (VPg-proteinase) domain is anchored to ER-derived membranes through the 6K domain. NIb (RdRp) would be recruited to these replication complexes through a protein–protein interaction with NIa, which would position it next to the VPg domain to stimulate polymerase activity and protein priming of RNA synthesis (57), analogous to what has been proposed for picornavirus RNA replication. Whether VPg acts as a protein primer in potyvirus replication remains to be demonstrated. CI, which *in vitro* has been shown to have the properties of a helicase (95,188), is also required for genome replication (42,177). Such helicase activity is generally needed by large RNA viruses for their replication.

Given the functions of NIa and NIb in replication, why are both found to accumulate in the nucleus? It may be a consequence of the viral polyprotein strategy. CP is needed in large amounts to encapsidate the genome for insect transmission as well as for cell-to-cell movement (see below). Thus, very large amounts of NIa and NIb, as well as the other viral-encoded proteins, will also be made. It has been proposed that targeting NIa to the nucleus may be a way to down-regulate the level of NIa proteinase activity in the cytoplasm by, in essence, removing NIa to a separate compartment. This could be mediated by the NLS within the VPg domain of NIa, or an association with NIb, which itself has two NLSs (57,207,303). Independently, NIb targeting to the nucleus would be a mechanism to decrease the amount of RdRp in the cytoplasm late in infection to favor encapsidation of viral genomes for movement and transmission. It has also been suggested that NIa might act in the nucleus to affect host cell functions such as transcription (303,363).

The reported multifunctional nature of the potyvirus proteins is striking, and whether this to some extent reflects the polyprotein strategy of these viruses is an interesting point to ponder. At least CI and CP are not so different in this regard from other plant virus–encoded proteins as their multifunctional nature may reflect the particular requirements for virus movement. As discussed in the section on movement proteins, studies during the past several years have broadened our view beyond these simply being proteins that move the virus genome across the cell wall: movement proteins coordinate replication of the viral genome with its directed movement within and between plant cells (194). Genetic and ultrastructural studies suggest that CI, the virus-encoded helicase, is the potyvirus MP, associated with cone-shaped structures that align with plasmodesmata in infected cells (42,293). CP is also required for movement apparently because the viral genome is transported either as virus particles or in an RNP-complex with CP (81). Its role in aphid transmission is similarly explained by the fact that virus particles are the form transmitted, in a process that requires the helper component N-terminal domain of HC-Pro (reviewed in 264). Genetic studies suggest that the VPg domain of NIa, as well as HC-Pro, are “factors” involved in virus long distance (systemic) movement as well as genome amplification (55,166,177,282,305), but such studies do not distinguish between these proteins directly or indirectly affecting these processes. For HC-Pro, the effects seem to be the consequence of its role as a “pathogenicity factor,” and indeed HC-Pro may be the most impressive example of a truly multifunctional protein (277,316). It is undoubtedly the helper component for aphid transmission and a proteinase for viral polyprotein processing. However, its effects on virus replication and systemic movement are now known to reflect an additional role of HC-Pro as a viral counterstrategy against a host RNA–mediated defense response (see Plant Responses to Virus Infection). Sorting out the effects of VPg mutations on long distance movement will require a better understanding of the forms in which the potyvirus genome moves, both from cell to cell and from leaf to leaf, and the mechanism of systemic movement.

Similar to the picornaviruses, the coding region of the potyvirus genome is flanked by untranslated sequences at its 5' and 3' ends. These regions appear to contain structural elements that regulate virus translation and replication. Similar to the picornaviruses, mutational analysis of PPV suggests that the 5'-untranslated region (5'-UTR) contributes to viral pathogenesis (211). However, whether there is an internal ribosome entry site (IRES) within this region, as there is in picornaviruses, remains unclear. Studies with PPV suggest that translation is initiated at the second in-frame AUG codon by a leaky-scanning mechanism (289). However, this region in TEV and pea seed–borne mosaic virus contains an AU-rich region that acts as a translational enhancer, and it has been suggested that this might contain an IRES (41,243). To date, there is no definitive evidence for this. The potyvirus 5'-UTR is less than 200 nt in length, much shorter that that found in picornaviruses, and it is predicted to be less structured (214). The 3'-UTR of the genome is predicted to contain secondary structures, and mutational studies with TVMV suggest that these may contribute to viral pathogenesis. Studies that compared the effects on virus replication of frameshift-stop codon mutations and deletions within the CP coding sequence of TEV suggest that translation through at least the first half of the CP coding sequence was required for efficient replication to occur (213), perhaps because passage of the translational apparatus alters secondary structure, which facilitates initiation of replication at the 3' end of the genome. These studies also identified a structural element within the 3' end of the CP coding sequence that was essential for replication, although whether this element interacts with *cis* or *trans* factors involved in genome replication or promotes stability of the viral genome remains to be determined (213).

Potexviruses: Subgenomic RNA and Leaky Ribosome Scanning

There are about ~30 viruses in the genus *Potexvirus,* of which PVX is the type member and one of the best characterized at the molecular level. In addition to its role in defining the potexvirus multiplication cycle, PVX has been important both historically and recently in studies on the synergistic enhancement of virulence that occurs when certain unrelated viruses co-infect a plant, and as a vector for gene expression studies in plants (47). Although individual potexviruses have a limited host range, as a group the potexviruses infect a range of monocotyledonous and dicotyledonous plants from bamboo and lilies to papaya, strawberry, cassava, and potato (1). The virions are flexuous filamentous rods ~14 nm in diameter and 470 to 580 nm in length (see Fig. 3) and appear to be one of the few groups of plant viruses directly

transmitted by the mechanical mode. The capsid is assembled from subunits of a single protein, the viral-encoded CP. The potexvirus genome is an unsegmented positive-sense ssRNA of ~5.8 to 7 kb with a 5'-terminal cap (m^7GpppG) and is polyadenylated at its 3' terminus.

The PVX genomic RNA contains five ORFs (151), which encode (in 5'-to-3' order) the viral RdRp (166K), a so-called triple gene block (TGB) cluster of three proteins required for virus movement (25K, 12K, and 8K), and the viral CP (25 kd) (Fig. 7). Following uncoating in a plant cell, the viral genomic RNA is translated as a monocistronic mRNA to produce the RdRp (encoded by the 5'-proximal ORF), which then copies the viral genomic positive-sense RNA to produce full-length complementary negative-stranded RNA. This is then transcribed by the RdRp to produce additional copies of full-length genomic positive-sense RNA, or if the RdRp uses internal subgenomic promoters, a set of 3'-collinear sgRNAs. Each of the sgRNAs contains sequences identical to the 3' end of the viral genome, and these function as the mRNAs for the viral movement proteins (TGB) and CP (see Fig. 7). A variety of *in vivo* and *in vitro* experimental studies support this mechanism for sgRNA synthesis and this view of potexvirus replication and expression (reviewed in 1 and 214). This strategy in general generates a family of 3'-co-terminal sgRNAs such that each gene to be expressed is located at the 5' end of one of the sgRNAs (i.e., they are each functionally a monocistronic mRNA). In the case of PVX, the largest (2.1 kb) and the smallest (0.9 kb, encoding CP) sgRNAs are monocistronic and the major sgRNAs, and these encode the 25-kd movement protein and CP, respectively. However, the third sgRNA (1.4 kb), which is less abundant, functions as a bicistronic mRNA. It is translated by leaky ribosome scanning to produce the 12K and 8K proteins (349).

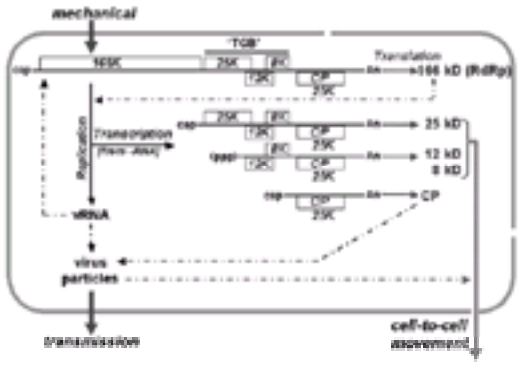


FIG. 7. Multiplication of potato virus X (PVX).

For PVX and other potexviruses, the first and largest ORF was originally predicted to encode the viral replicase (RdRp = 166K protein) based on sequence comparisons that showed it contained the conserved motifs characteristic of the methyltransferase/helicase/polymerase arrangement of the alphavirus supergroup (see Chapter 13). Mutational studies show that the 166K protein is the only viral protein that is absolutely required for PVX RNA synthesis. In particular, mutation of the GKS nucleotide binding motif characteristic of helicases or the conserved GDD motif found in viral RdRps eliminates virus replication and infectivity in plants (61,210). Viral genome length positive- and negative-sense ssRNAs and the three sgRNAs, as well as the corresponding dsRNA forms, have been identified in PVX-infected protoplasts or plants (80,236,271,349). Consistent with its function as an mRNA, the viral genomic RNA from PVX and other potexviruses is infectious and is translated *in vitro* into the RdRp encoded by the first ORF (23,136). The largest (2.1 kb) and smallest (0.9 kb) sgRNAs, as well as the viral genomic RNA, are capped and polyadenylated, and infectivity studies, as well as *in vitro* translation studies, show that the 5'-cap structure is necessary for efficient infection (1,136,236). Whether the low abundance PVX 1.4-kb sgRNA that encodes the 12K and 8K proteins is capped has not been directly determined. In general, sgRNAs commonly have the same 5' and 3' modifications as do the genomic RNA, but this is not always the case.

The PVX 1.4-kb sgRNA functions as a bicistronic mRNA, with the 12K and 8K proteins being translated by a leaky ribosome scanning mechanism. This was shown in *in vivo* studies using PVX engineered to express either GUS or GFP as a visual marker for virus movement, and transgenic plants that expressed different combinations of the TGB-encoded proteins (349). Because of the PVX expression strategy, the gene encoding GUS or GFP could be cloned just upstream of the *CP* gene so that it would be expressed from a duplicated copy of the CP subgenomic promoter. Thus, during infection the marker protein would be translated from an sgRNA separate from the other virus proteins (Fig. 8). Because PVX has a helical capsid, such engineered genomes can also be encapsidated into infectious virus particles.

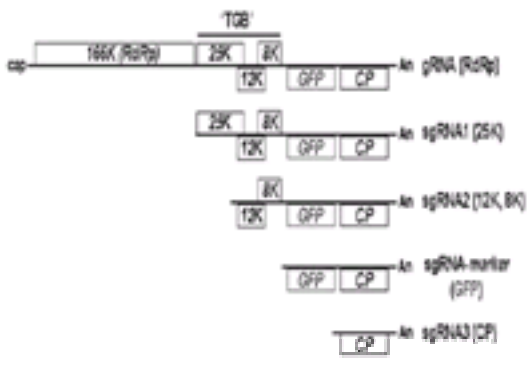


FIG. 8. Strategy to express a foreign gene using potato virus X (PVX).

Mutant PVX-GUS virus or transcripts that could not express either the 25K (D25K), the 12K (D12K) or the 8K protein (D8K, engineered by inserting a premature stop codon so that the overlapping *12K* ORF would not be affected) were not infectious when inoculated onto wild-type plants. Histochemical staining of inoculated leaves for GUS activity showed that each of these mutants is defective in cell-to-cell movement: GUS activity is restricted to the single initially infected cells. The cell-to-cell movement defect of each mutant could be rescued in *trans* when these were inoculated onto certain transgenic plants. In all cases, save one, each mutant was infectious on transgenic plants that expressed the corresponding ORF as a monocistronic mRNA or from a bicistronic or tricistronic mRNA in which this ORF was the 5'-most ORF in the transgene. Thus, for example, the D12K mutant was infectious on transgenic plants expressing either the *12K* ORF alone or the bicistronic *12K-8K* transgene but was not rescued on transgenic plants that expressed the tricistronic *25K-12K-8K* transgene in which the *12K* ORF was internal. The one exception was the D8K mutant: this was rescued on transgenic plants that expressed either the *8K* ORF alone or the bicistronic *12K-8K* transgene, thus showing that it is translated from a bicistronic mRNA (see Fig. 7). Inspection of the sequences surrounding the *12K* ORF initiation codon showed that it was in a suboptimal context for translation initiation, consistent with its being translated by leaky ribosome scanning. This was shown by constructing a set of PVX-GFP mutants in which the *12K* ORF was internally deleted (to remove any IRES), or its initiation codon was eliminated, or mutated to a stop codon or optimized initiation sequence. Because most of these mutations would eliminate translation of the 12K protein, each mutant was tested for infectivity on transgenic tobacco plants that expressed the *12K* ORF. The only mutant that could not be rescued on these transgenic plants was that in which the 12K initiation codon had been mutated to an optimized context. This mutant could be rescued only on transgenic plants that expressed the bicistronic *12K-8K* transgene, showing that the 8K protein is translated by leaky ribosome scanning (349). Thus, it appears that the amounts of the 12K and 8K proteins are regulated, relative to the 25K protein and other viral proteins, in at least two different ways. First, the bicistronic sgRNA encoding the 12K and 8K proteins is present at much lower levels in infected cells than are the mRNAs for the 25K protein, CP, and RdRp. Second, the relative amounts of the 8K and 12K proteins are regulated by leaky ribosome scanning. An interesting aspect of these PVX mutant studies is that although the appropriate transgenic lines could rescue PVX mutants for local cell-to-cell movement, in no case was there complementation for long distance systemic infection.

PVX sgRNAs, genomic RNA, and full-length complementary negative-sense RNA can be synthesized *in vitro* using membrane-containing extracts from infected plants (86). This suggests that PVX replicates in association with cytoplasmic membranes, like most if not all positive-sense plant and animal RNA viruses, including TEV and TMV (35). Consistent with this, virus replication appears to occur associated with virus-induced cytoplasmic laminate inclusions (“viroplasms”) in plant cells (1,62). PVX genomic RNA contains an 84-nt 5'-UTR and a 72-nt 3'-UTR. The 3'-UTR contains cis-acting sequence elements important for initiation of RNA negative-strand synthesis. These include a hexanucleotide sequence (5'-ACUUAA-3') that is conserved among potexviruses and a downstream U-rich motif (5'-UAUUUUCU-3') that has been identified in PVX and reported to bind host proteins from tobacco (322,372). The PVX 5'-UTR includes an AC-rich region (nucleotides 1 to 41) and contains cis-acting elements required for efficient translation (268,387) and presumably virus assembly, this last inferred from studies of papaya mosaic virus assembly (319). By analyzing positive- and negative-strand RNA accumulation in tobacco protoplasts that had been transfected with PVX transcripts, mutational studies have shown that multiple sequence and structural elements in the 5'-UTR affect the accumulation of both genomic RNA and sgRNA, without significantly affecting levels of negative-strand RNA (173,227). In particular, thermodynamic predictions and the analysis of solution structure by chemical modification and RNase digestion suggest that nucleotides 1 to 203 contains two well-defined stem-loop structures (SL1 and SL2), both of which terminate in stable GAAA tetraloops, a motif shown in other studies to enhance RNA stability (Fig. 9). Site-directed mutations introduced into SL1 show that both its sequence and structure are required for positive-strand RNA accumulation, with the internal loop C (closest to the terminal tetraloop) and its two flanking stems being particularly critical. Both the formation of and specific nucleotides within loop C were essential for positive-strand RNA accumulation, as was base-pairing within and the length of the flanking stems. Increasing the length or stability of these two stems drastically reduced positive-strand RNA accumulation (both genomic and sgRNA), suggesting that they may function to position other elements to interact with viral and/or host proteins. The sequence of the tetraloop was also important beyond just stabilizing structure (227).

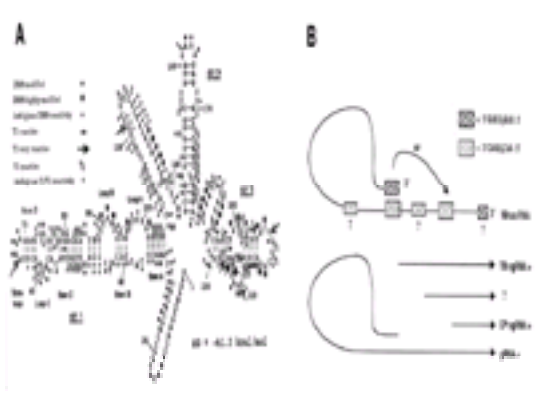


FIG. 9. A: Structure of the 5' end of potato virus X (PVX) genomic RNA. Shown are three predicted stem-loop structures (SL1, SL2, SL3) as determined by a variety of *in vitro* chemical methods (indicated by *symbols*). Labeled in SL1 are the tetraloop and specific stems and loops discussed in the text. **B:** Model for PVX positive-strand (+) RNA synthesis from the viral negative-strand (–) RNA. Shown are the PVX -RNA and the +RNA products (full-length and subgenomic RNAs (sgRNAs). The *black hatched box* corresponds to the element located 4 nucleotides from the 3' end of the –RNA (5'-GUUUAGUU-3'). The *gray hatched boxes* mark the locations of the octanucleotide elements (3'-CAAUUCAA-5') upstream of the triple gene block (TGB) and coat protein (CP) open reading frames (ORFs). The putative octanucleotide element near the 12K ORF and corresponding sgRNA for the 12K and 8K proteins are also shown (indicated by ?). An additional putative octanucleotide element within the 166K ORF is also marked with a “?” below it. (A from ref. 226, with permission; B from ref. 175, with permission.)

The relevance of these findings to virus genomic and sgRNA replication was shown by inoculating PVX mutants with specific alterations within SL1 in the 5'-UTR onto plants and analyzing revertants that accumulated following serial passages (226). Mutational studies using the protoplast replication assay also showed that conserved octanucleotide sequence elements located upstream of the two major PVX sgRNAs (2.1 kb and 0.9 kb) and the location of each element relative to the sgRNA start site were essential for sgRNA accumulation (174). Notably, these octanucleotide elements contain the conserved hexanucleotide element that is in the 3'-UTR of potexvirus genomic RNA, and they also exhibit complementarity to sequences in an apparently unstructured region of the 5'-UTR. The importance of this complementarity and of the sequence of the conserved octanucleotide elements was shown by inoculating protoplasts or plants with PVX transcripts that contained mutations within these elements, as well as compensatory mutations. Complementarity between the 5' terminus (which would be the 3' terminus of the negative-strand RNA) and the conserved octanucleotide elements is important for accumulation of both genomic RNA and sgRNA, and long distance interaction is required for all positive-strand RNA accumulation. In addition, the conserved octanucleotide sequences themselves are important for sgRNA accumulation.

Based on these studies, a model for PVX positive-strand RNA accumulation has been proposed in which the initiation events for synthesis of genomic RNA are a prerequisite for synthesis of all positive-strand RNA species (see Fig. 9). In this model, initiation of positive-strand RNA synthesis requires an interaction on the full-length negative-strand RNA template between the 3'-terminal element (defined by the element in the 5'-UTR of the genomic RNA) and a complementary element on the negative-strand RNA. The two octanucleotide elements upstream of the *TGB* and *CP* genes potentially act as partners in this process. Following initiation, sgRNA synthesis proceeds, possibly enhanced by additional interactions between the 3'-terminal element and the octanucleotide elements. Alternatively, the process can be modeled on the genomic positive-strand RNA: similar interactions would occur to generate truncated species during negative-strand RNA synthesis, with these then functioning as templates for sgRNA synthesis, as proposed for coronaviruses (see Chapter 35) and for the plant virus red clover necrotic mosaic virus (175). Another hypothesized role for the conserved elements is that they signal the replication machinery to pause or terminate RNA synthesis. In this model, the conserved element (GU-rich) at the 3' terminus of the replicating negative-strand RNA would act to terminate negative-strand RNA synthesis and allow the replication complexes to initiate positive-strand RNA synthesis. Subsequently, once the GU-rich conserved octanucleotide sequences upstream of the *TGB* and *CP* genes were synthesized as the positive-strand RNA was made, these would cause pausing or termination and thus reinitiation to synthesize the sgRNAs.

Viral CP is required for cell-to-cell movement of PVX, and several lines of evidence suggest that PVX moves from cell to cell in the form of encapsidated virus particles. When plants are inoculated with a PVX mutant in which the *CP* gene is replaced with the gene encoding GFP (PVX-GFPDCP) so that infected cells can be visualized by confocal microscopy, the virus is not infectious and is found to be limited to single inoculated cells (253). Mutational studies also show a correlation between the ability of altered CP to assemble virus particles and the ability of such mutants to spread on inoculated leaves (46). CP is detected in the central cavity of plasmodesmata when thin sections of leaves from PVX-infected tobacco plants are immunogold labeled to detect viral CP and examined by electron microscopy. In contrast, in transgenic tobacco plants that express the PVX CP, immunogold staining shows that CP is found throughout the cytoplasm and not localized to plasmodesmata. These transgenic plants make functional CP since the defective PVX-GFPDCP mutant that is missing the *CP* gene is rescued when inoculated onto the CP-expressing plants. Furthermore, in these infected plants CP is again found localized to plasmodesmata. Extending these studies, an affinity-purified antivirion antisera that can distinguish between CP subunits and assembled virions, labels plasmodesmata in PVX-infected plants, suggesting that PVX virions move from cell to cell (300).

Tobacco Mosaic Virus: Subgenomic RNA and Termination Suppression

The genus *Tobamovirus* comprises 15 members, of which TMV is the type member. TMV and the closely related tomato strain of TMV (now called tomato mosaic virus [ToMV]) are the best characterized on the molecular level. TMV, as already discussed, has been important historically in the investigation of viruses and the field of molecular biology. Beyond what has already been cited, its prominence in the study of plant viruses includes development of the local lesion assay, the first quantitative biological assay for plant viruses in which necrotic lesions on a leaf can be counted much like plaques; the use of viral replicase to engineer virus-resistant plants; and the nature of a dominant host gene that confers resistance to virus infection (Fig. 10). The economically most important tobamoviruses are TMV, which infects tobacco and other mostly solanaceous plants, ToMV in tomato, pepper green mottle virus, and odontoglossum ringspot virus in orchids. The virions are rigid helical rods approximately 18 nm in diameter and 300 nm in length (see Fig. 3) and, like potexviruses, are directly transmitted by the mechanical mode. The capsid is assembled from subunits of a single protein, the viral-encoded CP. TMV is extraordinarily stable: it not only persists in dead plant matter in the soil and on contaminated seeds, but has also been reported to exist for years in cigars and cigarettes made from infected tobacco leaves (5). The tobamovirus genome is an unsegmented positive-sense ssRNA of ~6.4 to 6.6 kb with a 5'-terminal cap (m^7GpppG) followed by an AU-rich 5'-UTR of about 70 nt. The 3'-UTR consists of sequences that can fold into a number of pseudoknots followed by a transfer RNA (tRNA)-like structure at the terminus, which in TMV and most tobamoviruses, can accept histidine *in vitro*. In TMV, the 3'-UTR contains five pseudoknots, two of which are in the tRNA-like terminus (204,313). Positive-sense RNA plant viruses in a number of genera have a 3' terminus that, like that of the TMV genome, can fold into a tRNA-like structure that encompasses pseudoknots: Watson-Crick base pairing between a loop and a single strand region in the same RNA molecule. This distinguishes them from tRNAs, which do not contain pseudoknots. The significance of pseudoknots is that they introduce structure into RNA molecules, which can potentially provide specificity in terms of interactions with proteins or catalytic reactions. The 3' tRNA-like structures in those positive-sense RNA plant viruses in which they are found have been shown to be important for negative-strand RNA synthesis. They can be aminoacylated *in vitro* and *in vivo*, but only three specificities have been reported for different plant viral RNAs: histidine, valine, or tyrosine.

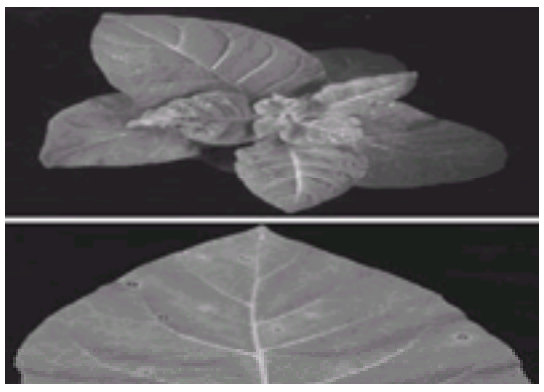


FIG. 10. Tobacco mosaic virus (TMV) infection of plants with or without the dominant *N* gene. **Top:** Systemic mosaic symptoms of TMV infection on *nn* tobacco plants. **Bottom:** Localized necrotic lesions typical of the hypersensitive response (HR) on a leaf from a TMV-infected tobacco plant carrying the *N* gene. (Photographs courtesy of Dr. Milton Zaitlin, Cornell University.)

The TMV genomic RNA contains four ORFs (119), which encode (5' to 3'): a methyltransferase/helicase and RdRp (126K and 183K, respectively), the MP (30K, referred to as the 30-kd protein), and the viral CP (17.5K) (Fig. 11). The viral genomic RNA is infectious (63,118), consistent with its initial role as an mRNA for translation of the viral RdRp (183K + 126K proteins). Following entry into the cell, the removal of a few CP subunits from the virion end that contains the 5' terminus of the genome appears to initiate uncoating. *In vitro*, this can be accomplished by incubating virions at pH 8.0 or for brief periods in low levels of sodium dodecylsulfate (SDS) (238,375). Because some cations can slow or prevent this process, it has been suggested that removal of Ca^{2+} when virions encounter the low Ca^{2+} concentrations inside the cell may induce a conformational change and removal of these few CP subunits. After this, disassembly of about three-fourths of the genome rapidly proceeds cotranslationally as ribosomes translate the 126K and 183K proteins. This was first shown to occur *in vitro* by incubating pH 8.0 treated TMV virions in rabbit reticulocyte cell-free protein synthesizing extracts. It was subsequently shown *in vivo*, both by examining extracts from tobacco epidermal cells that had been inoculated with radioactively labeled virions, and in time course studies in which reverse transcription and PCR were used to analyze the parts of the viral genome that remained encapsidated at different times following inoculation of tobacco protoplasts (314,375,380). Following this 5'-to-3' disassembly, the remainder of the genome is coreplicationally disassembled in the 3'-to-5' direction as replication of complementary negative-sense RNA is initiated. This was concluded from protoplast studies again, in which it was shown that particles from which large regions of the *126K* and *183K* ORFs have been deleted cannot undergo 3'-to-5' disassembly. However, these will fully disassemble if the protoplasts are co-inoculated with TMV genomic RNA that can provide the 126K and 183K proteins in trans. Furthermore, virions in which the genomic RNA is mutated in sequences in or near the conserved methyltransferase and helicase motifs within the 126K protein are also defective in final 3'-to-5' disassembly (379).

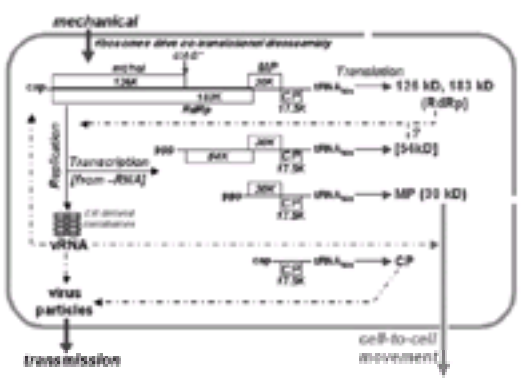


FIG. 11. Multiplication of tobacco mosaic virus (TMV).

As described previously, following initial 5'-to-3' disassembly, the 126K and 183K proteins are translated from the genomic RNA. The 183K protein is produced by readthrough translation approximately 5% to 10% of the time of the UAG codon that terminates the 126K protein. This was first shown *in vitro* using rabbit reticulocyte extracts primed with TMV genomic RNA (260), and it was subsequently reported that tobacco plants contain two suppressor $tRNA^{tyr}$ having a 5'-GYA-3' anticodon that can accomplish this (21). Readthrough (more properly termination suppression) is context dependent: in TMV the two codons just downstream of the suppressed UAG are critical for efficient readthrough *in vitro* and *in vivo* (320,344). As a result of this readthrough translation, the two proteins are read in the same frame and the amino acid sequence of the 126K protein is contained within the N-terminus of the 183K protein. The efficiency of readthrough regulates the relative amounts of the 183K and 126K proteins made. The sequence of the TMV genomic RNA first showed that the 126K (and thus the N-terminal region of the 183K) protein contains conserved motifs typical of methyltransferases and helicases, and the 183K readthrough domain contains in addition the GDD motif typical of a viral RdRp, with the arrangement of these motifs being characteristic of that found in the alphavirus superfamily (see Chapter 13). The 126K protein has also been shown to covalently bind [a-^{32}P]GTP (93). This, combined with the studies described previously and additional mutational studies in which deletion of the *MP* and *CP* ORFs was shown not to inhibit replication (225), led to the suggestion that the 183K protein, perhaps together with the 126K protein, is required for viral replication and that these two proteins make up the subunits of the replicase. Consistent with this, the 126K and 183K proteins are found in membrane-associated preparations of viral replicase and copurify with viral RdRp activity. Antibodies against different regions of the 126K protein also inhibit RdRp activity *in vitro* (256,257). Using a

complementing system in which the 183K and 126K proteins could be expressed from separate RNAs, it has recently been shown that the 183K protein is apparently the RdRp and can act in *trans* because mutants that express only this readthrough form can replicate all forms of viral positive- and negative-sense RNA, and can synthesize the viral proteins, move from cell to cell, and replicate defective deleted forms of the genomic RNA that themselves do not encode the 126K or 183K proteins (205). The 126K protein seems to increase the efficiency of replication approximately 10-fold, apparently acting in cis. Whereas expression of the 126K protein from a defective RNA does not increase replication of the helper virus expressing the 183K protein, the 126K-expressing defective RNA itself is efficiently replicated in the presence of this helper virus. This 126K-expressing defective RNA, however, is not efficiently replicated by wild-type TMV that expresses both the 126K and 183K proteins. This suggests that efficient TMV replication requires the 183K protein to form a heterodimer with the 126K protein that itself is already bound to the template RNA (205).

Following initial translation of the 126K and 183K proteins, these act to replicate full-length complementary negative-strand RNA. This then serves as the template for the synthesis of additional copies of the full-length genomic RNA or, if the RdRp (183K + 126K) recognizes internal subgenomic promoters, three subgenomic RNAs, each of which is functionally monocistronic (204,313) (see Fig. 11). Unlike the potexviruses, there are not any conserved sequences that aid in identifying the viral subgenomic promoters. The smallest sgRNA is the most abundant and is translated into the viral CP. The middle-sized sgRNA, which accumulates to much lower levels than the CP sgRNA, is translated into the viral MP. Mutational studies, and complementation studies using mutant viruses and transgenic plants that express the MP, show that MP is the only viral protein essential for cell-to-cell movement (71,225). The CP, although not required for cell-to-cell movement, is required for systemic infection of the plant through the phloem (64). As is the case for the potexviruses, the sgRNAs serve to regulate expression of the MP and CP both temporally and quantitatively. Whereas CP is produced late during infection and in extremely large amounts, MP is produced early, accumulates to low levels, and decreases at late times following infection (16,65). In addition to differences in the accumulated levels of the two sgRNAs, the sgRNA for the MP is not capped and has a long 5'-UTR. In contrast, the sgRNA for the CP is capped and has a short AU-rich 5'-UTR, which together appear to make it an efficient mRNA (204). The longest sgRNA found in TMV-infected cells accumulates to low levels and is predicted to encode a 54-kd protein with the same sequence as the readthrough domain of the 183K protein. Although such a protein is translated from this sgRNA *in vitro* , this 54-kd protein has never been detected in infected plants or in protoplasts (37,329). The predicted protein is also not detected in transgenic plants that are engineered to express this sgRNA, despite the fact that these plants are resistant to TMV infection (121). The studies that have been done suggest that this sgRNA for the 54-kd protein is translated: it is associated with polyribosomes in infected cells, and analysis of mutant forms in infected protoplasts indicates that its translation, not simply the presence of the sgRNA, is required to inhibit virus replication (37,329). Thus, although it stands as the first example of replicase-mediated resistance as an approach to engineer virus-resistant plants, the 54-kd protein remains enigmatic. Comparing TMV to another alphavirus, namely Sindbis (see Chapter 30), in which the replicase subunits are generated by a combination of readthrough and proteolytic processing by the viral-encoded proteinase nsp2, and considering that the predicted 54-kd protein would be the equivalent of nsp4 in Sindbis, it is tempting to speculate that the 54-kd protein is highly labile and may play a regulatory role in suppressing the synthesis of full-length negative-strand RNA during TMV infection while allowing the synthesis of all forms of positive-sense RNA (sgRNA and genomic RNA) to continue, analogous to what occurs during Sindbis replication. Just like Sindbis, TMV negative-strand RNA replication ceases early in infection, as the replication of genomic RNA and sgRNAs continues, so that late in TMV infection there is an asymmetric production of the viral positive-strand RNAs. Studies of TMV replication using recently described *in vitro* template-dependent membrane-associated RNA polymerase preparations from infected tomato plants (256) should help to solve this mystery of the 54K protein and better define the functions of the 183K protein alone, or as a heterodimer with the 126K protein, in regulating the synthesis of the different form of viral RNA.

Like other cytoplasmic positive-sense RNA viruses, TMV replication occurs in association with cytoplasmic membranes. This was first suggested by ultrastructural studies of so-called X bodies or viroplasms as sites of virus replication and protein synthesis (99), which were described as being composed of ER, ribosomes, virus rods, and bundles of tubules. Purified preparations capable of directing TMV replication in a template-dependent manner have now been shown to be membrane-bound complexes (256). The effects of TMV infection on the organization of the ER have been examined by infecting transgenic tobacco plants that expressed an ER targeted GFP fused to the retention signal HDEL so that GFP accumulated in the ER and ER structure could be examined by confocal microscopy (284). Early in infection, the ER tubular network is converted into large cytoplasmic aggregates. Remarkably, late in infection these aggregates disappear and the normal ER network is reformed (284). That these ER-derived membranes are sites of virus replication has been shown by indirect immune fluorescence staining with antibodies against the TMV RdRp and *in situ* hybridization for viral RNA. These approaches show that the replicase and viral RNA are found associated with ER membrane and the ER-derived aggregates (219,284). Based on localization of a TMV GFP:MP fusion protein in infected plant cells and infected protoplasts, it appears that the MP plays a central role in establishing these ER-associated replication sites and in the transient reorganization of the ER membrane network (see Virus Movement) (134,284). Two different approaches have also implicated host proteins in TMV replication. The analysis of TMV recombinant viruses and mutants that could induce or overcome the so-called gene-for-gene resistance response (see Plant Responses to Virus Infection) in tobacco plants that contain the dominant *N* gene have identified the helicase domain in the 126K/183K RdRp as being responsible for induction of the hypersensitive response that characterizes this type of host defense response (98,259). This suggests that the TMV RdRp interacts with the *N* gene product or other host factor in the *N* gene–mediated signal transduction pathway. The analysis of proteins that copurify with TMV RdRp activity from infected tomato plants has implicated a protein related to yeast GCD10, the RNA-binding subunit of yeast eIF-3, in virus replication. An antibody-linked polymerase assay was used to show that active TMV RdRp complexes bind to antibodies against the yeast GCD10 protein. Furthermore, these anti-GCD10 antibodies inhibited *in vitro* synthesis of viral ssRNA and dsRNA by the purified RdRp, and this could be reversed by prior addition of GCD10 itself (257).

Cis-acting sequences in the 5'-UTR and 3'-UTR of the TMV genome have been implicated in efficient viral translation and replication. The study of virus mutants and recombinant viruses has shown that within the 205 nt 3'-UTR, the $tRNA_{his}$ structure and the 3'-most pseudoknot just upstream of this structure are both involved in negative-strand RNA replication (63,337). Sequence elements in both the 68 nt 5'-UTR and the 3'-UTR, together with the 5' cap, are required for efficient translation of the viral genome. In this regard, the 3'-UTR appears to substitute for the lack of a poly(A) tail in synergistically interacting with the cap to facilitate translation. The 5'-UTR (also called the *leader sequence*) appears to

lack any stable secondary structure and acts as an efficient translational enhancer *in vitro* and *in vivo* when fused to a reporter gene (87,114). The 5'-UTR of many TMV strains contains two conserved regions—three copies of an 8 nt direct repeat element and a (CAA)n repeat region—and mutational studies suggest that these are functionally redundant in their effects on translation. By fusing different combinations of these two elements as a leader to a GUS reporter gene and expressing these mRNA constructs in carrot protoplasts, it has been shown that the core regulatory element consists of one copy of the 8 nt direct repeat and a 25 nt (CAA)n region, with the (CAA)n region being the more critical one. Furthermore, by combining such constructs with sequences from the TMV 3'-UTR, a 72 nt region that contains the three pseudoknots located just upstream of the $tRNA_{his}$ was also shown to be required for efficient translation (115,198). This synergism in translation involves interactions with both the 5' cap and the translational enhancer region within the 5'-UTR, with sequences within as well as the structure of the pseudoknots being important (198). These studies suggest that the 5' and 3' ends of the viral genome interact with each other to regulate translation, and perhaps replication. Consistent with this, a 102-kd protein that binds to both the (CAA)n region and the 72 nt 3'-UTR pseudoknot region has been identified in wheat germ extracts (339). The precise nature of these interactions, and whether the 3'-terminal $tRNA_{his}$ may also interact with sequences in or complexes formed at the 5' end of the genome to potentially regulate replication and translation, has not been determined. Further biochemical studies are needed to address these questions.

With the report in 1955 that TMV virus particles could be assembled *in vitro* from the component viral RNA and CP (105), this assembly process has been extensively studied *in vitro*. The *in vivo* studies that have been done (378) are compatible with the model that has been developed based on *in vitro* analyses (36,199). Encapsidation initiates at a sequence-specific stem-loop structure within an origin of assembly that is located within the *MP* ORF for most tobamoviruses (Fig. 12). Fusion of this assembly origin to nonviral RNAs will direct their assembly into virus-like particles. Loop 1 of the assembly origin interacts with and is threaded through the center of a two-layered disk composed of CP, with both the 3'-to-5'ends of the viral RNA trailing from one side of the disk. The association of the RNA with a central groove between the two layers of the disk causes a conformational change in the disk to a so-called lockwasher configuration (see Fig. 12). Elongation rapidly proceeds in the 3'-to-5' direction as additional disks are added and lengths of the genome bearing the 5' end are looped through the central cavity of the new disk and inserted into the central groove, inducing a change to the lockwasher configuration with each new addition as the helix is assembled. Encapsidation of the exposed 3' end of the genome appears to occur more slowly by the addition of smaller aggregates (the so-called A form) of CP subunits, although there is some disagreement as to how this occurs. A consequence of the assembly origin being located within the *MP* ORF (or the *CP* ORF for two tobamoviruses) is that the corresponding sgRNA is also encapsidated, the amount found in virus preparations being determined by the relative level of expression of the sgRNA.

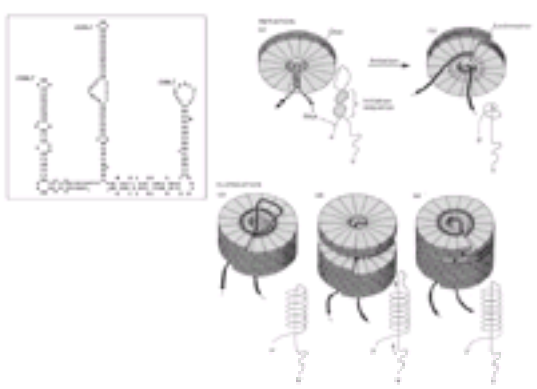


FIG. 12. Assembly of tobacco mosaic virus (TMV). **Inset:** Origin of assembly of TMV. (From Darnell JE, Lodish H, and Baltimore D. *Molecular cell biology,* 1st ed. New York: Scientific American, 1986, with permission; **inset** adapted from ref. 220, with permission.)

Turnip Yellow Mosaic Virus (TYMV): Subgenomic RNA, Polyprotein, and Independent Initiation

There are ~20 viruses in the genus *Tymovirus,* of which *t*urnip *y*ellow *mo*saic virus (TYMV) is the type member and best characterized on the molecular level. Tymoviruses are naturally found in uncultivated dicotyledonous plants and are transmitted by beetles. Each virus has a narrow host range, and none has been reported to cause economically important diseases of crop plants (117). TYMV has been important historically as one of the earliest examples of the infectious nature of viral RNA and as a model for investigating the structure of isometric virus particles (215,313). TYMV virions are T=3 icosahedral particles that are 29 nm in diameter and composed of 180 copies of a single protein, the 20-kd CP. Each CP subunit is folded into an eight-stranded antiparallel (jellyroll) b-barrel, and these are arranged in pentameric clusters at the vertices of the virus particles. The genome is an unsegmented positive-sense ssRNA of ~6.3 kb with a 5'-terminal cap (m^7GpppGp) and a 3' terminus that can form a tRNA-like structure (Fig. 13). TYMV was the first virus for which it was shown that this type of tRNA-like 3'-terminal end could accept an amino acid, in this case valine (263), and it has recently served as a model for unraveling the role of such structures in directing the viral RdRp to recognize the viral 3' end and initiate RNA negative-strand synthesis.

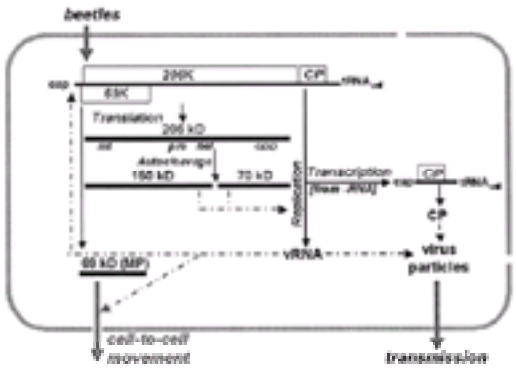


FIG. 13. Multiplication of turnip yellow mosaic virus (TYMV).

The TYMV genome contains three ORFs, which encode (5' to 3') the MP (*69K*), the RdRp (*206K*), and the viral CP (*20K*) (88) (see Fig. 13). It is a very compact coding arrangement. The 5'-UTR is 87 nt, with the *69K* ORF initiating at nucleotide 88. This ORF extensively overlaps the *206K* ORF, which initiates only 7 nt downstream in a different reading frame at nucleotide 95. There is only a short intergenic region (~15 nt, depending on the isolate) between the end of the *206K* ORF and the start of the CP (*20K*) ORF, which is followed by a 107 nt 3'-UTR. The 206-kd protein contains (starting from its N terminus) a methyltransferase motif, a helicase nucleotide binding site motif (GXGKT), a papain-like protease domain, and the GDD motif characteristic of a viral RdRp. Sequence similarities and the arrangement of the methyltransferase, helicase, and GDD motifs place TYMV in the alphavirus family. It has been suggested that upon entering a cell, deprotonation of histidine residues in the CP may aid in virion disassembly (117).

The TYMV multiplication cycle has been deduced based on *in vitro* translation of both virion RNA and infectious transcripts of cloned full-length genomic cDNA, as well as identification of viral RNA in infected plants. Consistent with the virion genomic RNA being infectious, the genome is first translated into the 69-kd and the 206-kd proteins. The 206-kd protein autocatalytically processes itself into a 150-kd protein (containing the methyltransferase, papain-like protease, and helicase domains) and a 70-kd protein (containing the GDD motif) (see Fig. 13). The 150-kd and 70-kd proteins together apparently constitute the RdRp, which uses the genomic positive-strand RNA as the template to replicate full-length complementary negative-strand RNA. This then functions as the template for the replication of viral positive-strand RNAs, both full-length genomic RNA or, if the internal subgenomic promoter is recognized, an sgRNA that is translated into CP. The CP sgRNA is encapsidated into virus particles; hence, its translation into CP was demonstrated *in vitro* using virion RNA in cell-free protein synthesizing lysates from rabbit reticulocytes or wheat germ. The CP sgRNA has also been detected associated with polyribosomes in infected plants (220). Viral RNA replication takes place in the cytoplasm, associated with membranes. However, unlike TEV, PVX, or TMV replication, these membranes appear to be derived from the chloroplast outer membrane. TYMV infection causes clumping of chloroplasts and invagination of the chloroplast outer membrane. Both viral RNA and RdRp localize to these chloroplast-associated peripheral vesicles in infected cells (220).

Most of the details of TYMV multiplication have been deduced using infectious transcripts of the cloned TYMV genomic cDNA, into which mutations could be introduced and studied for their effects on *in vitro* translation, replication in Chinese cabbage or turnip protoplasts, and infectivity in these same hosts. The full-length TYMV genomic RNA is translated *in vitro* into the 69-kd protein, which migrates at 75 kd apparently because it is highly basic (pI = 12.1), and the 206-kd protein, which is incompletely processed *in vitro* to the 150-kd and 70-kd proteins. In contrast to what one would expect for leaky ribosome scanning, mutations of the initiation codon for the 69-kd protein (nucleotides 88 to 90) have no effect on expression of the 206-kd protein, but mutations of the initiation codon for the latter (nucleotides 95 to 97) significantly increase expression of the 69-kd protein (88). It has been suggested that similar to influenza virus B segment 6, in which the initiation codons for the overlapping *NB* and *NA* ORFs are only 4 nt apart (see Chapter 46), translation of the 69-kd and the 206-kd proteins is initiated by random ribosome movement in which the 40S subunit moves both 5'®3' and 3'®5' until the AUG initiation codon is encountered. The identification of the *69K* ORF as encoding the viral MP is based on mutations that eliminated the initiation codon at nucleotide 88 or caused premature termination of its translation: such mutants replicate normally in protoplasts but fail to form local lesions on inoculated leaves or systemically infect plants, thus suggesting that they are defective in cell-to-cell movement. Consistent with its proposed role as an MP, antisera specific for the 69-kd protein has detected the protein associated with an insoluble cell wall fraction from TYMV systemically infected leaves, and it appears to be maximally expressed in young, expanding systemically infected leaves (29). The CP is not needed for local cell-to-cell movement. A TYMV mutant in which two-thirds of the *CP* ORF is deleted can replicate normally in protoplasts and produce chlorotic local lesions on inoculated leaves (88). However, this mutant does not systemically infect the host, showing that in addition to beetle transmisson, the CP is needed for systemic infection via the phloem.

Using antisera specific for the N- and C-terminal regions of the 206-kd protein, it was shown *in vitro* that the 206-kd protein is processed into a 150-kd protein (recognized by the N-terminal–specific antisera) and an apparently unstable 70-kd protein (recognized by the C-terminal–specific antisera) (88). This resembles the processing of Sindbis virus RdRp by autocatalytic proteolysis to nsp123 and nsp4, with the TYMV 70-kd protein corresponding to Sindbis nsp4 (see Chapter 30). Whether there is additional processing *in vivo* of the 150-kd protein, analogous to the processing of nsp123 during Sindbis infection, is not clear as there is disagreement about a viral 115-kd protein being a subunit of the RdRp. Further studies are needed to determine whether the 150-kd protein is further processed and what role, if any, processing of the 206-kd protein and its products plays in regulating the levels of newly synthesized viral positive- and negative-sense RNA. Mutational studies have mapped the protease domain (amino acids 670 to 885) and the cleavage site within the 206-kd protein. Consistent with its proposed function as a papain-like proteinase, mutation of Cys^{783} and His^{869} within the protease domain of the 206-kd protein prevent its autocatalytic cleavage to the 150-kd and 70-kd proteins (88). Furthermore, TYMV missense mutants in which the initiation codon for the *206K* ORF is eliminated, or the helicase or GDD motifs are mutated, are noninfectious and do not replicate in protoplasts. This is consistent with both

proteins being required for replication, with the 150-kd protein having helicase activity and the 70-kd protein being an RdRp. Complementation studies using the TYMV helicase and GDD mutants together, or each mutant complemented with a helper TYMV in which the CP was deleted, further suggest that the 150-kd and 70-kd proteins may function as a heterodimeric complex and act in a *cis*-preferential manner (371). The complementing helicase and GDD mutants can support the replication of each other in *trans*, but they do so poorly. When each mutant is complemented with the helper virus, they replicate to 20% or less of the level of the helper RNA. However, if a TYMV deletion mutant that cannot make the 70-kd protein is substituted for the GDD missense mutant that produces a defective 70-kd protein, the deletion mutant does accumulate to high levels, comparable to the helper virus. This deletion mutant could also complement the defective helicase missense (150-kd) mutant; however, again the 70-kd protein deletion mutant replicated to approximately 10-fold higher levels than did the helicase mutant. This suggests that the 70-kd protein can act in *trans* but that the 150-kd protein acts in a *cis*-preferential manner, leading to a model similar to that proposed for the TMV 183-kd and 126-kd proteins: the 150-kd protein binds to the negative strand promoter in *cis*, and the 70-kd protein, also showing some *cis* preference, forms a stable heterodimeric complex with the 150-kd protein, thus favoring replication of the RNA encoding the active complex.

It appears that TYMV virion RNA is rapidly packaged following replication so that it is not accessible to the host cell CCA-tRNA nucleotidyltransferase. Thus, the tRNA-like structure at the 3' end of the virion RNA terminates in 5'-CC-3' but is efficiently adenylated to 5'-CCA-3' *in vitro* by CCA-tRNA nucleotidyltransferase. In this form, it is an active *in vitro* substrate for aminoacylation with valine by valyl-tRNA synthetase from yeast or wheat germ (88). Valylated TYMV RNA has also been detected in infected plant cells. Although this tRNA-like structure has only limited sequence similarity to higher plant $tRNA^{val}$, it does have a valine -CAC- anticodon and an -ACAC- present in the anticodon loop of all eukaryotic $tRNA^{va}$, and it structurally resembles tRNAs on the secondary and tertiary levels. As discussed for TMV, the TYMV tRNA-like structure is distinct from cellular tRNAs in having a pseudoknot, which is involved in building the amino acid acceptor stem. The role of the TYMV $tRNA_{val}$-like structure in virus replication has been extensively investigated, using either the viral genomic RNA or an *in vitro* transcript comprising a 3'-terminal fragment of the genomic RNA. *In vitro*, the valylated TYMV tRNA-like structure tightly binds elongation factor eEF1a from wheat germ or EF-Tu from *E. coli.* Initial mutational studies showed a direct correlation between valylation and TYMV RNA replication in protoplasts or viral infectivity in plants. In addition, mutation of the TYMV aminoacylation specificity from valine to methionine, a novel specificity for viral tRNA-like structures, produced infectious virus, and there was now a direct correlation between the ability of mutated forms of this altered TYMV genome to accept methionine and replicate (88,90). Thus, the valine specificity does not appear to be crucial here, and the results suggested that aminoacylation might be required for efficient replication of TYMV RNA.

However, tymovirus genomic RNAs exhibit a wide range of tRNA mimicry, from highly developed, as in the case of TYMV, to minimal, and it was possible to construct infectious variants of TYMV that were not aminoacylated (89,122). The starting point for these variants was a chimeric TYMV genomic RNA in which the 3'-$tRNA_{val}$–like structure had been replaced with 3'-sequences from TMV. Although it could accept histidine *in vitro*, this chimeric TYMV-TMV genome was not infectious, but it did replicate to ~3% the level of wild-type TYMV in protoplasts. Adding back TYMV sequences from the anticodon loop and amino acid acceptor stem to the 3' region of this chimeric genome increased its replication a few fold. Two of these chimeric genomes could be serially passaged in plants, eventually giving rise to two highly infectious TYMV-TMV variant viruses which had accumulated some minor additional changes in their 3'-end sequences. Unexpectedly, these two infectious TYMV-TMV variants, both of which now contained the valine-specific anticodon loop, could be adenylated to produce -CCA- 3' ends *in vitro*, but they could not be aminoacylated. Thus, aminoacylation is not obligatory for virus replication. This suggests that a number of structural features of the viral 3' end can contribute to genome amplification and infectivity, and that these have the ability to compensate for lack of aminoacylation (122). Of course, some of the TYMV 3'-terminal sequences added back to these chimeras could have included critical promoter elements needed for negative strand synthesis that would be recognized by replicator complexes and overlap the determinants for valylation. However, the ability of the TYMV RdRp to efficiently transcribe a range of unmodified tRNAs and tRNA-like structures, some quite short, in addition to the heterogeneity in the 3'-terminal sequences of the TYMV-TMV chimeras themselves, brought into question the very existence of such specific promoter elements, either in terms of sequence or structure, to direct negative strand synthesis. Using a TYMV RdRp preparation from infected plants to transcribe different RNA templates *in vitro*, it was determined that a 27 nt fragment encompassing the 3' terminus and pseudoknotted acceptor stem, or a 21 nt fragment encompassing this 3' end and adjacent hairpin, were efficiently transcribed, each correctly initiating at the penultimate C in the unpaired terminal 5'-CCA-3' sequence (68,318). By testing additional truncated, mutated, and duplicated templates, as well as unstructured C-rich templates or a 12 nt (CCA)n template, the minimal template requirement for initiation of negative-sense RNA synthesis by the TYMV RdRp has been defined. The surprising answer is that the TYMV RdRp can recognize any sterically accessible 5'-CC(A/G)-3' triplet; indeed, it will accurately initiate at multiple internal sites on unstructured C-rich or (CCA)n templates. However, the introduction of nonspecific secondary structure into such templates can confer specificity in terms of the 5'-CC(A/G)-3' triplets at which the RdRp will initiate transcription (68,318). Thus, there are no specific promoter elements within the acceptor stem and upstream regions of the TYMV tRNA-like structure: the specific target for initiation by the TYMV RdRp appears to be 5'-CC(A/G)-3'. The function of the tRNA-like structure would appear to be to prevent internal initiation of negative-strand RNA synthesis and place the 3'-terminal correct initiation site (-ACCA-) in a very accessible location. In addition, tRNA mimicry becomes important here for viral negative-strand RNA synthesis. The TYMV genome terminates in 5'-CC-3'. However, the 3'-terminal A is essential for initiation of negative-strand RNA synthesis, and this is added by the host CCA-tRNA nucleotidyltransferase, which recognizes the viral 3' tRNA-like structure and adenylates it. Thus, tRNA mimicry creates the proper 5'-CCA-3' terminus for the viral RdRp to initiate negative strand synthesis. Recent studies show that the RdRp of turnip crinkle virus (a carmovirus in the family *Tombusviridae*) and phage Qb replicase can also, to varying degrees, initiate transcription *in vitro* at CCA sequences without unique promoter elements. Thus, initiation at CCA sites with specificity being conferred by preference for 3'initiation sites and RNA structure may be common among the RdRps encoded by positive-sense RNA viruses (384).

Luteoviruses: Subgenomic RNA, Frameshift, Leaky Ribosome Scanning, Termination Suppression, and Polyprotein

The viruses that were formerly grouped as luteoviruses (“luteo” is Latin for “yellow”, referring to a characteristic symptom

produced by these viruses) have recently been reclassified within two genera in the new family of *Luteoviridae:* *Luteovirus* (formerly subgroup I) and *Polerovirus* (formerly subgroup II) (347). *Enamovirus* is a third genus in this family and consists solely of pea enation mosaic virus type 1 (PEMV1). PEMV1 exists in an interdependent relationship with a second virus, PEMV2. The genome organization of PEMV1 resembles that of the poleroviruses; however, PEMV2 is classified as an umbravirus. Only the luteoviruses and poleroviruses will be discussed here. Viruses within these two genera are grouped as *Luteoviridae* because of their biological properties, and they are clearly similar in the genes encoded in the 3' halves of the viral genomes. However, the genetic organization of the 5' halves of the luteovirus and polerovirus genomes is quite distinct. The luteovirus-encoded RdRp is similar to the RdRp encoded by tombusviruses and umbraviruses, among others; but the polerovirus-encoded RdRp resembles that of the sobemoviruses. The divergent nature of the 5' halves of the luteovirus and polerovirus genomes has led to the suggestion that these two genera were generated by horizontal gene transfer (229).

Of the ~20 viruses in these two genera, barley yellow dwarf virus (BYDV) (luteovirus, although some isolates may be poleroviruses), and the poleroviruses potato leaf roll virus (PLRV), beet western yellows virus (BWYV), and cereal yellow dwarf virus (CYDV) occur worldwide and cause significant losses in crops of economic importance. Although PLRV infects only potatoes, it can cause devastating disease and high losses. BYDV and CYDV affect a wide range of grains, including barley, oats, wheat, and rye, causing significant losses in these crops annually. The viruses are transmitted in nature by aphids. With the exception of BWYV, most luteoviruses and poleroviruses have very narrow host ranges. This, combined with their phloem restriction, have made them difficult to study. Although complete cDNA clones have been generated for a few of the viral genomes (202,275,385), the phloem limitation of virus infection means that, in contrast to viruses that infect mesophyll cells, neither the cDNAs nor the virions can be mechanically transmitted to plants for experimental studies. Agroinfection has been successfully used to infect plants with the cDNA clone for BWYV (202), but this approach does not efficiently work for many monocotyledonous plants, such as those infected by BYDV. PLRV gene functions have been, in part, studied by expressing the viral genome or individual ORFs in transgenic plants (275). The virions are T=3 icosahedral particles that are 24 to 30 nm in diameter and composed of 180 copies of the 22-kd CP, some fraction of which has a C-terminal extension of ~50 kd (CP-AT) (229). BYDV-PAV (luteoviruses), and PLRV and BWYV (poleroviruses), are the best characterized *Luteoviridae* at the molecular level. Each virus has a genome of unsegmented positive-sense ssRNA of ~6 kb. The PLRV genomic RNA has a genome-linked viral-encoded protein (VPg) of 7 kd covalently attached to its 5' terminus. In contrast, the BYDV-PAV genome does not have this or any other known modification at its 5' terminus (12). The luteovirus and polerovirus genomes do not contain either a poly(A) tail or a tRNA-like structure at their 3' end. A combination of approaches has been used to investigate the multiplication of these viruses. These include transfection of protoplasts with virion genomic RNA and transcripts of cloned cDNA, expression of cloned ORFs in *E. coli* (which also provides viral proteins for generating specific antisera), hybridization studies with sequence-specific probes and immunoblot studies with specific antisera to detect viral RNAs and proteins in infected plants and protoplasts, and *in vitro* translation of genomic and sgRNAs. Given that infected protoplasts can produce virions, aphids can be fed on protoplast extracts through a membrane to acquire virions with mutated genomes and transmit these to plants. This has allowed for mutational and complementation studies to study the functions of some of the viral proteins in aphid transmission and in systemic infection of plant hosts.

BYDV-PAV genomic RNA encodes six ORFs, numbered 1 through 6 (5' to 3') (Fig. 14). ORF 2 (*60K*) is predicted to encode a protein containing the GDD motif characteristic of a viral RdRp. It is translated from the genomic RNA both *in vivo* and *in vitro* only as a C-terminal fusion to the protein (39 kd) encoded by ORF 1 by a ¯1 ribosomal frameshift (72,233). These are the only proteins translated directly from the genome. Consistent with the presence of the GDD motif within the C-terminus of this fused protein, deletion studies show that both ORFs 1 and 2 are essential for viral replication in plant protoplasts (233). Because the ribosomal frameshift appears to occur £8% of the time in BYDV-PAV, the majority of the time, ORF 1 is translated into a 39-kd protein, the function of which alone is unknown. Although the 39-kd protein has been proposed to be a helicase, it does not appear to have homology to any known helicases (233). By analogy to other positive-sense viruses, it is assumed that following translation of the genomic RNA, the RdRp (99 kd), perhaps together with the 39-kd protein, uses the genomic positive-strand RNA as a template to synthesize full-length complementary negative-strand RNA, which then serves as template for the replication of additional genomic positive-sense RNA and three 3' coterminal sgRNAs (see Fig. 14), all of which have been detected in infected plants (169). The largest of these (sgRNA1) functions as a tricistronic mRNA. It is translated into the CP (22 kd), a small amount of CP with a C-terminal 50-kd extension (CP-AT) generated by occasional ribosomal readthrough of the CP stop codon (50,102), and a 17-kd protein which appears to be the MP. ORF 4, which encodes the 17-kd protein, is completely contained within ORF 3 encoding the CP. The initiation codon of ORF 4 is in a better context than that of ORF 3, and mutational studies show that ORF 4 is translated into the 17-kd protein from sgRNA1 by leaky ribosome scanning (76). Mutational studies also show that the 17-kd protein is not required for viral replication or encapsidation in protoplasts, but is required for systemic infection of plants, thus suggesting that it may be a viral MP (48). The protein encoded by ORF 5 is translated only as a C-terminal extension fused to the CP by readthrough of the CP stop codon; thus, it is called *RTD,* for *readthrough domain.* Mutational and complementation studies show that it is required for aphid transmission (48), and it is referred to here as *AT* for the sake of clarity. Subgenomic RNA2 contains only ORF 6, which potentially encodes a small protein that is highly variable in length (4.3 to 7.1 kd) and sequence among different BYDV-PAV–like isolates. The protein encoded by ORF 6 has only been detected *in vitro* (169). Subgenomic RNA3 does not contain any ORFs. Nevertheless, both sgRNA2 and sgRNA3 accumulate to high levels in infected plant cells. It has been suggested that sgRNA2 and sgRNA3 may be regulatory RNAs (229,230).

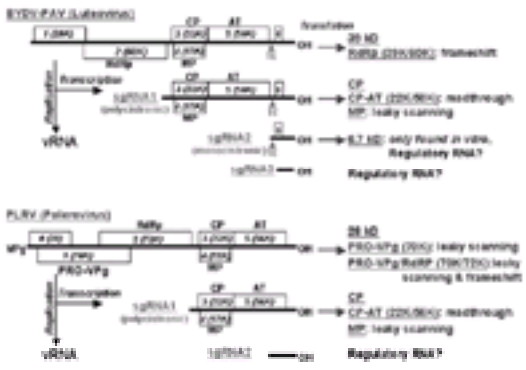


FIG. 14. Replication and transcription of barley yellow dwarf virus (BYDV-PAV strain) and potato leaf roll virus (PLRV). *TE* indicates the location of the translational enhancer.

Although *Luteoviridae* do not replicate in their aphid vectors, they do follow a circulative pathway that requires virions to be transported across three barriers following acquisition: the hindgut epithelium, across which virions enter the hemocoel, and the basal lamina and plasma membrane of the accessory salivary glands, to which they are actively transported from the hemolymph. Virions are then introduced into the phloem of a plant through the salivary duct as the aphids feed. Electron microscopic studies suggest that virion transport across the hindgut epithelium and accessory salivary gland plasma membrane may occur by receptor-mediated endocytosis (229). Mutational studies with BYDV-PAV suggest that the AT extension in the CP-AT subunits is required for transport of virions from the hemolymph into the salivary glands (48). This transport is an interesting example of the virus taking advantage of a symbiotic relationship. The aphid hemocoel contains a high level of proteins called *symbionins* that are produced by intracellular endosymbiotic bacteria (*Buchnera* species) and are homologs of the *E. coli* chaperonin groEL. Symbionins have been shown to bind the AT readthrough domain in BYDV-PAV, PLRV, and BWYV *in vitro*, and aphid transmission studies with viral mutants suggest that binding of symbionins to the virus particle may protect it from degradation in the aphid (101,143,345). The few CP-AT subunits incorporated into virions are found as a ~58-kd cleavage product that contains CP fused to the N-terminal half of AT, although full-length 72-kd CP-AT can be found in infected plant cells (48,50,233,365). Analysis of these BYDV virions and mutational studies of BWYV AT (33) show that the N-terminal half of AT, fused to CP, is required for aphid transmission. However, synthesis of the full-length 72-kd CP-AT fusion does appear to affect virus accumulation in infected plants (33,48,388).

PLRV also encodes 6 ORFs. The 5' half of the genome that encodes ORFs 0, 1, and 2 is organized quite differently than the corresponding region of BYDV-PAV (see Fig. 14). A viral-encoded VPg is covalently bound to the 5' end of the genome RNA. ORF 0 (*28K*) and ORF 1 (*70K*) may be translated by leaky ribosome scanning, but this has not been shown experimentally. The function of the ORF 0 encoded product is unknown. Mutational studies with BWYV suggest that ORF 0 is not needed, either for systemic infection or aphid transmission (388). Only ORF 1 and ORF 2 are essential for virus replication in protoplasts. The 70-kd protein encoded by ORF 1 has the catalytic triad characteristic of chymotrypsin-like proteases (PRO) in its N-terminal region and a helicase domain, between which is the VPg sequence. Consistent with VPg (7 kd) being produced by proteolytic processing, it has been identified in PLRV-infected plants at the N-terminus of a 25-kd apparent precursor protein that can bind RNA (274,276). ORF 3 (*72K*) encodes the putative RdRp since it is predicted to contain the GDD motif. It is only translated as a C-terminal fusion with PRO-VPg by infrequent ¯1 ribosomal frameshifting, reminiscent of the situation for ORF 1 and 2 in BYDV-PAV (187). Thus, in contrast to viruses like TEV and poliovirus, it would appear that PLRV VPg is produced in great excess over the RdRp.

In contrast to this divergence in the 5' end of the genome, ORF 3 (*22K*), ORF 4 (*17K*), and ORF 5 (*56K*) correspond to the same ORFs in BYDV-PAV and are also expressed from a subgenomic RNA (sgRNA1) to encode CP, a proposed MP, and AT, respectively (see Fig. 14), with AT only translated as the fused CP-AT as the result of occasional readthrough of the CP stop codon (143,223,228,334,336). There is also a second subgenomic RNA (sgRNA2), which in this case is not predicted to encode any ORFs. The PLRV 17K protein (ORF 4) is proposed to be an MP based on its having properties similar to those of other well-characterized MPs such as the TMV 30-kd protein. In particular, the PLRV 17-kd protein can nonspecifically bind single-stranded nucleic acid *in vitro*, is phosphorylated, and has been reported to localize to plasmodesmata in infected plants or when expressed in transgenic plants (309,335,336). This corresponds to the suggestion that BYDV-PAV ORF 4 encodes an MP. However, mutational studies of BWYV using agroinfection to introduce the mutated cDNAs into tobacco led to a very different conclusion: that ORF 4 was dispensable for virus systemic infection and aphid transmission, but that the C-terminal half of AT was required for systemic infection and a potential MP (33,388). Thus, the picture is confusing in terms of movement. Given the technical limitations involved in investigating luteovirus infection in plants, studies of BYDV-PAV, PLRV, and BWYV have not directly studied virus movement, nor do they distinguish between local cell-to-cell movement and systemic infection. In addition, the potential functions of the CP in local or long-distance systemic movement have not been addressed, although for BWYV it was concluded that the CP is needed for systemic infection (388). A better understanding of the phloem cell types between which these viruses move and the functions of CP in local and long-distance movement, coupled with direct investigation of luteovirus and polerovirus movement, should clarify this situation.

Luteoviruses and poleroviruses are notable for the sheer number of strategies they employ to regulate and maximize their gene expression: (a) sgRNAs, some of which appear to be regulatory molecules rather than mRNAs; (b) leaky scanning to produce CP and MP (ORF 4), and likely translate ORFs 0 and 1; (c) polyprotein synthesis and autocatalytic processing (poleroviruses) of PRO-VPg, and likely PRO-VPg-RdRp, to produce VPg, probably in different functional forms analogous to TEV and poliovirus; (d) ¯1 ribosomal frameshifting to produce RdRp; and (e) readthrough to produce CP-AT, the capsid subunit essential for aphid transmission. Beyond the number of strategies, there are some novel luteovirus twists here that have been reported in studies of BYDV-PAV. Frameshifting to produce RdRp occurs at a shifty heptanucleotide sequence (5'-GGGUUUU-3' in BYDV-PAV, 5'-GGGAAAC-3' in PLRV) and involves a downstream pseudoknot or hairpin structure (230), as reported for other viruses. However, a higher rate of frameshift was found when

the intact BYDV genome was translated *in vitro* in wheat germ extracts than when a reporter gene, in which the BYDV frameshift site was inserted so that the coding sequence was mostly in the −1 frame, was used (72). Mutational studies identified sequences 4 kb downstream in the 3'-UTR of the BYDV-PAV genome that appear to be required for efficient frameshifting (367). Readthrough to produce CP-AT in BYDV-PAV requires at least five copies of a series of tandem repeats of the sequence 5'-CCN NNN-3', beginning 12 to 21 nt downstream of the CP stop codon, with no apparent secondary structure in this region. Deletion studies further identify an additional element ~700 nt downstream within ORF 5 that is also needed for readthrough (32). Even given all of this, perhaps one of the most puzzling aspects of BYDV translation is its cap-independent nature, and the solution here may be the most novel of all.

A 109 nt sequence element located in the 3'-UTR of BYDV-PAV between ORFs 5 and 6 has been identified as a translational enhancer, and thus named 3'TE (see Fig. 14). It appears to mimic a 5' cap in interacting with eIF4F, either directly or indirectly, to facilitate efficient translation of uncapped mRNAs (366). This conclusion is based on *in vitro* and *in vivo* studies using a GUS reporter gene that contained different 5'-UTR sequences (BYDV, TMV, or plasmid) that were or were not capped, in combination with different 3'-UTR sequences [BYDV or plasmid with poly(A) tail]. Without a 5' cap, the GUS reporter gene is very inefficiently translated *in vitro*, whether or not a poly(A) tail is present. However, if the GUS reporter is flanked by the 5'-UTR from BYDV upstream and the 3'TE inserted in its 3'-UTR, it is efficiently translated *in vitro* without a cap, with the level of translation being comparable to that of GUS with a 5'-cap but lacking the 3'TE. The relative enhancing effect of the 3'TE is the same *in vitro* and *in vivo* in oat protoplasts, although additional BYDV 3'-UTR sequences are needed *in vivo* to substitute for a poly(A) tail, whether the 3'TE or a 5' cap is present. The 3'TE within the 3'-UTR enhances translation of uncapped mRNA when the BYDV 5'-UTR is present upstream of the GUS reporter, but has the same effect without the BYDV 5'-UTR sequences if the 3'TE itself is placed in the 5'-UTR region upstream of the GUS coding sequence. Thus, the 3'TE can functionally substitute for a 5' cap in this position and, in either position, it was found to decrease the amount of eIF4F needed for maximum translation *in vitro*. Furthermore, in competition studies, excess 3'-TE RNA inhibits *in vitro* translation of capped GUS mRNA in *trans*, and this effect is reversed by addition of excess eIF4F. Thus, the 3'TE appears to act to recruit translation factors and ribosomes by interacting with eIF4F, either directly or indirectly. The BYDV 5'-UTR is proposed to function for long distance 3'–5' interactions, probably by also interacting with eIF4F or other host factors rather than through direct base pairing, to position the recruited translation machinery at the 5' end of the viral genome (366). The significance of this translational enhancer being located between ORFs 5 and 6 (see Fig. 14) is that it would act to enhance translation of the genome and sgRNA1 to produce BYDV proteins. At the same time, its presence at the 5'-end of sgRNA2, which accumulates to high levels later in infection, could inhibit translation of genomic RNA by effectively competing for eIF4F and translation factors to favor replication and encapsidation. Of course, it could eventually act to inhibit translation of sgRNA1 as well.

Brome Mosaic Virus: Multipartite, Subgenomic RNA

The *Bromoviridae* comprise five genera: *Bromovirus, Cucumovirus, Alfamovirus, Ilarvirus,* and *Oleavirus* (347). All of these viruses have positive-sense tripartite genomes that consist of three separate segments of positive-sense ssRNA. The three genomic segments are separately encapsidated in distinct particles. This separation of gene function on physically distinct RNAs was historically a major advantage before the development of recombinant DNA approaches. Genetic reassortment of the genomic segments between different strains or mutants of the same virus, or different related viruses within these genera, made mapping gene functions relatively straightforward. In addition, studies of viral replication were facilitated by the components of the RdRp being encoded on two different segments distinct from the third genomic RNA that encodes the MP and CP. Thus, the RdRp activities could be mutated separately from promoters for positive-strand RNA and negative-strand RNA synthesis on the third genomic RNA, in effect using this third RNA as a reporter to identify essential replication functions in the RdRp and to investigate the mechanisms of genomic RNA, complementary RNA, and sgRNA synthesis. BMV, the type member of the bromoviruses, together with cowpea chlorotic mottle virus (CCMV) (bromovirus), cucumber mosaic virus (CMV) (cucumovirus), and alfalfa mosaic virus (alfamovirus) are among the best characterized viruses in the *Bromoviridae*. BMV illustrates many of the principles governing gene expression of multipartite plant viral genomes, and is the focus here. Although BMV combines expression of a sgRNA with the multipartite strategy, different multipartite positive-sense ssRNA plant viruses combine a number of the strategies that have been discussed, with their segmented genome approach, such as polyprotein synthesis and processing by a viral-encoded proteinase (comoviruses); readthrough and sgRNA (tobraviruses); or sgRNA, readthrough, and leaky scanning (hordeiviruses) (123). Multipartite viruses that use the polyprotein approach can have a viral VPg linked to the 5' ends of the genomic RNAs and 3' poly(A) tails, as do the comoviruses. The others, in general, have 5' caps combined with a poly(A) tail, a tRNA-like structure, or no known modification at the 3' ends of their genome segments.

BMV, although not of major economic importance, is historically important as the first RNA virus for which infectious genomic transcripts were generated *in vitro* from cDNA clones (8) and a model for investigating how positive-sense RNA plant viruses replicate their genomes and transcribe sgRNA (6,231). More recently, the demonstration that BMV can replicate in yeast has led to a novel approach to study virus replication *in vivo* and use yeast genetics to identify host factors involved in BMV replication (159). There appear to be no efficient invertebrate vectors for bromoviruses, although beetles and nematodes have been experimentally shown to transmit them. The viruses can be mechanically transmitted, in both the laboratory and field. The virions have T=3 quasi-icosahedral symmetry and are about ~28 nm in diameter. These are composed of 180 copies of the viral 20-kd CP, with each subunit having a b-barrel structure that is uncharacteristically perpendicular to the capsid surface so that the pentameric and hexameric subunit clusters have a bulging knoblike appearance (7,186). The N-termini of the CP subunits are internal and appear to interact with the encapsidated viral genomic RNA. The BMV genome consists of three distinct segments of positive-sense ssRNA that are 3.2 kb (RNA1), 2.9 kb (RNA2), and 2.1 kb (RNA3) in size, each with a 5' cap (m^7GpppGp) and a 3' tRNA-like structure that contains a pseudoknot and can accept tyrosine *in vitro* (Fig. 15). Density gradient centrifugation shows that these, together with the subgenomic RNA encoding the viral CP (RNA 4), are separately encapsidated into three different particles: (a) 3.2-kb genomic RNA (gRNA) in the highest density particles; (b) 2.9-kb gRNA in the intermediate density particles; and (c) 2.1-kb gRNA and 0.9-kb sgRNA in the lowest density particles (7). The CP sgRNA, like the genomic RNAs, has a 5' cap and a 3' $tRNA_{tyr}$-like structure (see Fig. 15).

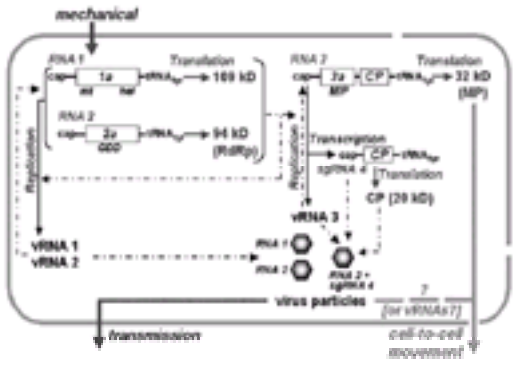


FIG. 15. Multiplication of brome mosaic virus (BMV).

How the virions are uncoated is not well understood. What is clear is that the three genomic RNAs and subgenomic RNA4 (hereafter [sg]RNA4) are each monocistronic and translated into the protein encoded by the single or the 5'-proximal gene. RNA1 and RNA2 are the only genome segments required for virus replication, as shown by direct transfection of plant protoplasts. These are translated, respectively, into proteins 1a (109 kd) and 2a (94 kd) (see Fig. 15), which coordinately act to replicate all three genomic RNAs by way of negative strand intermediates, as well as recognize an internal promoter on RNA3 to transcribe it into the 0.9-kb [sg]RNA4 that encodes the CP. RNA3 itself is translated into the viral MP (32 kd). Consistent with BMV having a positive-sense ssRNA genome and the previously described picture, the three genomic RNAs together are infectious, and each of these, as well as [sg]RNA4, is translated into the corresponding protein *in vitro* (6). This view of BMV multiplication and gene expression is supported by a variety of experimental studies that were facilitated by the generally high efficiency with which RNA can be transfected into protoplasts and the ability to prepare highly active template-dependent replicase-containing extracts from BMV-infected plants (232), in addition to the segmented nature of the BMV genome. Protein 1a contains conserved motifs characteristic of RNA methyltransferases (N-terminal domain) and helicases (C-terminal domain), and the central region of protein 2a contains the GDD motif characteristic of a viral RdRp. The arrangement and sequences of these resemble those in Sindbis and other alphaviruses, placing BMV in the alphavirus superfamily. Mutations in any of these conserved domains of 1a or 2a block or alter virus RNA replication and sgRNA transcription (6). Consistent with the N-terminus of 1a (methyltransferase) having a role in capping viral genomic and subgenomic RNA, 1a expressed in *E. coli* or yeast has been shown to methylate GTP at the 7 position of guanine in a reaction requiring *S*-adenosylmethionine and to form an adduct with m(7)GMP (10,178). Evidence further suggests that proteins 1a and 2a interact. Pseudorecombinant studies in which RNA1 and RNA2 from BMV and the bromovirus CCMV were exchanged first suggested that 1a and 2a had to be "compatible": such pseudorecombinants do not replicate in protoplasts, not even supporting the replication of BMV RNA3, which can be replicated by either homologous *1a/2a* gene combination (75). In addition, antisera against BMV 1a coprecipitates 1a and 2a from BMV RdRp-containing extracts (280). The C-terminal helicase region of 1a has been shown to interact with the N-terminal region of 2a by Far Western blotting and yeast two-hybrid assays (9,248), and recombinant studies suggest that the core RdRp region of 2a interacts with 1a as well (321). That the 32-kd protein encoded by the *3a* gene is the MP is suggested by several lines of evidence. RNA3 is not needed for viral replication in protoplasts but is essential for virus infectivity in plants. In addition, genetic studies show that deletions in the *3a* gene confine virus infection to individual cells; such mutations prevent the appearance of local necrotic lesions on the inoculated leaves of an appropriate plant host (234). The 32-kd protein also has properties reported for other viral MPs, such as the TMV 30-kd protein: it cooperatively binds single- stranded nucleic acids *in vitro* in a sequence-nonspecific manner, and it has been immunolocalized to plasmodesmata in BMV-infected plants (109,161).

Mutational studies also show that the CP is needed for systemic infection via the phloem, but whether it is required as well for local cell-to-cell movement has not been resolved. Mutations that disrupt the CP of BMV or CCMV have been reported to slow down, but not eliminate, virus spread within the inoculated leaf, based again on the rate of appearance and size of local lesions (7,103). However, mutation of the BMV CP appears to block cell-to-cell spread in some hosts, and studies with chimeric viruses in which the MP and/or CP was exchanged between BMV and CMV suggest a "compatibility" for MP–CP combinations in cell-to-cell movement, although in the latter studies an antagonistic effect of the CMV CP on the BMV MP could not be excluded (239). The important question is whether mutations in the CP affect BMV cell-to-cell movement directly or whether these inhibit local spread within the inoculated leaf through the minor veins. Studies with PVX have shown that infection of an inoculated leaf involves not only virus moving directly from cell to cell, but also virus entering the phloem and exiting locally (292). The question of the mode of cell-to-cell spread is particularly intriguing for BMV given its tripartite genome. Whether moving as encapsidated virus particles or viral RNAs directly complexed with MP, do the genomic RNAs move independently into adjacent uninfected cells until all three (and perhaps [sg]RNA4 as well) accumulate in the same cell, or is there a mechanism to coordinate movement of the three genomic RNAs as they move from cell to cell?

BMV replication occurs in the cytoplasm in association with ER-derived membranes, as reported for other positive-sense RNA viruses. Viral RdRp activity isolated from BMV-infected plants is membrane associated. In addition, immune staining with specific antisera against 1a, 2a, or ER marker proteins, or to detect 5-bromouridine incorporation into replicating viral RNA, show that 1a, 2a, and nascent viral RNA colocalize to cytoplasmic vesicles that also contain ER marker proteins (286). The BMV multiplication cycle is temporally regulated at the level of positive-sense RNA and negative-sense RNA synthesis, and the transcription of [sg]RNA4 to produce the CP, as has also been shown for PVX, TMV, and TYMV. The accumulation of positive-and negative-sense RNA is independently regulated such that late in infection, there is an ~100-fold excess of the genomic RNAs and [sg]RNA. Because [sg]RNA4 transcription is dependent on prior replication of complementary negative-strand RNA, it does not accumulate to high levels until later in infection, at which time efficient translation of [sg]RNA4 into CP appears to compete with that of the nonstructural proteins (1a, 2a, and particularly MP), inhibiting these while large amounts of CP are synthesized for encapsidation of virions (7). The cis-acting sequence elements involved in transcription of BMV [sg]RNA4 and replication of BMV negative-strand RNA and genomic positive-sense RNA have been well characterized in both *in vivo* studies that analyzed the effects of

specific mutations on the accumulation of different RNA species in protoplasts and *in vitro* studies with BMV template-dependent RdRp preparations from infected plants in which specific templates could be tested and initiation sites more precisely mapped. The 250 nt intercistronic region between the *MP* and *CP* genes on RNA3 in particular contains important sequence elements for the regulation of replication as well as transcription. Transcription of [sg]RNA4 initiates internally on negative strand copies of RNA3 within a 100 nt sequence in this intercistronic region located upstream of the *CP* gene (231) (Fig. 16). This subgenomic promoter consists of a core promoter of 20 nt located immediately upstream of the *CP* gene that directs specific basal transcription of [sg]RNA4, and additional upstream activating sequences that consist of an oligo(A) tract and partial repeats of core promoter sequences that act to increase levels of [sg]RNA4 transcription (108,216).

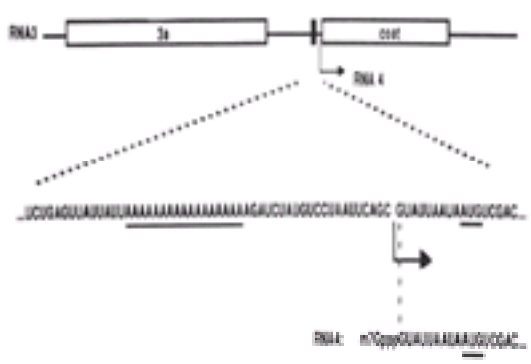


FIG. 16. Internal promoter for brome mosaic virus (BMV) subgenomic RNA4 ([sg]RNA4) encoding the capsid protein (coat or CP). The sequence of the intercistronic region is shown, below which is the 5'-terminal sequence of [sg]RNA4. AUG of the CP is underlined. (From ref. 220, with permission.)

Replication of BMV RNAs involves cis-acting sequences within the 5'-UTR and 3'-UTR of RNA1, RNA2, and RNA3, as well as internal sequences within the *2a* gene of RNA2 and a 150 nt sequence within the intercistronic region of RNA3. This 150 nt sequence is located upstream of the oligo(A) tract and most of the subgenomic promoter, overlapping sequences at the 5' end of the activating region. The promoter sequences for negative strand RNA synthesis have been mapped to the 3'-terminal 200 nt of the genomic RNAs that include the $tRNA_{tyr}$-like structure (6). Unlike TYMV, encapsidated BMV genomic RNAs terminate in 5'-CCA-3', but *in vitro* transcribed genomic RNAs that lack the 3'-terminal A residue can be adenylated by cellular CCA-tRNA nucleotidyltransferase *in vitro*. Like TYMV, BMV negative-strand RNA synthesis starts at the penultimate C within this terminal 5'-CCA-3' sequence, and thus it is assumed that adenylation of the 3' end of BMV positive strand RNAs by host CCA-tRNA nucleotidyltransferase must normally occur *in vivo*. The role of tRNA mimicry in BMV replication has not been as extensively studied biochemically as it has for TYMV. Interestingly, a recent study of the template requirements for initiation by the RdRp of TYMV and BMV reported that BMV RdRp can initiate synthesis internally at 5'-ACCA-3' sequences, but does so inefficiently. However, it could efficiently initiate at internal 5'-AUGC-3' sequences (69). The *cis*-acting sequences within the 5'-UTR regions of the BMV genomic RNAs are presumed to be involved in initiation of positive-strand RNA synthesis. These, and the 150 nt internal intercistronic sequence required for efficient accumulation of BMV RNA3, all contain a sequence similar to the motif 5'-GGUUCAAyyCC-3' (y = U or C), which is one of two consensus elements (Box B) found in cell RNA pol III promoters and also corresponds to the invariant residues of tRNA TYC loops (7). Mutations in this sequence within the 5'-UTR of RNA2 or the intercistronic region of RNA3 each reduce the accumulation of the corresponding RNA *in vivo*, and several proteins from barley extracts have been reported to bind to the Box B–like element within the 5'-UTR of RNA2 or the complement of this element within the 3'-UTR of the negative strand of RNA2 (92,265).

The involvement of Box B–like elements in the replication and/or accumulation of BMV RNA, the binding of host proteins to these elements, and the presence of cellular proteins in partially purified preparations of BMV RdRp (280,281) have been cited as circumstantial evidence for the involvement of host proteins in BMV replication. More direct evidence for this, as well as more detailed analysis of viral replication, has been obtained through studies of BMV replication in yeast (154,155,159). In this system, BMV proteins 1a and 2a are each expressed from separate yeast 2-μ plasmids having an *HIS3* (for 1a) or *LEU2* (for 2a) selectable marker so that the transformed yeast strain (*his3*$^-$ *leu2*$^-$ *trp1*$^-$ *ura3*$^-$) can be selected for growth in the absence of histidine and leucine. These are constructed such that the mRNAs encoding 1a or 2a are transcribed from the yeast *ADH* promoter; however, most of the 5'-UTR and 3'-UTR from RNA1 and RNA2 are missing, so these RNAs cannot be replicated. Replication is assayed by introducing RNA3, either by transfecting yeast coexpressing 1a and 2a with *in vitro* transcripts of RNA3, or by *in vivo* transcription of a RNA3 cDNA from a yeast *CEN4* plasmid (*TRP1* selectable marker) or chromosomal insertion (Fig. 17). In these latter two cases, RNA3 cDNA is flanked by the *GAL1* promoter and the self-cleaving ribozyme from hepatitis d virus. Growth in galactose induces transcription of RNA3, and the ribozyme cleaves the transcript at its natural 3' end. In the presence of BMV 1a and 2a, this RNA3 transcript, which is complete with its natural 5'-UTR and 3'-UTR, is correctly replicated in yeast via its complementary strand, and [sg]RNA4 is transcribed. For purposes of selection or screening, the *CP* gene on RNA3 can be replaced with the yeast *URA3* gene or the bacterial GUS or choramphenicol acetyltransferase (CAT) coding sequence. These will be translated from [sg]RNA4, thus allowing for selection of yeast replicating RNA3 (in the case of transfection of *in vitro* transcripts) based on uracil-independent growth of the appropriate yeast strain, or screening of RNA3 replication based on GUS or CAT assays. Selection against expression of the *URA3* gene (growth in 5-fluoroorotic acid) can also be used to isolate mutants defective in BMV 1a- and 2a-dependent replication of RNA3 (154). The *MP* coding sequence has also been replaced by *CAT* to assay for translation of RNA3 relative to its replication, the latter determined by Southern blotting with appropriate probes (160).

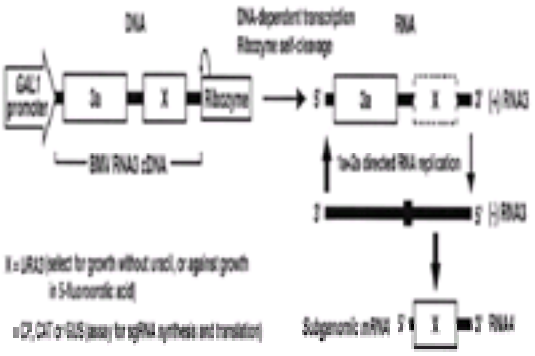


FIG. 17. A yeast system to study brome mosaic virus (BMV) RNA replication. (From ref. 154, with permission.)

The intriguing finding is that RNA3 is faithfully replicated and transcribed in the presence of 1a and 2a in yeast. This suggests that whatever host factors may be involved in BMV replication, yeast must contain equivalent factors that can substitute for those present in plants (159). Beyond this finding, the power of the yeast system is the ability to investigate the independent as well as the coordinate effects of 1a or 2a on replication and transcription of RNA3 and to select for host mutants that block different stages of RNA3 replication and transcription. Using this system, it was found that BMV 1a alone acts to stabilize RNA3 positive-sense transcripts expressed from the *GAL1* promoter, increasing their half-life from 5-to-10 minutes to greater than 3 hours. Using a *CAT* reporter gene in place of the *MP* coding sequence, this 1a stabilization appeared to block translation of RNA3, which led to the suggestion that 1a interacts with RNA3 to recruit it (and by inference RNA2 and RNA1) away from translation and into RNA replication (160). What sequences does 1a interact with in RNA3?: The intercistronic 150-nt "replication signal" that includes the consensus Box B–like element. Mutational analysis of RNA3 in this 1a-dependent yeast system shows that this 150-nt "enhancer of RNA3 replication" is required for this 1a stabilization of RNA3, and beyond this is sufficient to confer 1a-dependent stabilization when fused to the 5' end of b-globin RNA (328). Using 5-fluoroorotic acid to select against replication of a chromosomal insertion of RNA3 that expresses the *URA3* gene in place of the *CP* gene, recessive mutations in a number of yeast genes have now been identified that inhibit this BMV 1a- and 2a-dependent replication and transcription of RNA3. Characterization of these and other yeast genes that affect BMV replication and transcription in yeast is a powerful approach for deciphering the involvement of host proteins in RNA virus replication. One yeast protein, Lsm1p, a cytoplasmic protein related to core RNA splicing factors, has been shown to be involved in the 1a stabilization of RNA3 through interactions with the 150-nt element that contains the Box B–like consensus sequence (74). An additional interesting question is why does BMV replicate in yeast at all. This is apparently not true for all positive-sense RNA viruses: to date, only BMV and a few nodaviruses have been reported to replicate in yeast.

Enveloped Viruses

Given the plant cell wall and the movement of plant viruses from cell to cell without going through an extracellular phase, the existence of enveloped plant viruses raises a number of interesting questions in terms of viral adaptation. At what site(s)within the plant cell will these viruses bud to acquire their envelope? Surely the plant cell wall poses problems for budding at the plasma membrane, the site of maturation for most, although not all, enveloped animal viruses. Why would a plant virus have an envelope? Does the envelope and its associated glycoproteins have a role in infecting the plant host, or do they serve a separate function? What is the relationship between the form of the virus that moves from cell to cell to infect the plant and the virion? As already alluded to for tospoviruses such as TSWV (see Plant-to-Plant Transmission), and described in more detail later, the enveloped virion with its glycoproteins may represent an adaptation to a dual lifestyle: that of an "animal-infecting virus" as well as a plant virus. The genomic organization of the tospoviruses, and plant-infecting rhabdoviruses that have been characterized, suggests that they are "animal viruses" that encode one extra, unique protein—a movement protein needed for the virus to invade and infect plants. An important issue then becomes how virus maturation may be regulated in infected plant cells. Being negative-sense (nucleorhabdoviruses and cytorhabdoviruses) or ambisense (tospoviruses), transcripts of cDNA clones are not infectious. Thus, the interactions of these viruses with their hosts have been difficult to study, and a number of the details of their replication are sketchy or inferred by analogy to other, better characterized animal viruses within the same family. These particular plant viruses are included here, not to focus on the details of their replication, but to illustrate possible adaptations of enveloped viruses when infecting a plant.

Tomato Spotted Wilt Virus

Topsoviruses, a genus in the *Bunyaviridae*, have emerged as serious pathogens of crops in the past 20 years. In nature, the viruses are transmitted by thrips, but they can be mechanically transmitted in the laboratory. TSWV, the type member and best characterized of the tospoviruses, is in the current "top ten" of plant viruses in terms of economic losses worldwide (272). The recent emergence of TSWV, as well as a number of other tospoviruses, has been attributed to the increased worldwide prevalence of one of its major vectors, the western flower thrips (*Frankliniella occidentalis*), and the increased movement of ornamental plants as the result of increased world trade. TSWV infects >900 plant species of monocotyledonous or dicotyledonous plants, but other topsoviruses generally have more narrow host ranges (237). TSWV is a typical *Bunyaviridae* in terms of virion structure and overall genomic organization. Virions are spherical enveloped particles ~80-to-120 nm in diameter, with surface projections (spikes) composed of two viral-encoded glycoproteins, G1 and G2. The three genomic segments of the viral tripartite genome are associated with the nucleoprotein N and a few copies of the viral RdRp (L protein) to form three separate nucleocapsids within the enveloped particles (see Chapter 48). There is no matrix protein.

TSWV has three genomic ssRNA segments designated *L* (large), *M* (middle), and *S* (small) (Fig. 18) that are organized and expressed overall as are the genomic segments of the animal-infecting *Bunyaviridae* (see Chapter 48). The L

segment encodes a single protein, L (330 kd), shown to be the viral RdRp in several animal-infecting *Bunyaviridae.* The TSWV M segment encodes the viral glycoproteins, G1 and G2, on the complementary strand, and a nonstructural protein NSm (33.6 kd), which is translated from an sgRNA that is 5' coterminal with the viral genomic M segment. The S segment encodes the nucleoprotein N (30 kd) on the complementary strand, and a nonstructural protein NSs (54 kd), which like NSm, is translated from an sgRNA that corresponds to the 5' end of the viral genomic S segment. Thus, both the M and S segments of TSWV are expressed by an ambisense strategy (67,180,181) (see Fig. 18). All of the *Bunyaviridae* are principally negative sense viruses in terms of their replication strategy. Thus, TSWV, like other *Bunyviridae* and negative sense RNA viruses, contains the viral-encoded RdRp (L protein) associated with the nucleocapsids in the enveloped virus particles and in infected plant cells (346). L is the putative RdRp of TSWV based on sequence similarity to the RdRp of other *Bunyaviridae* and negative sense RNA viruses (66). Consistent with this, detergent-disrupted TSWV virions have been shown to contain an RdRp activity *in vitro* (4). The L protein is an endonuclease as well as an RdRp. The endonucleolytic activity is required because *Bunyviridae,* like the segmented negative strand influenza viruses and "ambisense" arenaviruses, require capped primers to initiate transcription of mRNAs. These are acquired from host mRNAs in the cytoplasm, not the nucleus, by a "cap snatching" mechanism (see Chapter 48). For TSWV, primer extension analysis has shown that the two mRNAs transcribed from the S segment contain nonviral sequences 12 to 20 nt in length at their 5' ends (184) (see Fig. 18). In addition, when plants are co-infected with TSWV and alfalfa mosaic virus (AMV) (a *Bromoviridae* with a genome organization like BMV), the heterogeneous 5' ends of the TSWV mRNAs can be acquired from the co-infecting AMV RNAs, thus clearly showing that this cap-snatching does occur in the cytoplasm. By analogy to other well-characterized negative strand RNA viruses such as vesicular stomatis virus (VSV), it is generally assumed that the TSWV N protein regulates the switch from mRNA transcription to viral genome replication (see Fig. 18). Consistent with this, both TSWV genomic and full-length complementary RNAs are found associated with N protein in RNP particles in infected plants (181). Using antisera generated against NSs expressed from a baculovirus vector in insect cells, NSs has been found associated with what has been described as fibrous structures or paracrystalline arrays in the cytoplasm of infected TSWV plant cells (182,237). Its function remains unknown. It is also not known how the viral nucleocapsids are released from virions following their introduction into plant cells by thrips. It is generally assumed by analogy with other enveloped viruses that they are taken up in cytoplasmic vesicles, with release occurring by fusion of the viral envelope with the vesicle membrane (Fig. 19).

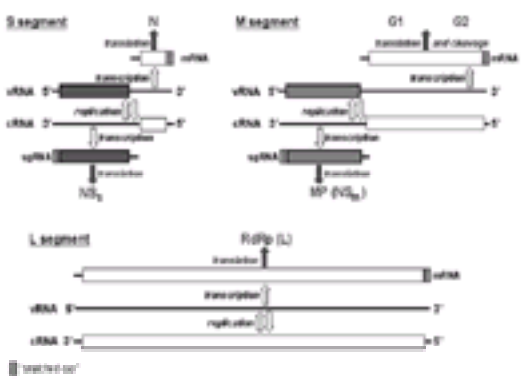


FIG. 18. Tospovirus replication and gene expression. (Adapted from ref. 272, with permission.)

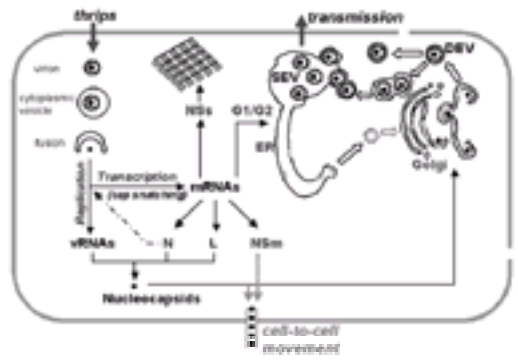


FIG. 19. Multiplication of tomato spotted wilt virus (TSWV). Stages of virion uncoating have been proposed based on analogy to animal bunyaviruses. DEV, double-enveloped virus; SEV, single-enveloped virus. See text for details. (Adapted from refs. 170 and 272, with permission.)

The distinctive feature of the genomic organization of TSWV when compared with that of the animal-infecting *Bunyaviridae* is the presence of an additional virus-sense gene in the M segment, which is expressed from an sgRNA (see Fig. 18). Evidence suggests that this encodes the viral MP. In infected plant cells, viral RNPs are seen associated with unique large tubular structures that extend into adjacent cells from what appear to be highly modified plasmodesmata. NSm immunolocalizes to these unique tubules, and has been shown to be necessary and sufficient to induce such tubules when transiently expressed in plant protoplasts or insect cells. In addition, NSm immunolocalizes to plasmodesmata in transgenic plants that express this protein (183,273,327). Although viral RNPs are associated with or within these tubules, transient expression studies in plant protoplasts show that N protein is not required for the formation of the tubules. Thus, it has been suggested that viral RNPs move from cell to cell in the plant through these MP-containing tubules (see Fig. 19). This picture of TSWV movement in infected plants envisions a pathway quite distinct from and clearly not involving enveloped virions with their associated G1 and G2 glycoproteins. As already discussed, the enveloped virion structure and G1 and G2 glycoproteins are required for thrips recognition and acquisition of TSWV but not for plant infection, as clearly demonstrated by the fact that plant-infectious TSWV lacking the envelope

structure and glycoproteins can be generated by repeated mechanical passage in plants (285) (see Plant-to-Plant Transmission). Only thrips in the larval stage can acquire the virus, which they ingest during feeding on plants. Ultrastructural studies show that virions are transported along the lumen of the thrips digestive tract to the midgut, where they appear to cross the midgut wall by endocytosis to enter the hemocoel. From the hemocoel, they invade the salivary glands. However, unlike the *Luteoviridae,* topsoviruses replicate in their thrips hosts, persistently infecting the insects without being pathogenic. Evidence for this includes the detection of the nonstructural protein NSs in infected thrips and the identification of viral inclusions in endothelial cells, muscle cells, and the salivary glands (237). Using gel overlay and immune labeling assays, two candidate thrips receptor proteins that recognize TSWV glycoproteins have been identified: a midgut protein that can bind both G1 and G2, and a protein found in all tissues except the midgut that recognizes G2 (17,171). The functions of these thrips proteins in virus acquisition and circulation has not been determined.

Where does TSWV virion assembly occur in infected plant cells? For those animal-infecting *Bunyaviridae* where it has been investigated, the viral G1/G2 polyprotein precursor has a signal sequence so that it is cotranslationally transported into the ER lumen, being processed to G1 and G2 by host cell peptidases and glycosylated as it transits from the ER to the Golgi stacks. The heterodimeric G1:G2 complex contains a Golgi-retention signal, so that virion assembly occurs by viral RNPs associating with G1:G2-containing areas of Golgi membranes and budding into the Golgi. These assembled virions are then transported to the cell surface in vesicles, where they are released from the cell as the vesicles fuse with the plasma membrane (see Chapter 48). Until recently, the pathway for TSWV virion morphogenesis in plant cells has not been clear. The TSWV G1/G2 precursor is predicted to contain a signal peptide and has been shown to be processed when expressed in insect cells, where it was also reported to be associated with cellular membranes, including the plasma membrane (3). However, this study done in insect cells does not answer the question concerning where virions mature in infected plant cells. Ultrastructural and immune labeling studies have reported virus particles or virion proteins associated with what morphologically appeared to be ER membrane in infected plants cells or Golgi-like structures in infected thrips cells, leading different authors to state that TSWV acquires its envelope by budding into the ER or the Golgi. However, the identity of these membrane structures was never determined by immune labeling for appropriate ER and Golgi marker proteins. A major advance in this area is the recent development of a plant protoplast system for investigating TSWV infection in which more than >50% of the cells (*Nicotiana rustica* tobacco cells) can be infected with TSWV particles and maintained for several days (172). Using this synchronous infection system for ultrastructural studies and immune staining for compartment-specific marker proteins, the TSWV glycoproteins G1 and G2 were found to accumulate in Golgi membranes. These areas of the Golgi then appear to wrap around viral RNPs in the cytoplasm to form double-enveloped particles (DEV) (see Fig. 19). At later stages of infection, both in the infected protoplasts and in cells in TSWV-infected plants, these cytoplasmic DEV double-enveloped particles can be found fused to each other and fusing to ER membrane to form single-enveloped particles (SEV, the virions) clustered in membrane-bound bodies in the cytoplasm or in the ER (170) (see Fig. 19). Thus, rather than be transported in Golgi-derived vesicles to the plant plasma membrane with its cell wall, TSWV appears to have adapted a strategy that allows mature virions to move back from the Golgi to the ER and accumulate there, as well as in the cytoplasm. It is suggested that when thrips feed, they acquire TSWV virions from these sites of accumulation in the ER (170).

Rhabdoviruses: Sonchus Yellow Net Virus and Lettuce Necrotic Yellows Virus

Plant viruses have historically been categorized as rhabdoviruses based on ultrastructural examination of infected plant cells or virus preparations, which identify enveloped virus particles that are bullet shaped in unfixed preparations or bacilliform when fixed (Fig. 20). Virions are 45 to 100 nm wide and 130 to 250 nm long, and in nature are transmitted by aphids, leafhoppers, or planthoppers, depending on the virus. Like the tospoviruses, these viruses infect and multiply in their insect vectors, thus leading a dual lifestyle as an animal-infecting and plant-infecting virus. Approximately 75 plant viruses are currently listed as possible rhabdoviruses, although it is not clear that all of these represent distinct viruses or are in fact rhabdoviruses. About 15 of these viruses have been sufficiently characterized in terms of virion structure and composition, host range, vector transmission, serology, nucleic acid hybridization, and ultrastructural alterations in infected plant cells to be placed in the genus *Cytorhabdovirus* or *Nucleorhabdovirus* (158). As these names imply, the plant rhabdoviruses are grouped based on the site of virus multiplication, as determined by electron microscropic examination of infected plant cells. Nucleorhabdoviruses form nuclear inclusions and appear to bud from the inner nuclear envelope to accumulate in perinuclear spaces. Cytorhabdoviruses are seen to form at cytoplasmic membranes and accumulate in the cytoplasm. Of these, sonchus yellow net virus (SYNV; a nucleorhabdovirus) and lettuce necrotic yellow virus (LNYV; a cytorhabdovirus) are the best characterized in terms of sequence analysis.

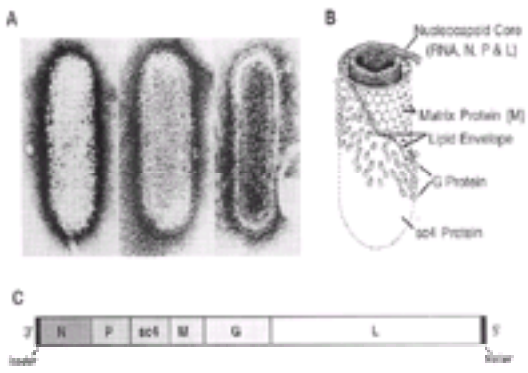


FIG. 20. Structure and genome organization of sonchus yellow net virus (SYNV), a nucleorhabdovirus. (From ref. 158, with permission.)

Electron microscopy of purified virions, combined with detergent disruption, gel analysis of viral proteins, and RNA analyses, show that plant rhabdoviruses, as typified by SYNV and LNYV, resemble VSV in structure and composition (158) (see Fig. 20). Virions are composed of a lipid envelope from which project spikes made of the viral-encoded G

glycoprotein, which is presumed to exist in a trimeric form as in VSV. Within the envelope, the virion core is composed of a helical RNP that consists of the viral genomic unsegmented negative-sense ssRNA (~11 to 14 kb) surrounded by N protein to form the nucleocapsid, in association with a few copies of the viral P and L proteins that make up the RdRp (see Fig. 20). The location of the matrix protein M has not been unambiguously determined, but like VSV, it is assumed to be involved in coiling the nucleocapsid and interacting with the internal domains of G (see Chapter 38). The available evidence suggests that replication of the plant rhabdoviruses resembles that of VSV. Treatment of LYNV virions with nonionic detergent activates a viral RdRp that transcribes RNAs complementary to the viral genome *in vitro* (158). The RdRp activity is associated with loosely coiled nucleocapsids that contain N, L, and P, in addition to the virion ssRNA. Interestingly, similar treatment of SYNV virions does *not* activate RdRp activity, but such an activity can be extracted from the nuclei of plant cells infected with SYNV (358). This SYNV RdRp activity co-sediments with viral nucleocapsid cores and contains the viral N, L and P proteins, based on copurification and immune precipitation studies (359). Antibody inhibition studies show that L is a functional component of the RdRp activity. Kinetic analysis of the products transcribed *in vitro* shows that like VSV, SYNV transcription occurs by a stop-start mechanism in which the individual mRNAs for each gene are sequentially transcribed starting at the 3' end of the viral genome and continuing through to the 5' end, with the RdRp pausing at intergenic regions between each gene to polyadenylate and terminate the upstream gene, and then transcribe the next gene in order (see Chapter 38). Also like VSV, the relative abundance of each mRNA is determined by the gene order in the viral genome, with the 3'-proximal N mRNA being most abundant and the 5'-proximal L mRNA being transcribed in the lowest amount (359). All of the mRNAs are polyadenylated by the viral RdRp activity. Like VSV, a positive-sense short leader RNA is transcribed from the 3' end of the genomic RNA; but unlike VSV, the SYNV leader RNA is also polyadenylated (359). Thus, while the picture is one of virions containing RNP cores that can initiate transcription upon uncoating, as one would expect for a negative-sense RNA virus, the SYNV core-associated RdRp appears to require activation within the host cell. This, along with the polyadenylation of the SYNV leader RNA, appears to reflect adaptations necessary for the nuclear multiplication of this virus.

The complete sequence of the SYNV genome shows an organization that is the same as VSV, with one additional gene *sc4* not found in VSV (reviewed in 358) (see Fig. 20). The 3' leader sequence is followed by the genes encoding N, P, M, G, and L in the same order as they occur in VSV (see Chapter 38). The *sc4* gene is located between the P and M genes (see Fig. 20). The 3' leader and 5'-proximal noncoding region of SYNV are distinct from those in VSV and other animal-infecting rhabdoviruses, but the intergenic gene junctions are highly conserved (158) (Table 4). The sc4 protein is found in virions (see Fig. 20) and appears to be phosphorylated *in vivo*. Although its precise location is unknown, it is solubilized from virions by treatment with nonionic detergents. It has been suggested that sc4 might be the viral MP, although it could also play a role in aphid transmission (158). SYNV induces the formation of viroplasms in the nuclei of infected plant cells. The viral N, L, and P proteins have been immunolocalized to these nuclear viroplasms, which appear to be the sites of viral replication and the source of the RdRp activity that can be extracted from nuclei of infected cells (217,359). N and P also appear to contain NLSs: when N or P are individually expressed from a PVX vector (see Fig. 8), each accumulates in the nucleus, and if expressed as fusions to GUS, the GUS:N and the GUS:P fusion proteins are also targeted to nuclei (217). Ultrastructural studies show SYNV virions budding at the inner nuclear membrane to accumulate in swollen perinuclear spaces between the inner and outer nuclear envelopes. Thus, the rather striking adaptation to replicate in nuclei, as opposed to VSV, which replicates in the cytoplasm, appears to be a strategy to acquire the viral envelope from the inner nuclear envelope rather than the plant cell plasma membrane. The current model for SYNV multiplication (Fig. 21A) proposes that upon entering a cell, the viral nucleocapsid cores are released into the cytoplasm, possibly in association with the ER, and these are transported into the nucleus, where the SYNV RdRp is activated and primary transcription occurs. Viral mRNAs are transported to the cytoplasm, where they are translated, and newly synthesized N, L, and P are transported into the nucleus to continue transcription of viral mRNAs and replicate the viral genome. Presumably, newly synthesized viral M and sc4 proteins also have to be transported to the nucleus: M for virion assembly and sc4 since it is present in virions. Because tunicamycin interrupts SYNV budding at the nuclear envelope and causes nucleocapsids to accumulate at the nuclear periphery, it is proposed that newly synthesized G is transported to the inner nuclear envelope, and virus buds at these sites into the perinuclear space between the inner and outer nuclear membranes (158). This model envisions that viral cores lacking an envelope are exported from the nucleus and move cell to cell, presumably through plasmodesmata, as an alternative to virion assembly (see Fig. 21), as is the case for TSWV.

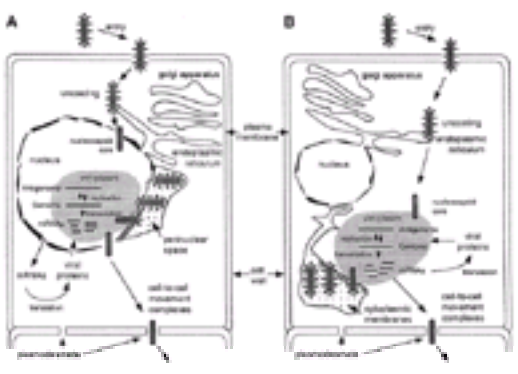
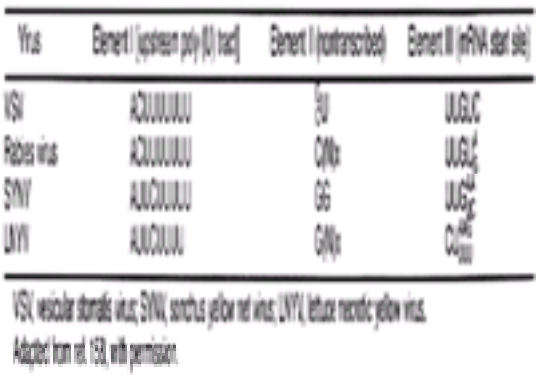


FIG. 21. Multiplication of **(A)** nucleorhabdoviruses and **(B)** cytorhaboviruses. (From ref. 158, with permission.)



Virus	Element I (upstream poly (A) tract)	Element II (untranscribed)	Element III (mRNA start site)
VSV	ACUUUUUU	3U	UUGUC
Rabies virus	ACUUUUUU	CUUa	UUGU$_C^G$
SYNV	AUUCUUUU	GG	UUG$_C^{GA}$
LNYV	AUUCUUU	GUUa	C$_{UUU}^{AG}$

VSV, vesicular stomatitis virus; SYNV, sonchus yellow net virus; LNYV, lettuce necrotic yellows virus.
Adapted from ref. 158, with permission.

TABLE 4. *Sequence elements in the intergenic regions of plant and animal rhabdoviruses*

In the context of the nuclear replication of SYNV, the presence of an inactive form of the RdRp within virions and the polyadenylation of the leader RNA make sense. Packaging of an inactive RdRp provides a mechanism to prevent premature transcription from occurring in the cytoplasm before nucleocapsid cores are transported to the nucleus. Host activation of the RdRp could involve a posttranslational modification, such as phosphorylation of L or P within the RdRp complex, or of the core structure such that it can be transported into the nucleus. Polyadenylation of the leader sequence may prevent this RNA from being degraded within the nucleus. Although there are some biochemical and immune localization studies to support aspects of this model, the proposed pathways for viral assembly and movement are primarily based on ultrastructural studies. Cell biological and additional biochemical studies are needed to investigate the interesting questions raised by this model. Essentially nothing is known about the form in which the virus moves from cell to cell to invade the plant, or the process of movement. Perhaps M is required only for virion assembly at the inner nuclear envelope, and flexible nucleocapsids, in association with sc4 as well as P and L, are the form that exits from the nucleus and moves from cell to cell. What regulates whether genomic RNPs are packaged into virions or transported from cell to cell? Could it be posttranslational modifications of sc4, such as phosphorylation or dephosphorylation? If sc4 is not the viral MP, what is the MP? As is clear from studies of TSWV, appropriate mutational studies and cell-based systems are needed to investigate viral morphogenesis and movement, for both the nucleorhabdoviruses and the cytorhabdoviruses. LYNV has not been completely sequenced, but genome mapping studies suggest that it has a genome organization similar to that of SYNV (158). Based exclusively on ultrastructural studies of LYNV and barley yellow striate mosaic virus, replication of cytorhabdoviruses is proposed to occur in the cytoplasm in association with what appear to be ER membranes, and virions appear to assemble by budding into vesicles that may be derived from the ER (158) (see Fig. 21). These studies suggest that cytorhabdoviruses also use internal membranes—perhaps the ER—for assembly, and it is again proposed that nonenveloped viral RNPs move from cell to cell by an alternate route. The development of appropriate model cell-based systems for investigating both these genera of rhabdoviruses should help to define the principles involved in the adaptation of enveloped viruses to infecting plants.

Viruses with DNA Genomes

Caulimoviruses: Cauliflower Mosaic Virus

The *Caulimoviridae* comprises the plant-infecting pararetroviruses. The viruses in this family, like the *Hepadnaviridae* (see Chapter 86), have circular dsDNA genomes and replicate by a reverse transcription mechanism by way of a positive-sense ssRNA template. The two genera of *Caulimoviridae*—*Caulimovirus* (12 viruses) and *Badnavirus* (13 viruses)—are distinguished based on virion morphology (icosahedral or bacilliform) and genome organization (347). Given its dsDNA genome, CaMV, the type member of the caulimoviruses, was the first plant virus to be cloned and sequenced. Thus, it has been extensively characterized in terms of its molecular biology and host interactions. CaMV, in addition to providing what is still generally the promoter of choice for generating transgenic plants (the 35S promoter), has been a model for investigating the replication and gene expression strategies of the caulimoviruses in particular, and mechanisms of translational control in general. Caulimovirus particles are icosahedral (T=7), 45 to 50 nm in diameter, with the capsid being composed of subunits of a single protein, CP, which in CaMV exists in two processed forms of 37 kd and 44 kd. CaMV particles also contain minor amounts of three additional viral-encoded proteins: a 15-kd nucleic acid–binding protein, and a protease (PR) and reverse transcriptase (RT) that are synthesized as the PRO-RT polyprotein product of a single ORF. Most caulimoviruses are transmitted in nature by aphids in a noncirculative manner, neither circulating in nor infecting the vector. Although each virus has a fairly narrow host range, individual caulimoviruses cause serious yield losses in economically important crops, such as cauliflower, turnips, and blueberries (144,310). CaMV infects crucifers, including *Brassica* species, such as turnip and cauliflower.

The CaMV circular dsDNA genome is 8 kb and contains three discontinuities: two in the viral positive strand (same polarity as viral mRNA transcripts) and one in the complementary viral negative strand (Fig. 22). There is a short 5' overlap at each discontinuity, with ribonucleotides often attached to the 5' ends. These discontinuities are formed by strand displacement synthesis during reverse transcription of the 35S pregenomic positive strand RNA. CaMV encodes seven major ORFs (I to VII). Mutational studies show that ORFs I through VI are essential for viability and infectivity. These encode: the viral MP (I, 45 kd), n aphid transmission factor (ATF, II, 18 kd), a minor capsid component (pIII, 15 kd), a precursor of the capsid protein (pre-CP = GAG, IV, 56 kd), a polyprotein (V, POL, 80 kd) that is processed to an aspartic proteinase (PR) and reverse transcriptase (RT) with an RNase H domain (RH), and a multifunctional translational transactivator (TAV, VI, 62 kd) (144) (see Fig. 22). These functional assignments are supported by a large number of experimental studies. ORF I is essential for cell-to-cell movement and systemic infection of the plant. The encoded MP has been localized to unique large tubules that contain virus-like particles and extend from plasmodesmata in infected cells, and it can induce these tubules when expressed in cultured cells (208,261,340). ATF forms inclusion bodies in infected cells and has been shown to bind to virus particles. CaMV ATF mutants are infectious when mechanically inoculated onto plants, but they cannot be transmitted by aphids. It is not precisely known how ATF acts. However, aphids which are fed ATF can acquire and transmit ATF-defective CaMV, suggesting that ATF may interact

with the cuticular lining of the aphid stylet (307,310). pIII, found associated with virus particles, can bind DNA in a sequence-nonspecific manner and appears to function as a tetramer. It has been suggested that it compacts the viral genomic DNA during virion assembly (60,200). GAG, or pre-CP, as synthesized, contains highly acidic N- and C-terminal regions, which are removed by the viral aspartic proteinase (PR) to form the mature capsid proteins of 44 kd and 37 kd: the 37-kd protein is contained within the 44-kd protein sequence. It is not known whether one or both of these CP forms is the functional capsid protein (144). The polyprotein product of ORF V (POL = PR-RT) if expressed in *E. coli* or translated *in vitro* in reticulocyte extracts, self-cleaves into the N-terminal PR (20 to 22 kd) and C-terminal RT (56 kd). PR contains the consensus motif found in aspartic proteinases, and mutation of this motif destroys proteolytic activity and is lethal for the virus. PR processes both the POL polyprotein to PR and RT and the pre-CP (GAG) (341). The expected RNA:DNA replication intermediates and RT, activity have been identified in the cytoplasm of infected cells, and the RT activity has been characterized (27,262). TAV is a translational activator required for the 35S pregenomic RNA to be translated as a polycistronic mRNA (112,311). It also forms inclusion bodies, distinct from the ATF inclusions, that appear to be the site of virus assembly and reverse transcription. Virus particles, pregenomic RNA, and reverse transcriptase all copurify with these inclusions (262,315).

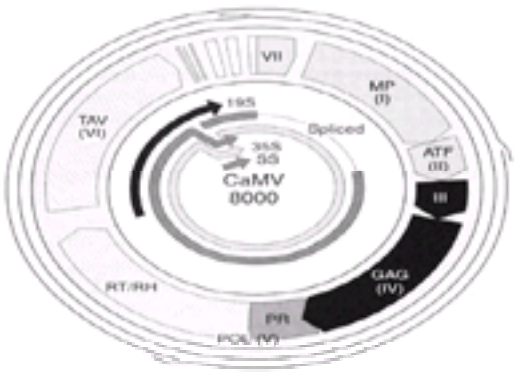


FIG. 22. Genome organization of cauliflower mosaic virus (CaMV). The open circular viral dsDNA with its overhangs (discontinuities) is indicated by the *outer two lines.* The arrangement of the open reading frames (ORFs) is shown on the main circle, with the six short ORFs within the 35S RNA leader indicated by *white boxes.* The 35S, 19S, short stop (SS) and essential spliced transcripts are each indicated by the *inner circular arrows.* (From ref. 144, with permission.)

Although the early events of caulimovirus uncoating are not well understood, it appears that upon infection, virus particles deliver the viral dsDNA to the nuclear pore, where it is released into the nucleus and the discontinuities are sealed by host enzymes to form a supercoiled minichromosome. Host RNA pol II complexes transcribe these minichromosomes. Transcription is unidirectional, using two viral promoters to produce two major capped and polyadenylated transcripts that are named for their sedimentation characteristics: the 35S RNA (also termed the *35S pregenomic RNA*), which is transcribed from the 35S promoter, and the 19S RNA, transcribed from the 19S promoter (Fig. 22 and Fig. 23). The relative amounts of these transcripts are determined by the promoter strength. The 35S promoter is very strong owing to the presence of several enhancer elements, which in combination make it a constitutive promoter in most plant cell types. The 19S promoter is relatively weak *in vitro*, but *in vivo* could be influenced by the enhancer elements within the 35S promoter region (54,91,129,249,250). The 19S RNA is monocistronic and is translated into TAV. The 35S RNA is translated into the remaining five essential proteins (MP, ATV, pIII, pre-CP [GAG], POL [PR-RT]) by two unusual mechanisms in eukaryotic cells: ribosome shunting (84,113) and reinitiation, the latter of which makes it functionally a polycistronic mRNA (112,311) (see Fig. 23). The 35S RNA has 180-nt terminal repeats and, similar to hepadnaviruses and retroviruses, functions both as the major viral mRNA and the template for reverse transcription to synthesize the viral dsDNA. The polyadenylation signal for both the 19S RNA and the 35S RNA is located 180 base pairs downstream of the transcription start site for the 35S RNA. The 180-nt terminal repeats at the ends of the 35S RNA are created by RNA pol II ignoring this polyadenylation signal on its first passage to transcribe around the circular genome and terminate on the second encounter with this signal. The polymerase will occasionally recognize the polyadenylation signal on its first encounter, thus producing a 180 nt “short stop” RNA, the function of which is not known (144) (see Fig. 22). Alternatively spliced transcripts of the 35S RNA have also been detected using reporter gene constructs in transiently transfected protoplasts and in CaMV-infected cells. All of these use an acceptor site within ORF II, but differ in using a donor site near the end of the RNA leader region or at sites within ORF I (Fig. 22). The function of these spliced transcripts is not known, but mutation of the common splice acceptor site suggests that splicing is essential for viral infectivity (176).

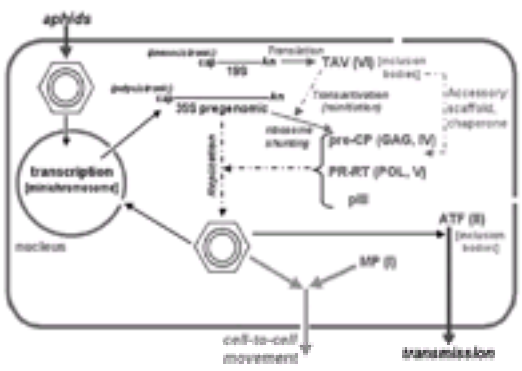


FIG. 23. Multiplication of cauliflower mosaic virus (CaMV).

Whereas the 19S RNA can be translated *in vitro*, the 35S RNA, and most of its spliced variants, cannot because of its polycistronic nature and a long highly structured 600-nt leader. This leader prevented *in vitro* translation of the 35S RNA until the recent development of a cell-free wheat germ system in which ribosome shunting could occur (138,308). Thus, most of the studies on the translation of the 35S RNA have been done by expressing appropriate dicistronic and polycistronic reporter gene constructs transiently in plant protoplasts or in transgenic plants. The earliest indication that the 35S RNA was polycistronic was the polar effects that certain mutations in ORF VII or *ATF* had on the expression of viral proteins encoded by the downstream ORFs (145 and citations therein). Most of the ORFs downstream of ORF VII are poorly expressed in protoplasts or transgenic plants unless TAV is present. In particular, transient expression studies with CAT or GUS reporter genes inserted in dicistronic constructs derived from the CaMV 35S RNA or at different sites along the 35S RNA transcript from figwort mosaic virus (also a caulimovirus) showed that these could be expressed, but only if TAV was provided in *cis* or in *trans* (112,311). Activation by TAV is specific for downstream ORFs and does not require virus-specific sequence elements, nor does TAV induce internal ribosome binding or ribosome shunting. Rather, TAV acts on ribosomes migrating through and apparently translating regions upstream of the reporter gene, thus suggesting that it acts by allowing ribosomes to reinitiate translation at a downstream ORF.

The 600-nt leader of the CaMV 35S RNA contains seven to nine short ORFs (sORFs) depending on the strain, and both chemical and enzymatic probing show this region to be highly structured (138) (Fig. 22 and Fig. 24). Normally in eukaryotic translation, the presence of such features will inhibit ribosome scanning unless there is an IRES (see Chapter 23) or the ribosomes can bypass or “shunt” around the sORFs and structured region. Expression studies with appropriate reporter gene constructs in protoplasts and transgenic plants first suggested that ribosome shunting is the mechanism by which translation is initiated on the first ORF (VII) in the CaMV 35S RNA (see Fig. 24). Of the six sORFs shown within the 35S RNA leader in Figure 24, A to D are only 2 to 8 codons each, but E and F are 26 and 33 codons, respectively. Transient expression studies first showed that some regions of the 35S leader are more inhibitory to translation than other regions or the intact 600-nt leader. In particular, the very short sORFs A to D do not inhibit translation of a downstream reporter gene, but E and F are strongly inhibitory to translation of ORF VII or a downstream reporter gene. Insertion of a stable stem-loop structure near the cap or within a duplication of the first 60 nt of the leader inhibits translation, but insertion of the stem-loop or the GUS coding sequence in the middle of the leader does not. That this is not a linear process was shown by expressing the capped 5' half of the leader and the 3' half fused to a CAT reporter gene as two distinct molecules in protoplasts: CAT was expressed only when both transcripts were expressed in the same cell. These studies suggested that ribosomes initiate translation on the 35S RNA by cap-dependent scanning, but can bypass or shunt around the central region of the leader. By inserting AUGs at different positions within the leader, the shunt donor site was located in the first 100 nt of the leader, past the termination codon of sORF A, and the shunt acceptor site was mapped to within sORF F (111,113). The development of an *in vitro* wheat germ translation system in which ribosome shunting can occur has led to a more detailed examination of the shunting mechanism (308). In particular, insertion of the leader between the coding sequences for GUS upstream and CAT downstream prevents expression of CAT activity, clearly showing that an IRES is not present within the leader sequence. By constructing an in-frame fusion of sORF F with the CAT coding sequence, the relative rates of scanning through the leader (translation of the longer F:CAT fusion protein) and shunting (translation of the shorter CAT protein) could be determined, and mutational studies showed that the structure of stem 1 (see *st1,* Fig. 24) is important for shunting to occur. Mutations that abolish stem 1 drastically reduce CAT expression, and compensatory mutations that restore stem 1 structure restore CAT expression. In fact, stem 1 and flanking sequences alone will allow shunting, and sORF A preceding stem 1 enhances shunting (84).

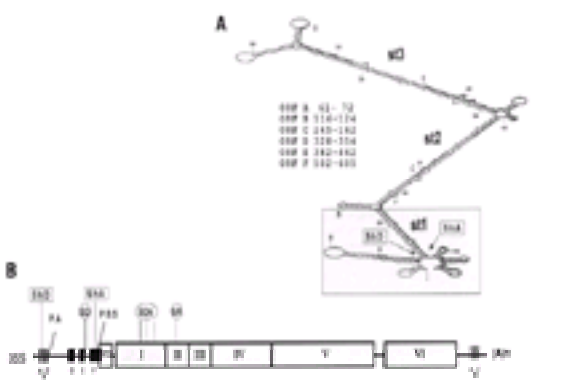


FIG. 24. A: Extensive secondary structure of the CaMV 35S RNA leader. Shunt donor (*ShD*) and Shunt acceptor (*ShA*) sites and stems (*st*) are indicated. **B:** CaMV 35S RNA. *Black boxes* represent small open reading frames. (From ref. 84, with permission.)

The importance of sORF A was further shown by constructing viruses with mutations that eliminated the various sORFs or structures within the leader. Viable mutants were obtained that were very delayed in the time of appearance of symptoms. Serial mechanical passage of these in turnip plants produced phenotypic revertants that resembled wild-type CaMV in their disease production. Analysis of these showed that sORF A and secondary structure within the central region of the leader are important for viral infectivity and thus presumably ribosome shunting (266). Further investigation of ribosome shunting by *in vitro* translation in reticulocyte as well as wheat germ extracts and expression of reporter constructs in plant protoplasts showed that proper translation and termination of a short upstream ORF is required for shunting to occur and that TAV increases shunting efficiency (137,267,295). Thus, the shunt-mediated translation appears to require reinitiation, which can be facilitated in the presence of TAV. As to the mechanism of ribosome shunting, a model has been proposed in which scanning 40S subunits and 80S ribosomes translating through sORF A stall when they encounter the stable hairpin structure. Proper termination of translation of sORF A by the 80S ribosomes promotes reinitiation, such that on release of the encoded peptide and dissociation of the 60S subunit, the reinitiation-competent 40S subunit skips over the stem and resumes scanning downstream (137). Why have this

elongated 480-nt hairpin in the middle of the leader in the first place, necessitating ribosome shunting at all? Yeast three-hybrid studies show that CP specifically interacts with a purine-rich sequence at the top of this elongated hairpin (128). Thus, ribosome shunting ensures, in the context of this structure, that translation of the 35S RNA and its encapsidation as a template for reverse transcription of the viral dsDNA can both occur.

CaMV replicates by reverse transcription of the 35S RNA. Unlike retroviruses, and more similar to hepadnaviruses, the CaMV dsDNA is packaged into virions, and it does not integrate into host chromosome during normal multiplication. Rather, it exists as autonomously replicating circular minichromosomes in the nuclei of infected cells. However, the mechanism of reverse transcription is more similar in several respects to that of the retroviruses (see Chapter 57) than the hepadnaviruses. Reverse transcription occurs in the cytoplasm. Yeast three-hybrid studies show that the zinc finger motif (C-X_2-C-X_4-H-X_4-C) and an adjacent basic domain in the central region of CP are essential for strong and specific interaction with the purine-rich sequence in 35S RNA leader. This is consistent with a model in which the 35S RNA is first encapsidated and then transcribed within the virus particle (128). Studies suggest that the acidic C- and N-terminal regions of pre-CP, which are cleaved by the viral PR, may regulate packaging of the 35S (pregenomic) RNA and nuclear targeting of the virus particles. Presence of the CP C-terminal acidic domain prevents interaction of the CP with the 35S RNA leader, suggesting that it may ensure that 35S RNA is available as mRNA until late in infection when sufficient virion proteins, including PR (ORF V), which cleaves this domain, are present (128). Cleavage of this C-terminal domain would allow CP binding to and encapsidation of 35S RNA, presumably together with bound RT. Like retroviruses, reverse transcription is initiated from a tRNA primer ($tRNA_{met}$), which binds to a primer binding site on the 35S RNA at a position ~600 nt downstream of the 35S promoter, which is ~400 nt downstream of the end of the first terminal repeat at the 5' end of the 35S RNA. Synthesis of the complementary DNA negative strand proceeds to the end of the 35S RNA, where the template switch occurs as the 3' end of this newly synthesized negative strand DNA melts from the 5' end of the 35S RNA template and anneals to the terminal repeat at the 3' end of the same or another 35S RNA. Synthesis of the DNA negative strand continues along the 35S RNA, passing the primer binding site and the 5' end of the of the molecule (overshooting) to create the 8-nt discontinuity or terminal repeat at the ends of the negative strand DNA. As this proceeds, regions of the RNA template that have been reverse transcribed are digested by RNase H, except for oligo(G) stretches—two for the genome shown in Figure 22—which are resistant and serve as primers for DNA positive strand synthesis. Again, the polymerase will overshoot, creating the two discontinuities or short terminal redundancies in the viral positive strand DNA (27,262) (see Fig. 22). Encapsidation of the 35S RNA and reverse transcription within the virus particles appear to occur within the TAV inclusion bodies, and it has been suggested that the TAV matrix may provide accessory scaffolding or chaperone functions important in virus assembly. At this point, the virus particles with their dsDNA can either target back to the nucleus, contributing to the transcription pool, or move from cell to cell, apparently through the plasmodesmal-associated tubules that contain the viral MP (see Fig. 23). The fully processed virus CP contains an N-terminal NLS, which is exposed on the surface of virus particles but is masked if the N-terminal acidic domain of pre-CP is present. Thus, the presence of the N-terminal acidic domain keeps pre-CP in the cytoplasm, where it can be targeted to inclusion bodies for virus assembly. Once particles are assembled, cleavage of this domain by the viral PR exposes the CP NLS, and the virus particle can be transported to the nucleus (201). The CP is also phosphorylated by a host casein kinase II, which apparently stabilizes it (201), and it has been reported to be methylated and glycosylated. Not addressed by this model is the question of whether the presence of the N-terminal acidic domain of pre-CP or other CP modifications may play a role in the virus particles being directed to move from cell to cell or bind ATF for aphid acquisition.

Geminiviruses

The *Geminiviridae* are named for their unusual capsid structure. Virions in this family have paired incomplete (T=1) icosahedral capsids (gemini = twin moons), ~18 × 30 nm, composed of subunits of a single CP (29 kd) (Fig. 25). The viral genomes are covalently closed circular ssDNA (~2.5 to 3.0 kb), with a single ssDNA molecule encapsidated in each geminate particle (193). *Geminiviridae* comprise three genera based on genome organization, vector specificity, and host range properties (Fig. 26). Mastreviruses (type member, maize streak virus [MSV]) have a single genomic component of ssDNA, are transmitted by different leafhopper species and, with two exceptions (bean yellow dwarf virus [BYDV] and tomato yellow dwarf virus [TYDV]), infect monocotyledonous plants. Begomoviruses (type member, bean golden mosaic virus) are transmitted by whiteflies (*Bemicia tabaci,* A and B biotypes), infect dicotyledonous plants and, with a few exceptions (e.g., some isolates of tomato yellow leaf curl virus [TYLCV]), have two distinct genomic components (A and B) that are both required for infectivity and separately encapsidated. TYLCV has a single genomic component that is similar in its overall organization and sequence of the CP to the A component of the begomoviruses. Curtoviruses (type member, beet *curly top* virus [BCTV]), like mastreviruses, are transmitted by leafhopper species (except for tomato pseudo-curly top virus, which is transmitted by planthoppers) and have a single genomic component, the organization of which looks like a hybrid of the mastrevirus virion-sense (V) ORFs and the begomovirus complementary-sense (C) ORFs (see Fig. 26). Like the begomoviruses, curtoviruses infect dicotyledonous plants (34). With the notable exception of BCTV, individual *Geminiviridae* have narrow host ranges. Nevertheless, they have emerged in recent years as one of the most serious groups of plant viral pathogens worldwide because of the widespread expansion of the B biotype of *B. tabaci* and the ability of viruses in mixed infection to give rise to new strains and isolates through recombination (141,150).

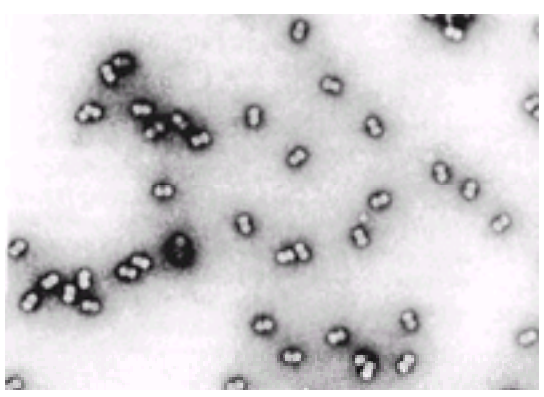


FIG. 25. Electron micrograph of virions of bean golden mosaic virus, a begomovirus. (From ref. 5, with permission.)

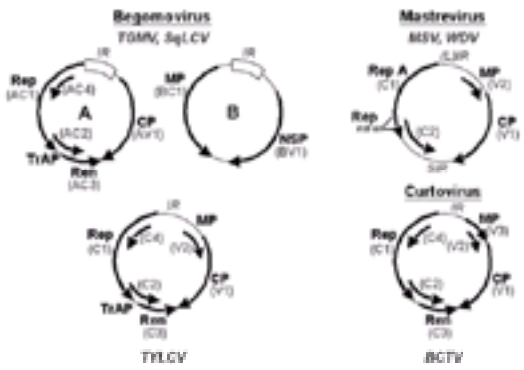


FIG. 26. Genome organization of different genera in the *Geminiviridae*.

Viral genes are transcribed bidirectionally from an intergenic region (IR), named the *long intergenic region* (LIR) in mastreviruses (see Fig. 26). Genome sequences for several begomoviruses first showed that this 200-nt IR was identical in the A and B components, and thus it has been called the *common region* in these bipartite viruses (193). The encapsidated ssDNA is the virion-sense positive strand. Viral ORFs were traditionally named based on their being encoded on the virion-sense (V) strand or the complementary-sense (C) strand, which for some viruses were referred to as the *rightward* (R) or *leftward* (L) transcribed ORFs (see *arrows* in Fig. 26). For example, AC1 and AL1 referred to the same ORF. Adding to this confusion, ORFs with the same name did not always encode the same function. Thus, CP was encoded by the AV1 or AR1 ORF in begomoviruses, but by ORF V2 in MSV. The nomenclature in Figure 26 represents an emerging consensus for naming the viral ORFs. For clarity, individual gene functions, when known, will be referred to. These are summarized in Table 5 for the different virus groups, based on mutational and infectivity studies, transient expression assays in protoplasts, and cell-based assays in infected and transgenic plants.

Function	Begomovirus[a] TGMV, SqLCV, ACMV	TYLCV	Curtovirus[a]	Mastrevirus[a]
Replication	Rep (AL1, AC1) Ren (AL3, AC3)	Rep (C1) Ren (C3)	Rep (C1) Ren (C3)	Rep (C1)[b] Rep A (C1)
Transcription				
Represses Rep	Rep (AL1, AC1), (AL4, AC4)			
Activates late (V) genes	TrAP (AL2, AC2)	TrAP (C2)		Rep A (C1)
Encapsidation and Insect Transmission	CP (AR1, AV1)	CP (V1)	CP (V1)	CP (V1)
Movement	MP (BL1, BC1) NSP (BR1,BV1)	MP (V2) CP (V1)	MP (V2) CP (V1)	MP (V2) CP (V1)
Host activation[c]	Rep (AL1, AC1)		C4	Rep A
Symptoms	MP (BL1, BC1)	C4	C4	MP (V2)
Suppress host defenses	TrAP (AL2, AC2)		C2	
Regulate viral ssDNA accumulation	CP (AR1, AV1)	CP (V1) MP (V2)	V2	CP (V1)

TGMV, tomato golden mosaic virus; SqLCV, squash leaf curl virus; ACMV, African cassava mosaic virus; TYLCV, tomato yellow leaf curl virus; WDV, wheat dwarf virus; MSV, maize streak virus; BCTV, beet curly top virus; PCNA, proliferating cell nuclear antigen.

[a]Functions have been determined based on mutational, infectivity, biochemical and cell biological studies with these, as well as several other, bipartite geminiviruses. For every function has been shown for each virus: TGMV, MSV, and ACMV have been models for replication. TGMV, MSV, and MSV have been models for transcription. SqLCV has been a model for virus movement. BCTV has been a model curtovirus. See recent reviews for additional details and references [24, 102, 132, and 194].

[b]Activation of PCNA transcription, induction of cell division, and/or trans-Rb based on studies with TGMV, BCTV, and [65].

TABLE 5. *Geminivirus gene function*

The bipartite begomoviruses, such as TGMV, ACMV, and squash leaf curl virus (SqLCV), encode proteins needed for replication (Rep, Ren), transcriptional regulation (TrAP), and encapsidation (CP) on their A component, and two essential movement proteins (MP and NSP) on the B component (see Fig. 26 and Table 5). In some begomoviruses, there is an additional ORF, *AC4,* which for TGMV has been reported to repress transcription of *AL1* weakly in transient expression assays in plant protoplasts. However, mutational studies of TGMV show that *AC4* is dispensable for virus infection (94,127,269). Mutational and transient expression studies show that Rep (replication initiation) is essential for virus replication. Ren (replication enhancer) is not essential for replication, but it interacts with Rep to increase the efficiency of viral replication (97,312,333). Using appropriate GUS reporter gene constructs in transiently expressing plant protoplasts, TGMV TrAP (transcriptional activator protein) has been shown to activate transcription of the V genes encoding CP and NSP on the A and B component, respectively. TrAP binds ssDNA in a sequence-nonspecific manner, and only weakly binds dsDNA (133,246,330). The biochemical properties of TGMV TrAP expressed from a baculovirus vector in insect cells have been studied. TrAP is a phosphoprotein that binds and requires zinc for optimal interactions with ssDNA. The highly acidic C-terminal domain of TrAP acts as a transcriptional activator. When fused to the DNA binding domain of the yeast transcriptional activator GAL4, it specifically activates expression of a *CAT* reporter gene driven by a minimal adenovirus E1B promoter with GAL4 binding sites in mouse cells, or of an *E. coli lacZ* gene expressed from the GAL1 promoter in yeast (133). Thus, it has been suggested that TrAP, like adenovirus E1A (289R) or herpes simplex virus VP16, may act as a transcriptional coactivator, interacting with cellular transcription factors bound to sequence-specific promoter elements to cooperatively recruit the basal transcription machinery. Transcriptional regulation by TrAP is complicated. Analysis of the expression of GUS reporter gene constructs in transgenic plants shows that TrAP activates transcription of the CP promoter in mesophyll cells but derepresses the expression of this promoter in phloem cells (331). CP is required for encapsidation, and studies in which the CP of the curtovirus BCTV was substituted for that of the begomovirus ACMV showed that CP is required for insect acquisition and transmission (31) (see Plant-to-Plant Transmission). The B component encodes two movement proteins because, like most animal DNA viruses, geminiviruses replicate in the nucleus. Thus, NSP and MP are required to cooperate in moving the viral genome across the nuclear

envelope, through the cytoplasm, and across the cell wall. Studies with SqLCV show that NSP is a *n*uclear shuttle *p*rotein that binds viral ssDNA and transports it between the nucleus and cytoplasm. Like many other rapidly shuttling nuclear proteins, including HIV Rev and adenovirus E1B-55 kd and E4-34 kd, SqLCV NSP contains nuclear localization signals and a leucine-rich nuclear export signal (NES). MP traps NSP-genome complexes in the cytoplasm and, analogous to the MPs encoded by the RNA viruses discussed in this chapter, acts to move these across the cell wall through what appear to be modified plasmodesmata (194). The bipartite begomovirus CP is not required for systemic infection of natural hosts. Mutational and functional studies for TYLCV, including the finding that TYLCV can complement a TGMV TrAP mutant in tobacco protoplasts, suggest that each of the ORFs on its single genomic component encodes functions analogous to the bipartite virus A component (133,164,246,247,370), except for the extra *V2* (MP) ORF and perhaps *C4* (see Fig. 26). Mutational studies suggest that *V2* may encode the MP, and in contrast to the bipartite begomoviruses, CP is also needed for viral movement. *C4* affects TYLCV infection, depending on the host, showing that it is does not encode an essential movement protein. It appears to play a role in the development of symptoms (163,370).

Mastreviruses, such as MSV and wheat dwarf virus (WDV), while having a distinct genome organization from the begomoviruses, encode proteins that carry out analogous functions in viral replication (Rep), transcriptional activation (Rep A and/or Rep), movement (MP), and encapsidation and insect transmission (CP) (see Fig. 26 and Table 5). One major distinction from the begomoviruses is the processing of viral transcripts. All geminiviral genomes are bidirectionally transcribed to produce mRNAs that are polyadenylated and are likely to be capped; and all of the viral promoters are in the IR (LIR), except those for *TrAP* and *Ren,* and perhaps *AC4,* in begomoviruses. For the TGMV A and B components, a single unspliced V-sense transcript is translated into CP or NSP. The pattern of C-sense transcripts is complicated, but only TrAP and Ren appear to be translated from a bicistronic mRNA, and none of the transcripts is spliced (34,132). In contrast, splicing is essential in MSV and WDV. Rep, which is essential for viral replication, is translated from a spliced transcript that fuses ORFs C1 and C2 (306,377) (see Fig. 26). The corresponding unspliced transcript is translated into Rep A (C1), which shares ~200 N-terminal residues with Rep and alone, or together with Rep, activates expression of the V-encoded genes (45,53,142). This is analogous to the role of TrAP in activating *CP* and *NSP* transcription, although the mechanisms may not be identical. For WDV, only a single V-sense transcript has been detected that would be translated into MP and CP, but how CP is translated is not known. In MSV, there are two V-sense transcripts that initiate either 1 or 142 nt upstream of the MP coding sequence. Both of these are spliced within the MP coding sequence. It appears that MSV CP is translated from an abundant spliced form of the former (shorter) transcript, and MP is translated from a low abundance unspliced form of the latter with a 142 nt leader sequence (377). Mutational studies show that both MSV MP and CP are needed for systemic infection (28,195), and transient expression studies in plant protoplasts or plants biolistically bombarded with a GFP:MP fusion suggest that MSV MP has an analogous function to MP in the bipartite begomoviruses, but CP carries out the nuclear shuttling role of NSP (185,209). MSV MP is predicted to have a transmembrane domain, making it quite distinct from the bipartite virus MP. The curtovirus BCTV, like the mastreviruses, produces several V-sense transcripts, consistent with its complex genome organization (see Fig. 26). Based on mutational and transient expression studies, BCTV Rep (C1), Ren (C3), and CP (V1) are analogous to these same proteins in begomoviruses or mastreviruses (148,149,324). Like the mastreviruses, BCTV MP (V3) and CP (V1) are both required for systemic infection and thus may perform analogous roles to the SqLCV MP and NSP, or the MSV MP and CP, respectively. BCTV C2 is not analogous to TrAP in that it is not a transcriptional activator. However, both BCTV C2 and TrAP may play a similar role in counteracting host defenses against infection (149,356) (see Plant Responses to Virus Infection). C4 appears to induce cell division in BCTV-infected vascular cells and could function in inducing cells into S phase, as does Rep (189). V2 is unique to the curtoviruses and has been reported to be involved in regulating viral ssDNA accumulation (324).

Following introduction by the vector, how the viral genome is uncoated and delivered to the nucleus is not known. The identification of viral circular dsDNA as well as genomic ssDNA in infected plants was the first indication that the viral genome replicates through a dsDNA intermediate, as one might expect (Fig. 27). Since virion ssDNA is infectious, it appears that the dsDNA replicative form is synthesized from the incoming viral ssDNA genome (the positive strand) by host DNA replication enzymes, with cellular DNA polymerase a-primase complex also synthesizing the primer for synthesis of the complementary DNA strand. In the mastreviruses, such a primer—a ~75-to-80 nt oligodeoxyribonucleotide that contains several ribonucleotides at its 5' end—is found bound to the viral genome within the *small intergenic region* (SIR) in virions (see Fig. 26). This serves as a primer for synthesis of the viral complementary DNA strand *in vitro* (34,193). Such a primer is not present in virion ssDNA of begomoviruses or curtoviruses, suggesting that it would have to be synthesized by host enzymes upon infection. Studies of ACMV replication intermediates suggest that such an RNA primer may be synthesized within the IR (302). As shown in Figure 27 for the begomoviruses, geminivirus multiplication involves the same nucleocytoplasmic trafficking of viral mRNAs and proteins, and the same temporal regulation of viral gene expression, as the multiplication of nuclear-replicating animal DNA viruses. It is assumed that host RNA pol II complexes transcribe the viral dsDNA template into viral mRNAs. These are then transported to the cytoplasm, where they are translated. The C-sense genes (*Rep, TrAP, Ren, MP,* and *C4,* if present) are transcribed first. With the exception of the plant virus–specific MP, these correspond to the early genes found in DNA animal viruses and phage, encoding nonstructural proteins for regulating viral gene expression and initiating viral DNA replication. Trap, Rep and Ren are transported to the nucleus, where Rep and Ren initiate synthesis of viral genomic ssDNA by rolling circle replication, apparently in much the same way as the ssDNA phage fX174 (see Chapter 22) (301,326). Probably early in infection, the newly synthesized viral ssDNA positive strands are copied to make additional dsDNA templates for transcription. TrAP (Rep A, Rep, or both, in mastreviruses) will activate transcription of the *CP* and *NSP* (late) genes, and as Rep accumulates, it will repress its own transcription (147,332). CP and NSP are synthesized in the cytoplasm and transported to the nucleus. Studies with SqLCV suggest that initially, CP sequesters genomic ssDNA away from the viral dsDNA replication pool without encapsidating it, to make it available for NSP binding (278). NSP–genome complexes are exported to the cytoplasm, where MP will trap them and move them across the cell wall. As CP accumulates, virus particles will be assembled in the nucleus (34,193). In mastreviruses, and perhaps curtoviruses, CP appears to serve a dual role of exporting newly replicated viral ssDNA genomes to the cytoplasm for movement by MP and encapsidating these into virions within the nucleus for insect transmission (185,209).

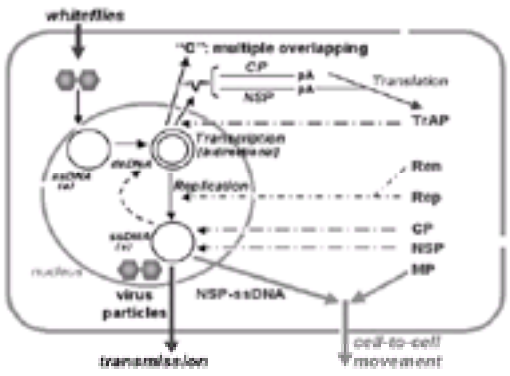


FIG. 27. Multiplication of begomoviruses.

The sequence identity of the IRs (common regions) in the A and B components of the bipartite begomoviruses such as TGMV and ACMV first suggested that the viral replication origin was located within this region. This was further reinforced by the fact that the IR (or LIR) in all geminiviruses contains a potential stem-loop structure with a conserved nonanucleotide sequence in the loop, 5'-TAATATTAC-3', which resembles the origin for positive strand synthesis in fX174 and bacterial plasmids that replicate by a rolling circle mechanism. Two approaches first showed that geminiviruses replicate by this mode. Two-dimensional gel electrophoresis of extracts from ACMV-infected plants identified the expected DNA intermediates for rolling circle replication (301). In separate studies, plants were inoculated with plasmids that contained heterodimers of WDV or ACMV, or the genomes of two strains of BCTV cloned as tandem direct repeats in the two possible combinations. In the BCTV studies, two predominant types of progeny genomes were recovered, depending on the order in which the two BCTV strains were cloned, and the sequences of these progeny were consistent with rolling circle replication initiating within the conserved stem-loop structure in the IR (326). The same conclusion was drawn in the WDV and ACMV studies (139,323), and the latter mapped the initiation site for viral positive strand ssDNA synthesis to the conserved nonanucleotide sequence TAATATT¯AC. *In vitro* biochemical studies show that Rep from WDV, TYLCV, and TGMV is an endonuclease that nicks virion sense ssDNA at this same position (140,191,255). Further biochemical characterization of WDV and TYLCV Rep using appropriately end-labeled ssDNA substrates show that Rep is also a ligase, capable of catalyzing a strand transfer reaction following cleavage at the viral replication origin *in vitro*. The reaction intermediate is Rep covalently linked to the 5'-end of the cleaved DNA through a phosphodiester bond to tyrosine-103, which identifies this residue as the one mediating DNA cleavage (140,190,191). Thus, analogous to fX174 rolling circle replication, these studies suggest that Rep nicks the viral origin in the positive strand of the dsDNA to initiate replication and remains bound to the 5' end of the nicked strand as a new virion-sense ssDNA is copied from the complementary strand, presumably by host DNA replication complexes. Rep is also the terminase, with its endonuclease and ligase activities resolving the nascent virion ssDNA into genome-length units and reinitiating synthesis of a new ssDNA positive strand after each round of rolling circle replication. Although TYLCV and TGMV Rep have ATPase activity *in vitro*, this activity is not DNA dependent, and Rep has only weak homology to consensus helicase motifs.

The conserved potential stem-loop structure containing the invariant nonanucleotide sequence is essential for replication (197,255) and the only element common to the positive strand origins of geminiviruses. However, geminivirus replication origins are, in general, virus specific, a consequence at least in part of Rep being a sequence-specific dsDNA binding protein. The TGMV Rep binding site is a 13-bp element that contains two pentanucleotide repeats (*underlined*): 5'-GGTAGTAATAGGTAG-3'. It is located upstream of the conserved potential stem-loop, between the TATA box and transcriptional start site for Rep mRNA, and Rep binding to this motif also represses Rep transcription (reviewed in 132). Related but not identical elements located in a similar position are also found in the IR of curtoviruses and other begomoviruses, and the Rep binding site is necessary, but not sufficient, for virus-specific origin recognition in these viruses (51,104). The Rep binding site and a palindromic AG motif, located between the binding site and conserved potential stem-loop, are also essential for origin function in TGMV, (132). Mutations in the TATA box of the Rep promoter, a G box motif, and a CA motif decrease the efficiency of TGMV replication, but whether the contribution of these sequences involves binding of transcription factors or other host factors has not been determined (254). TGMV appears to bind DNA as a dimer but exists in solution as an octamer.

The modular structure of the geminivirus positive strand replication origin is also evident in studies of WDV, although there are multiple rather than a single Rep-binding site (consensus: GTGTGAN$_{22\text{-}23}$GTG(G)TC) (130). A detailed study of WDV Rep binding to its own LIR using gel-shift assays, electron microscopy, and DNase footprinting identifies three distinct Rep-DNA complexes: two high-affinity complexes located in the regions of the two divergent TATA boxes for C-sense and V-sense transcription (see Fig. 26), and a low-affinity complex that assembles in the region containing the conserved stem-loop under conditions supporting site-specific cleavage of the invariant sequence TAATATT¯AC (45). The WDV stem-loop would form a dsDNA region containing the sequence GTGG(T)GG, and it is proposed that this half binding site directs formation of the low-affinity complex, which would be capable of producing the sequence-specific cleavage within the loop. It is also proposed that the high-affinity complexes in the regions of the V-sense and C-sense TATA boxes may function, respectively, in up-regulation of V-sense transcription and repression of C-sense transcription, although the latter has not been shown to be a property of mastrevirus Rep. The latter high-affinity complex is essential for origin activity, suggesting that a Rep oligomeric complex formed at this site could also contact the low-affinity site, thus bending the DNA and positioning the Rep complex to initiate replication by site-specific nicking of the positive strand (45). A similar bent structure has been proposed for TGMV Rep, mediated by Rep–Ren interactions (132). The DNA binding domain is present in both WDV Rep and Rep A. Thus, an interesting possibility is that Rep A–Rep hetero-oligomers, as well as homo-oligomers of each protein, have the potential to finely regulate different aspects of viral replication and transcription. The involvement of cellular proteins in any or all of these interactions is one area yet to be explored. The situation described for WDV Rep interacting with the LIR resembles in a number of respects that of SV40 large T interacting with the viral origin region (see Chapter 63).

A problem that geminiviruses have in common with the majority of animal DNA viruses is that fully differentiated plant cells lack detectable levels of the DNA replication enzymes that the virus will need for its own replication. DNA replication and cell division in mature plants is restricted to meristems at the growing points of the plant, developing leaves and roots, and the cambium that gives rise to the vascular system. Infection by a number of geminiviruses, such as SqLCV, BCTV, and Abutilon mosaic virus (AbMV), is restricted to the phloem. Studies with SqLCV suggest that, in this situation, the virus replicates in actively dividing procambial cells (developing phloem cells) that will differentiate into phloem (369). Other geminiviruses, such as TGMV and MSV, can infect a variety of differentiated plant cells, and studies suggest that these viruses, similar to DNA animal viruses, may induce cells to synthesize S-phase replication enzymes by a number of possible mechanisms, but without necessarily inducing cell proliferation. Studies of TGMV-infected tobacco plants and of transgenic plants that express TGMV Rep showed that Rep induces synthesis of proliferating cell nuclear antigen (PCNA), the processivity factor for DNA polymerase d (240), although the cells are not induced to divide. Whether this is a direct or indirect effect of Rep on PCNA transcription is not known. A common strategy of DNA animal viruses such as SV40, adenoviruses, and some human papillomaviruses, is to induce cells into S phase by encoding an immediate early or early protein with the consensus LXCXE motif that will bind to the pocket of the tumor suppressor Rb (and the related p107 and p130) to sequester Rb or target it for degradation (see Chapter 63 and Chapter 67). Plant proteins related to RB have been identified (termed *RBR*, for Rb-related protein). The Rep and Rep A of mastreviruses contain an LXCXE motif, and WDV Rep A (but not Rep) interacts with a plant RBR by means of this motif in yeast two-hybrid assays. The functional significance of this interaction is suggested by studies in wheat protoplasts. Mutation of the LXCXE motif in RepA/Rep complexes reduces the ability of WDV to replicate in these cells. Furthermore, wild-type WDV replication is reduced when these protoplasts are cotransfected with a plasmid that will produce high levels of either the maize RBR ZmRb1 or the human pocket protein p130 (381,382). TGMV Rep has also been reported to interact with a maize RBR in yeast two-hybrid studies, or using a GST pull-down assay to identify complexes when Rep is coexpressed with a GST:RBR fusion protein in insect cells (2). However, this TGMV Rep–RBR interaction appears to be about 10-fold weaker than that of SV40 large T with RBR (179). Begomovirus Rep proteins do not contain the pocket binding motif LXCXE. Deletion studies map the interaction domain in TGMV Rep to 80 residues that overlap the DNA cleavage/ligation, DNA binding, and oligomerization domains of Rep. These 80 residues have no homology to any known plant RBR binding proteins or any other proteins, except for begomovirus Rep proteins, suggesting that whatever the sequences involved in this RBR interaction, it is a novel or previously unrecognized motif (179). Additional studies should establish whether these Rep proteins directly interact with RBRs *in vitro*. To date, there is no evidence for direct interaction of WDV Rep or TGMV Rep with RBRs in plant cells, nor have the plant RBRs been shown to function in cell cycle control in plants as do Rb and other pocket domain proteins in mammalian cells. The geminiviruses are good models for investigating these aspects of cell cycle control, as well as regulation of DNA replication in plant cells.

VIRUS MOVEMENT

For virus infection to disseminate throughout the plant, the virus first moves from cell to cell within the inoculated leaf and enters the vascular system. For the majority of plant viruses, with the exception of phloem-restricted viruses like the luteoviruses and certain geminiviruses, this involves moving from infected mesophyll cells to bundle sheath cells, and from these into phloem parenchyma and/or companion cells, from which the virus will enter the sieve elements. From the sieve elements, the virus can infect additional sites within the inoculated leaf, as well as follow photoassimilate flow to move to distant leaves and systemically infect the plant (242) (see Fig. 5). Phloem-restricted viruses appear to move locally between companion cells, phloem parenchyma, and bundle sheath cells, and from leaf to leaf through the sieve elements. Some viruses also move from leaf to leaf through xylem. The study of plant virus-encoded MPs has revealed how viruses can alter plasmodesmata to move from cell to cell and enter phloem tissue. We understand less about how viruses enter into and exit from sieve elements or the xylem to systemically invade the plant.

Movement proteins do more than simply move the viral genome across the barrier of the plant cell wall. They coordinate replication of the viral genome with its directed movement within, as well as between, plant cells. In doing this, MPs use the cellular machinery for trafficking macromolecules. The final act of crossing the cell wall is preceded by a series of regulated events in which MPs interact not only with replicating viral genomes but also with the endomembrane system, the cytoskeletal network, and in the case of geminiviruses, the nuclear import and export machinery. Thus, the study of MP function in the past decade has been to a large extent an investigation of intracellular and intercellular transport in plants. Genetic studies have established that MPs are critical determinants of plant virus host range and pathogenic properties. Thus, as knowledge has been gained about MP function, this has also suggested new approaches for engineering virus-resistant plants.

Approaches to Investigating Virus Movement

A variety of approaches, each designed to address different questions, have been used to investigate MP function (reviewed in 43 and 194). Genetic studies identified the viral gene(s) encoding the MP(s) and defined the role of the MP(s) in infectivity, host range, and pathogenesis. MP null mutants were noninfectious in all host plants, but could replicate and assemble virions in protoplasts. Partially defective MP missense mutants, such as alanine-scanning mutants, could be altered in their host range phenotype, still infecting some host plants but not others, and produce attenuated symptoms. This role in pathogenesis was underscored by the finding that transgenic plants that expressed one of a number of viral MPs phenotypically resembled virus-infected plants. By inserting a *GUS* or *GFP* reporter gene into the viral genome, the precise stage at which virus infection was blocked could be determined. This approach identified the path followed by the virus in infecting a leaf and moving long distance from leaf to leaf, and the role of other proteins, such as CP, in affecting virus movement locally or systemically could also be examined (see Fig. 5). Immunogold labeling of systemically infected leaves or protoplasts, usually using antisera raised against MPs overexpressed from plasmids in *E. coli,* and subcellular fractionation of infected plant tissue provided information about MP function based on where the MP was found. In virus-infected or transgenic plants that expressed the MP, some MPs encoded by viruses like TMV or red clover necrotic mosaic virus (RCNMV; a dianthovirus) were localized to plasmodesmata that were not morphologically altered, and there was no evidence of virus particles being present. The MPs encoded by other viruses like CaMV and TSWV were localized to tubules that extended from what appeared to be altered plasmodesmata, and virus-like particles were associated with or within these tubules, consistent with a

requirement for CP in cell-to-cell movement. For PVX, virus particles were associated with “swollen” plasmodesmata, but no tubules were present. Consistent with this association with plasmodesmata, most viral MPs (with the exception of SqLCV NSP) cofractionated with cell wall and plasma membrane fractions from infected or transgenic plants. The TMV 30-kd MP and SqLCV MP also cofractionated with ER membrane. Indirect immune fluorescence staining of plant protoplasts infected with the virus or expressing the viral MP and confocal microscopy further showed association of the TMV MP with microtubules, actin filaments, and ER membrane, and the SqLCV MP and NSP with ER membrane and nuclei, respectively. *In vitro* binding assays showed that a common property of most viral MPs was the ability to bind nucleic acids cooperatively and in a sequence-nonspecific manner, consistent with their role in moving the viral genome. Thus, as first shown for the TMV and RCNMV MPs, the MPs encoded by many positive-sense RNA viruses bound ssRNA and ssDNA *in vitro*. The begomovirus SqLCV NSP was shown to be an ssDNA binding protein. For viruses, such as TSWV, in which CP is also required for local cell-to-cell movement, the MP (NSm) has been shown to interact with the nucleocapsid protein (N).

The dynamics of MP function and the effects of MPs on plasmodesmata have been investigated using two basic approaches in plants and model studies in plant protoplasts. Dye-coupling studies in which fluorescently tagged dextrans of different sizes were microinjected into tobacco mesophyll cells of transgenic plants that expressed an MP, or co-injected with an *E. coli*– expressed MP into mesophyll cells in leaves of wild-type plants, were designed to test a particular property of MPs, namely their ability to affect the gating properties of plasmodesmata in the absence of virus infection (Fig. 28). By microinjecting fluorescently labeled MP, or unlabeled MP together with RNA or DNA fluorescently labeled with TOTO-1 iodide, the movement of the MP itself or its ability to move nucleic acids in *trans* could also be tested. As first shown for TMV and RCNMV, the ability to increase the size exclusion limits of plasmodesmata was a common feature of MPs encoded by a number of plant viruses, and the MPs could themselves move from cell to cell and facilitate movement of nucleic acids as well (110,361,376). Concerns about possible artifacts as the result of the high pressure microinjection techniques used in these studies, as well as the effects of posttranslational modifications (most, if not all, MPs are phosphorylated), led to the examination of GFP:MP fusion proteins expressed within mesophyll cells from plasmids that were introduced by biolistic bombardment, or expressed from a PVX vector (Fig. 29**A**), with generally the same results (156). A complication in all of these studies was the growing recognition that the gating properties of plasmodesmata were developmentally regulated and that large proteins could move between mesophyll cells at certain developmental stages (156,252). This, and mutational and subcellular fractionation studies that showed that MP expression and function were regulated during infection, led to investigations of mesophyll cells in plants, and plant protoplasts, infected with a virus that expressed either free GFP or a GFP:MP fusion protein (reviewed in 194). Using confocal microscopy, in combination with immunostaining for appropriate marker proteins, the TMV MP was shown to form specific associations with microtubules and microfilaments, as well as the ER, both in infected protoplasts and plants (134,218,224,284). Time course studies could be done in single protoplasts embedded in agarose, or in expanding local lesions on leaves inoculated with the GFP-expressing TMV, because the visibly expanding necrotic lesion and GFP allowed the precise identification of the infected cells visually and by confocal microscopy (134,218) (Fig. 30). These studies showed that MP function was regulated. MP did not move beyond the leading edge of the infection front, nor were the gating properties of plasmodesmata altered in cells beyond the expanding lesion, in contrast to the results of microinjection and bombardment studies. In addition, the size exclusion limits of plasmodesmata early in infection (leading edge of the lesion) were increased so that 10-kd dextrans would move from cell to cell, but these had returned to normal late in infection (center of lesion). The associations of the MP with the cytoskeleton and endomembrane system were also found to be temporally regulated, as the MP associated with replicated viral RNA at ER-membrane derived sites and moved the genomes along microtubules and actin filaments to plasmodesmata (134,251,284). Other aspects of the dynamics of movement and subcellular associations could also be studied in protoplasts expressing viral MPs. The MP encoded by TSWV, CaMV, and other viruses was shown to induce the formation of tubules in transfected protoplasts (reviewed in 194), and the nuclear shuttling of SqLCV NSP and its interactions with MP to redirect NSP to the periphery of the cell were also shown (298,368).

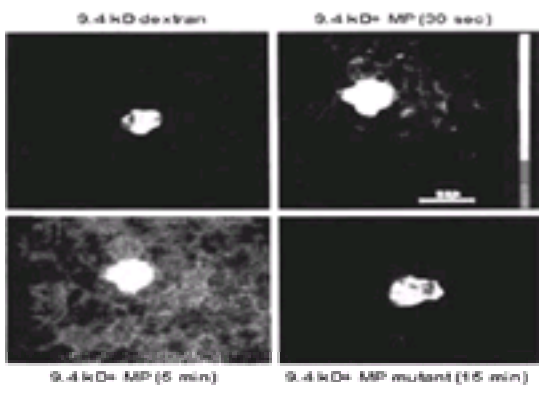


FIG. 28. Dye-coupling studies of red clover necrotic mosaic virus (RCNMV) movement protein (MP) co-injected with 9.4-kd fluorescently tagged dextran into tobacco mesophyll cells. The dextran, when injected alone or with a nonfunctional MP mutant protein, remains in the microinjected cell. When co-injected with wild-type MP, it rapidly moves to surrounding mesophyll cells, indicating that the plasmodesmal gating properties have be altered to allow the passage of larger molecules. (From ref. 110, with permission.)

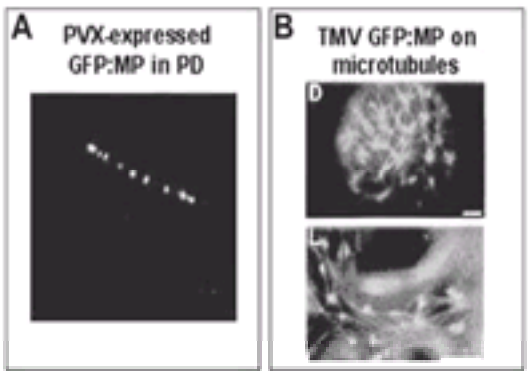


FIG. 29. Studies in living cells of green fluorescent protein (GFP)–movement protein (MP) fusion proteins expressed from an infectious potato virus X vector **(A)** or in tobacco mosaic virus (TMV)-infected protoplasts or plants **(B)**. (**A** courtesy of Dr. Karl Oparka, Scottish Crop Research Institute, Dundee; **B** from ref. 134, with permission.)

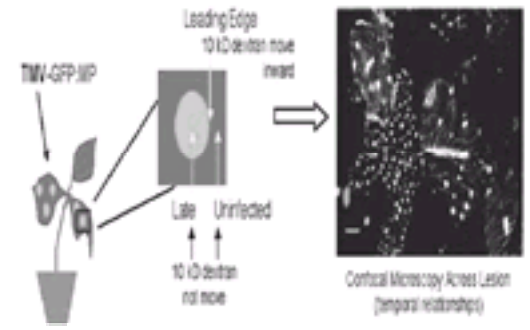


FIG. 30. Time course of tobacco mosaic virus (TMV) green fluorescent protein (GFP)–movement protein (MP) effects on plasmodesmal gating and associations with the cytoskeleton (microtubules and actin filaments) and endoplasmic reticulum (aggregates) in different cells within an expanding lesion. Because virus is moving outward from the center, cells at the leading edge are at early stages in infection, whereas those at the center are at late stages in infection. (Cartoon based on ref. 134, with permission; figure from Oparka KJ, Boevink P, Santa Cruz S. *Tr Plant Sci* 1996;1:412–418, with permission.)

These studies demonstrate that whereas different viruses may solve the problem of moving the viral genome across the cell wall in a number of different ways, there appears to be a finite number of solutions, and these are not unrelated. MPs, and CPs where these are also required, identify replicated progeny genomes and target these away from the replication pool and along a path toward the cell wall that is distinct from insect transmission. MPs alter plasmodesmata to facilitate movement of the viral genome from cell to cell, sometimes morphologically altering them or affecting their formation as well as function. For a few MPs where it has been investigated, specific associations with ER have been reported. This may not be coincidental since the desmotubule at the center of a plasmodesmata is originally derived from the ER. Similarly, given that actin filaments have been shown to be involved in regulating plasmodesmal function, specific associations of MPs with the cytoskeletal network where these have been documented are probably not coincidental either. Although the same studies have not been done for all plants viruses, there are four well-developed models for the function of MPs encoded by different virus groups. As additional studies are done, these models will be refined, and a few more may be added.

Cell-to-Cell Movement

Studies of the TMV 30-kd protein established the paradigm for MP function, first showing that contrary to a then commonly held view, plasmodesmata were not just boring holes in the plant cell wall, but rather could be modified by a viral MP. This presented a more dynamic view of plasmodesmata than had been previously held (78). TMV represents the simplest case of a virus that needs only one MP and does not require CP for cell-to-cell movement (Fig. 31A). It does require CP for systemic movement through the phloem. The TMV MP has been more extensively characterized than other viral MPs in terms of its effects on the size exclusion limits of plasmodesmata and the details of its regulation and cellular interactions during virus infection (194). In addition to binding viral RNA, the MP interacts with the ER, microtubules, and actin filaments at different stages during the progression of TMV infection. Immune colocalization studies have also localized the TMV MP to sites of viral replication (134,218). The model that has been proposed is that the TMV 30-kd MP binds to replicating viral genomic RNA at ER-anchored replication sites (so-called X bodies) and, at early stages, tracks these along microtubules to establish additional replication sites within the cell. It is suggested that this is the period during which the ER network is disrupted and forms aggregates. From replication sites established in the cortical region of the cell (the periphery), the MP bound to the viral genome is proposed to target it along actin microfilaments to plasmodesmata, where the MP acts to increase the size exclusion limits and move the viral genome into adjacent uninfected cells. The plasmodesmata do not appear to be altered morphologically, and studies suggest that the MP may act like a chaperone to extend the viral genome into a configuration that is compatible with passing through plasmodesmata. Mutational and expression studies show that TMV MP function is regulated in terms of both its synthesis and posttranslational modification. The TMV MP accumulates to high levels early in infection, after which it declines. This appears to be a common property of viral MPs: it has also been documented for other plant viral MPs, including SqLCV NSP and TSWV NSm (183,235,369). Mutational studies suggest that TMV MP function may be regulated by changes in its phosphorylation state (52,360). Temporal regulation of its activity is also evident from studies in expanding TMV lesions. Although the MP localizes to plasmodesmata at both early (leading edge) and late (lesion center) stages of

infection, it acts to increase the size exclusion limits of infected cells in the early stage cells at the leading edge, and not at the center (251) (see Fig. 30). Recent studies suggest that the TMV MP interacts with a cell wall–associated pectin methylesterase, and mutational studies indicate that this interaction is important for TMV MP function (49).

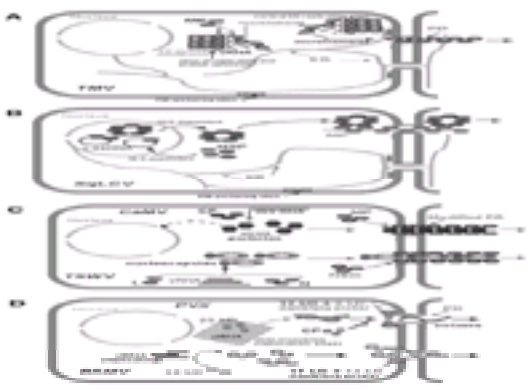


FIG. 31. Models for virus cell-to-cell movement. (**A**) TMV, (**B**) SqLCV, (**C**) CaMV and TSWV, where the MP forms tubules for the movement of encapsidated genomes, and (**D**) examples of triple gene block proteins for a virus that requires (PVX) or does not require (BSMV) CP for local movement. (Modified from ref. 194, with permission.)

The bipartite begomoviruses, such as SqLCV, encode two nonstructural movement proteins (NSP and MP) that are required for cell-to-cell movement and, like TMV, do not require CP for local movement. In contrast to TMV and many other plant viruses, these viruses also do not require CP to systemically infect their natural hosts, and it has been suggested that NSP–ssDNA genome complexes may be the form that moves long distance through the phloem, as well as from cell to cell (153,298). As already discussed, NSP and MP are both required to cooperate in moving the viral ssDNA genome from its site of replication in the nucleus, to and through the cytoplasm, and across the cell wall (see Fig. 31B). SqLCV is phloem limited, which is clearly shown by infecting plants with SqLCV engineered to express free GFP (196). Consistent with this, SqLCV NSP and MP are found only in phloem cells in systemically infected leaves, and it appears that SqLCV infects and replicates in procambial cells. In these immature phloem cells, immunogold labeling shows that MP is associated with unique tubules that extend from and cross the cell walls (369). There are no virus-like particles associated with these tubules, consistent with CP not being needed for movement. These tubules, which are not found in normal uninfected cells, are also labeled with antisera against the ER lumenal binding protein BiP, suggesting that they are derived from the ER. Consistent with this, SqLCV MP cofractionates with ER membrane from infected plants. The cooperative interaction of NSP and MP has been studied using transient expression assays in tobacco protoplasts, where it was also shown that this interaction is regulated (297,298). When each protein is expressed alone, NSP localizes to the nucleus, and MP is found in the cortical cytoplasm at the cell periphery, apparently the consequence of its targeting to the cortical ER (194). However, when NSP is coexpressed with MP in the same cells, NSP is relocalized from the nucleus to the cortical cytoplasm, where it now colocalizes with MP. This interaction is regulated. At late times after cotransfection of these cells, NSP no longer interacts with MP, but rather is found in the perinuclear region of these cells, although MP remains in the cortical cytoplasm (296,298). The model that has been proposed is that NSP, as a nuclear shuttle protein, binds newly replicated viral ssDNA genomes in the nucleus and moves these between the nucleus and cytoplasm. MP, associated with ER-derived tubules, traps these complexes in the cytoplasm and directs them to and across the cell wall. In adjacent uninfected cells, the NSP–genome complex is released, which then targets the viral ssDNA genome to the perinuclear region and delivers it across nuclear pores to initiate a new round of replication (194). The apparent ER origin of the MP-associated tubules and the fact that SqLCV is replicating in actively dividing procambial cells have led to the suggestion that the tubules may be the analog of the desmotubule core of plasmodesmata. Thus, MP is proposed to associate with regions of ER that are destined to form desmotubule, perhaps altering plasmodesmata as they are being formed in the walls of these cells (369). The regulated interaction of NSP with MP, as observed in protoplasts, further suggests that NSP is posttranslationally modified as a consequence of interacting with MP, and it has been proposed that this may involve alterations in the phosphorylation of NSP.

Unlike TMV and SqLCV, TSWV, CaMV, as well as comoviruses and nepoviruses, require CP in addition to a dedicated MP for cell-to-cell movement. All of these viruses, like TMV, infect fully differentiated mesophyll cells. The MP encoded by each of these viruses has been immunolocalized in infected plants to large tubular structures that extend from what appear to be modified plasmodesmata that lack a central desmotubule (see Fig. 31C). These tubules characteristically extend from the walls of infected cells into neighboring cells and have virus-like particles associated with or within them (reviewed in 43 and 194). The role, if any, of cellular components in the formation of these tubular structures remains unknown. Studies that have been done do not show an association with ER, microtubules, or microfilaments. The tubular structures have been reported to form in plant protoplasts or insect cells that transiently express the MP of TSWV (NSm), CaMV (I), or cowpea mosaic virus. These studies show that the MP in each case is necessary and sufficient to induce the formation of these tubules, and neither CP, plasmodesmata, nor any unique plant cell factors are apparently required for this process. CP is required for the formation of the virus-like particles within the tubules (167). The TSWV NSm has recently been shown to interact with the viral nucleocapsid (N) protein (321a). The model that has been proposed, as shown for CaMV and TSWV (see Fig. 31C), is that an encapsidated form of the viral genome—a subviral or viral particle—moves from cell to cell guided through the MP-containing tubular structures and altered plasmodesmata. For CaMV, icosahedral virus particles assembled in the cytoplasm and containing newly reverse transcribed viral genomic dsDNA appear to be the form that is transported from cell to cell. For TSWV, this would be assembled nucleocapsids, which are flexuous rods, containing L protein (the RdRp) as well as the viral genomic components. Presumably, specific interactions of the CaMV CP or TSWV N protein with the MP within the tubules is required to target the encapsidated genomes to the tubules and move these through into the next cell. It has been suggested that tubular structures are needed to accommodate the large diameter of the CaMV icosahedral particles or the TSWV RNPs with their L protein (300). An unresolved issue for both CaMV and TSWV is what regulates the choice between movement of the genome from cell to cell as opposed to an alternate path: the assembled virion targeting the viral genome to the nucleus or

associating with ATF, in the case of CaMV; or nucleocapsid complexes associating with Golgi stacks to assemble enveloped virions, in the case of TSWV. It might be that posttranslational modifications regulate which path is taken. Studies of TEV suggest that it, too, may use a similar mode to move its genome from cell to cell, with the CI cone structures that align with plasmodesmata performing a function similar to that of the large MP-containing tubular structures in CaMV and TSWV (42,293). CP is also required for cell-to-cell movement of TEV. Although it has not been determined whether the TEV genome is transported from cell to cell as virus particles or as a distinct RNA-CP complex, the finding that cell-to-cell movement requires an assembly-competent CP is consistent with virus particles being transported between cells (81,82).

Requiring CP for cell-to-cell movement does not necessarily require the formation of tubular structures. PVX requires CP, as well as the TGB-encoded proteins, for cell-to-cell movement but moves in the absence of any tubular structures. A number of distinct plant viruses encode an analogous set of essential TGB movement proteins, and it is intriguing that some of these, such as barley stripe mosaic virus (BSMV; a hordeivirus) do not require CP for cell-to-cell movement (192). In fact, like SqLCV, BSMV does not require CP for systemic infection of natural hosts. In the case of PVX, a viral mutant in which *GFP* replaced the *CP* gene was found to be confined to single inoculated cells, thus establishing the requirement for CP in cell-to-cell movement. Immune localization studies of infected plant cells and confocal imaging of plants inoculated with PVX that expressed a GFP:CP fusion show that CP localizes to plasmodesmata. Using a virion-specific antiserum, immune electron microscopy further identifies virions within the plasmodesmata of all PVX-infected cell types, leading to the conclusion that PVX virus particles move from cell to cell. However, dye-coupling studies using transgenic plants that express PVX CP show that CP does not affect the gating properties of plasmodesmata (253,299,300). The PVX TGB-encoded 25-kd protein (25K) does affect plasmodesmal gating properties. This was first shown in dye-coupling studies in which PVX viruses that expressed a GUS reporter gene were microinjected into tobacco trichome cells these viruses remained confined to single cells when the 25K protein was not expressed, and only the smallest sized dextrans (4.4 kd as opposed to 10 kd) could now move out of the infected cells (14). However, in infected plant cells, the 25K protein localizes to viral inclusion bodies in the cytoplasm, whereas the TGB-encoded 8-kd (8K) protein localizes to the cell wall. It has been suggested that both the TGB 8-kd and 12-kd proteins may function as membrane anchors since they contain potential transmembrane domains and associate with membranes. In recent studies, plasmids that expressed the PVX 25K protein fused to GFP (GFP:25K) were introduced by biolistic bombardment into transgenic plants that expressed CP, or the TGB 8-kd or 12-kd proteins. The GFP:25K fusion moved from cell to cell in wild-type tobacco plants and tobacco plants that expressed either the 8K or 12K proteins, but it did not move in transgenic plants that expressed either CP or both the 8K and 12K proteins (383).

One model that is consistent with the data is shown in Figure 31D, although other models are possible. This model is based, in part, on the 25K protein binding RNA *in vitro* and having properties similar to the largest TGB protein encoded by BSMV (58 kd) and other viruses. In this model, the 8K and 12K proteins act as membrane anchors. Replicated viral genomic RNA associated with the 25K protein is targeted to the plasma membrane and plasmodesmata through interactions of the 25K protein with the 8K/12K anchors. This interaction somehow facilitates exchange of the CP for the 25K protein bound to the viral genome, while the 25K protein acts to increase the size exclusion limits of the plasmodesmata and the assembled virions move into neighboring cells. The 25K protein is retained in the infected cell through binding to the 8K/12K membrane-anchored complexes and/or because viral CP occludes plasmodesmal sites to prevent its moving through. It has been suggested that early in infection, before CP and the 8K and 12K proteins accumulate, the 25K protein could itself move from cell to cell, creating a “preconditioned” state conducive to virus movement (383). It is also formally possible that the 35K protein and CP both associate with replicated viral genomic RNA as RNPs and, together with the 8K and 12K proteins, participate in assembly of virion complexes once the RNPs are targeted to the membrane sites, or that at sites of replication, CP and viral RNA assembled into virions associate with 25K, and these complexes are then targeted to the cell membrane and wall through interactions of 25K with the 8K/12K complexes. The BSMV TGB-encoded proteins that are analogous to those in PVX are 58 kd (bb), 17 kd (bc) and 14 kd (bd). In BSMV, the 58-kd protein is essential for cell-to-cell movement, and its ability to bind RNA appears to be required for this function (85). RNPs containing BSMV genomic RNA complexed with the 58-kd protein can be isolated from infected plant cells. The 17-kd and 14-kd proteins have potential transmembrane domains and appear to be similar to the PVX 12-kd and 8-kd proteins. A model consistent with the studies of BSMV (85,192) that also draws on parallels to PVX (see Fig. 31D) is that the 17-kd and 14-kd proteins act as membrane anchors. The 58-kd protein forms RNP complexes with replicated viral genomic RNA, which are targeted to the plasma membrane and plasmodesmata through interactions with the 17-kd/14-kd anchors. The 58-kd protein, like the TMV MP, then acts to increase the size exclusion limits of the plasmodesmata, and the 58-kd protein genome complexes move into adjacent cells. Other models are also possible, and as for PVX and other viruses encoding TGB proteins, biochemical and cellular studies of the TGB proteins are required to fully understand how these proteins function and interact to facilitate movement.

Long-Distance Movement

Little is understood about how viruses enter, move through, and exit from the phloem, owing largely to technical difficulties associated with investigating phloem cells without damaging them. Most of what is known is based on immunocytochemical and ultrastructural studies, and the analysis of virus mutants or host range variants (242). A complicating factor in determining the role of MPs in viruses entering the phloem and moving systemically is their requirement in cell-to-cell movement, which is a prerequisite for systemic infection. This is also an issue for those viruses in which CP is also needed for cell-to-cell movement. In addition, in analyzing viral mutants it is difficult to discern whether effects on phloem entry or systemic infection directly involve a stage in vascular transport or are the general consequence of a decrease in virus accumulation and efficiency of infection. This becomes an issue if a threshold level of virus accumulation must be reached for systemic spread to successfully occur, or if a lower efficiency in spread allows time for the host to mount a defense response. Some plant viruses are transported through the xylem. We know even less about this process than about phloem transport of viruses, and it will not be discussed here. Whether phloem-restricted viruses follow the same routes that evidence suggests are used by viruses not so restricted has also not been studied in detail.

It has generally been accepted that viruses that infect mesophyll cells and move through the phloem do so along with the flow of nutrients (photoassimilates), exiting from mature leaves by the same path and at the same sites that

photoassimilates are exported (the minor veins), and entering developing leaves by the same path and at the same sites (major or “lower order” veins) as photoassimilates are imported. This was suggested by classic studies that showed that plant viruses rapidly move toward the growing regions or young leaves in infected plants, and by more recent studies of CaMV infection in which virus accumulation in systemically infected leaves was found to be similar to that of photoassimilate import (203). This has now been directly shown for PVX infection in tobacco, by comparing spread of PVX expressing GFP with that of carboxyfluorescein (CF), a membrane-impermeant fluorescent solute that is transported through phloem in a manner similar to photoassimilate (292). Although virus transport was slower than that of CF, PVX exited from the same lower order veins (class III) into importing regions of developing leaves as did CF, and following infection of mesophyll cells, moved into the minor veins when these matured. The virus, as well as CF, was unable to enter regions of a leaf that were already actively exporting photoassimlate at the time of virus entry into the leaf.

Studies of the progression of infection for a number of plant virus variants and mutants have identified barriers or “control points” for virus movement from bundle sheath cells into phloem parenchyma cells, or from these into companion cells and then into the sieve elements. Plasmodesmal function between these cell types is also regulated (77); thus, not surprisingly, it appears that both viral and host cell factors control virus entry into the phloem. The majority of plant viruses require CP for long distance movement, whether they require CP for cell-to-cell movement or not. Analysis of CP mutants for a number of these viruses, including TMV, TEV, and RCNMV, suggests that CP is essential for entry into and/or spread through the sieve elements of the phloem, probably in the form of virions (242). In the case of TMV and RCNMV, the ability to assemble virions correlated with efficient systemic infection. Immunocytochemical analysis of infection by TMV CP mutants suggested that CP and encapsidation were not required for movement into minor veins but did appear to increase the efficiency of movement from companion cells into sieve elements (79,343). For PVX, a mutant that lacked CP but expressed GFP was inoculated onto transgenic plants that expressed viral CP, and its movement into grafted leaves that did not express CP was examined (300). The PVX mutant systemically infected the CP transgenic rootstock and moved into the grafted nontransgenic leaves, where it remained confined to cells close to major veins because of the lack of CP in the nontransgenic tissue. These studies showed that PVX CP, presumably assembled into virions, is transported through the phloem.

A detailed study using quantitative immune electron microscopy and an MP:GFP fusion to examine cucumber mosaic virus (CMV, a cucumovirus) progression into the minor veins of tobacco raised the interesting possibility that virions may be assembled in sieve elements (26). CMV needs MP (the 3a protein) and CP for cell-to-cell movement. The CMV MP was found associated with plasmodesmata whether it was expressed from infectious CMV or from PVX as an MP:GFP fusion. In minor veins, the MP was clearly present in plasmodesmata connecting companion cells and sieve elements, and in fibrillar formations within the sieve elements. However, in CMV-infected plants, virions were not detected in plasmodesmata, including those between the companion cells and sieve elements, but were found as large aggregates associated with the MP in the sieve elements. It was suggested that the viral genomic RNA, CP, and MP move into the sieve elements as an RNP complex and that virions are subsequently assembled within the sieve elements. Despite the evidence supporting the idea that where CP is required for systemic infection, virions may move through sieve elements, it remains an open point as to whether virions, as such, could pass through the plasmodesmata between sieve elements and companion cells to exit from the phloem.

It has been difficult to distinguish potential functions of MP in phloem entry and systemic movement from its role in cell-to-cell movement. A recent study with RCNMV took advantage of the ability to create partially defective MP mutants using alanine-scanning mutagenesis to identify functions of the viral MP in long distance movement that were distinct from its requirement for cell-to-cell movement (364). Immunocytochemical studies of three different host plants infected with different RCNMV MP mutants showed that MP was required for viral entry into the phloem for systemic infection, acting to facilitate viral movement from phloem parenchyma into companion cells independent of its function in cell-to-cell movement within mesophyll, bundle sheath, and phloem parenchyma.

PLANT RESPONSES TO VIRUS INFECTION

Although plants do not have an immune system, they are not completely defenseless in the face of virus infection. Plants can be nonhosts for or tolerant of infection by particular viruses. In the former instance, there is no evidence of virus replication in the plant, whereas in the latter, virus replicates and can move, but the host shows no or mild disease symptoms and continues to grow. Historically, it has been presumed that in these situations the infecting virus cannot interact with host factors that are involved in its replication or movement. While this may be true in a number of virus–host interactions, the recent demonstration of an innate plant defense response to virus infection suggests other possibilities as well. Potentially susceptible species of plants can also be resistant to infection by viral, bacterial, and fungal pathogens if they contain a dominant or recessive resistance gene, and these have been successfully used in breeding. Little is known about the action of recessive resistance genes; however, dominant resistance (*R*) genes have been shown to respond to a corresponding avirulence (*Avr*) gene in the attacking pathogen. This so-called gene-for-gene interaction induces a hypersensitive response that is usually evident by the local production of necrotic lesions (see Fig. 10) and involves a series of responses in the plant that include the local production of reactive oxygen species and induction of an apoptotic response, and systemic signaling to induce pathogenesis-related proteins. This hypersensitive response eliminates local infection and protects the plant against further attack. A number of *R* genes have been cloned in the past few years, and they appear to be involved in signal transduction pathways (reviewed in 56). One of the best characterized *R* gene responses to virus infection is that of the *N* gene in tobacco in conferring resistance to TMV, in which the corresponding *Avr* gene product has been shown to be the viral 126-kd component of the RdRp (258,259). The *N* gene has been cloned by transposon tagging and, in common with a number of other cloned *R* genes, it appears to encode a cytoplasmic protein that contains a nucleotide binding site and a region of leucine-rich repeats. Such repeats are known to be important in protein–protein interactions. Strikingly, the amino-terminal domain of the *N* gene–encoded protein is similar to the cytoplasmic domain of the *Drosophila* Toll protein and the mammalian interleukin-1 receptor, suggesting that it mediates resistance through a Toll–interleukin-1–type pathway (373).

Mixed virus infections in plants are common. If the two infecting viruses are related (e.g., different strains of a particular virus), one outcome can be the phenomenon of cross protection. As typified in studies of TMV, inoculating plants first with a mild tomato strain of TMV will protect plants against subsequent infection by more virulent strains of TMV. This

cross protection response involves the CP of TMV, and it provided the first example of so-called pathogen-derived resistance in which transgenic plants that expressed a viral gene or protein, in this case the TMV CP, were shown to be resistant to virus infection (reviewed in 20 and 70). Subsequently, other viral genes encoding CP, as well as different viral RdRps and MPs, have been used to engineer virus-resistance plants, as have genes encoding known plant antiviral proteins, such as pokeweed antiviral proteins, a class of ribosome-inactivating proteins found in a wide variety of plants (342). The studies of the mechanism of pathogen-derived resistance quickly made it apparent that although, at times, such as with transgenic plants expressing the TMV CP, the resistance is mediated by expression of a viral protein, in most cases it is conferred by RNA (RNA-mediated resistance). The expressed viral transgene induces a homology-dependent degradation of the infecting virus genome that is mechanistically related to posttranscriptional cosuppression of a nonviral transgene and corresponding cellular gene in transgenic plants, and homology-dependent posttranscriptional gene silencing in plants (PTGS) (18). These, and other studies (11,283), suggested that plant viruses could be initiators as well as targets of PTGS, also called *RNA interference* (RNAi), a phenomenon that occurs in plants, animals (e.g., *Caenorhabditis elegans* and *Drosophila*) and fungi. As can happen in science, these studies on RNA-mediated resistance and investigation of the phenomenon of plant virus synergy converged to show that PTGS is a plant defense against virus infection and that, not unexpectedly, plant viruses can encode suppressors of PTGS as a counterstrategy.

In contrast to the antagonistic interaction in cross protection, mixed infection of plants by two usually unrelated viruses can be synergistic, producing more severe disease symptoms than would be caused by either virus alone and enhancing the replication and/or spread of one or both viruses. This synergistic interaction occurs in a number of mixed plant virus infections. Many of these involve a potyvirus and any of a number of unrelated viruses, which suggested that rather than the potyvirus affecting replication of the co-infecting virus directly, it was more likely to do so indirectly by modulating host functions. Recent studies of the interactions of potyviruses PVY, TEV, and TVMV with PVX, using viral mutants and transgenic plants, showed that the P1/HC-Pro region of the potyvirus genome is responsible for this synergism (277,348). This was directly shown in two ways. Infectious PVX engineered to express the P1/HC-Pro or HC-Pro of TEV was found to have enhanced pathogenicity in tobacco plants (Fig. 32). Similarly, transgenic plants that express the P1/HC-Pro region of TEV or TVMV showed enhanced symptoms when infected with wild-type PVX, which normally produces only mild symptoms on wild-type tobacco. Mutational studies showed that the region of HC-Pro that had been implicated in affecting replication, long distance movement, and pathogenicity of TEV was necessary and sufficient to produce the synergistic increase in disease symptoms, and that both P1 and HC-Pro were required for the increased replication of PVX (277). At the same time, in an attempt to develop a transient assay for investigating PTGS, it was discovered that local injection of *A. tumefaciens* expressing a 35S-GFP construct not only would silence GFP expression in a transgenic plant locally in the inoculated leaf, as hoped, but also would induce PTGS of the GFP expression systemically throughout the entire plant. This was evident by the fact that these plants were no longer fluorescent under ultraviolet light (354). These and subsequent studies showed that PTGS in plants can be triggered by a systemically transmitted signal that is presumed to be nucleic acid (354,357). By infecting these GFP-silenced transgenic plants with PVX vectors expressing different forms of HC-Pro, or using transgenic tobacco plants that contain a preexisting silenced *GUS* transgene, it was shown that HC-Pro could suppress the PTGS of GFP or GUS (13,165,357). Small RNAs approximately 23 nt in length that correspond to both sense and antisense strands are associated with PTGS, and it has been proposed that these provide the specificity for targeted RNA degradation through association with an RNAse III–like enzyme (131,386). Additional studies have shown that encoding such a suppressor of PTGS is a strategy employed by a number of diverse plant viruses, including TMV, CMV (2b), TGMV (TrAP), cowpea mosaic virus (comovirus), a number of potexviruses (although not PVX), tobacco rattle virus (tobravirus), and tomato bushy stunt virus (tombusvirus, 19K) (356,357). In some cases, the viral-encoded suppressor has been identified (shown in parentheses). Thus, not every plant virus encodes such an RNAi suppressor, or counteracts this defense response in the same way. For viruses that do not, like PVX, it has been suggested that the virus can partially evade this host defense, although it clearly does not replicate at its maximal potential in those host plants in which this has been studied. The viral proteins identified as suppressing PTGS had each been identified in mutational studies as a “pathogenicity” factor that affected virus replication, movement, and/or pathogenesis, driving home the point that the roles of a number of these proteins, such as HC-Pro and CMV 2b, in systemic infection and phloem-dependent transport could be explained by suppression of the host PTGS defense response, rather than the protein directly facilitating active virus movement through plasmodesmata. This raises the possibility that some viral MPs could facilitate cell-to-cell movement by hindering intercellular communication required for spread of a PTGS defense (38). Recent studies with PVX suggest that the 25-kd protein, one of the TGB-encoded movement proteins, can block systemic spread of the PTGS signal (355), thus being responsible for the proposed PVX evasion of the PTGS response. Current studies are identifying host genes involved in PTGS. As the functions of these host genes are determined, we will begin to understand the signaling pathways by which PTGS is induced and how it can be suppressed or evaded.

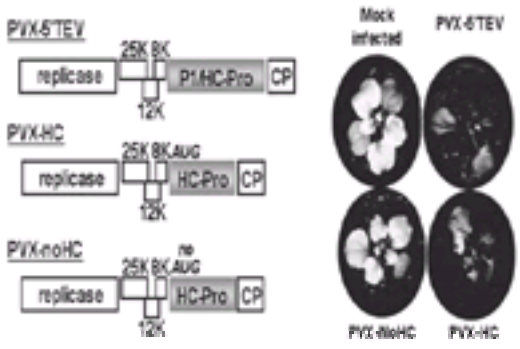


FIG. 32. PVX viruses that express TEV P1/HC-Pro or HC-Pro show enhanced pathogenicity (PVX-5'TEV, PVX-HC). Expression of P1/HC-Pro or HC-Pro protein is required, since PVX expressing HC-Pro without a start codon (PVX-NoHC) does not show enhanced pathogenicity. (From ref. 277, with permission.)

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CHAPTER 20 Insect Viruses

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HISTORY OF INSECT VIRUSES

It is likely that insect viruses have existed as long as the insects themselves and have therefore evolved for 350 million years. Insect viruses have long been of interest and concern to humans, as first indicated by scientific reports and literature of the 16th and 17th centuries, which describe insect diseases. In the early 19th century, crystalline polyhedral-shaped bodies called polyhedra were first discovered in association with the “wilting” disease of silkworms. It was subsequently established that the polyhedra were the causal agents of the disease, and by 1920, the silkworm disease was attributed to a filterable virus (reviewed in 14). Finally in 1947, Gernot Bergold (15) made the landmark discovery that the protein matrix of the polyhedra contained rod-shaped virions that are characteristic of the baculoviruses.

Studies in the 1940s and 1950s set the stage for development of insect pathogens as biologic pesticides. Important candidates were the insect viruses, especially the baculoviruses (246). The list of viral candidates was expanded in the 1960s through the discovery of new families of insect viruses, including the iridoviruses, nodaviruses, polydnaviruses, and entomopoxviruses. In the 1960s, significant progress also was made in establishing insect cell lines, and by 1973, a plaque assay for baculoviruses was available (100,131,132). Thus studies of the molecular biology and genetics of virus replication under controlled conditions were possible. Ultimately, the first insect virus (a baculovirus) was registered as a pesticide for use against the cotton bollworm by the United States Environmental Protection Agency in 1975 (reviewed in 192).

The mid-1970s marked the beginning of modern research on the molecular biology of insect viruses. Pioneering studies during this period triggered great interest in diverse applications of invertebrate viruses, including their use as vectors for foreign gene expression and as genetically improved biopesticides. To date, molecular studies on the replication, assembly, and host interactions of insect viruses have contributed enormously to molecular, cellular, and organismal biology.

CURRENT IMPACT OF INSECT VIRUSES

Insect Control

Insects comprise more than 80% of the existing animal species, outnumbering *Homo sapiens* 200 million to 1. Often considered the most successful animals on earth, many insect species are of serious biomedical concern to humans and represent significant pests in agricultural and forestry settings. It is not surprising that throughout history, humans have sought to control the insects. In nature, insect populations are held in balance by diverse factors, including predation, parasitism, and microbial (viral and fungal) infections. Of these regulatory forces, viral diseases can have profound effects.

Viruses can cause widespread epizootics and morbidity in insects, especially within dense populations. In contrast, some viruses have little or no overt effects, but can influence the “health” of a species by stressing the general population. Viral diseases occur in pest insects (gypsy moths, corn earworms, and mosquitoes, among others) as well as beneficial species (honey bees, silkworms, and parasitoids). Diseases of beneficial insects can cause economic and ecologic problems, whereas diseases of pest species can alleviate economic hardship and reduce medical concerns. Natural epizootics by viruses have been frequently observed in agricultural and forest settings, especially when insect populations are high. One of the most dramatic episodes of virus-mediated insect control occurred in North America during infestations of the European spruce sawfly. On accidental introduction of the sawfly baculovirus in the 1930s, sawfly populations declined sharply and remained low because of the continued presence of the virus (4). Such dependence on natural epizootics for insect pest control in agriculture, however, is generally impractical, because crop damage exceeds economically acceptable levels before virus-mediated reduction occurs.

Much effort has been invested at the academic and industrial levels in the development of virus-based biopesticides for the purpose of reducing agricultural dependence on broad-range, environmentally toxic chemical insecticides. Currently in Brazil, more than a million hectares of land are treated annually with a baculovirus to control the soybean looper. In Europe, a different baculovirus is used to control the codling moth, a common pest of fruit crops (192). In the South

Pacific, a nudivirus is used for effective control of the rhinoceros beetle on coconut plantations (297). Several baculoviruses are currently registered as pesticides in the U.S., but their implementation has been pursued primarily by government agencies such as the U.S.D.A. Forest Service. Industrial interest in commercial development of virus insecticides has been sporadic, in part because of restricted markets and limitations in efficacy of these biopesticides. However, as environmental concerns increase and insects acquire resistance to chemical pesticides, it is expected that viral insecticides will have an expanded role in integrated pest-management programs. In particular, the capacity to improve insecticidal efficacy by genetic manipulation of baculoviruses through the insertion of insect-specific toxin or hormone genes holds much promise (118,183,247,266). Field tests of genetically improved baculovirus pesticides have already been conducted in the U.S. and the United Kingdom and have involved participation by multiple companies (reviewed in 20,192).

Foreign Gene Expression Vectors

Baculoviruses are popularly known for their use as highly efficient vectors for foreign gene expression in eukaryotic (insect) cells (reviewed in 144,155,199). This important and widely used application is the direct result of molecular studies on these large DNA viruses and is a striking example of the utility of insect viruses for biotechnology and basic research. Baculovirus expression vectors are distinguished by their capacity to produce unusually high levels of biologically active foreign proteins. Posttranslational modifications and protein folding in infected insect cells parallel those in mammalian cells and thereby provide abundant proteins for applications in vaccine development, therapeutic and diagnostic purposes, and structure/function studies. To date, several thousand genes have been expressed by using baculovirus vectors, which themselves have been recently improved for ease of use and increased protein yields. The biologic activity of these foreign proteins often can be tested directly in the vector-infected insect cells, providing a reliable and efficient system for functional analysis. The unique properties of other insect viruses, including the entomopoxviruses, nodaviruses, densoviruses, and alphaviruses, also are being exploited for use as expression vectors.

Recently the nucleopolyhedroviruses (NPVs) have been evaluated as gene-transfer vectors in mammals and insects (reviewed in 155). Reporter genes placed under control of mammalian promoters and inserted into the virus genome are readily expressed after inoculation of certain mammalian cell lines (26,52,134). Moreover, it has been demonstrated that a recombinant NPV can be used to stably deliver functional genes to the germline of the silkworm *Bombyx mori* (291), suggesting that baculoviruses will provide a useful vector for targeted gene insertion and gene disruption in transgenic insects.

Cell Biology, Host–Virus Interactions, and Evolution

The study of insect viruses has provided unique insights into fundamental biologic processes. Examples include the discovery of the 7-methyl guanosine cap structure of eukaryotic mRNAs by using the insect cypoviruses of *Reoviridae* (89). Studies on translation of cricket paralysis–like viruses have revealed a new mechanism for initiation of protein synthesis (235,282). The polydnaviruses have revealed a novel symbiotic relationship between virus and host that involves virus-mediated immune suppression. Insect viruses also are providing unique opportunities to investigate antivirus defense strategies. Because of their capacity to induce and suppress apoptosis, baculoviruses have provided insight into the role played by programmed cell death as an antivirus defense mechanism and as a contributor to viral pathogenesis. Recombinant baculoviruses also have been used to trap host cell apoptotic regulators (243), providing information on regulation of programmed cell death. Last, apoptotic suppressors of these insect viruses are being used in antiapoptosis therapies involving transgenic organisms (58,133,270).

Insect viruses also have provided unique insight into virus evolution. The close association of insects with plants and higher animals has allowed the natural exchange of viruses, thus contributing to virus evolution. Many insect viruses within the families *Poxviridae, Rhabdoviridae, Reoviridae, Picornaviridae*, and *Parvoviridae* have vertebrate counterparts, spurring speculation that viruses of higher animals evolved from ancient insect viruses. It is therefore likely that understanding the evolution of vertebrate viruses will depend on knowledge of their invertebrate relatives. In addition, some insect viruses have coevolved with viruses of unrelated families. For example, the unusual conservation of specific genes found in baculoviruses, entomopoxviruses, and orthomyxoviruses suggests an evolutionary link between these diverse virus families. Last, certain insect viruses (baculoviruses) readily accommodate host transposons, which are especially abundant and diverse in arthropods. Stable insertion of such host-derived genetic elements accelerates virus evolution and may contribute to the horizontal exchange of genes between invertebrate species.

CLASSIFICATION OF INSECT VIRUSES

Insects are susceptible to highly diverse families of RNA and DNA viruses (Table 1). Many members of these insect virus families have counterparts in vertebrates. Others are unique to arthropods. Nonetheless, insect-specific members of these virus families have novel properties that are conserved between families. A striking example is the use of an occluded form of infectious virus that is required for virus transmission between insect hosts and is conserved among diverse virus families, including the baculoviruses, entomopoxviruses, and cypoviruses.

This chapter focuses on those virus families that are found primarily or exclusively in insects: *Baculoviridae, Nudiviridae, Polydnaviridae, Ascoviridae, Nodaviridae, Tetraviridae,* and the cricket paraly-sis–like viruses. Viruses in which insects are used as temporary hosts during transmission (e.g., arboviruses) are not included, nor are virus families that have well-characterized vertebrate members described in other chapters. These insect viruses and others (entomopoxviruses, iridescent viruses, densoviruses, and cypoviruses) are described by recent reviews (189).

Characteristics	Virus family	Genus	Representative member
dsDNA, enveloped	*Baculoviridae*	Nucleopolyhedrovirus (NPV)	Autographa californica sNPV (AcMNPV)
		Granulovirus (GV)	Cydia pomonella GV (CpGV)
	Nudiviruses[a]	Nonoccluded virus	Hz-1, Oryctes rhinoceros virus
	Polydnaviridae	Ichnovirus	Campoletis sonorensis virus (CsV)
		Bracovirus	Cotesia melanoscela virus (CmV)
	Poxviridae	Entomopoxvirus A	Melanoplus entomopoxvirus
		Entomopoxvirus B	Amsacta moorei entomopoxvirus
		Entomopoxvirus C	Chironomus luridus entomopoxvirus
	Ascoviridae	Ascovirus	Spodoptera frugiperda ascovirus (SfAV)
dsDNA, nonenveloped	*Iridoviridae*	Iridovirus	Chilo iridescent virus
		Chloriridovirus	Mosquito iridescent virus
ssDNA, nonenveloped	*Parvoviridae*	Densovirus	Galleria densovirus
ssRNA, nonenveloped	*Reoviridae*	Cytovirus	Bluetongue virus
		Cypovirus	Cytoplasmic polyhedrosis viruses (CPVs)
		Entomovirus	Colorado tick fever virus
ssRNA, enveloped	*Birnaviridae*	Birna virus	Drosophila X virus
	Togaviridae	Alphavirus	Sindbis virus
	Flaviviridae	Flavivirus	Yellow fever virus
	Rhabdoviridae	Vesiculovirus	Carajas virus
		Lyssavirus	Humpty Dau, Sigma virus
	Bunyaviridae	Bunyavirus	Anopheles A group
		Phlebovirus	Sandfly fever virus
		Nairovirus	Crimean-Congo hemorrhagic fever virus
ssRNA, nonenveloped	*Nodaviridae*	Alphanodavirus	Nodamura virus, Pariti Hooker Virus
	Tetraviridae	Betatetravirus	Nudaurelia capensis β virus
		Omegatetravirus	Nudaurelia capensis ω virus
	Cricket paralysis-like[a]	Cricket paralysis-like	Cricket paralysis virus
			Drosophila C virus

[a]These viruses are currently listed as unclassified by the ICTV.

TABLE 1. *Families of viruses infecting invertebrates*

DESCRIPTION OF INSECT VIRUS FAMILIES

Baculoviridae

Classification of Baculoviruses

Members of the family *Baculoviridae* are taxonomically characterized by their large, circular genome of double-stranded DNA, which is packaged into an enveloped, rod-shaped virion. The family name is derived from the Latin term *baculum,* meaning rod or stick, to describe the virion. Baculoviruses also are distinguished by their two morphologically distinct forms of infectious particles: occluded virus (OV), comprising enveloped virions embedded within a crystalline matrix of protein, and budded virus (BV), comprising a single virion enveloped by a plasma membrane. Baculoviruses are divided into two genera: the nucleopolyhedroviruses (NPVs) and granuloviruses (GVs), previously known as nuclear polyhedrosis viruses and granulosis viruses, respectively. For GVs, a single virion is embedded within each ovicylindrical OV particle, which collectively have a “granular” appearance in the light microscope. In contrast, NPVs have numerous (>20) virions per polyhedral OV particle (polyhedra) which are several microns in diameter and thus easily visualized by light microscopy. OV is produced in the nucleus of NPV-infected cells, whereas OV generally forms after loss of the nuclear membrane in GV-infected cells.

Baculovirus diseases have been reported in more than 500 different insect species. Individual viruses are most often named according to the species of host insect from which they were isolated. For example, *Autographa californica* nucleopolyhedrovirus (Ac*M*NPV) was isolated from the alfalfa looper *Autographa californica,* and *Cydia pomonella* granulovirus (CpGV) was isolated from the codling moth *Cydia pomonella*. In general, baculoviruses have distinct and relatively narrow host ranges that are limited to invertebrate species within a single genus or family. However, certain baculoviruses, including Ac*M*NPV, have broader host ranges, infecting more than 30 different lepidopteran (moth and butterfly) species.

Baculovirus Structure: OV and BV

Baculoviruses sequentially produce BV and OV in their unique, biphasic multiplication cycle. These infectious forms (Fig. 1) contain one or more virions comprising a rod-shaped nucleocapsid that is 250 to 300 nm long and 30 to 60 nm in diameter (recently reviewed in 88). The nucleocapsid is composed of a nucleoprotein core surrounded by a bacilliform proteinaceous sheath, referred to as the capsid. The capsid contains ring-like subunits stacked every 4.5 mm in the longitudinal axis (12). A distinctive morphology at the apical end of the capsid suggests a specialized function, for either DNA packaging, virion budding, nuclear entry, or uncoating (77,269). An essential 78-kd phosphoprotein is associated with this end structure (269). The capsid contains one major 35- to 40-kd capsid protein and additional minor proteins (213,258,264,269). The nucleoprotein core consists of a single molecule of circular, double-stranded DNA associated with protein. The major core protein is a 6.9-kd protamine-like protein, rich in arginine (150,268,285).

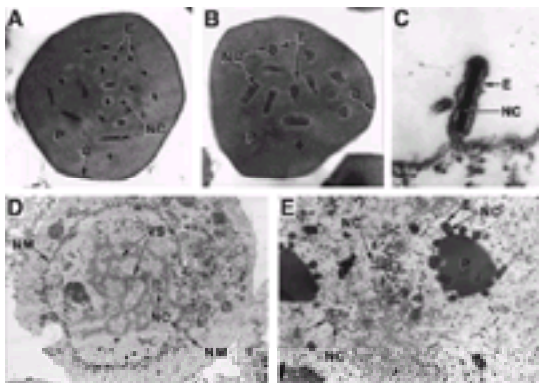

FIG. 1. OV and BV structure of the nucleopolyhedroviruses. **A:** Cross section of the OV particle (2-mm diameter) of a single capsid NPV (*S*NPV). Individual nucleocapsids (*NC*) within a unit membrane envelope (*E*) are embedded within a matrix of polyhedrin protein (*P*). The OV external surface is covered by the thin carbohydrate-rich calyx (*C*). **B:** Cross section of the OV particle (3-mm diameter) of a multicapsid NPV (*M*NPV). Envelopes (*E*) containing two or more nucleocapsids (*NC*) are embedded within the polyhedrin matrix (*P*). **C:** A nucleocapsid budding from the plasma membrane of an NPV-infected cell. The budded virus form *(BV)* of *M*NPVs and *S*NPVs contain a single nucleocapsid (*NC*) of the same dimensions (30 × 250 nm) as OV-derived nucleocapsids (**A, B**). **D:** Thin section of an *M*NPV-infected cell during the late phase of replication. The nucleus, surrounded by the nuclear membrane *(NM)*, contains the virogenic stroma (*VS*) with associated nucleocapsids (*NC*). **E:** Nucleus of an *M*NPV-infected cell at the beginning of the very late,

occlusion phase. Newly assembled nucleocapsids *(NC)* associate with intranuclear membrane envelopes (*E*), then become enveloped, and are embedded within a polyhedrin matrix (*P*). Electron micrographs were kindly provided by Dr. Malcolm J. Fraser, Jr., University of Notre Dame.

A distinctive feature of the baculoviruses is the large size (0.15 to 15 μm) and polyhedral or oval shape of the OV particles (see Fig. 1). The OV mediates virus transmission between insect larvae, the developmental stage most susceptible to viral infection. On ingestion by a larva, the OV protein matrix is dissolved by the alkaline pH of the midgut, releasing the occlusion-derived virions (ODVs), which initiate infection of the midgut epithelium. Within the OV particle, these virions are embedded in a crystalline matrix consisting of a single 29-kd protein, designated polyhedrin or granulin for the NPVs and GVs, respectively. Both matrix proteins are closely related. Within the matrix of NPVs (see Fig. 1A and Fig. 1B), either a single nucleocapsid (*S*NPVs) or multiple nucleocapsids (*M*NPVs) are bundled together and surrounded by a membrane envelope with virus-encoded proteins (88). The intranuclear membranes used for envelopment may be synthesized *de novo* or represent a modified form of the nuclear membrane (29,250). On their surface, mature OVs have an additional covering of protein and carbohydrate, known as the polyhedron envelope or calyx.

The BV form of baculoviruses consists of a nucleocapsid surrounded by a loose-fitting membrane envelope acquired by budding at the plasma membrane surface of the host cell (see Fig. 1C). The membrane envelope of BVs is thus distinct from that which envelops nucleocapsids embedded within OV. BV is the infectious unit responsible for cell-to-cell transmission within the insect and in cell culture. The envelope fusion protein (EFP) gp64 is the major virus-specific protein associated with the BV envelope of some NPVs (272). Antiserum to EFP gp64 neutralizes BV infectivity (275), suggesting that this protein participates in host cell–receptor interaction. Indeed, EFP gp64 is required for cell-to-cell infectivity (190). Envelope proteins unrelated to gp64 appear to mediate infectivity of other NPVs (211).

Baculovirus Genome Structure

The baculovirus genome is a large (90 to 160 bp) covalently closed, supercoiled circle of double-stranded DNA. To date, the complete nucleotide sequence of six baculoviruses has been reported, including that of *Autographa californica M*NPV (Ac*M*NPV), *Bombyx mori S*NPV (BmNPV), *Orgyia pseudotsugata M*NPV (Op*M*NPV), *Lymantria dispar M*NPV (Ld*M*NPV), *Xestia c-nigrum* granulovirus (XcGV), and *Cydia pomonella* granulovirus (CpGV) (2,3,95,126,163,200). Extensive transcriptional mapping of several baculoviruses has provided a broad picture of gene organization and regulation of expression. Ac*M*NPV is the best studied and represents the prototype NPV.

The AcMNPV genome (133,894 bp) consists almost entirely of unique DNA sequences, encoding 337 open reading frames (ORFs) of 150 nucleotides or more (3). The ORFs are closely spaced on both DNA strands with no apparent organization with respect to temporal expression during infection. Early, late, and very late genes are interspersed throughout the genome. Most ORFs are separated by only two to 200 nucleotides. These intergenic regions have a high A+T content and constitute the promoter and termination sequences for each gene (reviewed in 216). Illustrative of the economy of gene organization, the translational termination codon (usually **UAA**) often overlaps the polyadenylation signal, AA**UAA**A. There also are examples of overlapping ORFs. However, because splicing of baculovirus RNA is rare, there are few if any introns in the genome. An example of splicing involves expression of the *ie*-0/*ie*-1 gene (see later), which encodes the principal transcriptional regulator of baculoviruses (45,157,212). Frequently transcripts of one AcMNPV gene initiate from within or extend through an adjacent gene, a phenomenon especially prevalent among viral RNAs late in infection (81,179). Thus numerous genes are transcribed into bi- or multicistronic RNAs in which the upstream ORF is translated. In addition, there are multiple examples of the transcription of overlapping, antisense RNAs. The potential regulatory role of such antisense RNAs is unclear. Evidence exists that the interspersion of different temporal classes of transcripts may have functional significance. For example, activation of the very late promoter of *polh*, the gene encoding the major OV protein polyhedrin, down-regulates overlapping, antisense RNAs initiated downstream of *polh* (204). However, no evidence of this type of promoter interference occurs for a different set of overlapping RNAs that are all transcribed in the same direction (104).

A distinguishing feature of the baculovirus genome is the presence of multiple copies of interspersed repetitive sequences, known as homologous regions (*hrs*) (51,108). The *hrs* function as transcriptional enhancers (108,112) and are likely origins of viral DNA replication (154,210). Ac*M*NPV contains eight *hrs*, ranging in size from 30 to 800 bp and accounting for 3% to 4% of the genome. The *hrs* have a complex modular organization consisting of 60-bp repeats, each with a highly conserved 28-bp imperfect palindrome (28-*mer*). The 28-*mer* is the minimal sequence required for orientation- and position-independent enhancement of promoter activity and DNA replication (169,231). The *hrs* bind host- and virus-specific proteins. In particular, the transcriptional activator IE1 binds as a dimer to the palindromic 28-*mer*, which is required for IE1-mediated stimulation of enhancer activity (156,169,232). Deletion of a single *hr* (*hr*5) from the Ac*M*NPV genome reduces transcription of proximal genes, but has no obvious effect on virus replication, suggesting that these elements are functionally redundant (231). Nonetheless, the novel distribution and repetition of the *hrs* or *hr*-like elements within multiple baculovirus genomes suggest that these sequences play a critical role in the virus life cycle.

Stages of Baculovirus Replication

Primary and Secondary Infection

Baculovirus multiplication in the insect larva is divided into primary and secondary stages to describe the initial infection of midgut cells and the subsequent infection of secondary tissues, respectively (Fig. 2A and Fig. 2B). In both stages, infection is initiated by the binding of virus to the host cell. However, multiple aspects of primary and secondary infections differ. Secondary infection most closely resembles that which occurs in cultured cells after inoculation with BV, a process that is better understood with respect to individual replication events. Thus we first describe the known details of

Ac*M*NPV replication in a high-multiplicity infection of cultured SF21 (or SF9) cells, a well-studied and fully permissive cell line derived from the nocturnal moth, *Spodoptera frugiperda* (Lepidoptera; Noctuidae). Subsequently, aspects unique to primary infection in the midgut of permissive caterpillars are described. It should be noted that the timing of replication events varies with the virus and insect host.

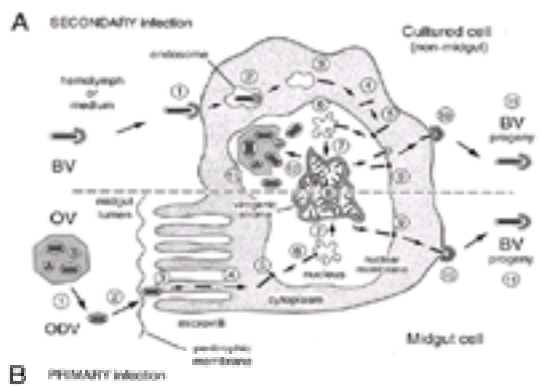

FIG. 2. Multiplication cycle of a baculovirus (*M*NPV). **A:** Infection of a cultured or nonmidgut cell by BV (secondary phase of infection). Enveloped BV attaches to receptors on the cell surface *(step 1)* and enters by endocytosis (*step 2*). As the endosome acidifies, virus and endosomal envelopes fuse (*step 3*), releasing nucleocapsids into the cytoplasm. Nucleocapsids proceed to the nucleus (*step 4*), where they interact end-on with the nuclear pore (*step 5*). On entering the nucleus, the DNA is released from the virion (*step 6*), and transcription begins (*step 7*). In association with the newly formed virogenic stroma, viral DNA is replicated and packaged into nucleocapsids (*step 8*). During the late phase, nucleocapsids exit the nucleus (*step 9*), migrate to the plasma membrane, and bud (*step 10*), thereby acquiring virus envelope proteins and producing infectious BV (*step 11*). During the very late phase, nucleocapsids are retained in the nucleus, acquire an envelope (*step 12*), and are embedded within a crystalline matrix of polyhedrin (*step 13*) to form OV particles. Mature OV are released by cell lysis. **B:** Infection of a midgut epithelial cell by OV (primary phase of infection). The polyhedrin matrix of an ingested OV particle dissolves in the midgut lumen *(step 1)*, releasing occlusion-derived virions (*ODV*), which crosses the peritrophic membrane (*step 2*) to gain access to the columnar epithelial cells. The enveloped ODV fuses with the microvilli membranes (*step 3*), releasing nucleocapsids into the cytoplasm, whereupon they migrate to the nucleus (*step 4*). The remaining replication events (*steps 5 to 11*) are similar to those in cultured cells (**A**).

Attachment, Endocytosis, and Uncoating

BV enters the host cell (see Fig. 2A) by what appears to be receptor-mediated endocytosis (reviewed in 280). However, the participating host-cell receptor(s) has not been identified. A second, less efficient, membrane-fusion mechanism also may allow virus entry (273). The major glycoprotein of BV, gp64 EFP, is required for receptor interaction and fusion with the endosomal membrane (25,127,274). After release within the cytoplasm, nucleocapsids migrate to the nucleus in conjunction with virus-induced actin polymerization (41). Ac*M*NPV nucleocapsids interact end-on with the nuclear pore, enter the nucleus, and uncoat. In this process, the capsid sheath of the nucleocapsid is released from the nucleoprotein core (268). It is unknown whether the 6.9K protamine-like core protein remains associated with the viral chromosome, but phosphorylation by a virus-encapsidated protein kinase may induce its release (284). Uncoating occurs rapidly because new synthesis of viral RNAs can be detected within the first 15 minutes after inoculation (45,113,140,160,195,260).

Early Phase

By definition, the early replication phase (from 0 through 6–9 hours) precedes virus DNA replication. During this period, baculoviruses express genes encoding transcriptional activators, a virus-specific RNA polymerase activity, DNA replication factors, apoptotic suppressors, and others. These early gene products prepare the cell for the enormous burden imposed by the synthesis of viral nucleic acid and structural components that can constitute more than a third of the infected cell's mass (Fig. 3A). The level and timing of both early and late gene expression is orchestrated to ensure proper temporal assembly of infectious BV and OV. Early viral gene products also block cell-cycle progression, causing infected cells to accumulate in S and G_2/M phases (28,142). In addition, the cell's cytoskeletal structure is dramatically rearranged. Both filamentous actin and microtubules are redistributed, leading to a characteristic hypertrophy of the nucleus and cell rounding during infection (40,41,234).

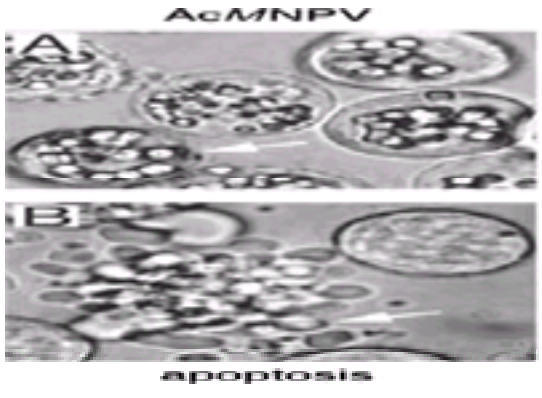

FIG. 3. Ac*M*NPV-infected SF21cells and apoptosis. **A:** Infection with wild-type Ac*M*NPV. Very late in infection the highly refractile OV particles *(arrow)* accumulate in the hypertrophied nucleus of each cell. **B:** Late stages of apoptotic blebbing. A single cell has undergone apoptosis forming a cell mass surrounded by membrane-enveloped apoptotic bodies

(arrow). Magnification, 200x. Micrographs provided by Gulam Manji, University of Wisconsin-Madison.

Baculovirus gene expression is regulated primarily at the level of transcription and involves a highly coordinated cascade of gene-activation events (recently reviewed in 80 and 178). Transcription of strictly defined early genes usually peaks between 6 and 12 hours after infection and declines thereafter, as late viral transcription accelerates. During this early time, the viral DNA genome adopts a nucleosome-like structure (286). The level of viral early RNAs is usually lower than that of late RNAs, which are transcribed by a novel virus-encoded RNA polymerase (RNA pol) specific for late genes. Suggesting that early transcription is mediated by host RNA pol II, early RNA synthesis is inhibited by a-amanitin (106,141), and early promoters are active in cell-free transcription extracts from uninfected cells (21,93,135,223). Moreover, the structure of early viral promoters resembles that of host RNA pol II promoters, complete with a consensus TATA element located ~30 bp upstream from the RNA start site (22,61,110,159,175,223,260). A tetranucleotide CAGT motif found at the RNA start site is common to early promoters and functions as an initiator element (21,222). These core promoter elements also cooperate with upstream activating elements or more distal transcription enhancers (*hr*s) to interact with sequence-specific transcription activators. The cis-acting transcriptional control elements for multiple Ac*M*NPV early genes have been studied in detail, including those for *ie-1, gp64* EFP, *pp31* (39K), *he65*, and *p35*. Viral DNA devoid of protein is infectious, indicating that viral structural proteins are not required for early gene expression. However, many early promoters are highly responsive to virus-encoded transactivators, including the multifunctional regulatory protein IE1 or its spliced gene product IE0 (reviewed in 80). Although it is likely that many host transcription factors also participate in promoting viral transcription, few cellular factors have been identified (92,151,159,228).

IE1 is the principal transcriptional regulator of early baculovirus genes. When assayed by plasmid transfections, IE1 stimulates early, but not late baculovirus promoters (22,110,111,158,162,175,195,209,229,260). This highly conserved, 67-kd transregulator has a modular structure consisting of an acidic transactivation domain(s) within its N-terminal half and DNA binding and oligomerization domain(s) at its C terminus (156,233,244). IE1 also augments expression of genes that are cis-linked to the *hr* elements by interacting with the 28-bp palindromes within these enhancers (108,112,156,169,195,232). By virtue of its interaction with the *hr*s, the co-localization of IE1 and DNA replication factors (202), and the requirement for IE1 in plasmid replication assays (see later), it is postulated that IE1 also contributes to viral DNA replication. However, the exact functions of IE1 during infection are still unclear (229).

Baculoviruses encode additional transcription activators, including IE0, IE2, and PE38. Although these proteins are conserved among baculoviruses, their role during infection is poorly understood. Derived by RNA splicing, IE0 is identical to IE1, except for 54 additional residues at its N terminus. It therefore has regulatory activities similar to those of IE1 (45,158,212,232). IE2 is a 47-kd leucine zipper, RING finger-containing transactivator that is also conserved among baculoviruses (35,261,293). Ac*M*NPV mutants containing loss-of-function *ie-2* alleles are viable, but exhibit delays in virus replication (218). On overexpression in transfected cells, *ie-2* causes cell-cycle arrest (220), but the significance of this activity is unknown. IE2 and the transregulator PE38 co-localize to punctate nuclear structures in transfected cells (161). PE38, which also contains a leucine zipper and RING finger, is capable of promoter-specific transactivation in plasmid transfection assays (160,175,209).

In general, the promoters for the immediate early baculovirus genes are highly active in the absence of virus proteins (158,161,223,261). As a consequence, several of these promoters including those for IE1 and IE2 have been used extensively for high-level gene expression in diverse lepidopteran and dipteran insects by stable or transient transfection protocols.

Late Phase

The late phase of the baculovirus life cycle is initiated by viral DNA replication, which begins between 6 and 12 hours after infection. The synthesis of new genomic DNA and the expression of late structural genes are necessary for the production of BV, the predominant infectious form generated during this period. Viral late gene expression is dependent on proper viral DNA replication. For example, inhibition of viral DNA synthesis by using a temperature-sensitive (*ts*) mutant in the helicase *p143* gene (see later) blocks DNA replication at the nonpermissive temperature and abrogates late gene expression and BV production (96). Likewise, treatment of infected cells with aphidicolin, which inhibits viral and host DNA polymerases, prevents viral DNA synthesis and blocks late viral transcription (230). The molecular basis for the coupling of late gene expression to DNA replication is unknown, but is common among large DNA viruses. At least five baculovirus genes (*ie-1, p143, lef-1, lef-2,* and *lef-3*) are required for DNA replication (Table 2). These genes were identified by using transient DNA replication assays conducted with plasmids containing an *hr* element/origin (152,177). The essential function of several replication genes during infection has been confirmed through the use of conditionally lethal mutants (see Table 2). Replication factors include a DNA-binding helicase (p143), a putative primase (LEF-1), a primase-associated protein (LEF-2), and a single-stranded DNA binding protein (LEF-3). The transactivator IE1 may stimulate transcription of the early replication genes and act as a DNA origin (*hr*)-binding protein (176). Five other genes (*dnapol, ie-2, lef-7, pe38*, and *p35*) directly or indirectly stimulate replication in this transient replication assay (reviewed in 176). A virus-specific DNA polymerase activity has been characterized (120,267), but the exact role of virus-encoded *dnapol* during infection is unclear. Viral factors localize to centers of viral DNA replication within the virogenic stroma appearing with the nucleus (see later) (202).

Gene	DNA replication	LATE expression	Function/Homology	Virus mutants
ie-1	+	+	Transcription transactivator; DNA-origin binding protein	tsB901
ie-2	+	+	Transcription transactivator; cell line–specific cell cycle regulator	
p47	–	+	Late RNA pol component	ts51-7
p143	+	–	Helicase; DNA binding; ATP binding motif	tsB
dnapol	?	+	DNA polymerase; 5′→3′ exonuclease	
pp31	–	+	Tumigenic virions association	
p35	+	–	Apoptosis suppressor	
lef-1	+	+	Primase homology (MIYDAD motif); LEF2 association	
lef-2	+	+	Primase-associated protein? Late, *trans* expression	tsL5-H
lef-3	+	+	ssDNA binding, p143 association	
lef-4	–	+	Late RNA pol component; guanyl transferase; 5′ triphosphatase	tsG30
lef-5	+	–	Unknown	
lef-6	+	–	Vaccinia virus RNA pol homology	
lef-7	+	+	Cell line–specific DNA replication	ref-7-KO
lef-8	–	+	Late RNA pol component	tsG1
lef-9	–	+	Late RNA pol component	
lef-10	–	+	Unknown	
lef-11	–	+	Unknown	
lef-12	–	+	Unknown	
p6.9	+	+	Transactivator of p143	
hcf-1	+	+	Cell line–specific, late, and VEL expression	
lef-3	–	–	ssDNA expression, nuclease/helicase/primase homology	tsB901?

TABLE 2. *Baculovirus DNA replication and late expression factors*

The mechanisms of baculovirus DNA replication are less well understood. Although the *hr* sequences are likely origins of viral DNA replication (154,169,210), it remains to be demonstrated that they act as origins during infection. The *hr*s are functionally redundant and do not affect late gene expression (210,229,231). Other non-*hr* sequences within the genome also have been implicated as replication origins (153,167,288). As suggested by the presence of multiple unit-length genome fragments and concatamers of defective genomes in cells (167,207), the large DNA genome may be replicated by a rolling-circle mechanism. Newly replicated, unit-length circles of DNA are packaged within nucleocapsids. During the late phase, the viral DNA adopts a nucleoprotein organization distinct from the host DNA that remains in a typical nucleosomal structure (286). The replicative or transcriptional function of this viral nucleoprotein structure is unknown.

Nineteen baculovirus genes are required for late gene expression (see Table 2), as first determined for Ac*M*NPV (173,177,227). These genes, designated late expression factors (*lef*s), were identified by their ability to support high-level expression of a reporter gene placed under control of a virus late promoter and cis-linked to an *hr* origin/enhancer element (209). The function and role of the *lef*s in baculovirus replication is the subject of ongoing research in this field (reviewed in 178). Because of the link between DNA replication and late gene expression, several genes required for late gene expression (*ie-1, p143, lef-1, lef-2*, and *lef-3*) are required DNA replication factors (see Table 2). Multiple *lef*s (*ie-2, lef-7*, and *hcf-1*) confer tissue or species specificity, because they are dispensable for late gene expression in certain cell lines. In addition, a variety of viral genes contribute to host range, including helicase *p143* and host range factor-1 (*hrf-1*) (42,53,148). Several *lef*s function directly or indirectly at the level of transcription or mRNA stabilization (see Table 2). Of these genes, four (*p47, lef-4, lef-8*, and *lef-9*) have been shown to encode components or subunits of the viral RNA polymerase (114) specific for late and very late gene expression (see later). The role of transcription-specific *lef*s during infection also has been characterized by using conditionally lethal mutations (see Table 2).

Unique among the nuclear-replicating DNA viruses, the baculoviruses switch from host RNA pol II for early transcription to a novel RNA polymerase for selective transcription of late and very late genes. The virus-encoded RNA polymerase retains its specificity for late virus promoters when assayed by using *in vitro* transcription extracts and is distinguished from other host RNA polymerases by its resistance to a-amanitin and tagetitoxin, inhibitors of RNA pol II and III, respectively (87,93,140,289). Chromatographic analyses of the late RNA polymerase activity indicated that the core of the complex comprises p47, LEF-4, LEF-8, and LEF-9, and is associated with additional proteins (13,114).

Late RNA transcripts initiate from a nucleotide motif containing the sequence (A/G/T)TAAG, which is the primary element for late and very late promoter activity (reviewed in 178). All genes encoding proteins synthesized in abundance during the late phase, including the major capsid protein vp39, the 6.9K basic core protein, and gp64 EFP, possess this late promoter motif. Nucleotide substitutions within the TAAG motif cause a dramatic loss of late promoter activity (191). Moreover, fewer than 18 nucleotides surrounding this initiation site are sufficient to direct high-level late gene expression. This simple promoter organization is reminiscent of yeast mitochondrial promoters and the T-odd bacteriophage promoters. Interestingly, certain late genes have multiple TAAG motifs, each serving as an independent RNA initiation site. For example, the *vp39* capsid gene is transcribed from three autonomous TAAG motifs at –57, –105, and –321 relative to the ORF (264). Such an arrangement may boost transcription levels needed for highly expressed genes. It is noteworthy that some early genes (*p35, gp64, pp31*, and *ie-1*) are also transcribed late in infection. This late transcription is most often accomplished by the use of late TAAG-containing elements that overlap the early promoter elements (22,110,195,222).

Very Late Phase

The final, very late stage of infection is characterized by the hyperexpression of occlusion-specific genes and the production of mature OV from 18 through 76 hours after infection or until cell lysis (Fig. 3A and Fig. 4). The very late phase is marked by a dramatic increase in transcription of *polh*, which encodes the major matrix protein of the OV. The temporally regulated hyperexpression of *polh* and its nonessential nature for BV production were the basis for development of the baculoviruses as highly efficient and productive vectors for foreign gene expression (reviewed in 144,155,186, and 199). In early vectors, foreign genes were placed under control of the *polh* promoter replacing the *polh* ORF to obtain high levels of expression very late in infection of cultured cells or whole larvae (180,215,245). More recently, the expression system has been extended to include other promoters, including that of the *p10* gene (see later).

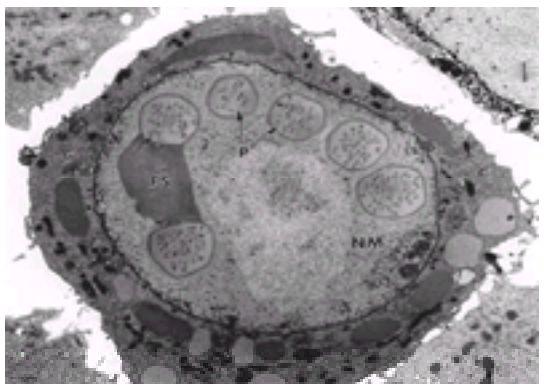

FIG. 4. The very late, occlusion stage of Ac*M*NPV infection. During the very late, occlusion phase, multiple OV particles of polyhedrin (*P*) form on the inside edge of the enlarged nucleus, defined by an intact nuclear membrane (*NM*). Dense fibrillar structures (*FS*) often form in the nucleus and the cytoplasm (*C*). Electron micrograph was provided by Dr. Peter Faulkner and Dr. Gregory Williams, Queens University.

Very late genes also are transcribed by the baculovirus a-amanitin-insensitive RNA polymerase. The promoters of these hypertranscribed genes, which include *polh* and *p10*, contain the late TAAG motif, which is the critical transcriptional element as shown by mutational analyses. However, these very late promoters also are positively regulated by the 50 nucleotides constituting the noncoding leader of their gene (191,205,279). This region between the TAAG and the ORF is responsible for the very late burst of transcriptional activity that distinguishes very late genes from late genes. Interestingly, insertion of the “burst” sequences immediately downstream from a late promoter was not sufficient to convert it to a very late promoter (191). These sequences may interact with activation factors responsible for the stimulation of transcription at this late time. Genetic evidence (184) suggests that virus-encoded very late factor-1 (VLF-1) is a likely candidate for the positive activator (see Table 2). VLF-1 forms a complex with the burst sequences, but the mechanism of transcriptional enhancement is not known (292). Host factors also may participate in transcription of very late genes (92).

The p10 gene is also the other hyperexpressed during the very late phase (reviewed in 196). It encodes a small coiled coil-domain protein (P10) that associates with expansive fibrillar structures that assemble in the nucleus and cytoplasm late in infection (see Fig. 4). P10 is nonessential for NPV replication, including OV formation (271,281). However, it may contribute to disruption of the nuclear membrane and may function at the tissue or organismal levels.

Virus Assembly, Budding, and Occlusion

Nucleocapsids assemble during the late and very late phases of infection. New capsids appear at the edge of the stromal matte located in the virogenic stroma (see Fig. 1D), a virus-specific nucleoprotein structure centered within the nucleus (123,294). Partially filled nucleocapsids with DNA strands connecting the core with the virogenic stroma have been observed, suggesting that the virogenic stroma may be the site of viral DNA replication, condensation, and encapsulation (77,294). A major viral protein associated with the virogenic stroma is phosphoprotein pp31, which is capable of DNA binding by a phosphorylation-regulated mechanism (30,31). RNA also may serve as a structural component of the virogenic stroma (294). Little else is known about this critically important virus-replication center. Newly assembled nucleocapsids exit the nucleus by budding or by transport through nuclear pores (see Fig. 2) (reviewed in 280). Nucleocapsid maturation is sensitive to the actin-depolymerizing drug cytochalasin D, suggesting a critical role for actin in this morphogenic process (40,201). Although enveloped nucleocapsids have been observed in the cytoplasm, the nuclear envelope is removed when the plasma membrane is reached. By interacting end-on with the plasma membrane that is associated with the envelope fusion protein EFP gp64, single nucleocapsids bud from the cell surface to produce extracellular BV (see Fig. 1C). EFP gp64 is required for budding of Ac*M*NPV (206). The rate of BV release from Ac*M*NPV-infected cells increases exponentially from 10 to 20 hours and then declines through 36 hours (166). The kinetics of BV production varies between baculoviruses.

OV particles accumulate in the nucleus of Ac*M*NPV-infected cells (see Fig. 3A and Fig. 4) starting ~18 hours after infection. OV production accelerates as BV production shuts down. Virion occlusion involves a number of processes, including virus-induced modification of the nuclear membrane and nuclear transport of polyhedrin, the OV matrix protein (145,280). Nucleocapsids align along nuclear membrane segments and acquire an envelope before condensation of polyhedrin, which encases the enveloped virions (see Fig. 1E). OV formation begins on the inside edge of the infected nucleus (see Fig. 4). Time-lapse video microscopy has indicated that the number of OV particles per cell is established early, and with time, each particle increases in size (181). Maturation of the OV includes deposition of the polyhedron envelope (calyx) over the particle's surface. OV is released from the nucleus on cytolysis very late in infection. Multiple virus genes are likely involved in OV production. For example, loss of function of the 25K protein gene of Ac *M*NPV causes the easily recognized “few polyhedra” (FP) phenotype, which is characterized by reduced OV accumulation, limited intranuclear envelopment of nucleocapsids, and increased production of BV (10,27,124). The exact function of the cytoplasmic 25K protein remains unknown. A gene involved in calyx formation also has been identified (94).

Disease Progression in the Insect

Horizontal transmission of baculoviruses occurs through the OV, the form of the virus that is most stable and resistant to environmental degradation. Primary infection occurs in the midgut epithelium of the insect larva (reviewed in 69). Infection is initiated by larval ingestion of virus-contaminated food. On entry into the midgut lumen (see Fig. 2B), the OV particles dissolve in response to the alkaline environment, thereby releasing the ODV. These virions pass through the peritrophic membrane, facilitated by the activity of virus-encoded proteases (enhancins), first discovered in the GVs (170). ODVs subsequently bind to the brush-border membrane of columnar epithelial cells and enter, apparently by direct

fusion with the plasma membranes (see Fig. 2B). Infection proceeds in a manner similar to that of BV-infected cell cultures, with the interesting exception that formation of OV in midgut cells of most lepidopteran hosts is rare (76). In addition, there is a distinct polarity in the intracellular transport of EFP gp64, which is directed primarily to the basal membrane side of the midgut cell (149). This directionality likely dictates nucleocapsid budding to the basal lamina, allowing access of BV to the hemocoel and tracheal epithelium for rapid systemic spread. Certain baculoviruses, such as the mosquito (Diptera) NPVs, are restricted to the midgut.

Secondary infection is achieved by BV produced in midgut cells (see Fig. 2A). Virus is spread by hemocytes (blood cells), the epithelial cells lining the tracheal network, or both (65,101). With highly susceptible hosts, secondary infection can affect virtually all the internal tissues of the larva (69). In the final phases, the larva is converted into a milky white liquid, consisting mostly of OV particles. This process, referred to as “melting” or “liquification,” is facilitated by a virus-encoded cathepsin and chitinase, which cooperate to break down the larval cuticle (skin) (125). In a typical Ac*M*NPV infection, more than 10^8 OV particles are produced per larva, constituting more than 10% of the dry weight of the insect. On rupture of the cuticular exoskeleton, OV is released into the environment for transmission to other insects.

It is noteworthy that not all baculoviruses are lethal. There are several reports of persistent or latent baculovirus infections in seeming healthy insects. Such viruses may be transmitted vertically. The most thoroughly described virus/host system is that of a *Mammestra brassicae* NPV that persists in a laboratory insect colony without causing significant mortality (139).

Baculovirus-Mediated Alterations of the Host

Baculoviruses have dramatic effects on the host cell, altering both cytoskeletal and nuclear structure (see earlier) and causing shutdown of host protein and RNA synthesis (36,203). However, these viruses also can control their insect hosts at the physiologic and behavioral levels (reviewed in 196). For example, infected larvae often display the abnormal behavior of wandering away from their food supply or climbing to the top of the vegetation, where they succumb hanging from a leaf or branch. This strategy may facilitate virus dispersal. In another example, baculoviruses arrest development of their larval hosts by controlling the activity of ecdysone, the steroid hormone that triggers molting (197). The baculovirus *egt* gene encodes an ecdysteroid uridine diphosphate (UDP)-glucose/galactose transferase, which is secreted from infected cells and transfers glucose or galactose to the hydroxyl group at position C22 of these ecdysteroids. Evidence exists that viral *egt* blocks larval molting (reviewed in 196). Larval developmental arrest is correlated with increased virus production, because on deletion of *egt*, infected larvae attempt to molt, but die sooner and produce less virus (198). The overall effect of *egt* depends on virus dose and the developmental stage at the time of inoculation.

Apoptosis and Apoptotic Suppressors

The replicative success of baculoviruses depends on active suppression of host-cell apoptosis, a process that may represent an antivirus defense strategy by the host. Apoptosis or programmed cell death (PCD) is a highly conserved mechanism for cellular self-destruction that is critically important in normal development, tissue homeostasis, and virus pathogenesis. To counteract the apoptotic suicide response and prolong cell viability, the baculoviruses encode novel apoptotic suppressors, which block virus-induced apoptosis (reviewed in 47 and 187). Because these same proteins function in phylogenetically diverse organisms, baculoviruses have provided important insight into the biochemical components of PCD and its regulation.

Ac*M*NPV mutants that lack PCD suppressors cause widespread apoptosis (48,49,129). Apoptosis of cultured *Spodoptera frugiperda* SF21 cells, a model system for studies of invertebrate PCD, is characterized by fragmentation of cellular and viral DNA and dismemberment of the infected cell into extracellular vesicles called apoptotic bodies (see Fig. 3B) (48,164,181). SF21 cells are highly sensitive to a variety of apoptotic stimuli, including UV radiation, actinomycin D, calcium ionophores, okadaic acid, and hydrogen peroxide (Fig. 5A). In cultured cells, apoptosis reduces the yield of infectious virus by as much as 10,000-fold (49,129). Moreover, the infectivity of apoptosis-causing Ac*M*NPV mutants in larvae is 25- to 1,000-fold lower than that of wild-type virus (49), suggesting that apoptosis impedes baculovirus multiplication in larvae. Interestingly, baculovirus-induced apoptosis is tissue or species specific. For example, cultured *Trichoplusia ni* TN368 cells fail to undergo apoptosis when infected by Ac*M*NPV mutants that cause apoptosis of SF21 cells (49,129). In *T. ni* larvae, the infectivity of these mutants is comparable to that of wild-type virus. The molecular basis of this cellular resistance to virus-induced apoptosis is not understood.

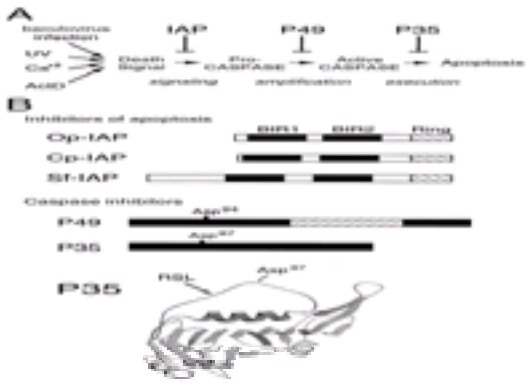

FIG. 5. Baculovirus suppressors of apoptosis. **A:** The apoptotic pathway. Apoptosis of S. *frugiperda* (SF21) cells is signaled by baculovirus infection and other agents which cause proteolytic cleavage of the inactive procaspases to their active forms. P35 functions to inhibit active caspases, including *Sf*-caspase-1, whereas P49 functions upstream to inhibit an apical caspase. Baculovirus IAP functions upstream of P49 to block procaspase activation. **B:** Comparison of baculovirus apoptotic regulators. The IAPs contain two BIRs (baculovirus inhibitor of apoptosis repeats) motifs and a C-terminal Ring finger motif. Viral IAPs, Op-IAP and Cp-IAP, closely resemble Sf-IAP, a cellular IAP from host

lepidopteran *S. frugiperda.* Caspase inhibitors P49 and P35 are 49% identical and contain aspartate residues (Asp ^{94} and Asp^{87}) required for caspase cleavage. P49 contains a 120 residue insertion (crosshatched) unrelated to P35. The P35 crystal structure (bottom) illustrates its reactive site loop (RSL) in which Asp ^{87} is located at the apex for caspase cleavage and interaction.

It is likely that multiple mechanisms are responsible for triggering apoptosis during baculovirus infection. Virus binding to cellular receptors is not sufficient. Rather, viral gene expression is required (50,164). After Ac*M*NPV infection, the earliest of detected apoptotic events (see Fig. 5A) is the activation of host-cell caspases (16,165). The caspases are a highly conserved family of **c**ysteinyl **asp**artate–specific prote**ases** that are critical and necessary effectors of apoptosis (reviewed in 265). Proteolytic activation of the proenzyme form of *Sf*-caspase-1, the principal executioner caspase in SF21 cells, coincides with the initiation of virus DNA replication (164,165,182,242). Thus apoptotic signaling must occur at or before this time, suggesting that viral DNA replication, late gene products, or shutdown of host RNA or protein synthesis may trigger apoptosis (50,164). Early gene expression also can contribute to apoptotic signaling (164). In particular, transactivator IE1 is sufficient to induce limited apoptosis in SF21 cells on overexpression by plasmid transfection (219). However, the mechanism by which IE1 induces apoptosis and its role in baculovirus-induced apoptosis are unclear (229).

The baculoviruses encode two mechanistically distinct types of apoptotic suppressors (see Fig. 5B), the inhibitors of apoptosis (IAPs) and the caspase inhibitors (P35 and P49). These proteins prevent premature host-cell death and thereby promote virus multiplication (reviewed in 47 and 187). Identification of functional *iap genes* in lepidopteran insects (see Fig. 5B) suggests that baculoviruses originally acquired these apoptotic regulators from the host cell (138,243). This possibility is consistent with the strong selective advantage conferred to the virus by the capacity to prevent virus-induced apoptosis (172). Although baculoviruses encode multiple apoptotic suppressors, usually one is sufficient per genome. For example, Ac*M*NPV encodes one functional copy of *p35* and two copies of *iap* (*iap1* and *iap2*), which are nonfunctional for blocking virus-induced apoptosis (50,103). Lacking *p35,* Op*M*NPV encodes one functional (*iap3*) and three nonfunctional *iap*s (19).

Baculovirus IAPs were the first discovered members of the IAP family, which now includes related proteins from invertebrates and vertebrates (reviewed in 47,59, and 187). IAPs are distinguished by their highly conserved Cys/His-containing motif called the BIR (**b**aculovirus **i***ap* **r**epeat) (see Fig. 5B). This defining ~65-residue motif coordinates a Zn atom and is required for IAP antiapoptotic function. Baculovirus Op-IAP (31 kd) and Cp-IAP (31 kd) from Op*M*NPV and CpGV, respectively, contain two BIRs and a C-terminal RING finger motif (19,54). Suggesting that they function at a conserved step in the apoptotic pathway, the viral IAPs prevent apoptosis induced by diverse signals in insect and mammalian cells (47,187). The BIR motif is required for interaction with cellular proapoptotic factors, including Reaper, Hid, and Grim from *Drosophila melanogaster* (276,277). In addition, the BIRs mediate homo-oligomerization of Op-IAP, which appears to be required for antiapoptotic activity (137). However, the molecular mechanism by which the BIRs contribute to viral IAP antiapoptotic activity is still unclear. More is known about mammalian IAPs, which directly inhibit activated caspases (59). Consistent with an anticaspase mechanism, Op-IAP prevents the appearance of caspase activity *in vivo* and blocks the proteolytic activation of *Sf*-caspase-1 by an upstream, apical caspase (165,182,242). Thus Op-IAP may function at or upstream of the step at which the caspases are activated in insects (see Fig. 5A).

First discovered in Ac*M*NPV (48), P35 is a universal suppressor of apoptosis. Ectopic expression of the *p35* gene prevents PCD in nematodes, flies, moths, and mice, as well as cultured human cells (37,50,58,133,143,224,225,257,270). Accounting for its broad effectiveness, P35 (35 kd) functions as a substrate inhibitor of the caspases. To block virus-induced apoptosis, P35 must be synthesized early in infection before the activation of host caspases (130). P35 is cleaved by the targeted caspase at Asp^{87} and forms a stable, stoichiometric complex (16,32,298). The 2.2-Å crystal structure of P35 reveals that the Asp^{87} cleavage site is located at the apex of a novel solvent-exposed reactive site loop (see Fig. 5B), the orientation of which is critical to P35's anticaspase activity (72,300). Identified in *Spodoptera littoralis* NPV, baculovirus P49 also blocks virus-induced apoptosis (63). At the amino acid level, P49 is 49% identical to P35 and contains a predicted reactive site loop with a cleavage site at Asp ^{94} (see Fig. 5B). Although P49 functions as a substrate inhibitor, it targets apical caspases that are upstream of and distinct from those inhibited by P35 (301). Thus baculoviruses have evolved multiple caspase inhibitors with different target specificities. Moreover, baculoviruses have evolved mechanisms to block virus-induced apoptosis at multiple steps in the death pathway (see Fig. 5A). No cellular homologs of *p35* or *p49* have been identified.

Host Transposons

The baculoviruses are one of the few families of animal viruses that accommodate the frequent and spontaneous insertion of host-derived transposable elements (reviewed in 79). By virtue of their nuclear replication and flexibility with respect to size of their DNA genome, baculoviruses are vulnerable to the insertion of resident transposons. Because of their relative abundance and mobility, arthropod transposons represent important insertional mutagens and can have a significant influence on virus and host evolution. Transposon insertion provides genetic diversity and potentially novel genes to the recipient virus. The baculovirus' capacity to act as a landing platform for these elements has led to the identification of new families of lepidopteran transposons.

Baculovirus mutants with transposon insertions are often distinguished by their altered plaque morphology, especially the “few polyhedra” (FP) phenotype, which exhibits reduced OV accumulation (see earlier). Several FP-causing lepidopteran transposons, including IFP2, IFP2.2, IFP1.6, TFP3, and hitchhiker, have been identified by their insertion at or near the FP locus that encodes the 25K protein gene (9,10 and 11,38,78). These middle to highly repetitive elements are characterized by their small size and terminal inverted repeats that sometimes flank a transposase-like ORF. Significantly, IFP2 (also called PiggyBac) is a 2.5-kb autonomous transposon from the moth *T. ni,* which is capable of stable germline transformation of the Mediterranean fruit fly from the order Diptera (119). Thus PiggyBac and other

lepidopteran elements have the potential for gene transfer in nondrosophilid insects.

Also discovered as a baculovirus insertion, TED (transposable element D) is a 7.5-kb, middle-repetitive retrotransposon of *T. ni* (82,185). TED has a retrovirus-like organization with *gag, pol,* and *env* genes that are flanked by long terminal repeats, and thus is a member of the gypsy family of retroelements (79). Unlike the smaller lepidopteran transposons, TED assembles virus-like *gag*-containing particles, complete with protease and reverse transcriptase activities (115,171). Moreover, the TED *env* gene encodes a membrane-associated glycoprotein that could function as a viral envelope protein (208). Thus TED and other *env*-carrying gypsy elements are likely insect retroviruses. As such, integration within the baculovirus genome could facilitate interspecies shuttling of these passenger viruses (79,185).

Nudiviruses

Classification and Structure of Nudiviruses

The nudiviruses were previously categorized as “nonoccluded” baculoviruses, but are currently unclassified. Like the baculoviruses, they have rod-shaped, enveloped nucleocapsids that contain a large circular, double-stranded DNA genome (recently reviewed in 33). The envelope is acquired in the nucleus, and in some cases, a second unit membrane is obtained by budding from the plasma membrane of the infected cell. In contrast to the baculoviruses, the nudiviruses lack an occluded form. This difference limits their survival outside the host and may contribute to their persistence or latency in infected insects. Because of the paucity of nucleotide sequence information, the relatedness between individual nudiviruses or baculoviruses is unknown. The nudiviruses could be an aggregate of genetically diverse viruses.

Nudivirus Transmission and Persistence

Nudiviruses can exert dramatic effects on insect populations. Oryctes virus, a nudivirus infecting the rhinoceros beetle *Oryctes rhinoceros,* causes a potentially fatal infection that initiates in the midgut of adult beetles and their larvae (295,296). This virus has been enormously successful as a control agent of the rhinoceros beetle in coconut plantations in the South Pacific (297). Virus transmission occurs primarily through contaminated food sources, but also can occur through mating. Another nudivirus, Gonad Specific Virus (GSV), infects gonadal tissue (ovarian and testicular organs) of adult moths and causes sterility (116,226). GSV appears to be transmitted by asymptomatic carriers within the insect population and can be vertically transmitted from eggs, probably in a persistent or latent state. Virus replication is hormonally activated in adult gonadal tissue. Hormonal or developmental activation also occurs with other viruses, including the polydnaviruses (see later). GSV can be propagated in cell culture, and persistently infected cell lines have been established (34).

Hz-1 is the most extensively studied nudivirus at the molecular level. This complex, nonoccluded virus has provided a useful model for insect virus persistence and latency (reviewed in 33). Hz-1 was originally isolated from a persistently infected cell line established from adult ovarian tissue of the corn earworm *Heliothis zea* (102). It has an unusually large (230 kbp) circular, double-stranded DNA genome. Hz-1 establishes persistent infections of cultured cells where replication occurs at low levels and produces defective interfering particles. Virus from persistent infections can cause lytic infection of naive cells not previously exposed to virus. The genome of persistently infected cells can harbor stably integrated Hz-1 DNA (174). The establishment or maintenance of viral persistence is associated with the synthesis of a single 2.9-kb RNA transcript from Hz-1, referred to as the persistency-associated transcript (PAT1) (39). The function of PAT1 and whether it encodes a protein(s) is unknown. Hz-1–infected cells also are resistant to superinfection. This immunity is not due to an acquired resistance to virus-induced apoptosis (168).

Polydnaviridae

Classification and Structure of Polydnaviruses

The polydnaviruses are distinguished by their obligate and mutualistic association with their host, the endoparasitic wasps (Order Hymenoptera). The family *Polydnaviridae* derives its name from the taxonomically characteristic “polydisperse” DNAs composing its multi-segmented genome. Virions contain numerous double-stranded, supercoiled DNA molecules with sizes ranging from 2 to 28 kbp (reviewed in 248 and 278). Depending on the virus, there are 15 to 30 different DNA molecules with an estimated genomic complexity of 75 to 200 kbp. Virion DNA is derived from proviral DNA integrated within the host wasp, where the virus is transmitted vertically. Current evidence indicates that the proviral DNA is also polydisperse within the host genome where certain DNAs are the linear equivalent of circular segments packaged within virions (55,74,105,290). Because polydnaviruses are not known to replicate outside the host wasp, it is not yet clear that virion DNA represents the entire virus genome.

The only characterized polydnaviruses are from endoparasitic wasps. These predatory insects lay their eggs inside the bodies of other living insects, often in the larval stage, where the eggs hatch and the young parasites develop. Polydnaviruses have been isolated from the Ichneumonidae and Braconidae families of wasps and are correspondingly divided into two genera, the ichnoviruses and bracoviruses (278). Although polydnavirus-like particles have been observed in other parasitoids, they remain unclassified.

The ichnoviruses and bracoviruses are morphologically distinct. The only feature shared by the two genera may be their polydisperse virion DNA and their novel life cycle. The ichnovirus virion is complex, resembling that of the ascoviruses (see later). The virions are ellipsoid or quasi-cylindrical, with a uniform size of 90 × 300 nm (Fig. 6A and Fig. 6B). The nucleocapsid is enveloped by two unit membranes, one derived from the nucleus during virion morphogenesis and the other by budding from the plasma membrane of the host cell. Resembling the baculoviruses, bracovirus nucleocapsids are rod-shaped with a uniform diameter (35–40 nm) and variable length (30–200 nm). They have a single membrane envelope, which is derived from the nucleus and surrounds one or more nucleocapsids (see Fig. 6C). Polydnavirus particles contain 20 different polypeptides. The minimal genetic information needed to encode these structural proteins is

about 30 kb. However, evidence exists that some structural proteins may be produced by the expression of integrated, proviral DNA, rather than virion DNA (278).

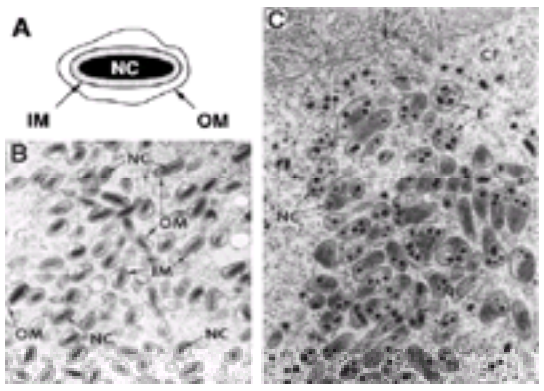

FIG. 6. Morphological features of polydnaviruses. **A:** Schematic view of the ichnovirus structure. The nucleocapsid (*NC*) is surrounded by an inner (*IM*) and outer (*OM*) membrane. **B:** Ichnovirus preparation. Lenticular nucleocapsids (*NC*) of uniform size are enveloped by inner and outer membranes (*IM* and *OM*). **C:** Bracoviruses in the calyx fluid (*CF*) of an adult female parasitoid wasp. Nucleocapsids (*NC*) are observed end-on and in lateral view. Multiple nucleocapsids are surrounded by a unit membrane envelope (*E*). An epithelial cell (*EC*) of the calyx is also observed. Electron micrographs provided by Dr. Donald B. Stoltz, Dalhousie University. Modified with permission from (251).

Obligate Mutualism of Virus and Host

The polydnaviruses play a critical role in the life cycle of their host wasp. This mutualistic participation is central to the multiplication strategy and genetic organization of these novel viruses. The polydnaviruses replicate asymptomatically in the oviducts of female wasps, as a normal step in the development of the reproductive tract (Fig. 7). Proviral DNA is present as dispersed, integrated segments and is inherited in a mendelian fashion (249). On replication, polydnavirus particles accumulate to high density in the calyx fluid of the oviduct. On parasitization, virus and the wasp's egg is delivered to the recipient caterpillar (see Fig. 7). Normally, the caterpillar mounts an immune response that is mediated in part by hemocytes of the open circulatory system, which encapsulate the intruding egg, thereby destroying it. However, in the case of polydnavirus-carrying ichneumonid and braconid wasps, encapsulation is counteracted or repressed by the accompanying virus. As a result, development of the parasitic egg is ensured. Demonstrating a definitive role for virus-mediated abrogation of the host immune response, the wasp egg is destroyed within the larva when introduced in the absence of virus (64). Thus the relationship between polydnavirus and wasp is mutualistic. The wasp requires the virus to provide protection against the immune response of the parasitized insect, and the virus requires the wasp for replication and survival of its genome.

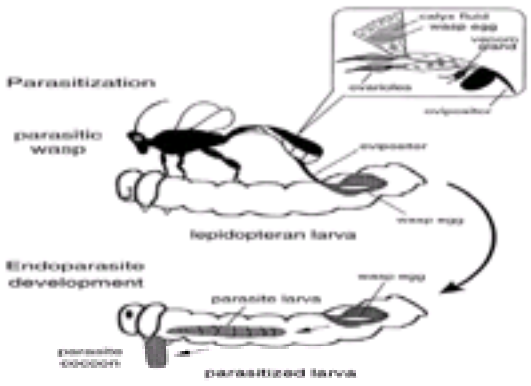

FIG. 7. Polydnavirus life cycle. Polydnavirus particles form in the epithelial cells of adult female ovaries and are released into the calyx fluid where they accumulate to high densities (*top inset*). As the first step in parasitization (*top*), the wasp egg along with virus and venom are injected into a lepidopteran larva during oviposition. During endoparasite development (*bottom*), polydnavirus activity suppresses the larval immune response allowing the wasp egg to develop within the parasitized larva. After development, the wasp larva emerges, spins a cocoon, pupates, and emerges as an adult to repeat the process in a new host. From Dr. Bruce A. Webb, University of Kentucky; modified with permission.

Polydnavirus Genome Organization

The polydnavirus genome is highly complex. Virion DNAs (designated by letters) are nonequimolar, polymorphic in length, and present in multiple topologic forms (superhelical, relaxed circular, etc.) (reviewed in 278). For example, the genome of *Campoletis sonorensis* polydnavirus (CsPDV), the ichnovirus prototype, consists of 28 to 30 different DNAs, ranging in size from 5.5 to 21 kbp, with a collective size of 200 to 250 kbp. It is not known how many viral DNAs are packaged within each virion or whether each virion contains all or a subset of DNA segments. Particles of CsPDV (see Fig. 6) have a uniform size and density and thus may have similar DNA contents. In contrast, bracovirus virions vary in length and may contain different quantities of DNA. In addition, certain ichnovirus DNA segments are likely derived from recombination events within larger segments, which are referred to as “nested” segments (55,290). The presence of nested segments may reduce the effective complexity of virion DNA. However, complete determination of genetic

complexity will require characterization of all genomic DNA segments.

From the few cases in which the relationship between virion and proviral DNA is known, the largest or “master” DNA segments within the wasp genome are flanked by direct repeats. The 16-kbp integrated W segment of CsPDV has long terminal repeats of 1.2 kb (55). Additional 350-bp repeats within this segment are apparently responsible for generating nested R and M segments through homologous recombination during amplification of circular DNA segments in the female wasp. Within the wasp genome, the termini of CsPDV segment B are flanked by 59-bp direct repeats and are removed during excision and circularization of this DNA (74). This finding and others suggest that proviral segments are excised from the wasp genome by recombination before amplification (105). Other sequences are repeated between CsPDV DNA segments, but may not be involved in recombination (262).

Virion DNA from the polydnaviruses carry genes that participate in suppression of the immune response of parasitized caterpillar. Several gene families have been identified (23,60,256,290). One family within CsPDV encodes proteins with sequence similarity to omega-conotoxins of marine snail venoms (60). One of the conotoxin-like proteins has been implicated in the suppression of the encapsulation response (reviewed in 278). Moreover, a functional relationship between the viral toxins and wasp venom peptides may explain the independence of the ichnoviruses from venom gland secretions of the wasp for immune suppression. Another gene family with sequence similarity to epidermal growth factors has been identified in *Microplitis demolitor* polydnavirus (MdPDV) (256). The nature and function of these gene products are unclear. It also is unknown whether virions contain all the genes required for virion assembly, because only a few polydnavirus structural genes have been characterized.

Polydnavirus Life Cycle and Host Effects

The formation of polydnavirus virions occurs primarily, if not exclusively, in the nuclei of epithelial calyx cells composing the oviducts of female wasps (see Fig. 7). Virus replication is under hormonal control and is first detected at a specific stage in wasp pupal development (reviewed in 278). Proviral DNA segments may be amplified in an “onion skin” fashion from within the wasp chromosome and then circularized. Alternatively, proviral DNA may be excised first, circularized, and then amplified. The presence of chromosomal sites lacking proviral DNA in tissues supporting viral replication is consistent with the latter mechanism (105). In mature females, the virions are released from the calyx cells into the oviduct lumen, either by budding (ichnoviruses) or by cell lysis (bracoviruses). The calyx fluid contains massive numbers of polydnavirus particles (see Fig. 6C), which are deposited along with the egg and wasp secretions such as venom from accessory glands during oviposition.

Despite the presence of high concentrations of virions, polydnaviruses cause no obvious pathology in wasps. RNA transcripts from some genomic segments have been detected in wasp ovaries, and there is evidence that some of the viral RNAs produced in wasps differ from those produced in parasitized hosts (23,262). Although virus particles are confined to the female reproductive tract, viral DNA also is present in many tissues of adult females and males (75). The predominant form of the viral DNA in males is the integrated proviral form, but a minor component of extrachromosomal DNA also has been detected.

The most obvious physiologic effect of the polydnaviruses is in the parasitized insect host, where larval immunity and development are altered (reviewed in 254 and 278). The mechanisms of virus-mediated immune suppression are not yet understood. Polydnavirus suppression of encapsulation involves physiologic changes in hemocytes that participate in capsule formation, including granulocytes and plasmatocytes (252). On virus entry, viral gene expression occurs in hemocytes and possibly other tissues. Many hemocytes survive throughout parasitization and continue to support virus gene expression. Some viral gene products are secreted and are thought to affect other hemocytes and tissues. In MdPDV, virus gene products induce apoptosis in a specific subclass of hemocytes, which are involved in the encapsidation response (255). Other aspects of larval physiology also are affected, either directly by polydnavirus-specific proteins or indirectly as result of infection of normal cells.

There is no evidence for polydnavirus replication in the parasitized larva. Neither synthesis of virion DNA nor assembly of virions has been detected. Nonetheless, virion DNA is long-lived, and viral RNA transcription occurs throughout parasitization. In the ichnovirus CsPDV, 12 different polyadenylated RNAs have been identified in parasitized or virion-injected larvae (23,24). Some of these viral RNAs are spliced. CsPDV transcripts are detected within 2 hours and persist throughout development and emergence of the parasite wasp 9 days after oviposition. Several virion gene products have been detected (reviewed in 278). A similar strategy of gene expression and persistence of the bracovirus MdPDV occurs in parasitized and virus-injected *Pseudoplusia includens* larvae (253,256). Viral DNA persists for 6 days, but is not amplified. At least six different classes of MdPDV RNA are synthesized primarily in hemocytes, which may be the principal target for immune suppression. The identification and functional characterization of polydnavirus gene products that modulate the lepidopteran immune system remain the areas of intense interest.

Ascoviridae

Classification and Structure

First discovered in the 1970s, the ascoviruses are classified as a single genus, ascovirus, within the family *Ascoviridae* (reviewed in 70 and 188). The name ascovirus is derived from the Greek word *askós*, meaning sac, to describe the large membrane-bound vesicles that are associated with virus development. The formation of these virion-containing vesicles is unique among viruses and represents the distinguishing feature of *Ascoviridae* (67). The virions are complex, double-enveloped particles having an allantoid (kidney-shaped), ovoidal, or bacilliform shape, depending on the species (Fig. 8A). The virions (~400 × 130 nm) have an electron-dense core that is surrounded by two unit membrane envelopes of unknown composition (see Fig. 8B). At least 12 different proteins, ranging in size from 11 to 200 kd, are present in virions (71).

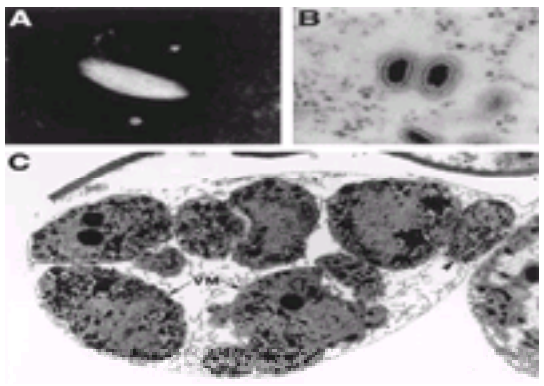

FIG. 8. Ascoviruses. **A:** An individual kidney-shaped nucleocapsid of the ascovirus from the moth *Trichoplusia ni.* The surface is reticular in appearance. **B:** Cross-section of two adjacent virions of the *Scotogramma* ascovirus. A multi-layered membrane envelope surrounds the nucleocapsid. **C:** Remnants of an ascovirus-infected cell. Dark-staining bodies are membrane-bound vesicles (*VM*) that contain *Autographa precationis* ascovirus particles (arrowheads). Different-sized vesicles are collectively bordered by a basement membrane. Provided by Dr. Brian A. Federici, University of California-Riverside, and Dr. J. J. Hamm, USDA-ARS, Tifton, GA; modified with permission from refs. 68 and 70.

Ascoviruses have a large, double-stranded DNA genome of ~100 to 180 kbp, as estimated by restriction fragment analyses (67,71). Evidence exists that the DNA genome is circular (18,44), but such forms have not been confirmed by electron microscopy. Limited nucleotide sequence information is available. Sequence comparisons of the *dnapol* gene of *S. frugiperda* ascovirus (SAV) with that of other viral *dnapol*s suggest that the ascoviruses are a unique virus family that is most closely related to the iridoviruses (214). Short imperfect repeats also have been identified within the ascovirus genome (18).

Replication Stages

Studies on ascovirus replication have been limited to cytologic observations in tissues of infected moth larvae (188). The first sign of infection is nuclear and cellular hypertrophy, in which the diameter of the infected cell increases from fivefold to 10-fold (67). As replication proceeds, the nucleus fragments, and the cell separates into 20 to 30 membrane-bound vesicles that contain assembling virions (see Fig. 8C). This unusual process involves extensive invagination of the plasma membrane, similar to that of a cell undergoing apoptosis. Once formed, the vesicles dissociate from the cell and are released into the hemolymph. As the vesicles accumulate, basement membranes surrounding the tissue are disrupted, and the hemocoel becomes filled with ~10^8 vesicles/mL, a process that turns the hemolymph a milky white color.

Ascovirus Transmission and Disease Progression

In the laboratory, ascovirus transmission is accomplished by pricking the host with a fine pin contaminated with virus-containing vesicles. A dose of 10 vesicles is sufficient to infect more than 90% of inoculated insects. In nature, ascoviruses are probably transmitted by female endoparasitic wasps through virus contamination of their ovipositor during egg laying. For example, *Diadromus pulchellus* ascovirus (DpAV) is vectored by the ichneumonid wasp *Diadromus pulchellus* during oviposition of leek moth pupae, where the virus multiplies during parasitization (18). DpAV can be found in the wasp reproductive tract, but appears to replicate poorly there. DpAV DNA is found in both male and female wasps. A mutualistic relationship between the ascovirus and its wasp vector is suspected, because survival of *D. pulchellus* wasps in the laboratory is positively correlated with the presence of DpAV (17). The host range of ascoviruses may include only a few closely related species or may extend to species in different families (117).

Ascoviruses cause a slow, chronic infection in lepidopteran (moth) larvae or pupae. The disease causes reduced feeding, aberrant development, and death within 2 to 6 weeks (reviewed in 188). During the early stages, there is little obvious pathology. In the later stages, the most notable disease symptom is developmental arrest. Some larvae exhibit a milky discoloration. Different ascoviruses exhibit different tissue tropisms. For example, SAV selectively multiplies in fat body, whereas other viruses multiply in diverse tissues.

Nodaviridae

Classification of Nodaviruses

The *Nodaviridae* are small riboviruses with a single-stranded, positive-sense genome of two RNAs. The bipartite genome is packaged within a single, nonenveloped virion (~30 nm diameter) with T=3 icosahedral symmetry (recently reviewed in 8,146, and 238). The genome organization and replication strategy of the nodaviruses is among the simplest of known viruses (Fig. 9). As such, the nodaviruses are powerful models for exploring strategies of virus replication, host interactions, and virion assembly.

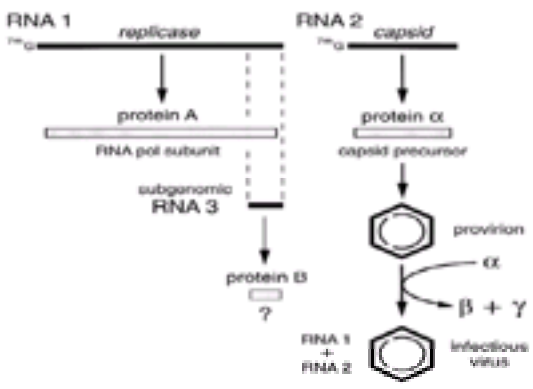

FIG. 9. Nodavirus replication strategy. The two genomic RNA segments (RNAs 1 and 2) encode the replicase (protein A) and capsid precursor protein (protein a), respectively. RNA 1 also specifies subgenomic RNA 3 which encodes protein B of unknown function. Assembled from capsid precursor a, provirions undergo a maturation cleavage producing capsid proteins b and k. Infectious virions contain both genomic RNAs.

The nodaviruses are divided into two genera (alpha and beta), which infect insects and fish, respectively. The family name originated from Nodamura virus, the first discovered family member isolated from mosquitoes near the Japanese village of Nodamura (236). The best-studied insect nodaviruses include Nodamura virus (NoV), black beetle virus (BBV), and flock house virus (FHV), each of which has been fully characterized at the molecular level. Other alpha members include New Zealand virus, Boolarra virus, Manawatu virus, and gypsy moth (*Lymantria ninayi*) virus. Most members of the alpha genus were isolated from insects of Australasia. NoV is the only nodavirus with the capacity to multiply in vertebrates (8). Nonetheless, on transfection of genomic RNA, nodaviruses can replicate in mammalian, yeast (*Saccharomyces cerevisae*), and plant cells (5,217,240).

Virion Structure of the Nodaviruses

The nodaviruses have provided enormous insight into icosahedral virus assembly and maturation. The structures of BBV, FHV, and NoV have been determined at atomic resolution (73,136,299). Each virion contains 180 copies of the same capsid protein (Fig. 10). Sixty triangular units, consisting of three similarly folded capsid proteins, form an icosahedral lattice with T=3 symmetry and a radius of 156 Å (recently reviewed in 146 and 238). The three capsid proteins form a prominent peak at the quasi-threefold axis (see images at www.bocklabs.wisc.edu/virusviz1.html). At the twofold axes, conserved C-terminal residues of the capsid protein make contact with duplexed genomic RNA that is highly ordered and stabilize the capsid protein interface (73). C-terminal residues of the capsid protein also are required for RNA encapsulation (237). At the fivefold axes, the capsid proteins form a pentameric helical bundle that has been proposed to participate in release of the genomic RNA on virus penetration into the host cell's cytosol (43).

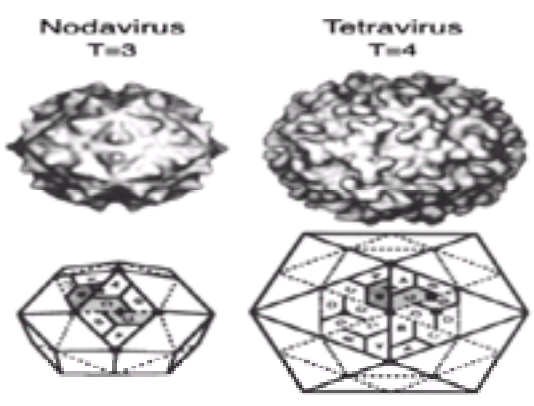

FIG. 10. Comparison of nodavirus and tetravirus structures. *Top:* Image reconstructions of the virion of a nodavirus (Flock House virus, *left*) and a tetravirus (Nudaurelia omega virus, *right*). The average particle diameter for the nodaviruses and tetraviruses is 320 Å and 415 Å, respectively. *Bottom*: Diagrammatic illustration of the subunit assembly of the T=3 nodavirus (*left*) and the T=4 tetravirus (*right*) virions. Each subunit (A, B, C, and D) is indicated by a trapezoid. Nodaviruses contain 60 subunits of three similarly folded capsid proteins that form a prominent peak at the quasi-three-fold axis (*top*). In contrast, tetraviruses contain 240 capsid protein subunits, twelve of which comprise each triangular face of the virion. Structural images are available at http://www.bocklabs.wisc.edu/virusviz1.html at the Institute for Molecular Virology, University of Wisconsin-Madison. Adapted with permission from Dr. John E. Johnson, Scripps Research Institute, La Jolla, CA.

The nodavirus provirion is assembled from 180 copies of the ~43-kd capsid precursor a. During virus morphogenesis (see Fig. 9), a is cleaved very near its C terminus to generate capsid proteins b and g (84,91,136). For FHV, this postassembly cleavage occurs autocatalytically between Asn^{363} and Ala^{364}, located on the inside surface of the virion. This cleavage is required for infectivity and virion stability (91,239). In addition, the maturation cleavage may be necessary for an undefined event occurring between cell attachment and cytosolic release of the RNAs.

Nodavirus genome organization

The nodavirus genome consists of two single-stranded, messenger-active RNAs (RNA 1 and 2) that are ^{7m}G-capped, but not polyadenylated (see Fig. 9). An unknown structure or factor blocks the 3' ends. RNA 1 (~3.1 kb) encodes the

functions required for RNA transcription and replication, whereas RNA 2 (~1.4 kb) encodes capsid precursor a. Both RNAs are required for infectivity and are packaged within the same virion (241). Infectious RNA transcripts from full-length cDNAs of the nodavirus genomic RNAs have been generated (57), and genetic functions have been determined by *in vitro* translation of the messenger-active RNAs and expression of cDNAs in heterologous systems, including baculovirus and vaccinia virus vectors and yeast cultures (5,62,84,217).

RNA 1 carries the information for at least two polypeptides, proteins A and B (see Fig. 9). Protein A (~112 kd) contains known RNA polymerase motifs and is required for nodavirus RNA replication. Most likely, protein A is an RNA-dependent RNA polymerase (RdRp) or a subunit thereof (7,90,109,217,287). En-coded by the 3' end of RNA 1 (see Fig. 9), protein B (~12 kd) is translated from subgenomic RNA 3 that is abundant in infected cells, but not packaged into virions (83,107). Although its exact function is unknown, protein B may play a role in RNA replication (8). RNA 1 encodes all the information necessary for autonomous replication (90). In contrast, RNA 2 encodes only capsid protein a. Thus the functions necessary for genome replication and virus transmission are segregated onto different genetic elements.

Stages of Replication

FHV and closely related BBV multiply prolifically in *Drosophila melanogaster* cell lines (86,91,241), thereby providing a highly tractable system by which to dissect virus replication. The mechanisms of nodavirus attachment and entry are unknown. Virus replication is cytoplasmic. Uncoated genomic RNA 1 is translated to produce the protein A–containing RdRp, which is insensitive to actinomycin D. Newly synthesized viral RNAs are detected by 2 hours after inoculation and continue to accumulate through 20 hours, eventually composing up to 50% of the total cellular RNA (84,85,91). Despite the presence of an equimolar ratio of RNA 1 and 2 throughout infection, synthesis of capsid protein a predominates because of the ability of RNA 2 to outcompete RNA 1 for rate-limiting translation factors (85). Nodavirus RNA synthesis also is regulated. RNA 1 and 2 replication is coupled, ensuring the production of an equal ratio of both genomic RNAs. During infection, RNA 1 must be generated before RNA 2 (6), which subsequently represses synthesis of subgenomic RNA 3 (90). Cis-acting sequences required for (+) and (–) strand RNA transcription and RNA 3 suppression have been identified (reviewed in 8). Virion assembly co-packages an equimolar ratio of RNA 1 and 2 by an unknown mechanism. Owing to the segmented genome, reassortment of genomic RNAs has been detected on mixed infections of cultured cells. Moreover, the nodaviruses can generate defective interfering particles at high multiplicities of infection and can establish persistent infections in cell cultures that are resistant to superinfection (56).

Tetraviridae

Classification of Tetraviruses

The tetraviruses, formerly known as the Nudaurelia b virus (NbV) group, contain a single-stranded, positive-sense RNA genome that is packaged into an nonenveloped, icosahedral virion. The virions (~40 nm diameter) have T=4 symmetry, which forms the basis of the family name (“tetra” from Greek *tettares,* meaning “four”). The tetraviruses are the only known nonenveloped viruses with T=4 symmetry (reviewed in 97,128, and 146). All tetraviruses have been isolated from insects of the order Lepidoptera (moths and butterflies). Nine viruses have been assigned to the family on the basis of serologic relatedness or the presence of a similar-sized capsid protein of more than 62 kd (97). Ten other viruses are likely members. The family is divided into betatetravirus and omegatetravirus genera. The genome of the betatetraviruses consists of a single (monopartite) RNA of ~5.5-kb, whereas the genome of the omegatetraviruses consists of two (bipartite) RNAs of ~2.5 and 5.5 kb. For example, the betatetravirus NbV has a single RNA, whereas the omegatetraviruses Nudaurelia omega virus (NwV) and *Helicoverpa armigera* stunt virus (HaSV) have two RNAs.

Virion Structure

Cryo-EM reconstructions have distinguished the basic structural features of the T=4 symmetry of the tetraviruses (see Fig. 10) (recently reviewed in 146). In addition, the structure of NwV has been resolved in atomic detail (193,194), providing the first structure of a T=4 virus. Tetravirus particles are composed of 240 capsid protein subunits. Twelve chemically identical capsid proteins compose each triangular face of the virion (see Fig. 10). The NwV capsid precursor is cleaved after Asn^{570} near the C terminus in a postassembly mechanism that is analogous to the cleavage of nodavirus capsid precursors required for virus infectivity (see earlier). The T=4 capsid protein arrangement of tetraviruses (~40 nm diameter) is readily distinguished from the T=3 arrangement of the nodaviruses (30 nm diameter), which have 60 fewer capsid proteins per particle (see Fig. 10). Nonetheless, the tertiary structure of capsid proteins of both virus families is strikingly similar. This conservation and the requirement for a postassembly cleavage of capsid proteins suggests that the tetraviruses and nodaviruses evolved from a common ancestor (146).

Tetravirus Genome Structure and Replication

The tetravirus RNA genome is either mono- or bipartite. The genomic RNAs are capped at their 5' end and have a unique tRNA-like structure at their 3' end (1,122). The genomes of several members of the beta and omega genera have been sequenced.

The 6,625-nucleotide NbV genome RNA contains two large ORFs (99). The 5' proximal ORF has the potential to encode a 214-kd protein containing domains characteristic of an RNA-dependent RNA polymerase with methyltransferase, nucleotide binding/helicase, and RNA polymerase motifs. The proteolytic processing steps of this predicted polyprotein are unknown. The 66-kd NbV capsid protein is encoded by the 3' proximal ORF, which is almost entirely overlapped by the replicase ORF, but in a different (+1) reading frame. It is therefore likely that the capsid protein is translated from a 2.5-kb subgenomic RNA that has been detected in preparations of virion RNA (99). *Thosea asigna* virus (TaV), a related betatetravirus, synthesizes a 2.5-kb subgenomic RNA, which also encodes the capsid protein (221).

The omegatetravirus HaSV contains two genomic RNAs of 5,300 and 2,478 nucleotides (98,122). RNA1 possesses a single ORF (1,704 codons) covering 96% of the RNA and likely encodes the RNA replicase. RNA2 encodes the 71-kd

HaSV capsid protein, which is closely related (66% identical) to the capsid protein of NwV (1). During assembly, the capsid precursor is cleaved near its C terminus to generate a 62-kd capsid protein and a smaller 7-kd sister polypeptide. RNA2 of HaSV also contains a 5' proximal ORF, which encodes a 17-kd protein of unknown function (122). It is not known whether both RNAs 1 and 2 of the omegatetraviruses are packaged within the same particle.

Stages of Replication

Tetravirus replication occurs in the cytoplasm of infected cells, and crystalline arrays of virus particles accumulate within cytoplasmic vesicles (128). However, because of the lack of permissive cell cultures, the stages of virus replication are unknown (97). Nonetheless, it is likely that tetravirus replication strategies resemble those of the nodaviruses and alphaviruses.

Tetravirus Transmission, Pathology, and Ecology

The tetraviruses are the only known RNA viruses restricted to insects, specifically those within the order Lepidoptera. Infection of larvae can be initiated by the ingestion of virus-contaminated foods (121). Virus multiplication is most often restricted to the foregut and midgut cells, even on injection of infectious virus into the hemolymph of larvae. Virus-mediated killing of midgut cells may account for the stunting pathology associated with HaSV. In general, susceptible insect larvae exhibit a wide range of pathogenic effects that include rapid death, an extended wasting disease, or a delay in pupation (97). NbV also has been isolated from pupae and adult moths. Larval death occurs within 4 to 7 days and is usually accompanied by discoloration, flaccidity, and sometimes liquefaction that resembles baculovirus-mediated melting. Tetravirus pathogenesis can be influenced by the presence of other viruses, latent or otherwise.

Tetravirus infections occur sporadically in natural populations. Thus ecologic aspects of virus spread and the existence of virus reservoirs are poorly understood. Nonetheless, these insect viruses function as natural biopesticides and have participated in natural control of certain insect pests. *Darna trima* virus and *Thosea asigna* (or *Setothosea asigna*) virus have been successfully used as pest-control agents in oil palm plantations. Although tetraviruses have been only isolated from insects, antibodies cross-reacting with some tetraviruses have been detected in wild and domestic animals and humans. Thus far, development of these viruses as biopesticides has been limited (97).

Cricket Paralysis–Like Viruses

Classification

The cricket paralysis–like viruses are small, nonenveloped riboviruses with icosahedral symmetry. Because their biophysical properties resemble those of the vertebrate picornaviruses, they were originally classified as members of *Picornaviridae* (recently reviewed by 46). However, more recent nucleotide sequence determinations (147,259,283) indicated that these invertebrate viruses are members of a taxonomically distinct family that remains unclassified (see Table 1). The best characterized members include Cricket paralysis virus (CrPV) and serologically related *Drosophila* C virus. Because of its ability to cause paralysis of the hindlimbs of early-instar cricket nymphs, CrPV is appropriately named. Other members of the CrPV-like virus family include *Plantia stili* intestine virus, himetobi P virus, and *Rhopulosiphum* virus (46).

Virion Structure

The physicochemical properties of the virions of the cricket paralysis–like viruses are strikingly similar to those of the vertebrate picornaviruses. These icosahedral riboviruses (<40 nm diameter, r =1.35 to 1.37 g/mL) contain four capsid proteins (VP1, VP2, VP3, and VP4) that are arranged in a pseudo T=3 lattice (259). The 2.4-Å resolution crystal structure of CrPV reveals a capsid conformation and virion morphology that is nearly identical to that of the vertebrate picornaviruses (259). However, unlike the human entero-viruses, CrPV lacks the distinguishing deep depression (canyon) at the fivefold axis or a VP1 pocket. These differences suggest that the mechanism of receptor binding is distinct from that of the picornaviruses. Interestingly, the proteolytic processing of VP0 ® VP3 + VP4 appears to be autocatalytic, which suggests that CrPV capsid assembly and processing more closely resemble those of the insect nodaviruses and tetraviruses than of the vertebrate picornaviruses (259).

Genome Organization and Novel Translation Initiation

CrPV and DCV possess single-stranded, messenger-sense RNA genomes of 9,185 and 9,264 nucleotides, respectively (147,283). The genomes are divided into two major ORFs (Fig. 11), which when translated generate two separate polyproteins that undergo proteolytic processing. Preceded by a 5' UTR, ORF1 encodes proteins with predicted RNA helicase, picornavirus 3C–like protease, and RNA-dependent RNA polymerase activities. ORF2 is preceded by an intergenic UTR (~200 nucleotides) and encodes the virion capsid proteins. Thus unlike the vertebrate picornaviruses, the capsid proteins are encoded within the 3' half of the genome, whereas nonstructural replication proteins are encoded within the 5' half (see Fig. 11).

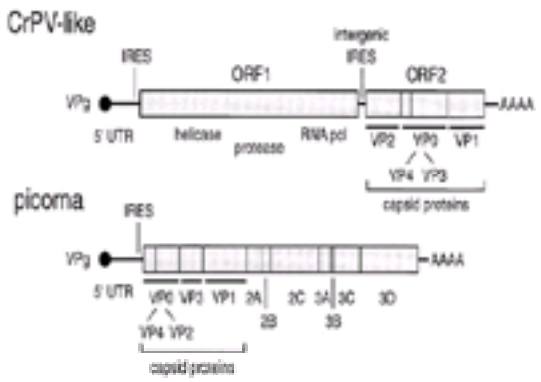

FIG. 11. Genome organization of the Cricket paralysis-like riboviruses. The single-stranded RNA genome of CrPV and DCV possess two open reading frames (ORF1 and ORF2) separated by a ~200 nucleotide spacer. Each ORF is preceded by an internal ribosome entry site (IRES). ORF1 encodes replicative functions (helicase, protease, and RNA-dependent RNA polymerase), whereas ORF2 encodes capsid proteins VP0, VP1, and VP2. VP3 and VP4 are generated by processing of precursor VP0 (259). VPg and an untranslated region (5' UTR) are located at the 5' end of the genomic RNA. The genome organization of the vertebrate picornaviruses differs from the CrPV-like viruses by possessing a single ORF which encodes capsid proteins and replicative functions at the 5' and 3' ends, respectively.

During infection, CrPV and DCV capsid proteins are produced in significant excess compared with the nonstructural proteins (46), a phenomenon that differs from that of the picornaviruses, which produce equimolar amounts of both structural and nonstructural proteins from their single ORF. Accounting for the differential levels of expression, CrPV possesses two internal ribosome entry sites, each located upstream from the nonoverlapping ORFs (283). Thus the CrPV genome represents a rare but naturally occurring bicistronic messenger RNA in eukaryotes. Most interestingly, ORF2 begins with a noncognate CCU triplet rather than AUG, suggesting a novel mechanism for translation initiation (283). Recent evidence indicates that the IRES within the intergenic UTR (see Fig. 11) can assemble an 80S ribosome in the absence of initiator Met-tRNA$_i$ and canonic initiation factors to initiate protein synthesis from the second triplet (GCU), which is positioned within the A site of the ribosome (235,282). This novel mechanism for translation initiation appears to involve the participation of a pseudoknot-like structure within the IRES that can substitute for the usual interactions between Met-tRNA$_i$ and the P-site initiator codon.

CrPV-like Virus Ecology and Transmission

CrPV was originally isolated in 1970 from Australian crickets *Teleogryllus oceanicus* and *T. commodus.* It has one of the widest experimental host ranges of the insect viruses and is capable of infecting invertebrate species of diverse orders, including the Diptera, Lepidoptera, Orthoptera, Hymenoptera, and Hemiptera (46). However, little is known of the distribution of CrPV in nature. Experimental inoculation by injection leads to virus multiplication and death of a number of insect hosts. CrPV transmission also can occur *per os* through contaminated food sources. In contrast to CrPV, DCV has a more restricted host range. DCV has been isolated only from wild or laboratory strains of *Drosophila melanogaster* (46). DCV can be transmitted *per* os to adult flies or larvae, causing death. In addition, DCV is transmitted horizontally between infected and uninfected adults.

This chapter is dedicated to the memory of Lois K. Miller, a friend and mentor, pioneer in baculovirology, and champion for studies of insect viruses and their application.

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CHAPTER 21 Viruses of Yeasts, Fungi, and Parasitic Microorganisms

Reed B. Wickner

Prions, retroviruses, ds (double-stranded) RNA viruses, ss (single-stranded) RNA viruses, and dsDNA viruses have all been found in simple eukaryote hosts, and the experimental advantages of these systems has made some of these the most thoroughly characterized of their viral classes. This is an overview of these infectious entities and their unique aspects, with some emphasis on recent developments.

One feature common to all the viruses, prions, and retrotransposons (or retroviruses) of yeast and fungi is their wholly intracellular life cycles. These viruses have forsaken the extracellular route of transmission, being passed from cell to cell either vertically or by cytoplasmic mixing, such as occurs in mating, or in hyphal anastomosis (fusion) of filamentous fungi. They resemble, in this regard, the plant cryptoviruses and the intracisternal A type particles (retroelements) and LINEs (retroposons) of mammals. It has been argued that the high frequency of mating and hyphal fusion of fungi in nature makes an extracellular phase dispensible for these viruses, and it is difficult to find strains of *S. cerevisiae* that lack either the L-A or L-BC dsRNA viruses or any of the Ty retrotransposons. Nor is the direct cell-to-cell route of infection completely neglected by mammalian viruses, those of the herpes group and human immunodeficiency virus being prime examples.

However, this mode of transmission means that these viruses must balance the need to spread and propagate against consideration for the viability of their host. Furthermore, the fact that most of these elements are widespread means that the hosts have been selected for the ability to protect themselves. This is clearly seen in the Ty elements, which tend to target sites that can tolerate an insertion. The Ty elements, particularly Ty3, regulate their transcription such that most transposition occurs on mating. This design optimizes the chances of infecting the potentially unoccupied genome of the mating partner. The copy numbers of several of the RNA replicons (L-A dsRNA, L-BC dsRNA, and 20S RNA) are repressed by the host Ski proteins acting in translation (185,226,259,285). Likewise, Ty1 retrotransposition is negatively controlled by the *RTT* genes, which appear to control particle and DNA stability and the structure of the chromatin target (54,138,148,167,175,219).

Most of the viruses and prions discussed here can best be viewed as selfish RNA or DNA (or selfish protein), but the killer satellite dsRNAs clearly benefit their hosts by allowing them to kill competitors. The [Het-s] prion of *Podospora* is unique among prions described to date in being necessary for a normal cellular function, namely, heterokaryon incompatibility (57).

It is striking that most of the viruses and retro-elements discussed use some form of ribosomal frame-shifting to make a Gag-Pol fusion protein. This is not surprising for the retroelements, but it was not expected for the dsRNA genomes. Ribosomal frameshifting (in contrast to splicing or RNA editing) provides a mechanism for viruses whose mRNA is their genome to obtain two proteins with overlapping sequence from one mRNA without producing mutant genomes (140). Other translational tricks used include readthrough of stop codons, and internal ribosome entry.

The use of *S. cerevisiae* as a model host for viruses of plants and insects promises to facilitate the understanding of

these viruses (141,218), but it will not be dealt with here.

DOUBLE-STRANDED RNA VIRUSES

The fungal dsRNA viruses were first detected in 1948 as “La France” disease of cultivated mushrooms in Pennsylvania, and its study led to the first description of fungal viruses in 1962. The antiviral agents helenine and statolon, discovered in *Penicillium* in the 1950s, later proved to be fungal virus dsRNA that was inducing interferon secretion (reviewed in ref. 31).

The dsRNA viruses are found in many fungal species (Table 1), and include the single-segment *Totiviridae*, the oligosegmented *Partitiviridae*, the potyvirus-like *Hypoviridae* that limit the virulence for chestnut trees of the chestnut blight fungus *Cryphonectria parasitica*, and reoviruses of the same species.

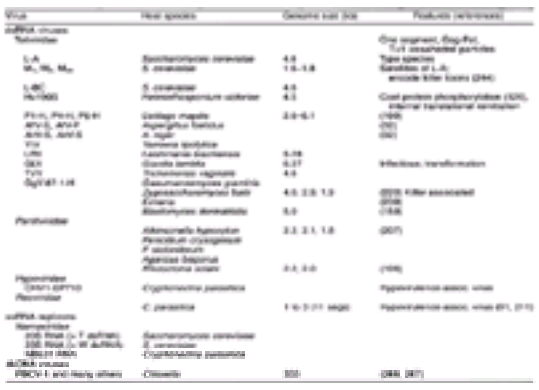


TABLE 1. *The dsRNA, ssRNA, and dsDNA viruses of simple ukaryotes*

L-A Virus of *S. cerevisiae*, Type Species of the *Totiviridae*

Some strains of *S. cerevisiae* secrete a protein toxin that is lethal to other strains, but to which they themselves are immune (Fig. 1) (182). This “killer character” of some strains is inherited as a non-Mendelian genetic element, and its study led to the discovery of the L-A dsRNA virus and its satellites M_1, M_2, M_{28}, and so on, each M encoding a different toxin-immunity specificity.

FIG. 1. The killer phenomenon of *S. cerevisiae*. A sensitive strain of yeast was spread as a lawn, and streaks of a killer strain (*above*) or a nonkiller strain (*below*) were applied. After 2 days' incubation, the lawn of the sensitive strain has not grown in a zone around the killer strain. The secreted protein killer toxin, and immunity to the toxin, are encoded by M_1, a satellite dsRNA of the L-A dsRNA virus.

L-A Virion Structure

P>The icosahedral structures of L-A and the *Ustilago* virus P4 are unusual in that they combine T=1 symmetry with 120 coat protein molecules per particle (Fig. 2) (35,41,98). This same symmetry has been found in the cores of all other dsRNA viruses examined, including reovirus, rotavirus, bluetongue virus, aquareovirus, and bacteriophage p6 (see Chapter 22 and Chapter 52, Chapter 53, Chapter 54, Chapter 55 and Chapter 56). Because sequence data suggest that dsRNA viruses arose independently from ssRNA viruses (161), rather than one being descended from the other, their common structure suggests that there is something about this structure that is well adapted to the intraviral replication pattern of dsRNA viruses.

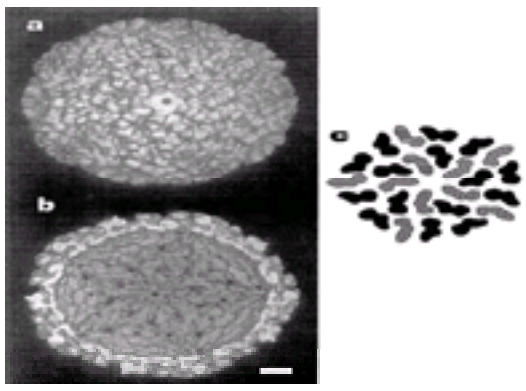


FIG. 2. L-A dsRNA virus capsid reconstruction at 16-Å resolution from cryoelectron microscopy (35). View of the outer (**a**) and inner (**b**) surfaces of the full capsid, viewed along a fivefold axis of symmetry. The internal contents were computationally removed to expose the inner surface (35). Bar = 50 Å. Schematic diagram (**c**) of the arrangement of Gag subunits around the fivefold axis. Note that solid and hatched subunits are identical in primary structure, but occupy distinct, nonequivalent environments (35).

The combination of 120 subunits and T=1 icosahedral symmetry implies that individual coat protein monomers can be in either of two environments. This expectation has been verified as subtly different morphology of the nonequivalent Gag protomers (35) (see Fig. 2). The packing of the dsRNA within the particles is less dense than is typical for dsDNA viruses, possibly reflecting the fact (see later) that in both replication and transcription, the genome must translocate sequentially past the RNA-dependent RNA polymerase immobilized on the particle wall, and this requires more space than is required by the static DNA genomes (35). The L-A virions also have 10- to 15-Å pores, which allow entry of nucleotides and exit of (+) strand transcripts but do not allow the dsRNA genome to exit or degradative enzymes to enter (35).

L-A Genome Structure

The L-A (+) strand has two overlapping open reading frames (ORF) (Fig. 3A) (140). They are *gag*, encoding the 76-kd major coat protein, and *pol*, encoding the 100-kd Pol domain of the Gag-Pol fusion protein (111,140). The pol ORF includes the consensus amino acid sequence patterns typical of viral RNA-dependent RNA polymerases. Three *in vitro* single-stranded RNA binding domains have also been localized to Pol (111,223,224), the central one cryptic unless an adjacent inhibitory region is deleted (see Fig. 3A). Pol residues 67 to 213 are necessary for packaging of the viral (+) strands (109,224). Neither 5' cap structure nor 3' poly(A) has been found on either strand of genomic L-A dsRNA or M_1 dsRNA (30), but there is, at each 3' end, an uncoded base that can be either A or G (24).

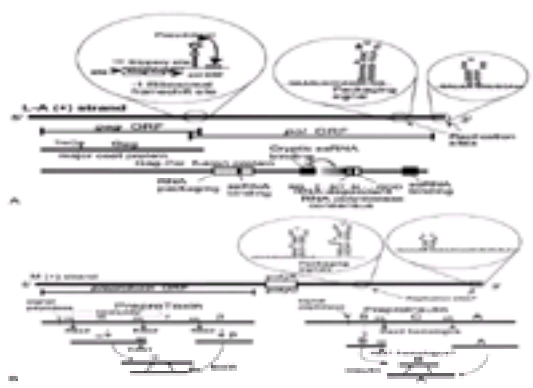


FIG. 3. A: Sites and encoded proteins of the L-A (+) strand. The sites responsible for –1 ribosomal frameshifting, (+) ssRNA packaging, and replication are shown. The pseudoknot makes the ribosome pause over the slippery site, where the tRNAs can unpair from the mRNA and re-pair in the –1 frame with correct base pairing of the nonwobble bases. Functional domains in the Pol region are also indicated. 7mGp indicates the cap-binding site at His154 of Gag. **B:** Coding and cis sites of M1 (+) strand. The analogous and homologous processing of the K1 preprotoxin encoded by M1 is compared to that of preproinsulin.

The M dsRNAs each encode, in their 5' portion, a preprotoxin protein (22,72,178,237) (see Fig. 3B). The M_1 (+) strand has an internal encoded polyA region whose length shows frequent clonal variation, presumably due to transcriptase stuttering (130), and a substantial 3' region that encodes no protein but contains essential *cis* sites (97,107,124,234,242). As will be detailed later, sites on the L-A and M_1 (+) strands necessary for packaging and (–) strand synthesis have been determined (see Fig. 3), and some limits have been set on the possible transcription signals.

Stages in the Replication Cycle

The L-A replication cycle and the closely related cycle of its satellites are shown in Figure 4 (98,108). L-A dsRNA-containing viral particles synthesize (+) ssRNA in a conservative reaction (108,134), and these new (+) strands are then extruded from the particle (98). There, they serve as mRNA for the production of the Gag and the Gag-Pol fusion proteins. These proteins then assemble with a viral (+) strand to form new particles. The newly assembled viral particles carry out the synthesis of (–) strands on the (+) strand template to form dsRNA and complete the cycle (108).

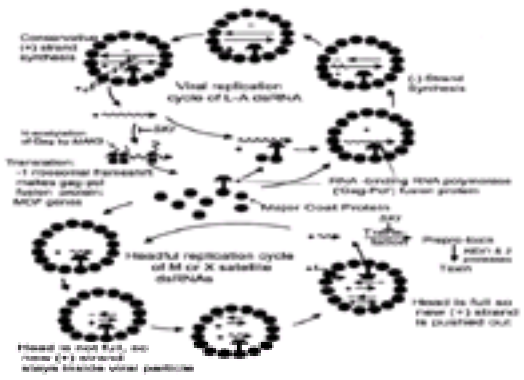


FIG. 4. Replication cycles of L-A virus and its satellite dsRNAs, M and X. The packaging region of the Pol domain of Gag-Pol binds to the packaging site of (+) strands as the Gag domain associates with other Gag molecules. This ensures packaging of viral plus strands (76,111). L-A particles have only one dsRNA molecule per particle. But the smaller M or X dsRNAs replicate within the viral coat until they fill the head (98,99).

In addition to their appropriating viral proteins from L-A, the replication cycle of the satellite dsRNAs, M_1, S (deletion mutants of M_1), and X [a 530- basepair (bp) deletion mutant of L-A] are similar to that of L-A itself except that they replicate more than once within the viral particle until it is full (see Fig. 4). Only a single (+) ssRNA is packaged per particle (107), so L-A and M are separately encapsidated. Because the particle capacity is determined by the structure of Gag to be sufficient to accommodate one full-length L-A dsRNA molecule, and the M and X dsRNAs are much smaller, new M or X (+) strand transcripts are often retained within the viral particles, where they are converted to new dsRNA molecules. Then, all new (+) strands are extruded from the now full particle (98,99). This is called the *headful replication mechanism* (in contrast to the headful packaging mechanism of many DNA bacteriophage) (98,99). This implies that extrusion of the transcripts is a mechanical consequence of the head being full, rather than an active process. Both (+) and (–) strands are synthesized within the viral particles, but at different points in the cycle, so the replication is conservative, intraviral, and asynchronous, and it fills the capsid (headful mechanism).

Transcription Reaction ((+) Strand Synthesis)

As in the *Reoviridae*, the transcription reaction for L-A is conservative (108,286), resulting in the overall process of viral replication being conservative (204). However, in *Aspergillus foetidus* slow virus and *Penicillium stoloniferum* slow virus (32), dsRNA transcription is semiconservative. The difference between conservative and semiconservative reactions concerns whether or not there is re-pairing of the template (–) strand with the parental (+) strand that was (in either case) displaced during the synthesis.

A template-dependent *in vitro* transcription reaction method has been developed for the L-A dsRNA virus (113). When L-A dsRNA-containing particles are treated with very low ionic-strength conditions, they rupture, releasing the dsRNA. These opened empty particles can be reisolated free of RNA and carry out a dsRNA template-dependent reaction that is, like the *in vivo* reaction, conservative (113). This reaction is highly template specific, using only L-A, M, and X dsRNAs (all physiologic templates), but not L-BC, p6, or rotavirus dsRNAs. Because X dsRNA retains only 25 bp of the end of L-A from which the transcription reaction starts (99), the signal recognized by the transcriptase is most likely within this region, perhaps within the terminal 6 bp that are in common among L-A, M_1, and M_2 (129,258). The template-dependent transcription reaction requires very high concentrations of polyethylene glycol (20%), suggesting that the transcriptase has a low affinity for the dsRNA template (113). Because the dsRNA is normally formed within and stays inside the viral particles, the RNA polymerase sees a very high effective concentration and need not have a particularly high affinity.

Translation

The translation apparatus is a prime battleground for the fight between an RNA virus and its host. For example, interferon acts primarily by blocking viral translation. This can also be seen in picornaviruses, where cleavage of a cap-binding component inactivates host translation (see Chapter 23), or influenza virus cap-stealing and antiinterferon measures (see Chapter 46). Studies of the replication mechanisms of the L-A virus and its satellites have likewise led to the suggestion that translation of viral proteins is the critical event determining the balance between virus and host.

Ribosomal Frameshifting. The *gag* and *pol* ORFs of L-A overlap by 130 nucleotides (nt), and the Pol protein is expressed only as a fusion protein with the major coat protein, Gag (see Fig. 3A) (111,140). The mechanism of formation of this Gag-Pol fusion protein is a –1 ribosomal frameshift (74), very similar to those described in Rous sarcoma virus, many mammalian retroviruses, coronaviruses, and several plant viruses (142, and reviewed in refs. 101,125).

There are two features of the mRNA that determine the frequency of –1 ribosomal frameshifts (see Fig. 3A). The “slippery site” is a sequence of the form X XXY YYZ, where the *gag* reading frame is shown. This sequence allows the tRNAs reading XXY and YYZ to each move back one base on the mRNA and still have their nonwobble bases correctly paired (142). X can be any base, but Y can be only A or U, probably because the frequency with which unpairing of the tRNAs from the 0 frame codons (XXY and YYZ) is also important, and because A site-pairing is stronger than P site-pairing (26,74). Z can be any base but G, suggesting that specific tRNAs are more able to frameshift than others (37,133). The second component determining frameshift efficiency is the presence of a strong secondary structure, usually an RNA pseudoknot, just downstream of the slippery site, to slow ribosomal movement at this point (25,142). The location of this secondary structure is particularly critical (27), and a pseudoknot is far more effective in promoting frameshifting than a simple stem-loop of the same overall melting energy. The pseudoknot, more than the simple stem-loop, should resist

melting of the first few bases of the stem, precisely positioning the ribosome with the slippery site in the A and P sites.

The efficiency of –1 frameshifting, and thus the ratio of Gag-Pol fusion protein to Gag protein produced, is critical for viral propagation (76), as is the efficiency of +1 frameshifting for Ty1 retrotransposition frequency (see later). A twofold change away from L-A's normal 1.9% efficiency results in failure to propagate the M_1 satellite dsRNA. L-A propagation is less sensitive, but the antibiotics sparsomycin, which increases ribosomal frameshifting, and anisomycin, which has the opposite effect, can cure L-A (75). The Gag-Pol to Gag ratio is likely to be important for viral assembly. Excess Gag-Pol (high frameshift efficiency) may result in starting too many particles and winding up with too little Gag to complete any of them. In contrast, excess Gag might result in particles closing before the packaging domain of Pol has had a chance to find a viral (+) strand (76).

Host genes affecting the efficiency of –1 frameshifting are called *MOF* (maintenance of frame) (76,77) and are expected to encode ribosomal components or translation factors. The differential effects of specific host mutations on frameshifting at specific slippery sites suggests that drugs similarly affecting frameshifting might target specific viruses (77). *MOF9* has been shown to be the 5S rRNA, showing a role for this 60S subunit component in maintenance of reading frame (78). *MOF4* is *UPF1*, one of the components of the nonsense-mediated decay system (61), and thus Upf1p plays roles in both monitoring reading frame and in the destabilization of mRNAs with premature termination codons. *MOF2* proved to be identical to *SUI1* [encoding a subunit of translation initiation factor 3 (eIF3)], and, like MOF4, it has effects on both nonsense mediated decay and maintenance of reading frame (62).

60S Subunits—Critical to Viral Propagation. Mutations in any of 20 chromosomal genes (called *MAK* for maintenance of killer) that result in diminished levels of free 60S ribosomal subunits also produce loss of M dsRNA and decreased copy number of L-A dsRNA (85,208). Mutations diminishing the supply of free 40S subunits generally had no such effect (208). The deficiency of 60S subunits results in selectively decreased rates of translation of mRNAs which, like L-A mRNA, lack 5' cap and 3' poly(A) (85). These *mak* mutations are suppressed by the *ski* mutations that derepress translation of non-poly(A) mRNAs (261).

Gag Decapping Activity—Necessary for M Expression. The L-A and L-BC Gag proteins each covalently bind the 5' cap from any RNA *in vitro* (17). L-A Gag covalently attaches ^{7m}GMP from the cap to His154 in a reaction that requires only Mg^{2+} (18). Modification of His154 on an L-A cDNA expression clone destroys Gag's ability to covalently bind cap, but it does not prevent the clone's ability to propagate the M_1 satellite virus (18). However, expression of the killer toxin encoded by M_1 is impaired by the His154 mutation. Mutation of the *SKI1*/*XRN1* gene encoding the 5' to 3'exoribonuclease, specific for uncapped RNAs and responsible for the major mRNA decay pathway (137), results in restoration of expression of killer toxin in spite of the mutation of His154 of Gag (185). It has been proposed that the virus decaps cellular mRNAs to decoy the Ski1p exoribonuclease from degrading the capless viral mRNA (185).

SKI *Antiviral System Blocks Translation of Viral [Non-poly(A)] mRNA.* The sole essential function of four of the SKI genes of *S. cerevisiae* is the repression of viral copy number, particularly control of M (14,15,222,226,245,259,285). The SKI proteins repress three unrelated viral systems: L-A and its satellites, L-BC (a dsRNA virus), and the ssRNA replicon called 20S RNA (10,188,259). Detailed studies of the *SKI2* gene suggested that the system acts by limiting translation of viral mRNA (285). Because all of these are cytoplasmic replicons, and none was known to have either 5' caps or 3' polyA structures, it was speculated that the *SKI* system recognized the absence of one or both of these structures typical of eukaryotic cellular mRNAs (285). That *SKI2* also represses translation of RNA polymerase I transcripts (probably lacking cap and/or polyA) supports this idea (285).

Electroporation of mRNAs into spheroplasts shows that the Ski2, Ski3, Ski6, Ski7, and Ski8 proteins inhibit the translation of mRNA specifically if it lacks a 3' polyA structure (13,14,185). Kinetic studies indicate that both the initial rates and the duration of translation are affected by the Ski proteins.

The *SKI2*, *SKI3*, *SKI6*, *SKI7*, and *SKI8* genes have been characterized, and only *SKI6* is essential for growth in the absence of M dsRNA (13,14,189,222,245,285). Ski2p has helicase motifs, a glycine-arginine-rich domain and it is highly homologous to two mammalian genes (205,285). Ski3p has the TPR an amino acid repeat pattern (222) while Ski8p has a different sequence repeat first identified in b-transducin (189). Ski2p, Ski3p, and Ski8p form a cytoplasmic complex (29). Ski6p is homologous to RNAse PH, a tRNA-processing enzyme (14), and it is part of a nuclear complex of exoribonucleases thought to have a role in rRNA processing (196). Indeed, *ski6* mutants produce abnormal 60S ribosomal subunits (13) and show abnormalities in processing of 5.8S rRNA (13). Ski7p has homology to EF1-a, an elongation factor, suggesting that it is involved in the translation process.

Recently, a yeast homolog of *SKI2* (called *YGR271w* or *SLH1*) has been found whose deletion (*slh1*D) depressed L-A copy number as does *ski2*D (184). The *ski2*D *slh1*D double mutants show dramatically in-creased L-A, L-BC and M_1 dsRNA copy numbers, but grow normally in the abscence of the L-A and M_1 viruses (238). Remarkably, the *ski2*D *slh1*D cells show the same *in vivo* translation kinetics of non-poly(A) mRNA as they do for poly(A)+ mRNA, in spite of the presence of normal amounts of competing poly(A)+ mRNA (238). Thus, the translation apparatus is indifferent to the 3' poly(A) structure except for the action of the Ski proteins and Slh1p. This suggests that the role of the eukaryote 3' poly(A), like that of Ski2p and Slh1p, is to allow cells to distinguish their own mRNAs from those of invading viral genomes.

Posttranslational Modification

KEX1, KEX2 *Proteases and Discovery of Prohormone Proteases.* The *kex1* and *kex2* mutants were first isolated because of their inability to produce the “killer” toxin encoded by the M_1 satellite dsRNA (KEX, from killer expression) (282). The *kex2* mutants were noted to have a defect in mating that was specific to cells of the a mating type, and *kex2*/*kex2* homozygous diploids were defective for sporulation (170). The a-specific mating defect was partially explained by the finding that the cells failed to secrete the a pheromone (170), a peptide that prepares cells of the opposite (**a**) mating type

for mating by arresting them in the G1 phase of the cell cycle. The failure to secrete killer toxin and a pheromone was explained by the finding that *KEX2* encodes a protease that cleaves C-terminal to pairs of basic amino acid residues (145), and that *KEX1* encodes a carboxypeptidase that can remove the pair of basic amino acids (55,79). These were the cleavages needed to convert the toxin and a pheromone proproteins to their mature forms (see Fig. 3B).

The specificities of the Kex proteases are the same as those needed to process preproinsulin (see Fig. 3B), preproopiomelanocortin, and other mammalian prohormones, but the enzymes responsible for these maturation cleavages had been elusive. Several genes and enzymes with homology to Kex2p were identified, and evidence that they are involved in these prohormone processing steps is accumulating (reviewed in ref. 246).

There is also evidence that the Kex2p-homologous enzymes are involved in the proteolytic processing of some mammalian viral proteins. Mutant CHO cells resistant to Sindbis virus and Newcastle disease virus were made sensitive by expression of the Kex2p-homolog, mouse furin, or by expression of Kex2p itself (197).

N-*Acetylation of* gag *by Mak3p—Necessary for Assembly. MAK3* is one of the three chromosomal genes known to be necessary for the propagation of the L-A dsRNA virus. Its sequence shows homology with *N*-acetyltransferases, particularly with the rimI protein of *Escherichia coli*, which acetylates the N-terminal of ribosomal protein S18 (256). In fact, the L-A Gag protein is normally blocked (presumably acetylated) and is unblocked in a *mak3* mutant host, resulting in failure of viral assembly (257). Mak3p recognizes the N-terminal four amino acid residues of Gag (255), and this signal can be transferred to b-galactosidase, producing *MAK3*-dependent N-terminal acetylation. Like L-A, the major coat proteins of Rous sarcoma virus, tobacco mosaic virus, turnip yellow mosaic virus, alfalfa mosaic virus, and potato X virus are N-terminally acetylated, but the enzymes responsible have not been identified, nor can the role of acetylation for these viruses be examined without altering the primary protein sequence.

Recently, a complex of Mak3p, Mak10p, and Mak31p has been detected (227). Mak10p, Mak31p, and Mak3p are the only three proteins known to be necessary for L-A propagation (16,169,260), and this indicates that Mak10p and Mak31p are also involved in *N*-acetylation of the coat protein. Both *mak3* and *mak10* mutants show slowed growth on glycerol or ethanol, further supporting the idea that their functions are related (73,169,255).

Viral Assembly

The headful replication mechanism implies that the coat protein determines the structure of the head, not the genome. This is typical of isometric viruses, and it has now been confirmed by the finding that expression of the Gag protein alone produces empty particles that are morphologically indistinguishable from normal L-A virions (109). Normal L-A particles have a T=1 icosahedral structure with an asymmetric unit consisting of a dimer of Gag (35; see preceding). Each particle has only one or two Gag-Pol fusion proteins (perhaps as a dimer). The requirement for the Mak3p-catalyzed *N*-acetylation of Gag for assembly suggests that the Mak3/10/31 complex is involved in this process (226).

The existence of a Gag-Pol fusion protein, the ssRNA binding activity of its Pol domain, and the fact that (+) ssRNA is the species encapsidated to form new viral particles, led to a model of assembly and packaging that has been supported by subsequent findings (Fig. 4) (111). The Pol domain of the Gag-Pol fusion protein recognizes and binds to a packaging site on the viral (+) strands. Then (or concomitantly), the Gag domain of the fusion protein associates with the free Gag protein. This leads to encapsidation of a single (+) strand per particle if there is only one Gag-Pol fusion protein (or one dimer) per particle.

In vivo, Pol residues 67 to 213 of the Gag-Pol fusion protein are necessary for packaging of (+) strands, but not for assembly of morphologically normal viral particles (109,224). One of the three single-stranded RNA binding domains of Pol is located within this region and is necessary for the packaging (224) (see Fig. 3A).

The *packaging site* recognized is an internal stem-loop sequence with an A residue bulging from the 5' side of the stem (see Fig. 3A) (107). This site, located about 400 nt from the 3' end of the L-A (+) strand, was first identified as necessary for binding of X (+) ssRNA to the opened empty particles (97). Binding requires the stem structure but not the sequence of the stem. In contrast, the sequence of the loop is important. The protruding A residue must be present and must be an A (107). A similar site, similarly located on the M_1 (+) strand, was found (107) by examining the predicted folded structure of the M_1 sequence (124), and another was found by studying sequences involved in exclusion of M_1 (242). Either the L-A (X) or M_1 stem-loops are sufficient for binding, but the addition of 10 bp from the 5' side of either one improves the binding substantially (107,242). Both the L-A and M_1 sites contain direct repeat sequences whose significance has not yet been determined. A site with the same structure has also been found in a similar location in the M_{28} satellite dsRNA (236).

The L-A and M_1 sites can each serve as a portable packaging signal *in vivo*, directing packaging of heterologous transcripts by L-A virus or by proteins produced from the L-A cDNA clone (107,109). The heterologous transcripts were packaged alone in viral particles, confirming the prediction of the headful replication model that a single (+) strand is initially packaged per particle.

The ratio of Gag-Pol fusion protein to Gag can be altered *in vivo* by adjusting the efficiency of ribosomal frameshifting and, as discussed, this ratio is important for M_1 satellite dsRNA propagation but less so for L-A itself (75,77). Whereas M_1 or X particle assembly can fail in a Gag-poor cell, an L-A (+) strand could be its own supply of Gag that will not be exhausted until completion of the particle makes the L-A (+) strand no longer available to the ribosomes. This form of *cis*-packaging could explain the differential effect of frameshift efficiency on M_1 and L-A propagation.

Replication [(–) Strand Synthesis]

Newly assembled viral particles contain an L-A (+) strand and are capable of converting it to the dsRNA form when

supplied with nucleoside triphosphates (108). The particles formed in this reaction have all the properties of mature L-A particles and can carry out the transcription reaction (110).

To study the detailed mechanism of the replication reaction, it was necessary to establish a template-dependent *in vitro* reaction. The opened empty particles, when supplied with viral (+) strands, Mg^{2+}, nucleoside triphosphates, and a low concentration of polyethylene glycol, carry out (–) strand synthesis to form dsRNA (112). Only L-A, M, or X (+) strand templates are active in this reaction, supporting the notion that it accurately reflects the *in vivo* reaction.

A maximally active template requires both sequences at the 3' end of the L-A (or X) plus strand and internal sequences (the internal replication enhancer) overlapping the packaging signal (97). The 3' end of L-A (+) strands has a stem-loop whose structure is necessary for template activity. While the sequence of the loop and that of the 3' terminal four nucleotides are important, that of the stem is not. In spite of the requirements for these structures and sequences in the context of L-A, the 3' terminal 33 nucleotides of M_1's (+) strand can substitute for the L-A 3' end, although there is little or no similarity between the two sequences (see Fig. 3) (97). The internal replication enhancer and the 3' end site must be bound together for optimal template activity in the replication reaction (114). However, they need not be covalently attached and can simply be hydrogen bonded. This suggests that the RNA polymerase binds first to the internal site and is thus brought close to the 3' end site where polymerization is to begin.

Because the RNA-dependent RNA polymerase consensus domains defined by Kamer and Argos (147) have since been found in essentially all (+) ssRNA and dsRNA viruses examined, the detailed mutagenesis of the most highly conserved of these regions was carried out using the L-A cDNA clone (225). This has defined the extent of the domains necessary for the propagation of the M_1 satellite dsRNA. Interestingly, homologous regions from Reovirus or Sindbis virus RNA polymerases could partially substitute for that of L-A (225).

Other Factors Affecting L-A, M, and L-BC Replication. Mutation of either *POR1*, encoding the major mitochondrial outer membrane porin, or *NUC1*, encoding the major mitochondrial nuclease, results in derepression of L-A copy number (73,177). A similar effect results from mutation of *scs1*/*lbc2* (122), encoding a subunit of serine palmitoyltransferase. *MKT1* is necessary for M_2 propagation, but not for M_1 (278,279). Several natural variants of L-A have been described based on their interactions' with *MKT1*, M_2 dsRNA, and *mak* mutations affecting levels of free 60S ribosomal subunits, but the mechanisms of these interactions have not yet been determined (reviewed in ref. 280).

Totiviridae *Using Internal Initiation to Make RNA Pol.* The 190S virus of *Helminthosporium victoriae* (Hv190SV) is a totivirus that does not make a coat protein–RNA polymerase fusion protein and does not use ribosomal frameshifting in its gene expression. Instead, the termination codon for the coat protein overlaps with the initiation codon for the RNA polymerase, the sequence being . . .GGA CA**A TG A**GT G. . . (139). The RNA polymerase is detected only as a separate protein, and it is suggested to be translated by the occasional reinitiation of ribosomes that have just terminated at the end of the coat protein part of the mRNA.

Two viruses of the filamentous fungus *Sphaeropsis sapinea*, a pathogen of pine trees, are closely related to Hv190SV in sequence, and they apparently use the same translation strategy to express their RNA-dependent RNA polymerase (217).

Leishmania dsRNA Viruses

Leishmania is a flagellated protozoan that causes cutaneous, visceral, or mucosal infections of man. *Leishmania* virus (LRV1-1) was first discovered as an RNA species associated with cytoplasmic, 32 nm virus particles (252). A similar virus (LRV1-4) was found in another strain and shown to be associated with an RNA polymerase activity (283). A survey of 71 *Leishmania* isolates showed related viruses present in 12, all of which were *L. braziliensis* or *L. guyanensis* originating in the Amazon basin (128). These species cause cutaneous and mucocutaneous forms of leishmaniasis, but not all such isolates carry the virus, nor is there evidence that the virus affects pathogenicity.

The replication cycle of LRV appears to be quite similar to that of the L-A virus of *S. cerevisiae* (276). The structure of LRV likewise closely resembles L-A (248). The two major ORFs, ORF2 and ORF3, overlap by 71 nt, with ORF3 in the +1 frame relative to ORF2 (Fig. 5) (248). ORF2 encodes the major coat protein (33), and ORF3 has the consensus patterns typical of viral RNA-dependent RNA polymerases. This suggests that a Gag-Pol fusion protein might be made (248), and an *in vitro* translation product of the predicted size (33) is a candidate for this protein. The sequence of the overlap region suggests a possible Ty1-like +1 shift site or a pseudoknot-promoted hop (248). The similarity of the LRV1 *pol* amino acid sequence with that of L-A of *S. cerevisiae* is striking, and it is far beyond that simply because they share the RNA polymerase consensus domains (248).

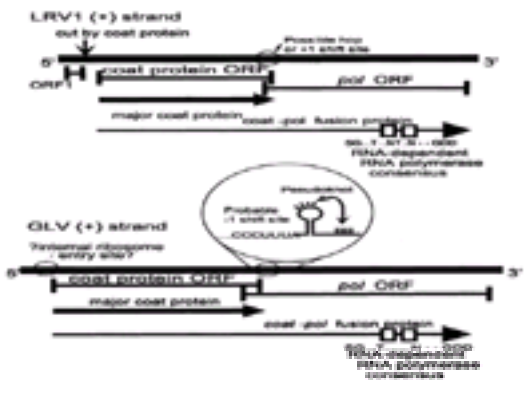


FIG. 5. Genome structure and expression of *Leishmania* virus, LRV1 (248), and the *Giardia* virus, GLV (273). There are several similarities of these viruses to each other and to the *Saccharomyces* virus, L-A, which has led to their

classification together as totiviruses.

A small ORF of 228 nt (ORF1) is present upstream of ORF2 in LRV1-1 (248), and two such small upstream ORFs are found in the closely related LRV1-4 (235). The absence of the spliced leader sequence common to all Leishmania cellular mRNAs suggests that this upstream region may be untranslated.

The capsid protein of LRV has an endonuclease activity that cuts viral (+) strands at GAUC*CG, 320 nt from the 5' end in LRV1-4 (180). The role of this unique activity is not yet clear.

Giardia lamblia Virus

The Giardiavirus (GLV) is an infectious totivirus, with a single-segment, 6.1 kb double-stranded RNA genome (271). Its host, *Giardia lamblia*, is a primitive eukaryote with two nuclei but no mitochondria, Golgi, or endoplasmic reticulum. This protozoan is a flagellated intestinal parasite of mammals with a wide distribution.

Unlike other known viruses of fungi or parasites discussed here, GLV is found in the culture supernatant and readily infects *G. lamblia* cells. Infected cells contain 36 nm isometric particles composed of a major 100-kd protein and a minor 190-kd protein (194). Purified viral (+) ssRNA can be reintroduced into a virus-free strain by electroporation, and a new infection can be initiated in this way (116). Inserting luciferase within an *in vitro* transcript of a cDNA clone of GLV, replacing most of the Gag ORF, and electroporating the hybrid RNA into GLV-infected *Giardia* trophozoites, resulted in stable expression of luciferase in the recipient cells and the production of viral particles able to transmit luciferase expression to newly infected cells (67).

The structure of the GLV genome (see Fig. 5) (271) has several features in common with other *Totiviridae* and with certain animal viruses. There are two major ORFs: The 5' ORF encodes the 100-kd major coat protein. The 3' ORF has the typical RNA-dependent RNA polymerase motifs, and it is expressed only as a fusion protein with the 5' ORF (271). Thus, expression in GLV resembles that of retroviruses, the *Saccharomyces* L-A virus, and the *Leishmania* dsRNA virus LRV-1 in forming a gag-pol fusion protein. The structure of the 210 nt overlap region of the *gag* and *pol* ORFs strongly suggests that the mechanism of fusion protein formation is by the same –1 ribosomal frameshift as that used by many animal retroviruses and the yeast L-A dsRNA virus. A slippery site C CCU UUA (*gag* frame shown) is followed closely by a predicted pseudoknot, as has been shown to be essential in these systems and in coronaviruses for programmed frameshifting.

The 5' noncoding region of the GLV genome has seven AUG codons, each followed by a very short reading frame (271). This feature appears to be common among the *Cryphonectria* hypoviruses, LRV1, and GLV. The 5' and 3' UTRs are necessary for replication of the luciferase hybrid viruses, but the 5' 265 nt of the Gag coding region further promotes expression of luciferase in these hybrids without affecting transcript levels, indicating a translational control mechanism (68). The results are consistent with an internal ribosome entry site bypassing the small upstream ORFs.

Trichomonas Virus and Host Phenotypic Variation

Many strains of *Trichomonas vaginalis* carry 33 nm viral particles composed of an 85-kd major coat protein and containing 5.5 kb dsRNA (272). This 5.5 kb dsRNA in fact consists of three distinct dsRNA species that do not cross-hybridize (152). Sequence analyses of two of the species indicate that each has a typical totivirus structure with overlapping 5' Gag and 3' Pol ORFs (250). Like the LRV1 totivirus, the Pol ORF is in the +1 frame relative to Gag (250).

Remarkably, the presence of the virus is correlated with a phenotypic variation of the host cells (270). Expression of a major surface antigen switches on and off in cells carrying the virus, but its transcription is always off in virus-free strains (153). Cells often lose the virus on serial passage, and loss of the virus is always accompanied by loss of phenotypic variation (270).

Partitiviridae

The *Partitiviridae* have a bipartite genome with the two segments separately encapsidated in particles containing the proteins encoded by both segments. This group includes viruses of the filamentous fungi *Penicillium*, and *Aspergillus*, and the mushroom *Agaricus*, as well as many other fungi. It also includes a large group of plant cryptoviruses (20,202), whose genome is dsRNA and whose biology is much like that of the systems discussed in detail here.

Atkinsonella hypoxylon *Partitivirus 2H.* This isolate of *Atkinsonella hypoxylon* strain 2H has three dsRNA segments of 2180, 2135, and 1790 nt. Segment 1 encodes an RNA-dependent RNA polymerase, and segment 2 encodes a 74-kd protein that may be the major coat protein (207). The third segment does not appear to encode a protein (207).

VIRUSES REDUCING VIRULENCE OF *Cryphonectria parasitica*

The American chestnut tree was devastated by the accidental introduction, in 1905, of the pathogenic fungus *Cryphonectria parasitica*, along with an oriental variety of the chestnut tree (reviewed in ref. 206). The fungus virtually eliminated the upper parts of the chestnut trees in the eastern United States, but the root systems are not killed and shoots continue to emerge, only to have their growth limited by the reattack of the fungus. *C. parasitica* had a similar effect in Europe, but in the 1950s, the emergence of fungal strains in Italy with markedly reduced virulence to the trees was noted. These “hypovirulent” strains (Fig. 6), later also found in Michigan, could transmit their hypovirulence by hyphal anastomosis to virulent strains, and this was used successfully for the biologic control of chestnut blight in

Europe. Hypovirulent strains also show decreased asexual spore formation, decreased production of laccase (a phenol oxidase possibly involved in pathogenesis), and reduction in pigment formation (reviewed in 206).

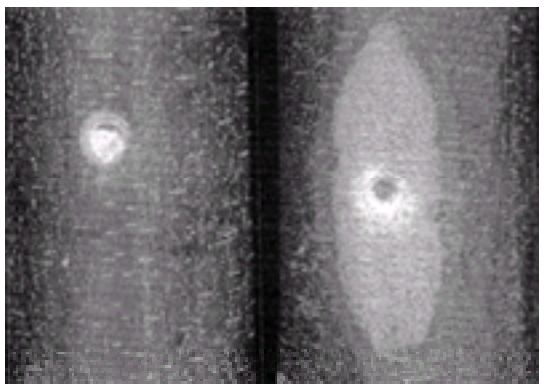


FIG. 6. Virulent (uninfected, *right*) and hypovirulent (virus-infected, *left*) *Cryphonectria parasitica* (chestnut blight fungus) inoculated into chestnut trees. Infection of the fungus by any of several hypoviruses attenuates its virulence toward the trees. (Photo courtesy of Dr. Donald Nuss.)

The cytoplasmically inherited factor that reduces the pathogenicity of the fungus and produces these hypovirulence-associated traits was identified as any of several apparently unrelated dsRNA replicons found in the hypovirulent strains and not in the virulent strains (6,66,135). Curing the dsRNAs by growth in the presence of cycloheximide was accompanied by the return of virulence (115).

Genome Structure

The most thoroughly studied hypovirulence-producing virus is CHV1-EP713, the L-dsRNA of hypovirulent *C. parasitica* strain EP713, a 12,712-bp molecule whose (+) strand has two long ORFs, ORFA and ORFB (Fig. 7) (240). Each ORF encodes a papain-like cysteine protease in its N-terminal portion that self-cleaves the primary translation products at least once, as shown in Figure 7 (49,241). The presence of related papain-like proteases and helicase domains, as well as RNA-dependent RNA polymerase motifs, has led to the suggestion that L-dsRNA of *C. parasitica* is related to the potyviruses, a group of (+) ssRNA viruses of plants (162).

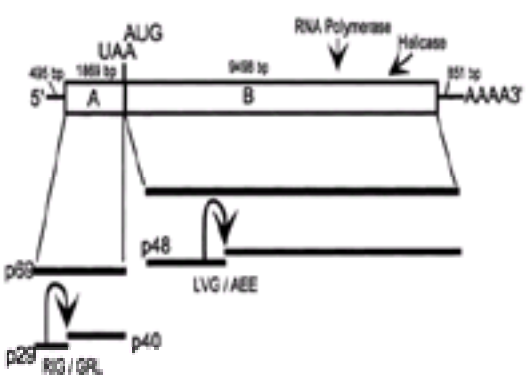


FIG. 7. Coding information and protein processing of the *Cryphonectria parasitica* virus, L. (Adapted from ref. 206, with permission.) The sites of action of the p29 and p48 proteases are indicated. This virus is now designated CHV1-EP713, the type member of the *Hypoviridae*.

The ORFA and ORFB overlap by a single nucleotide, with the UAA termination codon of the first overlapping with the AUG codon of the second (240). The mechanism by which ORFB is expressed has not yet been defined, but this structure implies that the mechanism will not involve ribosomal frameshifting or termination codon read-through. Rather, reinitiation is the most likely mechanism. The translation of ORFA also poses an interesting problem, as the 5' noncoding region contains six short ORFs (240). Whether ribosomes initiate internally as in the case of picornaviruses (see Chapter 23) or use these tiny ORFs for regulatory purposes, as in the GCN4 gene of *S. cerevisiae* (136), is not yet clear.

Virus Replication in Intracellular Vesicles

Unlike other mycoviruses, most of the *C. parasitica* dsRNAs described to date are not associated with virus particles. Rather, they are found in intracellular vesicles (80,131). These vesicles have an RNA polymerase activity that produces both ssRNA and dsRNA (100). Most of the label is incorporated into viral (+) strands (100). This is reminiscent of the membrane association of *in vivo* RNA synthesis of many (+) strand RNA viruses, supporting the notion that this virus is not closely related to other fungal viruses.

Virus Induction of Hypovirulence

CHV1-EP713 has very specific effects on certain genes. For example, the laccase gene encodes a phenol oxidase believed to be involved in pathogenesis. Transcription of the laccase gene is repressed by CHV1-EP713 infection (46,228), an effect that can be produced by expression of just the p29 protease encoded by part of ORFA (48). Although

p29 is sufficient to alter fungal phenotypes, it is neither necessary nor sufficient to cause hypovirulence (60). Deletion of p29 from the virus results in decreased induction of the hypovirulence-associated traits, but no decrease in hypovirulence itself. Moreover, it is not the protease activity of p29 that produces hypo-virulence (60). The symptom-determining domain of p29 has now been localized to the interval Phe25 to Gln73 of p29 (25). The effect of CHV1-EP713 on laccase appears to be transmitted by an influence on the inositol triphosphate–calcium signal transduction system of the fungus, possibly explaining the multiple phenotypic effects of hypovirulence (165).

Cryparin, a hydrophobic cell-surface protein, is also reduced in hypovirulent strains, an effect mediated at transcription (295). Another gene, *Vir2*, was isolated based on its decreased transcription in a hypovirulent strain. Although no effect was seen on virulence itself, deletion of this gene partially mimics some of the hypvirulent-associated traits, with decreased asexual sporulation and fruiting body formation and impaired sexual crossing ability (294). The hypovirulence-associated traits are thus caused by specific effects of viral gene products, rather than by the presence of a replicating dsRNA.

Recently, G protein signaling has been implicated in the induction of hypovirulence by CHV1-EP713. Hypo-virus infection leads to decreased expression of the host Gia (a inhibitory subunit of G protein) and a concomitant increase in cAMP levels (40,45). Moreover, disruption of *cpg-1*, the gene for Gia, results (in an uninfected strain) in elevated cAMP levels, total loss of virulence, and the development of the same associated phenotypes seen on virus-induced hypovirulence (118).

Infectious cDNA Clones and Biologic Control of Chestnut Blight

Introduction of complete cDNA clones of L-dsRNA under control of the *C. parasitica* glyceraldehyde-3-phosphate dehydrogenase promoter resulted in both a complete hypovirulence phenotype of the fungus and the launching of the RNA replicon in a form transmissible to other strains (47). In addition to its usefulness in studying the mechanism of the effects of CHV1-EP713 dsRNA on the cell, this infectious cDNA clone method is an important advance in attempts to control chestnut blight. Although artificial inoculation of trees with hypovirulent fungal strains in Europe resulted in spread of the hypovirulence dsRNAs to virulent strains and control of the blight, this approach has not succeeded in the United States. CHV RNA spreads by fusion of the growing fungal cells with other cells, a process called hyphal anastomosis, but not by sexual crosses (4). Hyphal anastomosis requires that strains have identical alleles at several different loci determining compatibility. The number of compatibility groups is apparently much greater in the United States, limiting the spread of the hypovirulence dsRNA.

In contrast, infectious cDNA incorporated into the *C. parasitica* genome, while generating dsRNA replicons, will also naturally spread to other mating and vegetative compatibility groups through sexual transmission (47,205). It is thus expected to be more effective in the biologic control of chestnut blight than the natural virus, and early results support this expectation (5).

A Reovirus of *Cryphonectria*

Two strains of *C. parasitica* have been found carrying reovirus-like elements (91,211), one of which (C18) is associated with hypovirulence (91). Strains C18 and 9B-2-1 each have 11 dsRNA segments ranging in size from about 1 to about 3 kb and associated with 60 nm virus particles, unlike the vesicles in which the potyvirus-like *Hypoviridae* are found. These 11 segments are present in equimolar amounts and transmission studies show that either all or none of the segments are transmitted, suggesting that they are parts of a single viral genome. Blotting experiments suggest that the C18 and 9B-2-1 genomes are not closely related, but both apparently cause hypovirulence. This system adds new dimensions to the study of both hypovirulence and the *Reoviridae*.

SINGLE-STRANDED RNA REPLICONS

20S RNA

In 1971, a stable species of RNA, intermediate in size between 18S and 25S rRNAs, dubbed 20S RNA, was found to appear specifically in cells exposed to the condition used to induce meiosis and sporulation—namely, when acetate is supplied as the carbon source in the absence of a nitrogen source (146). The ability to produce 20S RNA was then found to be inherited as a non-Mendelian genetic element, distinct from other known elements (123). It was not connected with meiosis, except that the same culture conditions are used to induce both (123). 20S RNA was finally proven to be an independent RNA replicon whose copy number is inducible in acetate (188). Its sequence shows that its 2,500 nucleotides encode a single 95-kd protein with some similarities to the RNA-dependent RNA polymerases of RNA phages and RNA viruses (190,230). W dsRNA, a minor species inducible by growth at high temperature (277), proved to be the replicative form of 20S RNA (190,230). Electron micrographs of purified 20S RNA showed about 50% circular molecules (188), but biochemical experiments indicate that the RNA itself is not circular (231). Recently, the terminal sequences of 20S RNA were shown to be 5'GGGGC . . . . . GCCCC-OH3' (232), suggesting that a circular structure may have been formed by hydrogen bonding.

20S RNA is an apparently naked cytoplasmic replicon with no coat protein (284). The 91-kd RNA polymerase is found bound specifically to the 20S RNA genome (119), and these complexes have RNA polymerase activity—that is, synthesizing 20S RNA and its complementary strand (120). Replication intermediates of 20S RNA include W dsRNA, which is a unit-length molecule, and larger, apparently double-length species (190). Single-stranded molecules of approximately dimer length have also been detected on denaturing gels (190).

23S RNA

T dsRNA was discovered as a minor species of dsRNA easily detected in cells lacking L-A and L-BC, and shown to be, like W dsRNA, an independent replicon inducible by growth of cells at high temperature (277). T is the replicative form of

23S ssRNA (96). 23S RNA has substantial homology with 20S RNA, and likewise appears to encode an RNA-dependent RNA polymerase (96), with which it is associated in extracts (95). All strains known to have T dsRNA also have W dsRNA, but many strains with W lack T (277). Whether T depends on W is not clear.

Control of 20S and 23S RNA Replication

The copy numbers for 20S RNA and 23S RNA are controlled by media conditions, requiring acetate as the carbon source and the absence of a nitrogen source for their 10,000-fold induction (96,146,188,277). Both are also induced by growing cells at 37°, and at least 20S is repressed by the *SKI* system (188,277). The *SKI* effect apparently reflects the absence of 3' poly(A) on 20S mRNA (see preceding).

The *Cryphonectria parasitica* Mitochondrial Replicon NB631 dsRNA

Polashock and Hillman (216) have described a mitochondrial dsRNA species in *C. parasitica* strain NB631. If one assumes the mitochondrial genetic code (UGA = Trp), then the NB631 dsRNA has a single long ORF that encodes an RNA-dependent RNA polymerase. This RNA polymerase is most closely related to those of 20S and 23S RNAs, and all are more closely related to RNA phage such as Qb, than to RNA viruses of higher organisms (215,229). The degree of similarity is such that these elements must be very close relatives. Nevertheless, 20S RNA and 23S RNA are primarily ssRNA replicons with small amounts of the dsRNA replicative form found in cells, whereas NB631 has been identified as a dsRNA element.

NB631 dsRNA is transferred by hyphal anastomosis, like other *C. parasitica* RNA replicons, but unlike those, and other viruses of filamentous fungi, it is also efficiently transmitted by meiotic spore formation if the female parent had the virus (215).

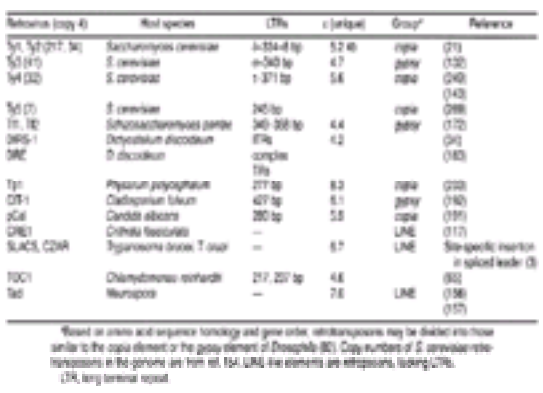
A dsRNA in *Rhizoctonia solani* Also Found as Chromosomal DNA

The M_2 dsRNA of the plant pathogen *Rhizoctonia solani* is a 3,750-bp molecule with a single ORF, and with a high similarity to the NB631 dsRNA. Some of the RNA-dependent RNA polymerase motifs are found, and the results indicate that this protein is an RNA-dependent RNA polymerase (RDRP) (164). Remarkably, the same sequences are present in the DNA genome of the fungus, and the original hypovirulent isolate carrying M_2 derives from a virulent strain having the DNA copy but lacking the free dsRNA (164).

RETROVIRUSES (RETROELEMENTS)

Retroviruses, Retrotransposons, Retroposons, and Retrointrons

Retroelements (Table 2) all share their use of reverse transcriptase in their propagation. The retrotransposons (Table 3) of fungi and parasitic microorganisms resemble mammalian retroviruses in all essentials except for their lacking an *env* (envelope) gene and, in part for this reason, are restricted to propagation without leaving the intracellular environment (Fig. 8). Nonetheless, the frequency with which these cells mate in nature is so high that most of these elements are widely distributed in their respective species. The retroposons are one step further removed in that they lack the long terminal repeat (LTR) structure, and their transposition process differs from those of the other groups. These elements resemble the mammalian LINE elements, but they have the advantage of readily detectable transposition and facile host genetics for their study. For example, Tad, of *Neurospora*, can retrotranspose between nuclei of a heterokaryon (156,157).



Element	*env*	LTRs	RT	Examples
Retrovirus	+	+	+	RSV, HIV
Retrotransposon	–	+	+	Ty1–5
Retroposon	–	–	+	LINEs, Tad
Retrointron	–	–	+	intron al1 of *COX1*

LTR, long terminal repeat; RT, reverse transcriptase; RSV, respiratory syncytial virus; HIV, human immunodeficiency virus.

TABLE 2. *Retrotransposons of simple eukaryotes*

TABLE 3. *Groups of retroelements*

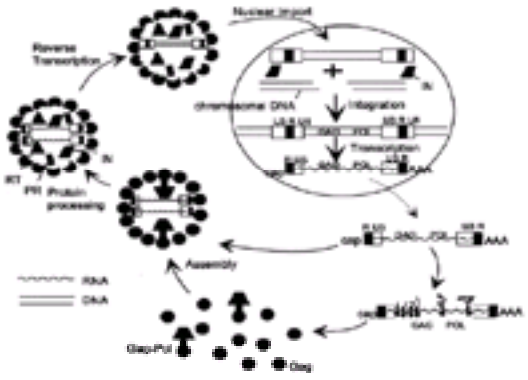


FIG. 8. The Ty replication cycle. It is likely that all retrotransposons follow this cycle.

A group of reverse-transcriptase-encoding introns in mitochondrial DNA of *S. cerevisiae* has also been identified (151). These are introns aI1 and aI2 of the COX1 (cytochrome oxidase subunit I) gene. These introns are capable of retrotransposition both into their normal location and into heterologous locations, both *in vitro* (151) and *in vivo* (199). These retroelements lack LTRs and have only a single ORF. Their properties suggest that introns may have begun as parasitic elements.

The retrotransposons are also divided based on amino acid sequence homologies and gene order in the *pol* domain into the *copia*-like and the *gypsy*-like elements. *Copia* and *gypsy* are retroelements of *Drosophila*. Because Ty3 is more similar to gypsy than to Ty1 (132,290), it is likely that Ty3 and Ty1 entered the yeast genomes at different times, and thus that horizontal transfer of these elements does occur.

Structure of Tys and Other Retroelements

The Ty elements of *S. cerevisiae* each have LTRs of 245 to 371 bp, separated by a unique region (called e) of 4.7 to 5.6 kb (Fig. 9 and Fig. 10) (21,51,132). The major Ty RNA transcript begins within the 5' LTR (at base 241 from the 5' end in Ty1) and ends at base 289 of the 3' LTR, 45 bp from the 3' end of the element (see Fig. 10) (90). This provides the basis for the conventional division of retroviral LTRs into the U3 region (present only at the 3' end of the Ty RNA, but located at the 5' end of the LTR), the R region (repeated at both ends of Ty RNA), and the U5 region (present only at the 5' end of Ty RNA, but at the 3' end of the LTR).

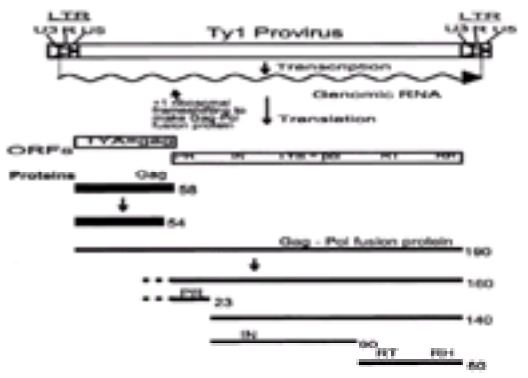


FIG. 9. Genome structure and expression of Ty1. Gag and Gag-Pol are the primary translation products, which are processed by cleavage with the viral protease to form the proteins shown. (Adapted from ref. 121, with permission.)

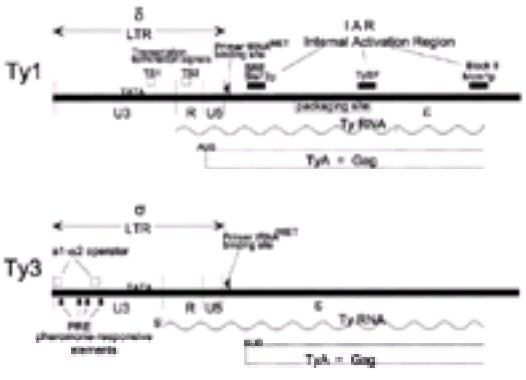


FIG. 10. Structure and functional regions of the Ty1 and Ty3 long terminal repeats (LTRs). Although the LTRs of Ty1 and Ty2 are similar, the control sequences within e, the unique regions, are different. SRE, Site-responsive element; TyBF, Ty binding factor site; Block II, the Mcm1p binding site. Ty38s control sequences are apparently all within the LTR, its primer binding site is two bases from the LTR, and its Gag protein initiation codon is within the unique region, not in the

LTRs.

Ty1 through Ty4 all have two overlapping ORFs, TYA, corresponding to *gag*, and TYB, homologous to the *pol* of mammalian retroviruses. Like mammalian retroviruses and *gypsy*, Ty3 has the gene order protease (PR)–reverse transcriptase (RT)–RNase H (RH)–integrase (IN) in the *pol* ORF (132), whereas Ty1 and Ty2 have the *copia* order PR-IN-RT-RH (51,275).

Replication Cycle of *S. cerevisiae* Ty Elements

The Ty replication cycle resembles that of mammalian retroviruses except that it begins and ends with the integrated form of the genome (see Fig. 8). Ty transcripts made with RNA polymerase II are translated to make the Gag (TYA) and Gag-Pol fusion (TYA-TYB) proteins. These proteins assemble, packaging the Ty RNA to make particles that are homologs of the core particles of retroviruses. The reverse transcriptase and RNase H make a dsDNA copy of the genome, and integrase inserts this into the genome, producing a short repeat of the chromosomal integration site.

Reverse Transcription

The reverse transcriptase and RNase H–homologous domains are present in the Ty1 particles as a 60-kd protein that is produced by proteolytic processing of the 190-kd Gag-Pol fusion protein (see Fig. 9) (121). Isolated Ty1 particles have reverse transcriptase that is active on either the endogenous Ty1 RNA or externally added templates. The Ty particles are also open to externally added enzymes, indicating that they are not impermeable shells but have pores. This porous structure may help explain how pseudogene formation can occur (71).

The mechanism of reverse transcription of Ty RNA is largely the same as that for mammalian retroviruses (but see ref. 166). The primer for reverse transcription is $tRNA_i^{Met}$, with 10 nucleotides of the 3' acceptor stem complementary to a site on the Ty1 (+) strand just 3' to the 5' LTR, called the (–) primer binding site (–PBS) (39). This tRNA is specifically packaged in viral particles (39). Interestingly, although the primer function of the initiator tRNAMet depends on its complementarity with the –PBS, its packaging is independent of this complementarity (39). This indicates that the $tRNA_i^{Met}$ is recognized by some other component of the Ty virus particle, and that it is probably not the acceptor stem of the tRNA that is recognized.

Integration

Ty3 shows a tight specificity for target sites, integrating 16 or 17 bp upstream of the 5' ends of tRNA coding regions (29,36). The DRE element of *Dictyostelium discoideum* and the spliced leader-specific elements SLACS, CZAR, and CRE1 of trypanosomes have a similar integration target specificity (3,117,183). Other genes transcribed by RNA polymerase III, such as 5S ribosomal RNA and U6 small nuclear RNA, are similarly targets for Ty3 (36). However, no common consensus sequence could be deduced for the insertion sites, suggesting that the integration apparatus was recognizing some aspect of chromatin structure or the RNA polymerase III transcription apparatus itself at these sites. Substitution of four purines with pyrimidines at the site of transcription initiation without moving the promoter did not change the site of Ty3 insertion. Eliminating transcription by destroying the promoter (box B inside the tRNA gene) eliminated target activity. But leaving the original transcription initiation site and Ty3 insertion site intact and moving box A of the promoter to a new site moved the site of transcription initiation and changed the Ty3 insertion site to a comparable position (36). This indicates that the integration apparatus recognizes the transcription apparatus but not the transcription initiation site or the promoter itself.

Other Tys are capable of insertion at many different sites, with Ty1 producing a five-bp duplication of the target DNA (102), but these sites tend to be within 750 bases of a tRNA gene or other RNA polymerase III–transcribed gene (144,154). Ty1 insertions in RNA *pol* II–transcribed genes tend to be in the 5' part of the *URA3*, *LYS2*, and *CAN1* genes (86,175,201), with control regions targeted far more often than the ORFs. Ty5 specifically targets regions of silent chromatin, such as the telomeric regions or the silent mating type loci (296).

An *in vitro* integration system has been established for Ty1 using Ty1 viral particles produced from an element carrying a copy of the *E. coli supF* gene and, as target, l DNA from a multiple amber mutant suppressible by *supF* (87,88). Using this system, it has been shown that linear double-stranded DNA substrates carrying the terminal 12 bp at each end of the LTR are sufficient for the integration reaction to occur at normal efficiency (88). There are also no nucleotide requirements. The substrate DNA must have 3' hydroxyls, suggesting that the reaction involves covalent attachment of these 3' ends to the target DNA. Unlike mammalian retroviruses, Ty1's IN does not remove two terminal bases in the process of integration (88), probably because in Ty1 the –PBS is immediately adjacent to the U5 part of the LTR. Ty3, however, resembles mammalian retroviruses in this regard (S. Sandmeyer, personal communication).

In fact, the purified IN protein is capable of carrying out an integration model reaction without other components of the viral particle (198). This suggests that it may be sufficient for IN and the reverse transcript to enter the nucleus for integration.

Expression

Control of Transcription

Ty1 transcription is controlled by MAT, the yeast mating type locus. MATa/MATa diploid cells have 20-fold lower Ty1 transcript levels than MATa or MATa haploid cells or MATa/MATa or MATa/MATa diploids (89). Exposure of cells to

mating pheromones, a step that immediately precedes mating itself, produces a posttranscriptional blockage of Ty1 transposition (293). Is this a control strategy adopted by Ty1 or imposed by the host, and why?

DNA damage, induced by ultraviolet irradiation or 4-nitroquinoline-1-oxide also induce Ty1 transposition by inducing transcription of the element (24). Is this effect adaptive for Ty1 as a first step in finding a new home, or is it a consequence of a failure of the host anti-Ty system?

Retroelements all must cajole the cellular RNA polymerase II into transcribing their proviral form to make viral RNA. They are constrained, however, to place the controlling sequences inside the limits of the element. While most mammalian retroviruses have enhancers in their LTRs upstream of the start site of transcription, Ty1 and Ty2 elements have major transcriptional control sequences downstream of both the transcript start site and the translation start site, most of them inside the unique region e (see Fig. 10). Ty1 has two major downstream sites responsible for its haploid-specific transcription: the sterile-responsive element (SRE) binds Ste12p, and another site (block II or PRTF) binds Mcm1p (53,92) (see Fig. 10). Ty2 has one upstream activation site (174), and at least two downstream activation regions, one of which (DAS1) responds to Gcn4p (103,264). Ty2 also has several downstream repression sites (DRS) (104).

Transcription of both Ty3 and Ty5 are, like that of Ty1, much higher in haploid cells than in diploids. Moreover, its transcription and transposition are derepressed by exposure of cells to the mating pheromones (52,150,265). Ty3 transposition is, in fact, induced in mating cells and Ty3 transposing from the genome of one mating partner to that of the other occurs at high rates (158). Ty3 transcription is induced before mating and repressed after mating by mating type control, but the particles synthesized before mating are sufficient to give a burst of transposition from one genome into the other (158). Here, the interpretation of preparation to transpose into the potentially Ty3-free genome of the mating partner seems clear.

In diploid *Saccharomyces* cells, the MATa1-MATa2 protein complex (encoded by the mating type locus) represses haploid-specific cellular genes. The regulation of Ty3 transcription involves two a1-a2 consensus recognition sites, determining mating type repression, and three PRE sequences, determining pheromone responsive expression, all located within the 5' LTR of the Ty3 element (16,265). An upstream repression sequence (URS) is located at the junction of the 5' LTR and the adjacent tRNA gene (16). Thus, in contrast to Ty1 and Ty2, most of whose control regions are located within the unique region downstream of the transcription and translation start sites, Ty3's transcription control regions are largely upstream within the U3 region of the LTR (see Fig. 10).

Effect of Ty Insertion on Cellular Genes

Insertion of Ty elements into the control regions of cellular genes can activate, inactivate, or alter the control of the target gene (93,287; reviewed in 21). The Ty insertions often move the normal regulatory sequences 5 kb away from the target gene, eliminating the normal regulation. Insertions of Ty whose 5' end is close to the 5' end of the target gene impose the Ty transcriptional control on the target gene. This produces a divergent transcription of the Ty element and the target gene, but both are under mating type control because of the effect of the Ty control region.

Chromosomal Genes Regulating Ty Transcription

Mutant cellular genes which have come under control of Ty1 have been used extensively to investigate the cellular genes affecting the transcription of Ty1 itself (50,288,289). Second-site mutations (suppressors) that restore the normal expression of the target genes have defined a large group of genes, called *SPT*, that include the TATA binding factor TFIID, the genes encoding histones, and many general transcription factors with effects on many genes (e.g., *SNF2*, *SNF5*, *SNF6*, *GAL11*, *SIN1*). In addition, the control of the target genes by mating type and mating pheromones have led to studies of effects of the mating type and pheromone control pathway genes on Ty itself. For example, *STE12* controls Ty1 transcription, as do the genes upstream of *STE12* in the mating type control kinase cascade (93,94).

+1 Ribosomal Frameshifting

Like mammalian retroviruses, Ty elements direct the synthesis of a Gag protein and a Gag-Pol fusion protein (Fig. 11). For reasons that are not yet clear, each of the *Saccharomyces* Ty elements uses +1 ribosomal frameshifting, whereas animal retroviruses all use –1 frameshifting (or readthrough of a terminator) to make Gag-Pol (reviewed in 101,125). As shown by studies of the L-A dsRNA virus, yeast can perform –1 ribosomal frameshifts by the same simultaneous slippage mechanism used by retroviruses.

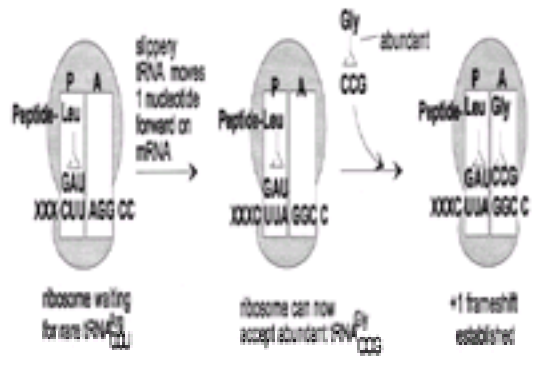


FIG. 11. Mechanism postulated for +1 ribosomal frameshifting of Ty1. (Adapted from ref. 12, with permission.) Ty3 frameshifting involves the new tRNA pairing in the +1 frame without a shift of the peptidyl tRNA as for Ty1 (268).

The mechanisms of the +1 frameshifts in Ty1 or Ty2 (see Fig. 11) and Ty3 all involve the combination of starvation for a rare tRNA and an unusual tRNA able to perform the frameshift (13,105). In the case of Ty1, the slippery site on the mRNA is CUU-AGG-C. The ribosomes are slowed by the AGG codon at the A ribosomal site. This codon is recognized by a $tRNA^{Arg}$ that is present in low abundance in yeast, so it is called a hungry codon. The $tRNA^{Leu}$, located at the P site while the A site is waiting for the AGG codon to be occupied, has as its anticodon UAG, and it is capable of pairing with either the 0 frame CUU codon or the +1 frame codon UUA. When it slips into the +1 frame, the GGC codon can be easily recognized by an abundant $tRNA^{Gly}$ species, and the ribosomes then continue in the +1 pol reading frame to make Gag-Pol fusion protein (13).

The efficiency of the Ty1 +1 ribosomal frameshift depends on the scarcity of the $tRNA^{Arg}$ recognizing the AGG codon. Thus, artificially oversupplying this tRNA lowers frameshift efficiency (13) and also lowers transposition frequency (291). Likewise, deletion of the gene for this $tRNA^{Arg}$ increases the efficiency of frameshifting and lowers the frequency of transposition (149). Starvation of cells for spermidine elevates the efficiency of Ty1 +1 ribosomal frameshifting and results in decreased transposition efficiency (8). Like the similar experiments done with the L-A dsRNA virus of *S. cerevisiae*, these results suggest that drugs affecting frameshifting efficiency might be useful as antivirals.

The Ty3 frameshift site is GCG-AGU-U, and, like that of Ty1, it is based on a hungry codon in the ribosomal A site, namely AGU (105). AGU is recognized by a low-abundance $tRNA^{Ser}$, so the ribosome pauses at this point. But the $tRNA^{Ala}_{CGC}$ that decodes the GCG codon in the P site cannot slip +1 and re-pair. Rather, it is believed that the valine tRNA simply pairs out of frame (268).

Proteolytic Processing and Phosphorylation

Ty1 Gag is expressed as a primary translation product of about 58 kd, most of which is processed by the Ty1 protease to form the 54-kd major particle protein (1). Gag is phosphorylated (193), and this phosphorylation is increased concomitant with the inhibition of transposition that occurs when cells are treated with mating pheromone (293). After Pol is synthesized as a 190-kd Gag-Pol fusion protein, it is processed through several intermediates to form a 23-kd protease, the 90-kd integrase, and the 60-kd reverse transcriptase–RNase H (see Fig. 9) (121).

Packaging: RNA Sites and Protein Requirements

The Ty1 RNA site determining packaging has been localized to within a 381-nt region between nt 239 and 620 (see Fig. 10) (292). The RNA structure recognized and the parts of TYA or TYA-TYB proteins that recognize this region have not yet been determined.

Debranching Enzyme and Ty1

Mutation of *DBR1*, encoding the enzyme that debranches the lariat structure produced by intron excision by cleaving the 2'-5' linkage at the branchpoint, results in a ninefold decrease in Ty1 transposition efficiency (38). However, the explanation of this unexpected finding remains unclear.

Host Limitations on Ty Transposition Efficiency

Although normal cells have about 35 chromosomal copies of Ty1, most of which are probably transposition competent (64), transposition is a relatively rare event. Most studies of the mechanism of transposition have used a high-copy plasmid with a GAL1-promoted Ty1 carrying a marker (such as *HIS3*) included in the Ty1 to facilitate detection of transposition (21). When Ty1 transposition is induced with such a plasmid, although Ty1 RNA is increased only a few fold over that derived from the normal chromosomal Ty1 copies, the frequency of transposition increases about 100-fold (63). One point at which transposition is blocked in uninduced cells is at the processing of viral proteins, particularly the PR and IN proteins encoded by TYB (63). Several host genes have been identified whose mutation derepresses Ty1 transposition, analogous to the effect of ski mutations on RNA virus copy number (see preceding).

Mutation of the cellular *rad6* gene, encoding a ubiquitin-conjugating enzyme, increases the frequency of Ty transposition at either *URA3* or *CAN1* (214). This effect was not caused by altered Ty transcript levels, and it was seen even when retrotransposition from a GAL1-promoted Ty1 was studied. The *rad6* mutation is believed to alter chromatin structure by failing to ubiquitinate histones, suggesting that its effect on Ty1 transposition is on the nature of the target. Indeed, Ty1's target-site specificity for gene control regions is apparently eliminated by the *rad6* mutation (148,175).

Another case of target-level regulation of Ty1 retrotransposition is found on mutation of *CAC3*, encoding a subunit of chromatin assembly factor, and *HIR3*, a histone transcription regulation gene (218). Here again, both the frequency of integration and the distribution of insertions is affected by alterations of the structure of the target (138).

Mutation of the *FUS3* mitogen-activated protein kinase (MAP kinase) results in an over 20-fold increased Ty1 transposition frequency (54). Ty1 transcript levels and primary translation products were not affected by *fus3*, but virus particle–associated processing products of TyB and Ty cDNA were each elevated 10-fold or greater in the mutant. The Fus3 kinase is activated by exposure to the mating pheromones via a cascade of kinases, so this explains the inhibition of Ty1 transposition by exposure to mating pheromones (293). Although Fus3 may act indirectly to block Ty1 transposition, several observations suggest a direct action (54). TyA is normally phosphorylated, but it is hyperphosphorylated when cells are treated with a pheromone, which is known to activate Fus3 (293). TyA has several consensus sites for Fus3 phosphorylation.

Also inhibiting Ty1 retrotransposition are components of the nucleotide excision repair/transcription factor TFIIH (167). *SSL2* and *RAD3* encode DNA helicases with roles in both nucleotide excision-repair and in the basal transcription factor

TFIIH. Mutants in either of these genes show elevated frequencies of Ty1 retrotransposition without alterations of the distribution of integration sites (167). There is no elevation of Ty1 proteins, but the level of Ty1 cDNA is elevated in either mutant (167). These results indicate that Ssl2p and Rad3p either inhibit reverse transcription or, perhaps more plausibly, destabilize Ty1 cDNA (167).

It is thus apparent that the host has several levels of defense against Ty1 attack: stability of viral particles, stability of cDNA, and structure of the target sites.

Schizosaccharomyces pombe Retroelements

The Tf1 and Tf2 elements of *S. pombe* are unusual in that a single ORF encodes both Gag and Pol (172). From the primary 140-kd translation product, the viral protease cleaves the proteins that form the viral particles (173). In view of the strict requirement for the ratio of Gag to Gag-Pol in Ty1 (149,291) and retroviruses, the assembly process in Tf1 and Tf2 must be significantly different. In log phase cells, the ratio of Gag to IN in particles is close to 1, whereas in stationary cells, it is quite high (7). Selective degradation of the non-Gag protein components alters the ratio after particle formation (7).

The priming mechanism of Tf1 is also unique. Instead of tRNA-priming as for other retrotransposons and retroviruses, Tf1 (–) strand DNA synthesis is primed by the first 11 nucleotides at the 5' end of the viral RNA, which is complementary to the primer-binding site (171,176). A hairpin is formed, which is nicked to separate the first 11 nucleotides from the 5' end of the RNA and give it a 3'OH that can serve as a primer for strong-stop DNA synthesis.

Candida albicans Retrotransposon as a Plasmid

Most retrotransposons have minute amounts of cDNA present in normal strains, but that of *C. albicans* (pCal) produces 50 to 100 copies per cell of linear dsDNA in a particular strain of *Candida* (191). pCal is a member of the Ty1/*copia* group found in integrated form in most strains, and the basis for its high free levels is not yet known. pCal is also unique among retrotransposons in that it uses readthrough of a stop codon to make its Gag-Pol fusion, as in murine leukemia virus (191).

Summary of Retroelements

The mating type and pheromone control of Ty transcription is clearly adapted to maximize transposition activity at the time when a potentially unoccupied genome becomes available (mating), and to minimize potential damage to the host, whose health is indispensable for survival. Posttranslational mechanisms, determined by the host, also limit transposition.

Among the many interesting questions about Ty elements are, Why do all Ty's use +1 frameshifting, but mammalian retroviruses use –1 frameshifting or termination readthrough to make their Gag-Pol fusion proteins? How does RNA polymerase II know to stop in the 3' LTR, but to keep going in the identical sequence in the 5' LTR? What does Dbr1, the debranching enzyme, do for Ty1? The bewildering array of retroelements continues to amaze.

DNA VIRUSES: *CHLORELLA* VIRUSES

Chlorella is a unicellular eukaryotic alga with a rigid cell wall and a single chloroplast (reviewed in ref. 266). Most *Chlorella* species are free-living, but several, called collectively zoochlorellae, live as endosymbionts (intracellular symbionts) of *Hydra* or *Paramecium*.

An attempt to isolate zoochlorellae free of their *Hydra* or *Paramecium* host often induces multiplication of a virus that grows and kills the zoochlorellae. These viruses are called HVCV (for *Hydra viridis Chlorella* virus) or PBCV (for *Paramecium bursaria Chlorella* virus) (reviewed in refs. 266,267). Hundreds of *Chlorella* viruses have been isolated directly from fresh water and are found throughout the world. They are grown on cultured zoochlorellae and form lytic plaques on agar plates.

Chlorella viruses are large (150 to 230 nm), polyhedral particles containing 5% to 10% lipid. The dsDNA genome of PBCV-1, the best studied *Chlorella* virus, is 333 kb, one of the largest viruses known. PBCV-1 DNA is linear with (a) terminal inverted repeats of 2.2 kb, (b) terminal hairpin structures, and (c) shorter direct repeats within the inverted terminal repeats (245). All three characteristics are in common with the poxviruses, vaccinia virus and African swine fever virus. Some other features are shared with the iridoviruses.

Chlorella virus DNA is heavily methylated, with 5-methylcytosine accounting for as much as 47% of C residues and 6-methyladenine for up to 37% of A residues. Surprisingly, the *Chlorella* viruses encode their own methylases. They also have been found to encode a variety of restriction endonucleases, similar in properties and, in many cases, in specificity to bacterial type II restriction endonucleases. The variety of such methylases and restriction endonucleases has only begun to be explored, but it is clear that a wide variety of specificities will be found (203). The function of these enzymes is completely unclear, and there is evidence against their being required for either degradation of host DNA or exclusion of other co-infecting viruses (reviewed in ref. 203).

It is not surprising to find glycosylated viral proteins, and the 54-kd major capsid protein of PBCV-1 has a carbohydrate component of about 5 kd. What is unexpected is that the virus encodes products that determine the glycosylation of Vp54 (274). Antivirus monoclonal antibodies directed against the carbohydrate moiety of Vp54 were used to select resistant mutants. The resulting virus mutants had altered glycoprotein moieties, but unaltered Vp54 amino acid sequence, indicating that the virus encodes either its own glycosyl transferases or products that control the host enzymes.

The complete sequence of PBCV-1 showed that the 330,742-bp genome encodes enzymes for synthesis of hyaluronic acid, aspartate transcarbamylase (uracil biosynthesis), an EF-3 homolog (translation elongation factor), 10 tRNA genes, genes with self-splicing type I introns, and numerous enzymes involved in nucleic acid metabolism.

Large DNA viruses of marine brown algae have also been described (reviewed in ref. 267).

PRIONS OF *SACCHAROMYCES* AND *PODOSPORA*

The concept of an infectious protein arose in studies of the transmissible spongiform encephalopathies, such as scrapie of sheep, mad cow disease, and Creutzfeldt-Jakob disease of humans (see Chapter 90). Based on genetic evidence (Fig. 12), two non-Mendelian genetic elements of *S. cerevisiae*, [URE3] and [PSI], were identified as prion (infectious protein) forms of Ure2p and Sup35p, respectively (281). This conclusion is supported by extensive further evidence and both appear to be transmissible amyloidoses, a remarkably close parallel to the mammalian disease.

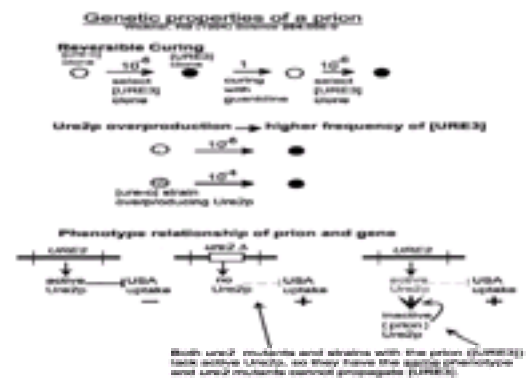


FIG. 12. Three genetic criteria for a prion. These are unusual features, expected of a prion but not of a nucleic acid replicon. (Adapted from ref. 281, with permission.)

[URE3]—A Transmissible Amyloidosis of Ure2p

In 1971, Lacroute described a non-Mendelian genetic element of *S. cerevisiae* called [URE3], which allowed cells to take up ureidosuccinate in spite of the presence of a rich nitrogen source such as ammonia (163). Ureidosuccinate is normally produced by aspartate transcarbamylase in the uracil biosynthesis pathway, and its uptake from the medium by Dal5p is repressed by ammonium. Chromosomal *ure2* mutants have the same effects as [URE3], but in addition they fail to propagate the [URE3] genetic element (2,281). Ure2p is inhibited by Mks1p (84) and posttranslationally inhibits the transcription activator, Gln3p (56). Gln3p positively regulates many genes in nitrogen metabolism, including *DAL5*, whose product is necessary for ureidosuccinate uptake (195,221):

NH_3—|Mks1p—|Ure2p—|Gln3p ® N catabolism genes

Genetic Evidence That [URE3] Is a Prion

Three lines of genetic evidence indicate that [URE3] is actually an altered form of Ure2p that has lost its activity in repressing nitrogen metabolic enzymes through Gln3p but has acquired the ability to convert the normal Ure2p to this altered form (see Fig. 12) (281). First, [URE3] is curable by growth of cells on guanidine HCl, but the curing is reversible in that [URE3] derivatives may again be selected from the cured strain (281). Second, overproduction of Ure2p leads to a 20- to 100-fold increase in the frequency with which [URE3] arises *de novo* (281). Third, the requirement of Ure2p for propagation of [URE3] and the identical phenotypes of *ure2*D and [URE3] are easily explained by the prion model, but not if [URE3] were a nucleic acid replicon—a virus or plasmid (281).

Ure2p is partially protease resistant in extracts of [URE3] strains, indicating that the Ure2 protein is altered, as predicted by the prion model (187). Deletion analysis showed that the part of Ure2p responsible for nitrogen regulation is residues 90 to 354, while the part responsible for the prion change is the N-terminal 80 residues (181,187) (Fig. 13A;. Moreover, the generation and propagation of [URE3] was unaffected by the repression or derepression of nitrogen regulation, eliminating some alternative models. [URE3] truly does arise *de novo*, and it is the Ure2 protein, not the *URE2* mRNA or the copy number of the *URE2* gene that induces [URE3] to arise (185).

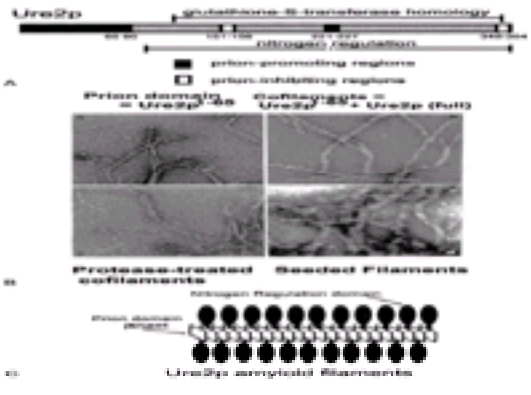


FIG. 13. A: Domains of Ure2p (181,186). **B:** Amyloid formation by Ure2p *in vitro* (253). **C:** Model of Ure2p amyloid structure (253).

Amyloid Formation In Vitro by Ure2p Promoted by the Prion Domain

Ure2p–green fluorescent protein fusions were found to be aggregated in [URE3] cells, indicating that aggregation is involved in the [URE3] change (83). Recent *in vitro* studies have shown that the Ure2p prion domain synthetic peptide can form amyloid, and that it can direct the conversion of the full-length native Ure2p into amyloid (see Fig. 13B) (253). The product of this reaction can seed further amyloid filament formation *in vitro*, suggesting that the [URE3] phenomenon is a transmissible amyloidosis of yeast (253). It appears that the conversion of Ure2p to amyloid inactivates the protein, and at the same time provides a seed for the further polymerization of newly synthesized Ure2p. It is proposed that the amyloid formed by Ure2p consists of a b-sheet core of the prion domain with the C-terminal nitrogen regulation domain attached as shown in Figure 13C.

[PSI]—A Transmissible Amyloidosis of Sup35p

In 1965, Cox described [PSI], a non-Mendelian genetic element of *S. cerevisiae* that increases the efficiency of nonsense suppression by classical tRNA suppressors (58,59). Like [URE3], [PSI] is reversibly curable, in this case either by guanidine or by high-osmotic-strength media (42,179,243,262). Overexpression of Sup35p increases the frequency with which [PSI] arises by 100-fold (42), like the induction of [URE3] appearance by Ure2p overproduction (see preceding). Finally, the phenotype of [PSI] is like that of *sup35* mutants, and *SUP35* is necessary for propagation of [PSI] (81,252), as *URE2* is necessary for propagation of [URE3]. This logical parallel with [URE3] led to the suggestion that [PSI] is also a prion form of Sup35p (281).

In support of the prion model for [PSI], it was shown that Sup35p is aggregated in [PSI+] strains, but not in [psi–] strains (210,212). Further, it was found that it was the overproduction of the Sup35 protein, not the *SUP35* mRNA or the gene in high copy number, that caused the *de novo* appearance of [PSI] (70).

Chaperone Involvement in [PSI] Propagation

Chernoff found that either overproduction of Hsp104p or deletion of the *HSP104* gene resulted in the inability of cells to propagate [PSI] (Fig. 14) (43,44). This finding is important because it at once supported the prion model for [PSI], it was the first proof of involvement of a chaperone in a prion phenomenon, and it provides a potential pathway to treatment of prion diseases. Because Hsp104 disaggregates denatured proteins *in vivo* (209), overproduction of Hsp104 probably cures [PSI] by disaggregating the aggregated Sup35p (see Fig. 14). Whether deficiency of Hsp104 results in loss of [PSI] because Hsp104 participates in the conversion of the normal to the prion form of Sup35p or because Hsp104 must partially disaggregate the prion form of Sup35p to ensure its segregation to both daughter cells remains unclear.

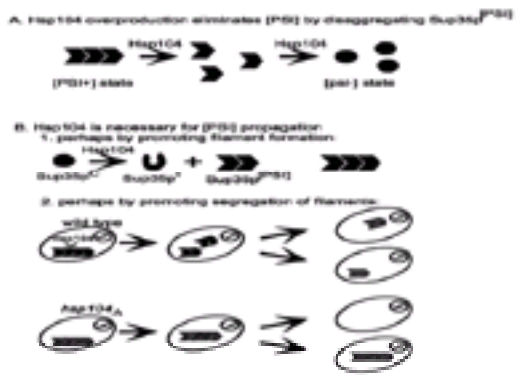


FIG. 14. Role of chaperone Hsp104 in propagation of the [PSI] prion of *S. cerevisiae* (43,44). Overproduction of Hsp104 cures the [PSI] prion by renaturing aggregates of Sup35p. Normal levels of Hsp104 are needed for [PSI] propagation, perhaps because it has a role in catalyzing Sup35p filament (aggregate) formation or in breaking up aggregates to ensure that each daughter cell gets at least one aggregate.

Amyloid Formation by Sup35p In Vitro

The *in vitro* aggregation of native soluble Sup35p seeded by the prion form from [PSI+] cells will continue indefinitely as long as fresh native Sup35p is supplied (213). Further, synthetic prion domain peptide spontaneously forms amyloid *in vitro* (155). In addition, the full-length Sup35p, made in *E. coli*, forms b-sheet-rich filaments spontaneously when diluted out of denaturant, a process that can be accelerated specifically by an extract from [PSI+] cells (127).

[Het-s]—A Prion of *Podospora* Responsible for a Normal Function

When two colonies of the same strain of filamentous fungus meet, they fuse their cellular processes (called hyphae) in a process called hyphal anastomosis or vegetative fusion. This allows exchange of cytoplasm and even nuclei and allows cooperation between the colonies in obtaining nutrients. However, a virus initially present in only one colony will spread

throughout the other colony, so the process is tightly controlled, requiring identity of the two strains at 6 to 10 special chromosomal loci, called *het* loci in *Podospora anserina*. Fusion of two strains that differ at a *het* locus begins, but it is quickly arrested with death of the few fused hyphae and formation of a barrier to further hyphal fusion. This is called the heterokaryon incompatibility reaction (Fig. 15) (11,229; reviewed in 10).

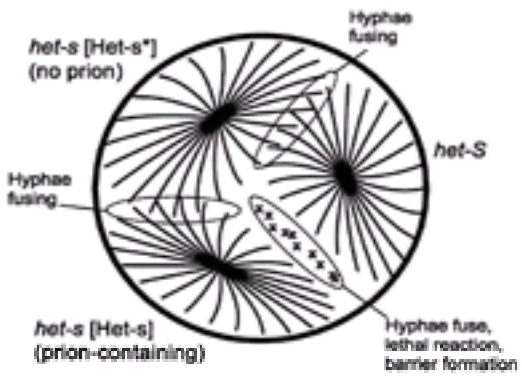


FIG. 15. The [Het-s] prion of *Podospora anserina* is required for heterokaryon incompatibility. (Reprinted from ref. 57, with permission.)

One of the het genes of Podospora is *het-s*, with alleles *het-s* and *het-S* encoding a 289-residue protein differing at 14 positions (263), although the difference at residue 33 is sufficient to trigger the incompatibility reaction (69).

Rizet found that *het-s* strains could show the incompatibility reaction only if they also carried a non-Mendelian genetic element, called [Het-s] (229). [Het-s] could be "cured" from a male strain by mating, but from the cured strain, [Het-s] cells arose at a frequency of about 10^{-7} (12). This is the *reversible curing* criteria for a prion.

Coustou et al. have made the case that [Het-s] is a prion of the protein encoded by *het-s* (57). They showed that overproduction of the Het-s protein increased the frequency with which [Het-s] arose, and that the Het-s protein is relatively protease resistant in strains carrying [Het-s]. Moreover, deletion of the *het-s* gene makes a cell unable to propagate the [Het-s] non-Mendelian genetic element (57).

Beyond its obvious interest as a new prion, [Het-s] is particularly important because it is the first case in which a prion appears to be responsible for a normal cellular function. Heterokaryon incompatibility is observed in most filamentous fungi, usually controlled by chromosomal genes that have no particularly unusual features. In this case, it requires the [Het-s] prion to be observed. This suggests that other prions may be found responsible for normal cellular functions.

PERSPECTIVE

The discovery of prions of yeast and fungi has resolved the debate over whether prions could exist at all and widened the scope of this concept, showing that it is not unique to a single mammalian protein. The possibility is now open for exploring the mechanisms of amyloid and prion generation and propagation using yeast, and using these systems to devise approaches to the many heretofore intractable amyloid diseases.

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CHAPTER 22 Bacteriophages

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HISTORY

Bacterial viruses (generally called bacteriophages or simply phages) were discovered independently by Twort and d'Herelle as filtrable, transmissible agents of bacterial lysis (21,68). Although their relevance to animal and plant viruses was not universally accepted, phages proved so tractable to experimentation that information about their nature was soon forthcoming. For example, their ability to form isolated plaques on bacterial lawns was noted, and d'Herelle correctly attributed the linearity of plaque assays to the self-replicating nature of the phage particles (as expected for a subcellular pathogen) rather than to the cooperative effect of many particles (as expected for a typical poison).

The early hope that phage might be used to combat pathogenic bacteria (either therapeutically or in decontamination of the environment, such as in purifying water supplies) did not materialize at that time, although efforts toward that goal have been renewed (51). Starting in the 1920s, phages were widely used in the typing of bacterial strains of medical interest.

A new era in phage research was initiated in the early 1940s by Max Delbrück, Salvador Luria, and their disciples. Their efforts laid the groundwork for the use of phage in the fundamental experiments of molecular biology, but the work itself is notable for its deemphasis of biochemistry and its focus on basic biologic information relevant to the mechanism of self-replication. The techniques used were simple, mainly microbiologic, and some of the questions investigated had already received indicative answers in the previous two decades. What was new was an emphasis on sharp, incisive answers, quantitative where appropriate, and the development of a few standardized systems.

Conditions were determined for the rapid, reproducible attachment of phage to bacterial cells, allowing synchronous infection of all the cells in a culture, with the resulting “one-step” growth. The effect of varying the ratio of phage to bacteria (multiplicity of infection) was analyzed as a problem in random statistics. It was also shown that infected cells pass through an eclipse phase, where infectious phage is unrecoverable from infected cells, and that infectious particles appear at a later time (3). The ability of one phage to exclude another, and of coinfecting phage to interact productively either genetically (by recombination) or phenotypically (by complementation or phenotypic mixing) or both (as in the production of viable phage from cells multiply infected with radiation-damaged particles) was rigorously documented. Quantitative analysis at a comparable level was introduced into animal virology, with the development of plaque assays on tissue monolayers. Thus, the foundations of modern animal virology (independent of its molecular aspects) go back to the phage work of the 1940s.

Central to the quantitative analysis of phage experiments is the random (Poisson) distribution, which states that, when a mean number s of objects (such as virions) are distributed randomly among containers (such as infected cells), then the fraction of containers with exactly r objects is $Pr = s^r e^{-s}/r!$, where e is the base of natural logarithms (2.718...) and $r! = 1 \times 2 \times ...(r\text{-}1)r$, with 0! = 1. Although s can be any nonnegative number, r is restricted to integral values (a cell cannot be infected with half a virion).

Of particular interest is the zero term $P_0 = e^{-s}$. Thus we expect that, when phage and bacteria are mixed at a ratio of 2:1, the fraction of infected cells should be e^{-2}, or about 0.14. To be exact, this calculation requires that every cell have the same large number of surface sites for viral attachment. Although this is not precisely true, the calculation accords fairly well with experiment.

The distribution also applied to the single burst experiment, where infected cells, before lysis, were diluted and distributed so that each tube received, on average, less than a single cell. For example, with an average number of 0.2 infected cells per tube, the fraction P_0 of sterile tubes is $e^{-0.2}$, or 0.818; the fraction P_1 of tubes with exactly one infected cell is $0.2e^{-0.2}$, or 0.162; the fraction with two infected cells is $0.2^2/2e^{-0.2}$, or 0.0162, and so on. Aside from pipetting errors or confounding factors like cell clumping, this calculation is precise.

Studies of phage genetics started in the 1940s with the construction of recombinational maps (36) and continued in the

1950s with Benzer's analysis of fine structure genetics of the T4 *r*II genes (10) and with the discovery and use of conditionally lethal mutations in l and T4, which allowed dissection of the developmental programs of phages (13,26). The rII work depended conceptually on the prior discovery of phenotypic mixing (54), which showed that when mutants of the same phage co-infect a cell, phage gene products are drawn from a common pool. And the later conditional lethal studies depended on the operational protocol laid out in the rII studies.

Serious phage biochemistry was initiated in the 1930s, when Schlesinger determined that phage particles were about 50% DNA and 50% protein (62). It did not enter into the mainstream of phage work until the 1950s, when Hershey and Chase showed by radiolabeling that the phosphorus (DNA) entered the cell during T2 infection but that most of the sulfur (protein) did not (35). The experiment followed the demonstration of the eclipse phase, and the use of electron microscopy to show that empty phage heads remain attached to the cell envelope and that the DNA of virions could be separated from the protein shell by osmotic shock (2). Concurrently, the enzymology of T4 infection was developed in detail, clearly indicating that the phage was introducing instructions for proteins present neither in the uninfected cell nor in the virion.

It was reported in the 1920s that many bacterial strains harbored phage permanently and secreted it into the medium, so that every culture contained phage. Such strains are called lysogenic. The Luria-Delbrück school initially chose to ignore lysogeny, in part because of the possibility that the phenomenon might be explainable simply by the adherence of extracellular phage particles to the bacterial surface and their occasional detachment from it. Increased rigor was introduced by Lwoff et al. around 1950 in a series of experiments showing first, that when individual cells were allowed to divide and daughter cells separated into different drops of liquid by micromanipulation, both daughters retained the ability to produce phage; second, that an occasional cell lysed and liberated a burst of phage (so that it was unnecessary to postulate secretion by living cells); and third, that certain treatments (such as ultraviolet light or other agents that induce the SOS response) induce mass lysis and phage liberation by all cells of the culture (49). As with infected cells in the eclipse phase, no infectious phage were found when lysogenic cells were artificially disrupted.

Phages capable of producing lysogenic complexes are called temperate, and the noninfectious form in which phage genetic information is transmitted is called prophage. Of the temperate phages, coliphage l has been studied most intensively, partly because it grows in the K-12 strain of *Escherichia coli*, for which conjugational and transductional mapping were possible by the 1950s. It was soon shown that prophage has a specific location on the bacterial map, and later that its genome is linearly inserted into the continuity of the bacterial chromosome (16,44).

From 1950 onward, the virtues of a simple identifiable genome with rigorous genetics that could be simultaneously introduced into (or induced to replicate in) all the cells of a culture made phage the favored experimental material for many of the classic experiments of molecular biology, such as the demonstration of DNA breakage and joining during genetic recombination, and the proof of the triplet code. In the 1960s, l, in particular, became the model for study of the genetic hierarchy controlling a simple developmental program. Although the initial investigations on conditionally lethal mutations were pursued in far more depth with T4 than with l, l had some inherent advantages: The initiation of the entire program of the lytic cycle from two promoters directly controlled by repressor, the uniformity of the DNA molecules extracted from virions, and the specific probes for different parts of the genome provided by specialized transducing phages and deletions or substitutions (whose locations could be seen via heteroduplex mapping) made the effects of genetic manipulation on molecular events readily interpretable; and the existence of alternative pathways toward lysis or lysogeny added to the interest.

Since 1970, phage work has merged with the rest of biology because artificial cloning allows the application to many systems of the techniques early available for phage. The background data on phage systems, plus their facile manipulation, continue to allow their study at a high level of sophistication.

VIRULENT PHAGES

Like viruses in general, phages can be divided into those with RNA genomes (mostly small and single stranded), those with small DNA genomes (generally less than 10 kilobases, mostly single stranded), and those with medium to large DNA genomes (30 to 200 kb). The last group includes most of the temperate phages, as well as the first virulent phages to be studied intensively.

Large DNA Phages

The classic T coliphages selected for study by the Luria-Delbrück school fall into four groups: T1 (about 50 kb); T2, T4, and T6 (about 170 kb); T3 and T7 (40 kb); and T5 (about 130 kb). The members of a group have similar virion morphology and can produce viable recombinants in mixed infection.

Phage T4

Virion

T4 was the object of many classic experiments. More than 200 T4 genes were identified through mutational studies. The virion contains 43 phage-encoded proteins, of which 16 are located in the head that encapsidates the DNA, and 27 form the tail through which DNA passes into the cell during infection. The head is an elongated T=13 icosahedron with an extra equatorial row of capsomeres. The tail consists of a hollow core (surrounded by a contractile sheath) that terminates in a baseplate to which are attached six fibers. The whole apparatus functions to inject phage DNA into the interior of the cell. The tips of the tail fibers make initial contact with lipopolysaccharide surface receptors on the bacterium. Once the phage is anchored by tail fiber attachment, random motion brings the baseplate into contact with the cell surface. Either this contact or the tail fiber attachment itself triggers conformational changes in the tail: The center of the baseplate opens like a shutter, allowing the tip of the core to pass through, and the sheath contracts, driving the core through the cell envelope, after which DNA is released into the cell. The double-stranded DNA is distinctive in containing

hydroxymethylcytosine (HMC) (heavily glucosylated) rather than cytosine. It is cyclically permuted and terminally repetitious—that is, the sequences of various molecules can be represented as ABCDEFGHAB, DEFGHABCDE, GHABCDEFGH, and so on. This is most simply demonstrated by allowing the DNA to separate into single strands and then reannealing with complementary strands from other virions, generating circular duplexes with single-stranded tails (Fig. 1).

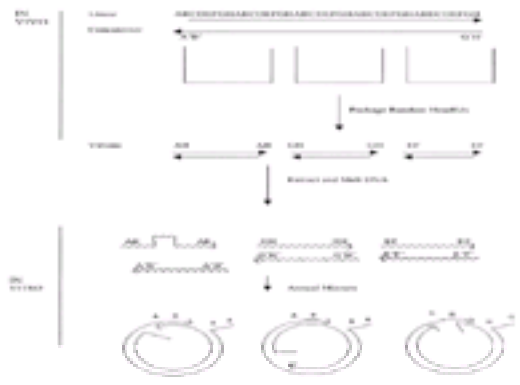

FIG. 1. Genesis (*in vivo*) and demonstration (*in vitro*) of circular permutation of T4. Within infected cells, replication generates a DNA network with linear concatemeric segments. From these, headfuls of DNA, cut randomly, are packaged. Thus DNA extracted from virions has random endpoints. When single strands reassociate, the complementary strands in general have different starting points and thus pair as shown.

DNA Transactions

Infection triggers a massive degradation of cytosine-containing host DNA with subsequent enzymatic conversion of deoxycytosine monophosphate to deoxyhydroxymethylcytosine monophosphate. The phage encodes enzymes that perform these and other conversions of deoxyribonucleotides, which are then used in phage replication. Bidirectional replication is initiated at several origins along the DNA, and elongation is performed by a complex of phage-encoded enzymes, leading- and lagging-strand synthesis proceeding coordinately from a single replication complex. Because there is no mechanism for priming synthesis at the extreme 5' end of the lagging strand, this first round of replication produces linear molecules with protruding 3' ends.

Soon after infection, these early replication origins cease to function (because replication there requires priming by RNA made by host RNA polymerase, whose promoter recognition specificity is altered). Late replication initiates at recombination intermediates formed by invasion of 3' ends into homologous double-stranded DNA. Because of the terminal redundancy, such homologous sequences are available, even in single infection. As replication proceeds, repeated invasions of this kind generate a complex network of intracellular viral DNA, which includes end-to-end concatemers (the preferred packaging substrate for T4). In packaging, empty heads are first assembled, then filled with DNA. Cutting is coordinated with packaging, so that headful lengths are cut to size after the head is filled. The packaging length exceeds the genome length, hence the terminal redundancy. The position of molecular ends along the concatemer is close to random.

This picture of the DNA transactions has genetic consequences; in fact, much of it was inferred from genetic studies that preceded the biochemistry and molecular biology. First, the recombination rate in T4 infection is very high, producing a linkage map thousands of centimorgans in length. It was early noted that this corresponded to about one recombination per replication cycle. Second, the linkage map is circular, as expected if virion DNA has random endpoints. Third, when cells are infected with phages of two different genotypes, some of the progeny particles can carry different information within the terminal duplication; for example, in a mixed infection between T4 and T4 *h*, some progeny plaques contain phage of both *h* and *h*+ genotypes. Another mechanism that generates mixed plaques is packaging of heteroduplex DNA recombination intermediates. Both types of heterozygous progeny are demonstrable (65).

Finally, there are genetic implications of headful packaging. If the length of the terminal overlap represents the difference between packaging length (determined by head size) and genome length, then that length should change in a predictable manner if either packaging length or genome length is deliberately altered. The first test was to alter genome length with a nonlethal deletion. The prediction that this would increase the length of the terminal overlap (and therefore the fraction of particles heterozygous for any locus in the genome) was fulfilled (67). Later experiments with other phages that use headful packaging, such as P22, showed that increasing the genome length by insertion of extra DNA has the reverse effect of decreasing the terminal overlap.

The opposite experiment, where genome length remains constant but packaging length changes, was accomplished through examination of virions with abnormal morphology. Preparations of T4 contain a small fraction of virions whose heads are isometric rather than elongated, separable from the majority type by ultracentrifugation. Certain treatments (such as phage development in the presence of amino acid analogs) induce formation of giant particles with the same diameter as normal virions but additional rows of capsomeres, approaching helical tubes in shape. Missense mutations in the major capsid protein can increase the proportion of both isometric and giant particles. Both types of abnormal particle inject their DNA into cells. The packaging capacity of isometric particles is about 67% that of normal virions; accordingly, single infection is unproductive, but multiple infection allows a normal cycle through recombination between molecules of less than genome length with random endpoints. Giant particles have longer than normal DNA (up to several genome lengths) and a correspondingly high degree of heterozygosity when coming from mixed infection. They are also highly resistant to ultraviolet light because of complementation and recombination between damaged genomes.

Regulation

The genes whose products are needed for phage DNA synthesis and host DNA breakdown, including those mediating nucleotide metabolism and the seven proteins of the replication complex, are all expressed immediately after infection, being transcribed from promoters recognizable by *E. coli* RNA polymerase with s70. With time, early synthesis shuts off and other genes that code for virion components and lysis genes are activated. The shutoff of early genes is effected at both the transcriptional and the translational levels. Translational control is sometimes autogenous, as for the DNA polymerase gp43 and the single-stranded binding protein gp32; many other early genes are controlled by a dedicated translational repressor, the product of the *regA* gene, itself an early gene subject to autogenous control (4,71). (In phage work, gp means gene product, not glycoprotein, as elsewhere in this book.)

Transcriptional control operates on three different types of T4 promoters. The early promoters have sequences approximating the consensus for *E. coli* s70 (with a small but apparently real difference in consensus). Middle promoters require *E. coli* polymerase with s70, in addition to the T4-specified positive regulators MotA and AsiA (1). Their hallmark is a sequence TGCTT at around –32. Late promoters follow –12 consensus TATAAATA. They are transcribed by *E. coli* holoenzyme, with a phage-coded s factor (gp55) replacing s70. In normal infection, late promoter activation requires concurrent DNA replication. *In vitro*, activation can be achieved by nicks or gaps on the nontranscribed strand near the promoter; treatments that create damage *in vivo* can also induce some replication-independent transcription.

In vitro, activation of late transcription by nicks or gaps requires three replication proteins, gp45 and gp44/62, which form a sliding clamp that loads at the nick or gap and moves along the DNA (29). An RNA polymerase-associated protein, gp33, is also required, as well as gp55. Transcription cannot be stimulated by bringing the DNA interruption spatially close to the promoter through catenation of DNA molecules; enhancement is seen only when all signals are on the same DNA double helix, as expected from the sliding clamp model. The exact relationship between these *in vitro* results and the *in vivo* dependence of late transcription on replication is unknown.

Some other ancillary changes during infection may reinforce the temporal sequence but are not essential for it. For example, the a subunit of RNA polymerase becomes adenosine diphosphate (ADP)-ribosylated a few minutes after infection by the action of either of two phage gene products: gp *alt*, which is activated during virion assembly and injected along with the DNA, and gp*mod*. ADP-ribosylation modifies polymerase specificity, but both successful infection and shutoff of host promoters occur in its absence (in *alt*/*mod* double mutants), perhaps because of a multiplicity of functionally overlapping shutoff mechanisms. Shutoff of host transcription is also effected by a T4-encoded transcriptional terminator protein (Alc), which recognizes cytosine-containing DNA.

Thus the chemical marking of phage DNA by HMC is used as a passover signal: Transcription of the host's cytosine (C)-containing DNA is first reduced by the Alc protein (39) and then C-containing DNA is degraded. T4 also has some noteworthy features with no known regulatory role, such as genes that are discontinuous because of intron splicing or ribosomal skipping (8,72).

Assembly

Once late genes are expressed, the stage is set for assembling virions. The T4 virion was the first biologic structure of comparable complexity whose pathways of assembly were worked out, from a combination of physical and genetic knowledge. The major technical factors facilitating the study were, first, the availability of conditionally lethal mutations in genes for individual virion proteins; second, the fact that the final steps of the pathway were readily executed *in vitro* so that reactions in the pathway could be distinguished from irrelevant interactions or side reactions that do not contribute to the yield of functional plaque-forming particles; and third, the facility of operations by then standard in phage work, such as assay for complexes stable to dilution.

The results of these studies suggested several general principles whose rationalization as effective strategies and extension to other phages has been subsequently assessed and updated. One of these is the repeated use of subassembly pathways rather than a single linear pathway. Thus, heads, tails, tail fibers, and soluble protein (gp63) catalyzing tail fiber addition are all made separately. Heads and tails combine first to form complexes that are both visible by electron microscopy and stable to dilution, and finally tail fibers are added to the complex. The tail consists of a baseplate (to which the fibers attach) composed of a central hub surrounded by six wedges. Hub and wedges are made separately, then combined to form the baseplate (Fig. 2). Likewise, in tail fiber assembly, the distal and proximal segments are assembled separately, then combined immediately before addition to the head–tail complex. The most obvious advantage of subassembly pathways is that mistakes in assembly can be rejected for each pathway separately, rather than ruining entire virions.

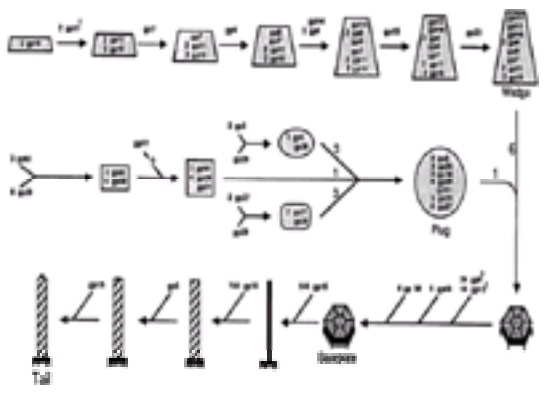

FIG. 2. Assembly pathway of T4 tails. Proteins must be added in the indicated order, except that gp11, gp12, and gp9

(indicated by †) can be added at later times as well. (From ref. 17, with permission.)

A second general rule is that linear elements are built from the end in (toward their junction with the rest of the virion). In tail assembly, baseplates are made first, then the core and sheath are added to them. Likewise, wedges are formed by adding different protein units successively, beginning with one of the outermost units, and finishing with the one that interacts with the hub. This strategy ensures that the intermediates of the subassembly pathway cannot compete with the final product for assembly into the virion. The order of assembly of the wedges themselves may be prescribed by allosteric changes on addition to the complex or by the recognition of binding sites that contain elements of more than one of the proteins already present. In fiber addition to head-tail complexes, fibers do not add efficiently to base plates until heads and tails are joined, apparently because the bend in the fibers contacts the head–tail junction and interacts with it. A protein (gp*wac*) in the collar serves this function *in vitro*, although it is not essential for plaque formation *in vivo*. In free virions, fibers are frequently wrapped around the tail sheath, becoming extended only under specific conditions.

The joining of fibers to head–tail complexes attracted attention early because it proceeds by catalysis of a noncovalent interaction. The catalyst (gp63) also functions as an RNA ligase that apparently acts during (but is not essential for) DNA synthesis. Protein catalysis of a one-step noncovalent interaction was novel at the time, and its discovery was too far in advance of later work on chaperonins to have wide impact.

Tail assembly raised the question of length determination. The core and sheath are both formed by polymerization of single protein species. Core assembly is initiated through nucleation on the baseplate, followed by addition of subunits to build the structure. How is the number of subunits counted, so as to ensure the observed uniformity among virions? The answer (better documented for l than for T4) is by use of a linear measuring stick protein that is embedded within the core and eliminated when assembly is complete (40).

The T4 tail fiber is a bent, linear structure with several specific protein monomers or dimers arranged end to end, terminating in a dimer of gp37. This protein at the tip of the tail fiber is so arranged that its amino acid sequence (and hence its genetic map) is colinear with the fiber axis (7). The specificity for host cell receptors and neutralizing antibodies lies in the distal portion, near the tip, which is also the location of mutations for altered host range.

The pathway of capsid formation in T4 (as for isometric capsids of any virus) is still understood in less depth than tail assembly, but several established features are noteworthy. Some of these are better understood for other phages (l, P22) and inferred for T4. The capsid is synthesized first, and it is filled with DNA later. This fits with the determination of DNA content by head size, but it is more directly established by the association of capsids assembled early with DNA made late in infection (47). Second, capsids are not built directly into their final form; rather, capsid proteins associate with an internal scaffolding protein, which fills the interior of the prohead and is later extruded through holes in the protein shell, which expands to its final shape before DNA enters. Both scaffolding protein (gp24) and the major head protein (gp23) have a shape-determining role [inferred from the aberrations in shape caused by certain missense mutations (12)]. Probably both proteins are added to the structure concurrently. How these proteins actually determine the elongated icosahedral shape is unknown, and the intermediate states are no better understood than for any other closed lattice. After the prohead expands and assumes a more obviously icosahedral shape, two decoration proteins, gp *hoc* and gp*soc*, are added at regular positions in the lattice; neither protein is essential for formation of functional virions. Third, assembly requires nucleation from one vertex, in which minor capsid proteins, noncapsid proteins, and host chaperonins (*gro*EL) all participate. This unique vertex becomes the portal vertex through which DNA enters the virion and to which the tail later attaches. DNA packaging requires two phage proteins (gp16 and gp17) that probably act at the portal vertex but are not present in the final virion. Protein gp17 has been identified as the endonuclease that cuts the DNA from the concatemer during packaging (11). Another packaging gene, gp49, is needed for processing the DNA substrate. Only linear DNA is packaged, but intracellular T4 DNA becomes highly branched during replication and recombination; gp49 cleaves Holliday junctions and other branched structures, creating packageable lengths of linear DNA (11). Fourth, processing of gp23 takes place concurrently with assembly. Figure 3 presents the general scheme for virion assembly in double-stranded DNA phages.

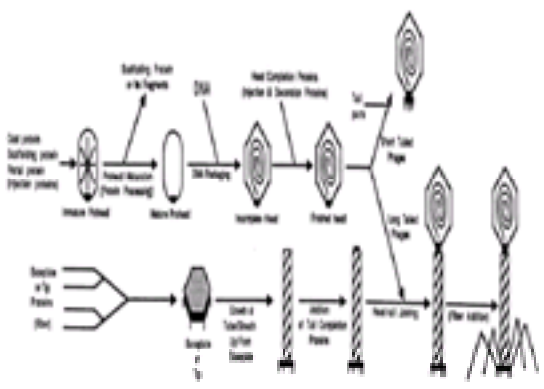

FIG. 3. Generalized assembly pathway for dsDNA phages with tailed virions, such as T4 and l. (From ref. 17, with permission.)

Lysis

The final stage of the T4 cycle is cellular lysis, releasing virions into the medium. Lysis requires two gene products: a

lysozyme (gpe), which attacks the bonds joining *N*-acetylglucosamines in the rigid murein layer, and a holin (gpt), which creates holes in the inner membrane, allowing the lysozyme to reach its substrate. Other genes affect the timing of lysis. The status of gpt as a holin is not fully established and rests largely on analogy to l gpS (75). In *e t*+ mutants, the cell dies and the membrane potential collapses at the normal time of lysis, whereas in *e*+ *t* mutants, the cell continues to produce lysozyme and intracellular phage long after the normal time of lysis.

It is clearly desirable to coordinate the time of lysis with the rest of the lytic cycle, but no regulatory system serving this function has been identified. The timing of lysis appears to be determined largely by the accumulation of gpt, formed coordinately with other late proteins. One genetic system that regulates time of lysis under certain conditions is the *r*II locus. T4*r* mutants were discovered by their ability to form larger than normal plaques, and the *r*II mutants have the related property of inability to plate on strains expressing the *rex* genes of l [which provided the selective conditions for Benzer's classical studies on fine structure genetics (9)]. However, their effect on lysis and plaque size is attributable to the absence, in *r*II mutants, of lysis inhibition. When a cell infected with T4 *r*+ is superinfected before lysis by another T4 particle, lysis is delayed and intracellular phage development continues, for a period of up to several hours beyond the normal latent period of 24 min. The rIIA and rIIB membrane proteins must mediate this response and must likewise destroy membrane damage inflicted by the l *rex* system in response to superinfection, but their mechanism of action is unknown.

Phage T7

Each group of T phages has unique properties. This brief account of phage T7 is intended to indicate some major differences from the T4 paradigm. The phage T7 virion contains 39,936 base pairs (bp) of linear double-stranded DNA (completely sequenced). It is terminally redundant (like T4) but not cyclically permuted. The length of the terminal overlap is 160 bp (25). Immediately after infection, host RNA polymerase initiates transcription from a promoter near the left end of the molecule, whose products include an antirestriction factor, a protein kinase that phosphorylates and inactivates *E. coli* RNA polymerase, a new T7-specific RNA polymerase, and a DNA ligase. After a few minutes, T7 polymerase replaces host polymerase and all transcription takes place on the 81% of the genome toward the right end. The first genes transcribed by T7 polymerase (nearer to the left end) include a DNA polymerase and recombination genes. Later, after replication ensues, the late genes encoding virion components and at least one lysis gene are expressed. Late gene expression does not require replication, as it does in T4, and the host polymerase is replaced rather than reprogrammed. Even without inactivation of host polymerase, the T7 RNA polymerase competes so effectively for nucleoside triphosphates that it redirects almost all transcription to T7 promoters, a feature that has been exploited in the design of T7-based expression vectors for DNA cloning.

The first cycle of DNA replication proceeds bidirectionally from a unique origin of the linear monomer. As with T4, concatemers are probably generated through strand invasion of terminal segments into other molecules within the 160-bp terminal repeat. Replication then generates concatemers in which the unique 39,616 viral sequences alternate with single copies of the 160-bp repeat. If complete virion DNA molecules with repeats at both ends were cut and packaged from such concatemers, half of the genomes would lack terminal repeats. It was suggested that T7 might solve the problem by cutting the DNA in opposite strands at the two ends of the repeat, creating protruding 160-base 5' ends, followed by DNA synthesis to produce flush ends.

The actual solution is apparently more complicated and wasteful. Replication of one strand initiated from a nick near the 160-bp segment proceeds leftward through the 160-bp segment to a reverse repeat beyond the segment. The displaced strand is then nicked at the end of the reverse repeat, which folds back and initiates synthesis rightward through the 160-bp segment. Thus, two copies of the 160-bp segment are present, which can be trimmed to size and packaged (19). Packaging proceeds from right to left processively along the concatemer (66). Because T7 packages the DNA between two specific sites, the amount of DNA per head is not fixed by head size. From study of T7 variants with deletions or duplications, the packaging limits of T7 are about 85% to 103% of normal genome length (F. W. Studier, personal communication).

Small DNA Phages

Some phages package small covalently closed circles of (+) strand genomic DNA in their virions. There are two groups: isometric and filamentous. Although the two groups are not detectably related in sequence and differ in modes of attachment, packaging, and egress, their replication mechanisms are similar.

Isometric Phages

The prototype is f × 174. The virion (25 to 35 nm) has an icosahedral T=1 capsid, composed of three polypeptide species: gpF (60 copies), gpG (60 copies), and gpH (12 copies). gpG and gpH are considered spike proteins and can be physically removed without disrupting the integrity of the capsid. The spike proteins are necessary for attachment to cells, and gpH enters the infected cell with the DNA and participates in phage development. Such participation is not essential for all productive infection, because transfection by pure (+) strand DNA from virions leads to some phage production.

Once within the cell, the DNA becomes coated with single-stranded binding protein (Ssb), then begins to replicate. The first cycle [parental single strand ® double-stranded replicative form (RF)] is mediated entirely by host enzymes. Transcription by host DNA–dependent RNA polymerase at a unique site near a DNA hairpin provides a primer, which is extended by the host replication complex and then digested away. At completion of the cycle, the new DNA strand is ligated to give a covalently closed circle (the RF). Transcription from RF by host RNA polymerase leads to production of phage proteins, among them a dimeric protein gpA, which works only in *cis* (i.e., an A- mutant cannot be complemented in *trans*, perhaps because the protein binds rapidly and irreversibly to DNA near the site of transcription). Rolling circle replication is initiated when gpA binds to RF at a specific origin site and nicks its parental (+) strand, which is then extended by the action of a host-specified DNA helicase (Rep) followed by the host elongation complex. The displaced 5' end is not free but remains bound to the growing point through the gpA dimer. The displaced strand is coated with Ssb

and remains single stranded (no lagging-strand synthesis). When one genome length of DNA has been synthesized and the growing point comes to the origin, the displaced DNA is cut from the newly synthesized DNA and its ends are ligated, giving a single-stranded circle and a double-stranded (RF) circle, both of which can recycle through further rounds of replication. Thus, a fraction of the (+) strands in infected cells are linear and from monomer to dimer length, whereas both (+) and (–) strands are present as monomer circles.

With time, virion proteins accumulate and form empty proheads, and concurrently a phage-specified DNA-binding protein (gpC) is made. On some of the (+) strands, gpC competes with Ssb for binding to the Rep-gpA complex, and these interact with proheads to become encapsidated into virions. Thus, the DNA is directed toward packaging before it encounters the prohead (5). The replicating pool then ceases to expand as (+) strands are packaged (Fig. 4). The multiplication cycle ends with lysis, mediated by the phage gpE protein. The mode of gpE action is uncertain, but it does not involve either a lysozyme or a holin (75).

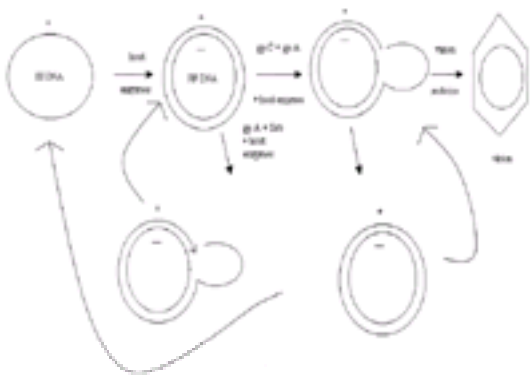

FIG. 4. Replication of fX174. The DNA injected from the virion as a closed ring of single-stranded (+) DNA is first replicated by host enzymes to give the supercoiled double-stranded replicating form (RF). Early after infection (*left*), the (+) strand is cleaved by the phage-coded gpA, and rolling circle replication is initiated. One cycle of replication produces one SS and one RF DNA. At late times, interaction with gpC initiates rolling circle replication, but here the SS DNA is packaged into virions. In early replication, SS DNAs are coated with the host single-strand binding protein Ssb; in late replication, they are coated with gpC until packaging.

Filamentous DNA Phages

The virions of filamentous phages, of which phage fd is the prototype, differ from those of most helical viruses in that the capsid surrounds a DNA molecule that is not linear but circular and therefore is doubled back on itself, although unpaired. The replication cycle of the filamentous DNA phages is similar to that of the isometric phages. Infection is initiated by adsorption of the end of a virion to the terminus of an F-coded pilus, used during bacterial conjugation. The details of DNA entry are obscure. As with f×174, ssDNA formed at late times is coated with a phage-specified DNA-binding protein (gp5) that directs the DNA to packaging rather than to recycling. Packaging is concomitant with egress. The major coat protein (gp8) is embedded in the cell membrane. As phage DNA enters the membrane, gp5 is exchanged for a helical capsid of gp8. Subunits of gp8 are proteolytically processed as they are added to phage DNA, and virions are extruded through the cell envelope without loss of cell viability. Except for a few enveloped phages, these are the only phages whose egress does not require lysis.

The filamentous phages (especially M13) are popular as cloning vectors because of their high yields, the absence of a packaging limit, and the single-strandedness of the product. Cloning is generally accomplished through cutting and ligation of RF DNA, followed by transfection. Filamentous phages have also had extensive use in phage display libraries.

RNA Phages

The common RNA phages have isometric T=1 virions, each containing a single linear single-stranded RNA molecule about 4 kb in length, encoding three to four genes and capable of assuming extensive secondary structure. They enter bacterial cells by attachment to the sides of F pili (rather than the ends, as with the filamentous DNA phages). Like the filamentous phages, they are only able to infect bacteria that harbor the F plasmid. The four genes of phage MS2 encode the coat protein (C), an RNA-dependent RNA polymerase (P), a lysin (L), and a fourth protein (A, for assembly) present in one copy per virion. The genes are arranged in the order 5'A-C-(L)-P3', where the L gene overlaps the 3' end of C and the 5' end of P.

In these phages, there is no known distinction between replication and transcription. All RNA copies are full length, and gene expression is controlled at the translational level. Nevertheless, they have developed a program of temporal control exquisitely tailored to their needs. The *A* gene at the 5' end is available for translation only in nascent RNA that has not yet folded into its most stable secondary configuration, a reasonable strategy for obtaining one copy per virion. The *C* gene, on the other hand, is available for translation at all times. The *P* gene is under a more complex control. Translation of the *C* gene is needed to open the secondary structure that otherwise sequesters the *P* ribosome binding site; but coat protein itself, binding to the RNA upstream of the *P* gene, inhibits polymerase translation. The first control delays the onset of phage replication whenever conditions for protein synthesis are poor; the second means that, as with T4 or T7, synthesis at late times is diverted to virion components (in this case, coat protein), with minimal competition from polymerase synthesis. The *L* gene lacks a ribosome binding site of its own. Lysin synthesis results from reattachment of ribosomes that initiated at the ribosome binding site for *C*, then disengaged as a result of high-frequency frameshifting and reattached at the AUG for L (41). By tying the accumulation of lysin to the rate of coat protein synthesis, this

arrangement should help coordinate the time of lysis with the rest of phage development.

In broad outline, the mechanism of RNA synthesis is similar to that of most linear single-stranded RNA viruses. RNA phage replication enjoys the distinction of being the first system in which *in vitro* replication of a nucleic acid was shown to initiate at a specific origin (33). In conjunction with three host proteins (elongation factors Tu and Ts and ribosomal protein S1, all part of the machinery for host protein synthesis), the phage polymerase initiates replication at the 3' end of a (+) strand. Generally, several subsequent initiations take place at the same end before the first (–) strand is completed. When completed (–) strands dissociate from their (+) strand templates, they are used in a similar manner as templates for (+) strand synthesis.

Because of their small size and rapid mutation rate, RNA phage genomes have been used in studies of *in vitro* evolution, where enzyme is supplied and those RNA molecules structurally capable of the most rapid rate of multiplication are selected (37). The most consistent result was selection for much smaller molecules, which always include the terminal sequences recognized by the polymerase. The results are instructive with respect to the defective interfering particles that accumulate *in vivo* during the high-multiplicity passage of many viruses. In the absence of added template, the replicase can also create short sequences *de novo* and copy them.

TEMPERATE PHAGES

Temperate phages are mostly large (>20 kb) double-stranded DNA viruses. Three prototypes can be distinguished based on their modes of establishment of lysogeny. The first, represented by coliphage l, inserts DNA into the chromosome at one or a few preferred sites. The second, represented by phage Mu-1, inserts it anywhere in the host chromosome by use of a phage-coded transposase. In the third, represented by phage P1, prophage DNA is not inserted into the chromosome but instead is maintained as a plasmid. Some other phages, such as satellite phage P4, can be maintained either as inserted prophages or as plasmids. The inserted state can be stably maintained only if the phage genes for autonomous replication are repressed, whereas in the plasmid state some viral functions are expressed without killing the cell.

Phage l

The development of l has become a paradigm for gene control in developing systems. An infected cell has one of two options: either to lyse and produce viral progeny or to survive as a lysogen. Once a cell is committed to one of these pathways, there are several safeguards to stabilize the decision.

The l virion is isometric, with a T=7 configuration of capsomeres and a long tail terminating in a single fiber. The virion DNA is a 48,502-bp linear molecule with complementary 12-base 5' overhangs. Once injected into the cell, the DNA ends pair and are ligated by host ligase to generate a covalently closed circle. Figure 5 shows a genetic map of l in its circular form.

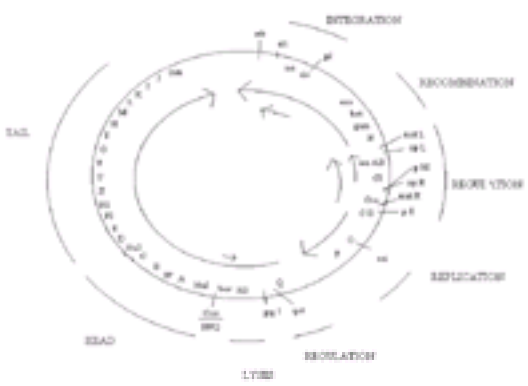

FIG. 5. Genetic map of l in its circular (replicating) form. Genes are shown inside the circle; sites are outside. Transcripts are shown inside the circle. Genes are clustered according to function in the phage life cycle. The *bor* and *lom* genes affect host phenotype in lysogens. Functions of most other phage genes and sites are discussed in the text. Some genes with minor or unknown functions are not shown.

Commitment

After infection, host RNA polymerase initiates transcription at three promoters: *p*L, *p*R, and *p*R'. The *p*L and *p*R transcripts each contain one gene (*N* and *cro*, respectively). The *p*R' transcript contains no genes. The Cro protein binds to the operators controlling *p*L and *p*R and represses transcription from them; however, because *cro* itself is transcribed from *p*R, the Cro concentration never reaches a level where repression is complete. The gpN protein is an antiterminator, which allows transcription at *p*L and *p*R to read through ordinary termination signals. The seminal early observations leading to this conclusion were, first, that the l gene *exo*, which is downstream of *N*, requires for expression both a functional gpN (suppliable in *trans*) and a derepressed *p*L (in *cis*), and second, that terminators of ordinary bacterial operons such as *trp* are read through when placed downstream of *p*L in the presence of gpN (28,48).

Antitermination by gpN is effected by binding to specific RNA sequences (*nut* sites) that are generated early in the transcripts from *p*L and *p*R. The *nut*R RNA includes a binding site (*boxA*) for host protein NusA, and a stem-loop sequence (*boxB*) specific for l's gpN. NusA (the principal elongation factor for *E. coli* RNA polymerase) is the product of one of the *nus* genes, isolated by screening for mutants with an "*N* undersupply" phenotype, so that even lN^+ behaves

like an N⁻ mutant on *nus* mutants. Various l-related phages have distinct N specificities determined by the sequence in the *boxB* loop. The gpN–NusA complex bound to *nut* RNA interacts with the RNA polymerase that has passed the *nut* site and is transcribing downstream from it and modifies transcription so that normal termination signals are no longer recognized. The complex N⁻NusA–RNA polymerase –nut^R site is stabilized by interaction with other host factors: NusB, NusG, and ribosomal protein S10 (50).

The result of antitermination is that both leftward and rightward transcription proceed beyond the primary terminators (*t*L1 and *t*R1, respectively). The leftward transcript includes several genes whose functions are not essential to either lytic or lysogenic development, although some of them play ancillary roles in DNA replication (*exo*, b) or lysogenization (*cIII*). The rightward transcript includes *cII* (whose product promotes lysogenization), replication genes *O* and *P*, and gene *Q*, the antiterminating positive regulator of late transcription.

The CII protein is a positive effector of transcription initiating at site pE within *cII* and proceeding leftward beyond *p*R through genes *cI* and *rexAB*. The CI protein (repressor) binds to the same operator sites as Cro and represses transcription from *p*L and *p*R, thus stopping all transcription characteristic of the lytic cycle.

If CII were formed at high concentration in all infected cells, they all should become lysogenic. In fact, among cells under the same ambient conditions, some commit to lysis and others to lysogeny, presumably because of stochastic fluctuations in early molecular events. Mathematical modeling with measured kinetic parameters adequately predicts the observed proportions of cells committed to the two pathways as a function of the phage-to-bacterial ratio (6). The critical biochemical players are CII (which is metabolically unstable because of proteolysis; CIII (which stabilizes CII in a manner dependent on the phage-to-bacterial ratio); and Cro, which accumulates with time and represses synthesis of CII. Cro accumulation thus functions as a developmental clock; when its concentration becomes high, CII decays and the cell is committed to lysis.

In those cells where repression is established, further events are needed to ensure stable lysogeny. First, repressor synthesis must continue in the lysogen so that the lytic cycle functions are shut off permanently. Once repression is established, *p*R transcription stops and CII disappears. Repressor does not disappear as well, because *cI* transcription commences from the maintenance promoter pM immediately to the left of *o*R.

The *o*R and *o*L operators are tripartite, with three binding sites separated by short spacers. Both repressor and Cro recognize these binding sites, but with different relative affinities. Cro binds most tightly to *o*R3 (which represses leftward transcription from *p*M) and less tightly to *o*R2 and *o*R1 (repressing rightward transcription from *p*R). Repressor, on the other hand, binds tightly and cooperatively to oR2 and oR1, which both represses transcription from pR and stimulates transcription from pM. At high repressor concentration (unsustainable in the steady state) repressor also binds to oR3 and represses its own synthesis (Table 1). Thus, once a high concentration of repressor is achieved through transcription from pE, repression is self-sustaining (58). The inhibition by Cro of spontaneous initiation at pM reinforces the decision to follow the lytic pathway.

Leftward transcription from pM	oR3	oR2	oR1	Rightward transcription from pR
(N+)				(Cro+)
Low	–	–	–	On
On	–	R	R	Off
Off	R	R	R	Off
Off	C	–	–	On
Off	C	C	–	Off
Off	C	–	C	Off
Off	C	C	C	Off

Source: Adapted from ref. 51, with permission.

TABLE 1. *Gene regulation by repressor (R) and Cro (C) at one oR*

Commitment to lysogeny requires not only repression of lytic cycle genes but also insertion of phage DNA into the host chromosome. Insertion is mediated by the phage-coded integrase (Fig. 6). The structural gene for integrase, *int*, is transcribed from two promoters: the major leftward promoter *p*L and a CII-activated promoter *p*I. The latter is the important promoter for lysogenization. Its control by CII ensures that insertion occurs in those cells where repression is established.

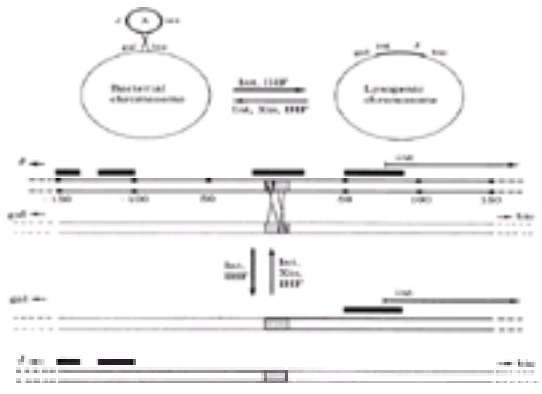

FIG. 6. Insertion of l DNA into the bacterial chromosome. **Top:** Overall process. **Bottom:** Detail of action at the crossover

point. The 15-bp identity between phage and host DNA is *stippled.* The *heavy bars* overlying the phage DNA are Int-binding sites. The 3' end of the *int* gene overlaps the right site. (Reproduced from ref. 14, with permission.)

Integrase promotes reciprocal recombination between sites on phage and host DNA, which inserts the phage DNA into the continuity of the host chromosome. The recombination occurs within a 15-bp sequence that is identical in phage and host. Both *in vivo* and *in vitro*, the minimal length of specific sequence required in the *attB* partner is 21 bp, and that in the *attP* partner is about 240 bp. A linear *attB* molecule functions *in vitro*, but *attP* must be supercoiled for optimal activity.

Integrase has two DNA binding sites. The strong binding sites (in the N-terminal part of the molecule) recognize arm sites, which are located on *attP* at positions –130, –80, +60, +70, and +80 (where 0 is the center of the 15-bp identity) (Fig. 7). Integrase molecules bound to these sites on DNA, bent appropriately by the host-encoded DNA bending integration host factor (IHF) protein, are so positioned that they can bind (in their C-terminal domains) to core sites, which are symmetrically disposed about the crossover point and approach the consensus 5'CAACTTNNT3'. Core sites are present in both *attP* and *attB*. The actual crossover then takes place by an exchange on the left between the “top” strands of the two double helices to create a Holliday junction, and resolution by exchange between the bottom two strands. The nicking site in the top strands is 7 bp to the left of the nicking site in the bottom strands. The process was once imagined to require branch migration of the Holliday junction between the two sites. However, recent data favor strand swapping between nicking and sealing, so that the Holliday structure exists only at the midpoint between the two sites (55). The left-right orientation here is determined by the positions of the arm sites on the *attP* partner, not by the core sequences themselves (41). Host and phage sites are aligned by protein–protein and DNA–protein interaction, and DNA–DNA recognition is restricted to the 7-bp overlap segment where branch migration occurs.

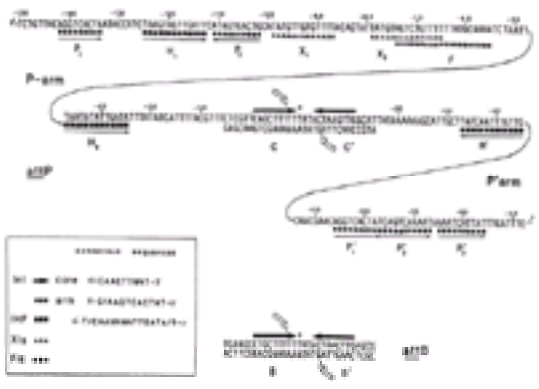

FIG. 7. Sequence of l *attP* and l *attB*, showing binding sites for proteins required for insertion and/or excision. P_1, P_2, P_1', P_2', P_3' (arm binding sites for Int); C, C', B, B' (core binding sites for Int); H_1, H_2, H' (IHF binding sites); X1, X2 (Xis binding sites); FI (Fis binding site). *Curved arrows* indicate portions of strand exchange in core. (Reproduced from ref. 43, with permission.)

Commitment to lysogeny is further reinforced by a third CII-activated promoter *p*AQ, located within gene *Q* reading leftward. By inhibiting *Q* translation, this antisense transcript apparently minimizes *Q*-promoted late gene transcription characteristic of the lytic cycle.

Lysogeny

Once established, the lysogenic state is quite stable. Lysogenic cells divide to give lysogenic progeny. Lysogeny can break down either through spontaneous switching to the lytic cycle (which happens about once every 10^4 cell divisions in an actively growing culture) or by spontaneous loss of prophage with cell survival (which happens less than once every 10^6 cell divisions). As long as repressor is present, *cI* transcription from *p*M continues, and more repressor is made. If repressor concentration rises so high as to saturate *o*R3, then repressor synthesis ceases until it returns to a lower level. Likewise, once the prophage is stably inserted into the chromosome, it remains so; there is a low rate of integrase synthesis from the *p*I promoter (not activated by CII) but essentially no excisionase (a phage-coded protein required for excision, though not for insertion).

Some ancillary factors reinforce this stability. One of these is the need for antitermination by gpN to initiate lytic development. This requirement puts a double lock on the system, because any transcripts made during a momentary unblocking of the operator sites will not get anywhere until the gpN that has been made by the first transcript can extend other transcripts. Because gpN is unstable and accidents should be rare, this will seldom happen. Stabilization of lysogeny is one reason that has been suggested for the use by l of antitermination (rather than simply mutating the termination sites). Poising the lysis/lysogeny decision to depend on molecular fluctuations may be another reason.

Escape from Lysogeny

About once every 10^4 cell divisions, stability breaks down. Detailed knowledge of what happens in those rare cells is unavailable, but the rate is strongly depressed in *recA*$^-$ hosts. This fact suggests that these spontaneous transitions to lytic development have the same primary basis as the mass induction of this transition by exposure to ultraviolet light or other DNA-damaging agents. In that case, the initial event is the activation of the proteolytic activity of RecA protein by products of DNA degradation, such as single-stranded DNA. RecA is not a conventional protease, but it accelerates the

specific cleavage of the host LexA protein, which can autocatalyze the same cleavage under extreme conditions. LexA is a repressor for many DNA repair functions, and RecA-promoted LexA cleavage turns these genes on. Like LexA, l repressor is sensitive to RecA-enhanced autoproteolysis (45).

RecA activation is one means of inducing the switch to lysis in almost all cells of a lysogenic culture. Another method makes use of l *cIts* mutants, which form a thermolabile repressor. With an appropriate mutant, lysogeny is quite stable at 30°C, but a shift to 43°C rapidly inactivates repressor in all cells. After derepression, transcription initiates from *p*L and *p*R. One early consequence of *p*L transcription is excision of the prophage from the chromosome. Whereas insertion requires only one phage-coded protein, integrase, excision requires a second protein, excisionase, as well. Excisionase is encoded by a gene *xis*, upstream from *int* and overlapping it by 20 bp. The CII-dependent *p*I promoter used during lysogenization lies at the 5' end of *xis*, and the start site is within *xis* so that the *p*I transcript makes only integrase. From the *p*L transcript, *int* and *xis* are expressed coordinately.

The mechanism of excisionase (Xis) action is not fully understood. Excisionase binds to two specific sites in the left arm of *attR*, bends the DNA, and interacts with integrase; one of the two excisionase molecules can be replaced by a host DNA-bending protein, Fis. Conventional enzymes are pure catalysts, and the nature of the catalysts cannot determine the direction of the reaction. Superficially, excisionase appears to reverse the integrase reaction; however, in excision as in insertion, cutting and resealing on the left precede cutting on the right; therefore, excision is not a true reversal of insertion. What remains unsolved is why integrase alone fails to promote the true reversal.

The *p*L transcript is subject to posttranscriptional control. In infected cells, either before commitment or after commitment to lysis, *int* and *xis* are cotranscribed; however, little integrase is made. This is because of exonucleolytic (3' ® 5') degradation of the transcript after cleavage by host enzyme RNase III at a site (*sib*) downstream of *att*. This process (retroregulation) prevents untimely or wasteful synthesis of integrase. Retroregulation does not affect *p*L transcripts made from a derepressed prophage, because the *sib* site is at the other end of the prophage. It also does not affect *p*I transcripts, which terminate within *sib* and therefore are resistant to RNase III. Because the *p*L transcript is made by an RNA polymerase molecule operating in the antitermination mode and reading through *sib*, it is degraded; this is another function for antitermination in the l life cycle. Despite the elegance and apparent utility of the retroregulation mechanism, deletion or mutation of the *sib* site has no observed effect on l development as normally studied in the laboratory.

The potentiality of controlling insertion and excision differentially provides a rationale for the use of arm sites by l integrase. Some related site-specific recombinases recognize only core sites, but in those cases there is no recognition of directionality.

When lytic development is induced in a lysogen, a small fraction (about 10^{-5}) of the particles produced result from abnormal excision that follows from breakage and joining of DNA at sites other than the *att* sites. If the excised DNA includes the *cos* sites and is within the packaging limits of the l virion, it can be packaged and used to infect other cells. Thus, a lysate produced by induction can transfer host genes that lie close to the l insertion site (such as *gal* or *bio*) into recipient cells. This specialized transduction is a form of natural cloning. The transduced bacterial DNA segment need not replace its homolog in the recipient; instead, the cell becomes lysogenic for the specialized transducing phage and is diploid for the transduced segment (Fig. 8 and Fig. 9).

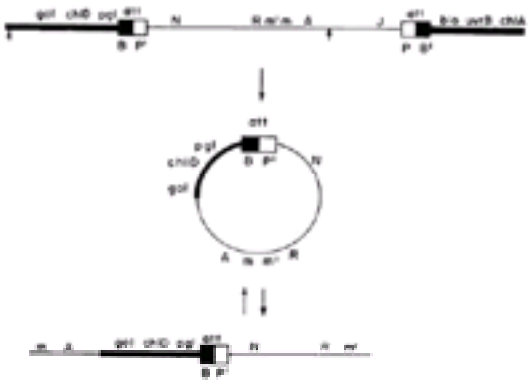

FIG. 8. Genesis of l *gal* specialized transducing phage. *Arrows* indicate positions of breaking and joining of heterologous DNA in a particular isolate. The m and m' indicate the ends of mature l DNA (equivalent to *cos*). (Reproduced from ref. 14, with permission.)

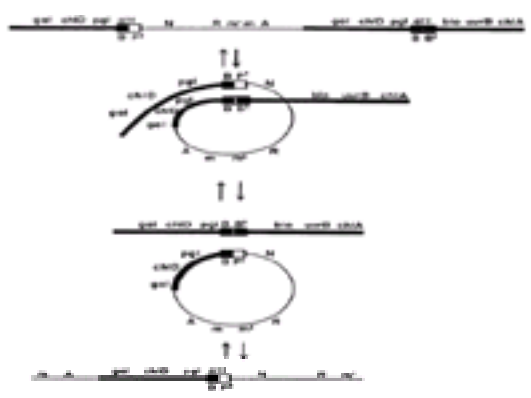

FIG. 9. Lysogenization by l *gal* (*bottom line*) to produce a heterogenote with two copies of *gal* separated by l DNA. (Reproduced from ref. 14, with permission.)

The genesis of specialized transducing phages may be comparable to the acquisition of oncogenes by retroviruses. Although the mechanisms may differ substantially, specialized transduction has provided a useful model because the entire process can be followed in the laboratory. With retroviruses, until very recently the critical events have generally occurred in nature from progenitors of inferred structure.

Lytic Development

Lytic development can be studied either in infected cells or in derepressed lysogens. Because of the high degree of synchrony obtainable, the method of choice has usually been thermal induction of lysogens carrying *cIts* prophages. The genetic program and its readout are well understood. At early times, most transcription is from *p*L and *p*R. The only protein of the *p*L transcript that is essential for lytic development is gpN. The pR transcript contains three important genes: *O* and *P* (replication) and *Q* (late gene activation). As time proceeds, gpQ accumulates and causes antitermination of the short transcript starting at *p*R' (downstream from *Q*). The mechanism of antitermination differs from that performed by gpN. The recognition elements for antitermination overlap the promoter. *Q* antitermination also requires a pause site close to the start site. The gpQ binds to the complex of DNA and paused s70-containing RNA polymerase, and it replaces s70 in the complex. The gpQ-containing polymerase then transcribes downstream DNA without stopping at ordinary termination signals (60).

At any rate, as gpQ accumulates, transcription of genes for lysis and virion formation becomes increasingly rapid. Concurrently, the rate of transcription from *p*L and *p*R decreases as Cro accumulates, producing an orderly transition from early gene expression to late gene expression.

Bidirectional DNA replication initiates from an origin within gene *O*. The gpO protein recognizes the origin and also recognizes gpP, which in turn recruits the host DnaB helicase into the initiation complex. Disassembly of the initiation complex to leave an elongation complex requires three host heat shock proteins (DnaJ, DnaK, and GrpE). Elongation proceeds, as in the host, by the combined action of DnaB helicase, DnaG primase, and Pol III holoenzyme, the polymerase. Theta-form replication can reproduce monomer circles, but a switch to rolling circle replication occurs in some molecules. l rolling circle replication (which predominates at late times) differs from that of fX in two major respects. First, the tails are double stranded rather than single stranded because lagging-strand synthesis takes place on the displaced strand. Second, tails can grow to multigenomic lengths rather than stopping when a single genome has been spun off.

Multigenomic tails are the primary substrates for l packaging. Empty proheads are first assembled, composed mainly of the major capsid protein gpE. Later, DNA enters the prohead, which increases in size and becomes more conspicuously icosahedral, and the smaller decoration protein gpD is added to the shell, with gpD hexamers interspersed among gpE trimers in a T=7 arrangement.

Efficient DNA packaging requires the presence of specific sequences (called *cos*, for cohesive site) at both ends of the packaged genome; hence, multigenomic tails or multimeric circles are good packaging substrates and monomer circles are poor ones. Cutting at *cos* is effected by a heterodimeric protein, terminase, whose subunits are encoded by l genes *Nul* and *A*. DNA molecules are packaged from left to right so a DNA monomer can be cut from the concatemer at its left end early in packaging and at its right end after the DNA has entered the virion. The *cos* site (about 200 bp in length) has three components: a binding site (*cos*B), recognized by gpNul before the DNA is cut at either end; a nicking site (*cos*N), at which DNA is cut at sites 12 bp apart in the two strands to generate the packaged single-strand overhangs of virion DNA; and a termination site (*cos*Q), required for the final nicking at the right end (20). Packaging is processive. After one genome is packaged, the next genome to the right can be packaged with no requirement for recognition of *cos*B on the second or subsequent genomes (27).

Because l packages the DNA between two *cos* sites, the amount of DNA per head can be changed by deletions or insertions (as in T7). The packaging limits are from about 79% to 110% of normal l length. Internal *cos* sites artificially placed within l at less than or near the lower limit can be packaged uncut, as though scanning for *cos* sites becomes more efficient as the head fills. l-based *in vitro* packaging systems have been used for efficient delivery of *cos*-containing plasmid-cloning vectors (cosmids) into *E. coli* cells.

Table 2 indicates the packaging limits for some phages described in this chapter. Those with headful packaging are listed with the same upper and lower limits. In fact, there is some random variation around the mean value. Alternative forms with different head sizes, such as the isometric and giant particles of T4 or the l virions formed by D $^{-}$ mutants, are not considered.

Phage	Packaging substrate	First cut	Second cut	Packaging limits (% of genome)
T4	Concatemer	Random	Head(full	102
P22	Concatemer	pac site	Head(full	109
λ	Concatemer	cos site	cos site	78-110
T7	Concatemer	Terminal overlap	Terminal repeat	85-103
φX174	Dimer ss of rolling circle	Replication origin	Replication origin	Limited
M13	Dimer ss of rolling circle	Replication origin	Replication origin	Unlimited
Mu-1	Inserted monomer	Near left end	Head(full	105
P1	Concatemer	pac site	Head(full	110

TABLE 2. *DNA cutting and packaging*

Genome Organization

The functional clustering of genes on the l map is notable. Not only do genes acting in a single pathway (head formation, tail formation, replication) lie together on the map, but genes for DNA binding lie close to their target sites, products of adjacent genes in a cluster frequently interact directly, and even within genes determinants are ordered so that they lie close to the elements with which they interact. For example, the *cos*B site is at the end of *cos*, next to the *Nul* gene whose product recognizes *cos*B; *Nul* in turn lies next to *A*, whose product forms the terminase heterodimer with gpNul. In both head and tail clusters, the same tendency is observed, the tail fiber gene *J* being most distal from the head cluster. The *att* site is adjacent to the *int* gene, and *cI* and *cro* are close to their target sites. The N-terminus of gpO recognizes the *ori* site that lies within the 5' end of *O*, whereas the C-terminus interacts with the product of the adjacent *P* gene. The clustering of major functional groups could increase regulatory efficiency, but the finer orderings may be more related to the disruptive effect of recombination on coevolved functions.

Phage Mu-1 as a Model Transposon

Mu-1 first attracted attention as a phage capable of inducing bacterial mutations at a high rate. The mutations proved to be insertions of Mu-1 into random sites on the host chromosome, disrupting the genes or operons into which it inserted.

Like l, Mu-1 can follow two alternative developmental pathways, leading on the one hand to a transcriptionally quiescent prophage and on the other to a lytic cycle with replication, packaging, and lysis. In l, the DNA transactions in these two pathways are distinct. Insertion plays no role in l replication, which proceeds normally if either the *int* gene or the *att* sites are deleted. With Mu-1, on the other hand, replication and insertion are minor variations of a common pathway, transposition. In Mu-1 replication, transposition happens many times during each cycle of infection, which has made it a system of choice for studying the biochemistry of transposition (compared with most bacterial transposons, which transpose naturally at rates such as 10^{-6} per cell division).

Mu-1 DNA is inserted into host DNA at all stages of its life cycle, even in the virion. When linear DNA from the virion is injected into a cell and the Mu-specific transposase is made, the first step in replication is transposition of Mu-1 DNA into the chromosome. Among bacterial transposons, the transposition mechanism may be either replicative (generating transposon copies in both the donor and the target site) or conservative (excising the transposon from the donor and inserting it into the target site). In cells destined to become lysogenic, the initial transposition is conservative rather than replicative. In cells destined to lyse, Mu-1 undergoes repeated rounds of replicative transposition.

The initial steps of replicative and conservative transposition are the same. The 3' end of the two transposon strands undergo strand transfer reactions with target DNA at positions displaced by five base pairs. Replication then initiates from the recessed 3' ends of the target DNA. In conservative transposition, the nicked donor DNA adjacent to the transposon is cut in the other strand and digested away; replication fills the gap, and ligation incorporates the parental transposon bodily into the target site (Fig. 10). In replicative transposition, the donor strands remain attached to the 5' transposon ends, and replication proceeds through the transposon with ligation to the free 5' donor ends at the opposite end of the transposon. This produces two semiconserved transposon copies, each joined to donor DNA at one end and target DNA at the other (52). Replicative transposition from a chromosomal donor produces two inserted copies accompanied by chromosomal rearrangement (inversion or excision); if replicative transposition happened from a linear infecting molecule (unaccompanied by subsequent recombination), it would linearize the chromosome.

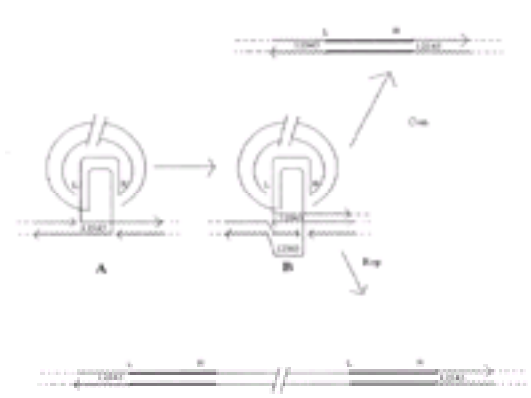

FIG. 10. Transposition of Mu phage. **A:** The stage immediately following strand transfer. **B:** The oligonucleotide sequence (12345) of the target molecule has been replicated. In conservative transposition (Con), the donor DNA is digested away and the Mu DNA is inserted into the target DNA, flanked by a direct oligonucleotide repeat; this is the usual fate of infecting DNA, where the donor is linear DNA (Mu and some flanking host sequence). In replicative transposition (Rep), replication from the 3' ends shown in (**B**) continues through the Mu, terminating with ligation to the free 5' end of donor DNA. This is the mechanism of Mu replication in the infected cell, where donor and target DNAs (both circular) are derived from the host chromosome. Mu DNA, with left and right ends indicated; , donor DNA; , target DNA.

In Mu-1 packaging, the left end of the inserted DNA is recognized, and cutting takes place within the flanking DNA, 50 to

150 bp to the left of the left end. Packaging is then by headfuls, and the headful length is longer than the genome length. Therefore, 500 to 3,000 bp of DNA on the right end of the virion molecule is host DNA. Thus virion DNA molecules have random segments of host DNA at their termini. When virion DNA is separated into single strands and reannealed, the host sequences are visibly unpaired when viewed in the electron microscope.

An internal segment of Mu-1 DNA about 3 kb in length is invertible through the action of a phage-coded integrase, so reannealed molecules are sometimes unpaired in this segment as well. The gene for the tail fiber protein spans one boundary of the invertible segments so that two types of tail fibers with different host specificities can be formed, depending on the orientation. Thus, if Mu-1 grown on *E. coli* K-12 encounters an alternative host such as *Citrobacter*, the rare particle that attaches to *Citrobacter* can invade and replicate. The rate of inversion is about 10^{-5} per generation. Certain other phages, such as P1, have related invertible segments.

In lysogens, inserted Mu-1 can occasionally transpose without induction of a full lytic cycle. Thus, inserted Mu-1 behaves in all respects as a bacterial transposon.

Phage P1 as a Model Plasmid

The P1 virion contains about 100 kb of linear double-stranded DNA in an isometric T=16 capsid. As in T4, the DNA is cyclically permuted and terminally redundant. Unlike T4 (but like *Salmonella* phage P22, a relative of l), processive packaging does not initiate at random but instead at specific *pac* sites. After infection, reciprocal recombination within the terminal overlap creates a circular DNA molecule of about 90 kb. Some of this recombination is mediated by a site-specific recombinase (Cre) whose target site (*lox*) is close to the *pac* site. The Cre–*lox* system has proven useful for generating controlled deletions and rearrangements during development of multicellular eukaryotes.

The cell can now enter either a lytic cycle or a lysogenic cycle. Unlike l or Mu-1, the lysogenic alternative entails replication of the circular P1 DNA as a plasmid and therefore requires expression of phage-coded replication genes. In lysogens, P1 is maintained at low copy number (about one per chromosome) and has an efficient partitioning mechanism so that both daughters of a dividing cell receive a P1 copy. The mechanism is similar to that used by other stringently controlled low copy number plasmids, such as the fertility factor F, and in some respects to that of the bacterial chromosome. The partitioning mechanism could potentially become ineffective if homologous recombination created a dimeric molecule, which could not pass into both daughter cells; this problem is greatly diminished by the site-specific *lox*–Cre recombination, which rapidly and reversibly equilibrates monomers with dimers. The elements for replication and partitioning (except *lox*–Cre) are localized in 1.5 kb of P1 DNA that can replicate as a plasmid when circularized; it includes a specific replication origin (*oriR*), its cognate replication initiator (*repA*), and a DNA sequence (*incA*) that controls replication by binding and sequestering *repA* (72).

In cells destined to lyse, P1 DNA replication (like that of l) proceeds in both a theta form and a rolling circle mode, the latter providing the packaging substrate. The replication origin used is distinct from the plasmid origin *oriR*. Rare mistakes lead to occasional packaging of random fragments of host DNA instead of phage DNA. Such fragments are responsible for the generalized transduction of bacterial genes. Generalized transduction at detectable frequencies is observed for most phages that use headful packaging, but not by wild-type l, probably because in l both cutting (at both ends) and injection require the specific *cosN* sequence, whereas in P1 or P22 initial cutting at sequences resembling *pac* sites can initiate processive packaging. Mutations that relax the specificity and thereby increase the frequency of transduction have been isolated in both P22 and P1 (63,71).

DEFECTIVE PHAGES AND PHAGELIKE OBJECTS

The fact that Mu-1 can behave as a transposon and P1 as a plasmid underscores some of the commonality between phages and nonviral elements. Transposable elements are common in bacteria, and most of them seem to use a biochemistry similar to that of Mu-1. Most bacterial transposons are smaller than Mu-1, going down to simple insertion sequences a few hundred base pairs long; and typical transposons carry genes affecting conspicuous bacterial traits such as antibiotic resistance (15). Transposition is usually conservative for some elements, replicative for others. Those using replicative transposition frequently resolve the resulting cointegrate structures through use of a site-specific recombinase that acts on a sequence internal to the element. Some transposons integrate and excise by a recombination mechanism similar in some respects to l insertion (64).

Bacterial plasmids fall into two general groups: elements with small genomes, present in many copies per cell and replicating randomly with respect to the cell division cycle, and larger elements similar in size to phage P1, present in one or a few copies per cell and partitioned regularly at cell division. Conjugative plasmids resemble viruses as autonomous elements capable of infecting cells, but they differ from them in the absence of an extracellular phase.

DNA sequences closely related to those of temperate phages are found in the genomes of many natural bacteria. For example, Southern hybridization of enteric bacteria with l DNA usually turns up some l-related sequences (59). Most of these are probably defective prophages: remnants of previous lysogenization that have since lost phage function through deletion or mutation. The K-12 strain of *E. coli* is naturally lysogenic for l but also harbors four l-related elements: DLP12, Rac, e14, and Qin. Such elements could in principle be of some value to the host or could even be precursors rather than descendants of complete phages; however, each defective prophage contains an array of genes from different functional clusters of the phage genome, frequently in the same order found in active phages, so that a role as host elements seems contrived. Defective lysogens arise from active lysogens in the laboratory by prophage mutations, and they frequently retain superinfection immunity, SOS-induced lysis, and production of structures resembling phage heads, tails, or complete virions.

Many natural strains liberate (or produce on induction) such phagelike or phage-related particles, which have not been shown to be infectious. Many bacteria also produce proteins (bacteriocins) that are toxic to other strains of the same bacterial species but not to the producing strain. Bacteriocins are sometimes encoded on plasmids and act through

diverse mechanisms. Operationally, some phagelike objects qualify as bacteriocins.

For example, many natural strains of *Bacillus subtilis* and several other *Bacillus* species harbor a prophage-like element, phage of *Bacillus subtilis* (PBS), that has been localized on the chromosome. After SOS induction, the cells lyse and liberate many virion-like particles with tails and DNA-filled heads. This is not simply a phage whose sensitive host remains to be discovered, because the large majority of particles contain random segments of host DNA with no significant preference for packaging prophage DNA. Like a bacteriocin, these particles kill other *Bacillus* cells. The producing strain is resistant, at least sometimes because the particles fail to be adsorbed. There are several varieties of PBS, each with a different killing specificity (76).

PBS has some similarity to mutants of generalized transducing phages that have lost (in this case, completely) their extreme preference for initiating packaging at phage-specified *pac* sites. Its wide distribution and the variety of killing specificities suggest that the bacteriocin-like activity confers a selective advantage to the host. Inasmuch as some bacteriocins are single protein molecules, a DNA-filled virion seems an especially clumsy and expensive kind of bacteriocin to have been so successful in evolution.

EVOLUTION AND NATURAL BIOLOGY OF PHAGES

For historical reasons, the coliphages have become workhorses of molecular biology and remain among the most thoroughly understood biologic objects. Phages are found throughout the prokaryotic world in a variety comparable with that of eukaryotic viruses, including enveloped virions with double-stranded RNA genomes (69), linear DNA genomes with protein covalently bound to their 5' ends (61), and satellite viruses like P4 [which forms virions with the capsid protein of its helper virus P2, induces late gene function from P2 prophage, lysogenizes P2 lysogens using its own integrase but can establish a plasmid state in P2 nonlysogens, and represses its own genes by the use of antisense RNA rather than a protein repressor (45)]. Compared with our knowledge of phage development in infected cells, little is known about their natural ecology or population biology. Some aspects are worthy of note.

Abundance

In some natural environments, phagelike particles are much more numerous than one might suspect from plaque assays on susceptible hosts. In estuaries, concentrations as high as 10^8 have been reported. If most of these are infectious (and they may very well not be), they potentially could play a major role in the natural cycling of the biosphere (31).

Host Defense Mechanisms

Bacteria have evolved mechanisms for phage resistance, and phages have evolved counterstrategies, presumably indicating in some measure the significant impact that phage infection can have. A host defense mechanism unique to prokaryotes is restriction/modification. Restriction enzymes damage DNA that has not undergone chemical modification at specific sites (most frequently methylation) by cleaving it either at those sites or nearby; nonspecific exonucleases then degrade the resulting linear fragments. Cells protect their own DNA from the restriction enzymes they make by modifying it. A phage that escapes restriction becomes modified and is fully infectious on a restricting host in the next cycle; so the strategy can be effective for the host only if the strains sensitive to a given phage have various restriction specificities. A strain with a rare restriction specificity will exclude almost all phage it encounters and will be favored over one with a common specificity; so eventually the rare specificity will become common. The recognition sites for restriction enzymes are frequently symmetrical, even if the cleavage takes place at a variable distance from the recognition site; this allows the modification of either strand to prevent cleavage, which ensures that, during semiconservative replication, a new strand that has not yet been modified is protected from destruction.

Phages have many strategies for avoiding restriction. The first level is a paucity of recognition sequence. Phages of *Bacillus*, where many known restriction enzymes recognize four-base palindromes, have few such palindromes in their DNA; coliphages are deficient in six-base palindromes, which are sites for some of the enzymes they naturally encounter (38). Various phages also have enzymes that destroy 5-adenosylmethionine, a cofactor for restriction (T3), expedite modification of phage DNA before restriction enzymes can cleave (l), or block recognition sites by wholesale nonspecific DNA modification (T4).

Some restriction enzymes are encoded by chromosomal genes, but many are encoded by phages or plasmids. Many resistance mechanisms contributing to defense of the host may in fact be manifestations of competition between extrachromosomal elements preserving their cellular territories.

One element may exclude others by diverse mechanisms, and in many cases exclusion may be an incidental side effect of the phage life cycle. The repressors of temperate prophages (essential for the internal stability of lysogens) render the lysogens immune to superinfection by phage of the carried type, and this superinfection immunity probably selects for diversification of repressor specificities. Certain phages, such as the e phages of *Salmonella*, encode enzymes that alter the polysaccharide surface receptors used in phage attachment, so that lysogens not only are immune but also cannot be penetrated by DNA of the same type. The alteration also takes place during the lytic cycle and reduces phage loss from attachment to fragments of cell envelope after lysis. The l-related phage HKO22 has a variant of the l *N* gene that competes with l*N* when l infects an HKO22 lysogen and causes termination rather than antitermination of early l transcripts (55). The l Rex proteins trigger a collapse of membrane potential when a l lysogen is infected with certain other phages (such as T4 *rII* mutants), preventing the production of viral progeny (57). The Lit protease (encoded by the defective l-related prophage e14) is activated by the major head protein of T4 to hydrolyze its specific substrate, elongation factor Ef-Tu (30). These last three mechanisms do not protect the infected cell but may protect other cells in a colony by impeding viral replication.

Natural Recombination

Third, the study of natural isolates has provided some perspectives on variation and the recombination among phages in nature. The l-related (lambdoid) phages are a good example.

The way that phage workers have approached taxonomy differs substantially from the method used by many medical virologists. In medical virology, once a virus such as measles is identified as a disease agent, many isolates from different clinical sources are examined, and those sharing some acceptable degree of similarity to the type virus are classified as measles virus. For epidemiology, some such approach is necessary. Phage workers are not epidemiologists, and their interest in natural variation is relatively recent. Many investigators have been more interested in developing highly defined experimental systems. To that end, each isolate is given its own name. l refers only to the prophage present in *E. coli* K-12 and its laboratory descendants; K-12 refers to one bacterial isolate, not to other natural strains with similar properties. Workers with a different perspective might have classified all lambdoid phages as natural variants of l, and the same can be said of other phage groups such as T2, T4, and T6.

The lambdoid phages have a common genetic map and the ability to generate viable hybrids when crossed in the laboratory. They came from various sources (usually from lysogenic bacteria) isolated over the past 70 years from Europe, Asia, and the United States. Genetic variation among the lambdoid phages is apparent from both functional specificity and DNA sequence. For example, phages with at least a dozen repressor specificities have been isolated; where the sequences are known, homologies between heterospecific repressor genes are barely discernible. Phages with different repressors have corresponding differences in their *cro* genes and operator sites.

Lambdoid phages also exhibit major variations in virion structure. Those phages (such as l) that plate on *E. coli* K-12 have long, flexible tails terminating in a single fiber that attaches to the *malB* chemotaxis protein of the outer membrane. Phages such as P22 (from *Salmonella*) have short, stubby tails and attach to the lipopolysaccharide 0-antigen of smooth strains of *Enterobacteria*, including *E. coli* (22). And in certain phage heads (such as HK97's), morphologically similar to that of l, the major capsid proteins are covalently joined into multimers that are catenated to create an indissoluble chainmail configuration (24).

If the l sequence is compared with that of any other lambdoid phage (or, equivalently, if heteroduplexes of the two phages are constructed), the general result is that the sequences match closely in some parts of the genome, whereas homologies are weak or absent in others. This is not because some sequences are highly conserved, because another phage pair shows a different set of matching segments; instead, the whole pattern strongly indicates that it is generated by frequent natural recombination so that in any phage pair, some portions of their genomes are of recent common ancestry. When defective prophages are compared with known lambdoid phages, they likewise are closely related to different phages in different segments. For example, the defective prophage DLP12 has an *int* gene and a partially deleted *xis* gene related to phage P22, followed by segments homologous to l's *exo* and P/*ren* genes, followed by analogs (*qsr*') of l QSR unrelated to any known l phage, followed by *cos* DNA similar to l's. Thus, all the lambdoid phages, including the defective prophages, seem to be drawn from a common gene pool.

Among the lambdoid phages, some genes that may serve viral functions have been borrowed from outside the l pool. Gene 12 of P22 occupies the same position in the genome as l gene P, but gene 12 is a homolog of the bacterial *dnaB* gene, and l gpP recruits the host DnaB helicase to participate in phage replication. Wild-type l has a gene for side tail fibers (*stf*) that facilitate phage attachment. (In the l commonly used in laboratories, *stf* has been inactivated by a frameshift.) The *stf* gene is closely related to tail fiber genes of other groups of coliphages (32). Most lambdoid phages encode a true lysozyme that hydrolyzes glycosidic bonds; l makes instead a transglycosylase that attacks the same bonds but has no detectable homology to lysozyme (75). The l lysozyme may have been appropriated from some external source, as yet unidentified.

Sequence comparisons of a wide variety of phages and prophages indicate that most double-stranded DNA phage genomes are chimeric products of both ongoing and ancient gene exchange (34). Thus, even apparently unrelated phages seem to have occasionally drawn genes from a common pool.

The natural function of genetic recombination is not obvious. In l, as in sexual eukaryotes, it is clear that recombination has a substantial evolutionary impact. But that does not tell us at what level natural selection acts to preserve recombination as a process, or whether recombination is an accidental byproduct of gene activities selected to function in repair or replication. One classic argument for the value of recombination is that an asexual line deteriorates through accumulation of deleterious mutations, which can be corrected by recombination with other lines that are the wild type for the loci of those particular mutations (53). The first experimental demonstration of this Muller's ratchet effect came from phage f6 (18); corroborative evidence from animal viruses has since appeared (23). Muller's ratchet may be central to the evolution of certain human viruses such as influenza, where the clonally selected epidemic strains are eventually replaced by reassortants with avian viruses.

In their natural ecology, phages exhibit many of the same features observed in eukaryotic viruses, and they will probably find increasing use as model systems.

PHAGE-BORNE GENES FOR BACTERIAL TOXINS AND OTHER PROTEINS AFFECTING HOST PHENOTYPE

In the prototypical temperate phages (l, Mu), most of the genome (especially genes needed for lytic growth) is strongly down-regulated in the prophage. For this reason, lysogeny is generally not an overt property of a bacterial strain, and many natural bacteria harbor prophages that remain unrecognized unless they show inducible lysis (not seen with all temperate phages) or unless a phage-sensitive bacterial host is available. However, lysogeny by some phages markedly affects host phenotype—a phenomenon known as lysogenic conversion.

Lysogenic conversions frequently entail changes in the bacterial surface, and they sometimes affect the virulence of

pathogenic bacteria. Their existence raises the questions of how the relevant genes became phage-associated, what advantage (if any) they confer on the phage, and how expression of the acquired genes is influenced by the phage's biology.

Gene Acquisition

The genesis of transducing phages by abnormal excision (see Fig. 8) is one way that a phage may acquire host genes. This mode places the acquired genes next to the phage *att* site. Other genes (such as those for antibiotic resistance) may enter the phage genome in transposons. The phage-borne genes for some bacterial toxins (diphtheria, cholera) are adjacent to *att* sites, whereas others, such as Shiga toxin, are not.

Selective Value for the Phage

Some conversions (such as those described earlier by the l phages of *Salmonella*) may benefit a phage by altering phage receptors on the bacterial surface. Some lambdoid phages have a gene (*nmpC*) with homology and partial functional equivalence to the major *E. coli* outer membrane protein gene *ompC* (whose product is a receptor for many phages). NmpC may replace OmpC, altering the cell's adsorption characteristics. Both these changes affect the antigenic specificity of the lysogen and could influence its ability to survive in a human host.

Phage l makes two proteins (Bor and Lom) that may increase a lysogen's ability to colonize the human intestine, thereby indirectly expanding the phage's habitat.

Relation to Phage Biology

Most of the genes described previously are expressed in lysogens, using promoters distinct from those that transcribe phage genes. Thus the *bor* gene (see Fig. 5) is transcribed in the opposite direction from the l late operon in which it is embedded. Transcription of the genes for diphtheria and cholera toxins is controlled by host transcription factors and is responsive to cues such as iron concentration.

Unlike most other temperate phages, the cholera phage (CTXf) is a single-stranded filamentous DNA phage similar to M13. Toxin expression from the integrated prophages is controlled by host factors (ToxR and ToxT). However, when phage replication is induced, the excised phage genome makes large amounts of toxin independently of ToxR and ToxT. The genes for Shiga toxin are in a lambdoid phage downstream of pR' (see Fig. 5). Although they can be expressed in the prophage from an iron-regulated promoter, high-level expression requires induction of the phage lytic cycle with concomitant Q-mediated antitermination. Phage-mediated lysis may also promote the liberation of toxin into the medium (70). Thus the phage lytic cycle may contribute significantly to the pathogenesis of toxin-producing bacteria.

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CHAPTER 23 *Picornaviridae:* The Viruses and Their Replication

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The Picornaviridae are nonenveloped viruses with a single-stranded RNA genome of positive polarity. This virus family contains many important human and animal pathogens, including poliovirus, hepatitis A virus, foot-and-mouth disease virus, and rhinovirus. The name of the virus family was intended to convey the small size of the viruses (pico, a small unit of measurement [10^{-12}]) and the type of nucleic acid that constitutes the viral genome (RNA).

Picornaviruses have played important roles in the development of modern virology. Foot-and-mouth disease virus was the first animal virus to be discovered, by Loeffler and Frosch in 1898 (193). Poliovirus was isolated 10 years later (182), a discovery spurred by the emergence of epidemic poliomyelitis at the turn of the 20th century. Forty years later, the discovery that poliovirus could be propagated in cultured cells led the way to studies of viral replication (84). The plaque assay, an essential method for quantification of viral infectivity, was developed with poliovirus (80). The first RNA-dependent RNA polymerase identified was that of mengovirus (20), a picornavirus, and the synthesis of a precursor polyprotein from which viral proteins are derived by proteolytic processing was first identified in poliovirus-infected cells (297). The first infectious DNA clone of an animal RNA virus was that of poliovirus (259), and the first three-dimensional structures of viruses determined by x-ray crystallography were those of poliovirus (142) and rhinovirus (272). Poliovirus RNA was the first messenger RNA (mRNA) shown to lack a 5' cap structure (139,231). This observation was subsequently explained by the finding that the genome RNA of poliovirus, and other picornaviruses, is translated by internal ribosome binding (155,244), a process now known to occur on cellular mRNAs (158,195).

Because they cause such serious diseases, poliovirus and foot-and-mouth disease virus have been the best-studied picornaviruses. Research on poliovirus has led to the development of two effective vaccines, and it is likely that poliomyelitis will be eradicated from the globe within the first 5 years of the new millennium. The World Health Organization has established a future goal of cessation of vaccination, at which time all poliovirus stocks must be destroyed. As a result, all research on this virus will cease. Poliovirus is truly a virus with a brilliant past, but with no future.

CLASSIFICATION

The family Picornaviridae consists of six genera: aphthovirus, cardiovirus, enterovirus, hepatovirus, parechovirus, and rhinovirus (Table 1), which all contain viruses that infect vertebrates. Other picornaviruses of invertebrates, such as cricket paralysis virus and *Drosophila* C virus, have not been assigned a genus.

Genus	Number of serotypes	Type species	Other members
Aphthovirus	7	Foot-and-mouth disease virus O	Foot-and-mouth disease viruses A and C, Asia 1, SAT 1, SAT 2, SAT 3
Cardiovirus	2	Encephalomyocarditis virus[a]	Theiler's murine encephalomyelitis virus
Enterovirus	100	Poliovirus 1	Polioviruses 2 and 3 Bovine enteroviruses 1 and 2 Human coxsackieviruses A1–A22,[b] and A24 Human coxsackieviruses B1–B6[c] Human echoviruses 1–7,[d][e] 9,[e] 11–21, 24–27,[f] 29–33 Human enteroviruses 68–71[c] Porcine enteroviruses 1–13 Simian enteroviruses 1–18 Vilyuisk human encephalomyelitis virus
Hepatovirus	2	Human hepatitis A virus[g]	Simian hepatitis A virus
Parechovirus	2	Human parechovirus 1	Human parechovirus 2
Rhinovirus	100	Human rhinovirus 1A	Human rhinoviruses 2–100 Bovine rhinoviruses 1–3

[a]Strains of this virus include mengovirus, Columbia SK virus, and mouse Ellerfeld virus.
[b]Coxsackievirus A23 was shown to be echovirus 9.
[c]Swine vesicular disease virus is similar to coxsackievirus B5.
[d]Echovirus 8 was shown to be echovirus 1.
[e]Echovirus 10 was shown to be reovirus 1.
[f]Echovirus 22 and 23 have been moved to a separate genus, Parechovirus, and renamed parechovirus 1 and 2; echovirus 28 was shown to be rhinovirus 1A.
[g]Formerly enterovirus 72.
SAT, South African type.

TABLE 1. *Members of the family Picornaviridae*

The aphthoviruses (foot-and-mouth disease virus) infect cloven-footed animals, such as cattle, goats, pigs, and sheep,

but rarely infect humans. Seven serotypes of this virus have been identified, and within each serotype are many subtypes. These viruses are highly labile and rapidly lose infectivity at pH values of less than 7.0.

There are two “clusters” of cardioviruses. The encephalomyocarditis-like viruses (encephalomyocarditis virus, Columbia SK virus, maus Elberfeld virus, and mengovirus) are murine viruses but can also infect other hosts, including humans, monkeys, pigs, elephants, and squirrels. The second cluster includes the Theiler's murine encephalomyelitis viruses. Strains such as GDVII cause a disease in mice that resembles poliomyelitis. The TO strains are less virulent and cause a chronic demyelinating disease that resembles multiple sclerosis. Vilyuisk human encephalomyelitis virus, or Vilyuisk virus, originally classified as an enterovirus, was recently transferred to the Theiler's cluster of the cardioviruses. Vilyuisk virus is believe to cause a neurodegenerative disease among the Yakuts people of Siberia and was originally isolated from the cerebrospinal fluid of a patient with a chronic case of encephalomyelitis (191). Nucleotide sequence analysis of Vilyuisk virus RNA indicates that it is a divergent Theiler's virus (258), justifying its transfer to the cardioviruses.

As the name implies, enteroviruses replicate in the alimentary tract, and as might be expected, they are resistant to low pH. This genus includes poliovirus (3 serotypes), coxsackievirus (23 serotypes), echovirus (28 serotypes), human enterovirus (4 serotypes), and many nonhuman enteric viruses. Formerly a member of the enterovirus genus, human hepatitis A virus (1 serotype) has been reclassified into a separate genus, hepatovirus. This reclassification was based on a number of unique properties of the virus, including nucleotide and amino acid sequence dissimilarity with other picornaviruses, replication in cell culture without cytopathic effect, and the existence of only one serotype.

The parechovirus genus contains two serotypes of human parechovirus that were previously classified as echoviruses types 22 and 23. Studies of the nucleotide sequence of the viral genome revealed no greater than 30% amino acid identity with other picornaviruses. In addition, the capsid consists of three, not four, capsid proteins. These and other differences led to the reclassification of these viruses (294).

There are 103 serotypes of rhinoviruses, so named because they replicate in the nasopharynx. These viruses, which are important agents of the common cold, are extremely acid labile.

VIRION STRUCTURE

Physical Properties

Picornavirus virions are spherical in shape with a diameter of about 30 nm (Fig. 1). The particles are simple in that they are composed of a protein shell surrounding the naked RNA genome. The virus particles lack a lipid envelope, and their infectivity is insensitive to organic solvents. Cardioviruses, enteroviruses, hepatoviruses, and parechoviruses are acid stable and retain infectivity at pH values of 3.0 and lower. In contrast, rhinoviruses and aphthoviruses are labile at pH values of less than 6.0. Differences in pH stability are related to the sites of replication of the virus. For example, rhinoviruses and aphthoviruses replicate in the respiratory tract and need not be acid stable. As discussed later, the acid lability of these viruses plays a role in uncoating of the viral genome. Cardioviruses, enteroviruses, hepatoviruses, and parechoviruses pass through the stomach to gain access to the intestine and therefore must be resistant to low pH. The structural basis for the acid lability of foot-and-mouth disease virus is partly understood (see Entry by Endocytosis).

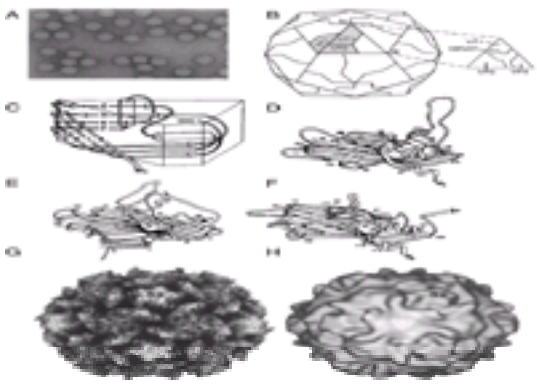

FIG. 1. Structural features of picornaviruses. **A:** Electron micrograph of negatively stained poliovirus, magnification 270,000. (Courtesy of N. Cheng and D. M. Belnap, National Institutes of Health.) **B:** Schematic of the picornavirus capsid, showing the pseudoequivalent packing arrangement of VP1, VP2, and VP3. VP4 is on the interior of the capsid. The biologic protomer (*gray*) is not the same as the icosahedral asymmetric subunit (*triangle expanded at right*). **C:** Diagram of how the eight b-strands of each capsid protein form a wedgelike structure with loops connecting the strands. These loops decorate the surface of the particle and give each subunit distinctive morphology and antigenicity. In many picornaviruses, the neutralizing antigenic sites are located in the BC loops of VP1 and VP3 and in the EF loop of VP2. These loops can accommodate extra amino acids without affecting the framework of the capsid (45). **D to F:** Ribbon diagrams of polioviruses VP1, VP2, and VP3, showing the common b-barrel structure that is also common to many other viral capsid proteins. The N- and C-termini of each capsid protein are separated by almost 50 angstroms. (C-F, adapted form ref. 142, with permission.) **G:** Model of poliovirus type 1, Mahoney, based on x-ray crystallographic structure determined at 2.9 Å (142). The model is highlighted by radial depth cuing so that portions of the model farthest from the center are bright. The 5-fold axis of symmetry is marked; it is characterized by a prominent star-shaped mesa. Surrounding the 5-fold axis is the canyon, which is the receptor-binding site. At the 3-fold axis is a propeller-shaped feature. **H:** Model of poliovirus type 1, Mahoney, made by image reconstruction from cryoelectron microscopy data. The resolution is 20 Å. The star-shaped mesa, canyon, and propeller are clearly visible.

The buoyant densities of picornaviruses are quite different (Table 2). Cardioviruses and enteroviruses have a buoyant

density of 1.34 g/mL, that of foot-and-mouth disease virus is 1.45 g/mL, and rhinoviruses have an intermediate value (1.40 g/mL). The reason for the difference lies in the permeability of the viral capsid to cesium. The capsid of poliovirus does not allow cesium to reach the RNA interior; hence, the virus bands at an abnormally light buoyant density (90). In contrast, aphthovirus capsids contain pores that allow cesium to enter (2). The rhinovirus capsid is permeable to cesium, but the presence of polyamines in the capsid interior limits the amount of cesium that may enter, explaining the intermediate value (90).

Genus	pH stability	Virion buoyant density	Sedimentation coefficient
Aphthovirus	Labile, d	1.43–1.45	145–146S
Cardiovirus	Stable, 3–9	1.34	160S
Enterovirus	Stable, 3–9	1.34	160S
Hepatovirus	Stable	1.34	160S
Rhinovirus	Labile, d	1.40	160S

TABLE 2. *Physical properties of some picornaviruses*

Ratio of Particles to Infectious Viruses

The ratio of particles to infectious virus is determined by dividing the number of virus particles in a sample (determined by electron microscopy or spectrophotometric measurements) by the plaque titer, yielding the particle-to-plaque forming unit ratio. This ratio is a measurement of the fraction of virus particles that can complete an infectious cycle. The particle-to-plaque forming unit ratio of poliovirus ranges from 30 to 1,000, and that of other picornaviruses is in the same range. The high particle-to-plaque forming unit ratio may be caused by the presence of lethal mutations in the viral genome. This explanation, however, probably does not apply to all picornaviruses; it has been shown that the infectivity of aphthovirus RNA approaches one infectious unit per molecule. An alternative explanation is that all viruses do not successfully complete an infectious cycle because they fail at one of the many steps—attachment, entry, replication, and assembly—that a virus must go through to complete the infectious cycle.

High-Resolution Structure of the Virion

The capsids of picornaviruses are composed of four structural proteins: VP1, VP2, VP3, and VP4. The exception is the parechoviruses, which contain only three capsid polypeptides: VP1, VP2, and VP0, the uncleaved precursor to VP2 + VP4. In the 1960s, Caspar and Klug, elaborating the principles of virus structure, concluded that the best way to build a shell with nonidentical subunits is to arrange the proteins with icosahedral symmetry (51). An icosahedron is a solid composed of 20 triangular faces and 12 vertices (see Fig. 1). The smallest number of subunits that can be used to compose such a solid is 60. The results of x-ray diffraction studies, electron microscopic observations, and biochemical studies of virus particles and their dissociation products led to the hypothesis that the picornavirus capsid contains 60 structural proteins arranged into an icosahedral lattice (274). Our understanding of the structure of picornaviruses took an enormous leap in 1985 when the atomic structures of poliovirus type 1 (142) and human rhinovirus 14 (272) were determined by x-ray crystallography. Since then, the high-resolution structures of many other picornaviruses have been determined.

The basic building block of the picornavirus capsid is the protomer, which contains one copy each of VP1, VP2, VP3, and VP4. The shell is formed by VP1 to VP3, and VP4 lies on its inner surface. VP1, VP2, and VP3 have no sequence homology, yet all three proteins have the same topology: they form an eight-stranded antiparallel b-barrel (also called a b-barrel jelly roll or a Swiss-roll b-barrel) (see Fig. 1). This domain is a wedge-shaped structure made up of two antiparallel b-sheets. One b-sheet forms the wall of the wedge, and the second, which has a bend in the center, forms both a wall and the floor. The wedge shape facilitates the packing of structural units to form a dense, rigid protein shell. Packing of the b-barrel domains is strengthened by a network of protein–protein contacts on the interior of the capsid, particularly around the 5-fold axes. This network, which is formed by the N-terminal extensions of VP1 to VP3 as well as VP4, is essential for the stability of the virion. VP4 is quite different from the other three proteins in that it has an extended conformation. This protein is similar in position and conformation to the NH2-terminal sequences of VP1 and VP3 and functions as a detached NH2-terminal extension of VP2 rather than an independent capsid protein.

The main structural differences among VP1, VP2, and VP3 lie in the loops that connect the b-strands and the N- and C-terminal sequences that extend from the b-barrel domain. These amino acid sequences give each picornavirus its distinct morphology and antigenicity. The C-termini are located on the surface of the virion, and the N-termini are on the interior, indicating that significant rearrangement of the P1 precursor occurs on proteolytic cleavage.

Resolution of the structures of poliovirus and rhinovirus yielded a great surprise: the b-barrel domains were strikingly similar in structure to those of plant viruses whose structures had been solved 5 years earlier, southern bean mosaic virus and tomato bushy stunt virus. Yet the capsid proteins of these viruses bear no sequence homology with those of the picornaviruses. It has since become apparent that similar protein topology is found in the capsid proteins of many plant, insect, and vertebrate positive-stranded RNA viruses as well as in the DNA-containing papovaviruses and adenoviruses. These findings suggest that either the polypeptides evolved from a common ancestor or that the b-barrel domain is one of the few ways to allow proteins to pack to form a sphere.

Surface of the Virion

Resolution of the structures of poliovirus and human rhinovirus revealed that the surfaces of these viruses have a corrugated topography; there is a prominent star-shaped plateau (mesa) at the 5-fold axis of symmetry, surrounded by a deep depression (“canyon”) and another protrusion at the 3-fold axis (see Fig. 1). It was originally proposed that the canyon is the receptor binding site, and this hypothesis has been proved for poliovirus and rhinovirus. However, not all picornaviruses have canyons. The surfaces of aphthoviruses and cardioviruses lack canyons and are much smoother in appearance. As discussed later, other features of these viruses serve as receptor-binding sites.

Interior of the Virion

A network formed by the N-termini on the interior of the capsid contributes significantly to its stability. At the 5-fold axis of symmetry, the N-termini of five VP3 molecules form a cylindrically parallel b-sheet. This structure is surrounded by five three-stranded b-sheets formed by the N-termini of VP4 and VP1. The myristate group attached to the N-terminus of VP4 mediates the interaction between these two structures (61). Interactions among pentamers are stabilized by a seven-stranded b-sheet, composed of four b-strands of the VP3 b-barrel and one strand from the N-terminus of VP1 that surround a two-stranded b-sheet made from the N-terminus of VP2 from a neighboring pentamer (86).

An extra 1500 nucleotides can be added to the poliovirus genome and successfully packaged, indicating that the interior of the capsid is not completely full (7). It has been suggested that picornaviral capsids are stabilized by interactions with the RNA genome, based on findings with bean pod mottle virus, which is related to picornaviruses. In this virus, ordered RNA can be observed at the 3-fold axis, and packaging of viral RNA stabilizes subunit interactions (58,188). Unfortunately, little information is available about the arrangement of the RNA genome of picornaviruses because the nucleic acid is usually not visible. Nevertheless, a few nucleotides have been tentatively identified in a similar location in the structures of P3/Sabin and rhinovirus type 14 (18,86). In the atomic structure of poliovirus P2/Lansing, RNA bases have been observed stacking with conserved aromatic residues of VP4 (185). This interaction could play a role in capsid stability or uncoating.

The Hydrophobic Pocket

Within the core of VP1, just beneath the canyon floor of many picornaviruses, is a hydrophobic tunnel or pocket. In poliovirus types 1 and 3, the pocket is believed to contain sphingosine (86); an unidentified lipid has been found in human rhinoviruses types 1A and 16, and a C16 fatty acid has been modeled in the pocket of coxsackievirus B3 (127,168,225). In contrast, the pocket of human rhinovirus type 14 is empty (18).

The same hydrophobic pocket is also the binding site for antipicornavirus drugs such as the WIN compounds produced by Sterling-Winthrop (290) as well as similar molecules produced by Schering-Plough (Kenilworth, NJ) and Janssen Pharmaceutica (Titusville, NJ) (13,68). Some of these drugs have been evaluated in clinical trials (1). These hydrophobic, sausage-shaped compounds bind tightly in the hydrophobic tunnel, displacing any lipid that is present. As discussed later, such drugs inhibit either binding or uncoating. Drug-dependent mutants of poliovirus spontaneously lose infectivity at 37°C, probably because they do not contain lipid in the pocket (224).

Myristate

Myristic acid (*n*-tetradecanoic acid) is covalently linked to glycine at the amino-terminus of VP4 of most picornaviruses (61). This residue is an integral part of the viral capsid. The N-termini of VP3 intertwine around the 5-fold axis to form a twisted tube of parallel b-structure (142). The five myristyl groups extend from the N-termini of VP4 and cradle the twisted tube formed by VP3 (61). The myristyl groups interact with amino acid side chains of VP4 and VP3. Mutagenesis of VP4 has revealed a role for myristic acid modification in virus assembly and in the stability of the capsid (14,196,197 and 198,220).

Neutralizing Antigenic Sites

As shown in Table 1, there are many different serotypes in the picornavirus family. Although the atomic structures of many picornaviruses have been solved, they provide no insight into why some picornaviruses, such as rhinovirus, occur in so many serotypes, whereas there are only three serotypes of poliovirus. However, serotype is determined by the connecting loops and C-termini of the capsid proteins that decorate the outer surface of the virion. These contain the major neutralization antigenic sites of the virus. Such sites are defined by mutations that confer resistance to neutralization with monoclonal antibodies directed against the viral capsid (213,214,286).

GENOME STRUCTURE AND ORGANIZATION

General Features

The genome of picornaviruses, a single positive-stranded RNA molecule, is infectious because it can be translated on entry into the cell and produce all the viral proteins required for viral replication. The picornavirus genome is unique because it is covalently linked at the 5' end to a protein called VPg (virion protein, genome linked) (88,184). VPg is covalently joined to the 5'-uridylylate moiety of the viral RNA by an O4-(5'-uridylyl)-tyrosine linkage. All picornavirus genomes are linked to VPg, which varies in length from 22 to 24 amino acid residues. The tyrosine that is linked to the viral RNA is always the third amino acid from the N-terminus. VPg is encoded by a single viral gene in all picornaviruses except the genome of foot-and-mouth disease virus, which encodes three VPg genes (89). VPg is not required for infectivity of poliovirus RNA; if it is removed from viral RNA by treatment with proteinase, the specific infectivity of the viral RNA is not reduced. VPg is not found on viral mRNA that is associated with cellular ribosomes and undergoing

translation; these mRNAs contain only uridine 5'-phosphate (pU) at their 5' ends. The lack of VPg is the only difference between poliovirus virion RNA and mRNA (230,249). VPg is removed from virion RNA by a host protein called *unlinking enzyme* (9). It is not known whether removal of VPg is a prerequisite for association with ribosomes or is a result of that association. Although VPg RNA can be translated in cell-free extracts, it is possible that VPg is rapidly cleaved from the RNA such that only RNAs lacking VPg are translated (112,217,316). VPg is present on nascent RNA chains of the replicative intermediate RNA and on negative-stranded RNAs, which has led to the suggestion that poliovirus RNA synthesis uses VPg as a primer (229,248). The role of VPg in viral RNA synthesis figures prominently in recent models of poliovirus RNA replication and is discussed in subsequent sections.

Nucleotide sequence analysis of many picornavirus RNAs has revealed a common organizational pattern (Fig. 2). The genomes vary in length from 7,209 to 8,450 bases. The 5'-noncoding regions of picornaviruses are long (624 to 1,199 nucleotides) and highly structured (Fig. 3). This region of the genome contains sequences that control genome replication and translation (see Genome Replication and mRNA Synthesis). The 5'-noncoding region contains the internal ribosome entry site (IRES), an element that directs translation of the mRNA by internal ribosome binding. Based on the RNA structure, two major classes of IRES elements have been defined (see Fig. 3). The 5'-untranslated regions of aphthoviruses and cardioviruses contain a poly(C) tract that varies in length among different virus strains (80 to 250 nucleotides in cardioviruses, 100 to 170 nucleotides in aphthoviruses). Among cardioviruses, longer poly(C) length is associated with higher virulence in animals (79,129).

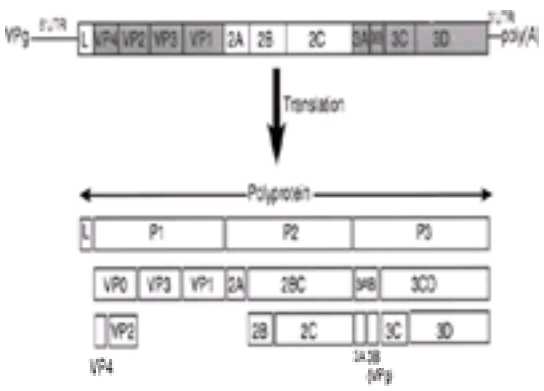

FIG. 2. Organization of the picornavirus genome. **Top:** Diagram of the viral RNA genome, with the genome-linked protein VPg at the 5' end, the 5' untranslated region, the protein coding region, the 3' untranslated region, and the poly(A) tail. L is a leader protein encoded in the genomes of cardioviruses and aphthoviruses but not other picornaviruses. Coding regions for the viral proteins are indicated. **Bottom:** Processing pattern of picornavirus polyprotein. The coding region has been divided into three regions, P1, P2, and P3, which are separated by nascent cleavage by two viral proteinases, 2A and 3C. Intermediate and final cleavage products are indicated. The proteinase responsible for cleavage of VP0 has not been identified. Proteins L and 2A carry out cleavages described in Figure 10. The remaining cleavages are carried out by $3C^{pro}$ and its precursor, $3CD^{pro}$.

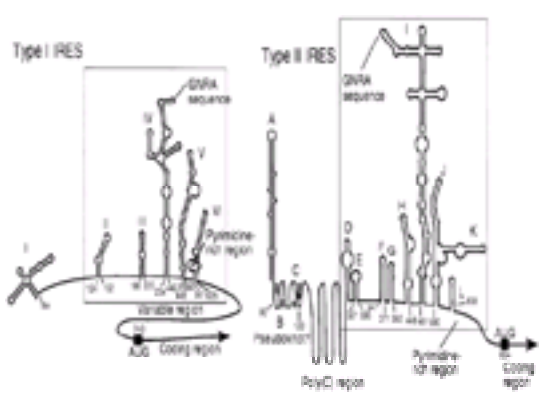

FIG. 3. Two major classes of picornavirus internal ribosome entry site (IRES). **Left:** The 5' untranslated region of poliovirus. **Right:** The same region from encephalomyocarditis virus. The IRES is indicated by a box. Predicated secondary and tertiary structures (RNA pseudoknots) are labeled. The poliovirus IRES is a type I IRES, also found in the genomes of other enteroviruses and rhinoviruses. The genomes of cardioviruses and aphthoviruses contain a type II IRES. The GNRA loop and pyrimidine-rich region are features that are conserved in both the type I and type II IRES. The IRES of hepatoviruses differs from the type I and type II IRES and probably constitutes a third class. (Adapted from Stewart SR, Semler BL. RNA determinants of picornavirus cap-independent translation initiation. *Semin Virol* 1997;8:242,243,244,245,246,247,248,249,250,251,252,253,254 and 255, with permission.)

The 3'-noncoding region of picornaviruses is short, ranging in length from 47 nucleotides for human rhinovirus 14 to 125 bases for encephalomyocarditis virus. This region also contains a secondary structure, notably a pseudoknot, that has been implicated in controlling viral RNA synthesis (154). The entire 3'-noncoding region of poliovirus and rhinovirus is not required for infectivity, although RNA lacking this sequence has low infectivity, and the resulting viruses replicate poorly (301). Both poliovirus virion RNA and mRNA contain a 3' stretch of poly(A) (326). Negative-stranded RNA contains a 5' stretch of poly(U), which is copied to form poly(A) of the positive strand (325). The average length of the poly(A) tail varies from 35 nucleotides in encephalomyocarditis virus to 100 nucleotides in aphthoviruses (5). Viral RNA from which the poly(A) tract is removed is noninfectious (293).

The results of biochemical studies of poliovirus-infected cells had predicted the presence of a single, long, open reading frame on the viral RNA that is processed to form individual viral proteins (297). This hypothesis was proved when the nucleotide sequence of the poliovirus genome was determined, which revealed a single open reading frame in the viral RNA (173,260). A similar strategy, in which a single polyprotein is synthesized, is used by all picornaviruses. The polyprotein is cleaved during translation, so that the full-length product is not observed. Cleavage is carried out by virus-encoded proteinases to yield 11 to 12 final cleavage products. Some of the uncleaved precursors also have functions during replication.

To unify the nomenclature of picornavirus proteins, the polyprotein has been divided into three regions: P1, P2, and P3. Aphthoviruses and cardioviruses encode a leader (L) protein before the P1 region. The P1 region encodes the viral capsid proteins, whereas the P2 and P3 regions encode proteins involved in protein processing (2A ^{pro}, 3C^{pro}, 3CD^{pro}) and genome replication (2B, 2C, 3AB, 3B^{VPg}, 3CD^{pro}, 3D^{pol}).

Infectious DNA Clones of Picornavirus Genomes

Recombinant DNA techniques make it possible to introduce mutations anywhere in the genome of most animal viruses. Progress in virus research has been greatly expanded by the availability of infectious DNA clones—a double-stranded copy of the viral genome carried on a bacterial plasmid. Infectious DNA clones, or RNA transcripts derived by *in vitro* transcription, can be introduced into cultured cells by transfection to recover infectious virus. The first infectious DNA clone of an animal RNA virus was that of poliovirus (259). The mechanism for infectivity of cloned poliovirus DNA is unknown but might result from production of RNA in the nucleus, by cellular DNA-dependent RNA polymerase, from cryptic, promoter-like elements in the plasmid. The initial transcripts produced are longer than poliovirus RNA but can be translated and replicated. The extra sequences must be removed or not copied because viruses produced from cloned DNA have authentic 5'- and 3'-termini.

The infectivity of cloned poliovirus DNA (10^3 plaque-forming units per μg) is much lower than that of genomic RNA (10^6 plaque-forming units per μg). The development of plasmid vectors incorporating promoters for bacteriophage SP6, T7, or T3 RNA polymerase for the production of RNA transcripts *in vitro* enabled the production of infectious picornavirus RNA from cloned DNA (309). Such RNA transcripts have an infectivity approaching that of genomic RNA.

THE REPLICATION CYCLE

Replication of picornaviruses takes place entirely in the cell cytoplasm. The first step is attachment to a cell receptor (Fig. 4). The RNA genome is then uncoated, a process that involves structural changes in the capsid. Once the positive-stranded viral RNA enters the cytoplasm, it is translated to provide viral proteins essential for genome replication and the production of new virus particles. The viral proteins are synthesized from a polyprotein precursor, which is cleaved nascently. Cleavages are carried out mainly by two viral proteinases, 2A ^{pro} and 3C^{pro}/3CD^{pro}. Among the proteins that are synthesized are the viral RNA-dependent RNA polymerase and accessory proteins required for genome replication and mRNA synthesis. The first step in genome replication is copying of the input positive-stranded RNA to form a negative-stranded intermediate; this step is followed by the production of additional positive strands. These events occur on small membranous vesicles that are induced by several virus proteins. Once the pool of capsid proteins is sufficiently large, encapsidation begins. Coat protein precursor P1 is cleaved to form an immature protomer, which then assembles into pentamers. These assemble with newly synthesized, positive-stranded RNA to form the infectious virus. Empty capsids are also found in infected cells; these are likely to be a storage form of pentamers.

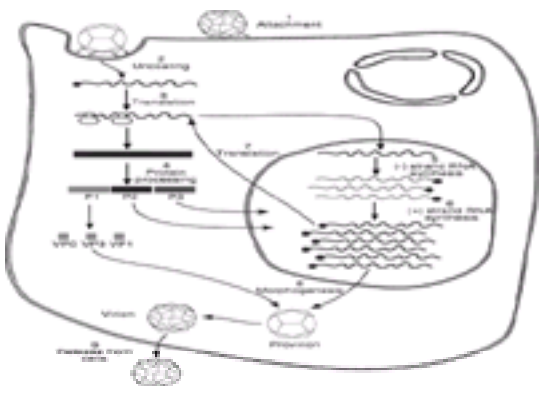

FIG. 4. Overview of the picornavirus replication cycle. Virus binds to a cellular receptor *(1)* and the genome is uncoated *(2)*. VPg is removed from the viral RNA, which is then translated *(3)*. The polyprotein is cleaved nascently to produce individual viral proteins *(4)*. RNA synthesis occurs on membrane vesicles (not drawn to scale in this diagram). Viral (+) strand RNA is copied by the viral RNA polymerase to form full-length (–) strand RNAs *(5)*, which are then copied to produce additional (+) strand RNAs *(6)*. Early in infection, newly synthesized (+) strand RNA is translated to produce additional viral proteins *(7)*. Later in infection, the (+) strands enter the morphogenetic pathway *(8)*. Newly synthesized virus particles are released from the cell by lysis *(9)*.

The entire time required for a single replication cycle ranges from 5 to 10 hours, depending on many variables, including the particular virus, temperature, pH, host cell, and multiplicity of infection. Many picornaviruses are released as the cell loses its integrity and lyses. Other picornaviruses, such as hepatitis A virus, are released from cells in the absence of cytopathic effect.

Attachment

Cellular Receptors and Coreceptors

Picornaviruses initiate infection of cells by first attaching to the host cell membrane through a host cell receptor. The nature of picornavirus receptors remained obscure until 1989, when the receptors for poliovirus and the major group rhinoviruses were identified (121,212,296). Since then, receptors for many other members of this virus family have been identified (Table 3). Examination of picornavirus receptor usage illustrates a number of important points. First, picornaviruses use a wide variety of cell surface molecules for cellular receptors. Second, many picornaviruses share cellular receptors. For example, the cell surface protein CD55 is a receptor for certain coxsackieviruses A and B, echoviruses, and enterovirus 70. Third, for some picornaviruses, a single type of receptor is sufficient for entry of viruses into cells. Viruses in this category include polioviruses and rhinoviruses. For other viruses, a second molecule, or coreceptor, is needed for virus entry into cells. For example, coxsackievirus A21, which attaches to CD55, requires intercellular adhesion molecule 1 (ICAM-1) for entry into cells (283). The precise role of coreceptors in picornavirus entry is not yet known.

TABLE 3. *Cell receptors for picornaviruses*

One Type of Receptor Molecule for Virus Binding and Entry

The poliovirus receptor (Pvr, CD155) was identified by DNA transformation (212). It was known that mouse cells are not susceptible to poliovirus infection because they lack cellular receptors (208). Mouse cells are permissive to poliovirus replication, however, because introduction of poliovirus RNA into these cells leads to the synthesis of infectious viruses (143,144). Poliovirus-susceptible mouse cells were produced by transforming mouse cells with human genomic DNA (211). Such cells were identified using a monoclonal antibody directed against the poliovirus receptor. Cloning of the human poliovirus receptor gene revealed that the receptor is a integral membrane protein and a member of the immunoglobulin (Ig) superfamily of proteins, with three extracellular Ig-like domains (212) (Fig. 5). The first Ig-like domain contains the site that binds poliovirus. This conclusion comes from three different types of experiments. First, cells expressing the first Ig-like domain of Pvr, either alone or as a hybrid with other Ig-like proteins, are susceptible to infection with poliovirus (177,218,280,281). Second, mutations in the first Ig-like domain of Pvr interfere with poliovirus binding (15,36,219). Mutations in the C'C'' loop, the C-terminus of the D-strand, and the DE and FG loops of Pvr domain 1 cause alterations in virus binding to the receptor. Third, models of the poliovirus–Pvr complex produced from cryoelectron microscopy data indicate that only domain 1 contacts the virus surface (32,136,323) (see Fig. 5). Mutations that affect receptor binding map to the virion–Pvr interface as determined by these structural studies.

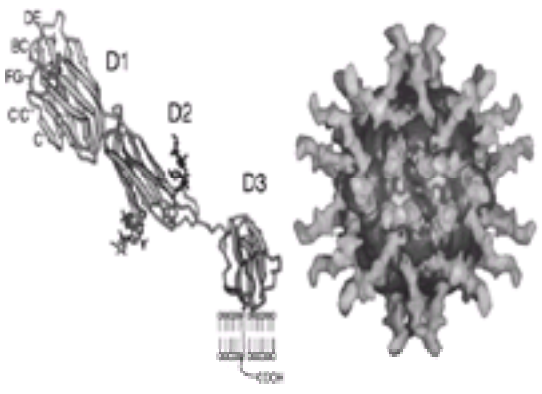

FIG. 5. Interaction of poliovirus with its cellular receptor. **Left:** Model of the poliovirus receptor produced from homology modeling and the density map from cryoelectron microscopy data of a virus-receptor complex (32). Immunoglobulin-like domains are labeled. The receptor contacts the virus only through domain 1; principal loops involved in the contact are labeled. Carbohydrate side chains have been modeled on domains 1 and 2. **Right:** Image reconstruction of poliovirus type 1 and a soluble form of Pvr, lacking the transmembrane and cytoplasmic domains (32). Domain 1 of Pvr binds in the canyon of the virus; no other receptor domains contact the particle. There are 60 receptor binding sites on each viral capsid; the complexes were produced with saturating amounts of Pvr, and all binding sites on the capsid are occupied.

A mouse model for poliomyelitis was developed by introducing the human Pvr gene into transgenic mice (179,262). Such

mice develop poliomyelitis after inoculation with poliovirus by different routes. Pvr expression in different tissues of transgenic mice is not always sufficient to permit virus replication. For example, Pvr is expressed in the kidney of transgenic mice, but the kidney is not a site of poliovirus replication (263). Furthermore, Pvr transgenic mice are not susceptible to infection by the oral route, the natural route of infection in humans, probably because of a postreceptor block to replication in mouse intestinal cells (332).

Expression of Pvr on cultured cells derived from different animal species leads to susceptibility to poliovirus infection. It therefore seems likely that Pvr is the only molecule required for poliovirus binding and entry. At one time, it was believed that the lymphocyte homing receptor, CD44, might be a coreceptor for poliovirus because a monoclonal antibody against this protein blocks poliovirus binding to cells (284,285). It was subsequently shown that CD44 is not a receptor for poliovirus and is not required for poliovirus infection of cells that express Pvr (43,93). It seems likely that Pvr and CD44 are associated in the cell membrane (94) and that anti-CD44 antibodies block poliovirus attachment by blocking the poliovirus-binding sites on Pvr.

The normal cellular function of Pvr is unknown. Sequence homologs of Pvr have been identified in humans (Prr1/Nectin-1 [81,194]), monkeys (Agm1 and Agm2 [178]), mice (Prr1, Mph/Prr2/Nectin-2, Nectin-3 [218,277]), and rats (pE4 [52]). Only Pvr, Agm1, and Agm2 encode proteins that can function as cell receptors for poliovirus (178,211,218; V. Racaniello, unpublished data, 1999). Pvr, Nectin-1, and Nectin-2 are coreceptors for alphaherpesviruses (102,287,321). Nectin-1 and Nectin-2 are homophilic adhesion proteins that interact with the actin cytoskeleton through a cytoplasmic linker protein called *afadin* (299). Mice lacking the Nectin-2 protein are sterile and produce sperm with cytoskeletal and nuclear defects (42). It is not known whether Pvr is also an adhesion molecule; it lacks the sequence in the cytoplasmic domain that is responsible for binding of Nectin-1 and Nectin-2 to afadin (299).

The cell surface receptor for the major group of human rhinoviruses (about 91 serotypes) was identified using monoclonal antibodies directed against the cellular binding site to isolate the receptor protein from susceptible cells. Amino acid sequence analysis of the purified protein revealed that it is ICAM-1, an integral membrane protein that is also a member of the Ig superfamily of proteins (121,296,303). The normal cellular functions of ICAM-1 are to bind its ligand, lymphocyte function–associated antigen 1 (LFA-1) on the surface of lymphocytes and to promote a wide range of immunologic functions (308). Erythrocytes infected with *Plasmodium falciparum* gain the ability to bind ICAM-1 on endothelial cells, allowing the parasite to become sequestered in tissues. ICAM-1 is expressed on the surfaces of many tissues, including the nasal epithelium, which is the entry site for rhinoviruses.

Mutagenesis of ICAM-1 DNA has revealed that the binding site for rhinovirus is located in the first Ig-like domain (204,261,295). The binding sites for two other known ligands of ICAM-1, LFA-1 and *Plasmodium falciparum*–infected erythrocytes, are also located in the first domain. A model of the rhinovirus–ICAM-1 complex produced from cryoelectron microscopy data, combined with x-ray structures of both components, confirms that only domain 1 of ICAM-1 contacts the virus surface (30,50,180,236). The residues of ICAM-1 that contact rhinovirus are located in the BC, CD, DE, and FG loops of the first aminoterminal domain. These findings agree with the results of mutational analysis that identify amino acids crucial for virus binding.

A comparison of the structures of virus–receptor complexes of two different picornaviruses reveals significant differences. The orientation of the receptors bound to their viruses are strikingly different. The long axis of ICAM-1 is perpendicular to the capsid surface, whereas Pvr binds at a glancing angle. In addition, the “footprint” of Pvr on poliovirus is significantly larger than that of ICAM-1 on rhinovirus, and the receptor sites on the poliovirus capsid are more exposed. The extra surface area on poliovirus includes the knob of VP3 and the C-terminus of VP1 from the fivefold-related promoter in the southeast corner of the road map describing the contact of Pvr on poliovirus. As discussed later, these differences may account for some of the differences in kinetic and affinity constants between the two virus-receptor complexes.

Receptors and Coreceptors Needed for Infection

Many enteroviruses bind to decay-accelerating factor (DAF, or CD55), a member of the complement cascade (see Table 3). For most of these viruses, however, interaction with CD55 is not sufficient for infection. For example, coxsackievirus A21 binds to CD55, but infection does not occur unless ICAM-1 is also present (283). Some coxsackie B viruses that bind CD55 may require $a_v b_6$-integrin as a coreceptor (4), whereas some echoviruses that bind integrin $a_2 b_1$ or CD55 are believed to require b_2-microglobulin as a coreceptor (320).

Alternative Receptors

Certain viruses bind to different cell surface receptors, depending on the virus isolate or the cell line (see Table 3). Foot-and-mouth disease virus A12 attaches to cells through the integrin $a_v b_3$ (226). However, the O strain of foot-and-mouth disease virus, which has been passaged many times in cell culture, cannot bind to $a_v b_3$; instead, this virus uses cell surface heparan sulfate (152). The type A12 strain cannot infect cells that do not express $a_v b_3$, even when heparan sulfate is present on the cell surface. It is believed that laboratory adaptation of foot-and-mouth disease virus in cultured cells expressing low levels of $a_v b_3$ leads to the selection of a virus that binds an alternative receptor.

How Picornaviruses Attach to Cell Receptors

As discussed previously, the capsids of picornaviruses are composed of four proteins arranged with icosahedral symmetry. Among the family members, the capsid proteins are arranged similarly, but the surface architecture varies. These differences account for not only the different serotypes but also the different modes of interaction with cell receptors. For example, the capsids of polioviruses, rhinoviruses, and coxsackieviruses have a groove, or canyon, surrounding each fivefold axis of symmetry. In contrast, cardioviruses and aphthoviruses do not have canyons.

The canyons of poliovirus and rhinovirus have been shown to be the sites of interaction with cell receptors. Mutation of

amino acids that line the canyons of poliovirus and rhinovirus can alter the affinity of binding to receptors (65,66 and 67,134,189). Models of the interaction of poliovirus and rhinovirus with their cellular receptors have been produced from cryoelectron microscopy and x-ray crystallographic data (30,32,50,136,180,236,323). As discussed previously, these models reveal that only domain 1 of Pvr or ICAM-1 penetrates the canyon of the respective virus. For both rhinovirus and poliovirus, the footprint of the receptor defined by structural models agrees with the results of mutational data. More detail is available about the ICAM-1–rhinovirus interaction because the x-ray crystallographic structure of ICAM-1 has been determined (30,50). This information, however, does not explain why major and minor group rhinoviruses recognize different receptors. Minor group rhinoviruses have no obvious amino acid sequence differences from members of the major group; indeed, the major group rhinoviruses 14 and 16 are more distantly related to each other than to the minor group rhinoviruses 1A and 2.

It was originally believed that the picornavirus canyons were too deep and narrow to allow penetration by antibody molecules, which contain adjacent Ig domains (271). This physical barrier was believed to hide amino acids crucial for receptor binding from the immune system. Structural studies of a rhinovirus–antibody complex, however, revealed that antibody does penetrate deep into the canyon, as does ICAM-1 (289). The shape of the picornavirus canyon is therefore not likely to play a role in concealing virus from the immune system.

Beneath the canyon floor is the hydrophobic pocket that opens at the base of the canyon and extends toward the fivefold axis of symmetry (see The Hydrophobic Pocket, earlier). The pocket appears to be occupied in many picornaviruses with a fatty acid or related compound. Certain antiviral drugs, such as the WIN compounds, displace the lipid and bind tightly within the pocket (290). Binding of such drugs to rhinovirus 14 causes conformational changes in the canyon that prevent attachment to cells (250). In contrast, drug binding to rhinoviruses 1A, 3, and 16 and to poliovirus causes smaller structural changes in the capsid (120,126,140). Inhibition of rhinovirus 16 binding by these compounds is probably not a consequence of altering the receptor binding site, but rather the result of preventing conformational changes required for receptor binding. Such compounds do not inhibit binding of poliovirus, but rather uncoating (209).

The capsids of some picornaviruses, including coxsackievirus A9 and foot-and-mouth disease virus, do not have prominent canyons. These viruses are believed to attach to cell receptors through surface loops. In foot-and-mouth disease virus, an Arg-Gly-Asp sequence in the flexible bG-bH loop of the capsid protein VP1 is recognized by integrin receptors on cells (91). Arg-Gly-Asp–containing peptides block attachment of foot-and-mouth disease virus (28), and alteration of this sequence interferes with virus binding (200). In coxsackievirus A9, the Arg-Gly-Asp sequence is present in a 17–amino acid extension of the C-terminus of VP1 and is also the site of attachment to cell receptors (53,268). Alteration of this sequence does not abolish binding to cells, however, suggesting that the virus can bind to another cell surface receptor (147).

As discussed previously, foot-and-mouth disease virus uses alternative receptors, either integrin or heparan sulfate, depending on the virus isolate. The binding site for heparan sulfate on cell culture–adapted foot-and-mouth disease virus is a shallow depression on the virion surface, where the three major capsid proteins, VP1, VP2 and VP3, are located (96). Binding specificity is controlled by two preformed sulfate-binding sites on the capsid. Residue 56 of VP3 is a critical regulator of receptor recognition. In field isolates of the virus, this amino acid is histidine. Adaptation to cell culture selects for viruses with an arginine at this position, which forms the high-affinity heparan sulfate–binding site.

Kinetics and Affinity of the Virus–Receptor Interaction

The affinity and kinetics of picornaviruses binding to soluble forms of their receptors have been studied by surface plasmon resonance. Such parameters are important because they describe the interaction of virus with receptor, which enables a better understanding of the reaction and its comparison to other systems. There are two classes of receptor-binding sites, with distinct binding affinities, on the capsids of poliovirus and rhinovirus (49,205,323). The association rates for the two binding classes are 25- and 13-fold higher for the poliovirus–sPvr interaction than for the rhinovirus–sICAM interaction at 20°C. The greater association rate of poliovirus and Pvr may be due, in part, to differences in the extent of contact between virus and receptor (see earlier). In contrast, whereas there are two dissociation rate constants for the poliovirus–Pvr interaction, only one has been reported for the rhinovirus 3–ICAM interaction. The dissociation rates for the poliovirus–sPvr interaction are 1.5-fold and 2.0-fold faster than for the rhinovirus–sICAM interaction, indicating greater instability of the former complex. The affinity constants for the poliovirus–sPvr interaction are 19-fold and 6-fold greater than those reported for the rhinovirus–sICAM-1 complex.

In contrast to the observations with poliovirus and rhinovirus, a single class of binding site was found on echovirus 11 for a soluble form of its receptor, CD55 (183). The affinity of this interaction is at least 4 times lower than either of the binding sites on poliovirus for sPvr. The association rate for the interaction between echovirus 11 and CD55 is faster than that of poliovirus–sPvr and rhinovirus–sICAM-1. One explanation for these findings is that the contact between echovirus 11 and CD55 is more extensive than that of the other two virus-receptor complexes. The binding site for CD55 on echovirus 11 may also be more accessible than those of Pvr and ICAM-1. The dissociation rate for the echovirus–CD55 interaction is at least 97 times faster than that of either the poliovirus–sPvr or the rhinovirus–sICAM-1 interaction. These findings are consistent with a more accessible binding site for CD55 on echovirus 11, compared with the receptor-binding sites on poliovirus and rhinovirus. Atomic interactions between CD55 and echovirus 11 may be weaker than between the other two viruses and their receptors. The faster dissociation rate of the echovirus 11–CD55 complex may be related to the finding that the interaction does not lead to structural changes of the virus particle (257), as occurs with poliovirus and rhinovirus.

Why do poliovirus and rhinovirus have two classes of receptor binding sites? The receptors for both viruses make contacts at two major sites on the virus surface, one in a cleft on the south rim of the canyon, and a second on the side of the mesa on the north rim. These two contact sites may correspond to the two classes of binding sites. Two classes of binding sites may also be a consequence of the structural flexibility exhibited by both viruses, which may cause exposure of different binding sites. Normally internal parts of the poliovirus and rhinovirus capsid proteins have been shown to be transiently displayed on the virion surface, a process called *breathing* (186,187). As discussed later, the interaction of poliovirus and rhinovirus with their cellular receptors leads to irreversible and more extensive structural changes. In

contrast to the findings with poliovirus and rhinovirus, binding of echovirus 11 with CD55 can be described by a simple 1:1 binding model. Such behavior, which would be expected for the interaction of two preformed binding sites, is consistent with the fact that the echovirus–CD55 interaction does not result in detectable structural changes in the capsid (257).

Entry Into Cells

Once picornaviruses have attached to their cellular receptor, the viral capsid must dissociate to release the RNA genome, which must enter the cytoplasm, the site of picornavirus replication. For some picornaviruses, interaction with a cell receptor serves only to concentrate virus on the cell surface; release of the genome is a consequence of low pH or perhaps the activity of a coreceptor. For other picornaviruses, the cell receptor is also an unzipper and initiates conformational changes in the virus that lead to release of the genome.

Formation of a Pore in the Cell Membrane

The interaction of poliovirus with its receptor, Pvr, leads to major structural changes in the virus. The resulting particles, called *altered,* or *A, particles,* contain the viral RNA but have lost the internal capsid protein VP4. In addition, the N-terminus of VP1, which is normally on the interior of the capsid, is on the surface of the A particle (95). This sequence of VP1 is hydrophobic, and as a result, the A particles have an increased affinity for membranes compared with the native virus particle. In one hypothesis for poliovirus entry, receptor binding leads to these conformational changes; the exposed lipophilic N-terminus of VP1 then inserts into the cell membrane, forming a pore through which the viral RNA can travel to the cytoplasm (Fig. 6). The finding that A particles, when added to lipid bilayers, induce the formation of ion channels supports this hypothesis (304). A model of the A particle produced from cryoelectron microscopy provides more insight into uncoating of the poliovirus genome (31). Compared with the native virus, capsid domains in the A particle have moved up to 9 Å, in a process that has been compared to the movements of Earth's tectonic plates. These movements produce gaps between the capsid subunits, and it is hypothesized that the gaps in the area where VP1, VP2, and VP3 meet could allow the emergence of VP4 and the N-terminus of VP1. In this model, VP4 and the VP1 N-terminus are extruded from the bottom of the canyon and are arranged around the mesa at the fivefold axis of symmetry. There, the five predicted amphipathic helices at the N-termini of VP1 would be in position to insert into the plasma membrane. A pore is formed, and then VP3, which forms a plug blocking the fivefold axis, is moved, allowing the RNA to exit the particle.

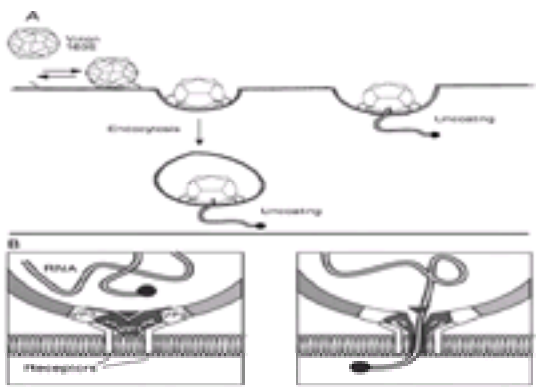

FIG. 6. Model for poliovirus entry into cells. **A:** Overview of poliovirus entry. The 160S native virus particle binds its cellular receptor, Pvr, and at temperatures greater than 33°C, undergoes a receptor-mediated conformational transition, leading to the production of altered *(A)* particles. These particles, which may be entry intermediates, are hydrophobic and have lost capsid protein VP4. The N-terminus of VP1, normally on the interior of the capsid, is translocated to the surface. The viral RNA, shown as a *curved line,* may exit the particle from the plasma membrane or from within endosomes. **B:** Hypothetical mechanism for translocation of poliovirus RNA across the cell membrane. At the left is a cross-section of a virus particle that has just bound Pvr at the cell surface. The viral RNA is in the capsid, and lipid occupies the hydrophobic pocket. Pvr docks on the capsid in the canyon, above the hydrophobic pocket. At right, the capsid has undergone the receptor-mediated conformational change. Docking of the receptor in the canyon leads to loss of the lipid in the hydrophobic pocket, allowing the capsid to undergo conformational changes. The extruded N-termini of VP1 form a pore in the cell membrane through which the viral RNA may pass. The dark triangle is the VP3 b-cylinder, which could move out of the way like a float valve (31).

The ultimate disposition of VP4 released from the viral capsid is not known, but it is clear that this protein is required for an early stage of cell entry. A virus containing a mutation in VP4 can bind to cells and be converted to A particles, but these are blocked at a subsequent step in virus entry (221). VP4 and VP2 are produced, during virus assembly, from the precursor VP0, which remains uncleaved until RNA encapsidation. Cleavage of VP0 can therefore be viewed as a way of priming the capsid for uncoating because cleavage separates VP4 from VP2.

Although the A particle has biochemical and structural properties consistent with an entry intermediate, its role in entry has been questioned by the results of experiments demonstrating that poliovirus can replicate at 25°C, a temperature at which A particle formation cannot be detected (77). Perhaps the A particle detected at higher temperatures is a stable end product and an exaggerated form of an uncoating intermediate. The uncoating intermediate may be unstable and difficult to detect. However, the A particle is likely to be similar to the uncoating intermediate, and thus warrants further study.

Although it is believed that poliovirus RNA crosses the cell membrane, it is not known whether that event occurs at the plasma membrane or from within endosomes (see Fig. 6). Drugs, such as bafilomycin A1, that block acidification of

endosomes through their inhibition of proton transport into the vesicles, do not inhibit poliovirus infection (245). These drugs effectively inhibit infection by viruses that enter the cytoplasm from acidic endosomes, such as influenza virus and Semliki Forest virus. Although it is likely that poliovirus RNA could enter at either the plasma or endosomal membrane, it is clear that endocytosis alone is not sufficient to trigger poliovirus uncoating. For example, antibody-coated poliovirus particles cannot efficiently infect cells expressing Fc receptors, which are efficiently endocytosed (16,199). This finding suggests that Pvr-mediated conformational changes in poliovirus are crucial to the uncoating process.

Entry by Endocytosis

Some picornaviruses, such as foot-and-mouth disease virus and rhinovirus, enter cells by receptor-mediated endocytosis, and uncoating is triggered by acidification of the endosomal, pH-dependent pathway (47,200). Infection of cells by these viruses is inhibited by weak bases and ionophores that block the acidification of endosomes (47,48). Consistent with this mechanism of entry, foot-and-mouth disease virus that has been coated with antibody can bind to and infect cells that express Fc receptors, in contrast to poliovirus (see earlier) (199). The cell receptor for foot-and-mouth disease virus is therefore a “hook”: it does not induce uncoating-related changes in the virus particle, but rather serves only to tether the virus to the cell and bring it into the endocytic pathway.

The mechanism by which low pH causes disassembly of the foot-and-mouth disease virus capsid has been illuminated by structural and genetic studies. At a pH of about 6.5, the viral capsid dissociates to pentamers, releasing the viral RNA (315). Examination of the atomic structure of the virus revealed a high density of histidine residues lining the pentamer interface (2). These residues confer stability to the capsid; because the pKa of histidine is 6.8, close to the pH at which the virus dissociates, protonation of the side chains of the histidines might cause electrostatic repulsion, leading to disassembly (70). To test this hypothesis, a histidine residue at position 142 of VP3 was changed to arginine by mutagenesis. The resulting capsids were more stable at low pH than wild-type capsids (83), supporting the proposed role of the histidine residue in acid-catalyzed disassembly.

Regulation of Uncoating by Cellular Molecules

The hydrophobic pocket located below each protomer surface appears to be a critical regulator of the receptor-induced structural transitions of poliovirus. In poliovirus type 1, each pocket is occupied by a natural ligand that is believed to be a molecule of sphingosine (Fig. 7) (86,324). The capsids of rhinoviruses 1A and 16 each contain lipids that are shorter than sphingosine (167,235), whereas that of coxsackievirus B3 has been modeled as a C16 fatty acid (225). The icosahedral symmetry of the capsid would allow each virion to contain up to 60 lipid molecules. In some picornaviruses, the pockets are apparently empty, such as in rhinovirus types 3 and 14 (18,333).

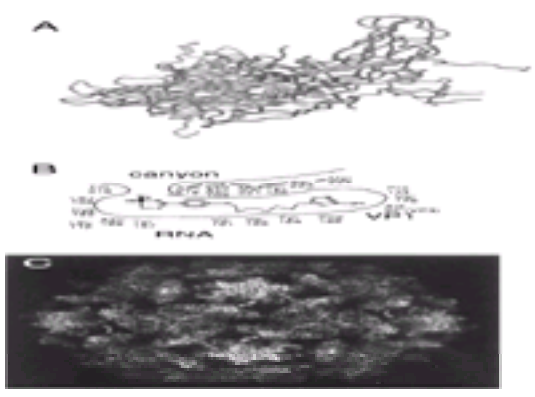

FIG. 7. The hydrophobic pocket in the picornavirus capsid. **A:** Cellular lipid bound in the hydrophobic pocket of poliovirus type 1. Side view of a protomer, consisting of one copy each of VP1, VP2, VP3, and VP4. RNA is below, and the 5-fold axis of symmetry is at the upper right. Gray spheres represent what is believed to be a molecule of sphingosine bound in the hydrophobic pocket. The lipid is just below the canyon floor. **B:** WIN52084 bound in the hydrophobic pocket. These drugs displace the lipid from the pocket, thereby blocking infectivity. **C:** Drug bound in the poliovirus pocket. R78206, a WIN-like compound, bound in the poliovirus capsid. The bound drug is the small white molecule at the base of the canyon. The drug appears to be exposed on the surface but is actually in the hydrophobic pocket. The capsid model is shown in a radial depth-cued representation, in which atoms are colored according to whether they are near the center of the virus (*black*) or far from the center (*white*). Residues at the bottom of the canyon are dark because they are at a position of low radius. The bound drug is not depth cued.

These lipids are thought contribute to the stability of the native virus particle by locking the capsid in a stable configuration and preventing conformational changes. Removal of the lipid is probably necessary to provide the capsid with sufficient flexibility to undergo the changes that permit the RNA to leave the shell. This hypothesis comes from the study of antiviral drugs, such as the WIN compounds, that displace the lipid and bind tightly in the hydrophobic pocket. These antiviral compounds block breathing of the rhinovirus capsid, the process by which normally internal parts of the capsid proteins are transiently displayed on the virion surface (186). Polioviruses containing bound WIN compounds can bind to cells, but the interaction with Pvr does not result in the production of A particles (92,331). WIN compounds appear to inhibit poliovirus infectivity by preventing Pvr-mediated conformational alterations that are required for uncoating. Additional support for the role of lipids in uncoating comes from the analysis of poliovirus mutants that cannot replicate unless WIN compounds are present (224). Such WIN-dependent mutants spontaneously convert to altered particles at 37°C, in the absence of the cell receptor, probably because of the absence of lipid in the hydrophobic pocket. The lipids can be considered to be switches that determine whether the virus is stable (lipid present) or will uncoat (lipid absent). It is not known what causes the lipid to be released from the capsid. Pvr docks onto the poliovirus capsid just above the hydrophobic pocket (see Fig. 6), which suggests that the interaction of the virus with receptor may initiate structural

changes in the virion that lead to the release of the lipid. Incubation of poliovirus with Pvr for short periods at low temperatures appears to result in release of the lipid (32).

Translation of the Viral RNA

Internal Ribosome Binding: The Internal Ribosome Entry Site

Once the picornavirus positive-stranded genomic RNA is released into the cell cytoplasm, it must be translated because it cannot be copied by any cellular RNA polymerase and no viral enzymes are brought into the cell within the viral capsid. Several experimental findings led to the belief that translation of the picornavirus genome was accomplished by an unusual mechanism. The positive-stranded RNA genomes lack 5'-terminal cap structures; although virion RNA is linked to the viral protein VPg, this protein is removed by a cellular “unlinking” enzyme on entry of the RNA into the cell (8). Furthermore, picornavirus genomes are efficiently translated in infected cells despite inhibition of cellular mRNA translation. Determination of the nucleotide sequence of poliovirus positive-stranded RNA revealed a 741-nucleotide 5'-untranslated region that contains seven AUG codons (174,260). Such 5'-noncoding regions, which were subsequently found in other picornaviruses, contain highly ordered RNA structures (265,288). These features led to the suggestion that ribosomes do not scan through the picornaviral 5'-untranslated regions, but rather bind to an internal sequence. The 5'-untranslated region of poliovirus positive-stranded RNA was subsequently shown to contain a sequence that promotes translation initiation by internal ribosome binding, or the IRES (Fig. 8).

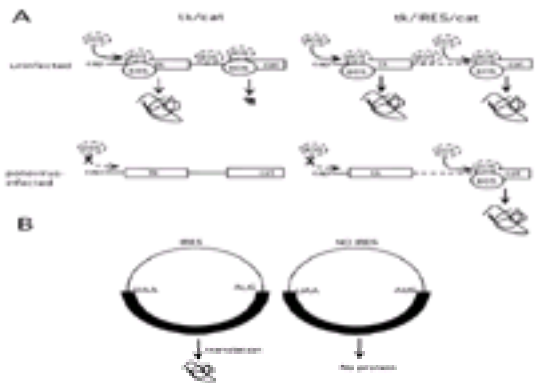

FIG. 8. Discovery of the internal ribosome entry site (IRES). **A:** Bicistronic mRNA assay used to discover the IRES (244). Plasmids were constructed that encode two reporter molecules, thymidine kinase (tk) and chloramphenicol acetyl transferase (cat), separated by an IRES (*solid line*) or a spacer (*dotted line*). After introduction into mammalian cells, the plasmids give rise to mRNAs of the structure shown in the figure. In uninfected cells (*top line*), both reporter molecules can be detected, although cat synthesis is inefficient without an IRES and is probably due to reinitiation. In poliovirus-infected cells, 5' end–dependent initiation is inhibited, and no proteins are detected without an IRES, demonstrating internal ribosome binding. **B:** Circular mRNA assay for an IRES. Circular mRNAs were constructed and translated *in vitro* in cell extracts. In the absence of an IRES, no protein is observed because 5'-end initiation requires a free 5'-end. Inclusion of an IRES allows protein translation from the circular mRNA, demonstrating internal ribosome binding (56).

The picornavirus IRES contains extensive regions of RNA secondary structure that is conserved within the type I (enteroviruses and rhinoviruses) and type II (cardioviruses and aphthoviruses) IRES (see Fig. 3). One sequence motif that is conserved among these two types of IRES is a GNRA sequence (N, any nucleotide; R, purine) in stem-loop IV of the type I IRES and in stem-loop I of the type II IRES. Another conserved element is an Yn-Xm-AUG motif, in which Yn is a pyrimidine-rich region and Xm is a 15- to 25-nucleotide spacer followed by an AUG codon. In the type II and hepatitis A virus IRES, this codon is the initiator AUG; in the type I IRES, the initiator is located 30 to 150 nucleotides further downstream. Translation initiation through a type 1 IRES involves binding of the 40S ribosomal subunit to the IRES and scanning of the subunit to the AUG initiation codon. The 40S subunit probably binds at the AUG initiation codon of a type II IRES.

Mechanism of Internal Ribosome Binding

Translation initiation directed by an IRES requires the same set of initiation proteins necessary for the translation of mRNAs by 5' end–dependent initiation. The exception was believed to be eIF4G, which is cleaved in picornavirus-infected cells, inactivating the translation of most cellular mRNAs. We now know that the C-terminal fragment of eIF4G, which contains binding sites for eIF3 and eIF4A (Fig. 9), stimulates IRES-mediated translation (44,234). In one model of the mechanism of internal ribosome binding, the 40S ribosomal subunit is recruited to the IRES through interaction with eIF3 bound to the C-terminal domain of eIF4G (see Fig. 9). Binding of eIF4G to the mRNA does not occur through eIF4E because picornavirus mRNAs are not capped. However, eIF4G may bind to the IRES either directly or through a “linker” protein (see Fig. 9). It has been reported that eIF4G mRNA contains an IRES (100,101), suggesting a mechanism for its synthesis in picornavirus-infected cells, in which 5' end–dependent translation initiation is inhibited. Stimulation of IRES function by the C-terminal proteolytic fragment of eIF4G may explain the observation that IRES activity is enhanced in cells in which the poliovirus protease $2A^{pro}$ is expressed (131). Protease $2A^{pro}$ is one of the picornaviral proteinases responsible for cleavage of eIF4G. Although the IRES of most picornaviruses functions with cleaved eIF4G, that of hepatitis A virus requires intact eIF4G (41).

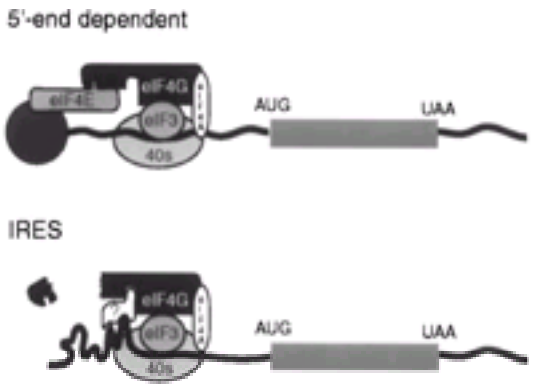

FIG. 9. Models for initiation complex formation. In 5'8 end–dependent initiation, the 40S subunit is recruited to the mRNA through its interaction with eIF3, which binds eIF4G. The latter initiation factor is part of eIF4F, which also contains eIF4A, a helicase to unwind RNA secondary structure, and eIF4E, the cap-binding protein. Binding of eIF4E to the cap thus positions eIF4E at the 5'-end and positions the 40S subunit on the mRNA. In IRES-dependent translation, a 5'-end is not required. The eIF3–40S complex is believed to be recruited to the RNA by the interaction of eIF4G with cellular IRES-binding proteins, marked by ?. No such universal IRES-binding protein has been identified.

Only one IRES, from cricket paralysis virus (322), has been shown to function *in vitro* in wheat germ extracts, and many IRESes function poorly in reticulocyte lysates; capped mRNAs can be translated efficiently in both types of extract. These observations may be explained if ribosome binding to the IRES requires cell proteins other than the canonical translation proteins, perhaps to link the IRES to eIF4G, as discussed previously. The search for such proteins has involved using mobility-shift assays to identify proteins that bind to the IRES and determining whether such proteins are necessary for IRES function in cell-free extracts. One host protein identified by this approach is the autoantigen La, a cellular protein that plays a role in the maturation of RNA polymerase III transcripts (210). It is present in low amounts in reticulocyte lysates; addition of the protein to such lysates stimulates the activity of the poliovirus IRES (210). La autoantigen is also required for efficient function of the encephalomyocarditis virus IRES (169).

Another protein that binds the picornavirus IRES is polypyrimidine tract-binding protein, a regulator of pre-mRNA splicing (138). Removal of this protein from a reticulocyte lysate with an RNA affinity column inhibits the function of the encephalomyocarditis virus IRES but does not affect translation by 5' end–dependent initiation (161). The deficiency in IRES function is restored by adding the purified protein back to the lysate. The depleted lysate, however, still supports the function of the IRES from Theiler's murine encephalomyocarditis virus, another picornavirus. It was subsequently shown that the requirement for polypyrimidine tract-binding protein by the encephalomyocarditis virus IRES depends on the nature of the reporter and the size of an A-rich bulge in the IRES (162). Polypyrimidine tract–binding protein is required for the function of the IRES of poliovirus and rhinovirus (149).

HeLa cell extracts also contain unr, an RNA-binding protein with five cold-shock domains that is required for IRES function (148). Recombinant unr stimulates the function of the rhinovirus IRES in the reticulocyte lysate and acts synergistically with recombinant polypyrimidine tract–binding protein to stimulate translation mediated by the rhinovirus IRES. However, unr does not augment the stimulation of the poliovirus IRES by polypyrimidine tract–binding protein.

Ribosome-associated poly r(C)–binding proteins bind at multiple sites within the poliovirus IRES (38,98). One binding site for these proteins has been identified within stem-loop IV of the poliovirus IRES (see Fig. 3) (38). Mutations in this region that abolish binding of poly r(C)–binding proteins cause decreased translation *in vitro.* Furthermore, depletion of poly r(C)–binding proteins from HeLa cell translation extracts results in inhibition of poliovirus IRES function (39). When this assay was used to survey a wide range of picornaviral IRES elements, it was found that poly r(C)–binding proteins are required for function of the type I, but not the type II, IRES (318). These results provide the first evidence of a difference in the manner in which the type I and type II IRES function. A second binding site for poly r(C)–binding proteins has been identified within a cloverleaf RNA structure that forms within the first 108 nucleotides of the positive-stranded poliovirus RNA genome (98,239). The interaction of poly r(C)–binding proteins with this part of the RNA has been proposed to regulate whether a positive-stranded RNA molecule is translated or replicated.

From the preceding discussion, it is clear that no single protein has been identified that is essential for the function of every IRES. It is certainly reasonable to propose that certain types of IRES have different requirements for cellular proteins. A different view is that cellular proteins, such as polypyrimidine tract–binding protein, may act as RNA chaperones, maintaining the IRES in a structure that permits it to bind directly to the translational machinery (151). IRESes that do not require such chaperones may fold properly without the need for cellular proteins. In support of this hypothesis, the IRES of hepatitis C virus, a flavivirus, has been shown to bind directly to eIF3 and the ribosomal 40S subunit, without a requirement for eIF4F (246).

Processing of the Viral Polyprotein

Picornavirus proteins are synthesized by the translation of a single, long, open reading frame on the viral positive-stranded RNA genome, followed by cleavage of the polyprotein by virus-encoded proteinases (Fig. 10; see Fig. 2). This strategy allows the synthesis of multiple protein products from a single RNA genome. The polyprotein is not observed in infected cells because it is processed as soon as the protease coding sequences have been translated. The polyprotein precursor is processed cotranslationally by intramolecular reactions (in *cis*) in what are called primary cleavages, followed by secondary processing in *cis* or in *trans* (intermolecular). Picornavirus genomes encode three proteinases: L^{pro}, $2A^{pro}$, and $3C^{pro}$, which carry out cleavage of the polyprotein.

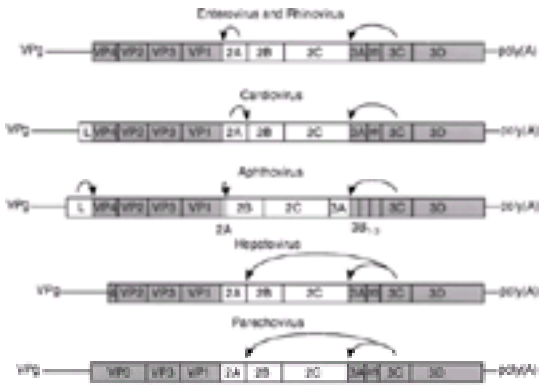

FIG. 10. Primary cleavages of picornavirus polyprotein. In cells infected with enteroviruses and rhinoviruses, nascent P1 is cleaved from P2 by $2A^{pro}$. In cells infected with cardioviruses and aphthoviruses, the P1–P2 junction is cleaved by $3C^{pro}$. The 2A proteins of these viruses are not proteinases, but they cause their own release at the junction with 2B. In all viruses shown, the P2–P3 cleavage is carried out by $3C^{pro}$. In hepatoviruses and parechoviruses, $3C^{pro}$ also carries out primary cleavage at the 2A–2B junction. The L^{pro} proteinase of foot-and-mouth disease virus catalyzes its release from VP4.

In contrast to other picornaviruses, the first protein encoded in the genome of cardioviruses and aphthoviruses is the L protein (see Fig. 2 and Fig. 10). The L protein of foot-and-mouth disease virus is a proteinase that releases itself from the polyprotein by cleaving between its C-terminus and the N-terminus of VP4. Based on sequence analysis, it was suggested that foot-and-mouth disease virus L^{pro} is related to thiol proteases (118). This prediction was supported by the results of site-directed mutagenesis, which showed that Cys-51 and His-148 are the active-site amino acids (252,266). The atomic structure of L^{pro} reveals that it consists of two domains, with a topology related to that of papain, a thiol proteinase (123) (Fig. 11). The active-site His is located at the top of the central a-helix, and substrate binds in the groove between the two domains (see Fig. 11). Besides releasing itself from the polyprotein, L^{pro} also cleaves the translation initiation factor eIF4G to effect inhibition of cellular translation (73). In cardioviruses, the L protein, which does not have proteolytic activity, is released from the P1 precursor by $3C^{pro}$ (238).

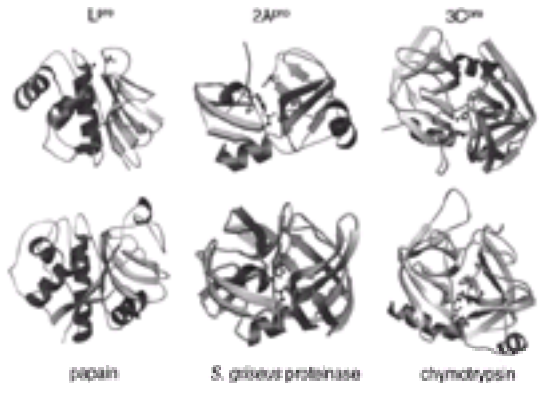

FIG. 11. Three dimensional structures of hepatitis A virus $3C^{pro}$, rhinovirus $2A^{pro}$, and L^{pro} of foot-and-mouth disease virus. Below each is the cellular proteinase that is structurally similar to each viral enzyme. Only the globular domain of L^{pro} is shown because the remaining residues have no counterpart in papain. Catalytic residues are drawn as *balls* and *sticks.* The rhinovirus $2A^{pro}$ structure is a model that was predicted before the crystal structure was solved. (Adapted from Seipelt J, Guarne A, Bergmann E, et al. The structures of piconaviral proteinases. *Virus Res* 1999;62:159,160,161,162,163,164,165,166,167 and 168), with permission.)

In cells infected with enteroviruses or rhinoviruses, the primary cleavage between P1 and P2 is mediated by $2A^{pro}$. In addition, $2A^{pro}$ cleaves the host cell protein eIF4G (see Cleavage of eIF4G). In the protein precursor of rhinovirus, poliovirus, and some other enteroviruses, the cleavage site for $2A^{pro}$ is between tyrosine and glycine. Other sites cleaved by $2A^{pro}$ include threonine-glycine and phenylalanine-glycine in certain coxsackieviruses and echoviruses. Based on sequence alignments, it was suggested that the structure of $2A^{pro}$ would resemble that of small bacterial chymotrypsin-like proteinases such as *Streptomyces griseus* proteinase A and would possess a catalytic triad consisting of His-20, Asp-38, and an active-site nucleophile of Cys-109 rather than serine (29). The results of site-directed mutagenesis and the resolution of the atomic structure of rhinovirus $2A^{pro}$ confirm that these residues compose the active site and that the fold of $2A^{pro}$ is very similar to that of *S. griseus* proteinase A (247,292,329) (see Fig. 11). However, $2A^{pro}$ differs from all known chymotrypsin-like proteinases in that the N-terminal domain is not a b-barrel, but rather a four-stranded antiparallel b-sheet. Another unusual feature of $2A^{pro}$ is a tightly bound zinc ion located at the beginning of the C-terminal domain. Biochemical and structural studies indicate that zinc is essential for the structure of the enzyme (247,291,317).

The 2A protein of hepatoviruses and parechoviruses does not have proteolytic activity and has no known function. The primary cleavage between the capsid protein precursor and the P2 region of these viruses is carried out by $3C^{pro}$ (see Fig. 10) (157,278,279).

The 2A protein of aphthoviruses and cardioviruses does not have proteolytic activity and is not related to other picornavirus 2A proteins. In cells infected with these viruses, the VP1/2A cleavage is not carried out by 2A, but rather by

3C^{pro} (238). Aphthovirus 2A protein is only 18 amino acids in length, yet this protein can catalyze autocleavage at the junction with 2B, even in heterologous polyproteins (275). The 2A protein of cardioviruses is 150 amino acids long, yet only the last 19 amino acids, plus the first amino acid from 2B, proline, are required for cleavage from 2B (76). Mutations within the conserved amino acid sequence Asn-Pro-Gly-Pro, which contains the cleavage site Gly-Pro, disrupt cleavage (238). The mechanism of aphthovirus- and cardiovirus 2A–mediated cleavage is not understood and represents a novel form of peptide bond scission probably involving a single turnover event.

All picornaviruses encode 3C^{pro}, which carries out a primary cleavage between 2C and 3A (see Fig. 10). In hepatoviruses and parechoviruses, 3C^{pro} also cleaves between 2A and 2B (see Fig. 10). Unlike the other picornavirus proteinases, 3C^{pro} also carries out secondary cleavages of the P1 and P2 precursors. Poliovirus 3C ^{pro} cleaves only at the Gln-Gly dipeptide; however, 3C^{pro} of other picornaviruses has less strict cleavage specificities and cleaves at other sites, including Gln-Ser, Gln-Ile, Gln-Asn, Gln-Ala, Gln-Thr, and Gln-Val. There are clearly other determinants of cleavage because not all such dipeptides in picornavirus polyproteins are cleaved by 3C ^{pro}. Additional determinants include accessibility of the cleavage site to the enzyme, recognition of secondary and tertiary structures in the substrate by the enzyme, and amino acid sequences surrounding the cleavage site. For example, efficient cleavage of poliovirus Gln-Gly pairs requires an Ala at the P4 position (Gln is residue P1, numbering is toward the N-terminus) (37).

Sequence comparisons with cellular proteinases led to the prediction that 3C ^{pro} folds similarly to the chymotrypsin-like serine proteinases (29,114,115,116 and 117). The putative catalytic triad was believed to consist of His-40, Asp-71 (aphthoviruses and cardioviruses) or Glu-71 (enteroviruses and rhinoviruses), and Cys-147 as the nucleophilic residue, in contrast to serine in cellular serine proteinases. These predictions have been confirmed by site-directed mutagenesis and by resolution of the atomic structures of rhinovirus, hepatitis A virus, and poliovirus 3C ^{pro} (55,122,132,166,201,222,247). The viral enzyme folds into two equivalent b-barrels like chymotrypsin (see Fig. 11) but differs in some of the connecting loops, the orientation of the catalytic residues, and in areas needed for transition-state stabilization. The acidic member of the catalytic triad, Glu or Asp, points away from the active-site His and is therefore not believed to assist in catalysis. However, 3C^{pro} also binds viral RNA (see Genome Replication and mRNA Synthesis), and this binding site is distal from the active site of the enzyme. The presence of this RNA-binding site imposes evolutionary constraints on 3C^{pro} that are not found in other proteinases.

Both 3C^{pro} and 2A^{pro} are active in the nascent polypeptide and release themselves from the polyprotein by self-cleavage. After the proteinases have been released, they cleave the polyprotein in *trans.* The cascade of processing events varies for different picornaviruses. In cells infected with rhinovirus and enteroviruses, the initial event in the processing cascade is the release of the P1 precursor from the nascent P2–P3 protein by 2A^{pro}. The activity of 2A^{pro} does not depend on whether it is cleaved from the precursor (137), but further processing of P1 by 3CD^{pro} does not occur unless 2A^{pro} is released from P1 (228,328). Next, 3CD^{pro} is released from the P3 precursor by autocatalytic cleavage; it is this proteinase, and not 3C^{pro}, which carries out secondary cleavage of glutamine-glycine dipeptides in poliovirus P1 (160,327). Both 3C^{pro} and 3CD^{pro} can process proteins of the P2 and P3 regions. 3D may be required to recognize structural motifs in properly folded P1, allowing efficient processing of the 3C ^{pro} part of the enzyme. The presence of multiple activities in a single protein is not found among eukaryotic proteinases and is an example of how the coding capacity of small viral genomes can be maximized. Not all picornaviruses require 3CD ^{pro} to process P1; aphthoviruses, cardioviruses, and hepatoviruses produce 3C ^{pro} that can cleave P1 without additional viral protein sequence.

An advantage of the polyprotein strategy is that expression can be controlled by the rate and extent of proteolytic processing. Alternative use of cleavage sites can also produce proteins with different activities. For example, because 3CD^{pro} is required for processing of the poliovirus capsid protein precursor P1, the extent of capsid protein processing can be controlled by regulating the amount of 3CD ^{pro} that is produced. Because 3CD^{pro} does not possess RNA polymerase activity, some of it must be cleaved to allow RNA replication.

A final processing step occurs during maturation, when VP0 is cleaved to form VP4 and VP2. This event is discussed later.

Genome Replication and mRNA Synthesis

In the 1950s, it was believed that the genome of RNA viruses was replicated by the cellular DNA-dependent RNA polymerase, through an intermediate DNA strand. The replication of RNA viruses was therefore thought to occur entirely in the cell nucleus. In the early 1960s, studies of mengovirus showed that virus infection results in the induction of a cytoplasmic enzyme that can synthesize viral RNA in the presence of actinomycin D (21). This observation suggested that viral genome replication occurred through a virus-specific RNA-dependent RNA polymerase because cellular RNA synthesis is DNA dependent, occurs in the nucleus, and is sensitive to actinomycin D. Shortly thereafter, a similar cytoplasmic, actinomycin D–resistant genome replication system was discovered in poliovirus-infected cells (19). Since then, most studies of picornavirus RNA synthesis have been carried out with poliovirus.

In poliovirus-infected cells, the positive-stranded genome is amplified through a negative-stranded intermediate, resulting in about 50,000 genomes per cell. Three forms of RNA have been identified in the cell: single-stranded RNAs, replicative intermediate (RI), and replicative form (RF). Single-stranded RNA is the most abundant form and is exclusively positive-stranded; free negative strands have never been detected in infected cells. RI is full-length RNA from which six to eight nascent strands are attached. RI is largely of positive polarity with nascent negative strands, although the opposite configuration has been detected. RF is a double-stranded structure, consisting of one full-length copy of the positive and negative strands. Viral RNA synthesis is asymmetric in that the synthesis of positive strands is 25 to 65 times greater than the synthesis of negative strands (106,232).

The Viral RNA-Dependent RNA Polymerase, 3D^{pol}

The first evidence for a viral RNA-dependent RNA polymerase activity came from experiments in which lysates from cells

infected with mengovirus or poliovirus were assayed for viral RNA polymerase activity by the incorporation of a radioactive nucleotide into viral RNA (21). Initial experiments demonstrated that the viral RNA polymerase is associated with a cellular membrane fraction, subsequently shown to be smooth membranes, which was called the *RNA replication complex* (109). A major component of the replication complex was a viral protein that migrated at 63,000 daltons on polyacrylamide gels (therefore called p63), which was suggested to be the viral RNA-dependent RNA polymerase. However, other viral and host proteins, including 2BC, 2C, 3AB, and 3C ^{pro}, were detected in the RNA replication complexes.

A limitation of this early work was that replication complexes did not respond to the addition of template RNA, but rather copied viral RNA already present in the complex. Therefore, attempts were made to purify a template-dependent enzyme from membrane fractions of infected cells, using a poly(A) template and an oligo(U) primer. A poly(U) polymerase activity was purified from poliovirus-infected cells, which could also copy poliovirus RNA in the presence of an oligo(U) primer. Highly purified preparations contained only p63, the major viral protein found in membranous replication complexes (87,310). This protein is the poliovirus RNA polymerase, now known as 3D ^{pol}. In the absence of an oligo(U) primer, 3D ^{pol} cannot copy poliovirus RNA. Recombinant 3D ^{pol} purified from bacteria or insect cells also requires the presence of an oligo(U) primer to copy poliovirus RNA (227,256). 3D^{pol} is therefore a template- and primer-dependent enzyme that can copy poliovirus RNA. Its molecular weight predicted from the amino acid sequence is 53 kd.

The structures of three of the four types of polymerases, DNA-dependent DNA polymerase, DNA-dependent RNA polymerase, and RNA-dependent DNA polymerase (reverse transcriptase), are characterized by analogy with a right hand, consisting of a palm, fingers, and thumb. The palm domain contains the active site of the enzyme. The first structure of an RNA-dependent RNA polymerase is that of poliovirus 3D ^{pol}, which was determined at 2.6 Å resolution by x-ray crystallography (133). The enzyme has the same overall shape as other polymerases, although the fingers and thumb differ (Fig. 12). The palm domain is similar to that of other polymerases and contains four conserved amino acid motifs that are found in other RNA-dependent polymerases. 3D ^{pol} also contains a fifth motif between the palm and thumb subdomains, which is found in RNA-dependent but not DNA-dependent polymerases.

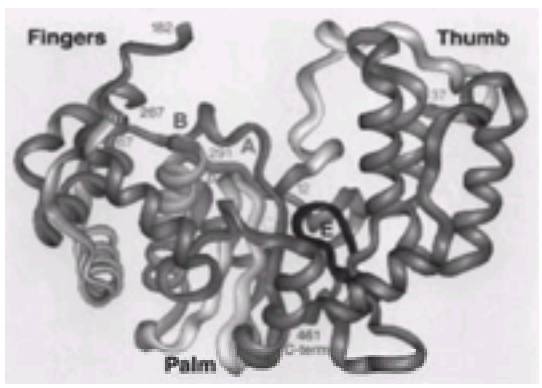

FIG. 12. Three dimensional structure of poliovirus 3D ^{pol}. Thumb, palm, and fingers subdomains are labeled. The two white regions have no counterparts in other polymerases. The region connecting these sequences is disordered (amino acids 33 to 66); hence, it is not known whether these residues are connected across the active site (considered unlikely) or whether amino acids 14 to 35 are from a separate molecule. If the latter is correct, such interactions may lead to oligomerization. (Adapted from ref. 133, with permission.)

In addition to its enzymatic activity, poliovirus 3Dpol is also a cooperative single-stranded RNA-binding protein and can unwind duplex RNA without the need for adenosine triphosphate hydrolysis (59,240). Consistent with these findings, two regions of polymerase–polymerase interaction have been observed in 3D ^{pol} protein crystals. The first, called interface I, is extensive and involves more than 23 amino acid side chains on two different surfaces of the protein (Fig. 13). This interaction occurs in a head-to-tail fashion and results in long fibers of polymerase molecules. When RNA is modeled into the 3D^{pol} structure based on the location of DNA in the crystal structure of DNA bound to human immunodeficiency virus type 1 reverse transcriptase, it falls along the fibers formed by interface I interactions. Interface II is formed by N-terminal polypeptide segments, which may lead to a network of polymerase fibers in combination with interactions at interface I. These N-terminal polypeptide segments may originate from different polymerase molecules. The N-terminus of 3D^{pol} is required for enzyme activity, which would support the idea that interface II interactions are of functional consequence. The viral nucleoproteins of negative-stranded RNA viruses are also cooperative single-stranded RNA-binding proteins. The function of these proteins is to allow multiple rounds of RNA synthesis by keeping the RNA single stranded and preventing base-pairing between template and product. The single-stranded RNA–binding properties of 3D^{pol} may have a similar function in infected cells.

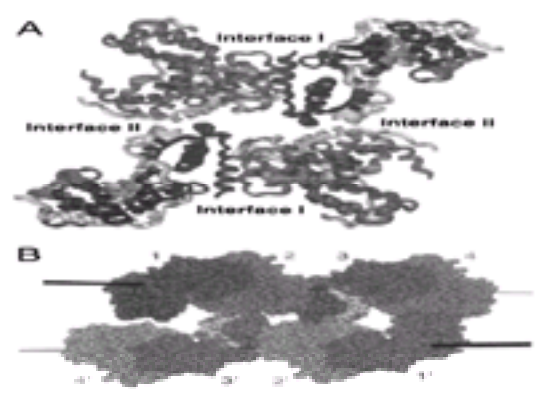

FIG. 13. Interactions among $3D^{pol}$ molecules in protein crystals. **A:** The manner in which four polymerase molecules interact is shown. Interface I, between the front of the thumb subdomain of one molecule and the back of the palm subdomain of another, leads to head-to-tail fibers of the enzyme. Interface II, formed by interactions between the two N-terminal regions of the polymerase (*white*), is a result of contacts between polymerase molecules in neighboring fibers. **B:** Model of how $3D^{pol}$ might oligomerize on RNA. Shown are two fibers formed by interface I interactions. Each fiber contains four polymerase molecules. The two fibers interact across interface II; the axis of each is shown in *black.* (Adapted from ref. 133, with permission.)

Viral Accessory Proteins

The poliovirus capsid proteins are not required for viral RNA synthesis; the region of the viral genome that encodes these proteins can be deleted without affecting the ability of viral RNA to replicate in cells (163). However, the capsid coding regions of rhinovirus, Theiler's virus, and mengovirus contain a cis-acting RNA sequence required for genome replication (192,206,207). A cis-acting RNA sequence required for RNA replication has also been identified in the poliovirus protein 2C coding region (113). Genetic and biochemical studies implicate most proteins of the P2 and P3 regions of the genome in RNA synthesis.

2A Protein

As discussed previously, $2A^{pro}$ protein is necessary for proteolytic cleavage of the polyprotein. Whether this is the sole requirement for this protein in viral replication was addressed by placing a second IRES element in the poliovirus genome before the $2A^{pro}$ coding region, effectively alleviating the need for the processing activity of the enzyme. Such viruses are viable (216). However, deletion of part of the coding region for $2A^{pro}$ is lethal. These results suggest that $2A^{pro}$ also plays a role in viral RNA replication, although its function in this process is not known.

2B Protein

Mutagenesis of 2B protein of poliovirus and coxsackievirus results in viruses with defects in RNA synthesis (159,311). The C-terminus of 2B contains a hydrophobic region and a conserved putative amphipathic a-helix that appear to be crucial to the function of the protein (312). However, the role of 2B in RNA synthesis is not known. Protein 2B has been shown to induce cell membrane permeability (6,276,313,314), which may play a role in release of virus from cells. Protein 2B is also partly responsible for the proliferation of membranous vesicles in infected cells, which are the sites of viral RNA replication. Because protein 3A has the same effect, it is not clear whether altered membrane proliferation is responsible for the RNA-defective phenotype in 2B mutants.

2C Protein

Mutations responsible for resistance of poliovirus and echovirus to guanidine hydrochloride, which blocks viral RNA replication, are located in the 2C protein (175,253,254). This compound has been shown to inhibit specifically the initiation of negative-stranded RNA synthesis and has no effect on initiation of positive-stranded RNA synthesis or on elongation on either strand (25). Protein 2C binds RNA, has nucleoside triphosphatase (NTPase) activity (267) that is inhibited by guanidine (251), and shares amino acid homology with known RNA helicases, proteins encoded by most positive-stranded RNA viruses with large genomes. These enzymes are believed to be necessary to unwind double-stranded RNA structures that form during RNA replication. Purified protein 2C, however, does not have RNA helicase activity (267). Alteration of conserved amino acids within the NTPase domain of 2C results in loss of viral infectivity. 2C may therefore have two functions during viral RNA synthesis: as an NTPase, and directing replication complexes to cell membranes. Expression of 2C leads to the formation of membrane vesicles and may contribute to the formation of the replication complex (60).

3AB Protein

Protein 3AB is believed to anchor VPg in membranes for the priming step of RNA synthesis. The purified protein greatly stimulates $3D^{pol}$ activity *in vitro* (241,255,264) as well as the proteolytic activity of $3CD^{pro}$ (215). 3AB interacts with $3D^{pol}$ and 3CD in infected cells and with $3D^{pol}$ in the yeast two-hybrid system (145). Mutations in $3D^{pol}$ that disrupt its interaction with 3AB result in viruses with defects in protein processing and viral RNA synthesis (104,105). A complex of 3AB and 3CD also binds the 3'-terminal sequence of poliovirus RNA. 3AB is therefore a stimulatory cofactor for $3D^{pol}$.

Cellular Accessory Proteins

Early studies of poliovirus replication using purified components suggested that a host cell protein is required for copying the viral RNA by $3D^{pol}$ in the absence of an oligo(U) primer (72). Although this protein was never identified, the concept of a host factor required for poliovirus replication endured, largely because there was ample precedent for the participation of host cell components in a viral RNA replicase. The best studied example is the RNA replicase of the bacteriophage Qb, which is a multisubunit enzyme consisting of a 65-kd virus-encoded protein and three host proteins: ribosomal protein S1 and translation factors EF-Tu and EF-Ts. The 65-kd viral protein has no RNA polymerase activity in the absence of the host factors but has sequence similarity with known RNA-dependent RNA polymerases. A subunit of the translation initiation factor eIF-3 is part of the polymerase from brome mosaic virus. Two different experimental systems have recently been used to provide additional evidence that poliovirus RNA synthesis requires host cell components.

When purified poliovirus RNA is incubated *in vitro* with a cytoplasmic extract prepared from cultured, permissive cells, the

viral RNA is translated, the resulting protein is proteolytically processed, and the genome is replicated and assembled into new infectious virus particles (217). When guanidine, an inhibitor of poliovirus RNA synthesis, is included in the reaction, complexes are formed, but elongation cannot occur (25). The preinitiation complexes can be isolated free of guanidine, and when added to new cytoplasmic extracts, RNA synthesis occurs. In the absence of cytoplasmic extract, preinitiation complexes do not synthesize viral RNA (24). These results indicate that one or more soluble cellular components are required for the initiation of viral RNA replication. A similar conclusion derives from studies in which poliovirus RNA is injected into oocytes derived from the African clawed toad, *Xenopus laevis.* Poliovirus RNA cannot replicate in *Xenopus* oocytes unless it is co-injected with a cytoplasmic extract from human cells (97).

Cellular poly r(C)–binding proteins are required for poliovirus RNA synthesis. These proteins bind to a cloverleaf-like secondary structure that forms in the first 108 nucleotides of positive-stranded RNA (Fig. 14). Binding of poly r(C)–binding protein to the cloverleaf is necessary for the binding of viral protein 3CD to the opposite side of the same cloverleaf (10, 11, 98, 239). Formation of a ribonucleoprotein complex composed of the 5' cloverleaf, 3CD, and a cellular protein is essential for the initiation of viral RNA synthesis (10). The human cell protein required for replication of poliovirus RNA in *Xenopus* oocytes has also been identified as poly r(C)–binding protein (98). A model of how these interactions lead to viral RNA synthesis is shown in Figure 14.

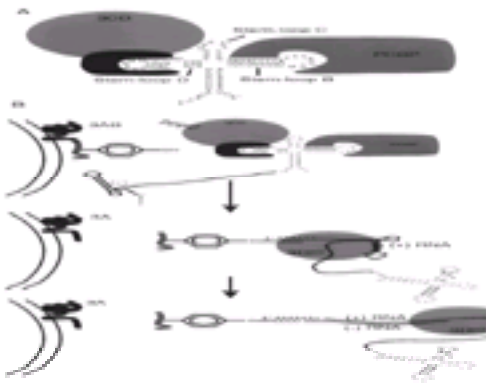

FIG. 14. Model for the synthesis of poliovirus (–) strand RNA. **A:** Schematic of the ribonucleoprotein complex that forms on a cloverleaf-like structure in the first 108 nucleotides of the viral (+) strand RNA. Binding of poly r(C) binding protein to stem-loop B is required for binding of viral 3CD to stem-loop D. This complex is required for synthesis of (+) and (–) strand RNA. **B:** Model for the initiation of (–) strand RNA synthesis. **Top:** the ribonucleoprotein complex has formed around the cloverleaf-like structure on the (+) strand and interacts with membrane-bound 3AB. Middle: 3AB is uridylylated and cleaved to release VPg-pUpU, and 3CD is cleaved to $3C^{pro}$ and $3D^{pol}$. Uridylylation and cleavage are carried out by 3CD. The $3D^{pol}$ then uses VPg-pUpU to prime RNA synthesis at the 3'-end of the viral (+) strand. A pseudoknot in the 3'-untranslated region may facilitate recognition of the 3'-end. **Bottom:** Elongation of a (–) strand RNA. Once this strand is complete, a cloverleaf forms at the 5'-end, leading to formation of another ribonucleoprotein complex and (+) strand RNA synthesis. Thus, many initiation reactions can occur on the original (+) strand RNA while (–) strands are being elongated.

Another candidate for a host protein that is involved in poliovirus RNA synthesis is Sam68 (Src-associated in mitosis, 68 kd), which is associated with $3D^{pol}$ in poliovirus-infected cells (203). Sam68 is normally located in the cell nucleus but becomes redistributed to the cytoplasm during poliovirus infection. Sam68 can also be isolated from infected cells using an antibody directed against viral protein 2C, which is present in membranous RNA replication complexes. These results suggest that Sam68 may have a role in poliovirus RNA synthesis, although no direct experimental support for this hypothesis has emerged.

A Protein-Linked Primer for RNA Synthesis

As discussed previously, poliovirus $3D^{pol}$ is a primer-dependent enzyme that will not copy poliovirus RNA *in vitro* without an oligo(U) primer. The discovery of VPg linked to poliovirus genome RNA, as well as to the 5' end of newly synthesized positive- and negative-stranded RNAs, suggested that VPg might be involved in the initiation of RNA synthesis. This hypothesis was supported by the finding that both VPg and a uridylylated form of the protein, VPg-pUpU, can be found in infected cells (69). Furthermore, VPg-pUpU can be synthesized *in vitro* in a membrane fraction from poliovirus-infected cells (300). It was also argued that a precursor of VPg, known as 3AB, is likely to participate in the initiation reaction. 3AB is a membrane-bound polypeptide and therefore an ideal candidate to act as a VPg donor in membranous replication complexes. Viruses containing a mutation in 3AB that decreases its hydrophobicity are defective in initiation of RNA synthesis, *in vitro* uridylylation of VPg, and *in vivo* synthesis of positive-stranded viral RNAs (104, 105). Because no protein larger than VPg has been detected linked to nascent RNA strands, it is likely that 3AB is rapidly cleaved by the proteinase $3C^{pro}$ to form VPg RNA. Additional evidence that VPg can serve as a primer for poliovirus RNA synthesis comes from experiments in which synthetic VPg is first uridylylated *in vitro,* then added to an *in vitro* polymerase reaction containing a poly(A) template and $3D^{pol}$ (242). The labeled, uridylylated VPg is extended to form poly(U). Thus, poliovirus RNA replication is primed by a genome-linked protein, a mechanism also involved in adenovirus DNA replication. A model for VPg priming of poliovirus RNA synthesis is shown in Figure 15.

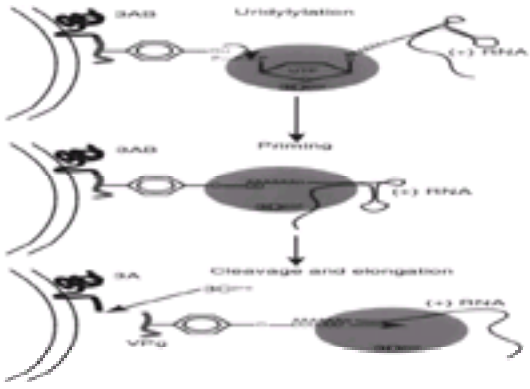

FIG. 15. Model for VPg priming of poliovirus RNA synthesis. 3AB is a hydrophobic, membrane-bound protein that is a precursor of VPg and probably participates in the initiation reaction. Uridylylation of membrane-bound 3AB is carried out by $3D^{pol}$ and is dependent on RNA template. Priming of RNA synthesis by 3AB-pUpU and cleavage to release VPg-pUpU probably occur in rapid succession because no protein larger than VPg linked to nascent RNA strands has been detected.

The Cellular Site of RNA Synthesis

Messenger RNA and genome synthesis of picornaviruses, like that of most RNA viruses, occurs in the cytoplasm of the cell. Cells infected with poliovirus accumulate small membranous vesicles that are the sites of poliovirus RNA synthesis (109,223). The accumulation of these vesicles is believed to be a result of the inhibition of membrane vesicle transport from the endoplasmic reticulum to the cell surface. Expression of the viral proteins 2B or 3A is sufficient to block the transport of glycoproteins to the cell surface, and it has been suggested that this inhibition leads to the accumulation of vesicles on which viral RNA synthesis occurs (74,75,314). Proteins 2BC and 2C might also contribute to the accumulation of such vesicles because expression of these proteins leads to membrane proliferation in cells (60).

Two other findings indicate the importance of vesicles in viral RNA synthesis. First, poliovirus RNA synthesis is inhibited by cerulenin, an inhibitor of lipid synthesis (124). Viral RNA replication is also inhibited by the fungal metabolite brefeldin A, which blocks the transport of proteins from the endoplasmic reticulum to the cell surface (150,202). Brefeldin A inhibits protein transport by blocking the movement of membrane vesicles from the endoplasmic reticulum to the Golgi apparatus and among the compartments of the Golgi. In the presence of brefeldin A, membranous vesicles derived from the endoplasmic reticulum do not accumulate in infected cells, leading to an inhibition of viral RNA synthesis. Poliovirus, therefore, appears to produce the membrane vesicles important for its replication by inhibiting an intracellular protein transport pathway. Because poliovirus is nonenveloped, its replication does not require the protein glycosylation and secretion functions of the endoplasmic reticulum and Golgi apparatus.

Two viral proteins, 2C and 3AB, bring the replication complex to membranous vesicles. As discussed previously, 3AB is a hydrophobic protein that anchors the protein primer VPg in the membrane for RNA synthesis. Protein 2C has an RNA-binding domain, which might anchor viral RNA to membranes in the replication complexes (82).

Translation and Replication of the Same RNA Molecule

The genomic RNA of picornaviruses is not only mRNA but also the template for synthesis of negative-stranded RNA. How does the viral polymerase, traveling in a 3' to 5' direction on the positive strand, avoid collisions with ribosomes translating in the opposite direction? Perhaps a mechanism exists to avoid the two processes occurring simultaneously. *In vitro* experiments using inhibitors of protein synthesis demonstrate that when ribosomes are frozen on the viral RNA, replication of the RNA is inhibited. In contrast, when ribosomes are released from the viral RNA, its replication is increased (26). These results suggest that replication and translation cannot occur on the same template simultaneously.

It has been proposed that the cloverleaf structure in the 5'-noncoding region of poliovirus positive-stranded RNA can regulate whether the RNA is translated or replicated (99). In this model, early in infection, binding of poly r(C)–binding protein to the cloverleaf structure stimulates translation. Once 3CD has been synthesized, it binds to the cloverleaf structure (see Fig. 14), represses translation, and stimulates RNA synthesis. Although this model has some experimental support, it does not account for the fact that viral RNA translation and RNA replication both occur for several hours in infected cells.

Whether there exist mechanisms to regulate translation and replication of an RNA, there is experimental evidence that some ribosome and RNA polymerase collisions do occur. This conclusion is based on the isolation of a poliovirus variant whose genome contains an insertion of a 15 nucleotide sequence from 28S ribosomal RNA (54). Apparently, the RNA polymerase collided with a ribosome, copied 15 nucleotides of rRNA, and then returned to the viral RNA template.

Discrimination of Viral and Cellular RNA

The RNA-dependent RNA polymerases of picornaviruses are template-specific enzymes. Poliovirus $3D^{pol}$ copies only viral RNAs, not cellular mRNAs, in infected cells. However, the purified enzyme will copy any polyadenylated RNA if provided with an oligo(U) primer. This observation has led to the suggestion that template specificity probably resides in the interaction of replication proteins with sequence elements in the viral RNA. The 3'-noncoding region of the viral positive-stranded RNA contains an RNA pseudoknot conserved among picornaviruses that is believed to play a role in the specificity of copying by $3D^{pol}$ (154). Disruption of the pseudoknot by mutagenesis produces viruses that have impaired RNA synthesis, indicating the importance of the structure in the synthesis of negative-stranded RNA. $3D^{pol}$ or 3CD cannot bind to the 3' end of poliovirus RNA unless 3AB is present. Thus, the $3AB–3D^{pol}$ complex may determine the

specificity of binding to the 3' pseudoknot. Despite experimental results that underscore the importance of the pseudoknot in RNA synthesis, polioviruses from which the entire 3'-noncoding region of the viral RNA has been removed are able to replicate, albeit inefficiently (301). It seems likely that there are other primary determinants of template selection by the polymerase, such as cis-acting RNA sequences within the coding region and membrane association of the RNA polymerase, which would lead to increased local concentrations of essential components. Template specificity may also be conferred by the position of the $3D^{pol}$ gene at the very 3' end of the viral RNA; translation places the polymerase at the 3' end of the genome, ready for initiation. Early in infection, the pseudoknot structure might facilitate template selection when few polymerase molecules are available and membrane association has not yet provided highly concentrated areas of replication components.

A cloverleaf-like structure that forms in the 5'-noncoding region also plays an important role in template specificity. The finding that a mutation in a cloverleaf-like structure in the 5'-noncoding region that affects RNA synthesis could be complemented by a suppressor mutation in 3C led to the suggestion that $3C^{pro}$ might bind the cloverleaf and play a role in viral RNA replication (12). It was subsequently found that 3CD binds the cloverleaf structure in the positive strand, together with a cellular protein, now known to be poly r(C)–binding protein, that is required for complex formation (see later) (10,11,239). The RNA-binding domain of 3CD is contained within the 3C portion of the protein, on the opposite face of the molecule from the site involved in proteolysis. Mutations within this domain abolish complex formation and RNA replication without affecting viral protein processing. 3CD, therefore, plays an important role in viral RNA synthesis by participating in formation of a ribonucleoprotein complex at the 5' end of the positive-stranded RNA. A role for these interactions in viral RNA replication is suggested in the model in Figure 14.

Recombination

Recombination, the exchange of nucleotide sequences among different genome RNA molecules, was first discovered in cells infected with poliovirus, and was subsequently found to occur with other positive- and negative-stranded RNA viruses. The frequency of recombination, which is calculated by dividing the yield of recombinant virus by the sum of the yields of parental viruses, can be relatively high. In one study of poliovirus and foot-and-mouth disease virus, the recombination frequency was 0.9%, leading to the estimation that 10% to 20% of the viral genomes recombine in one growth cycle. When poliovirus recombination is studied by quantitative polymerase chain reaction, obviating the necessity to select for viable viruses, the recombination frequency for marker loci 600 nucleotides apart was 2×10^{-3}, which is similar to estimates obtained using selectable markers (156). RNA recombination also occurs in natural infections. For example, intertypic recombinants among the three serotypes of Sabin poliovirus vaccine strains are readily isolated from the intestines of vaccinees; some recombinants contain sequences from all three serotypes (46). The significance of these recombinants is unknown, but it has been suggested that such viruses are selected for their improved ability to replicate in the human alimentary tract compared with the parental viruses.

Poliovirus recombination mainly occurs between nucleotide sequences of the two parental genome RNA strands that have a high percentage of nucleotide identity and is called base pairing dependent (Fig. 16). RNA recombination is believed to be coupled with the process of genome RNA replication: it occurs by template switching during negative strand synthesis, as first demonstrated in poliovirus-infected cells (172). The RNA polymerase first copies the 3' end of one parental positive strand, then switches templates and continues synthesis at the corresponding position on a second parental positive strand (see Fig. 16). Template switching in poliovirus-infected cells occurs predominantly during negative strand synthesis because the concentration of positive-strand acceptors for template switching is 25- to 65-fold higher than that of negative-strand acceptors. This template-switching mechanism of recombination is also known as *copy-choice.* A prediction of the copy-choice mechanism is that recombination frequencies should be lower between different poliovirus serotypes, a prediction that has been verified experimentally. For example, recombination between poliovirus types 1 and 2 occurs about 100 times less frequently than among type 1 polioviruses (the different poliovirus serotypes differ by about 15% in their nucleotide sequences) (170). The cause of template switching is not known, but it might be triggered by pausing of the polymerase during chain elongation.

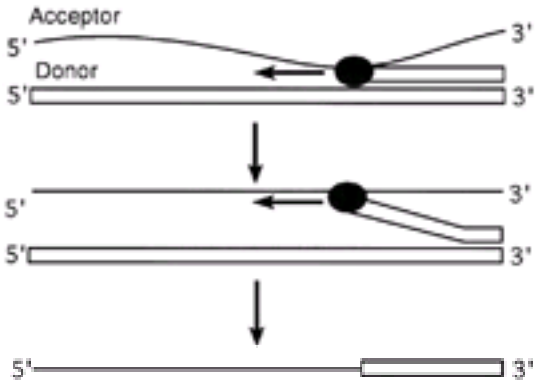

FIG. 16. RNA recombination. Schematic diagram of RNA recombination in picornavirus-infected cells by template switching, or copy-choice. Two parental genomes, the acceptor and donor, are shown as a *line* and *box.* The RNA polymerase, a *black oval,* is shown copying the 3' end of donor RNA and switching to the acceptor genome (middle). As a result of this template switch, the recombinant RNA shown at the bottom is formed.

Assembly of Virus Particles

Morphogenesis of picornaviruses has been studied extensively because the 60-subunit capsid is relatively simple and the assembly intermediates can be readily detected in infected cells (Fig. 17). During the synthesis of the P1 protein, the capsid protein precursor, the central b-barrel domains form, and intramolecular interactions among the surfaces of these

domains leads to formation of the structural units. Once P1 is released from the 2A protein, the VP0–VP3 and VP3–VP1 bonds are cleaved by proteinase $3CD^{pro}$. These cleavage sites are located in flexible regions between the b-barrels; there is considerable movement of the aminotermini and carboxyltermini after cleavage, but the contacts between b-barrels are not disturbed (142). In the mature capsid, the carboxyltermini of VP1, VP2, and VP3 are on the outer surface of the capsid, whereas the aminotermini are on the interior, where they participate in an extensive network of interactions among protomers. This process produces the first assembly intermediate in the poliovirus pathway, the 5S protomer, the immature structural unit consisting of one copy each of VP0, VP3, and VP1. Five protomers then assemble to form a pentamer, which sediments at 14S. Cleavage of P1 is probably required for assembly of the pentamer (237). This conclusion is supported by examination of the virion structure. The b-cylinder at the fivefold axis of symmetry is formed from the N-termini of neighboring VP3 molecules; the cylinder is surrounded by a bundle composed of the aminotermini of VP0 and VP1. For these interactions to occur, proteolytic cleavage of the capsid proteins must occur to allow movement of the aminotermini.

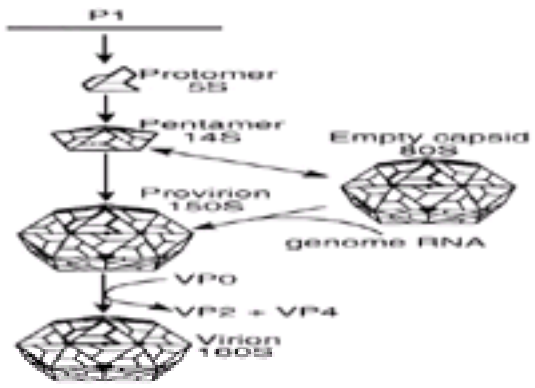

FIG. 17. Morphogenesis of picornaviruses. The capsid protein precursor, P1, folds nascently, is cleaved from P2, and then is further cleaved to VP0 + VP3 + VP1 by $3CD^{pro}$. Protomers (5S) self-assemble into 14S pentamers, and pentamers assemble into 80S empty capsids. In one model of encapsidation, RNA is threaded into the empty capsid, producing a 150S provirion in which VP0 is uncleaved. Another possibility is that pentamers assemble around the RNA genome and that empty capsids are storage depots for pentamers. Cleavage of VP0 is the final morphogenetic step that produces the infectious 160S virion. The proteinase responsible for the cleavage of VP0 is unknown. (Adapted from Hellen CUT, Wimmer E. *Human enterovirus infections.* Washington, DC: ASM Press, DC, 1995, with permission.)

Pentamers are important intermediates in the assembly of all picornaviruses (40,269). They can self-assemble *in vitro* or *in vivo* into 80S empty capsids; in one model of assembly, newly synthesized viral RNA is inserted into these particles to form the provirion (153), in which the capsid protein VP0 remains uncleaved. This assembly model would seemingly require an opening in the empty capsid through which the RNA can enter. Examination of the high-resolution x-ray crystallographic structure of these particles does not provide evidence for such an opening (27). This finding does not exclude this morphogenetic pathway because the pore might be dynamic and not observed in the crystals. In an alternative morphogenesis pathway, 4S pentamers assemble with virion RNA to form provirions. In this model, for which there is some experimental support (233), the empty capsids found in infected cells serve as storage depots for 14S pentamers.

The final morphogenetic step involves cleavage of most of the VP0 molecules to VP4 + VP2. The proteinase that carries out this final maturation cleavage has not been identified. The VP0 scissile bond is located on the interior of empty capsids and mature virions and is inaccessible to viral or cellular proteinases. The presence of a conserved serine in VP2 near one of the cleaved termini of VP2 led to a model that cleavage occurs by a novel autocatalytic serine protease-like mechanism in which basic viral RNA groups serve as proton abstracters during the cleavage reaction (17). However, replacement of ser-10 does not impair VP0 cleavage (135). More recently, it has been suggested that a conserved histidine in VP2 may be involved in catalysis, together with the viral RNA (27,71).

The structure of the 80S particle reveals differences in the network formed by the N-terminal extensions of the capsid proteins on the inner surface of the shell, compared with the native virion (27). In empty capsids, VP4 and the entire N-terminal extensions of VP1 and VP2 are disordered, and many stabilizing interactions that are present in the mature virion are not present. Thus, cleavage of VP0 establishes the ordered N-terminal network, an interlocking seven-stranded b-sheet formed by residues from adjacent pentamers. This network results in an increase in particle stability and the acquisition of infectivity.

The picornavirus encapsidation process is highly specific, resulting in packaging of only positive-stranded RNA, and not viral mRNA, negative-stranded viral RNA, or any cellular RNA (230,232). VPg is probably not an encapsidation signal because VPg-containing negative-stranded RNA is not packaged. The coupling of encapsidation to viral RNA synthesis may explain the selective packaging of viral positive-stranded RNA. In infected cells, newly synthesized RNA is packaged into virions within 5 minutes, whereas incorporation of capsid proteins in virions requires at least 20 minutes (22). The pool of viral RNA available for packaging is therefore small, whereas the pool of capsid proteins is large. If capsid formation is inhibited with *p*-fluorophenylalanine, the accumulated RNA cannot be packaged after removal of the inhibitor (130). These results suggest that packaging of the viral genome is linked to RNA synthesis, and would explain why only RNAs containing VPg are encapsidated.

During its synthesis, the P1 capsid protein precursor is linked to myristic acid at the aminoterminal glycine residue of VP4 that is exposed after removal of the initiation Met residue (61). The myristate groups, which form part of a network of interactions between subunits that form when protomers assemble into pentamers, cluster around the fivefold axis of symmetry and stabilize the b-cylinder that is made by the aminotermini of five copies of VP3. Mutagenesis indicates that

the myristate group plays a role in stabilizing pentamers and therefore virions (14,196,197 and 198,220).

Effects of Viral Multiplication on the Host Cell

Inhibition of 5' End–Dependent mRNA Translation

Cleavage of eIF4G

In cultured mammalian cells, poliovirus infection results in inhibition of cellular protein synthesis. By 2 hours after infection, polyribosomes are disrupted, and translation of nearly all cellular mRNAs stops and is replaced by viral mRNA translation (Fig. 18). Poliovirus mRNA, but not capped mRNAs, can be translated in extracts from infected cells. In such extracts, one translation initiation factor, eIF4F, has been inactivated by cleavage of the eIF4GI/II component (85,270,305). Cleavage of eIF4G prevents eIF4F from recruiting 40S ribosomal subunits to capped mRNAs because it releases the N-terminal domain of eIF4G, which binds eIF4E, which in turn binds the 5' cap of cellular mRNAs (Fig. 19). The synthesis of poliovirus proteins is not affected because the viral RNA is translated by internal ribosome binding, a process that does not require eIF4E. However, optimal function of the IRES does require the C-terminal fragment of eIF4G, which, as discussed previously, is necessary to anchor 40S ribosomal subunits to the IRES. Cleavage of eIF4G by picornaviruses inhibits cell translation and is also a strategy for stimulating IRES-dependent translation. Although both eIF4GI and eIF4GII are cleaved in poliovirus- and rhinovirus-infected cells, the kinetics of shutoff of host translation correlates with cleavage of eIF4GII and not eIF4GI (119,298).

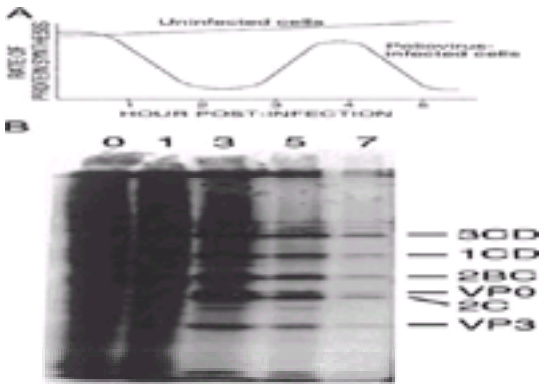

FIG. 18. Inhibition of cellular translation in cells infected with poliovirus. **A:** Protein synthesis in poliovirus-infected and uninfected cells at different times after infection. Poliovirus infection results in inhibition of host cell translation beginning about 1 hour after infection. The increase in translation beginning 3 hours after infection is due to the synthesis of viral proteins. **B:** Polyacrylamide gel showing inhibition of cellular translation. At different times after infection (indicated at the top of each lane), cells were incubated with ^{35}S-methionine for 15 minutes; the cell extracts were then fractionated on an SDS-polyacrylamide gel. By 5 hours after infection, host translation is markedly inhibited and replaced by the synthesis of viral proteins, identified at the right.

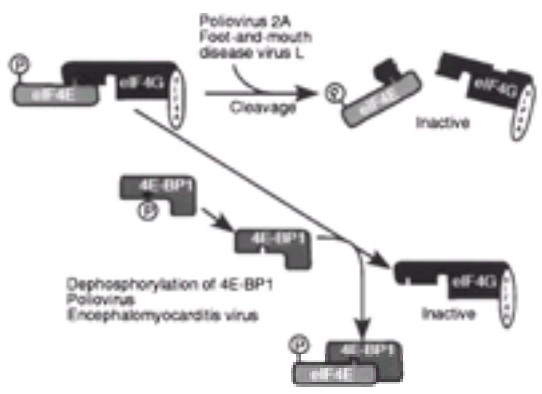

FIG. 19. Two mechanisms for regulating eIF4F activity in picornavirus-infected cells. The proteins 4E-BP1 and 4E-BP1 bind eIF4E and prevent it from interacting with eIF4G. 4E-BP1 binds eIF4E when it is dephosphorylated, an event that occurs in cells infected with poliovirus and encephalomyocarditis virus. Cleavage of eIF4G takes place in cells infected with poliovirus, rhinovirus, and foot-and-mouth disease virus, among others. Cleavage removes the binding site in eIF4G for eIF4E. As a result, eIF4F is unable to recruit ribosomes to the mRNA through the 5'-cap structure, and translation by 5' end–dependent initiation is inhibited.

Both forms of eIF4G are cleaved by protease 2A^{pro} of poliovirus and rhinovirus (119,298). *In vitro* cleavage of eIF4G by purified 2A^{pro} of rhinovirus is inefficient unless eIF4G is bound to eIF4E (128). This finding indicates that eIF4G is not cleaved as an individual polypeptide, but rather as part of the eIF4F complex. Binding of eIF4E to eIF4G may induce conformational changes in eIF4G that make it a more efficient substrate for the protease. Poliovirus 2A ^{pro} efficiently cleaves eIF4GI, but not eIF4GII, consistent with the differential cleavage of these proteins during virus infection (119). Cleavage of eIF4G in cells infected with foot-and-mouth disease virus is carried out by the L ^{pro} protein. The cleavage sites in eIF4G for the two proteinases are different: L ^{pro} cleaves between Gly-479 and Arg-480, whereas 2A^{pro} cleaves between Arg-486 and Gly-487 (171,181).

Modulation of eIF4E Activity

Two related low-molecular-weight cell proteins, 4E-BP1 and 4E-BP2, bind to eIF4E and inhibit translation by 5' end–dependent scanning, but not by internal ribosome entry (see Fig. 19) (243). 4E-BP1 is identical to a protein called PHAS-I (phosphorylated heat- and acid-stable protein regulated by insulin), which was previously known to be an important phosphorylation substrate in cells treated with insulin and growth factors (190). Phosphorylation of 4E-BP1 *in vitro* blocks its association with eIF4E. Binding of either 4E-BP1 or 4E-BP2 to eIF4E does not prevent it from interacting with the 5' cap but does inhibit binding to eIF4G. Consequently, active eIF4F is not formed. eIF4G and the 4E-BPs have a common sequence motif that binds eIF4E. Treatment of cells with hormones and growth factors leads to the phosphorylation of 4E-BP1 and its release from eIF4E. Those mRNAs with extensive secondary structure in the 5'-untranslated region, which are translated poorly, are preferentially sensitive to the phosphorylation state of 4E-BP1. As expected, translation by internal ribosome binding is not affected when 4E-BP1 is dephosphorylated. The nature of the signaling pathway through which binding of extracellular ligands leads to phosphorylation of 4E-BPs has not yet been elucidated (107).

Infection with several picornaviruses causes alteration of the phosphorylation state of 4E-BP1 and 4E-BP2 (see Fig. 19). Infection of cells with encephalomyocarditis virus causes inhibition of cellular translation, but in contrast to the shutoff that occurs in poliovirus-infected cells, shutoff cellular protein synthesis occurs late in infection and is not mediated by cleavage of eIF4G. Infection with encephalomyocarditis virus induces dephosphorylation of 4E-BP1, which then binds eIF4E to prevent it from forming eIF4F (108). Translation of cellular mRNAs is inhibited, but that of the viral RNA is not inhibited because it contains an IRES. Dephosphorylation of 4E-BP1 also occurs late in cells infected with poliovirus, but this event does not coincide with inhibition of cellular translation, which occurs earlier in infection (108).

Inhibition of Cellular RNA Synthesis

Infection of cells with picornaviruses leads to a rapid inhibition of host cell RNA synthesis catalyzed by all three classes of DNA-dependent RNA polymerase. RNA polymerases I, II, and III from poliovirus-infected cells are enzymatically active, suggesting that accessory proteins may be the target of transcriptional inhibition. Studies of *in vitro* systems have demonstrated the inhibition of specific transcription factors required by each of the three RNA polymerases. The RNA polymerase factor TFIID, which is a multiprotein complex, is inactivated in poliovirus-infected cells (176). This inactivation appears to be due, at least in part, to cleavage of a subunit of TFIID, the TATA-binding protein, by protease $3C^{pro}$ (63). A pol III DNA-binding transcription factor, TFIIC, is also cleaved and inactivated by $3C^{pro}$ (62). The target of $3C^{pro}$ is the a subunit of TFIIIC, which contacts the pol III promoter. An unidentified pol I transcription factor is cleaved by $3C^{pro}$, resulting in inhibition of pol I transcription in infected cells (273).

Cytopathic Effects

When cells are productively infected with poliovirus, they develop the characteristic morphologic changes known as *cytopathic effects.* These include condensation of chromatin, nuclear blebbing, proliferation of membranous vesicles, changes in membrane permeability, leakage of intracellular components, and shriveling of the entire cell. The cause of cytopathic effects is unknown. One hypothesis is that leakage of lysosomal contents is partly responsible (125). Although cellular RNA, protein, and DNA synthesis are inhibited during the first few hours of infection, they cannot account for cytopathic effects.

When poliovirus reproduction is hindered by certain drugs or other restrictive conditions, cell death occurs through induction of apoptosis (302). Although certain manifestations of cytopathic effects and apoptosis are similar, such as chromatin condensation and nuclear deformation, the pathways leading to their induction differ (3). During productive infections of cultured cells with poliovirus, apoptosis is blocked by a virus-encoded inhibitor (302). However, viral replication and central nervous system injury in mice infected with poliovirus are associated with apoptosis (110). Expression in cells of poliovirus protein 2A or 3C leads to induction of apoptosis (23,111). The viral inhibitor of apoptosis has not been identified. The ability of different strains of Theiler's murine encephalomyelitis virus to induce apoptosis may be a determinant of disease. The TO strain of Theiler's virus, which causes a persistent demyelinating disease in mice, encodes an additional protein, L* (57). This protein is produced by initiation from an AUG that is 13 nucleotides downstream from the initiator AUG of the polyprotein, in a different reading frame. In contrast, nondemyelinating strains of the virus, such as GDVII, do not encode L*. It was subsequently found that L* has antiapoptotic properties in macrophages and is critical for virus persistence (103). The ability of L* to inhibit apoptosis may be a key factor in determining whether infection of mice results in acute disease or persistence and demyelination.

Perspectives

Since the isolation of poliovirus in 1908, research on the virus has waxed and waned. With each lull in activity, questions were raised about whether it was productive to continue research on the virus. Each time, new technologies emerged that allowed the field to advance and become active once again. With global eradication of poliomyelitis looming on the horizon, research on poliovirus is as vibrant as ever. Many questions remain about nearly every stage of the replicative cycle, and an unprecedented array of experimental techniques and reagents are available to address them. We are on the verge of understanding the dynamics of the virion as it binds to the receptor and releases its nucleic acid. Why has poliovirus selected its particular receptor? Does the interaction lead to intracellular signaling that is beneficial for the virus? How is the genome extruded from the virus particle? Does the mechanism of RNA synthesis in infected cells resemble that which occurs *in vitro?* Why does RNA synthesis occur on membranes, and how is translation and replication regulated so that the processes do not collide? What other host proteins are involved in RNA synthesis, and what are their roles? How does the viral RNA enter the virion, and what causes the dramatic reorganization of the interior during the final stages of maturation? The study of poliovirus provides a unique opportunity to address fundamental questions in virology. However, we may not be able to answer these and many other interesting questions before the time comes to destroy all stocks of poliovirus, as part of the polio eradication effort (78). Fortunately, the same questions

apply to other members of the Picornaviridae, and it is anticipated that research activity on these viruses will rapidly intensify in the next few years. Although future versions of this chapter may not contain as much information about poliovirus, the lessons we have learned will not be forgotten.

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CHAPTER 24 Enteroviruses: Polioviruses, Coxsackieviruses, Echoviruses, and Newer Enteroviruses

Mark A. Pallansch and Raymond P. Roos

Human enteroviruses are members of the Enterovirus genus of the family *Picornaviridae* (Table 1). Although most enterovirus infections are asymptomatic, there are estimates of as many as 5 to 10 million symptomatic enterovirus infections each year in the United States (308). The majority of enterovirus infections do not cause significant disease, but infection can lead to serious illness, especially in infants and the immunocompromised. In addition, this group of viruses is the most common cause of aseptic meningitis, the most frequent central nervous system (CNS) infection. Enteroviruses have been implicated in chronic as well as acute diseases. These chronic diseases include dermatomyositis, polymyositis, dilated cardiomyopathy, and diabetes mellitus.

Genus and species	Number of serotypes
Genus Enterovirus	
Poliovirus	3
Human enterovirus A	12
Human enterovirus B	36
Human enterovirus C	11
Human enterovirus D	2
Bovine enterovirus	2
Porcine sapelovirus A	1
Porcine enterovirus B	2
Genus Hepatovirus	1
Genus Rhinovirus	
Human rhinovirus A	18
Human rhinovirus B	3
Unassigned	82
Genus Cardiovirus	
Encephalomyocarditis virus	1
Theilovirus	2 or 3
Genus Kobuvirus[b]	1
Genus Teschovirus[b]	
Porcine teschovirus	10
Genus Erbovirus[b]	1
Genus Aphthovirus	
Foot-and-mouth disease virus	7
Equine rhinitis A virus	1
Genus Parechovirus	3
Recognized picornaviruses not yet placed in genera	At least 20

[a]The new classification scheme that is shown is from the International Committee for the Taxonomy of Viruses Picornavirus Study Group (323) and divides serotypes into different species, at least partly on the basis of genome organization and sequence similarity. The particular enteroviruses that make up the species are detailed in Tables 6–8.
[b]Proposed as a genus.

TABLE 1. *Picornavirus genera, species,[a] and serotype*

HISTORY

The history of enteroviruses is very much the history of poliovirus. In fact, many of the poliovirus milestones are landmarks not only in the study of enterovirus but for all of virology.

Poliomyelitis is believed to be an ancient disease. It has been suggested that the depiction of a young man with an atrophic limb on an Egyptian stele from the second millennium B.C. represents a sequela of poliomyelitis (153). The first

clinical descriptions of poliomyelitis were made in the 1800s, with reports of cases of paralysis with fever. In 1840, von Heine (119) published a monograph more specifically describing the affliction. His contributions and those published later by Medin from Sweden (202) led to paralytic poliomyelitis being referred to as Heine-Medin disease. Another early report, by Charcot and Joffroy (50), described the pathologic changes in the anterior horn motor neurons of the spinal cord in poliomyelitis.

The 1900s began a new era in poliomyelitis investigations, and the beginning of an understanding of the infectious nature of this disease. Wickman (331) and others recognized the communicable nature of poliomyelitis, the importance of asymptomatic infected individuals in transmission of poliovirus, and the role of enteric infection in disease pathogenesis. The role of the gastrointestinal tract in the initiation and spread of poliovirus infection was later confirmed by Trask and colleagues (320). In a classic study, Viennese investigators Landsteiner and Popper (178) proved the infectious nature of poliomyelitis by successfully transmitting the clinical disease and its pathology to monkeys following inoculation of CNS tissue homogenates from human cases.

Despite this progress, there were a number of unfortunate misconceptions about poliomyelitis that initially confused scientists and misdirected efforts for control. These misconceptions included a belief that the virus was exclusively neurotropic, that the nasopharynx was a major site for virus entry into the CNS, and that the virus spread to the nervous system prior to viremia and by way of the olfactory nerve. As a result of these misconceptions and the failure of several poorly conceived immunization attempts, some with rather disastrous results (250), there was an atmosphere of pessimism by the middle of the 20th century concerning the eventual control of poliomyelitis, even among scientists working in the field. In 1945, Burnet (44) wrote, “The practical problem of preventing infantile paralysis has not been solved. It is even doubtful whether it ever will be solved.” The eventual realization that virus entered via the oral/gastrointestinal route and that CNS disease followed a viremia did much to boost hopes for effective immunization (38).

Building on studies of others, Enders and colleagues (89) performed a landmark study showing that poliovirus could be propagated in nonneural tissue culture. These investigations had implications for all of virology because they indicated, first, that poliovirus grew in various tissue culture cells that did not correspond to the tissues infected during the human disease, and second, that poliovirus destroyed cells with a specific cytopathic effect. Neutralization tests showed that poliovirus has three serotypes (39), and serologic tests (22) confirmed the fact that most infected individuals do not manifest clinical disease. These investigations laid a critical framework for the preparation of a vaccine, and they clarified a host of confusing data, such as the apparent presence of second attacks of poliomyelitis.

A variety of vaccines were subsequently produced, with the most well-known being the Salk inactivated vaccine delivered via the intramuscular route (licensed in 1955 in the United States) and the Sabin live, attenuated vaccine delivered via the oral route (licensed in 1961–1962). The importance of these vaccines and the individuals who produced them can begin to be realized by noting that more Americans knew the name of Jonas Salk than the President of the United States. The real impact of these vaccines will ultimately be felt with the complete global eradication of poliomyelitis.

Poliovirus work has had a continuing significant impact on the field of molecular virology. Poliovirus was the first animal virus completely cloned and sequenced (169,262), the first RNA animal virus for which an infectious clone was constructed (261), and the first human virus that had its three-dimensional structure solved by x-ray crystallography (128). In 1989, Racaniello and colleagues (211) identified the poliovirus receptor (PVR), a finding that was followed by the generation of mice carrying PVR as a transgene (173,267).

Coxsackieviruses (group A) were first isolated during a poliomyelitis epidemic in 1948 from the feces of paralyzed children in Coxsackie, New York (70). These isolates were obtained by inoculation of suckling mice, the pathogenicity in mice clearly differentiating these viruses from poliovirus. In the following year, coxsackievirus group B was isolated from cases of aseptic meningitis (210). The original coxsackievirus group A isolates produced myositis with flaccid hind limb paralysis in newborn mice, whereas the group B coxsackieviruses produced a generalized infection in newborn mice, with myositis as well as involvement of the brain, pancreas, heart, and brown fat.

In 1951, echoviruses were first isolated from the stool of asymptomatic individuals (277). Echoviruses received their name because they were *e*nteric isolates, *c*ytopathogenic in tissue culture, isolated from *h*umans, and *o*rphans (i.e., unassociated with a known clinical disease). Subsequent studies have shown that echoviruses do, in fact, cause a variety of human diseases.

INFECTIOUS AGENTS

Physical and Chemical Properties

Enteroviruses are distinguished from other picornaviruses on the basis of physical properties, such as buoyant density in cesium chloride and stability in weak acid. Many aspects of enteroviral pathology, transmission, and general epidemiology are directly related to the biophysical properties and the cytolytic life cycle of these viruses. The infectious virus is relatively resistant to many common laboratory disinfectants, including 70% ethanol, isopropanol, dilute Lysol, and quaternary ammonium compounds. The virus is insensitive to lipid solvents, including ether and chloroform, and it is stable in many detergents at ambient temperature. Formaldehyde, gluteraldehyde, strong acid, sodium hypochlorite, and free residual chlorine inactivate enteroviruses. Concentration, pH, extraneous organic materials, and contact time affect the degree of inactivation by these compounds. Similar inactivation is achieved when virus is present on fomites, although conditions may not be exactly comparable (2). In general, most reagents that inactivate enteroviruses are dependent on active chemical modification of the virion, while most extractive solvents have no effect.

Enteroviruses are relatively thermostable but less so than hepatitis A virus. Most enteroviruses are readily inactivated at 42°C, although some sulfhydral reducing agents and magnesium cations can stabilize viruses so that they are relatively stable at 50°C (8,83). The process of thermal inactivation appears to involve several steps culminating in the irreversible

release of RNA from the altered virion particles. The relative sensitivity to modest elevations in temperature makes it possible to use pasteurization to inactivate enteroviruses in many biologically active preparations (124).

As with other infectious agents, ultraviolet light can be used to inactivate enteroviruses, particularly on surfaces. In addition, the process of drying on surfaces significantly reduces virus titers. The degree of virus loss by drying is related to porosity of the surfaces and the presence of organic material (1).

Many studies of enterovirus inactivation have been conducted using poliovirus as a model enterovirus. A report describing strain-specific differences for gluteraldehyde inactivation among echovirus 25 isolates, however, implies that the assumption that poliovirus is representative of all enteroviruses may not be valid (49). The inactivation of infectivity may not be directly related to the destruction of the viral genome, because the polymerase chain reaction can be used to amplify viral RNA even after inactivation of virus has occurred (193). This would suggest that reactivation of infectivity may be possible in some circumstances. In fact, some examples of recovered infectivity have been reported through increased multiplicity of infection in cell culture (301,345), but the practical significance of these observations is not clear.

Antigenic Characteristics

As described in Chapter 23, the picornaviruses are among the simplest RNA viruses, having a highly structured capsid with little place for elaboration. Yet, despite the limited genetic material and structural constraints, evolution within the picornaviruses has resulted in a very large number of readily distinguishable members. This variability has been categorized antigenically as serotype. Each of the serotypes correlates with the immunologic response of the human host, protection from disease, receptor usage, and, to a lesser extent, the spectrum of clinical disease. These correlations, however, have only a partial relationship with their naming scheme. This limitation is derived from the original classification of enteroviruses into polioviruses, coxsackie A or B viruses, and echoviruses, based on the specific human disease they cause (poliomyelitis), pathogenicity in suckling mice (coxsackieviruses), and growth in cell culture (echoviruses). Within each of these groups, isolates can be readily distinguished on the basis of antigenicity as measured with antisera raised in animals. Reference antisera are obtained through the World Health Organization, Geneva, or directly from the World Health Organization Collaborating Center Virus Reference and Research, Copenhagen, by prior arrangement.

These antigenic groupings, which define the serotypes, became more complicated as the number of different viruses increased. The distinction between serotypes also began to blur as isolates were discovered that were weakly antigenically related at a lower level to known serotypes. Despite these limitations, the serotype remains the single most important physical property that distinguishes the different enteroviruses. The prototype enterovirus strains are maintained in the American Type Culture collection, Rockville, Maryland, and in the World Health Organization Collaborating Reference Laboratories. A new classification scheme divides the members of the enterovirus genera into species, at least partly on the basis of genome organization and sequence similarity (see Table 1).

Although the classification system distinguishes enteroviruses on the basis of differences in antigenicity and mouse pathogenicity, the coxsackie B viruses and many of the echoviruses are genetically related to each other, as are the polioviruses and some of the coxsackie A viruses. In addition to the genetic relatedness, many enterovirus serotypes share some antigenicity. For example, polioviruses 1 and 2 share a common antigen, and antigenic relationships also exist between coxsackieviruses A3 and A8, A11 and A15, A13 and A18, A16 and enterovirus 71, and A24 and enterovirus 70, and between echoviruses 6 and 30, and 12 and 29. When virions are disrupted by heating, particularly in the presence of detergent, nonsurface antigens are exposed that are shared broadly among many enteroviruses (212).

In addition to the 64 prototype strains, antigenic variants exist for several enteroviruses. One type of antigenic variant is the prime strain, identified for many enteroviruses, including coxsackieviruses A17 and A24; echoviruses 1 to 6, 9, 11, 29, and 30; and enterovirus 70 (3,149,294). A prime strain is defined by the following properties relative to the prototype strain (294): reference antisera to the prototype strain neutralize the prime strain poorly, or not at all; antisera prepared to the prime strain neutralize the prime strain and the prototype strain equally well; the prototype and prime strains share complement-fixing antigens. A prime strain thus appears to elicit a broader spectrum of neutralizing antibodies, suggesting the presence of additional antigenic epitopes compared to its corresponding prototype strain.

A second type of antigenic variant, referred to as an intratypic variant, has also been identified for some of the enteroviruses, including coxsackieviruses A24, B1 to B4, B6; echoviruses 4, 9, and 33; and enterovirus 70 (4,198,293,309). Such variants generally have an antigenic spectrum that is different from (possibly narrower, and overlapping) their corresponding prototype strains, and thus cannot be classified as prime strains. The reference antisera and antisera prepared against the intratypic variant have a reciprocal, reduced neutralization activity with the heterologous strain compared to the homologous one.

The origin of prime and intratypic variant strains is speculative, but they may result from either antigenic drift or recombination. The specific epitope changes among these antigenic variants have not been described, and therefore the structural changes involved are not known. In addition, it is not understood why some serotypes have numerous prime strains, others have antigenic variants, and still others are antigenically homogeneous. It is possible that different serotypes have different degrees of structural constraint on their capsid and therefore have different capacities for antigenic variation.

Despite this lack of understanding of molecular variation in virus structure as measured by polyclonal antibodies, high-resolution studies of the virion surface have been particularly useful in identifying the targets of neutralization of enteroviruses by monoclonal antibodies (see later section Pathogenesis) (129,249). In addition, there are other antigenic sites that elicit immune responses that are not neutralizing. The observed structure of the antigenic sites explains why antigenicity of the virus is destroyed by disruption of the virion structure.

The poliovirus neutralizing antibody response is serotype specific, with the exception of some minor cross-reaction

between poliovirus 1 and 2. A monoclonal antibody has been described that reacts with this shared site that is not found on poliovirus type 3 (322). As noted, heat-disrupted virions, particularly those heated in the presence of detergent, induce antibodies that react with many enteroviruses. These broadly reacting antibodies are generally not neutralizing, and at least one of these epitopes has been mapped to the amino-terminal region of capsid protein VP1 (288). Antisera raised in animals to each of the enteroviruses are largely type specific and are used for the determination of serotype in a neutralization assay. The virus also contains more than one T-cell epitope, but the role of cell-mediated immunity in controlling infection is not well understood (see the section Pathogenesis).

Although there are measurable *in vitro* differences in antigenic properties among strains within a serotype, the biologic significance of these differences has not been determined. Several polioviruses isolated during outbreaks have demonstrated different antigenic properties when compared to the reference vaccine strains (113,146). In all cases, immunity derived from vaccination has been sufficient to provide protection and control of these strains (41). In addition, even in the face of massive immunization campaigns, no antigenic “escape mutants” resistant to neutralization have ever been observed, and successive genotypes of poliovirus have been eliminated. Natural antigenic variants have also been identified with panels of monoclonal antibodies for several nonpolio enteroviruses (116,251).

Propagation and Assay in Cell Culture

One of the prominent characteristics of enteroviruses is the cytolytic nature of growth in cell culture. For many years, poliovirus was the prototype of a lytic viral infection. At the microscopic level, infection is usually manifest within 1 to 7 days by the appearance of a characteristic cytopathic effect, which features visible rounding, shrinking, nuclear pyknosis, refractility, and cell degeneration (Fig. 1 and Fig. 2). The earliest effects may be seen in less than 24 hours if the inoculum contains a large number of infectious particles. With fewer numbers of virions, however, visible changes are not recognizable for several days, even though a sufficient number of cells are infected. In addition, some enteroviruses either do not cause cytopathic effect at all, or do so only after several passages. In general, once focal cytopathic effect is detected, there is rapid spread of infection throughout the cell sheet with total destruction of the monolayer, sometimes in a matter of hours (see Fig. 2). The nature of the cytopathic effect in various cell cultures is so characteristic that a cautious presumptive diagnosis of enterovirus infection can often be made at initial detection of cytopathic changes (see later section Diagnosis). However, some components of a sample inoculated onto cell cultures may produce cell toxicity in the first 24 hours, which may be confused with viral cytopathic effect. To distinguish toxicity from virus cytopathic effect, an additional passage can be performed so that toxic components of a specimen can be diluted; the passage will allow cells to maintain viability or, alternatively, for the virus to amplify and produce cytopathic effect.

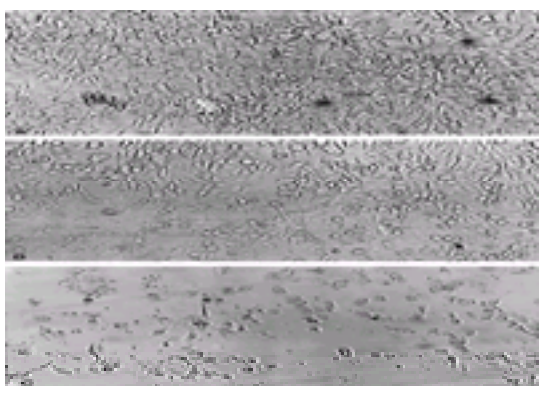

FIG. 1. Normal and poliovirus-infected monkey kidney cells in culture (cells neither fixed nor stained; ×150). **A:** Monolayer of normal unstained monkey kidney cells in culture. **B:** Unstained monkey kidney cell culture showing early stage of cytopathic effect typical of poliovirus infection. Approximately 25% of the cells in the culture show cytopathic effect (especially rounding) indicative of virus multiplication (1+ cytopathic effect score). **C:** Unstained monkey kidney culture illustrating more advanced cytopathic effect (3+ to 4+ cytopathic effect). Almost 100% of the cells are affected, and most of the cell sheet has come loose from the wall of the culture tube. (From ref. 207, with permission.)

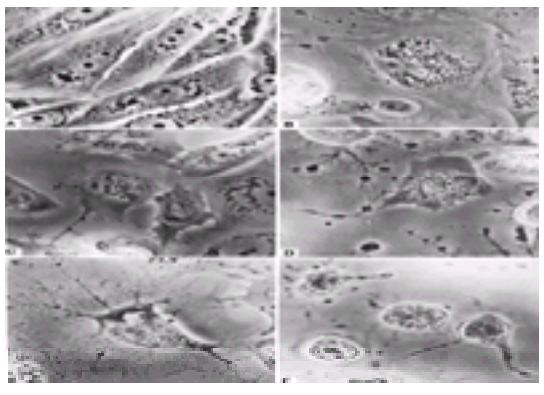

FIG. 2. Micrographs of living cells as seen in the phase microscope. Normal and poliovirus-infected monkey kidney epithelial cells in culture (×600). **A:** Normal monolayer, uninfected. **B:** Cell in early phase of infection with poliovirus type 1. In the cell in the center of the field, the nucleus is forced aside to the left edge of the cell by a clump of cytoplasmic material. **C:** Cells from the same culture at a slightly more advanced stage. The cells are beginning to pull away from each other. **D:** Another field of the culture at a more advanced stage. In the cell in the center of the field, the outlines of the nucleus are obscured by the paranuclear mass. Clear, filamentous areas are seen in the cytoplasm (*a*). They appear to arise in the central zone of the cytoplasm, and they constantly change their shape and location, moving toward the clear peripheral zone. The cell has lost contact with its neighbors and exhibits threadlike cytoplasmic extensions that contain dense swellings. **E:** Culture at a slightly more advanced stage. The cell has begun to round up, and large clear vacuoles (*V*) are seen in the upper left of the cytoplasm. The nucleus is masked by the cytoplasmic paranuclear mass

(*P*). **F:** Last stages of cytopathic degeneration induced by poliovirus. (From ref. 201, with permission.)

Viruses may be quantified and the cytopathic effect visualized by plaque formation on cell cultures under an agar overlay. Poliovirus plaques are very characteristic, with sharply defined, circular, clear-centered holes. Coxsackievirus B plaques may appear more slowly and do not have distinct edges. Coxsackievirus A plaques, when present, are variable in appearance. The speed at which plaques appear is often an intrinsic property of a specific serotype and cell culture type, and some viruses that form plaques readily on one cell culture line will not form recognizable plaques on another.

All known enteroviruses can be propagated in cell culture or in suckling mice. Most of the serotypes can be grown in at least one or more human or primate continuous cell cultures. No cell line, however, is able to support the growth of all cultivable enteroviruses. Even after many years of experimentation, a few serotypes (e.g., coxsackievirus A1) can be propagated only in suckling mice. The typical host range of human enteroviruses in cell cultures or animals is shown in a broad, generalized way in Table 2.

Virus	Antigenic types[a]	Cytopathic effect: Monkey kidney tissue culture	Cytopathic effect: Human tissue culture	Illness and pathology: Sucking mouse	Illness and pathology: Monkey
Polioviruses	1-3	+	+	−	+
Coxsackieviruses, group A	1-24[b]	±	±	+	−
Coxsackieviruses, group B	1-6	+	+	+	−
Echoviruses	1-34[c]	+	±	−	−
Enteroviruses	68-71	+	+	−	−

[a] Many enterovirus strains have been isolated that do not conform to these categories.
[b] New types, beginning with type 68, are now assigned enterovirus type numbers instead of coxsackievirus or echovirus numbers. Types 68–71 have been identified.
[c] Type 23 has been found to be the same as echovirus 9.
Echovirus 8 was determined to be the same serotype as echovirus 1; echovirus 10 has been reclassified as a reovirus type 1; echoviruses 22 and 23 as parechoviruses types 1 and 2; echovirus 28 as human rhinovirus 1A; and echovirus 34 as coxsackievirus A24.
Source: Adapted from refs. 51 and 227.

TABLE 2. *Usual host range of human enteroviruses: animal and tissue culture spectrum*[a]

Infection of target cells depends on viruses binding to specific receptors on the cell surface. Collectively, the enteroviruses use a large number of different receptors. These have been identified for numerous serotypes, and included PVR, decay accelerating factor, and intercellular adhesion molecule 1 (which is also used by some human rhinoviruses). At this time, however, there is no clearly recognizable pattern determining which serotypes use which receptors. In some cases, a single receptor seems to be used by a relatively well defined subset of the serotypes. For example, the three serotypes of poliovirus are the only enteroviruses that can use the PVR to infect cells. The coxsackie B viruses can use the coxsackievirus and adenovirus receptor (177). Some serotypes, however, are clearly able to use more than one receptor, whereas some individual isolates within a serotype may not be able to use the same receptor as the prototype strain for that serotype (31). Furthermore, even though an identified receptor is required for attachment to the target cell, another protein may be required for entry into the cell (299). Although a theoretical possibility, it has not been demonstrated that viruses are able to readily adapt receptor usage to that of a related virus. For example, even at high multiplicity, it is not possible to select mutants of coxsackie A viruses closely related to polioviruses that will use the PVR to infect cells. Nevertheless, the multiplicity of receptors within the enterovirus genus implies that on an evolutionary time scale viruses must be able to modify receptor usage. The structural correlates related to receptor binding are under active investigation.

A practical adaptation of the identification and genetic cloning of enterovirus receptors is the introduction of the receptor into animals and cells that do not normally permit virus infection. This approach has resulted both in advances in understanding the pathogenesis of poliovirus infection in a nonprimate animal model system, and in its practical application in the diagnostic laboratory. The L20B cells, which are murine cells that express PVR, are now used routinely to selectively isolate poliovirus (see the later section Diagnosis) as part of the global poliovirus laboratory network supporting the poliomyelitis eradication initiative (253).

Infection in Experimental Animals, Host Range

Man is the natural host for all human enteroviruses. Although serologically distinct picornaviruses with the same physical properties as enteroviruses have been found in many animals, human beings do not usually have recognizable infections with these animal enteroviruses. On the other hand, some animals are susceptible to experimental infection with human enteroviruses. These include nonhuman primates and PVR transgenic mice for polioviruses, mice and some monkeys for coxsackieviruses A and B, and monkeys for echoviruses. Human enterovirus infection of nonhuman primates appears to be largely subclinical, perhaps related to the homology that several simian enteroviruses share with human viruses (257). Among higher primates, chimpanzees and gorillas appear to be able to acquire poliovirus infection and develop disease from humans through natural exposure (84). At least one human enterovirus, coxsackievirus B5, is closely related antigenically to the porcine enterovirus causing swine vesicular disease, with about 50% genetic homology. Genetic studies of a number of strains of swine vesicular disease virus and epidemiologic information gleaned from outbreaks strongly suggest that a human coxsackievirus B5 was specifically introduced into swine several decades ago (349).

Even though most coxsackie A viruses have been successfully grown in various cell culture systems, isolation from clinical specimens is sometimes unsuccessful, necessitating the inoculation of suckling mice. Inoculation of suckling mice and subsequent virus identification is a process analogous to that of cell culture inoculation. The two groups of coxsackieviruses can be distinguished by the distinct pathology that they cause in mice (Fig. 3 and Fig. 4). With

coxsackievirus A infection, newborn mice develop flaccid paralysis and severe, extensive degeneration of skeletal muscle (sparing the tongue, heart, and CNS), and they may have renal lesions. Death usually occurs within a week. Coxsackievirus B infection proceeds more slowly and is characterized by spastic paralysis and tremors associated with encephalomyelitis, focal myositis, necrosis of brown fat pads, myocarditis, hepatitis, and acinar cell pancreatitis. Echoviruses, except for some isolates of echovirus type 9, do not generally cause disease in mice. A blind passage in mice may be necessary if the amount of coxsackievirus is very low, or possibly because passage of the virus is needed for it to adapt to growth in mice. Once a virus is growing in suckling mice, the specific enterovirus type can be identified by serum pool neutralizations in other litters of suckling mice, analogous to the process in cell culture (see section Diagnosis).

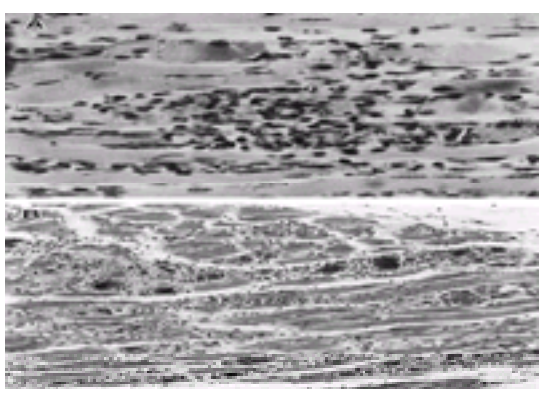

FIG. 3. Photomicrographs of lesions of striated muscle in suckling mice infected with coxsackieviruses. **A:** Two sarcolemmic tubes with numerous mononuclear phagocytes and remnants of hyaline material within the sarcolemmic sheaths. Acute stage of infection with Conn-5 strain, prototype of coxsackievirus B1 (×450). **B:** Partly mineralized residual masses surrounded by fibrous capsules, and sometimes by giant cells. The remainder of the muscle is completely restored. Texas-1 strain of coxsackievirus A4, 11 days after onset of paralysis (×180). (From ref. 207, with permission.)

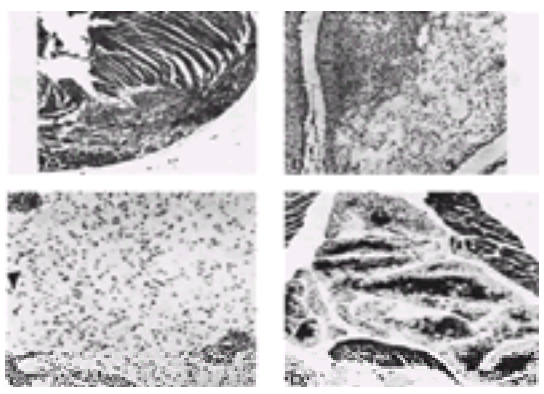

FIG. 4. Photomicrographs of lesions in heart, brain, and fat lobules of suckling mice in acute stage of infection with Conn-5 strain of coxsackievirus B1 (90). **A:** Heart. Large zone of myocardial necrosis at apex of left ventricle (×170). **B:** Brain. Rarefaction after necrosis in cerebrum (×140). **C:** Brain. Encephalitis showing marked perivascular cuffing and leptomeningitis (×190). **D:** Fat lobes. Interlobular edema and acute necrotizing steatitis, illustrating the selective destruction of the peripheral parts of the lobules [shown as pale margins, with preservation of the central parts (dark fuchsinophilic areas)] (×72). (From ref. 207, with permission.)

PATHOGENESIS

Entry, Site of Primary Replication, and Spread

It is appropriate to discuss the pathogenesis of poliovirus as a prototype for enteroviruses that enter the body via the oral route, as it is presumed that the general principals governing poliovirus pathogenesis will also be in effect with respect to nonpolio enteroviruses. However, some enteroviruses have other routes of infection, which may be associated with distinctive disease phenotypes. For example, coxsackievirus A21, a major enteroviral cause of respiratory infections, spreads via the intranasal or aerosol route through contaminated respiratory secretions, which can contain infectious virus. In addition, enterovirus 70, a cause of acute hemorrhagic conjunctivitis, spreads either directly through ocular and respiratory secretions or indirectly through contaminated items.

A discussion of the pathogenesis of poliomyelitis has benefited from study of the natural disease in humans, experimental disease in nonhuman primates, and experimental disease in mice that carry PVR as a transgene (77). Intracerebral inoculation of these transgenic mice with any of the three serotypes of poliovirus leads to a clinicopathologic syndrome similar to human paralytic poliomyelitis. The insensitivity of these mice to oral inoculation (see the section Animal Models, Poliovirus) may be one reason that many questions still remain about the pathogenesis of poliomyelitis.

The patterns of virus shedding and routes of transmission for polioviruses are similar to those seen for other enteroviruses. Poliovirus is resistant to the acidity of the stomach, making the small bowel a suitable milieu for viral replication following ingestion of the virus. Poliovirus is very efficient in establishing infection, and as few as 100 tissue

culture 50% infective doses ($TCID_{50}$) can infect an infant by the oral route (216). Fecal shedding of virus occurs for a prolonged period of time, sometimes for more than 6 weeks. Shedding may be intermittent and is affected by the immune status of the individual; the quantity and duration of virus shedding may be significantly reduced as a result of immunity from a prior natural infection with wild poliovirus, vaccination with oral poliovirus vaccine or the enhanced inactivated poliovirus vaccine, or because of competing enteric infections. Virus can be recovered from throat swabs and washings during the early acute phases of infection, but secretion of virus in saliva occurs for a much shorter time than fecal shedding. It is likely that person-to-person spread was the principal mode of transmission during an outbreak of poliomyelitis in Finland in 1984 involving an estimated 100,000 people during a 1-month period, because freezing during this winter month allowed little opportunity for significant fecal-oral transmission through exposure to contaminated surface or drinking water.

Poliovirus is thought to multiply initially in mucosal tissue, specifically Peyer's patches and the tonsils (Fig. 5); replication in these sites is usually detectable within 1 to 3 days. Virus then multiplies in deep cervical and mesenteric lymph nodes, leading to a “minor” viremia with subsequent invasion of systemic reticuloendothelial tissues, including lymph nodes, bone marrow, liver, and spleen. The CNS can be invaded at this stage, but CNS disease is more likely to occur after amplification in the systemic reticuloendothelial tissues followed by a subsequent “major” viremia. Most individuals infected with poliovirus or other enteroviruses control infection prior to the major viremia, resulting in an asymptomatic infection.

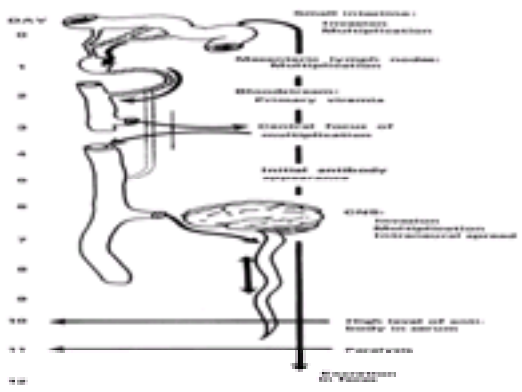

FIG. 5. This schematic illustration represents a longstanding view of the pathogenesis of poliovirus. Note that additional data emphasize the importance of retrograde axonal flow of virus into the central nervous system. Virus enters by way of the alimentary tract and multiplies locally at the initial sites of virus implantation (tonsils, Peyer's patch) or the lymph nodes that drain these tissues, and virus begins to appear in the throat and in the feces—leading to virus shedding into the environment. At this time, it is possible that virus may spread to the CNS by peripheral or cranial nerve axonal flow. Secondary virus spread occurs by way of the bloodstream to other susceptible tissues—namely, other lymph nodes, brown fat, muscle, and the CNS. Virus can then also spread into the CNS at this time by means of peripheral or cranial nerve retrograde axonal flow—for example, from muscle. If a high level of virus replication occurs within the CNS, motor neurons die and paralysis ensues. (Modified from Fenner by Melnick in ref. 207, and used with permission.)

There has been a longstanding controversy concerning the means by which poliomyelitis enters the CNS. The fact that paralytic poliomyelitis follows a viremia and that circulating neutralizing antibodies can prevent poliomyelitis clearly supports the importance of viremia in CNS spread (232). Although viremia appears critical for the delivery of virus into the CNS, a number of epidemiologic findings and experimental studies (see later) suggest that poliovirus is also capable of spreading into the CNS via peripheral or cranial nerves, presumably by retrograde axonal flow.

The importance of the peripheral nerve as a potential route for CNS invasion has been tragically demonstrated in the Cutter's vaccine incident, as well as by “provocation poliomyelitis” (307). In the Cutter's incident, intramuscular injections of incompletely inactivated poliovirus vaccine led to paralytic disease that tended to be initially localized to the extremity that had received the injection (233). Provocation poliomyelitis refers to an increased risk of paralytic disease in individuals who receive intramuscular injections during the incubation period of natural poliovirus infection. The term was more recently used for a situation in which intramuscular injections affected the outcome of vaccination with attenuated oral poliovirus vaccine (rather than affecting the outcome of natural poliovirus infection) (307). It is presumed that the intramuscular injection allowed the vaccine strain to gain entry into the peripheral nerve endings and then to spread to the spinal cord by retrograde axonal flow; however, it remains possible that the injection increased vascular permeability of the muscle to the virus, enhancing the viremia, with a subsequent blood-borne local spread into the spinal cord region that innervated the injected limb. It is likely that a situation resembling provocation poliomyelitis may underlie the finding that more severe paralytic disease and an increased incidence of bulbar poliomyelitis are associated with performance of a tonsillectomy during the incubation period of poliovirus infection; it may be that the tonsillectomy fosters entry of the virus into the brainstem via a cranial nerve (in contrast to entry into the spinal cord via a peripheral nerve in provocation poliomyelitis).

The possibility of virus spread by a peripheral nerve initially gained xperimental support from studies in nonhuman primates performed by Nathanson and Bodian (231) and others. More recent investigations of this issue have been carried out in PVR transgenic mice. In an attempt to reproduce the phenomenon of provocation poliomyelitis, PVR transgenic mice received an intramuscular injection at the same time as receiving poliovirus intravenously (111). These mice developed paralysis initially localized to the limb that received the intramuscular injections. This localization was not seen if there was an ipsilateral sciatic nerve block, demonstrating the critical role for retrograde axonal flow in this model system. The intramuscular injection caused an increase in poliovirus replication, presumably the result of an up-regulation of the human PVR or an increase in invading inflammatory cells susceptible to the infection.

Studies of Ren and Racaniello (268) and Ohka and colleague (242) have demonstrated that poliovirus can indeed spread into the CNS of PVR transgenic mice by retrograde axonal flow following virus inoculation of an extremity. Transection of the sciatic nerve before inoculation and within 4 hours after inoculation prevented paralysis (242). Poliovirus antigen as well as 160S intact viral particles were detected in the axons of the sciatic nerve, demonstrating that virions were being transported in the peripheral nerve following inoculation of the hindlimb.

The preceding data suggest that poliovirus is capable of entering the CNS following muscle infection via retrograde axonal flow. It is not unlikely that this occurs to some extent following the natural (oral) route of poliovirus infection, as studies have shown a low level of infectious virus in muscle following oral administration of virus to PVR transgenic mice (236) and chimpanzees (37). In addition, it is possible that following ingestion poliovirus also spreads from the intestines to the brainstem via the vagal nerve by means of retrograde axonal flow, as shown for reovirus (226). Axonal flow may also be important in association with the viremia. It is possible that viremia seeds muscle, which then leads to spread of the virus into the CNS via the axon. Recent data generated from PVR transgenic mice indicate that poliovirus is efficiently delivered across the blood–brain barrier (338), a further piece of evidence suggesting that the most common natural route of entry into the CNS is by the blood. In summary, viremia is important in the spread of poliovirus into the CNS, but it is likely that other means of virus spread, such as axonal flow, are also involved.

Cell and Tissue Tropism

An unexplained feature of poliovirus pathogenesis is the selective vulnerability of the motor neuron to poliovirus infection. Although the expression of PVR influences the distribution of poliovirus infection [e.g., glial cells do not express PVR and are not infected by poliovirus (77,236,338)], there appears to be a very imperfect correlation between PVR expression in a particular tissue and the distribution of virus in that tissue following intracerebral and intravenous inoculation. For example, there are higher levels of PVR expressed in human liver, lung, and heart than in the brain, despite the fact that these visceral tissues are not normally thought to be infected following poliovirus infection (236). Further cellular localization of specific spliced forms of PVR using appropriate probes or monoclonal antibodies may clarify these issues. The findings to date indicate that other factors besides PVR play an important role in determining the distribution of viral infectivity (77,338). Additional determinants of the tissue and cellular distribution of poliovirus (and of other enteroviruses) include the route of inoculation, host tissue- and cell-specific factors, and viral genomic elements. Regions of the poliovirus capsid proteins that interact with the cellular receptor can also affect tropism. In addition, elements in the viral genome that influence the efficiency of translation initiation or replication affect virus growth in specific cells. The internal ribosome entry site, a part of the viral 5' untranslated region that affects the efficiency of translation initiation, has a key role in neurovirulence (110): cell-specific factors bind to the internal ribosome entry site, affect the efficiency of translation initiation, and can therefore influence tropism and virulence (see the Virulence section later).

Immune Response

B-Cell Response

The efficacy of the immune response is key to the outcome of enteroviral infections. The humoral immune response in enteroviral infections is especially important for protection and life-long immunity. For example, a high level of poliovirus neutralizing antibody response following natural infection or vaccination protects against disease, although infection of the gut can occur—that is, there is no “sterilizing immunity.” The importance of the humoral immune response in enteroviral infections is demonstrated by the occurrence of persistent enterovirus infections in agammaglobulinemics (see the Meningitis and Encephalitis section), and in the increased susceptibility to enteroviral infection as well as the increased severity of enteroviral disease among neonates and infants (see Neonatal and Infant Disease in the section Clinical Features).

The neutralizing antibody response against poliovirus has been characterized by generating neutralizing monoclonal antibodies, then growing virus in the presence of the antibody to produce monoclonal antibody-resistant virus, and finally identifying the neutralizing epitope for the virus by sequencing the coding region for the capsid proteins of the virus. The neutralization sites are generally nonsequential conformational antigenic sites that correspond to exposed loops of the capsid proteins rising from the surface of the virions (128); some regions that are internally located in the native virion, however, can also function as neutralization sites (280). Using this approach, a limited number of major neutralizing antibody sites have generally been found in the case of poliovirus type 1: the BC loop of VP1; a complex site including residues 220 to 222 from VP1 (site 2a) and residues 169 and 170 and others of VP2 (site 2b); a complex site including residues 286 to 290 from VP1 (site 3a); and residues 58 and 59 and others on VP3 (site 3b) (214). Although only a limited number of studies have been performed to identify neutralization sites of nonpolio enteroviruses, the results have suggested similarities in the location of their antigenic sites to those found with poliovirus.

Results from investigations of enterovirus neutralizing monoclonal antibodies may not be easily extrapolated to answer questions related to neutralizing sites for polyclonal antibodies in human serum. Responses in humans may differ from those seen in inbred mice, and they may vary from one person to another. Another potential shortcoming of studies with poliovirus neutralizing monoclonal antibodies is that these antibodies have been raised against capsid proteins. Therefore, it has not been possible to identify neutralizing antibodies that are directed against nonstructural proteins, if they exist. Last, it is uncertain how well *in vitro* neutralization of poliovirus by monoclonal antibodies corresponds to protection from natural disease.

T-Cell Response

There are several ways in which T cells may lead to protection and immunity from enterovirus infection. T cells may have a role in providing help for the B-cell humoral immune response, which, as just noted, plays a critical role in the clearance of enteroviruses. In addition, cytolytic T cells may clear the virus directly by causing lysis of virus-infected cells or by means of cytokine release. Infecting mice that have varied mutations in their genetic background has further delineated

the importance of different arms of the immune response in clearance of enteroviruses and in enterovirus-induced disease.

Although the T-cell response is believed important in the clearance of many viruses, it has been investigated to only a limited extent in the case of human enterovirus infections. In one study of peripheral blood lymphocytes from humans vaccinated against poliovirus, T-cell epitopes were found in a region between VP1 amino acids 161 and 221, and another between VP1 244 and 264 (306). Other investigations have made use of mice immunized with inactivated poliovirus (180) or with Sabin vaccine strains (197).

To further clarify the correlates of immunity in the case of poliovirus, experiments were conducted in PVR transgenic mice (197). T cells were established from spleens of the transgenic mice infected with neurovirulent poliovirus type 3 Leon strain or from normal BALB/c mice immunized with attenuated Sabin type 3 vaccine strain. The primary cytokines secreted by the clones were typical of those found with helper T-cell subtype 1 (Th1). Adoptive transfer of the Th1 clone along with primed B cells was necessary for protection against poliovirus challenge, demonstrating the importance of antibody production by these B cells in virus protection. The findings of this study were surprising considering the generally held beliefs that secretory IgA is critical for gut immunity, and that Th2-type cells are responsible for providing help for IgA antibody production. A potential explanation for the findings may be that the transgenic mice were challenged by the intravenous rather than the more natural oral route. In other words, it may be that the more natural oral challenge would have generated Th2 rather than Th1-type cells.

In studies of coxsackievirus B3 infection of C3H/HeJ mice, Beck and colleagues (28) identified a shared T-cell epitope present in a number of enteroviruses as well as a comovirus. The results suggested that this epitope was present in a nonstructural protein, 2C. Infection of C3H/HeJ (H-2k) with coxsackievirus B3 showed that structural proteins, as expected, also contain T-cell epitopes (21).

Immunopathology

Although the B- and T-cell response in enterovirus infections has a role in virus clearance, there are indications that it may also lead to disease. There are several reasons for considering a role for immunopathology in some enterovirus-induced diseases: (a) the enterovirus-induced diseases have inflammatory pathology; (b) the experimental animal models for these enterovirus-induced diseases demonstrate the importance of genetic susceptibility in their causation, and the efficacy of treatment with immunosuppressive drugs; and (c) the enterovirus-induced diseases are related to human diseases that are considered to have an autoimmune contribution. An immune-mediated disease process has been most vigorously proposed in the case of enterovirus-induced chronic dilated cardiomyopathy lesions, chronic myositis, and diabetes.

A role for both molecular mimicry and bystander damage has been proposed in virus-induced autoimmune disease. In the former, an immune response directed against the virus cross-reacts with a cellular protein and causes disease. In the case of bystander damage, a virus up-regulates nonspecific immune responses that lead to pathology. Recent data regarding coxsackievirus-induced insulin-dependent diabetes mellitus and coxsackievirus-induced myocarditis suggest the importance of bystander damage rather than molecular mimicry (see later section Diabetes) (134,135). The activation of autoreactive resting T cells could be induced by cytokines or other factors, such as the release of self-antigen (134). The presence of proinflammatory cytokines would then activate Th1 cells or macrophages that foster immunopathology. Once damage occurs, new cellular epitopes that are not normally exposed and that are antigenic may trigger additional reactive epitopes, a process called epitope spreading.

Cardiac Disease

The evidence for enterovirus infection as an etiology for human acute and chronic cardiac disease is summarized in the section Clinical Features: Cardiac Disease . Most of the investigations related to a possible immune contribution to these cardiac diseases have been explored in experimental model systems, especially related to coxsackievirus B3 infection. Coxsackievirus B3 produces an experimental acute myocarditis that can progress to chronic dilated cardiomyopathy in particular susceptible mouse strains. As discussed later, the pathogenesis of experimental cardiac lesions induced by coxsackievirus B3 is complicated and controversial (140), with evidence that direct viral lysis as well as the immune system contribute to pathology.

Studies with mice that have severe combined immunodeficiency disease, which are susceptible to myocardiopathy, have emphasized the importance of direct viral lysis as a cause for cardiac tissue damage (55). It may be, however, that one cannot extrapolate the results obtained from immunocompromised mice to immunocompetent animals, and that disease pathogenesis varies depending on the intactness of the immune system (i.e., a direct viral lytic infection may be more important as a cause for cardiac lesions in immunocompromised than in normal mice).

In mouse strains that develop chronic myocarditis, coxsackievirus B3 RNA persists in the infected heart (and can be detected by *in situ* hybridization) in the absence of infectious virus (170). This persistent viral infection may trigger an immune response that can foster the disease (142). Some investigators have suggested that the immune system may be important because of antigenic mimicry, a situation in which the immune response against the virus cross-reacts with the heart. The possibility of a cross-reactive autoantigen is of interest because immunization with cardiac myosin induces an autoimmune myocarditis in mice (234). Studies have reported that monoclonal antibodies that neutralize coxsackievirus bind to heart muscle (63), and that adoptive transfer of similar antibodies leads to myocarditis (95). Similarly, T-cell hybridomas derived from the hearts of coxsackievirus B–infected mice respond to cardiac myosin as well as to the virus (296). On the other hand, a recent study failed to find support for a role for molecular mimicry in coxsackievirus B3–induced myocarditis (135). These investigators found that heart disease did not develop in transgenic mice that express interferon-g in their pancreatic cells (and therefore have a low level of virus replication in the heart), even though the animals generated a similar immune response to that seen in nontransgenic; they concluded that the immune system's involvement depends on damage to the heart by the virus. A role of T-cell inflammation in the development of

disease is supported by studies that demonstrate that knock-out of a chemokine, monocyte chemoattractant protein-1 (MIP-1a), interferes with the development of coxsackievirus-induced myocarditis (60).

Studies have suggested that there is a differing immunopathogenesis of disease depending on the genetics of the host (141). A recent study using A/J mice demonstrated a decrease in mortality and cardiac tissue inflammation in mice that lacked both CD4+ and CD8+ T cells, but not an absence of either CD4+ or CD8+ T cells alone (246). In other strains of mice, the CD8+ T-cell response rather than the CD4+ T-cell response appears to play a pathogenic role, whereas in still others, the CD8+ T-cell response has a protective role (122). DBA/2 mice infected with coxsackievirus B3 develop an anti-heart IgG antibody response associated with the myocarditis, whereas CD8+ T cells appear important in the pathogenesis of myocarditis in BALB/c mice; the differences seen following infection of BALB/c mice versus DBA/2 may have resulted from the fact that Th1 cells are preferentially activated in the case of BALB/c mice (providing helper activity for CD8+ cytolytic T cells), whereas Th2 cells are activated in the case of DBA/2 mice (inducing cytokines that lead to B-cell activation) (296). Another possible reason that humoral factors do not seem as pathogenic in BALB/c mice as in DBA/2 mice may be that BALB/c mice have significantly less myosin in their cardiac extracellular matrix—that is, there is a genetically determined decrease in target organ antigen (186).

As Schwimmbeck notes, “Despite the demonstration of autoimmune phenomena in patients with myocarditis and dilated cardiomyopathy, there is no proof of the autoimmune pathogenesis of these diseases” (296). It is clear that the role of the immune system in enterovirus-induced cardiac disease is complex and poorly understood, and that more studies are needed to clarify this subject. Disease is also influenced by host genetic (143) and cardiovirulent genetic determinants of the coxsackievirus strain and of the particular isolate (319) (see later section Coxsackievirus and Myocarditis). Recent studies have also suggested a role for apoptosis during enterovirus infection (123,339).

Inflammatory Muscle Disease

The immune system is believed to play a role in the inflammatory myopathies (see later section Clinical Features), and CD8+ T cells, CD4+ T cells, cytokines, and autoantibodies have been implicated in their pathogenesis (185). Some investigations have focused attention on experimental infections with the Tucson strain of Coxsackievirus B1, because it can produce a chronic form of muscle disease following intraperitoneal inoculation of certain mouse strains. Interestingly, the disease progresses despite the disappearance of infectious virus from muscle by 2 to 4 weeks after inoculation (312). In some cases, viral RNA persists for as long as a year after infection. A recent study reports evidence of viral double-stranded RNA, perhaps increasing stability and fostering persistence of the viral genome (313). A role for the immune system and especially T cells has been proposed but remains incompletely delineated.

Diabetes

Coxsackievirus B4 has been isolated from rare cases of fatal diabetes in humans (271). In addition, certain strains of this virus induce a pancreatitis and islitis in particular strains of mice (297). Viral RNA can persist for months in the pancreas in the absence of infectious virus or viral protein antigen. Islet cell inflammation and diabetes generally continue in these mice for over a year. These observations suggest that experimentally induced diabetes is caused by a persistent restricted viral infection with a contribution from the immune system to the disease process. The mechanisms involved in the pathogenesis of this experimental coxsackievirus-induced diabetes mellitus and its relationship to human insulin-dependent diabetes mellitus remain unclear.

An intriguing finding related to insulin-dependent diabetes mellitus is the sequence similarity between coxsackievirus B protein 2C and glutamic acid decarboxylase (GAD_{65}) (159), which is expressed in the pancreas and CNS. Antibody to GAD_{65} is associated with insulin-dependent diabetes mellitus (and with a rare neuromuscular disease called the stiff-man syndrome). The appearance of antibodies to GAD_{65}, as well as to islet cells and insulin, frequently precedes the onset of human diabetes. In addition, recent studies in transgenic mice suggest that there is a correlation between GAD_{65} expression and the development of diabetes in the nonobese diabetic (NOD) mouse, which is a genetically determined, immune-mediated mouse model of diabetes (344). These results raise the possibility that coxsackievirus infection leads to an immune response against 2C that cross-reacts with GAD_{65} and induces diabetes through a mechanism of molecular mimicry. A cross-reactivity between protein 2C and GAD_{65}, however, has not been consistently found (275), and some investigators have found that the immunodominant T-cell epitope is in a region of GAD_{65} that is distinct from the one with 2C sequence homology (191). In summary, although the proposal that antigenic mimicry between GAD_{65} and protein 2C is an intriguing one, its importance in the pathogenesis of insulin-dependent diabetes mellitus remains unproven.

A recent report demonstrating the complicated role of enteroviruses in diabetes mellitus favors a role for bystander damage rather than molecular mimicry. This study involves the use of BDC2.5 mice, which carry a transgene encoding a diabetogenic T-cell receptor specific to an islet granule antigen that is distinct from GAD_{65} and does not cross-react with coxsackievirus B4 (134). Inoculation of this mouse with coxsackievirus B4 led to the rapid development of diabetes mellitus. The infected BDC2.5 mice were found to have infiltration of the islet cells with lymphocytes carrying the transgenic T-cell receptor, even though the target cell for this virus is the exocrine pancreas and not the islet cells. It is presumed that this acceleration of diabetes is caused by bystander cells that respond to the release of proinflammatory cytokines, leading to activation of T cells that destroy the islet cells. Molecular mimicry did not seem involved in disease pathogenesis because coxsackievirus B4 virus failed to accelerate the development of insulin-dependent diabetes mellitus in the infected NOD mouse despite the known cross-reactivity of GAD_{65} with the coxsackieviral 2C protein; in addition, there was no increase in T-cell reactivity against GAD_{65} in splenocytes from these mice. The results suggest that enteroviral infections involving the pancreas (or other organs) may provide an environmental trigger that induces autoimmune disease in genetically susceptible animals. The implications of this study are that cytokines or islet cell antigens were released during the infection, causing activation of resting islet-specific memory lymphocytes through bystander mechanisms. These observations may have relevance to the human situation, where coxsackievirus infections of the pancreas could activate autoreactive T cells leading to insulitis and diabetes mellitus. Other transgenic mouse studies have also demonstrated complex and variable requirements during the development of experimental

insulin-dependent diabetes mellitus (324).

Animal Models

Poliovirus

Nonadapted poliovirus infects only primates, at least partly related to the presence of PVR in primates and not in other species. Studies of poliovirus neurovirulence that have made use of nonhuman primates have been limited by the relatively small number of animals that can be used as a result of ethical and financial concerns. The Lansing strain of poliovirus type 2 was adapted to mice to circumvent these difficulties; however, this mouse-adapted strain of poliovirus binds by a receptor that is not yet identified and is distinct from PVR. Of greater importance in the study of poliovirus disease pathogenesis is the PVR transgenic mice.

The use of PVR transgenic mice to study human poliovirus infection has been extraordinarily valuable. However, as in all animal models, infections in these mice are not a perfect model for the human disease. The mice are not as sensitive as humans to poliovirus infection by the oral route (173). Therefore, investigations of disease pathogenesis in PVR transgenic mice have generally involved routes of infection distinct from that seen with human poliomyelitis. This shortcoming is probably not secondary to the low expression of the PVR in intestinal epithelial cells, because transgenic mice that carry a promoter for rat intestine fatty acid binding protein to direct expression of PVR fail to have a more efficient gastrointestinal infection or an increase in the virus titer in feces after oral challenge (350). There are also differences between mice and humans in the organization of the CNS, including the motor pathways, which are likely to influence the character and development of the paralytic disease.

Nonpolio Enteroviruses

There are several examples of experimental models of nonpolio enterovirus infections. Although coxsackieviruses can cause a myocarditis in many species, including monkeys, the best-studied experimental enterovirus-induced acute and chronic heart disease is caused by coxsackievirus B3 infection in mice (see previous section Immunopathology: Cardiac Disease).

Strains of coxsackievirus A and B and echovirus cause myositis in mice (88). One of the best-studied experimental models of myositis is infection of mice with the Tucson strain of coxsackievirus B1 (see previous section Immunopathology: Diabetes).

A number of enteroviruses can infect the acinar cells of the pancreas in mice, but only a select group of enteroviruses infect the islet cells and produce diabetes. The latter group includes several members of coxsackievirus B group following passage in the pancreas of mice or in mouse beta cell cultures (317). Most experimental studies of enterovirus-induced diabetes have made use of the E2 strain of coxsackievirus B4. The latter virus produces an acute pancreatic infection with islet cell inflammation and necrosis in certain strains of mice (297). Some investigators have studied the NOD mouse following infection with coxsackievirus (see previous section Immunopathology: Diabetes) (324).

Both enteroviruses 70 and 71 can produce paralytic disease in monkeys (117). These experimental animals may be important in identifying virulence determinants and also in vaccine studies.

Virulence

Poliovirus and Neurovirulence

The molecular basis for viral determinants of neurovirulence is a topic of great interest, especially in the case of poliovirus. This subject has been best studied for poliovirus, at least partly because of the availability of well-characterized attenuated vaccine strains of poliovirus generated by Sabin.

Sabin developed attenuated vaccine strains for all three poliovirus serotypes through passage of virus in animals and in cell culture, with the concomitant assessment of the neurovirulence potential of the passed virus following inoculation of nonhuman primates. These vaccine strains grow well in tissue culture but poorly in the CNS of monkeys, which is presumably the reason that they are attenuated. The vaccines are inoculated into monkeys to assess their neurovirulence and ensure that they are sufficiently attenuated (Fig. 6).

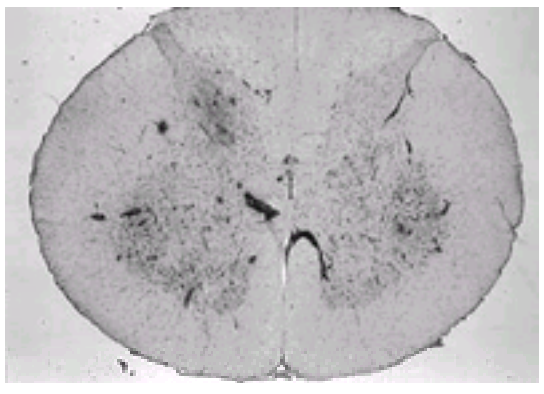

FIG. 6. Section of rhesus monkey spinal cord 17 days after inoculation with poliovirus type-3 in the anterior lumbar cord. There was evidence of hemorrhage and neuronal degeneration in the gray matter. (Courtesy of Dr. Philip J. Snoy, Center for Biologics Evaluation and Research, Food and Drug Administration.)

Despite the excellent safety record of Sabin oral poliovirus vaccine, vaccine-associated paralytic poliomyelitis occurs, albeit infrequently. Most of the cases have occurred after the first dose of the oral vaccine. The overall frequency of cases associated with the first dose of vaccine was reported as one case per 520,000. Of 105 cases of vaccine-associated poliomyelitis that occurred in the United States from 1973 through 1984, 14 occurred in immunocompromised individuals; 35 cases occurred in vaccinees, 50 in contacts of the vaccinees, and 6 in people with no apparent history of vaccination or exposure to vaccines (235).

Individuals with humoral immunodeficiencies have an increased risk of developing poliomyelitis, and therefore oral poliovirus vaccination of this group is contraindicated. These patients are unable to clear the vaccine virus from the intestinal tract and can excrete virus from the gut for prolonged periods of time, sometimes for over a decade. CNS disease and paralysis can occur in these individuals because virus persistence in the gut is associated with a low level of neutralizing antibody (295). At times, poliovirus can cause a slowly progressive neurodegenerative process (71). The use of immunoglobulin therapy in immunocompromised individuals is at least partly effective in preventing (and treating) these complications. Recent studies suggest that pleconaril may also have a place in this treatment (238). In addition, the use of inactivated poliovirus vaccine rather than the live poliovirus vaccine avoids the risk of vaccine-associated paralytic poliomyelitis.

Vaccine-associated paralytic poliomyelitis results from a reversion of the vaccine strain to a more neurovirulent form. Mutation is frequent, because poliovirus, like other positive-stranded RNA viruses, has an error-prone RNA-dependent RNA polymerase. For this reason, RNA viruses exist as a quasi-species, and mutations are even present in plaque-purified stocks of virus. Studies of normal immunocompetent individuals following vaccination have shown that there is rapid reversion of excreted virus to a genotype containing neurovirulent determinants (see later); in addition, intertypic recombinant viruses containing segments from the different poliovirus serotypes are generated in the gut and excreted in the feces (213). A recent reverse transcriptase–polymerase chain reaction analysis of isolates from the stool and the CNS of vaccine-associated paralytic poliomyelitis demonstrated that many varied poliovirus genotypes exist in a patient at the same time, including recombinants from the three poliovirus serotypes (98). Inoculation of these isolates into PVR transgenic mice failed to clarify the reasons why particular genotypes were present in the CNS or the gut. In other words, the CNS isolates were not more neurovirulent than isolates found in the gut, suggesting that random selection might determine whether an isolate spreads from the gut to the CNS. Not only do poliovirus serotypes recombine, but there is evidence of recombination between poliovirus and nonpolio enteroviruses, as demonstrated most recently in an outbreak in the Dominican Republic and Haiti (18).

Vaccine-associated paralytic poliomyelitis usually results from reversion of poliovirus type 3, less commonly from reversion of poliovirus type 2, and rarely with type 1 vaccine strains. The paucity of differences between the parental neurovirulent poliovirus type 3 and the vaccine strain (11 nucleotide and 3 amino acid changes) is presumed responsible for the frequent reversion of this serotype to a neurovirulent phenotype with the subsequent development of paralytic disease. The relatively small number of vaccine-associated paralytic poliomyelitis cases among vaccine recipients for types 1 and 2 may result from the many differences between the vaccine and parental neurovirulent strain (at least 56 nucleotide changes for type 1, and 23 nucleotide changes for type 2). In addition, the parental type 2 strain has a relatively decreased neurovirulence on its own.

A comparison of the sequences of neurovirulent parental poliovirus strains, the attenuated vaccine strains, and neurovirulent revertants from cases of vaccine-associated paralytic poliomyelitis, coupled with an investigation of engineered viruses generated from segments of infectious clones interchanged between the vaccine and neurovirulent parental strain, have identified changes critical for determining the phenotype of attenuated Sabin strains. All three Sabin poliovirus serotypes are thought to have a mutation in a major determinant for neurovirulence in a similar location in the internal ribosome entry site in the 5'-untranslated region: nucleotides 480 (type 1) (160), 481 (type 2) (195), and 472 (type 3) (90). It has been hypothesized that these mutations destabilize the secondary structure of the RNA (194). The destabilization is believed to perturb binding of neural cell-specific factors to the internal ribosome entry site in the viral RNA genome, so that there is inefficient translation initiation of the viral genome with a subsequent decrease in virus growth in neurons (leading to an attenuated phenotype). In other words, a factor in neurons binds the Sabin strain's mutated internal ribosomal entry site (IRES) far less effectively than the IRES of the virulent wild type, and therefore translation of the RNA from the vaccine strain is decreased in neurons, and subsequent virus growth in neurons is attenuated.

The importance of the 5'-untranslated region and cell-specific viral translational regulation in neurovirulence has also been shown in studies in which the rhinovirus type 2 IRES has been substituted for a similar region of poliovirus type 1 (110). The chimeric virus grew well in HeLa cells but was attenuated in both PVR transgenic mice and monkeys.

Recent data have modified our understanding of the relative importance of mutated nucleotides in the 5'-untranslated regions of the Sabin strains of type 1 and 2 (272). Studies of the Sabin strain of type 1 demonstrated that there is indeed frequent reversion of nucleotide 480 (and 525) following passage in cell culture and after vaccination into humans or monkeys; however, the presence of these mutations in as high as 17% of the vaccine lot did not consistently cause failure of the lot in a monkey neurovirulence test. On the other hand, the presence of the mutation in over 70% of the lot of virus did lead to an enhanced neurovirulence (272). These results suggest that nucleotide 480 in poliovirus type 1 has a less dramatic effect on neurovirulence in monkeys than previously suspected. Similar observations were made with Sabin strain type 2. In this case, inoculation of Sabin strain type 2 (with an additional single mutation at nucleotide 481 from A to a G) into a larger group of monkeys than was used in earlier studies (256), as well as into transgenic mice, showed no enhanced neurovirulence (273).

Additional determinants for attenuation besides those in the 5'-untranslated region have been identified (263). Transgenic mouse studies have shown that there are additional attenuating mutations in the case of Sabin poliovirus type 1 at amino acid 65 of VP4, 225 of VP3, 106 of VP1 (and also more weak determinants at 134 and 109 of VP1) (42).

In the case of Sabin poliovirus type 2, studies in monkeys and mice have demonstrated a determinant for attenuation at amino acid 143 of VP1 (270). Studies in monkeys have identified additional attenuating determinants in the case of Sabin poliovirus type 3, at amino acid 91 of capsid protein VP3 (330) and amino acid 6 of capsid protein VP1 (316). Other studies in monkeys found that there may be additional attenuating determinants in the polymerase 3D and 3'-noncoding regions (56,244) that were not found in studies with the transgenic mice (42). These results suggest that results obtained from one species may not be easily extrapolated to another species.

It remains unclear why so few vaccinated individuals develop paralytic poliomyelitis, given the relatively few changes in sequence between the parental and vaccine strains coupled with the frequency of reversion of the vaccine strain to more neurovirulent variants. The safety of the vaccine is even more remarkable given the fact that excreted virus from vaccinees contains revertants with wild-type neurovirulent determinants. The reason(s) may be as complicated and poorly understood as the reason that only 1% of the individuals exposed to wild-type poliovirus develop paralytic disease. Most likely, the outcome of the exposure depends on the dosage of the virus, the virus's neurovirulence determinants, genetic factors of the host and virus that influence virus growth, and the quality and rapidity of the immune response. It is also possible that differences in the inherent neurovirulence of wild and vaccine strains are not as important to the outcome of infection as differences in transmissibility and replication of particular isolates, for which there are no good models for study.

Coxsackievirus and Myocarditis

In an approach analogous to those used to clarify the neurovirulence determinants of poliovirus, studies have been performed to identify cardiovirulent determinants in coxsackievirus B3. Tu et al. (321) tested the effect of infection of mice with viruses generated from chimeric coxsackievirus B3 cDNAs in which segments of a myocarditic and amyocarditic strain (derived by tissue culture passage of the myocarditic strain) were recombined. The cardiovirulent phenotype was linked to nucleotide 234 (U in cardiovirulent and C in avirulent) in the 5'-untranslated region of coxsackievirus B3. However, sequencing studies of the 5'-untranslated region of coxsackievirus B3 isolates from humans found that this nucleotide was unlikely to be a major determinant in nature, because many coxsackievirus B3 isolates contain a U at nucleotide 234 despite marked differences in cardiovirulence (96). Other studies found that a single mutation at VP2 position 165 in the “puff” loop was critical for cardiovirulence (171), or that nucleotide 2916 was important for cardiovirulence (46). Some of these studies also involved somewhat artificially generated mutant viruses (96,171), and therefore they may have identified determinants of cardiovirulence that do not vary among natural isolates (87). A recent study involving recombinant viruses engineered from cDNA segments derived from two clinical coxsackievirus B3 isolates with marked differences in cardiovirulence identified the 5'-untranslated region as a major determinant of the cardiovirulence phenotype (87). It is clear that cardiovirulence may depend on multiple determinants throughout the genome. The identification of these sites and an understanding of their relative importance to the phenotype await continuing study.

A recent study of interest found that expression of coxsackieviral proteins in the heart as transgenes in mice, independent of any viral replication, induced dilated cardiomyopathy (329). Subsequent *in vitro* studies showed that purified coxsackievirus protease 2A cleaves dystrophin (25), a key protein component of heart muscle that is frequently mutated in inherited forms of dilated cardiomyopathy. In addition, the investigators found that coxsackievirus infection of cells in culture or mice leads to dystrophin cleavage, supporting a role for protease 2A in the disease process. If this model is correct, the similar specificity of the proteolytic activity of 2A in different enteroviruses would imply that multiple enterovirus serotypes have the potential to induce cardiomyopathies. Further studies are needed to validate the importance of coxsackievirus 2A in the pathogenesis of cardiomyopathy. For example, it will be important to verify that the presence of active 2A as a transgene, in the absence of other viral proteins, induces dilated cardiomyopathy; there may be difficulties, however, in ensuring 2A expression while avoiding host-cell translation shut-off with subsequent cell death.

Coxsackievirus and Infection of the Pancreas

Coxsackievirus A serotypes produce a widespread myositis in newborn mice, whereas group B viruses induce a focal myositis with infection of other tissues. Studies related to virulence associated with coxsackievirus B4 infection of the pancreas have been performed in mice with a virus strain that causes acinitis rather than islitis (264). Investigations using recombinant viruses demonstrated that a single amino acid in VP1 was critical for the virulent phenotype; however, these animals died of an acinitis in the pancreas, so there is no information at present regarding the identification of viral determinants critical in inducing diabetes.

Persistence

Enteroviruses are generally considered a cause of acute infections rather than persistent infections. It is clear, however, that persistent enterovirus infections occur in humans with humoral immunodeficiency because of a failure to clear virus. For example, agammaglobulinemic patients vaccinated with the Sabin strain of poliovirus may excrete virus for prolonged periods from the gut and develop paralytic disease after a long incubation period. These patients may also develop a chronic neurodegenerative-like disease with evidence of chronic brain and spinal cord inflammation (71). Other patients with immunodeficiency, especially X-linked agammaglobulinemia, can develop a chronic nonpolio enterovirus-induced aseptic meningitis or meningoencephalitis (see later section Meningitis and Encephalitis); these patients may have an associated dermatomyositis-like syndrome with inflammation of multiple organs (201). Echovirus strains are the predominant nonpolio enterovirus isolated from these cases for reasons that are not understood.

There have also been reports demonstrating persistence of enterovirus or the enterovirus genome in immunocompetent humans and animals with several diseases, including chronic dilated myocardiopathy, inflammatory muscle disease (myositis), and diabetes (see relevant sections of Clinical Features). In addition, enteroviruses, including poliovirus, have been implicated in the etiology of postpolio syndrome and amyotrophic lateral sclerosis (see later section Clinical Features: Poliomyelitis: Postpolio Syndrome and Amyotrophic Lateral Sclerosis). Additional studies will be important to

clarify how enterovirus infections may lead to chronic human diseases, and to determine whether the immune response or other factors are important in their pathogenesis.

EPIDEMIOLOGY

Demographics

Despite the nearly ubiquitous nature of enterovirus infections and the wide variety of clinical presentations, the demographics of the various infections and diseases have some consistent characteristics. In particular, several factors including age, sex, and socioeconomic status have largely predictable effects.

One of the most important determinants of enterovirus infection outcome is age. Different age groups have different susceptibilities to infection, severity of illness, clinical manifestations, and prognoses following enterovirus infection. Understanding these age effects on outcome of infection is complicated by the widely divergent prior history of infection and resulting immunity. Nevertheless, it is possible to make certain generalizations.

The largest amount and duration of virus shedding occurs on primary infection with a given enterovirus serotype. Because infection is so common, most primary infections occur during childhood. For these reasons, young children are probably the most important transmitters of enteroviruses, particularly within households. The greater exposure of children to virus during infection may make them more likely to have significant clinical symptoms. For example, in outbreaks of meningitis, children typically have higher rates of disease than adults (103,148). Unfortunately, most studies do not separately determine age-specific infection rates and disease rates, and the relative rates at which adults are infected are not generally known.

The incidence of poliomyelitis is relatively low for the first 4 to 6 months of life in developing nonindustrialized countries (in which control through vaccination has not yet been achieved) because of the frequent presence of protective maternal antibody. In these countries, there is an increased incidence of paralytic disease in children older than 6 months compared to children in wealthier industrialized countries, presumably related to an earlier exposure to virus as a result of poor sanitary conditions. Ironically, areas with improved hygiene may have a decrease in infant exposure, leaving an older (unexposed) population susceptible to epidemic disease, with high rates of paralytic disease during an outbreak (260). Adults are more likely to be severely affected in both nonindustrialized and wealthier industrialized countries, tending to acquire paralytic poliomyelitis rather than nonparalytic CNS disease (i.e., aseptic meningitis), abortive illness, or asymptomatic infection (132). The reason for the increase in severity later in life is unknown. A possible reason relates to the finding that fast axonal flow—which appears important in the spread of poliovirus within the CNS (155)—increases with age. In addition, it may be that PVR expression or host factors important in replication change with age.

Severity of a number of enteroviral diseases besides poliomyelitis may be strikingly age-related. There is an indirect indication that a delay in first infection with a number of enteroviruses increases risk of more severe disease. For example, exanthema associated with coxsackie A viruses and echoviruses are for the most part milder in children than in adults. On the other hand, some enteroviruses cause more severe disease in newborns than in older children and adults, possibly inducing a fulminant “viral sepsis” with myocarditis, encephalitis, and sometimes death (see later section Clinical Features: Neonate and Infant Disease) (52,150). In addition, recent outbreaks of hand-foot-and-mouth disease caused by enterovirus 71 have been associated with a significant CNS complication, fatal brainstem encephalitis, that was restricted largely to young children (Table 3) (see later section Meningitis and Encephalitis) (174,192).

Year	Location	Clinical findings
1969–1973	California	Aseptic meningitis, encephalitis
1972	New York	Aseptic meningitis, encephalitis, hand-foot-and-mouth disease
1972	Australia	Aseptic meningitis, rash, polyneuritis, acute respiratory infection
1973	Sweden	Aseptic meningitis, hand-foot-and-mouth disease
1973	Japan	Hand-foot-and-mouth disease, aseptic meningitis
1975	Bulgaria	Aseptic meningitis, encephalitis, acute myocarditis, acute flaccid paralysis
1978*	Hungary	Aseptic meningitis, encephalitis, acute flaccid paralysis
1985	Hong Kong	Micropiegia
1986	Australia	Central nervous system involvement
1987	USA	Acute flaccid paralysis, meningitis, encephalitis
1988	China	Hand-foot-and-mouth disease
1987	Malaysia	Fatal encephalitis, acute flaccid paralysis, hand-foot-and-mouth disease
1998	Taiwan	Fatal encephalitis, hand-foot-and-mouth disease

*A mixed epidemic of tick borne encephalitis (chiefly in adults) and enterovirus 71 disease (chiefly in children).

Source: Adapted from ref. 297, with permission.

TABLE 3. *Some enterovirus 71 outbreaks, 1969 TO 1989*

In general, encephalitis and aseptic meningitis caused by enteroviruses appear to be most frequent among those 5 to 14 years old rather than those older or younger. In a 10-year surveillance summary from the United States (222), adults tended to be overrepresented among cases of severe disease (paralysis, encephalitis, meningitis, carditis) when compared with the age distribution of the enterovirus-infected population as a whole. In another study, the mean age among patients with coxsackievirus B meningitis (7.7 years) or pericarditis (9.9 years) was greater than the mean age of patients with coxsackievirus B gastroenteritis (1.3 years) (80).

Enterovirus diseases, and possibly also enterovirus infections, occur more frequently in males than in females (103,223), although some exceptions have been described (78). In numerous reports, the male-to-female ratio appears to generally range between about 1.2 and 2.5:1; that is, approximately 55% to 70% of such diseases occur in males. Male predominance tends to be greater for the more severe diseases, for example CNS disease or carditis, than for less severe disease, for example pleurodynia, hand-foot-and-mouth disease, respiratory disease, acute hemorrhagic

conjunctivitis, rash, or undifferentiated febrile illness.

The apparent predominance of enteroviral infections among males may have both sociologic and biologic explanations. Population-based measurements of infection such as serosurveys, which should be gender neutral, have not consistently demonstrated a higher infection rate for males. Several additional explanations for the male predominance have been proposed on the basis of studies of infections in healthy children (97): a longer duration of virus excretion occurs in males than in females (leading to increased chance of identifying infected males); a higher virus titer occurs in the feces of males (leading to a similar increase in diagnosis). Another possibility is that there may indeed be more frequent infections in males because of a greater exposure to the pathogen, perhaps because there are differences in the parental treatment and play habits of younger boys, and greater activity among older boys. An additional possibility is that males are more likely to develop a serious illness from a given enterovirus infection than females. For example, the reason that human myopericarditis is more common in adolescent and adult males than in females (except in pregnant and postpartum women) (333) could be caused by sex-related endocrine effects leading to differences in disease susceptibility.

Enterovirus infections are more prevalent among persons of lower socioeconomic status and those living in urban areas (115,152). In a study utilizing active surveillance of healthy children for enterovirus infections in West Virginia during 1951 to 1953, the rate of isolations among children in a lower socioeconomic setting was 2 to 7 times higher than among children in a higher socioeconomic setting (131). A similar study in Ghana during 1971 to 1973 further indicated that enterovirus isolations were significantly more frequent from children in areas with poorer sanitation and in urban areas during both rainy and dry seasons (247).

Paradoxically, poliomyelitis and possibly other enterovirus diseases tend to be “diseases of development.” In the case of poliomyelitis, improvement in a country's hygienic and socioeconomic conditions (before vaccination programs) successfully reduces the incidence of paralysis due to poliovirus and leads to a transitional period in which there is a delay in age of first infection with a subsequent temporary increase in the paralysis-to-infection ratio. Prior to the introduction of the poliovirus vaccine in the United States and other developed countries, paralytic poliomyelitis was disproportionately a disease of the middle and upper socioeconomic classes; this disease distribution was a result of infection at an older age, when paralysis was a more frequent complication. Ironically, the delay was a result of improved hygiene. The infant mortality rate, a general indicator of a country's level of health development, may be inversely correlated with the age-specific incidence of poliomyelitis (205).

Transmission

Enteroviruses can be isolated from both the lower and upper alimentary tract and can be transmitted by both fecal-oral and respiratory routes. Fecal-oral transmission may predominate in areas with poor sanitary conditions, whereas respiratory transmission may be important in more developed areas (133). The relative importance of the different modes of transmission probably varies with the particular enterovirus and the environmental setting. It is believed that almost all enteroviruses, except possibly enterovirus type 70, can be transmitted by the fecal-oral route; however, it is not known whether most can also be transmitted by the respiratory route. Enterovirus 70 and coxsackie A24 variant, the agents that cause acute hemorrhagic conjunctivitis, are seldom isolated from the respiratory tract or stool specimens, and are probably primarily spread by direct or indirect contact with eye secretions (175). Enteroviruses that cause a vesicular exanthem can presumably be spread by direct or indirect contact with vesicular fluid, which contains infectious virus.

It is likely that enteroviruses are transmitted in the same manner as are other viruses causing the common cold—that is, by hand contact with secretions (e.g., on the hand of another person) and autoinoculation to the mouth, nose, or eyes. Direct bloodstream inoculation, usually by laboratory accidents such as needle sticks and shattered glass vials, may result in enterovirus infection; however, neither blood transfusion nor mosquito or other insect bites appear to transmit infection. The isolation of enteroviruses from flies has led to a suspicion that houseflies (*Musca domestica*) and various filth flies may be vehicles of mechanical transmission. There is no evidence that venereal transmission is important.

Transmission within households has been well studied for both polioviruses and nonpolio enteroviruses. Small children generally introduce enteroviruses into the family, although young adults make up the majority of index cases in some outbreaks of acute hemorrhagic conjunctivitis (290). Intrafamily transmission may be rapid and relatively complete, depending on duration of virus excretion, household size, number of siblings, socioeconomic status, immune status of household members, and other risk factors (115). Transmission has been generally found to be greatest in large families of lower socioeconomic status with a greater number of 5- to 9-year-old children and with no evidence of serologic immunity to the virus type. Not surprisingly, infections in different family members may result in different clinical manifestations.

Observations of household transmission of various enteroviruses have documented that many infected contacts do not become ill and that the extent of secondary transmission varies with different enteroviruses. Household secondary attack rates in susceptible members may be greatest for the agents of acute hemorrhagic conjunctivitis (enterovirus type 70 and coxsackievirus A24 variant) and for the polioviruses, and of lesser magnitude for the coxsackieviruses and echoviruses. In some studies, secondary attack rates may be 90% or greater, although they are typically lower. New York Virus Watch data indicate that enterovirus infections were more frequent among 2- to 9-year-old children and that secondary coxsackievirus infections were more frequent in mothers (78%) than in fathers (47%) (172). In the same study, coxsackieviruses spread to 76% of exposed susceptibles versus 25% of exposed persons who had detectable antibody to the infecting type; echoviruses infected 43% of susceptibles and only one person with antibody. The greater spread of polioviruses and coxsackieviruses may derive from longer periods of virus excretion.

Transmission occurs within the neighborhood and community, particularly where people congregate. In addition, like many other viruses, enteroviruses can be rapidly transmitted within institutions when circumstances permit (e.g., crowding, poor hygiene, or contaminated water). School teams or activity groups and institutionalized ambulatory retarded children or adults may be at special risk (12). Despite crowding, enterovirus transmission is not usually

accelerated to a noticeable degree in institutions where good sanitation is found.

As a result of widespread but incomplete poliovirus immunization, rare poliovirus-susceptible enclaves have arisen. These usually consist of unvaccinated religious groups in countries with an otherwise high prevalence of poliovirus immunity. Despite the barrier of millions of immune persons, poliovirus outbreaks have occurred in some of these enclaves (245). The frequency and ease of international travel may result in the continuous introduction of wild poliovirus in all regions of the world, indicating that a large proportion of the population must be vaccinated if poliomyelitis epidemics are to be prevented. This suggests that herd immunity may be of only limited value in protecting groups of susceptible persons who have regular contact with outside populations, and it raises questions about the risks that such groups may pose to the community at large.

Nosocomial transmission of various coxsackieviruses of groups A and B and the echoviruses has also been well documented, typically in newborn nurseries. Hospital staff may have been involved in mediating transmission in some of these outbreaks. Enterovirus type 70, as well as coxsackievirus A24 variant, is highly transmissible and may cause outbreaks in ophthalmology clinics when instruments are inadequately cleaned between patients. An apparent outbreak of coxsackievirus A1, which included some fatal cases, has been reported in bone marrow transplant recipients (318).

Although human enteroviruses have been isolated from various environmental sources, man is thought to be the only important natural reservoir (85,91). There is not known to be any extrahuman reservoir. Survival beyond a few weeks does not generally occur, although enteroviruses can survive for months in favorable environmental conditions; these favorable conditions include neutral pH, moisture, and low temperatures, especially in the presence of organic matter, which protects against inactivation.

Although there is little evidence that enteroviruses found in the environment are of public health importance, concern has been expressed about possible dangers of contaminated sources of water (Fig. 7). Recreational swimming water has been investigated in several studies, and enteroviruses have been isolated from swimming and wading pools in the absence of fecal coliforms and in the presence of “recommended” levels of free residual chlorine. Coxsackievirus B5 was isolated from an unchlorinated lake swimming area during an outbreak at a boy's camp in Vermont, although the outbreak itself was explained by person-to-person transmission. In one study, the relative risk of enterovirus infection among children was found to be significantly higher for beach swimmers, especially for those under 4 years old (68). These reports suggest that swallowing of contaminated pool or lake water may theoretically account for transmission, but there is no proof that this type of transmission is significant in many typical recreational settings.

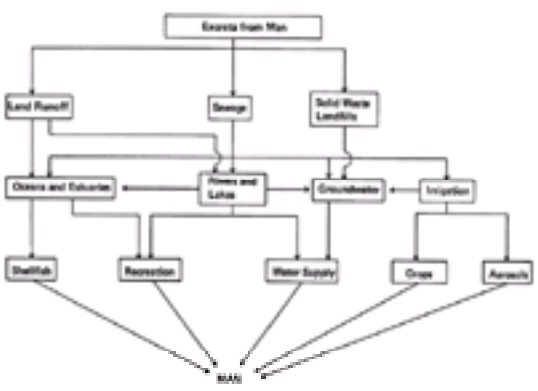

FIG. 7. Routes of potential enteric virus transmission in the environment. (From ref. 201, with permission.)

Enteroviruses have been found in surface and ground waters throughout the world. In the tropics, virus survival is more prolonged in ground water because it is cooler than surface water. As in the case with swimming pools, enteroviruses can be found in these waters even after chlorination and even in the absence of fecal coliforms. In industrialized countries, enterovirus transmission from potable water is apparently uncommon but is a constant source of concern for public health investigators, because the usual conditions under which city drinking water is chlorinated may be insufficient to completely inactivate enteroviruses.

Enteroviruses have been isolated from raw or partly cooked mollusks and crustacea, and their overlying waters (105). Shellfish rapidly concentrate many viruses, including enteroviruses. These viruses may survive in oysters for 3 weeks at temperatures of 1° to 21°C. To date, there has not been an outbreak of enterovirus disease attributed to consumption of shellfish. Other food-borne transmission has been documented but is thought to be uncommon. A 1976 outbreak of aseptic meningitis attributed to echovirus type 4 was apparently caused by consumption of contaminated coleslaw at a large picnic (Centers for Disease Control, unpublished data, 1976).

Enteroviruses, especially polioviruses, are regularly found in sewage. Enteroviruses are more prevalent in sewage from areas with low socioeconomic conditions or with large proportions of young children. In addition, sewage workers have been shown to have a higher prevalence of serum antibodies to enteroviruses than highway maintenance workers, consistent with an occupational risk (58).

Soil and crops also provide conditions favorable to enteroviruses. Enteroviruses survive well in sludge and remain on the surface of sludge-treated soil and even on crops. Air samples from aerosolized spray irrigants using contaminated effluents have also been found to contain enteroviruses (221). Survival of enteroviruses on vegetable food crops exposed to contaminated water or fertilizer has not been proven to be associated with virus transmission.

Prevalence and Disease Incidence

Incidence data about diseases caused by particular enterovirus types may be derived from prospective longitudinal surveillance of a defined population, or a sample of the population, in which the occurrence of disease and/or infection can be reliably determined. The Virus Watch program in the 1960s in United States cities exemplifies this type of surveillance study, in which specimens from subject children were obtained every 2 weeks for virologic evaluation (61,172,308). While difficult and extremely expensive, such prospective cohort studies avoid many of the pitfalls of passive surveillance, and they allow interpretations about both infection and disease incidence.

Less useful information is based on passive case finding. Ascertainment may be incomplete because the surveillance system is likely to identify a case only if it is easily recognizable and diagnosed by someone who decides to report it. Since such data indicate neither how many ill persons were not reported, nor how many ill persons had negative laboratory tests, the information is mostly of qualitative value; however, it may be useful in indicating trends. Despite these limitations, occasional reports do appear (29,308).

In the United States, enterovirus surveillance data are collected and analyzed by the Centers for Disease Control and Prevention. The data have been reported irregularly since the beginning of the program in 1961 (222,225). In the United States, the only notifiable enteroviral diseases are poliomyelitis and encephalitis. These are reportable by diagnosis only (e.g., encephalitis) and not by etiology (e.g., echovirus encephalitis). Such disease-based surveillance is the most accessible but the least representative of all surveillance data.

Enterovirus excretion does not necessarily imply association with disease, since most such excretion is asymptomatic. This word of caution applies particularly to developing countries where enteroviruses are ubiquitous and childhood infections commonplace and characteristically silent.

Enterovirus activity in populations may be either sporadic or epidemic, and certain enterovirus types are associated with both sporadic and epidemic disease occurrences. The reported incidence or prevalence of a given enterovirus disease may be actually and/or artifactually increased in an outbreak situation when there is a sudden focus of attention that improves diagnosis and reporting of cases, but this may also increase reporting of “noncases.” In addition, there may be a tendency for other strains to be excluded when a particular strain is predominant in a community; however, large communities with summer enteroviral disease typically support co-circulation of several different types simultaneously and in no particular pattern.

An important concept in understanding the epidemiology of the enteroviruses is variation: by serotype, by time, by geographic location, and by disease. This concept is illustrated in surveillance studies of nonpolio enterovirus infections. For example, Figure 8 summarizes the data for the years from 1970 to 1998 for coxsackievirus B3, echovirus 11, and echovirus 30 isolates in the United States collected and analyzed by the Centers for Disease Control and Prevention. These data illustrate endemic and epidemic patterns of enterovirus prevalence. The epidemic pattern, as typified by echovirus 11 and echovirus 30, is characterized by peaks in numbers of isolations followed by periods with few isolations. These peaks may be sharp (1- or 2-year) or broad (multiyear) periods of increased virus isolations. For example, during the study period there were several major epidemics of echovirus 30 in the United States: outbreaks from 1981 to 1982, 1990 to 1994, and 1997 to 1998. By contrast, endemic viruses such as coxsackievirus B3 are isolated nearly every year and in similar numbers each year. Even with endemic viruses, larger outbreaks do occasionally occur, as with coxsackievirus B3 in 1980. Similar endemic and epidemic patterns are seen for the other echoviruses and coxsackie A viruses.

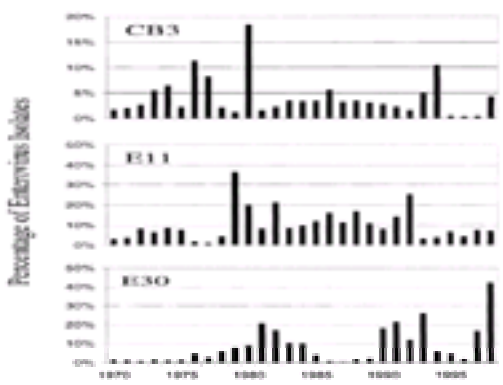

FIG. 8. Reported enterovirus isolations in the United States, 1970–1998. The graphs represent the percentage of total enterovirus isolations in a given year for each of three common serotypes, coxsackievirus B3 (CB3), echovirus 11 (E11), and echovirus 30 (E30). Note that full scale for the CB3 panel is 20%, whereas it is 50% for both of the echoviruses.

Variation by location is also a major characteristic of enteroviruses. Outbreaks can be restricted to small groups such as schools and day care centers or to selected communities, or they may become widespread at the regional, national, or even international level. Outbreaks in small groups can sometimes be linked epidemiologically to a breakdown in hygiene practices. Even during national outbreaks of a specific serotype, the location of virus activity may not be uniform. During the period of 1990 to 1993, echovirus 30 was the most commonly isolated enterovirus in the United States (Fig. 9). As can be seen in Figure 9, not all parts of the country had echovirus 30 isolates during the entire period. Some areas, such as the New England states, had extensive circulation in only one year, whereas other areas, such as the entire western United States, had extensive virus circulation for three or more of the four years. It is important to note, therefore, that

aggregate national data can obscure significant regional and local variation in viral prevalence.

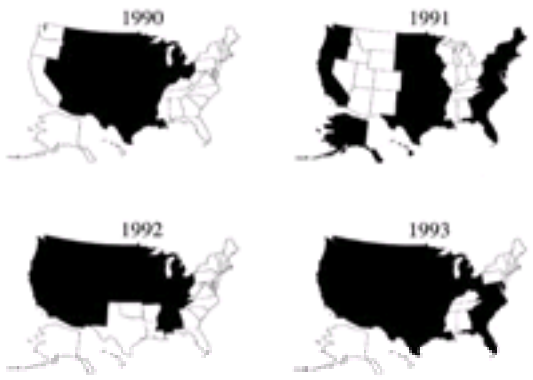

FIG. 9. Geographic distribution of echovirus 30 isolates in the United States, 1990–1993. Maps represent regions of the United States where echovirus 30 was one of the three most common enterovirus isolates for the given year. States are shaded on a regional basis, because not all states report enterovirus isolation data.

In temperate climates, enteroviruses are characteristically found during the summer and early autumn, although outbreaks may continue into the winter. In fact, naturally occurring enteroviruses have a distinct seasonal pattern of circulation that varies by geographic area; in contrast, live attenuated polioviruses (mostly vaccine strains) are isolated year round, reflecting the routine administration of poliomyelitis vaccine to children. In tropical and semitropical areas, circulation tends to be year-round or associated with the rainy season. In the United States, 10 years of surveillance indicated that 82% of enterovirus isolations were made during the five summer/fall months of June to October. In a 6-year study of viral CNS disease, 85% of enteroviral disease, compared with 12% to 26% of diseases caused by other viral agents, occurred between June and November (222).

Many studies have examined the prevalence of antibodies to the enteroviruses in specific populations (reviewed in refs. 206,220) (see later section Diagnosis), with several important conclusions. First, the number of persons who have neutralizing antibody to any given enterovirus is large, indicating a high incidence of past infection. A high incidence of recent infection is also suggested by surveys of IgM antibodies to enteroviruses, which typically show 4% to 6% positivity. Second, infections with one serotype of enterovirus can boost antibody titers to other enterovirus serotypes as measured by either IgM or neutralization. The pattern of the heterotypic response varies by serotype and among individuals. The nature of this heterotypic response has been recently explored through the identification of specific epitopes using monoclonal antibodies and peptide antisera (114,288,337). Third, the pattern of antibody prevalence by serotype varies by geographic location, by time, and by age. Thus, prevalence data from different years and locations are not directly comparable. These three points must be considered when interpreting the findings of serologic studies of associations between enterovirus infection and disease.

Enterovirus isolates are routinely characterized by serotype using polyclonal animal sera, and by this criterion, the serotypes appear to be quite stable and well defined. However, studies using monoclonal antibodies prepared against coxsackievirus B3 and coxsackievirus B4 suggest that there can be considerable antigenic variation within a serotype of coxsackievirus B (131,251). Monoclonal antibodies have been used to study coxsackievirus B strains, plaque variants of the prototypes, and clinical isolates (137,259,327). In all cases, multiple differences between strains were detected at many epitopes, although no correlation with clinical symptoms was made. This antigenic diversity among the multiple viral epitopes has so far yielded very little additional understanding about the biology of infection and pathogenesis.

Molecular Epidemiology

Study of the molecular variation of viral proteins or nucleic acid may contribute significant epidemiologic information on viral diseases (see later section Diagnosis). For the enteroviruses, and in particular poliovirus, three types of molecular studies have been used to generate epidemiologic information: analysis of antigenic variation of virion proteins using monoclonal antibodies, examination of changes in the viral genome using oligonucleotide fingerprinting, and direct analysis of genomic variation using nucleic acid sequencing.

All three approaches have been utilized in addressing the classic problem of differentiating wild from vaccine-derived poliovirus (62,92,113). The first successful test intended to differentiate between wild and vaccine viruses depended on cross-absorbed antibody. This approach was subsequently refined, and it is now possible to distinguish between wild and vaccine polioviruses using cross-absorbed rabbit IgG in an enzyme-linked immunoabsorbent assay (ELISA) (100,101).

The advent of monoclonal antibody technology provided the possibility of generating vaccine-specific antibodies. There now exists a collection of monoclonal antibodies that are capable of uniquely recognizing the Sabin vaccine strains (151). These antibodies have also been used to show changes in the antigenic properties of vaccine virus excreted by a primary vaccinee, demonstrating that the virus can become antigenically less vaccine-like (215). This antigenic drift to a less-vaccine-like virus helps explain some of the difficulties in using antigenic tests to differentiate wild from vaccine virus. It thus appears that enteroviruses may undergo continuous antigenic drift in natural host populations, although it is not known whether this occurs in response to the selection pressures of gut immunity. All studies looking at antigenic properties of enteroviruses emphasize the significant variation observable at individual epitopes despite consistent serotype stability.

At about the same time that monoclonal antibodies were first being produced, oligonucleotide mapping began to be applied to the study of poliovirus (118,237). These oligonucleotide maps appear to be characteristic for given strains of

virus and have been referred to as fingerprints. Studies of wild and vaccine polioviruses have revealed many aspects of poliovirus molecular epidemiology. First, fingerprints of poliovirus strains that are epidemiologically distinct (i.e., those that are isolated sporadically or from persons in different epidemics) are unequivocally different. Second, poliovirus genomes appear to spontaneously and randomly mutate during replication in humans, resulting in variation in fingerprint types isolated during epidemics, presumably reflecting single and multiple generations of person-to-person transmission.

Several studies using oligonucleotide fingerprinting have examined isolates of enterovirus 70. Fingerprints of isolates obtained from multiple sites worldwide during the 1981 to 1982 pandemic showed that all of the isolates were very closely related, supporting a single agent as the etiology of the global outbreak (165). Further studies demonstrated that the changes in the genome of enterovirus 70 were progressive, with increased numbers of changes with time over a 10-year period. Quantitative analysis of these fingerprint data suggested a common origin for all enterovirus 70 isolates, with an estimated date of origin of 1966 to 1967, most likely in West Africa, 2 or 3 years prior to the first known pandemic of this disease (218,311).

Both monoclonal antibodies and oligonucleotide fingerprinting are limited by their ability to show similarities and small differences only among relatively closely related viruses. However, neither technique is able to detect any patterns among seemingly unrelated virus isolates. The introduction of the technique of genomic nucleic acid sequencing and its application to the study of wild poliovirus isolates from different parts of the world has significantly extended the epidemiologic power of molecular studies (276). By analyzing the random mutations that occur in the genome of different polioviruses, closely related viruses were easily detected and, in addition, more distantly related viruses were clustered into distinct geographic groupings of endemic circulation. This approach allowed epidemiologic links to be extended beyond those identified with other techniques.

Nucleic acid sequencing technology has been most comprehensively applied to studies of poliovirus, where the information has proved valuable for supporting the global poliovirus eradication program (163,164). From these studies, it is possible to determine (a) if an isolated poliovirus is related to vaccine virus, (b) similarities among isolates in an epidemic, and (c) differences among isolates from different geographic areas. By comparing the changes that are observed between virus strains, the geographic and temporal origin of a virus can be determined. Building on a nucleic acid sequence database of poliovirus strains worldwide, it has been possible to develop rapid approaches to tracking wild poliovirus strains (9,190,230,276,351).

Studies on the molecular epidemiology of nonpolio enteroviruses have focused on the evolutionary inference derived from the comparison of virus isolates within a serotype over time, as well as the comparison of isolates from different serotypes and even between different genera within the *Picornaviridae*. Molecular epidemiologic studies using sequencing have been reported for coxsackievirus B1, coxsackievirus B5, echovirus 30, and enterovirus 71 (43,86,176,239,353). One of the studies of coxsackievirus B5 isolates examined the pattern of genetic changes over three separate outbreaks in the United States. The nucleotide sequence from multiple isolates from the epidemics showed that each of the epidemics was caused by a single genotype. The genotype of coxsackievirus B5 observed in the 1967 epidemic showed more similarity to the virus observed in the 1983 epidemic than to viruses isolated during the intervening years (176), suggesting discontinuous transmission of the epidemic coxsackievirus B5 in the United States during this time. In an analogous manner, echovirus 30 genotypes have demonstrated an overlapping succession among the isolates characterized in the United States (239). More than one genotype may be found in certain periods, and the displaced genotype can be found in other parts of the world after isolations have ceased in the United States for many years. In studies of enterovirus 71 isolates, three distinct genotypes have been characterized (43). Unlike the situation with echovirus 30 and more similar to the coxsackievirus B5 example, the transition from one genotype to another occurred during a single year, 1987, and the older genotype has not been isolated since in the United States despite isolation in other parts of the world.

CLINICAL FEATURES

Asymptomatic Infections

The great majority of enterovirus infections are asymptomatic. On the other hand, these viruses can cause a spectrum of clinically distinct syndromes when they lead to disease. Table 4,Table 5,Table 6,Table 7 and Table 8 list the prototype enterovirus strains and the illness if any in the person from whom the prototype virus was isolated. Individual serotypes generally lead to varied symptomatology and disease processes. Similarly, individual clinical disorders can generally be caused by a number of different enteroviruses (Table 9). On occasion, however, particular syndromes are associated with specific enteroviruses. For example, acute hemorrhagic conjunctivitis is usually caused by coxsackievirus A24 variant or enterovirus 70. Acute flaccid paralysis is usually caused by poliovirus or enterovirus 71. The occasional isolates from cases of diabetes are usually coxsackievirus B serotypes.

Type	Prototype strain	Geographic origin	Illness in person yielding prototype virus	Species
1	Brunhilde	Maryland	Paralytic poliomyelitis[a]	PV
2	Lansing	Michigan	Fatal paralytic poliomyelitis[b]	PV
3	Leon	California	Fatal paralytic poliomyelitis[b]	PV

[a]Recovered from feces.
[b]Was recovered from spinal cord.
PV, Poliovirus species.
Source: Adapted from et al. 2007, with permission.

TABLE 4. *Polioviruses*

Type	Prototype strain	Geographic origin	Illness in person yielding prototype virus[a]	Species
1	Tompkins	Cokeaskie, NY	Poliomyelitis[c]	HEV C
2	Fleetwood	Delaware	Poliomyelitis[c]	HEV A
3	Olson	New York	Meningitis	HEV A
4	High Point	North Carolina	Sewage of polio community	Not yet assigned
5	Swartz	New York	Poliomyelitis	HEV A
6	Schultz	New York	Meningitis	Not yet assigned
7	Parker	New York	Meningitis	HEV A
8	Donovan	New York	Poliomyelitis[c]	HEV A
9	Bozek	New York	Meningitis	HEV B
10	Kowalik	New York	Meningitis	HEV A
11	Belgium-1	Belgium	Epidemic myalgia	HEV C
12	Texas-12	Texas	Fever or polio-similarity	HEV A
13	Flores	Mexico	None	HEV C
14	G-14	South Africa	None	HEV A
15	G-9	South Africa	None	HEV C
16	G-10	South Africa	None	HEV A
17	G-12	South Africa	None	HEV C
18	G-13	South Africa	None	HEV C
19	NIH 8659	Japan	Guillain-Barré syndrome	HEV C
20	9A-28	New York	Infectious hepatitis	HEV C
21	Kuykendall, Cox	California	Poliomyelitis,[c] mild respiratory illnesses[d]	HEV C
22	Chulman	New York	Vomiting and diarrhea	HEV C
23	Senne ex echovirus 9			HEV B
24	Joseph	South Africa	None	HEV C

[a]Cross-reactivity has been observed between A2 and A8, A11 and A13, and A12 and A18; QN-19 antiserum partially neutralizes coxsackievirus A24. But A24 antiserum does not neutralize CN-19 virus, although it reacts with the virus in complement fixation and gel diffusion tests.

[b]All isolates were from stools, except for prototypes of A4 and A12, which were isolated from sewage and flies, as indicated. Numerous strains of each of these types were isolated from stools also.

[c]When coxsackieviruses have been isolated from patients with paralytic poliomyelitis, the patient has often been found to have a dual infection, the poliomyelitis presumably being responsible for the paralytic illness.

[d]The Colo virus was isolated from throat washings.

HEV-A, human enterovirus A; HEV-B, human enterovirus B; HEV-C, human enterovirus C; species.

Source: Adapted from ref. 207, with permission.

TABLE 5. *Group A coxsackieviruses*[a]

Type	Prototype strain	Geographic origin	Illness in person yielding prototype virus	Species
1	Conn-5	Connecticut	Meningitis	HEV B
2	Ohio-1	Ohio	Summer grippe	HEV B
3	Nancy	Connecticut	Minor febrile illness	HEV B
4	J.V.B.	New York	Chest and abdominal pain	HEV B
5	Faulkner	Kentucky	Mild paralytic disease with residual atrophy	HEV B
6	Schmidt	Philippine Islands	None	HEV B

[a]All isolates were from stools.

HEV-B, human enterovirus B.

Source: Adapted from ref. 207, with permission.

TABLE 6. *Group B coxsackieviruses*[a]

Type	Prototype strain	Geographic origin	Illness in person yielding prototype virus	Species
1	Farouk	Egypt	None	HEV B
2	Cornelis	Connecticut	Meningitis	HEV B
3	Morrissey	Connecticut	Meningitis	HEV B
4	Pesanochi	Connecticut	Meningitis	HEV B
5	Noyce	Illinois	Meningitis	HEV B
6	D'Amori	Rhode Island	Meningitis	HEV B
7	Lee	Ohio	None	HEV B
8	Burgess	Connecticut	Meningitis	HEV B
9	Wallace	Ohio	None	HEV B
10	Bryson	Ohio	None	HEV B
11	n/d	Ohio	None	HEV B
12	Gregory	Ohio	None	HEV B
13	Travis	Philippine Islands	None	HEV B
14	Del Carmen	Philippine Islands	None	HEV B
15	Trial	Rhode Island	Meningitis	HEV B
16	CH 30-24	West Virginia	None	HEV B
17	Harrington	Massachusetts	Meningitis	HEV B
18	CHHC-29	Mexico City	None	HEV B
19	Metcalf	Ohio	Diarrhea	HEV B
20	Re/Na	Ohio	Diarrhea	HEV B
21	Au-1	Washington, DC	Fever	HEV B
22	Faetna	Massachusetts	Meningitis	HEV B
23	Dalldorp	Ohio	Diarrhea	HEV B
24	Au-4	Washington, DC	Diarrhea	HEV B
25	Conmel	Philippine Islands	None	HEV B
26	Wood	Philippine Islands	None	HEV B
27	Au-10	Washington, DC	None	HEV B
28	Beaumont	New York	Meningitis	HEV B
29	Cydezell	Kansas	Meningitis	HEV B
30	PR-10	Puerto Rico	Meningitis	HEV B
31	Toluca-3	Mexico	None	HEV B

[a]Types 1 and 9 share antigens; type 1 having the broader spectrum. Type 10 was soon excluded from this group. It turned out to be a simple RNA virus and was reclassified as a prototype reovirus. Type 28 was reclassified as rhinovirus type 1. Type 22 and 23 have been reclassified as members of the genus Parechovirus and are named parechovirus 1 and 2. These viruses share only superficial virus replicative traits with members of this new genus. Type 24, Dn-19, is now considered a prime strain of coxsackievirus A24, rather than a distinct prototype.

TNI isolates were from stools.

HEV-B, human enterovirus B.

Source: Adapted from ref. 207, with permission.

TABLE 7. *Echoviruses*[a]

Type	Prototype strain	Geographic origin	Illness in person yielding prototype virus	Species
68	Fermon	California	Lower respiratory illness (pneumonia and bronchiolitis)	HEV D
69	Toluca-1	Mexico	None	HEV B
70	J670/71	Japan and Singapore	Acute hemorrhagic conjunctivitis	HEV D
71	BrCr	California	Meningitis[a]	HEV A
72	(Hepatitis A virus, provisionally identified as type 72, but now classified as prototype of a distinct picornavirus genus)			

[a]An identical strain was isolated from the brain of a fatal encephalitis case in the same local outbreak of central nervous system disease. Strains of the same serotype have since been isolated from patients during epidemics of poliomyelitis, meningitis, and hand-foot-and-mouth disease (see Melnick [205]).

HEV-A, human enterovirus A; HEV-B, human enterovirus B; HEV-D, human enterovirus D.

Source: Adapted from ref. 207, with permission.

TABLE 8. *High-numbered enterovirus types*

TABLE 9. *Clinical syndromes associated with infections by enteroviruses*

Poliomyelitis

As is true for nonpolio enteroviruses, infection of the vast majority of patients with poliovirus does not result in disease or lead to any symptomatology. The most common symptomatic disease caused by poliovirus, known as abortive poliomyelitis, is a mild febrile illness with gastrointestinal signs that occurs in 4% to 8% of individuals. The incubation period from infection to the onset of abortive poliomyelitis is usually 1 to 3 days, although symptoms may be seen as late as 5 days after infection. Less frequently, poliovirus infection results in aseptic meningitis. This nonparalytic illness has the typical features of viral meningitis, with fever, headache, and meningeal signs but an absence of signs of CNS parenchymal involvement. The meningitis has a self-limited course and lasts for a few days to 2 weeks. Only 1 in 200 poliovirus infections in a fully susceptible population results in the paralytic disease known as poliomyelitis. The incubation period from infection to the onset of paralysis is usually 4 to 10 days, although it can be as short as 3 days or longer than a month. The paralysis generally occurs 2 to 5 days after headaches and peaks within a few days. There is usually a prodrome with sensory complaints and shooting or aching pains in muscle. The muscle pains may reflect growth of the virus in this tissue, which is known to occur (269). In children, one can see a biphasic or “dromedary” course of neurologic involvement, and paralysis can occur as the initial symptom.

Paralysis is classified as either spinal or bulbar depending on whether the spinal cord or brainstem, respectively, is involved. Not infrequently, the spinal form becomes associated with the bulbar form during the course of the disease, resulting in so-called bulbospinal polio. Spinal polio is usually asymmetric, flaccid, and limited to the extremities and trunk, and it varies from a mild weakness to a complete quadriplegia. Only about 10% to 15% of poliomyelitis cases are bulbar, a term indicating involvement of the motor cranial nerves or medullary centers controlling respiration and the vasomotor system. The most frequent cranial nerves affected are IX and X, leading to paralysis of the pharyngeal and laryngeal muscles with resultant difficulty swallowing and talking. Involvement of other cranial nerves may lead to weakness of the face (VII) and tongue (XII). Most dreaded is involvement of the brainstem reticular formation, resulting in respiratory compromise, potentially requiring ventilatory support. One can see evidence of autonomic involvement, manifest as abnormalities of sweating, urination, defecation, and blood pressure control. Recovery can be very delayed. Not infrequently, one extremity is left severely weak and atrophic, with a relatively normal contralateral limb.

The pathology of poliomyelitis is one of inflammation and destruction of the gray matter of the CNS, especially of the spinal cord. Motor neurons up and down the neuraxis can be infected by poliovirus, including “upper” motor neurons located rostrally in the brainstem and cerebral hemispheres in addition to the anterior horn “lower” motor neurons in the spinal cord. The widespread nature of the gray matter infection demonstrates that the disease is frequently a polioencephalomyelitis (i.e., inflammation of the gray matter of the brain and spinal cord) rather than merely a poliomyelitis (inflammation of the gray matter of the spinal cord). Interestingly, the perivascular mononuclear inflammatory cells can persist for months, although virus is difficult to culture from the spinal cord after a week (36). Although the focus of pathology in the spinal cord is in the anterior horn, there are also abnormalities outside the motor system in the posterior horn and intermediolateral column. Similarly, the brainstem shows involvement of a number of sensory cranial nerve nuclei and of the reticular formation in addition to the motor cranial nerve nuclei. Neurons die with evidence of chromatolysis followed by neuronophagia. Recent investigations of PVR transgenic mice have demonstrated that spinal cord neurons die by apoptosis (99); studies in cell culture have also shown evidence of apoptosis of cells following poliovirus infection (10).

Poliomyelitis or acute flaccid paralysis may occur as a result of infection with other enteroviruses besides poliovirus. Enterovirus 71 is emerging as the most important virulent neurotropic enterovirus during the poliomyelitis eradication period. This virus causes epidemics of poliomyelitis-type disease, including bulbar disease (57). In an enterovirus 71 epidemic in Bulgaria in 1975, a paralytic disease occurred in as many as 21% of approximately 700 patients, with a case fatality rate approaching 30% (303). Enterovirus 71 is also associated with epidemic encephalitis and meningitis (see later section Meningitis and Encephalitis). Enterovirus 70, a cause of epidemics of acute hemorrhagic conjunctivitis, can lead to a severe and acute paralytic disease (325). The incidence of paralysis is probably 1 in 10,000 infections (153). These patients can also have cranial nerve palsies, autonomic abnormalities, and sensory signs. Sometimes, enterovirus 70 infections may cause isolated cranial nerve palsies, most commonly involving the facial nerve. The eye disease is usually spread by direct or indirect contamination of the eye rather than the fecal-oral route. Enteroviruses 71 and 70 can both produce paralytic disease in monkeys (153), although this is not a consistent finding for all isolates. Some echoviruses and coxsackieviruses have also been associated with acute flaccid paralysis. For example, coxsackievirus A7 virus has been reported to cause outbreaks of paralysis (108).

Postpolio Syndrome and Amyotrophic Lateral Sclerosis

Patients with postpolio syndrome complain of new weakness, fatigue, and pain decades after paralytic poliomyelitis. One

subgroup of this syndrome, called postpoliomyelitis progressive muscular atrophy, is an uncommon primary neurologic disorder manifested by slowly progressive atrophy of muscles with evidence of on-going motor nerve damage (47). Some investigators have reported a persistent poliovirus infection in the spinal fluid or CNS tissue from postpoliomyelitis progressive muscular atrophy patients (183,184,227,253,300), but others have failed to confirm these findings (156,203,287). One of the groups that originally reported enterovirus genome in postpolio syndrome patients later claimed that both patients and controls had evidence of enteroviral genome in the CNS (228). Although the poliovirus genome of a mouse-adapted mutant poliovirus has been reported to persist at low levels following experimental infection of mice (81), the only consistent evidence for persistent poliovirus infection in humans has been found in immunocompromised individuals (see previous section Pathogenesis, and the next section Meningitis and Encephalitis). We await more convincing data demonstrating that a persistent infection underlies the postpolio syndrome.

The issue of whether poliovirus can persist in postpoliomyelitis progressive muscular atrophy has raised questions about whether poliovirus or another enterovirus is involved in amyotrophic lateral sclerosis, a chronic progressive noninflammatory disease of unknown etiology associated with the death of motor neurons. A 1994 study reported evidence of enteroviral sequences in spinal cord tissue from patients with motor neuron disease (334). One later study was generally negative (274, but a recent investigation using reverse transcriptase polymerase chain reaction and reverse transcriptase *in situ* hybridization with primers from the 5'-untranslated region reported finding enterovirus sequences resembling echovirus 6 and 7 in CNS tissue from 13 of 17 amyotrophic lateral sclerosis patients (and a low percentage of controls) (32). Although a role for enteroviruses in amyotrophic lateral sclerosis seems unlikely given the immunocompetence of these patients and the noninflammatory pathology of the disease, it is clear that further studies are indicated using blinded samples and probes from throughout the enteroviral genome.

Meningitis and Encephalitis

Aseptic meningitis is a nonbacterial inflammation of the meninges associated with fever, headache, photophobia, and meningeal signs in the absence of signs of brain parenchymal involvement (284). The syndrome is the most common CNS infection, with 7,000 cases of aseptic meningitis reported per year in the United States and an actual incidence believed to be 10-fold higher (283). In one survey from Finland, aseptic meningitis occurred in 219 of 100,000 children under 1 year of age, and in 19 of 100,000 children aged 1 to 4 years, demonstrating a decreased incidence with increase in age (266).

Enteroviruses are the main recognized cause of aseptic meningitis in both children and adults in developed countries, and enteroviruses were identified in 85% to 95% of cases in which a specific pathogen was cultured (284). In one study (158), 62% of infants less than 3 months old with aseptic meningitis had coxsackievirus group B as the etiologic agent. In addition, aseptic meningitis is the most common clinical syndrome caused by enteroviruses that results in medical attention.

Enteroviruses should be suspected as the causative agent for aseptic meningitis occurring in the summer and fall in temperate zones. The seasonal increase in incidence of enteroviral aseptic meningitis may result because the fecal-oral route of transmission is facilitated in warm periods when less clothing is worn (153). Fever is common in patients with enterovirus-induced aseptic meningitis, as with most cases of aseptic meningitis. At times, the fever has a biphasic pattern, initially associated with constitutional symptoms and then returning with meningeal signs. One may see nonneurologic abnormalities associated with enteroviral meningitis, such as a rash, which may be helpful in the diagnosis and identification of the particular enteroviral serotype (153) (see later section Respiratory Infections, Herpangina, and Hand-foot-and-mouth Disease). For example, rashes have been associated with CNS disease caused by coxsackieviruses A5, A9, and A16, and echoviruses 4, 6, 9, and 16. The rash associated with echovirus 9 meningitis can be petechial, resembling meningococcemia.

Encephalitis signifies that the brain parenchyma is infected, with the possibility of a disturbed state of consciousness, focal neurologic signs, and seizures. The distinction between an aseptic meningitis and an encephalitis is an important one because the absence of parenchymal involvement in aseptic meningitis suggests a more benign condition and a more favorable prognosis. Encephalitis is usually associated with aseptic meningitis, so that one is usually dealing with a meningoencephalitis.

Patients with enterovirus encephalitis usually have a global neurologic depression in function, although one can occasionally see focal neurologic signs, resembling herpes simplex virus encephalitis (219). Cases associated with an acute cerebellar ataxia have been reported in children infected with various enteroviruses, including polioviruses, echoviruses 6 and 9, and coxsackieviruses A2 and A9 (153). Other uncommon clinical manifestations that have been reported include acute hemiplegia, opsoclonus-myoclonus, movement disorders, and Guillain-Barré syndrome (153). At times, the spinal cord rather than the brain is involved (i.e., there is a myelitis) (see preceding section Poliomyelitis).

The meningeal syndrome is usually a self-limited and benign one, with evidence of improvement in days to a week. Deaths have been reported, however, following infection with a number of enteroviruses, including coxsackievirus B1 and echoviruses 9, 17, and 21 (153). Morbidity and mortality are also increased in the neonate. The neonate's disease tends to be especially severe when the disease appears soon after birth, perhaps reflecting perinatal spread of virus to the fetus from the mother. Mortality is increased in the neonate partly because the aseptic meningitis can be associated with systemic disease, such as hepatitis and myocarditis, as well as encephalitis. The largest study of recovered patients failed to find any neurodevelopmental abnormalities above levels seen in controls (281).

Isolation of enteroviruses may be unsuccessful because a particular enterovirus may grow poorly in cell culture or because inhibitory factors, such as neutralizing antibody, may be present in the spinal fluid. With the use of reverse transcriptase polymerase chain reaction, enterovirus genome may be found in a significant number of culture-negative spinal fluid samples from cases of aseptic meningitis. Using reverse transcriptase–polymerase chain reaction, the New York State Department of Health found evidence of the enterovirus genome in the spinal fluid from 13 patients in a series of 33 cases of CNS infection occurring from July 1997 to November 1998 (138). In another series, 23 of 39 adult patients

diagnosed with aseptic meningitis between August 15 and October 31, 1998, had evidence of enteroviral genome by reverse transcriptase–polymerase chain reaction (284). The potential usefulness of reverse transcriptase–polymerase chain reaction can be seen in a recent report of a Swiss epidemic of aseptic meningitis caused by echovirus 30, when enterovirus genome was identified by using this technique in 42 of 50 spinal fluid cultures (84%) that were negative for virus by cell culture (104). On the other hand, the use of reverse transcriptase—polymerase chain reaction may be unsuccessful because some so-called generic reverse transcriptase–polymerase chain reaction primers may fail to hybridize well to particular enteroviruses because of a difference in target sequence.

Enteroviruses appear to vary in their neurotropism and neurovirulence, and some types are overrepresented as etiologic agents of aseptic meningitis. In the past, poliovirus was a major cause of meningitis, but this infection is now rare except in association with vaccine-associated paralytic poliomyelitis. In the United States, the 15 most common enterovirus isolates make up over 80% of all the United States isolates (308). Recent enteroviruses implicated in aseptic meningitis in the United States have included coxsackievirus B5 and echoviruses 4, 6, 9, 11, and 30 (283), although from time to time most enteroviruses have been the cause of occasional cases.

Although a specific virus is not usually identified in most cases of encephalitis, and although the number of cases in which an enterovirus has been identified as the cause of encephalitis is low, enteroviruses nevertheless rank second to herpes simplex virus and comparable to arboviruses as a recognized cause of encephalitis in the United States. The New York State Department of Health found evidence of enterovirus genome by polymerase chain reaction in the spinal fluid of 3 of 41 cases of encephalitis over the period from July 1997 to November 1998 (138); herpes simplex virus genome and arbovirus genome were each found in five cases.

In some areas of the world, epidemics of enterovirus 71 (57, 303) have been associated with a high incidence of aseptic meningitis as well as CNS parenchymal involvement; the CNS infection has caused acute flaccid paralysis as well as a more varied clinical symptomatology (204). As noted previously, enterovirus 71 is emerging as the most significant neurotropic enterovirus in some areas of the world. This virus circulates in the United States, and 26% of adults tested in a seroepidemiologic study in New York in 1972 had antibody (79). An epidemic of enterovirus 71 infection in 1998 in Taiwan involved frequent CNS disease and 129,106 reports of hand-foot-and-mouth disease or herpangina (see later section Respiratory Infections, Herpangina, and Hand-foot-and-mouth Disease), which may correspond to 1 million actual cases of enterovirus infection (126). The hand-foot-and-mouth disease patients had vesicular lesions on their hands, feet, mouth, and at times buttocks, whereas the patients with herpangina had vesicular lesions of the palate and pharynx. Of special concern was the finding of 405 severe cases with associated complications, 78 of which were fatal. Among the more severe cases, the case fatality rate in different areas ranged from 7.7% to 31.0%. Most of the hospitalized cases (80%) and the deaths (91%) were in children younger than 5 years of age. The most frequent complication was encephalitis. Other serious complications, which were associated at times with encephalitis, included aseptic meningitis, myocarditis, and pulmonary edema and hemorrhage. It is unclear whether the pulmonary signs were related to viral invasion of the lungs or to brain injury from the viral encephalitis.

A recent review (139) of 41 hospitalized children with neurologic complications and culture-confirmed enterovirus 71 infection during the Taiwan epidemic in 1998 demonstrated an unusual and rather distinctive neurologic manifestation, brainstem encephalitis or rhombencephalitis. The mean age of the 41 children was 2.5 years (with a range from 3 months to 8.2 years). There was frequently an associated condition: hand-foot-and-mouth disease in 68%, or herpangina in 15%. The neurologic disease usually followed the initial illness of fever and skin or mucosal lesions, and it was manifested as rhombencephalitis (37 patients), aseptic meningitis (3 patients), and acute flaccid paralysis (associated with the disease in 4 patients, and following rhombencephalitis in 3 of the 4). At times, breathing difficulties and coma were seen in the rhombencephalitis patients, progressing to death in five of them. The brainstem and spinal cord pathology was presumably related to direct viral injury, as virus was cultured from the spinal cord and brainstem of the one patient who underwent autopsy. Five of the patients who survived had neurologic sequelae. Enterovirus 71 brainstem involvement was also seen during an epidemic in Malaysia in 1997, but not in earlier epidemics. In contrast, hand-foot-and-mouth disease and herpangina did not occur during the previous epidemics in Bulgaria and Hungary. It is not clear whether the changes in enterovirus 71 disease phenotype represent the emergence (or reemergence) of a more virulent strain, or whether it is related to the serologic status of the at-risk populations.

There are likely to be epidemiologic, host genetic, and viral genetic factors that determine which enteroviruses are more neurotrophic than others. For example, some epidemics of aseptic meningitis may be caused by infection of a seronegative population with a particular rare enterovirus. Host factors in neural cells may also affect the replication or translation of some enteroviruses differently from other enteroviruses. There may be determinants in the viral genome that affect neurovirulence (see previous section Pathogenesis).

The presence of immunodeficiency predisposes to a syndrome of enterovirus-induced aseptic meningitis or meningoencephalitis, at times producing a persistent CNS infection. McKinney and colleagues (201) reviewed over 40 cases of chronic enteroviral meningoencephalitis in patients with congenital immunodeficiencies, most commonly X-linked agammaglobulinemia. Clinical features were remarkably varied and included headaches, seizures, ataxia, a disturbed state of consciousness, motor deficits, personality changes with cognitive decline, and sensory disturbances. At times, patients had other associated abnormalities, such as a dermatomyositis syndrome (in 21 of 41 patients; see later section Muscle Disease), rashes (16 of 35 cases), edema (20 of 40 cases), and hepatitis (15 of 32 patients). Although some cases started abruptly, most tended to be slowly progressive, at times lasting years, but with a not infrequent fatal outcome. Most patients had a spinal fluid mononuclear cell pleocytosis with an increase in protein. Some cases at autopsy had evidence of inflammatory infiltrates in the heart, lungs, kidneys, adrenals, thyroid, and pancreas, in addition to the CNS.

In the series of McKinney and colleagues (201), 37 of 41 immunocompromised patients with an associated CNS enterovirus infection had echovirus infection, with echovirus type 11 isolated in 11 cases. Other isolates besides echovirus included single cases with coxsackievirus A11, A15, B2, and B3. The reason for the preponderance of echoviruses, especially echovirus 11, remains unclear. Some patients had evidence of more than one sequential or simultaneous enterovirus infection. A more recent study reported that spinal fluid from 18 of 27 immunocompromised

patients with an associated CNS enterovirus infection had polymerase chain reaction evidence of enterovirus genome, although enteroviruses could be cultured from only two (328). The low incidence of enterovirus isolation in this series contrasts with the high frequency (93%) of culture-positive enteroviruses noted in a previous large review. Rotbart (283) questions whether this discrepancy may be a result of the more frequent and probably earlier use of intravenous immunoglobulin as treatment for patients in the more recent study. Intravenous immunoglobulin treatment may decrease the level of infectious virus but not clear it completely. The patients from the recent series had more frequent evidence of normal spinal fluid protein and cells, suggesting that the clinical spectrum of treated disease may have changed from previous descriptions. Last, it is likely that the more frequent and early institution of intravenous immunoglobulin treatment will alter the spectrum of enteroviruses that are seen—that is, strains that are less common in the population (and not neutralized by the pools of immunoglobulin used for therapy) may more likely be present.

Cardiac Disease

Myocarditis is an inflammation of the myocardium associated with damage that is unrelated to an ischemic injury. Myocarditis is frequently self-limited and subclinical, with few if any sequelae. On the other hand, the acute disease may lead to significant morbidity and even death. On the basis of well-established criteria, one study found evidence of myocarditis in approximately 1% of autopsies (107).

In some cases, the myocardial inflammation may persist, producing a chronic myocarditis that can progress to dilated cardiomyopathy (reviewed in refs. 48,157,200,282). In dilated cardiomyopathy, the heart is large, with impaired function and evidence of heart failure but with little or no inflammation. The incidence of dilated cardiomyopathy in the United States has been reported to be 6 cases per 100,000 (59), and approximately 100,000 new cases occur each year. Patients with dilated cardiomyopathy are frequent recipients of heart transplants, making up perhaps 50% of all cardiac transplant patients. Of note is the observation that nonpolio enteroviruses, especially coxsackievirus B3, cause acute and chronic myocarditis in animals, and that a contribution from the immune system to the chronic disease has been proposed (see earlier section Pathogenesis).

Acute Cardiac Disease

Enteroviruses have been implicated as an etiologic agent of viral myocarditis in humans. It is believed that 1.5% of enteroviral infections, including 3.2% of coxsackievirus B infections, result in overt cardiac signs or symptoms (108). Myocarditis is a very frequent autopsy finding in children who die of overwhelming coxsackievirus infection (158). Coxsackievirus B5 has been implicated in cardiac disease, although other coxsackieviruses of group B or group A or echoviruses have also been identified. The peak age group in which myocarditis caused by coxsackieviruses of the B group occurs is young adults, primarily between 20 and 39, with a higher prevalence among men (335).

A summary of data from varied sources showed that the prevalence of enteroviral infection in acute myocarditis on the basis of serologic studies is 34% (214 positives in 636 patients) compared to 4% (44 in 1,139) of controls (199). However, there are a number of potential shortcomings to these studies. The serologic studies demonstrating seroconversion with an enterovirus do not prove that enteroviruses are involved in the pathogenesis of cardiomyopathy, as they may result merely from an unrelated infection (see later section Differential and Presumptive Diagnosis). In addition, there is evidence that multiple enteroviruses can circulate at the time of an epidemic, so that isolation of an enterovirus does not prove its role in a disease; for example, more than 10 enteroviruses were recovered from the community during an echovirus 9 epidemic (153).

A metaanalysis of data obtained from molecular studies (slot blot hybridization, *in situ* hybridization, and reverse transcriptase–polymerase chain reaction) published in 12 reports found 23% of cases (68/289) and 6% of controls (14/216) had evidence of enterovirus genome in heart tissue, giving an odds ratio of 4.4 with a 95% confidence interval of 2.4 to 8.2 (24). These data, coupled to reports of positive virus isolations from the heart, indicate that enteroviruses may represent a common cause of acute myocarditis. The surprisingly large number of positive results in controls is presumed to result from difficulties in obtaining appropriately controlled heart tissue.

Chronic Cardiac Disease

Human dilated cardiomyopathy has multiple causes, both inherited and sporadic. A prior history of enteroviral infection of the heart, especially with coxsackievirus B, has been implicated in sporadic human dilated cardiomyopathy (23,308). A summary of serologic data from multiple sources found that the prevalence of enteroviruses in dilated cardiomyopathy was 24% (64/260) compared to 10% of controls (26/255) (199). These serologic data suffer from the same drawbacks that were just noted.

In contrast to acute myocarditis, there are no reports of isolation of enteroviruses from chronic dilated cardiomyopathy, suggesting that the virus may have a restricted expression or disappear following the acute infection. These possibilities led to studies probing affected tissues for persistent enteroviral genome. A metaanalysis of data from molecular studies described in 17 published reports found 23% of the patients and 7% of controls had evidence of enteroviral genome, giving an odds ratio of 3.8 with a 95% confidence interval of 2.1 to 4.6 (24). The metaanalysis data are suggestive of an association between enterovirus infection and chronic cardiac disease; however, it remains a possibility that RNA from other laboratory-based enteroviral studies contaminated test samples in the highly sensitive polymerase chain reaction studies. Most of the hybridization and polymerase chain reaction studies did not include sequencing of the viral genome, and therefore failed to more directly prove that an enterovirus was involved. In a few cases, however, there was partial sequencing of the RNA found in clinical samples; for example, Archard et al. (19) identified the amplified sequence as coxsackievirus B.

Not all the viral genomic studies have been positive. One recent investigation involving a nested polymerase chain reaction failed to find evidence of enterovirus RNA in 287 heart biopsy specimens from 38 patients with dilated cardiomyopathy and 39 patients with heart failure of unknown cause (74). There are at least two other studies that have

also been negative (187,298). The lack of consistent reproducible results regarding the presence of enteroviral genome in tissue from patients with cardiomyopathy indicates that further studies are needed under careful, blinded conditions.

Arrhythmogenic right ventricular dysplasia/cardiomyopathy is a variation of dilated cardiomyopathy in which there is fatty or fibrous tissue replacement of the right ventricular myocardium. About 65% of cases have evidence of inflammatory infiltrates (27). A recent reverse transcriptase–polymerase chain reaction investigation reported that heart tissue from three of eight patients had evidence of a slightly varying RNA sequence that was most similar to coxsackievirus B2 (112). At the same time, these investigators found a positive result by reverse transcriptase–polymerase chain reaction in 7 of 23 patients with myocarditis or dilated cardiomyopathy (and in none of five patients with noninfectious cardiomyopathy). Further studies of this disease process are indicated.

Muscle Disease Including Pleurodynia

The relationship of enteroviruses to inflammatory muscle diseases was initially recognized because of the myotropism of coxsackieviruses in suckling mice. This observation was fueled by the association of these viruses with epidemic pleurodynia on the Danish island of Bornholm. The latter disease, called Bornholm disease, has occurred as an epidemic and also sporadically in various locales. Bornholm disease is an acute febrile illness with myalgia, especially involving the chest and abdomen, but without muscle weakness. Relapses may occur. Coxsackievirus B3 and B5 are the most frequently recognized etiologic agents, although other enteroviruses have also been isolated (347). The limited information from muscle biopsy findings suggests that the inflammation in this disease may be confined to the endomysial part of the muscle and is therefore not a true myositis (67).

Inflammatory muscle diseases can be acute or chronic. Acute inflammatory muscle diseases are generally called acute polymyositis or acute myositis. These acute diseases are characterized by fever with myalgia, elevated muscle enzymes, and, at times, myoglobinuria. Chronic inflammatory muscle diseases are primary inflammatory diseases of the muscle that are generally classified as polymyositis, dermatomyositis, or inclusion body myositis. These diseases cause a subacute to chronic progressive weakness with a distinctive pathology on muscle biopsy. Dermatomyositis is distinguished from the other two inflammatory myopathies because it is associated with a characteristic rash.

The causes of chronic inflammatory myopathy are generally unknown. Hypotheses concerning the etiology of polymyositis and dermatomyositis include a direct virus infection, especially an enterovirus infection, or an autoimmune process in which the virus infection triggers a reaction against muscle (see earlier section Pathogenesis). The fact that coxsackieviruses cause acute and chronic inflammatory myopathy in experimental animals provides additional support for their involvement in human inflammatory muscle diseases. Investigations of this model system may help clarify the pathogenesis of these diseases in humans.

Enteroviruses have been implicated in polymyositis on the basis of serologic and electron microscopic studies (66). The findings from serologic studies have varied interpretations, however, and may simply reflect an intercurrent enterovirus infection that is unrelated to the acute myositis. Similarly, ribosomes or glycogen have an ultrastructural appearance that resembles an enterovirus, and therefore electron micrographs implicating enteroviruses may have been misinterpreted.

Enteroviruses have been isolated from cases of inflammatory myopathies, but these isolations have generally been rare and from single cases with acute (33) or atypical clinical pictures (315). The inability to isolate virus from cases of chronic inflammatory myopathy has suggested that there is a restricted expression of the virus with little infectious virus present. For this reason, investigators have probed muscle tissue from patients with inflammatory myopathy for picornaviral genome. Some reports involving slot blot or *in situ* hybridization studies of muscle tissues from patients with inflammatory myopathies have shown positive results using enterovirus-specific probes (346). In some cases, the product amplified in a reverse transcriptase–polymerase chain reaction has been identified as coxsackievirus group B sequence (20). In contrast, other studies using reverse transcriptase–polymerase chain reaction and nucleic acid hybridization have been negative (125,181,182,248). It remains a possibility that an enterovirus could trigger an autoimmune inflammatory muscle disease and then disappear. Additional studies firmly indicating enteroviral involvement in the pathogenesis of inflammatory muscle disease are needed. Studies regarding the possible role of enteroviruses in chronic fatigue syndrome have similarly failed to find reproducible evidence of enterovirus involvement (reviewed in ref. 67).

Of special interest in the issue of enterovirus involvement in inflammatory muscle disease is the observation that patients with immunodeficient states can manifest a dermatomyositis-like disease with an accompanying persistent echovirus infection (326,332). It should be noted, however, that questions have been raised as to whether these patients had a true myositis (i.e., inflammation of the muscle) or a fasciitis with interstitial inflammation in the endomysium (67). The inflammatory muscle disease is associated with a chronic encephalomyelitis as well as a more disseminated disease in which enteroviruses, especially echoviruses, can be cultured from the spinal fluid (see previous section Meningitis and Encephalitis) (352). Although virus has been isolated on occasions from affected muscle, it remains unclear whether the dermatomyositis syndrome in immunodeficient patients is a result of direct invasion of the muscle by the virus or an immune-mediated disease associated with virus persistence. The existence of this dermatomyositis-like syndrome demonstrates that echoviruses, and perhaps other enteroviruses, may be able to produce a chronic myositis in humans that is associated with a persistent infection and chronic inflammation.

Diabetes

Both genetic and environmental factors, including enterovirus infection, have been implicated in the etiology of insulin-dependent diabetes mellitus. A number of epidemiologic and serologic studies have demonstrated a relationship between enterovirus infection and the development of diabetes. D'Allessio (69) found that 15 of 84 cases (17.8%) of newly diagnosed insulin-dependent diabetes mellitus had evidence of IgM antibody against coxsackievirus B, compared to 5 of 71 controls (7.0%). The individuals with IgM antibody were especially common in association with HLA-DR3 positivity. A recent prospective study in Finland found a greater incidence of serologic conversion of enterovirus antibodies among children who developed insulin-dependent diabetes mellitus than among those who did not (147). The

antibody present in the prediabetic period was directed against a number of different enterovirus serotypes, including coxsackievirus A9, B1, B2, B3, and B5 (279).

There have been rare but well-documented isolations of coxsackievirus B4 from the pancreas of patients with acute-onset as well as from fatal cases of insulin-dependent diabetes mellitus (265,271). Some of the isolates have been demonstrated to be diabetogenic when inoculated into certain mouse strains (342) and nonhuman primates (343). In addition, patients dying from coxsackieviral myocarditis can have an associated pancreatitis including islitis. More recent investigations have involved probing tissues from patients for evidence of enterovirus genome. Andreoletti et al. (13) reported that peripheral blood mononuclear cells from 42% of newly diagnosed patients with insulin-dependent diabetes mellitus, and from no controls, had evidence of enteroviral genome with a sequence similar to that of coxsackievirus B3 and 4; this study clearly needs to be reproduced. Although it is likely that enteroviruses are capable of causing diabetes mellitus in animals and humans, it remains unclear how often this occurs in humans, and what if any immune mechanisms are involved.

Eye Infections

Acute hemorrhagic conjunctivitis is characterized by a short incubation period of 24 to 48 hours preceding a rapid onset of uniocular or binocular symptoms and signs. Patients manifest excessive lacrimation, pain, periorbital swelling, and redness of the conjunctiva (from subconjunctival petechiae to frank hemorrhages) (340). Keratitis with accompanying pain and possible visual impairment as well as anterior uveitis may be seen. The epidemic disease can also be associated with nonophthalmic symptoms and signs, such as neurologic dysfunction (see previous section Poliomyelitis) and respiratory and gastrointestinal disturbances. The disease usually resolves without sequelae in 1 to 2 weeks.

The first pandemic of acute hemorrhagic conjunctivitis was recognized in 1969 in Africa. Within 2 years, two enteroviruses, a new antigenic variant of coxsackievirus A24 and a previously unknown enterovirus serotype designated enterovirus 70, were implicated as etiologic agents responsible for the epidemics of this disease (189). During the first pandemic from 1969 to 1971, millions of people were likely to have been infected. Subsequent epidemics of acute hemorrhagic conjunctivitis have continued in various locations throughout the world. Other enteroviruses have also been recognized as causes of acute hemorrhagic conjunctivitis (e.g., echovirus type 7) (289) and as causes of sporadic conjunctivitis and keratoconjunctivitis (161). The ocular disease caused by enteroviruses is often indistinguishable from acute hemorrhagic conjunctivitis caused by various adenovirus serotypes.

Respiratory Infections, Herpangina, and Hand-Foot-and-Mouth Disease

Enteroviruses are a common cause of respiratory illnesses. A recent review notes that enteroviruses were isolated in 1.7% of 3,119 respiratory specimens submitted for viral culture from 1983 to 1994 (54), and in 6.4% of all the viruses that were isolated from these specimens (54/838). Respiratory illness occurred in 15% of enterovirus infections collected by the World Health Organization from 1967 to 1974 (108) and 21% of cases from the Centers for Disease Control obtained from 1970 to 1979 (16). The enteroviruses implicated in these respiratory infections included coxsackie type A viruses (12.4%), coxsackievirus type B (20.3%), and echoviruses (12.6%) (108).

Enteroviral respiratory infections are more frequently associated with upper respiratory infections, such as the common cold, croup, and epiglottitis, than lower respiratory infections, such as pneumonia. The infections are frequently subclinical, but if they cause clinical disease it tends to be self-limited and mild, with a short incubation period of 1 to 3 days. More severe lower respiratory infections may be related to a higher dose of virus in the inoculum. In a series of infants with coxsackievirus B infections, 30 of 77 patients had marked abnormalities (e.g., interstitial inflammation and hemorrhage), and 12 had virus isolated from the lung (158).

Herpangina is a febrile illness of relatively sudden onset with complaints of fever and sore throat. There are characteristic lesions on the anterior tonsillar pillars, soft palate, uvula, tonsils, and posterior pharynx. The illness, which has a predilection for the young, is usually self-limited and disappears within a few days. At times, the disease is associated with more significant clinical abnormalities such as meningitis. Hand-foot-and-mouth disease is an illness associated with vesicular lesions of the hands, feet, mouth, and, at times, buttocks.

A number of enteroviruses have been identified as causes for herpangina, including coxsackievirus A and B serotypes, echovirus types 6, 9, 11, 16, 17, 22, and 25 (54), and enterovirus 71. The main causes of hand-foot-and-mouth disease are coxsackieviruses A10 and A16 and enterovirus 71 (108,222). The pathogenesis of the lesions seen in herpangina is not clear; however, experimental infection of rhesus monkeys with coxsackievirus A4 may provide a model system for the study of the pathogenesis of herpangina. In this system, ingestion of the virus by the oral route leads to multiplication in the lower gastrointestinal tract, followed by viremia and then multiplication in the oropharynx (305).

Neonate and Infant Disease

Neonates are at increased risk from enteroviral infections. This increased susceptibility is present in humans as well as experimental animals infected with varied enteroviruses (55).

The increased risk of neonates to enterovirus infection was apparent in the prevaccine era with respect to poliovirus. Although the incidence of paralytic disease among neonates approached 40% born to mothers with poliomyelitis at the time of delivery, the overall incidence of neonatal poliomyelitis was an infrequent occurrence, perhaps related to the protective effect of maternal antibody (336). Neonatal poliomyelitis generally had a shorter incubation period and a higher case fatality rate than found with disease later in life, demonstrating the increased susceptibility of immature hosts to enterovirus infection. Infection of the mother early in gestation was associated with an increased risk of abortion, stillbirth, and prematurity (55).

Nonpolio enteroviruses are a not infrequent cause of infection in neonates and infants. In a series from the Centers for

Disease Control, enterovirus isolates from patients less than 2 months old included echoviruses (51%), coxsackievirus B (45%), and coxsackievirus A (4%) (224). Echovirus serotypes included 4, 9, 11, 17 to 20, 22, and 31 (201).

The most frequent presentation in the neonate infected with nonpolio enteroviruses is asymptomatic disease (152). Symptomatic cases most commonly manifest a self-contained febrile illness, at times associated with irritability and nonspecific signs of infection, making enterovirus infection a leading cause of fever in the infant. A rash of variable character is seen in over 30% of cases (5). More serious infections can be seen, with the peak of symptoms correlating with viremia (65). Nonpolio enteroviruses are the most frequently identified cause of aseptic meningitis in infants less than a month old, and are believed responsible for more than a third of these cases (302). In a small series of patients with pneumonia in the first month of life, enteroviruses were implicated in 15% of cases (6/40) (6). There is usually a history of fever or respiratory symptoms in the mother a week prior to the delivery, although mothers can be asymptomatic or have a more severe disease.

One of the serious infections caused by enteroviruses is a sepsis-like disease. In one series, enteroviruses were implicated in 65% of infants less than 3 months old admitted for suspected sepsis (64). In another, more recent series, there was evidence of enteroviral genome in 80 of 345 infants less than 90 days old admitted to a medical center with suspected sepsis (45). A number of other studies have yielded similar results, emphasizing the importance of enteroviruses as a cause for a sepsis-like syndrome among neonates and infants (65)—in fact, enteroviruses tend to be a more common cause of this syndrome than bacteria in the summer and fall (45). There may be extensive multiorgan involvement in severely affected cases, especially ones that go on to a fatal outcome; the organs affected include liver, lung, heart, pancreas, and brain.

The incidence and severity of enterovirus infections among infants can be better appreciated by reviewing a study conducted in Nassau County, New York, from 1970 to 1979 (158). Out of 153,250 live births, 77 infants younger than 3 months required hospitalization for group B coxsackievirus infection. These 77 cases were from a pool of 602 infants who tested positive for group B coxsackievirus, demonstrating the high frequency of group B coxsackievirus infection in infants. The attack rate might have been even higher than was found, because the positive samples from this study were from only a limited number of infants hospitalized in the community. Twenty-four mothers had evidence of a viral-like infection occurring within a period from 10 days prior to delivery to 5 days after delivery. The most common syndrome that was seen was aseptic meningitis. Some of the infections in the infants were very serious, as evidenced by the finding that eight children died from overwhelming group B coxsackievirus infection. All but one of these eight had evidence of myocarditis.

Severe enterovirus disease in the young is associated with an early age of illness, prematurity, a more severe illness in the mother, multiorgan disease, low socioeconomic status, bottle-feeding, specific enterovirus types, and an absence of neutralizing antibody to the pathogen (5). The level of maternal neutralizing antibody appears to be important both for determining the risk of developing infection and for modulating the severity of disease (7). The importance of antibody in determining the outcome of disease suggested a role for administration of intravenous immunoglobulin in the treatment of neonatal infections, especially with a pool of immunoglobulin that contains a high level of neutralizing antibody to the particular virus involved (7). Long-term sequelae following neonatal myocarditis or CNS infection appear infrequent (5,283).

Nonpolio enteroviral infection may affect the fetus, inducing clinical abnormalities and causing overt disease in the neonate. There are reports of abortion and stillbirth associated with maternal enteroviral infections, although these are infrequent (5). The relationship between maternal enterovirus infection and congenital anomalies in the newborn remains unclear.

There are a number of routes by which enteroviruses can cause neonatal disease. Studies have found evidence of placentitis and viral infection in fetal tissues (5). Virus may enter the placenta or fetus from contaminated maternal blood, feces, or vaginal secretions; this may occur during pregnancy, with virus penetrating the fetal membranes, or at the time of the delivery. In addition, infection of the neonate may result from a viremia, because enteroviruses have been cultured from cord blood (154), but it is unclear how often this route of transmission occurs. Infection can also occur from virus shed by other neonates or hospital staff in the nursery. In cases involving nursery outbreaks, implementation of infection controls, such as strict hand washing and the isolation of affected patients, has a role in prevention of new cases.

DIAGNOSIS

Differential and Presumptive Diagnosis

The process of diagnosing an enterovirus infection or establishing that an enterovirus infection produced a particular clinical syndrome can be complicated and challenging. This problem results from the biology and epidemiology of enterovirus infections, as well as from limitations in current diagnostic methodologies. Although it is possible to demonstrate that a person is infected with an enterovirus, this association does not necessarily prove likely disease causation. On the other hand, a presumptive diagnosis in certain epidemiologic and clinical situations can be possible with a high degree of certainty on clinical grounds alone.

There are several related biologic properties that complicate the diagnosis of enterovirus-induced disease. The first is that most virus replication and infection typically occurs in the respiratory and gastrointestinal tract. These infections are often asymptomatic, with few if any systemic clinical signs. Since these infections are extremely common, even random sampling of healthy individuals can demonstrate enterovirus infections at substantial rates. For this reason, the simple recovery or detection of virus from certain nonsterile sites does not establish a firm linkage to disease, and it may be merely coincidental.

A second difficulty is that even when illness results from the enterovirus infection, most of the signs and symptoms are relatively generic and usually lack specificity. This is often the reason that a diagnosis of enterovirus-induced disease is

not considered until other etiologies are excluded, after which optimal specimens are often unfortunately depleted or no longer available. The collection of appropriate clinical specimens for virus isolation or detection is critical to laboratory confirmation of enterovirus infection. At times, the appropriate specimens are not obtained, even in the case of serious disease processes where enteroviruses are clearly a potential etiologic agent, such as meningitis, encephalitis, or myocarditis. The exclusive use of CNS or cardiac specimens to diagnose these conditions limits the sensitivity for detection of infection. For example, even though it is possible to detect enteroviruses in the cerebrospinal fluid from meningitis cases, it is uncommon to find virus in the cerebrospinal fluid from cases of encephalitis where there is a reasonable suspicion of enterovirus as the cause. There are some exceptions: sometimes the virus has readily been recovered from cerebrospinal fluid, such as during the recent outbreaks of fatal encephalitis associated with enterovirus 71 (14). In general, however, the specimen with the highest sensitivity for establishing an acute infection is a stool specimen, regardless of clinical presentation (217). The difficulty with virus isolation is further compounded because virus shedding from the gastrointestinal tract may stop prior to the onset of symptoms, and therefore sensitivity for virus isolation may be lower.

There are circumstances, however, where a presumptive diagnosis can be made on the basis of limited information. This is because of the pronounced seasonality of enterovirus infection in temperate latitudes and the tendency for enteroviruses to cause community outbreaks. For example, cases of aseptic meningitis occurring during the late summer and early autumn have a high probability of enterovirus etiology. In this situation, a presumptive diagnosis of an enterovirus infection could be made on the basis of signs and symptoms, exclusion of other nonviral pathogens, and evidence of characteristic cytopathology of inoculated cell cultures. Another example pertains to epidemic acute hemorrhagic conjunctivitis; in this case, the identification of an enterovirus (either coxsackievirus A24 variant or enterovirus 70) precludes further testing of specimens from other patients with similar symptoms.

Laboratory Diagnosis

Virus Isolation

Many of the detailed procedures for the established laboratory diagnosis of enterovirus infections have been described previously (106). The traditional techniques for detecting and characterizing enteroviruses rely on the time-consuming and labor-intensive procedures of viral isolation in cell culture and neutralization by reference antisera. Isolation of enteroviruses from specimens using appropriate cultured cell lines is often possible within 2 or 3 days and remains a very sensitive method for detecting these viruses. The best specimens for isolation of virus are, in order of preference, stool specimens or rectal swabs, throat swabs or washings, and cerebrospinal fluid. Throat swabs or washings and cerebrospinal fluid are most likely to yield virus isolates if they are obtained early in the acute phase of the illness. For cases of acute hemorrhagic conjunctivitis, the best specimens are conjunctival swabs (340), although occasionally virus can be isolated from tears (341). Since the major pandemic in 1981, however, isolation of enterovirus 70 from patients with acute hemorrhagic conjunctivitis has been very difficult, and molecular methods provide the only sensitive method of detection for this agent (304).

With the accelerating efforts to eradicate poliovirus, virologic surveillance has become a critical part of the program (144,254). For this reason, specimens of good quality are important for confirmation of the diagnosis of suspected poliomyelitis. Isolation of the virus also makes it possible to determine whether the virus is wild type or vaccine related. This information is important to public health officials in areas considered to be free of wild poliovirus, because decisions must be made as to whether control programs are needed to prevent further cases of poliomyelitis. Laboratory studies of isolates can also be used to support the diagnosis of poliovirus infection when the patient has no knowledge of any exposure or has an atypical disease presentation. In the case of suspect cases of poliomyelitis, fecal specimens for viral isolation should always be obtained, because the virus is shed the longest and in highest titer from the intestinal tract. Early in the acute phase of the illness, virus is frequently isolated from the throat. Isolation of virus from cerebrospinal fluid provides the most direct link to disease but is usually less successful. The earlier in the course of illness that cerebrospinal fluid specimens are collected, the more likely virus will be isolated.

The procedure for virus isolation involves inoculation of appropriate specimens onto susceptible cultured cells. Unfortunately, there is no single cell line capable of growing all human enteroviruses. It is common practice to use several types of human and primate cells to increase the spectrum of viruses that can be detected (53). Even with a variety of cells, however, there remain several coxsackievirus A serotypes that fail to propagate in culture. The coxsackieviruses, including those that do not grow in cell culture, can be isolated and propagated in suckling mice.

As a consequence of current poliovirus eradication activities and the importance of poliovirus as a public health problem, specific diagnostic procedures have been developed to detect this virus. In general, poliovirus grows quite well on a variety of primate and human cell culture lines, but it cannot be distinguished from other enteroviruses solely on the basis of cytopathic effect. Polioviruses are unique in the use of a specific receptor to infect cells, PVR, which is distinct from receptors used by all other enteroviruses to infect cells. This receptor has been transfected and expressed in a murine cell line that normally cannot be infected by most enteroviruses but is permissive to viral replication when the viral genome is present within the cell. One of these transfected murine cells, L20B, can grow poliovirus, and it has been exploited to selectively isolate poliovirus, even in the presence of other enteroviruses (136). When a specimen is inoculated onto these cells and a characteristic enterovirus cytopathic effect is seen, the virus can be presumptively identified as a poliovirus. However, there are a few strains of certain nonpolio enterovirus serotypes that are able to grow on the parent murine cells, and therefore growth on L20B cells is not a definitive identification of poliovirus, and confirmatory testing is required.

In routine diagnostic testing, all enterovirus growth in cell culture is detected by its cytopathic effect, and the isolate is typically confirmed as a specific enterovirus by neutralization with type-specific antisera. The most widely used reagent antiserum was prepared in horses and can be obtained from the World Health Organization (208); however, supplies are now becoming limited. As there are at least 66 different known enteroviruses, it would be impractical to attempt neutralization using all possible reference antisera individually. Type-specific antisera are therefore combined in intersecting pools in such a way that antibody to any one type is present in only a limited number of pools. In the Lim,

Benyesh-Melnick (LBM) pool scheme (209), antibodies to 42 different enteroviruses are sorted into eight pools (labeled A through H) (Table 10 and Fig. 10). The typical neutralization test consists of mixing approximately 100 $TCID_{50}$ of an unknown virus with an antiserum pool and incubating at 37°C for 2 hours. Next, the virus–antiserum mixture is inoculated onto a susceptible cell culture system, which is then observed for several days. A separate virus–antiserum mixture is made for each of the available antiserum pools. The pool that neutralizes the unknown isolate identifies the virus. For example, if only LBM pools E and F neutralize the unknown virus, it is presumptively identified as echovirus 18, because only antisera to this virus are found in both of these pools and not in the others. If only pool A neutralizes the isolate, the identity is echovirus 15. Other pool schemes have been devised and used successfully, but they are less widely used due to limited reagent availability. If the virus cannot be identified by use of the A to H pools, other pools and single antisera can be tested. Despite these efforts, the neutralization assay may still fail. Additional procedures may need to be performed, including filtration, treatment with chloroform, and resolution of mixtures by terminal dilution or plaque purification. Even with all of these procedures, “untypable” enteroviruses may still be encountered. They may represent mixed infections, aggregates of viruses, extreme antigenic drift, or undiscovered types. In theory, it should be possible to identify or characterize all of these, but such laborious and time-consuming processes are rarely justified. For clinical management of routine cases, it is seldom critical to identify the specific nonpolio enterovirus type. A high index of suspicion for an enterovirus infection can be developed by reflecting on the clinical picture, the virus isolate's cytopathic effect and cell culture systems utilized, and knowledge of basic enterovirus epidemiology.

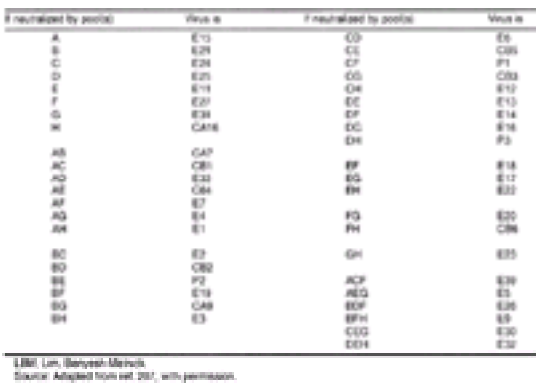

TABLE 10. *Code for reading results of neutralization tests using LBM antiserum pools A–H*

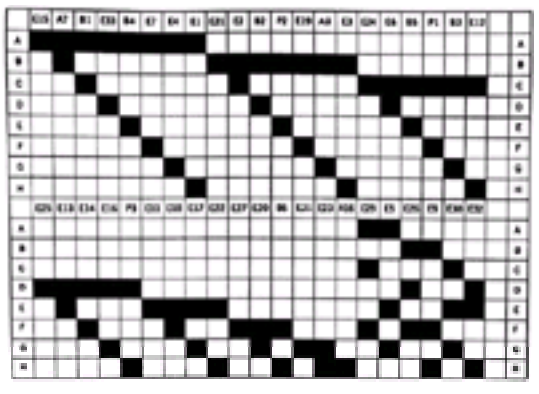

FIG. 10. Schema of antiserum pools. Multiple antisera are incorporated into different pools; a virus isolate can be specifically typed by its pattern of neutralization in tests against the pools. With the antiserum combinations shown, new isolates of 42 enteroviruses can be identified in tests against only eight pools (188,209).

Antibody Tests

Serologic diagnosis of enterovirus infection is made by comparing titers in acute and convalescent phase (“paired”) serum specimens. If an enterovirus isolation and identification have been made, paired serum can be tested against the patient's own isolate. If not, it still may be possible to establish a likely enterovirus infection if a particular serotype is suspected. In this case, a reference strain maintained in the laboratory may be used in a neutralization test. In general, however, enterovirus serodiagnosis is more relevant to epidemiologic studies than to clinical diagnosis (see earlier section Epidemiology). The most basic serologic test is that of neutralization in cell culture. The mechanics of the assay are similar to the test used for identification of isolates, except that a known virus is mixed with serial dilutions of antisera from a patient. There are other, less widely accepted serologic methods, all with particular strengths and weaknesses, including plaque reduction neutralization, complement fixation, passive hemagglutination, hemagglutination–inhibition, and immunofluorescence. A fourfold or greater rise in type-specific neutralizing antibody titer is considered diagnostic of infection. Unfortunately, antibody may already be present at the time the original specimen is obtained because of the extended incubation period and prodromal period of many enteroviral illnesses, complicating interpretation of results.

Many serologic studies rely on the detection of IgM antibody as evidence for recent enterovirus infection, and this is now widely used as an alternative to the neutralization and complement-fixation test. In the last decade, several groups have developed an ELISA for enterovirus-specific IgM (30,196). These tests have been found positive for nearly 90% of culture-confirmed coxsackievirus B infections, and can be performed rapidly. The ELISA has been successfully applied for epidemiologic investigations of outbreaks (102), as well as for specific diagnostic use (72).

In most cases, the IgM ELISA test is not completely serotype specific. Depending on the configuration and sensitivity of

the test, from 10% to nearly 70% of serum samples show a heterotypic response caused by other enterovirus infections. This heterotypic response has been exploited to measure broadly reactive antibody, and the assay used to detect enterovirus infection generically (40,310). In attempting to characterize the exact nature of the response using different antigens, it is clear that the human immune response to enterovirus infection includes antibodies that react with both serotype-specific epitopes and shared epitopes (93). Despite this problem, which is inherently biologic, there remains a fairly high concordance of results between assays of different configurations (127). In summary, the IgM assays that are generally used in epidemiologic studies have very good sensitivity and appear to be very specific for enterovirus infection; however, these assays detect heterotypic antibodies resulting from other enterovirus infections, and therefore cannot be considered strictly serotype specific. Attempts to improve the serotype specificity of these assays have, as expected, either reduced the sensitivity of the assay or decreased the convenience. A positive result with either the neutralization test or IgM ELISA indicates a recent viral infection; however, the infecting serotype found with the IgM assay may not be the same one determined by the neutralization test.

Molecular Detection

The application of molecular biology techniques to clinical virology has begun to significantly change approaches to enterovirus diagnostics (see also the earlier section Epidemiology). Because of distinct advantages in speed, many of these procedures have already supplanted traditional methods of detection and characterization. Continued development and refinement of these procedures will undoubtedly lead to even further application of the methodologies to a broader spectrum of enterovirus diagnostic problems and ultimately into routine primary usage.

As with virus isolation and serotyping, the molecular methods attempt to detect the presence of enteroviruses in a specimen and, in some procedures, to further characterize the detected virus. The techniques can be grouped on the basis of their infrastructure and technical requirements and the types of specimens to which they are applicable. Additionally, the tests can be grouped on the basis of the type of answer the test provides and the predictive value of a positive and a negative result. The first broad group of tests is based on the polymerase chain reaction, which is used primarily to detect enterovirus genome in cell cultures, clinical specimens, and biopsy or autopsy tissues. The second group of tests is based on nucleic acid probe hybridization and is used for both detection and characterization, although usually with less sensitivity than polymerase chain reaction. The third and newest procedure utilizes genomic sequencing for characterization of enteroviruses at the highest levels of specificity.

By far the most common use of polymerase chain reaction for enterovirus diagnosis is the direct detection of virus in clinical specimens (162,243,286). There are numerous variations on the details of the procedures, but all methods that are able to generically detect enteroviruses have several common features. The most important property of these tests is that the primers are targeted to amplify the 5'-untranslated region of the virus genome. Many different primers targeted to this region have been published, and they target different sequences and provide different product sizes. Unfortunately, many of these primers have not been completely evaluated on a large number of clinical isolates to confirm reactivity with all enterovirus serotypes and strains within serotypes, and therefore they have not been validated sufficiently for diagnostic use (229). The major advantage of the pan-enterovirus polymerase chain reaction is that rapid detection of an enterovirus is possible, even with very small amounts of clinical specimens such as spinal fluid. It is also possible to detect enteroviruses that do not readily grow in cell culture. As with all polymerase chain reactions, the sensitivity of amplification of RNA from biologic specimens is extremely variable, depending on the nature of the specimen. Although the polymerase chain reaction procedure can be shown to give a positive result even from only one or a few copies of viral RNA, it is not unusual for the sensitivity to be many orders of magnitude lower in certain specimens (e.g., stool). For this reason, despite the theoretical advantage of the polymerase chain reaction to greatly improve enterovirus detection from clinical specimens, the gains in practice are much more modest (e.g., with spinal fluid) or nonexistent (e.g., with stool) (11,314).

By changing the target for amplification, it is possible to characterize a particular enterovirus using the polymerase chain reaction. Because the antigenic property of viruses that defines serotype is a property of the viral capsid proteins, there are sequences that correspond to this conserved antigenic property within the capsid-coding region of the virus. Extensive studies of the capsid region of poliovirus isolates have led to the design of primers that can selectively amplify isolates from a single serotype but not from other isolates of heterologous serotypes (168). This is accomplished, despite the high rate of synonymous nucleotide substitutions, through the use of inosine in the primer synthesis. In an analogous manner, the ability of poliovirus to uniquely bind to the PVR is also an intrinsic property of the capsid proteins. The amino acids that are involved in receptor binding have been well characterized from studies of the three-dimensional structure of the virion. When primers are designed that correspond to these presumably poliovirus-specific sequences, amplification is observed with all poliovirus isolates but not with other enteroviruses (167). It should be possible to extend these principles to other enterovirus receptor groups and serotypes, and to provide tools for rapid serotype characterization of enteroviruses without the requirement for cell culture procedures.

Nucleic acid probes, the first application of molecular techniques developed for detecting enterovirus genomes, are largely being replaced by the polymerase chain reaction. These early probes were made by synthesizing cDNA from coxsackievirus B viral RNA. The cDNA was cloned into bacteria, amplified, and radiochemically labeled to provide a reagent for detecting enterovirus genomes by nucleic acid hybridization. Further refinement of this technique included the use of RNA probes to improve sensitivity, introduction of nonradiochemical labeling of probes, and the application of these techniques to *in situ* hybridization in myocardial tissue (130). This technique has been particularly useful for understanding the cytopathology of enterovirus infection, especially in studies of myocarditis. The technique, however, cannot be used to identify the serotype of the enterovirus in a specimen because of probe binding to related serotypes.

Nucleic acid probe hybridization has also been extensively used in the poliovirus eradication program as a basic technique to determine whether an isolate is a vaccine-derived or a wild poliovirus. For wild polioviruses, probes can identify the specific strain and therefore its probable geographic origin (73).

A goal in virus identification is knowledge of the sequence of the viral genome. Encoded within this sequence are determinants for all the biologic properties that are attributable to a given virus. Therefore, the nucleic acid sequence

information of a virus represents its ultimate characterization. All important information about a virus could potentially be obtained directly by polymerase chain reaction in conjunction with nucleic acid sequencing if all the molecular correlates of viral phenotypic determinants were understood. At present, however, the genetic location for many properties of the virus remains uncertain. Nevertheless, it is possible at present to use sequence information to assign an enterovirus isolate to a particular serotype (240). The molecular typing system is based on reverse transcriptase–polymerase chain reaction and nucleotide sequencing of the 3' half of the genomic region encoding VP1. The serotype of an unknown isolate is inferred by comparison of the partial VP1 sequence to a database containing VP1 sequences for the prototype and variant strains of all 66 human enterovirus serotypes. Strains of homologous serotypes are easily discriminated from heterologous serotypes (Fig. 11). This method can greatly reduce the time required to type an enterovirus isolate, and it can be used to type isolates that are difficult or impossible to type using standard immunologic reagents. The technique is also useful to rapidly determine whether viruses isolated during an outbreak are epidemiologically related.

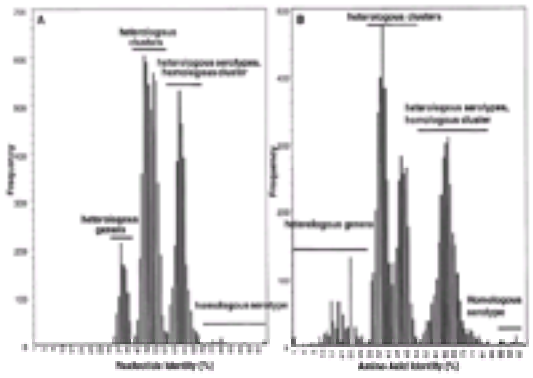

FIG. 11. Serotype identification using sequence data. Frequency distribution of pairwise identity scores for comparison of VP1 nucleotide and deduced amino acid sequences. **A:** Nucleotide sequence distribution. **B:** Amino acid sequence distribution (240). Serotype identification of an isolate can be achieved by comparing the sequence of the isolate with known sequences in VP1 for all serotypes and looking for identities of greater than 75% in nucleotide sequence or greater than 88% in amino acid sequence.

PREVENTION AND CONTROL

Treatment

There is no currently available drug treatment for enteroviral infections in clinical use. However, the effectiveness of a variety of drugs *in vitro*, as well as in animal models, has been documented, and a variety of clinical trials are in progress. In addition, pooled immunoglobulin has been used as a therapy for prevention and treatment of enteroviral disease in immunocompromised individuals.

There are several therapies (e.g., interferon and anti-viral antibody) that target early stages in the virus life cycle, such as spread. Interferon alpha and beta have been found to be effective in coxsackievirus A24 *in vitro* infections (179). Pooled immunoglobulin has been effectively used in enterovirus-induced CNS infections in immunocompromised patients, both by the intravenous route as well as via a shunt into the spinal fluid. Intravenous immunoglobulin appears to stabilize and improve some of these infections, although the disease can progress even in the face of intrathecal immunoglobulin. In some cases, efficacy may be limited by inadequate amounts of the relevant antibody in the immunoglobulin pool (e.g., if the infection involves an unusual and rare enterovirus serotype), as well as problems in the delivery of adequate levels of antibody to the appropriate infected cells. Pilot studies have been conducted administering intravenous immunoglobulin in neonates suspected of having enteroviral infection (7).

Other antiviral drugs have been developed to target specific enteroviral proteins. The WIN compounds, which were originally shown to be effective against rhinovirus, have attracted the most attention. These drugs bind a hydrophobic site near the surface of the virion called the pocket (348), which lies in the floor of the “canyon ”—the region believed to bind the virion to the cellular receptor. By binding to the pocket, these compounds are believed to interfere with viral uncoating and/or adsorption. Variations in activity of WIN compounds against different picornaviruses are presumably related to the particular fit of the drug into the pocket of a specific enterovirus strain. Oral administration of WIN 54954 significantly decreased the number of upper respiratory infections following challenge with coxsackievirus A21 (i.e., 3 of 27 patients in the treated group had an upper respiratory infection versus 15 of 23 in the placebo group) with decreased associated symptoms and viral titers (292); adverse reactions, however, curtailed further investigations with this drug. Pleconaril or VP 63843 is a more recently developed WIN-type compound with a broad *in vitro* inhibitory activity against 95% of the 215 nonpolio enteroviruses that were tested (252). Pleconaril integrates within the hydrophobic pockets of the virus capsid, interfering with capsid functions such as attachment. Significant activity was noted against some serotypes, such as echovirus 11. A randomized, double-blind study involving the administration of pleconaril following a challenge with coxsackievirus A21 showed statistically significant decreases in viral shedding in nasal secretions, nasal mucus production, and total respiratory illness symptom scores in pleconaril-treated patients compared to placebo-treated subjects (291). Another phase II trial of the same drug against enteroviral meningitis showed a statistically significant decrease in disease duration (9.5 days in the placebo group versus 4.0 days in the treatment group), headache duration, and use and amount of analgesia (285). A recent report found that pleconaril treatment cleared an enteroviral gastrointestinal infection in two infants with subacute combined immunodeficiency (238). Trials involving the use of pleconaril in other enteroviral infections including neonatal sepsis are in progress.

Mutant viruses resistant to the WIN compounds can arise. In the case of resistance to rhinovirus, the mutant viruses tend to have bulky amino acid substitutions that sterically block entry of the drug into the pocket (120). These mutant viruses

may not be as significant a problem as expected, as drug-resistant coxsackievirus B3 mutants that appeared in tissue culture following exposure to the WIN compounds tended to be attenuated when inoculated into mice (109).

Enviroxime is an antiviral drug that targets nonstructural protein 3A, leading to a block in the synthesis of plus-strand viral RNA. Although this compound inhibits enterovirus and rhinovirus *in vitro* infections, it is toxic and not effective in humans (82). Resistance to enviroxime is determined by changes in the amino acid at position 30 in protein 3A (121).

Other possible targets for antiviral agents include the viral receptor, the viral proteases (2A or 3C), and the viral replicase (3D).

Poliovirus Vaccine and Eradication

The poliovirus field was fortunate to have more than one excellent vaccine, since both the killed intramuscular vaccine of Salk and the oral attenuated vaccine of Sabin were available. Both of these vaccines led to the production of antipoliovirus antibody (Fig. 12) (234) with subsequent protection from disease. Although there has been a continuing advocacy for one or the other vaccine over the years, it is clear that each has advantages and disadvantages (Table 11 and Table 12)— and that there are appropriate circumstances for the use of each. Now, with the incidence of poliovirus declining dramatically, new guidelines from the Centers for Disease Control and the Advisory Committee of Immunization Practices for vaccine use have been developed in the United States, with a switch from live vaccine to the exclusive use of killed vaccine (at 2 months, 4 months, 6 to 18 months, and 4 to 6 years of age). The main expected benefit for this change is the elimination of vaccine-associated paralytic poliomyelitis.

Advantages
Safe because inactivation ensures that there is no infectious virus
Can be used in immunodeficient and immunosuppressed individuals
Provides excellent systemic immunity
Disadvantages
Requires intramuscular injection with repeated doses
More expensive than live vaccine
Potential hazards because of the use of wild seed virus in production
Reduced intestinal immunity compared to natural infection

TABLE 11. *Killed polio vaccine: advantages and disadvantages*

Advantages
Confers both systemic and intestinal immunity
Relatively inexpensive
Provides herd immunity because the virus is excreted into the environment
Oral delivery
Relatively safe
Disadvantages
May mutate to neurovirulent form causing vaccine-associated paralytic poliomyelitis
Contraindicated in immunodeficient and immunosuppressed individuals
Requires monkeys for safety testing

TABLE 12. *Live polio vaccine: advantages and disadvantages*

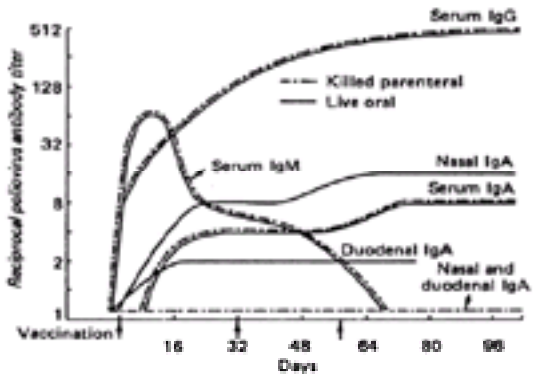

FIG. 12. Serum and secretory antibody responses to oral administration of live attenuated polio vaccine and to intramuscular inoculation of killed polio vaccine (241).

The poliovirus vaccination program has been remarkably successful. As a result of the universal use of Sabin oral poliovirus vaccine, no case of paralytic poliomyelitis from indigenous wild-type poliomyelitis has occurred in the United States since 1980 or in the entire region of the Americas since 1991, laying a framework for the global eradication of

poliovirus. The ultimate eradication of poliovirus seems likely, given the biologic properties of poliovirus and the availability of effective vaccines. Of note, poliovirus is dependent on nearly continuous person-to-person transmission for its survival, because there are no nonhuman reservoirs and because the virus persists for a relatively short time in the environment. Routine immunization in most developed countries was successful in eliminating the indigenous circulation of virus, but many developing countries remained endemic decades after the introduction of poliovirus vaccines. Then, in little more than two decades after the widespread availability of the oral poliovirus vaccine, disease eradication was undertaken, beginning in the Americas (76).

A progress report on the global efforts to eradicate poliovirus is provided on a Web site, http://www.polioeradication.org/. There are four fundamental components of the strategy to eradicate poliovirus (145). First is the achievement and maintenance of high levels of routine immunization. With the accelerated activities resulting from the Expanded Program on Immunization of the World Health Organization, routine coverage with three doses of oral poliovirus vaccine in children under 1 year of age reached nearly 90% of the world's children by 1990.

The second element of the strategy is the use of National Immunization Days for the delivery of vaccine to all children under 5 years of age in a very short period of time, usually from one to a few days (34). The mass immunization interferes with the spread of wild poliovirus. This strategy was used in many early immunization efforts in the 1960s, and it was applied successfully in Cuba to achieve and maintain the elimination of poliovirus from that country following three successive years of annual campaigns (278). The current National Immunization Days are carried out in two rounds about 1 month apart, and they are targeted for the “low season” of poliovirus circulation, when poliovirus transmission is presumably least efficient and therefore most easily disrupted. The National Immunization Days have grown in an extraordinary way as more countries adopt this strategy. Some campaigns now represent the largest public health activities on record. For example, in India in 1999, more than 125 million children were vaccinated in a single day in two rounds 1 month apart. During the same year, more than 450 million children were vaccinated in these mass campaigns worldwide.

The third basic element is the use of surveillance based on cases of acute flaccid paralysis (35). One of the major differences between the smallpox eradication program and the efforts to eradicate poliovirus is the low rate of clinical disease with poliovirus. Because less than 1% of infected susceptible individuals will develop paralytic illness, most infections are not clinically recognized. Therefore, unlike smallpox, where almost all infections were symptomatic, poliovirus is quite difficult to detect; this is certainly a challenge to eradication efforts. To improve the sensitivity of detecting poliovirus infection and yet achieve a practical system, surveillance was developed around the unique clinical presentation of paralytic poliomyelitis. To avoid the requirement of extensive neurologic examinations, which are not feasible in many developing countries, the surveillance was simplified to include any case of acute-onset flaccid paralysis. This system reports many other diseases in addition to poliomyelitis, such as Guillain-Barré syndrome, transverse myelitis, and traumatic neuropathy. However, this loss of specificity is compensated for by a gain in sensitivity, because most cases of true poliomyelitis are reported. It remains unclear how stable the incidence of nonpoliovirus-induced flaccid paralysis will be.

The remaining part of the surveillance is focused on detection of the virus. Two stool specimens are collected from all cases of acute flaccid paralysis and tested in a global network of laboratories to attempt isolation of poliovirus if it is present (144). The major advantages of this surveillance system are simplicity, practicality, and reasonable sensitivity for detection of poliovirus. The major disadvantages are the requirement to rapidly collect, transport, and test a large number of specimens from all areas of the world, and the requirement of high-quality laboratory testing.

The last element of the eradication strategy is the use of “mopping-up” activities (75). This strategy focuses on an area or country where the previous three parts of the program have successfully reduced the number of poliovirus cases to a small number, and where surveillance has localized the remaining reservoirs of transmission. It is then possible to intensify immunization activities in those targeted communities that contain the remaining circulating virus, or the last “chains of transmission.” These intensified activities usually involve active searches in communities for children, including house-to-house or boat-to-boat immunization (15). Teams visit all residences in the area and ensure that children are not missed.

These elements of poliovirus eradication strategy have proven successful in large parts of the world. Since the program began, many countries are now free of indigenous poliovirus circulation. This can be seen in Figure 13, where in 1988 poliovirus was found on all the continents (except Australia), with estimates of more than 350,000 cases of poliovirus each year. In 1999, the number of countries with proven or suspected poliovirus was only 50, and all of the Americas, Europe, and the Far East are believed to be free of poliovirus (17). The remaining countries with large numbers of poliomyelitis cases include countries in south Asia (India, Bangladesh, Pakistan, and Afghanistan) and sub-Saharan Africa (Nigeria, Ethiopia, Democratic Republic of Congo, Angola, Ivory Coast, and other countries in central and western Africa).

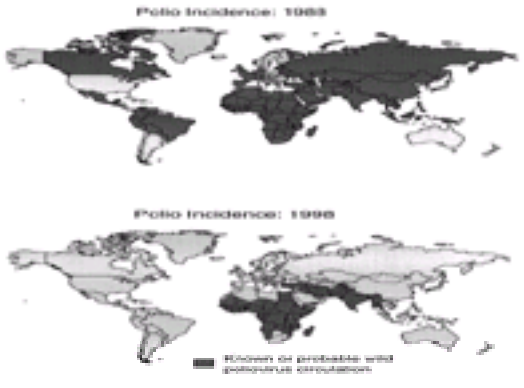

FIG. 13. World map depicting the circulation of wild poliovirus for 1988 and 1998 as reported to the WHO. (Adapted from

Centers for Disease Control, unpublished data.)

In 1988, in part based on the rapid progress of eradication activities in the Americas, the World Health Assembly unanimously adopted a resolution calling for the global eradication of poliovirus before the end of the 20th century. This resolution was reaffirmed in May of 1999, and an acceleration of activities was urged. These accelerated activities include additional rounds of National Immunization Days in large endemic countries, intensified surveillance in high-risk communities, and mopping-up in focal reservoirs.

The achievement of eradication is only the first step in a process that, like smallpox, will lead to the cessation of vaccination and realization of savings that will exceed $1.5 billion per year (26). After wild virus circulation is interrupted, the only remaining viruses will be in the laboratories of the world. Therefore, it will be necessary to institute proper containment of poliovirus, and shift studies to other viruses where possible. A plan for these steps of containment has been drafted and will be implemented in phases as eradication proceeds. In the first pre-eradication phase, preparation for later phases is to be undertaken by conducting an inventory of all institutions and laboratories that have wild polioviruses or potentially infectious materials. Wherever possible, use of vaccine strains of poliovirus should be substituted for wild strains. In the second phase, which begins 1 year after the last wild virus has been isolated anywhere in the world, all work with wild polioviruses will be done at the higher Biosafety Level 3/polio level of containment. At this point, the main risk of reintroduction is from the laboratory. Other possible concerns involve the excretion of virus from immunodeficient individuals. It is sometime during this phase, once all countries have been certified as being free of circulating poliovirus and all laboratories have properly contained, referred, or destroyed their poliovirus stocks and potentially infectious materials, that the Global Certification Commission will declare poliovirus eradicated. The last phase of containment will occur sometime in the future when the decision is made to stop vaccination with oral poliovirus vaccine. At this point, all infectious polioviruses will need to be in maximum containment.

There are numerous unknowns and potential obstacles that remain to be faced in the eradication efforts. Because of the inherent insensitivity of detecting poliovirus infection, it is important that surveillance quality be achieved and maintained for a period of time. Although the exact length of time required to be assured of success is not known in all circumstances, the minimum requirement is 3 years. Previous experience indicates that if excellent surveillance is achieved and maintained, poliovirus genotypes that have not been detected for a period of 1 year are not detected ever again. This has been true for more than a dozen genotypes that have been eliminated from circulation (Fig. 14).

FIG. 14. World map depicting type 1 poliovirus genotypes in different regions from 1992 to 1999 and the years in which the eradication occurred. (Adapted from Centers for Disease Control, unpublished data.)

One potential problem with poliovirus eradication pertains to individuals with immune deficiencies, who may continue to excrete poliovirus for periods of over a decade. Prolonged excretion of vaccine-related poliovirus has been observed in several patients with humoral immune deficiencies, such as agammaglobulinemia, but not with wild polioviruses (166). It is not clear whether this lack of persistent wild virus infection is caused by the greater neurovirulence of wild polioviruses—leading to a uniformly, fatal outcome—or by the lack of exposure to wild virus by patients with severe immune deficiencies who are largely residents of poliovirus-free countries. There is currently no evidence that patients with cell-mediated deficiencies are at increased risk for chronic poliovirus infections; however, studies are being undertaken to specifically look at prolonged infection in human immunodeficiency virus–infected children and adults.

The last potential obstacle will be the final cessation of vaccination with oral poliovirus vaccine. Since oral poliovirus vaccine strains retain the demonstrable ability to revert to neurovirulence and the potential to regain the increased transmissibility of wild viruses, they pose a continual threat for reintroduction of a new wild poliovirus strain into circulation. The analysis of the risks of different strategies for stopping vaccination are complicated by the lack of information about reversion of vaccine strains. For example, it is not known exactly which genetic determinants are responsible for the property of transmissibility, although it is fairly clear that these are different determinants from the ones controlling neurovirulence.

A recent outbreak of poliomyelitis in the Dominican Republic and Haiti in the summer of 2000 underscores the importance of maintaining excellent surveillance, the need to maintain high vaccine coverage, and the critical importance of developing strategies for the cessation of vaccination with the oral poliovirus vaccine (18). The outbreak affected at least 19 children and was confirmed by virus isolation from 6 of the cases. A type 1 poliovirus that was isolated was clearly related by nucleic acid sequencing to the vaccine strain and was distinct from all wild type and reference strains of poliovirus. Preliminary characterization of the isolates showed reversion of the type 1 attenuation markers and apparent

recombination with an unknown nonpolio enterovirus. The extent of change from the parental vaccine strain was compatible with circulation of this strain for approximately two years prior to the first recognized cases. The intermediate isolates were likely missed because of inadequate surveillance in the affected regions.

The world has endured the crippling effects of poliovirus for millennia, yet the end of these particular viruses is approaching. It is possible that the next edition of this chapter will treat poliovirus as a historic footnote, rather than a major focus of public health activities.

PERSPECTIVES

For 25 to 50 year prior to the identification of acquired immunodeficiency syndrome, much of virology was focused on poliovirus. With the eradication of poliovirus imminent, the picornavirology field is about to change and will never be the same again. It is somewhat ironic to realize that despite the incredible advances in our understanding of the molecular aspects of poliovirus and the relatively recent availability of extraordinarily powerful molecular tools for studying poliovirus, the eradication has been made possible through the use of vaccines that were developed prior to the molecular virologic revolution and without any of the new knowledge or technology. The eradication of poliovirus will become a reality as a result of the use of the Salk and Sabin vaccines, vaccines that were generated empirically prior to the breakthroughs in our understanding of the molecular biology of poliovirus.

But perhaps it is a bit unfair to belittle the achievements of modern techniques in the eradication of poliovirus. Certainly, the tools of molecular biology were and will continue to be important in monitoring and tracking poliovirus and identifying strains. In fact, it is likely that the need for continuing poliovirus surveillance will foster the development of novel diagnostic methods that will not only assist in surveillance of poliomyelitis but also can be used to identify new enteroviruses—for example, the efficient detection of viruses that may not grow in cell culture. Additionally, these tools will provide better and more accurate descriptions of the epidemiology of enterovirus infections.

What new directions will drive the field of picornavirology? One enterovirus that is likely to be targeted from a medical perspective is enterovirus 71. The ability of this virus to mount huge epidemics and to be a major cause of neurologic disease, especially acute flaccid paralysis, is likely to have already generated special interest with respect to vaccine development.

It is clear that the coxsackieviruses will also be a subject for increased study among picornavirologists. The large number of diseases that the coxsackieviruses cause also makes this group of viruses of great interest from the point of view of vaccine development. In addition, investigations of coxsackieviral diseases may clarify the pathogenesis of virus-induced autoimmune diseases.

The next decade is likely to see the development of a number of antiviral drugs that are effective in enteroviral infections. Some of these new drugs will probably take advantage of the receptors used by different viruses as well as our increased understanding of nonstructural enteroviral proteins. Enteroviruses will still be very much with us, even after the eradication of poliovirus.

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CHAPTER 25 Rhinoviruses

Robert B. Couch

Rhinoviruses are the most commonly isolated viruses from persons experiencing mild upper respiratory illnesses (common colds). The name applied to this genus of *Picornaviridae* designates the primary site of infection with these viruses. In contrast to the enteroviruses, rhinoviruses do not appear to replicate in the intestinal tract. They also differ from enteroviruses in their greater species specificity and fastidious growth requirements.

HISTORY

The long association of colds with humans provided the basis for allusions to the malady in ancient history. Hippocrates believed therapeutic bleeding for common colds was a worthless treatment; and in the first century, Pliny the Younger recommended “kissing the hairy muzzle of a mouse” as therapy for colds. Benjamin Franklin spoke strongly against cold exposure and atmospheric dampness as being responsible for development of a cold; he believed that colds were acquired from other persons. Recordings of freedom from colds while at sea and occurrence of colds among voyagers and isolated populations when contact with continental populations was reestablished supported a belief in the contagious nature of colds.

In the early 1900s, colds were induced in volunteers by intranasal inoculations with filtrates of nasal secretions from other volunteers with colds. As a result of these various studies, colds were presumed to be caused by filterable agents (viruses).

Long-term studies of experimental colds in volunteers were begun by Andrews (5) in the 1940s at a Common Cold Research Unit in Salisbury, England. A great deal of understanding of the properties and spread of colds was provided by these studies and by similar studies at a later time by Jackson in Chicago (63,130,131). Andrews was able to propagate an agent in lung explants that later proved to be a rhinovirus; many of the nasal secretions used for inducing colds in volunteers at Salisbury and at Chicago also were subsequently shown to contain rhinoviruses. Long-term studies on the epidemiology of colds and other acute respiratory illnesses were begun by Dingle et al. (53) in the 1940s among families in Cleveland. These studies demonstrated the role of the family in the spread of colds and emphasized the greater incidence among children than adults.

The initial isolations of a rhinovirus were accomplished by Pelon et al. (175) and Price (188) using rhesus monkey kidney tissue cultures and nasopharyngeal washings from persons with colds. The initial isolates, strains 2060 and JH, were shown to be identical and were designated echovirus type 28; a subsequent definition of properties resulted in these strains being reclassified as rhinovirus type 1A. In 1960, Tyrrell and Parsons (228) at Salisbury isolated strain HGP by reducing the incubation temperature of cultures to 33°C, lowering the pH of tissue culture media to around 7.0, and gently rotating cultures during incubation. Use of these “conditions of the nose” and human tissue cultures then led to isolations of rhinoviruses in many locations. When the sensitive semicontinuous strains of human embryonic fibroblast tissue cultures described by Hayflick and Moorehead (117) and the conditions described by the Salisbury group were used routinely, a profusion of rhinovirus serotypes was detected.

Epidemiologic studies by Hamre et al. (111,112), Mogabgab (158), Gwaltney et al. (96,97 and 98,100,122), Monto et al. (160,161,162,163 and 164), Fox et al. (66,75,76,146), and others documented the major importance of rhinoviruses as a cause of mild acute respiratory illness and described many of the features of their occurrence in various human populations. Couch et al. (44) noted the surprisingly small amount of virus required to initiate experimental infections in

volunteers. Couch, Cate, and Douglas, with others (30,31,32 and 33,44,58,61,62), and Gwaltney, Hendley, and others (91,95,101,102,119,120) provided considerable information on the pathogenesis and immunology of rhinovirus infections.

The name “rhinovirus” was originally suggested by Andrews, and a description of the group was proposed by Tyrrell and Chanock in 1963 (227); this was adopted by a nomenclature committee. In 1967, a collaborative program (141) assigned numbers 1A through 55 to the known rhinovirus types. In 1971, a second phase added types 56 through 89 (142), and a third phase increased the number to 100 (106).

Both inactivated virus vaccines (60,177) and attenuated viruses (59) were tested in volunteers in the 1960s and 1970s, but the multiplicity of rhinovirus serotypes and lack of established methods for optimizing mucosal immunization soon led to a cessation of vaccine development. Efforts to develop antivirals has characterized research on control of the common cold for the past two decades. This effort was facilitated by the description of the three-dimensional structure of rhinovirus type 14 by Smith et al. (207), and of compounds capable of binding to the receptor site on the virion surface.

The introduction of molecular and structural biology into research led to identification and description of the cell receptors of rhinovirus and the structural biology of cell receptor and antibody interactions with the virion surface (Chapter 23). Recent epidemiologic studies, using nucleic acid amplification techniques for detecting rhinoviruses, have further increased the significance of rhinoviruses as causes of upper respiratory illnesses and documented a role for them in induction of serious disease in elderly persons and those with asthma and chronic lung disease.

INFECTIOUS AGENTS

Propagation and Assay in Cell Culture

Initial isolations were accomplished with rhesus monkey kidney tissue cultures (types 1A, 1B), but a clear superiority of human tissues for isolation and growth was shown later. Efficient growth of rhinoviruses occurs only in human and some primate cells; the presence of receptors on cell surfaces influences this tissue specificity (38). Three different cell-surface receptors are used by rhinoviruses (229). Type 87 alone uses a receptor other than the minor group receptor (low-density lipoprotein [LDL] receptor) used by 10 serotypes (1A, 1B, 2, 29, 30, 31, 44, 47, 49, and 62) and the major group receptor intercellular adhesion molecule 1 (ICAM-1)] used by the remaining described serotypes.

A variety of human cells are available for growth of rhinovirus. Those reported to be useful include human embryonic kidney, human amnion, diploid fibroblast lines from human embryonic lung, tonsil, liver, intestine, skin, adult fibroblast lines from aorta and gingiva, and the L132, KB, HEP-2, and HeLa continuous cell lines (94). Some strains may be detectable only with polymerase chain reaction (PCR) testing (140). Organ cultures of nasal and tracheal epithelium are required for growth of some fastidious strains (126), but whether strains detected only with PCR will all grow in organ culture is not known.

The most commonly used cells for rhinovirus growth are the WI-38 strain and the MRC-5 strain (133) of diploid fibroblasts (117), fetal tonsil cells (75), and rhinovirus-sensitive lines of HeLa cells (39). Comparative information on these different cell cultures for isolation of rhinoviruses from clinical specimens has indicated superiority of different cultures in different laboratories (10,43,150). Available data show that maximal isolation of rhinoviruses requires simultaneous use of several cell-culture systems.

Optimal conditions for growth of rhinoviruses include a concentration of bicarbonate in media of less than 0.35 g/L (pH 7.0–7.2), incubation temperature of 33°C, and slow rotation of cultures. Eagle's minimum essential medium is most commonly used for maintenance and is supplemented with 2% fetal calf serum and antibiotics. The low pH is less critical with use of HeLa and human fibroblast cultures than with monkey and human kidney cultures.

Growth of virus in cell monolayers is usually detected by appearance of cytopathic effects (CPEs); on rare occasions, interference with a second virus has been used. CPEs are similar to those produced by enteroviruses (Fig. 1). They initially occur in foci where cells become rounded, and some may swell; nuclei become disorganized, and the focus of the CPEs will contain cellular debris and both large and small rounded cells with pyknotic nuclei.

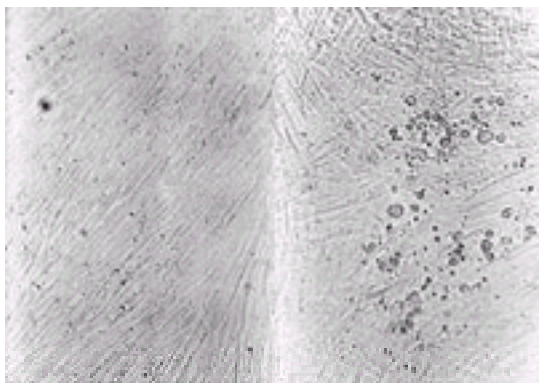

FIG. 1. Cytopathic effects in human fibroblast tissue cultures that are caused by a rhinovirus. **Left:** Normal WI-38 human embryonic lung fibroblast tissue culture. **Right:** A culture inoculated 2 days earlier with a rhinovirus. Note the variable cell size and numerous small, rounded cells.

Plaques can be obtained in a variety of cell lines with most described techniques by using purified agar or methyl

cellulose with diethylaminoethyl (DEAE)-dextran in the overlay (40,54,67). Different plaque sizes and appearances are a function of the overlay medium used. Added magnesium enhances plaque formation of many but not all rhinoviruses in HeLa cells (68).

Biologic, Chemical, and Physical Characteristics

The property used to distinguish rhinoviruses from enteroviruses is their acid lability. Inactivation occurs for all rhinoviruses below pH 6, and rapid and complete inactivation occurs at pH 3 (94).

In contrast to enteroviruses, most rhinoviruses are thermostable. Types 3 through 12, 15, 18, and 19 are relatively stable at 50°C for 1 hour; types 14, 16, and 17 are partially inactivated; type 13 is completely inactivated (147); and types 1A, 1B, and 2 are variable in their response. One molar magnesium chloride will stabilize most rhinoviruses but will not stabilize type 30. Rhinoviruses are relatively stable at 24°C to 37°C and will survive for hours to days on environmental surfaces (123); they are stable for years at –70°C.

Rhinoviruses are resistant to 20% ether, 5% chloroform, and sodium deoxycholate solutions; they are not destroyed by blending with fluorocarbon (94). A variety of disinfectants are virucidal (91,124). Types 3 through 6 and 14 were shown to be trypsin resistant, but types 1A, 1B, 13, 15, 16, 17, 32, and 33 were trypsin sensitive (148).

Description of Key Antigens

Rhinovirus infectivity can be neutralized by specific antisera. Structural analysis of a monoclonal neutralizing antibody indicated that bivalent binding by antibody is critical for strong neutralizing capacity and that both receptor-binding inhibition and capsid stabilization contribute to neutralization (208,209). Studies with neutralizing monoclonal antibodies against types 2 and 14 revealed three and possibly four or five sites on the contiguous halves of two icosahedral asymmetric units (7,202,203). One and possibly two sites are on VP1 toward the apex of fivefold symmetry, one spans the receptor canyon (see Chapter 23), and one and possibly two are near the base of the asymmetric unit and are on VP1 and 2. In recent studies, it has been shown that antibody may undergo conformational changes and actually penetrate the canyon (206). Although neutralization of virus in *in vitro* testing can occur with monovalent antibody binding, as well as binding away from the receptor canyon, bivalent binding that spans the canyon receptor site with high affinity characterizes the most potent neutralizing antibodies (35,125).

Designation of new serotypes is based on the neutralization reaction; absence of cross-reactivity of polyclonal antisera with established serotypes constitutes the criterion for a new serotype. These criteria resulted in acceptance of 100 serotypes (Table 1), but an unknown number of other serotypes exist. Work with hyperimmune rabbit sera revealed a number of cross-reactions between serotypes and led to the proposal that subgroups of rhinoviruses that share common epitopes must exist (42). The concept of shared epitopes is supported by occurrences of heterotypic antibody responses among humans infected with a number of rhinoviruses (70,159). Thus cross-reactions, some minor and some major, appear to be common among the different rhinovirus serotypes. Sequencing data on types 1A, 2, 14, 16, and 89 rhinoviruses indicate amino acid identity of capsid proteins between serotypes ranging from 41% to 83%; type 14 was least similar to the other four types (172). Sequencing of the genome region that included the major neutralizing antibody–binding site of 21 serotypes revealed at least 70% amino acid identity between serotypes with 84% identity between types 3, 14, and 72 (128).

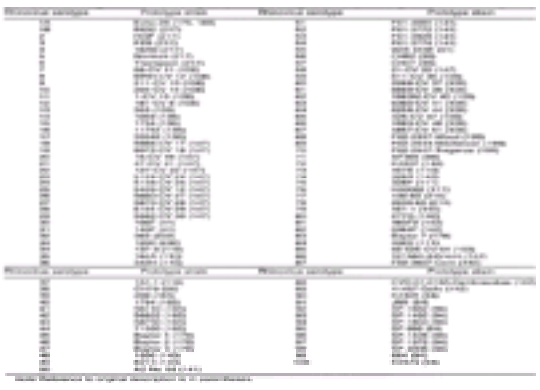

TABLE 1. *Rhinovirus prototypes*

Antigenic variants have been described for rhinovirus types 22 and 51 (200,216). Prime strains for a number of types also have been described (108,200). Prime strain antigenic variants of type 17 emerged from cell-culture passage in the presence of polyclonal antisera, but such variation did not occur for 15 other serotypes treated similarly (174). Description of intertypes further supports antigen variation as a property of rhinoviruses.

Treatment of rhinoviruses at pH 5 and 56°C produces particles that cross-react in immunodiffusion and complement-fixation tests with antisera to other rhinoviruses and with enteroviruses (94). Exposure of these common epitopes appears to result from a conformational alteration of the virus particle. This sharing of epitopes among picornaviruses is in keeping with the demonstration of nucleic acid homology between rhinoviruses and enteroviruses (204,212). Percentage homologies for the capsid proteins VP1 through 4 of type 2 rhinovirus and poliovirus type 1 are 17%, 45%, 49%, and 58%, respectively, whereas those for type 14 and polio 1 are 39%, 49%, 41%, and 58% (204).

Infection in Experimental Animals: Host Range

A major characteristic of human rhinoviruses is a high degree of species specificity, attributable for major group viruses primarily to presence of the receptor ICAM-1. Chimpanzees have been infected with types 14 and 43 and gibbons with types 1A, 2, and 14; no overt illnesses were observed in the infected animals (50,181). Inoculation of vervet and rhesus monkeys with M (monkey kidney grown) strains of virus did not produce infection. Infection was not produced in rabbits, guinea pigs, weanling mice, or 1-day-old mice injected with human rhinoviruses by the subcutaneous, intraperitoneal, or intravenous routes. Similarly, intracranial injections of monkeys, hamsters, or baby mice did not induce either infection or disease (108,148). Intranasal inoculation of ferrets, hamsters, and newborn mice also was without effect. One of the animal rhinoviruses, equine rhinovirus, can infect other species including humans (187); a hamster model for use in screening of antiviral compounds uses this virus. One of the human rhinoviruses, type 2, was adapted to grow in L cells and used in a mouse model of rhinovirus infection in which growth was demonstrated (236).

PATHOGENESIS AND PATHOLOGY

Elucidation of the pathogenesis and pathology of rhinovirus infections has been provided primarily through study of experimentally induced infections in normal adult volunteers. Descriptions of infections and illnesses are similar to those reported for natural colds.

Mechanisms and Route of Transmission

Rhinovirus infections are spread from person to person by means of virus-contaminated respiratory secretions. A clear demonstration of the feasibility of direct-contact transmission of rhinovirus infections was provided in studies using experimental infections (91,95,101,102). High concentrations of virus in nasal secretions and a propensity for ill persons to contaminate their hands and environmental objects with virus were demonstrated. Rhinovirus was recovered from 40% to 90% of hands of persons experiencing colds and from 6% to 15% of diverse environmental objects such as door knobs, dolls, coffee cups, and glasses (101,123). Rhinovirus exhibits good survival on many environmental surfaces for hours after contamination, and infection is readily transmitted by finger-to-finger contact and by finger to contaminated environmental surface if the newly contaminated finger is then used to rub an eye or touch the nasal mucosa (95). Hand transmission was interrupted experimentally by pretreatment of fingers with 2% aqueous iodine (102) or treatment of contaminated environmental surfaces with a phenol/alcohol disinfectant before finger contact (91). In separate observations, Hendley et al. (123) noted that frequent rubbing of the eye or “picking” at the nose represents normal human behavior. Thus there seems little doubt that rhinoviruses may be transmitted by direct contact.

Despite the availability of data supporting the possibility of direct-contact transmission, investigators have had difficulty demonstrating transmission by this route in natural circumstances (46,134). Indeed, one investigator (134) concluded that “spread of colds is unlikely to occur via objects contaminated by the hands of the virus-shedder.” Dick (52) suggested that failure to detect significant airborne transmission of rhinovirus was attributable to insufficient intensity of exposure. He developed a model exposure circumstance in which infected and ill volunteers slept and interacted closely, as in card games (156). There was a direct relationship between donor-hours of exposure and transmission rate to susceptible exposed volunteers in this model situation. Dick then restrained susceptible volunteers so they could not touch their faces (airborne exposure only) and found the transmission rate in this group to be the same as that among unrestrained volunteers (airborne and contact exposure). Exposure of another group to virus-contaminated playing cards did not lead to transmission. Thus airborne transmission of rhinoviruses can clearly occur.

A study of transmission of rhinovirus infection among childless couples, after one partner was artificially infected, showed that conditions associated with most transmissions were (a) presence of high titers of virus in nasal secretions, (b) presence of virus on hands and anterior nares, (c) presence of a moderately symptomatic illness, and (d) spending of many hours together (47).

The study of colds in Antarctica among persons completing 6 months of isolation and those just arrived from the United States has not shown differences in susceptibility of the two groups (231). Moreover, rhinovirus spread among susceptible persons has been slow and deliberate. The authors suggested that their experience supports effectiveness of good ventilation of buildings and proper disposal of nasal secretions in the prevention of spread of colds.

Infection and Illness

Infection can be initiated with less rhinovirus than is detectable in optimal assay systems. The 50% human infectious dose (HID_{50}) for antibody-free volunteers given rhinovirus type 15 by nasal drops corresponded to 0.032 median tissue culture infective dose ($TCID_{50}$) of virus when assayed in human embryonic fibroblast tissue cultures (44). Similar low HID_{50} values were obtained for rhinovirus type 13 (0.1 $TCID_{50}$) (55), type 16 (0.28 $TCID_{50}$) (46), and type 39 (0.4 $TCID_{50}$) (113), whereas the HID_{50} was 5.7 $TCID_{50}$ for a type 14 inoculum (120). The HID_{50} was increased if volunteers possessed prechallenge serum antibody (120). When the same type 15 inoculum referred to earlier was given by small-particle aerosol, the HID_{50} was increased to 0.68 $TCID_{50}$ (44); this 20-fold disparity in infectious dose between nose drops and inhaled particles suggests that the lower respiratory tract is less susceptible to rhinovirus infection than is the nasopharynx. Support for initiation of infection in the nasopharynx was provided by comparative 50% doses for infectious type 16 of 0.28, 1.39, 2,260, and 11,000 for the nasopharynx, anterior nares (internal), tongue, and anterior nares (external), respectively (46).

The incubation period to onset of virus shedding into nasal secretions is 1 to 4 days (58). Virus in secretions will increase rapidly to peak concentrations in most susceptible persons, and after a 2- or 3-day interval, titers will decrease to lower levels (Fig. 2); virus may remain detectable for 3 weeks. Persons with this early-onset high-concentration pattern of virus shedding are likely to develop respiratory illness, whereas persons who have a later onset tend to exhibit a slower increase in titer of virus in secretions and to remain clinically free of symptoms (58). Thus there is a direct correlation between titer of virus in secretions and occurrence, as well as severity of illness. Using biopsy and a method of

processing that ensured that detected virus was intracellular, Douglas et al. (56) also showed that the number of sites in the nasal mucosa where virus could be detected correlates with the titer of virus in nasal secretions at that time, indicating that the titer of virus in secretions is a reflection of the extent of mucosal involvement with infection.

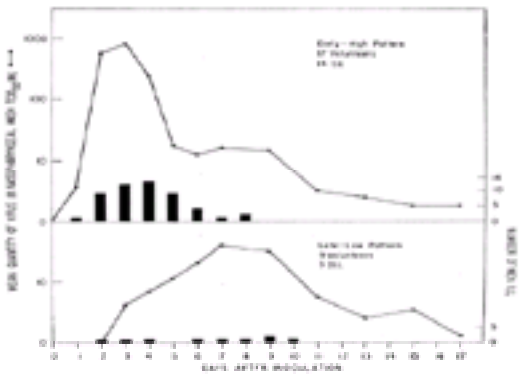

FIG. 2. Temporal quantitative virus shedding in nasopharyngeal wash specimens. Results are means for serum antibody–negative volunteers inoculated by the intranasal route with rhinovirus type 15. Volunteers in the early-high group began to shed virus on day 1 or 2; volunteers in the late-low group began to shed virus on day 3 or later. **Bars:** Number of men ill each day. From ref. 58, with permission.

Examination of nasal mucosa biopsy specimens that yielded virus and nasal polyp organ cultures in which virus replicated to high titers did not reveal histologic abnormalities (56,105,234,235). Nevertheless, virus recovery data (56), demonstrations of specific immunofluorescence and immunoperoxidase staining of columnar epithelial cells in nasal washings from infected volunteers (226), and replication of rhinovirus in ciliated epithelial cells detected by *in situ* hybridization (8,17) indicate that the primary site of infection is the epithelial surface of the nasal mucosa. Morphologic changes were noted in some but not all infected human tracheal organ cultures, but abnormalities were documented (192) in scanning electron microscopy of bovine tracheal epithelium infected with a bovine rhinovirus. Studies of the number of ciliated epithelial cells in nasal secretions from infected volunteers revealed a pattern of increase and decrease that paralleled symptom scores, but a correlation between number of cells in secretions and symptom score on the peak day of cellular shedding was not apparent (223). This absence of histopathology despite presence of virus in the nasal mucosa led to the postulate by Gwaltney et al. that chemical mediators of inflammation might be responsible for nasal symptoms (168).

Explant cultures inoculated with rhinovirus failed to develop CPEs, whereas those inoculated with influenza virus and adenovirus did (234). Full-thickness biopsies of the nasal mucosa during colds revealed marked edema of subepithelial connective tissue with sparse infiltration of inflammatory cells. Hyperemia, exudation of seromucinous fluid, and narrowing of nasal passages are known to accompany the histologic changes. During the height of illness, there is considerable transudation of serum proteins into nasal secretions (26), and this is accompanied by impaired mucociliary flow rates (198). An increase in kinins, enzymes with arginine esterase activity, albumin, and neutrophils has been detected in nasal washings from those who developed experimental or natural colds (168,190). There was no change in histamine levels in those studies. These studies supported the notion that kinins may induce the edema and transudation that partially accounts for the symptoms of the common cold. Nevertheless, a reduction in kinin levels that occurred during treatment of common colds with oral steroids was not accompanied by a reduction in symptoms (90). Search for other mediators of the pathophysiologic changes noted led to identification of elevated levels in nasal secretions of interleukin-1b (IL-1b), interleukin 6 and 8 (IL-6, IL-8), and tumor necrosis factor a (TNF-a) (218,221,225,237,238). IL-8 and other cytokines also have been detected in rhinovirus infection of primary and continuous bronchial epithelial cell lines (20,137,189,201,218,237). IL-8 is proposed as the mediator promoting neutrophil infiltrations into submucosal and epithelial sites, a finding in both nasal secretions and nasal biopsies of common cold patients. There is a correlation between IL-8 levels, neutrophils in nasal secretions, and severity of colds (225,234). Nevertheless, the relative importance of IL-8, other cytokines, neutrophils, other cells and kinins in the pathophysiology of common colds is yet to be determined, but the absence of histopathology in the nose strongly supports the notion that some constellation of mediators and neurogenic reflexes accounts for the physiologic abnormalities characterizing common colds.

Analysis of colds among experimentally infected subjects indicated that psychosocial factors influenced responses (37). Persons who had experienced stress and displayed mood alterations were more susceptible to infection and experienced more illnesses, whereas those with diverse social ties were more resistant. Such factors could influence symptom reporting as well as release and response to physiologic mediators.

Colds are more common in temperate climates during the colder months of the year, and a commonly held belief is that cold exposure and chilling will “cause a cold.” Early studies on the effect of “chilling” on causing colds and increasing susceptibility to virus challenge failed to detect evidence of an effect of cold (5). In a systematic approach to the question, Douglas et al. (61) examined the effect of chilling and transient lowering of body temperature on susceptibility to rhinovirus type 15 infection, on development and severity of illness, and on the pattern of recovery. No effect of either chilling or lowering of body temperature on the different phases of a rhinovirus cold was noted. Thus modern studies have failed to find an effect of cold and chilling on the development and course of colds.

The major site of symptoms, the nose, also is the major site of infection with rhinoviruses. Virus may be recovered from throat and oral secretions but never as frequently or in as high a concentration as in nasal secretions (30). A rare recovery of rhinovirus may be obtained from rectal swab or fecal specimens, but direct inoculation of the small intestine of volunteers with large doses of virus failed to result in establishment of infection (31). This finding indicates that the primary reason for the difference between rhinoviruses and enteroviruses in occurrence of intestinal infection does not

reside in destruction of rhinovirus during passage through the acid environment of the stomach. Viremia is not detected in healthy adults with colds, but viremia with a rhinovirus was reported in two infants who experienced unexpected crib deaths (230).

Rhinoviruses also can infect the lower respiratory tract and thereby account for lower respiratory symptoms. Rhinovirus inoculations of volunteers by small-particle aerosol has produced tracheobronchitis (29); rhinovirus is the only virus recovered from some infants with pneumonia; natural colds in healthy persons are often accompanied by lower respiratory symptoms and abnormal lung function (80); and colds may cause wheezing in asthmatic children (157) and exacerbations of chronic bronchitis in adults (213). These findings all suggested that infection of the lower respiratory tract can occur. Direct virologic evidence for growth in the lower tract was found recently in biopsy and bronchoalveolar specimens from infected volunteers (18,84) and immunocompromised patients (85).

Considerable effort has been devoted recently to defining the role of rhinovirus infection in lower respiratory disease and episodes of asthma and breathlessness in patients with chronic lung diseases. These studies have indicated that the majority of asthmatic attacks and a significant portion of exacerbations in patients with cystic fibrosis and chronic obstructive lung disease (COPD) occur in association with a viral respiratory infection; rhinovirus infections predominate in these episodes (15,25,138,139,171,191,210). Additionally, rhinovirus infections in elderly patients exhibit a high frequency of lower respiratory symptoms (169). Acute tracheobronchitis, a lower respiratory disease syndrome, was reported among healthy volunteers given rhinovirus 1A by small-particle aerosol in 1965, whereas similar inoculations with rhinovirus 15 did not exhibit this syndrome (44). The combination of clinical reports and evidence of infection of lower respiratory tract epithelium indicates that rhinovirus infection and disease in the lower respiratory tract can occur. Nevertheless, the infrequency of significant lower disease in healthy persons supports occurrence of this disease as primarily a complication experienced by certain populations. A possibility of risk for lower tract disease only accompanying certain strains was suggested by studies of comparative growth at 34°C and 37°C, in which some serotypes grew well at both temperatures, but others did not (173).

A major effort has been devoted to elucidating the pathogenesis of rhinovirus-induced attacks of asthma. Repeated demonstrations have been provided of enhanced responsiveness of the lower airways to histamine and acetyl choline analogs among rhinovirus-infected persons (36,71,78,82,89). In addition, inflammation in the lower airways, a cardinal feature of asthma (104), was documented in bronchial biopsies in rhinovirus-infected asthmatics (78). Notable in studies focused on detecting parameters of allergic disease (eosinophil infiltration, eosinophil cationic protein, evidence of an atopic state) has been their frequent identification in wheezing rhinovirus-infected asthmatics (78,81,88,136,186,191,219). An increase in neutrophils and IL-8 also has been noted in sputum of asthmatics (89,136,186). Thus evidence supports the notion that rhinovirus infection of the lower respiratory tract can lead to lower tract disease in persons with an increased risk for such an illness because of an underlying condition.

Rhinoviruses also have been recovered in pure culture from maxillary sinus fluids collected from persons with acute sinusitis (182), and Gwaltney et. al. (103) have demonstrated extensive abnormalities of the upper airways, including occlusion and abnormalities in sinus cavities during colds. Similarly, rhinoviruses have been recovered in pure culture from the middle ear of young children with otitis media (185). These findings have led to the proposal that rhinovirus infection can be described as a rhinosinusitis (93) and that otitis media in rhinovirus-infected children could result from rhinovirus infection of the middle ear (183).

Immune Responses

Neutralizing antibody to the infecting virus develops after infection in serum and secretions of the majority of persons. Among infected volunteers, detectable serum antibody usually developed between 7 and 14 days after inoculation, but in initially antibody-free volunteers, it might first be detected on day 21 (33). Reported frequencies of responses for natural infections have been as low as 37% and as high as 92%. With a sensitive assay using human tissues, Gwaltney et al. (97) documented an increase in serum antibody in 77% of persons from whom a rhinovirus was isolated. Fox et al. (75,76 and 77) indicated an effect of age on responsiveness because 59% of adults in their virus watch studies developed a response, whereas only 42% of children did so. Studies using experimental infections and low doses of virus in serologic tests usually documented a response in more than 90% of volunteers (30).

Using an enzyme-linked immunosorbent assay (ELISA), Carey et al. (28) indicated that the dominant serum antibody response to infection was in the immunoglobulin G1 (IgG1) subclass, followed by IgG3, IgG4, and IgG2; the dominant IgA antibody was IgA1. With blocking monoclonal antibodies, they showed that the serum antibody response was directed toward each of three separate sites. Neutralizing antibody responses in nasal secretions also develop in most persons infected with a rhinovirus, and the time of appearance parallels that for antibody in serum (33). Studies in experimentally infected volunteers have revealed such responses in 70% to 100% of infected persons (33,62). Antibody in secretions mostly sediments in the 9S to 14S region along with IgA, although 7S IgG and 19S IgM antibody may be detected (196).

During the illness phase of an acute rhinovirus infection, Butler et al. (26) documented a considerable transudation of serum proteins into secretions; if present before infection, this transudation will include specific IgG antibody. After the acute illness period, transudation ceases, and then the concentration of IgA progressively increases. By 7 days, this increase may be twice that detected in baseline specimens, even through specific antibody is not detectable for an additional 7 to 14 days. Douglas et al. (62) showed that neutralizing antibody also developed in tears and parotid saliva, even though no virus shedding in those secretions is detectable. Neutralizing antibody in serum and secretions may continue to increase in titer for 4 to 5 weeks after primary infection (33).

Serum antibody persists for at least 2 to 4 years after natural infections, but a decrease in titer of two- to 16-fold will occur in some persons; it is probable that antibody persists for many years. Measurements of nasal immunoglobulin and rhinovirus antibody among persons placed in Antarctic isolation revealed a decrease for a period of 5 months despite a stability of serum antibody titers during this time (127). Disparities in serum and secretion antibody ratios at the time of rhinovirus challenge (33) indicate that a disproportionate decrease in titer must have occurred for rhinovirus antibody in

secretions. Cate (33) reported a ratio of neutralizing antibody titer in nasal secretions to titer in serum of 1:2 after recent infection but a ratio of 1:16 remotely after infection. Nevertheless, the titer of antibody in serum always correlates with the titer in nasal secretions whether acquired by experimental or by natural infection. Other serologic methods have been used for detecting antibody to rhinoviruses, but none has been studied systematically.

Because of the late appearance of antibody in serum and secretions, it is believed that recovery from infection and illness is not dependent on its appearance (33). This belief is supported by the seemingly normal recovery exhibited by persons with isolated IgA deficiency and by those with hypogammaglobulinemia. Interferon is detectable in nasal secretions of one third of infected volunteers (32); it is usually present 1 or 2 days after the peak virus titer and therefore may play a role in recovery. However, it is detected only in those volunteers with highest titers of virus and most severe illness; for this reason, many believe other recovery mechanisms may be more important. Nevertheless, administration of interferon before challenge with rhinoviruses can prevent or reduce the intensity of infection (197). Cessation of virus shedding is directly related to time of first detection of neutralizing antibody in nasal secretions, suggesting that antibody accomplishes final clearance of infection (33).

Gern (83) isolated virus-specific clones of responsive lymphocytes and showed that cells from most persons are activated by a number of serotypes, indicating shared epitopes by different rhinoviruses. T cells from tonsils of children also responded to multiple serotypes; responses were by $CD4^+$ cells, and T_H1-type cytokines were released (233).

Immunity

Epidemiologic studies have revealed an increase in frequency of serotypes to which serum antibody is detectable with increasing age. Analysis of 184 sera from persons of various ages revealed an increase throughout childhood and early adult years, with a plateau after 35 to 40 years (107). This reduction corresponds to a decrease in infection rates among older persons. It seems likely that these findings are partly attributable to a reduced exposure to children in the home, but immunity to reinfection is probably operative as well.

Rates of rhinovirus infection and illness are lower among those with preexisting antibody than among those free of detectable antibody. In an early study among families in Wisconsin, the rhinovirus infection rate for the group with serum antibody was half that of the group with no antibody (46). About 20% of Seattle family members possessed preexisting serum antibody to the serotype producing infection in the family; the infection and illness rates were reduced by about 50% in this group (75,76). Similar reductions in infection frequency were noted among college students and military personnel with preexposure antibody.

A clear indication that homologous immunity develops after a rhinovirus infection was provided in studies using artificial challenge among volunteers. Increasing titer of preexisting serum antibody was accompanied by reduced frequency of infection and illness (30). When rechallenge was performed 1 month after infection, however, protection was greater than that seen with antibody acquired by a prior remote infection (30). This led to a serial evaluation of challenges with homotypic and heterotypic virus, which revealed a transient, nonspecific resistance to rechallenge that was manifested by a reduced frequency and magnitude of infection (69). Whether this represented transient cross-reacting antibody protection or a nonspecific effector mechanism was not determined. Nevertheless, protection against homologous reinfection was better early after infection than late after infection.

The probable natural history of antibody responses to rhinovirus infection is described in Fig. 3. As noted in the first panel, the titer of antibody in nasal secretions was about half that of serum antibody early after infection; at this time, immunity to homologous rechallenge had been complete. As indicated in panel 2, however, another group of volunteers with a similar serum antibody level but much lower nasal secretion antibody titer was reinfected. The third group of volunteers who resisted infection had higher serum antibody titers, and their nasal secretion antibody titers were similar to those seen early after infection in the primary infection group. The current prevailing concept of immunity is that both nasal secretion and serum antibody increase after primary infection, and in the first few months, antibody in secretions prevents reinfection. Antibody in secretions decreases with time, and renewed susceptibility results, so that reinfection occurs (panel 2). Eventually, sufficient antibody is present in both sites to prevent reinfection.

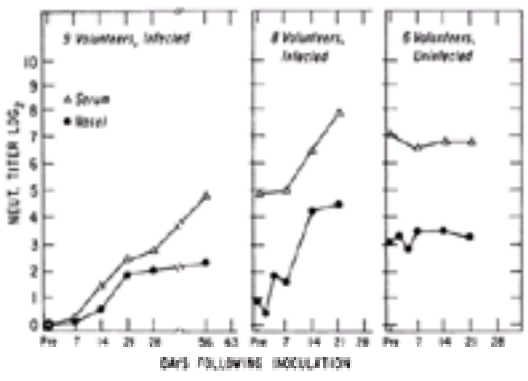

FIG. 3. Geometric mean neutralizing antibody titers against rhinovirus type 15 of nasal wash and serum specimens collected before and after inoculation with this rhinovirus. From ref. 33, with permission.

Attempts to prove that the IgA antibody in secretions is the mediator of resistance to infection have not produced clear results. Serum and nasal-secretion antibody titers correlate with each other, and both correlate with resistance to infection. To dissociate the two antibodies, volunteers were given intranasal or intramuscular vaccine with an inactivated rhinovirus type 13 vaccine and then challenged with infectious type 13 virus (176). A significant reduction in frequency of

illness and virus shedding was seen only among those given vaccine intranasally; in addition, antibody titers in secretions significantly related to occurrence of illness, whereas antibody in serum did not. A clear relation to prevention of infection was, however, not detected. Although quantitation of virus was not a part of that study, these results are best interpreted as showing partial but incomplete immunity attributable to IgA antibody.

Other data, however, support a primary role for IgA in resistance to rhinovirus infection. Studies on synthesis have indicated that more than 95% of this immunoglobulin is produced by local submucosal B lymphocytes. Concentrations of IgG in nasal secretions are only about one-tenth those of IgA, and about 50% of this IgG is produced locally. Thus although definitive data have not been provided, the primary mediator of resistance to rhinovirus is likely to be specific IgA antibody in nasal secretions. During acute illness, there is a considerable transudation of serum proteins, including serum IgG, into secretions, so a contribution of serum antibody to modifying an established infection and any accompanying illness is possible.

EPIDEMIOLOGY

Relation to Age, Sex, Season, and Location

Rhinovirus infection rates are highest among infants and children and decrease with increasing age. Actual virus isolation rates for a group of Seattle families and insurance company employees in Virginia are shown in Table 2. Such rates depend on sensitivity of methods used and reflect minimal estimates. Rates of rhinovirus infections among infants are thought to be highest because of the extreme susceptibility of this age group to any rhinovirus brought into the household. The mean rate for Seattle families was 0.68 infections per person-year (75); rates were intermediate among young children and lowest among mothers. Rates in Virginia for the same age group as mothers in Seattle were higher, although rates in that study also decreased with increasing age (96). Adjustment of the Seattle rates to reflect the Virginia rates would indicate that infants and small children average more than one rhinovirus infection per year. Rates for young women in Virginia and in a Tecumseh, Michigan, survey were higher than those for men of similar age (96,164). A greater exposure to young children is thought to account for this difference.

Population group	Age (yr)	Mean isolations per person-year	Mean illnesses per person-year[a]	
Seattle families (52)	0–1	0.84	0.75	
	1–2	0.64	0.87	
	2–5	0.64	0.74	
	6–9	0.64	0.31[b]	
	Mothers	0.25	0.54	
	Fathers	–	0.35	
			Males	Females
Charlottesville working adults (65)	18–25	0.56	0.47	0.60
	26–35	0.59	0.48	0.52
	36–45	0.57	0.69	0.53
	45+	0.39	0.40	0.26

[a]Includes all family-episode illnesses for Seattle families.
[b]Ages 6–19 years.
Source: From refs. 76 and 96, with permission.

TABLE 2. *Relation of age to rhinovirus isolation and illness rates*

Rhinovirus illness rates also are highest for small children and decrease with increasing age (see Table 2). Estimates of rates for Seattle families were near 0.8 per person-year for young children and 0.54 for mothers. The illness rates for mothers in Seattle and women of comparable age in Virginia were similar and were greater than those for young men. Compensation for this higher illness rate among young women in early adult years appears to ensue in later years, when illness rates are higher among men (96).

When compared with respiratory illnesses caused by other viruses, a general mildness of rhinovirus illness is reflected in a low percentage of persons seeking health care for illness. In the Michigan community surveillance, rhinoviruses represented 38.5% of viruses isolated from all acute respiratory illnesses but only 23.3% of illnesses that caused a person to consult a physician (164).

Rhinoviruses are isolated from persons experiencing respiratory illness in temperate climates in all months of the year. However, studies in a variety of populations in various parts of the U.S. indicate an increased frequency in early fall and late spring (75,76,96,162,164). Increased prevalence in the fall was noted in each of 4 surveillance years in Seattle, Washington, although an increase was noted during spring in only 2 of 4 years (75). The same fall and spring prominence was noted in Charlottesville, Virginia, but an increase during fall months was most common (96). Rhinovirus isolations may be prominent during winter months, but this appears to be uncommon; although present, prevalence rates are regularly lowest in summer.

Rhinoviruses have been clearly demonstrated as common causes of colds in the U.S. and in Western Europe. Although epidemiologic studies associating rhinoviruses with respiratory illness in other parts of the world are limited, serum antibody prevalence data have indicated that rhinovirus infections are widespread throughout the world (22). A 29-month surveillance of young children in an impoverished urban population in Brazil revealed a high incidence of respiratory illness and a dominance of rhinoviruses in specimens from the children (12).

Morbidity in Specific Population Groups

Families

The family unit is established as a major site for spread of rhinoviruses in contemporary society. Considering the index case as the introducer among families in Seattle led to a conclusion that the highest rate of introductions was attributable to young children and primarily to those younger than 2 years (75,76). Introductions, their rate, and the calculated relative risk for this event indicated that 50% of introductions were by children younger than 2 years old and about 75% were by preschool children. The basis for such a high frequency for infants and small children is uncertain but is believed to reflect their high degree of susceptibility, so that exposure to infected baby-sitters, friends of the family, and fomites contaminated by playmates or siblings results in a high infection rate. In a smaller group of working families in Virginia, the school-aged child was a more common introducer, and adults also were prominent. Family size is directly related to number of family episodes of rhinovirus infections (75).

Secondary infections within the family are most common among young children and mothers but occur in all members of the household; these infections appear at 2- to 5-day intervals, but most will appear within a few days of onset of illness in the index case (75,76). Secondary attack rates in the family have varied between 30% and 70%, but most studies have revealed rates of about 50%. When antibody-free (susceptible) members only are considered, secondary rates of 70% are common. Secondary infection rates in families decrease with increasing age and are higher among mothers than fathers, presumably because of their close exposure to children in the family.

Characteristics of the introducer influence the secondary attack rates in families. Among Seattle families, the secondary attack rate was 71% if the introducer was ill but only 27% if he or she was well, a finding similar to that noted for spread between spouses (75).

Schools

Efficient spread of rhinoviruses among children in a nursery school was demonstrated by Beem (19), who detected rhinovirus infections more commonly than all other respiratory virus infections combined. Rhinoviruses clearly spread among university students, medical students, boarding school populations, and probably other groups in dormitory-type situations who have little or no contact with children (100,111,112,158). The manner of spread of rhinoviruses and features of their epidemiologic behavior in these populations have not been described.

The simultaneous occurrence of respiratory illness peaks among children and adults, as well as among working adults with and without children, suggested that something other than opening of school accounted for the fall increase (122). Attempts to identify a correlative meteorologic event were not successful. Nevertheless, once infected, children were instrumental in introducing rhinoviruses into families.

Military

Rhinoviruses cause a major portion of afebrile respiratory disease in military populations. The season of peak occurrence has been more varied for this population (21,158). In a prospective study among marine and navy recruits for a 4-week period in November, Rosenbaum et al. (195) reported an attack rate of almost 90%, with many recruits experiencing two infections during this interval. Accompanying illness was typically an afebrile upper respiratory illness, and this occurred an average of 1.2 times per person during the 4-week period. This extraordinary attack rate has not been reported by others; relative frequencies of antibody to rhinoviruses were similar among university students and a population of young airmen, suggesting that rhinovirus infection rates relate better to age than to population group (158).

Epidemiology of Serotypes

Initial observations on serotyping of rhinovirus isolates indicated that a multiplicity of serotypes existed (95), that surveillance of a population for several months would result in isolation of several distinct types, and that prevalent serotypes would change from year to year. This multiplicity of serotypes of presumed medical significance provided the primary basis for the belief that conventional vaccination approaches for prevention of rhinovirus illness were not reasonable. Nevertheless, it was noted in early reports that a small number of the serotypes caused most of the illnesses. In the nursery school population described earlier, 14 serotypes were detected in a 10-month period, but only seven of the 14 spread to another child, and only three spread extensively. Studies in other populations also suggested a varying degree of transmissibility for the different serotypes. Mogabgab (159) noted that only eight of 37 serotypes recovered from military and university students accounted for 81% of 301 isolates. In New York families, 11 of 39 serotypes accounted for 55% of isolates; in Seattle families, 15 of 59 serotypes accounted for 57% of 456 isolates; and in Virginia industrial employees, 10 serotypes accounted for 53% of 197 isolates (74). Although an Antarctica population was equally susceptible to two different rhinoviruses, only one spread in the population (231). Characterization of rhinovirus serotypes in subsequent surveillance periods (76,160) has continued to show prevalence of a limited number of serotypes. However, only some of the prevalent types were prevalent in earlier surveillance periods, so that the number of serotypes producing most disease may still be large. Andries (6) systematically evaluated capsid-binding antivirals against all serotyped rhinoviruses and identified two cluster groups on the basis of antiviral sensitivity; one group contained 33 serotypes, and the other contained 67 serotypes. Viruses within a group exhibited sequence similarities for viral protein I (VPI) and the antiviral binding site. Published data for 1,018 isolates suggested a natural occurrence of infections with the 67-serotype group at 2.37 times the expected rate.

A change in distribution of serotypes recovered over a 7-year period in Virginia consisted of an increase in higher-numbered serotypes and in nontypeable strains with time (27). Analysis of serotypes occurring at New York, Seattle, and Michigan during this same period also suggested antigenic variation (74). Antigenic characterization of later isolates, however, indicated that rhinoviruses identified earlier were still prevalent. Antisera to types 1 through 89 identified 15 (94%) of 16 isolates in Boston in 1982 through 1983, 752 (95%) of 790 isolates in Seattle in 1975 through 1979, and 194 (93%) of 209 isolates in Tecumseh, Michigan, in 1976 through 1981 (108).

Prevalence

Illness induced by rhinoviruses probably represents the most common acute infectious illness of humans. National Health Survey data for the U.S. indicate that acute respiratory illness accounts for about half of all acute conditions on an annual basis. Of absenteeism among insurance companies at Charlottesville, Virginia, 36% was attributable to respiratory illness; in a group of Seattle families containing young children, 86% of all illnesses over a 4-year period were respiratory. The most commonly isolated viruses from persons experiencing acute respiratory illness are rhinoviruses. The prevalence of these viruses among persons in disparate populations in the U.S. who were experiencing acute respiratory illness indicates that a greater frequency of illness is caused by rhinoviruses than by other common viral respiratory pathogens. With conventional isolation tests, 42% to 77% of isolations were rhinoviruses (19,21,66,111,158,161,166). Estimates of the proportion of acute respiratory illnesses caused by rhinoviruses varied between 10% and 40% among various populations. These varying frequencies reflect the population studied, period of observation, and methods used. In recent reports, addition of the PCR for detecting rhinoviruses has increased the rhinovirus proportion from 20% to as much as fourfold (10,15,129,140,152). Because serology for rhinoviruses is not used in most surveillance studies, it is reasonable to suggest that one third to one half or more of all acute respiratory illnesses are caused by rhinoviruses.

CLINICAL FEATURES

Primary Illness

The typical illness accompanying rhinovirus infection is a common cold. Predominant symptoms are sneezing, nasal obstruction, nasal discharge, and sore throat, but other prominent symptoms are headache, cough, and malaise. The percentage of persons in a variety of different populations who reported various symptoms during a rhinovirus infection is shown in Table 3. Notable is the fact that, despite a prominence of symptoms in the upper respiratory tract, symptoms referable to the lower respiratory tract, gastrointestinal system, and other organ systems are not uncommon. Fever varies in frequency of occurrence but is generally seen in no more than 10% to 20% of persons (see Table 3). Physical examination usually confirms the presence of nasal obstruction and discharge and reveals a hyperemic or pale edematous nasal mucosa. Minimal erythema of the pharynx and tender cervical nodes also may be present, but otherwise abnormal findings are not usually detected (30). Nasal secretion weights in experimental colds averaged 8.5 g for the maximal period of rhinorrhea but varied between 0 and 85 g (30). An elevation in neutrophil and a decrease in lymphocyte counts are seen in peripheral blood leukocytes on the first day of illness, but counts return to normal in 1 or 2 days (30). About one third of experimentally infected volunteers developed a transient elevation in erythrocyte sedimentation rate.

Symptoms	Percentage reporting symptoms				
	Military recruits	Health care employees	English schoolboys	Industrial employees	Children OPD pts.
Nasal obstruction and/or discharge	90	97	100	90	67
Sneezing	49				
Eye irritation	31		17	10	7
Sore throat	71	66	61	50	33
Cough	86	71	39	40	100
Chest pain	50				
Fever	22	6	11	20	60
Chills	45	17	22		
Headaches	62	34	61	60	7
Vomiting	14			5	33
Abdominal pain	13		17	0	7
Diarrhea	12			5	
Anorexia	43			5	7
Myalgia	25	17	28		
Reference	(75)	(166)	(145)	(104)	(104)

OPD, outpatient department patients.

TABLE 3. *Symptoms associated with rhinovirus infections in persons from whom virus was isolated*

About two thirds of antibody-free volunteers will develop an illness with an experimentally induced infection (30,44,58), and similar percentages of naturally occurring infections will be accompanied by illness. Illness occurred in 63% and 60% of infected family members (75,146), 69% of infected employees of an insurance company (96), 88% of infected medical students (111), and 74% of infected military recruits (21). Frequency of colds among infected persons appears unrelated to age.

Illness is maximal for 2 or 3 days; then symptoms progressively improve. The median duration of illness in the insurance company population listed earlier was 7 days, with symptoms persisting for 2 weeks in 25% of persons and occasionally for as long as a month. In cigarette smokers, the frequency of cough was increased, and the duration of illness prolonged (97).

Complications

Colds are believed to predispose to development of sinusitis and otitis media because persons with these latter diseases frequently give a history of a simultaneous or preceding upper respiratory illness. Gwaltney (103) showed sinus disease as frequently present in persons with clinical colds only, with involvement of maxillary and ethmoid sinuses most common. For this reason, he considered rhinovirus colds to be cases of acute rhinosinusitis. Detection of rhinovirus in middle ear and sinus fluid and other studies associating rhinovirus with otitis media in children argue strongly for a role of rhinoviruses in this complication (182,184,185). Rhinovirus isolations were present in 6% to 8% of sinus aspirates of adults with acute sinusitis, but reverse transcription-PCR (RT-PCR) testing of sinus brushings in one study revealed a rhinovirus in 40% of specimens (183,186). Similarly, abnormalities in middle ear pressure are common in persons experiencing colds (24,64), and rhinovirus isolations have been obtained from middle ear fluid of children with clinical

otitis media (179). When RT-PCR testing was applied to such fluids, 19% and 24% of specimens were positive for rhinovirus (184,185).

Acute bacterial sinusitis or otitis media is presumed to result from obstruction of the orifice of the eustachian tube or of a sinus, owing to the local edema accompanying virus infection, a finding demonstrated among infected and ill volunteers (154). Specimens from persons with acute sinusitis or otitis have yielded virus only, bacteria only, and both (182,185). The role of each or both in disease is not yet clear.

Lower respiratory disease has been reported to occur more commonly among infected healthy infants and children than among infected adults (194), and rhinoviruses have been associated with atypical pneumonia in airmen (158) and recovered from the lung and lower respiratory tract of immunocompromised persons with pneumonia (45,85). Rhinoviruses are commonly isolated from young children experiencing serious lower respiratory disease, including pneumonia (2,155), and have been reported to occur in up to 40% of exacerbations of chronic bronchitis (213), and 16% to 18% of exacerbations in children with cystic fibrosis (183,210).

Special attention has been given recently to respiratory viral infection as a cause of exacerbations of asthma. Most cases are associated with a respiratory viral infection, and rhinovirus infections are detected most commonly (15,138,139,157,171,191). Asthmatic children also reportedly experience more rhinovirus infections than their siblings, and illnesses last longer. In a study of wheezing among children, rhinovirus infection was associated with 71% of episodes in children aged 2 to 16 years, and comparisons of wheezing children with contacts indicated a significant risk for wheezing if rhinovirus-positive cases also were positive in radioallergosorbent tests (RASTs) for allergen-specific IgE, demonstrated nasal eosinophilia, or elevations in eosinophil cationic protein (191).

DIAGNOSIS

Clinical

The typical constellation of signs and symptoms leading to diagnosis of a common cold is well known. Although many symptoms may be reported, nasal obstruction and/or nasal discharge must be present and be the dominant symptoms. When contrasted to colds caused by other viruses, no distinctive features of colds caused by rhinoviruses emerge that permit an etiologic diagnosis from clinical findings alone. The epidemiologic setting of the illness may, however, permit a presumptive diagnosis of rhinovirus common cold. Features suggesting a rhinovirus etiology are occurrence of a cold in early fall or late spring, particularly in an older child or adult while schoolmates, playmates, or family members are experiencing similar illnesses. Rhinoviruses may cause other acute respiratory illness syndromes, but the predominance of other viruses in these syndromes makes a rhinovirus etiology unlikely.

Laboratory

Viral Isolation and Identification

Specimens containing nasopharyngeal secretions are best for virus isolations. Oral secretions and sputum specimens (when available) are low-yield specimens. Rhinoviruses are rarely detected in rectal swab or stool specimens.

Studies in volunteers indicated virus was present in highest concentrations in nasal secretions, and maximal isolation frequencies were obtained on day 1 or 2 of illness (30,58). Virus was isolated from more than 50% of specimens obtained from persons with naturally occurring rhinovirus colds between the day before and 6 days after onset of illness, but maximal frequencies were on the first five illness days (75).

Isolation of rhinoviruses requires use of cell cultures. No experimental animal is suitable, and growth does not occur in embryonated hen's eggs. Cell cultures of human origin are preferred; human embryonic fibroblast cell cultures and rhinovirus-sensitive lines of HeLa cells have been used most extensively (see Propagation in Cell Culture). Organ cultures of nasal and tracheal epithelium are required for growth of some fastidious rhinovirus strains, but routine use of such cultures is impractical (126).

Comparisons of the rhinovirus-sensitive line of HeLa cells and various sources of human embryonic fibroblasts have not shown a clear superiority of a single tissue culture for isolations (10,43,150). Although the optimal tissue culture for rhinovirus isolation is unclear, most investigators use either the WI-38 or MRC-5 strains of human embryonic fibroblasts. Use of more than one type of cell culture is required for optimal isolation. Addition of antiinterferon antibody to 256 specimens obtained from children with respiratory illness led to isolation of a rhinovirus from 40 specimens, whereas only 19 were positive when this antibody was not present (79).

Tissue cultures are observed frequently for CPEs (see Propagation in Cell Culture). CPE is usually detected in the first week and is frequently detected the second or third day after inoculation of cultures. When a culture system such as WI-38 cells is used, observation beyond 10 to 14 days is not necessary.

Presumptive identification of an isolate as a rhinovirus may be made on the basis of the tissue cultures supporting growth, the pattern of CPE, and demonstration of lability at pH 3 (94). A PCR assay using specific primers is an acceptable alternative to an acid lability test for differentiating between rhinoviruses and enteroviruses (14). Definitive identification requires neutralization of 32 to 100 $TCID_{50}$ by specific antisera. Although described for only a few serotypes, typing was accomplished with PCR followed by restriction enzyme analysis of a 380-bp fragment from the 5' region and by hybridization with 3' region probes (73,220).

Direct Detection of Rhinoviruses

Dearden (49) described an ELISA assay using rabbit hyperimmune serum to type 2 and an untyped strain as both a capture and detecting antibody. A degree of cross-reactivity of antigen was present, suggesting that a single system could detect about two thirds of 57 different serotypes. Sensitivity appeared high, and amplification overnight in tissue culture appeared to provide some advantage. Use of the assay for direct detection of type 2 virus in secretions from infected volunteers revealed a sensitivity greater than virus isolation (3). The same group also described use of oligonucleotide probes for detecting rhinoviruses in clinical samples (23).

Reports from a number of sources have now documented the fact that use of RT-PCR for detecting rhinoviruses invariably leads to higher numbers of proven infections (13,15,129,140,170). The increased proportion attributable to PCR has varied considerably, presumably because of varying sensitivity of the culture and PCR methods used.

Serologic Diagnosis

The multiplicity of rhinovirus serotypes and the relative lack of cross-reacting antigens prevent routine use of serologic means for diagnosis of rhinovirus infection. A variety of serologic methods have been described, however, and may be used for a serologic diagnosis if the infecting serotype is known. Neutralization tests are sensitive for detecting a serologic response, provided low doses of virus (6–16 $TCID_{50}$) are used in the tests. Both macro- and microneutralization tests that use tissue cultures have been described (94). Plaque-reduction neutralization tests also have been described for the latter procedure; a purified agar or methyl cellulose overlay is needed for plaque formation, and size is enhanced by addition of magnesium and DEAE.

A description of hemagglutination of sheep erythrocytes by certain rhinoviruses led to the development of a hemagglutination-inhibition test for serum and nasal secretion antibody (193). Correlation of titers with those detected in neutralization tests was good, but seroconversion was detected in fewer than half of volunteers who shed virus. An indirect hemagglutination-inhibition test also has been described (94). Although complement-fixing (CF) rhinovirus antigens have been described, use of the CF tests for detecting antibody in humans resulted in frequent detection of responses to heterotypic rhinoviruses and detection of responses after enterovirus infections (94). Moreover, CF antibody did not parallel neutralizing antibody titers. A sensitive ELISA assay using antibody-captured rhinovirus as antigen can detect IgG and IgA in serum and IgA antibody in unconcentrated secretions (16).

PREVENTION AND CONTROL

There are no established methods for effective prevention or treatment of rhinovirus infections. A variety of approaches have been evaluated, most with little or no effectiveness. Nevertheless, certain principles emerging from these studies provide direction to development efforts.

Nonspecific Measures

The commonness of common colds accounts for the multitude of approaches suggested to prevent or treat colds by lay-persons, scientists, and physicians. These approaches include various medicaments, nutritional supplements, and recommendations of various types of human behavior that supposedly enhance host resistance. None of these is accepted as being of proven value for prevention or treatment of colds.

In the card-game exposure situation described earlier (see Transmission), transmission was completely prevented when citric acid/malic acid–impregnated tissues were used frequently, and donors were prompted to blow their noses gently and smother all coughs and sneezes. The need for the chemical impregnation was not established, so it is possible that attention to the hygienic measures alone would be effective for preventing spread. Similarly, the reduction in transmission in the home with use of iodine on fingers supports the view that hygienic measures would be beneficial (121).

A number of studies have been performed on the effect of various treatments for rhinovirus colds. Included have been analgesics, antiinflammatory compounds including oral and topical steroids (1,65), decongestants (205), anticholinergics (92), antihistamines (151), vitamin C (34,118), zinc lozenges (132), extracts of *Echinacea* (87), and inhalation of hot humidified air (119). Beneficial effects on symptoms have been described, and it is accepted that reductions in rhinorrhea, nasal obstruction, cough, and so on can be achieved with use of some of these medications.

Vaccines

Monovalent and multivalent inactivated vaccines have been evaluated against artificial challenge in volunteers (60,177). A problem with such vaccines appeared to be antigenic dose, because the systems used for preparation of viral antigen did not yield doses comparable to those used for poliovirus vaccines. Nevertheless, a few potent preparations induced titers comparable to those after natural infection (60,177); reductions in virus shedding and severity of illness resulted, but neither infection nor illness was prevented. These findings contributed to concepts of the relative significance of serum and nasal secretion antibody in immunity to rhinovirus infection and illness described earlier, because little or no secretion antibody appeared after intramuscular immunization.

A live-attenuated strain of rhinovirus type 15 was developed, and differing plaguing properties of the wild parent and the attenuated progeny were described (59). Use of attenuated viruses for immunization would require use of multivalent live preparations. Despite some promise, the live-virus vaccine approach has not been pursued.

Problems for all immunization approaches are the multiplicity of serotypes and the suggestion that most have epidemiologic significance (160).

Antivirals

Because of the problems with vaccination as an approach to control of rhinovirus infections, most effort has focused on development of effective antivirals. A great variety of compounds and types of compounds have exhibited *in vitro* activity, but few have demonstrated effectiveness at the clinical level (11).

Early studies using various interferon inducers demonstrated production of interferon in nasal secretions and a degree of prophylactic effectiveness that was related to the amount of interferon induced (57). The description of the three-dimensional structure of rhinoviruses permitted precise assessment of drug interaction with capsid proteins. A number of compounds were described that bind to the pocket at the floor of the canyon, displacing the floor so that receptor binding could not occur; some also inhibited a step during eclipse (207). Compounds exhibiting this mechanism of action include a variety of isoxazole derivatives, flavinoids, pyridazines, and others. These compounds have contributed to understanding the receptor-binding process through three-dimensional structure analyses, but testing for effectiveness in humans has been almost uniformly discouraging. Active compounds that were well absorbed after oral administration lacked a beneficial effect on infection or illness (180,222). The lack of effect was attributed to insufficient drug in nasal secretions, a finding for some other candidate antirhinoviral compounds. Topical administration of canyon inhibitors also has failed to provide a beneficial effect, but when incorporated into a cyclodextrin, a relatively insoluble compound, R61837, exerted some clinical effect (4). Pleconaril, another canyon inhibitor, had clinical benefit in one dosage group (114), whereas topical pirodavir, one of the most potent and broadly reactive canyon inhibitors, led to reduced viral shedding but no clinical benefit (115). Other antirhinoviral compounds with different mechanisms of action, such as the benzimidazide derivative enviroxime, have exhibited similar problems in demonstrating value (180). An ICAM-1 inhibitor (224) and a protease inhibitor were shown recently to be effective in volunteers challenged with a rhinovirus (9,144,149,167,224). Thus the problem in development of antivirals for rhinovirus infection is not in identifying potent and safe drugs but in developing methods for optimal administration to the nasal mucosal cells. Greenberg et al. (86) provided insight into the problem with a series of physiologically oriented studies using interferon. Structure, direction of air flow, chemical nature of secretions, and mucociliary clearance are all important factors in design of optimal approaches for topical administration of antivirals. Mechanisms of action that require constant presence of the antiviral in secretions can impair use. Other problems for antiviral use include development of resistant strains (9,48) and obtaining effectiveness for treatment as well as prevention because the peak viral shedding and illness periods are brief.

The availability of recombinant DNA–produced interferon led to evaluation of larger doses of interferon for prophylaxis of rhinovirus infection. Use of a 5-day schedule beginning before challenge resulted in a high degree of effectiveness (80%) in prevention of illness if intranasal doses were at least 10^7 units per day (197). When these doses were used for longer periods, however, local erosions, nasal stuffiness, and blood-tinged secretions occurred (197). Studies with interferon indicate a greater effectiveness for interferon a than interferon b for preventing rhinovirus infection (211). When used for short periods in contacts of a family member with a cold, effectiveness for prevention of rhinovirus infection was demonstrated (113). Although it is possible that practical ways to use this approach for prevention of rhinovirus infection might be developed, use of interferon has not been pursued. Administration of very high doses for therapy of established infection has not been effective (116).

PERSPECTIVE

The emergence of rhinovirus infections as the most common cause of common colds, a relatively minor illness in healthy persons, led to the belief that causing loss of time from work and school constituted their medical significance. In the past few years, they have emerged as infections also causing more severe disease. Currently they are the most common cause of episodes of asthma, are a cause of exacerbations of illness in persons with underlying lung disease, and convey a risk for lower respiratory disease in elderly persons. Moreover, they may directly and indirectly cause many cases of acute sinusitis and otitis media; a quantitative definition of the role of rhinoviruses, bacteria, and simultaneous virus and bacterial infection in causing sinusitis and otitis media is needed. However, developing effective control of rhinovirus infections constitutes the most important need for future disease-related research. Because conventional approaches for vaccine development do not appear reasonable for prevention of infection and clinically useful antivirals have not yet been identified, continued efforts to define more precisely the mediators and/or mechanisms for disease causation is desirable not only to understand better the pathogenesis of disease but also to permit design of inhibitors of disease. A reduction in disease among infected persons could have benefit at several levels: shortening the course of illness, reducing the risk of complications, contributing to relief of asthmatic episodes, and reducing spread of virus to susceptible persons. Although not specifically directed toward the infection, such a modality might also be useful for preventing illness in exposed persons' families and other population groups.

Continued efforts toward identifying antivirals effective against most rhinoviruses remains as an important effort. Although the record of the past is not encouraging, an antiviral useful for prevention of infection and/or illness in exposed persons would have value, and one that is safe and effective for both prevention and treatment would constitute a major medical accomplishment.

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CHAPTER 26 Hepatitis A Virus

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HISTORY

Ancient Chinese literature made reference to the occurrence of jaundice [reviewed by Zuckerman (455)], as did a letter from Pope Zacharias to Archbishop St. Boniface of Mainz during the 8th century AD (279). However, it was not until the 17th, 18th, and 19th centuries that scattered outbreaks of jaundice affecting diverse populations were recorded more frequently. The disease was especially common among the military and was called *campaign jaundice.* Although the prevailing view was that all types of jaundice were obstructive, an infectious etiology was suspected by a number of clinicians, especially for the milder, more contagious forms of jaundice called *epidemic catarrhal jaundice.*

An accurate accounting of epidemic jaundice was made by Cockayne (58) in 1912, who believed that the disease reached the liver through the blood, although the aerosol route was erroneously considered to be the principal mode of spread. Cockayne used the term *infectious hepatitis* to describe the epidemic form of the disease. McDonald (272) postulated that acute yellow atrophy of the liver may be caused by a virus acting on a “previously damaged liver.” Support for a viral etiology of infectious hepatitis gained favor from that point forward.

In 1923, Blumer (37) summarized the pattern of illness found in 63 outbreaks of epidemic jaundice reported in the United States between 1812 and 1922. Most infections occurred in children or young adults, developed 7 to 10 days after exposure to a jaundiced patient, showed a peak incidence in the fall and winter, and appeared to be transmitted largely by person-to-person contact. The relatively short incubation period seen in many cases probably reflected co-primary events, rather than secondary cases, or failed to take into account the period of communicability that precedes clinical disease. Nevertheless, these early observations established the existence of one of the major forms of viral hepatitis, now known as *viral hepatitis type A.*

Epidemiologic studies and human volunteer experiments performed during and after World War II confirmed the viral etiology of this disease and demonstrated its uniqueness from homologous serum jaundice, another form of hepatitis having a longer incubation period (38, 162, 163, 213, 249, 273, 276, 423). In 1947, MacCallum (246) introduced the terms *hepatitis A* and *hepatitis B* to categorize these diseases. These terms have been adopted by the World Health Organization (WHO) Committees on Viral Hepatitis (441, 442) to replace the myriad descriptions previously applied.

Between the late 1950s and early 1970s, the studies of Murray (289) and Krugman et al. (213) were instrumental in further defining the seroepidemiologic relationships between hepatitis A and B and eventually led to the evaluation of new methods for the immunoprophylaxis of these diseases (214). Two distinct types of hepatitis were differentiated at the Willowbrook State School for the Mentally Handicapped (see also Chapter 87). One of the hepatitis agents, designated *MS-1,* was transmitted primarily by the fecal–oral route but also could be spread by inoculation of blood obtained during a transient stage of viremia that occurred during the incubation period. The disease was highly infectious, had a relatively short incubation period of 4 ± 2 weeks, and closely resembled classic viral hepatitis type A. The MS-1 strain of hepatitis A virus (HAV) was used to infect adult volunteers in whom the disease was reproduced (38), and the virus was clearly identified for the first time by immune electron microscopy of their feces, as reported by Feinstone et al. in 1973 (108). Over the next 3-year period, highly sensitive immunoassays were developed for detecting the antigen and its antibody, culminating in the development of immunoglobulin M (IgM)-specific anti-HAV tests to distinguish recent HAV infection from a previous infection (reviewed in 172). In 1979, Provost and Hilleman (322) were successful in cultivating and serially passaging HAV in cell culture, an important step that contributed to the development of a vaccine.

INFECTIOUS AGENT

There has been a rapid expansion of knowledge pertaining to HAV since it was unequivocally identified in 1973 (108). A comprehensive summary was published by Gust and Feinstone in 1988 (146). In addition, several recent reviews pertain to the biology and replication of HAV (92, 223, 235, 338).

Biologic Characteristics

Classification and Comparison with Other Viruses

In the early 1980s, HAV was provisionally classified as enterovirus type 72 because its biophysical characteristics are like those of enteroviruses and cardioviruses, and its biochemical features resemble those of enteroviruses (see Table 1 and Chapter 23 and Chapter 24). However, later studies have demonstrated several characteristics that distinguish HAV from other picornaviruses: (a) HAV nucleotide and amino acid sequences are dissimilar from those of other picornaviruses, as are the predicted sizes of several HAV proteins; (b) HAV is difficult to adapt to growth in cell culture and usually replicates very slowly without cytopathic effect; (c) HAV is resistant to temperatures and drugs that inactivate many picornaviruses; (d) HAV is stable at pH 1 (350); (e) HAV has only one serotype, and one neutralization site is immunodominant; and (f) an enterovirus-specific monoclonal antibody does not react with HAV (449) (see also Table 1). It is concluded that HAV is a unique member of the family *Picornaviridae,* resulting in its classification into a new genus, Hepatovirus (Fig. 1) (280).

TABLE 1. *Viral characteristics: comparison of examples among the picornaviral genera*

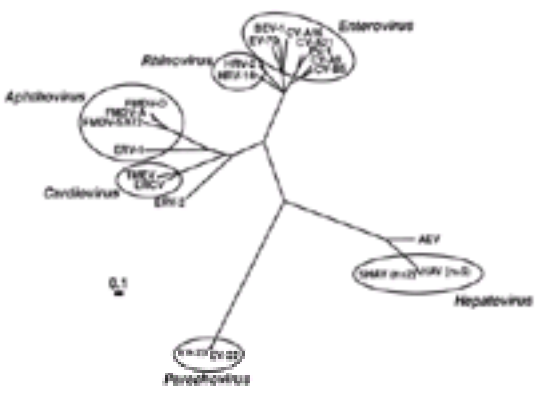

FIG. 1. Phylogenetic relationship of hepatitis A virus (HAV) and simian HAV (SHAV) to representative species of the mammalian picornavirus genera and to avian encephalomyelitis virus. The relationship was examined by the major-joining algorithm using amino acid similarities of the capsid precursor polypeptide P1. The bar represents distance as units of the expected fraction of amino acids changed. From ref. 261, with permission.

The knowledge that the antigens and nucleotide sequence of HAV are distinct from those of all other known viruses is especially relevant for clinical assays that detect evidence of infection. There is no serologic or hybridizing cross-reactivity between HAV and other agents of viral hepatitis, including hepatitis E virus (HEV), the other known agent of enterically transmitted hepatitis (see Chapter 89).

Strains and Genotypes

An HAV isolate that can be distinguished from other HAV isolates is defined as a *strain.* Viruses recovered from different locations often represent different strains, whereas viruses from individuals in the same outbreak usually represent the same strain. However, viruses from two or more locations may represent the same strain, or more than one strain may be present at a given location and time (334). Identical partial sequences for viruses from clinical specimens suggest an epidemiologic link (296).

The best-characterized HAV strains include HM175 (Australia, 1976), CR326 (Costa Rica, 1960), MS-1 (New York, 1964), SD11 (California, 1974), LA (California, 1976), HAS15 (Arizona, 1979), MBB (northern Africa, 1978), GBM (Germany, 1976), and H2 (China, 1982), all collected from humans, and PA21 (from an owl monkey, Panama, 1980) and AGM27 (from an African green monkey, Russia, 1985). These have been distinguished from each other on the basis of different growth characteristics (34,45,366) but are most reliably distinguished by genomic sequencing (47,63,141,292,293,308,334,417).

The identification of HAV and much of its initial characterization were done with strain MS-1 (108,212). Wild-type strain HM175 (63,72) has been adapted to yield the widest range of mutants in cell culture, including those with attenuated (61,200), persistently infecting (71,373), cytopathic (11,71,235), and neutralization-resistant (69,235,316,384) phenotypes.

Infectious cloned cDNA has been manipulated to yield mutants of HM175 with various phenotypes in cell culture and experimentally infected primates (60,83,92,93,95,118). Attenuated mutants of HM175, CR326, and H2 are the best characterized candidates for live virus vaccines, but variants of strains GBM and MBB (111,177,200,255,319) and chimeras between simian AGM27 (15) and human HM175 strains (329) also are being evaluated.

Sequence comparison of HAV isolates has been accomplished by using efficient methods for nucleic acid amplification [polymerase chain reaction after reverse transcription of RNA (RT-PCR) from virus purified by capture with monoclonal antibody] (334). Analysis of a 168-base region of 152 HAV strains resulted in the designation of seven *genotypes* (£85% sequence identity between genotypes and no more than 15% divergence within a genotype, a criterion used for polioviruses) (236,334). Two genotypes (I and III) were further divided into subgenotypes A and B (86–92.5% identity) (Fig. 2). The vast majority of human strains were genotype I. These included CR326, MS-1, SD11, HAS-15 (subgenotype IA), and HM175 and MBB (subgenotype IB). Genotype III represented most of the remaining human strains as well as the "simian" strain PA21 (subgenotype IIIA). The five other genotypes were each represented by only one strain, two from humans and three from monkeys. Clusters within genotypes predominate in certain geographic regions, such as a group of subgenotype IA strains with more than 97% identity that represented nearly all of the viruses from the U.S. Other regions, such as Europe and Japan, had a greater genetic mixture of viruses, suggesting regular introduction of strains by travelers returning from endemic regions.

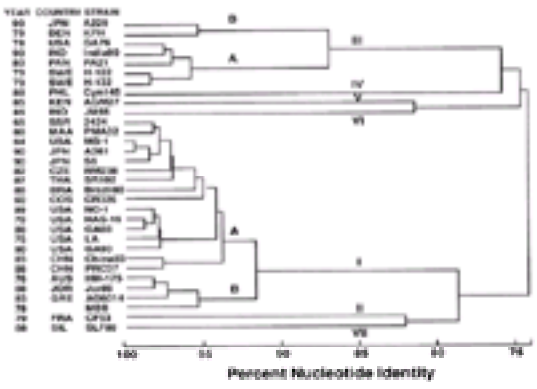

FIG. 2. Dendrogram depicting nucleotide sequence identity (relatedness) among 32 representative hepatitis A virus (HAV) strains. Identity was determined by pairwise comparison of sequences within a 168-nucleotide segment at the predicted junction of the 1D and 2A genes of HAV HM175/wild-type (nucleotide 3,024 to 3,191). A node connecting two strains indicates the approximate extent of identity between the strains. Genotypes (defined by £85% identity) are indicated by *roman numerals,* whereas subgenotypes (86–92.5% identity) of genotypes I and III are indicated by *A* and *B.* Genotype I (which represents most of the strains analyzed to date), and genotypes II and VII are known to include only human strains. Genotype III includes human strains and PA21, a strain that was collected from a New World monkey. The only known representatives of genotypes IV, V, and VI were collected from Old World monkeys. From ref. 236, with permission.

Several characteristics of HAV are common to strains collected from humans in widely separated geographic regions. These include the single serotype of HAV and similar reactivity of different strains with monoclonal anti-HAV antibodies (232,384). Nucleotide and amino acid sequences are highly conserved (>90% and >98% identity, respectively) throughout the genomes of strains HM175, LA, MBB, CR326, and HAS-15 in genotype I.

Strain PA21, recovered from a New World monkey, is biologically indistinguishable from human strains and has sequence similarity with genotype III human strains (334), suggesting that it may be a human rather than a simian strain. Strains that were recovered from Old World monkeys (genotypes IV, V, and VI) may be true simian strains: they have relatively distinct sequences and may have important biologic differences (see Fig. 2) (292,417). Some amino acid differences correspond to those that confer neutralization–resistance on experimentally selected mutants (236,316,334). Although the genotype V virus is not recognized by certain monoclonal antibodies that neutralize human HAV, the virus elicited an anti-HAV response in chimpanzees that protected against disease after challenge with the virulent human-derived HM175 strain (96). Because the simian virus did not cause hepatitis in chimpanzees, the experimental host most like humans, it is a candidate Jennerian vaccine. Chimeric recombinant viruses constructed by substituting a portion of a simian gene for the analogous region of the HM175 genome also may be promising vaccine candidates (329).

Morphology and Physicochemical Properties

Morphology

As first described by Feinstone et al. (108), HAV is a nonenveloped virus, 27 to 32 nm in diameter, that is morphologically indistinguishable from other picornaviruses (323). By electron microscopy (Fig. 3), "full" and "empty" particles appear to be spherical and featureless, but fine structural analysis has demonstrated icosahedral symmetry.

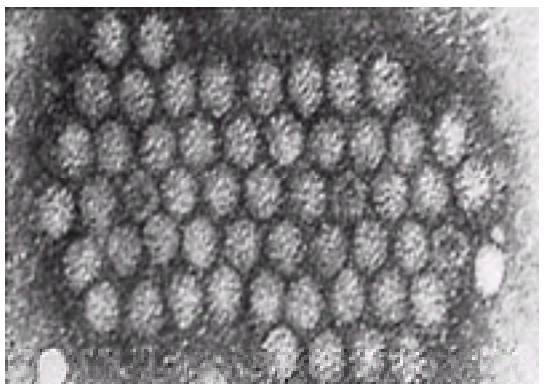

FIG. 3. Hepatitis A virus (HAV) particles observed by immune electron microscopy. Virus-like particles, 27 to 32 nm in diameter, are arranged in a crystalline array, with antibody coating their surfaces. These HAV particles appear to have cubic symmetry and have capsomeres measuring 8 to 12 nm (×130,650). From ref. 244 ×130,650, with permission.

Characteristics in Density Gradients

Hepatitis A virions have a primary buoyant density of 1.32 to 1.34 g/cc in CsCl and a sedimentation coefficient of 156S to 160S in neutral sucrose solutions (44,323,369). Additional populations of virus with different sedimentation characteristics have been isolated from human feces and cell culture. Empty particles (lacking RNA) have been detected at 1.20 and 1.29 to 1.31 g/cc, with sedimentation coefficients ranging from 50S to 90S, predominantly 70S. These are abundant in feces collected during early infection (44) or as a variable proportion of particles from cell culture (233) and are believed to represent procapsids (35) and perhaps other premature or defective particles. These alternative structures are morphologically indistinguishable from typical HAV and have the same major surface antigens (86), but their significance is unknown. Capsid-like structures have been produced by inserting the open reading frame of HAV into the genome of vaccinia virus (218) or baculovirus (337); these banded at 1.29 g/cc, sedimented at 70S, and were morphologically similar to empty HAV capsids.

Genome and Proteins

The virion is composed of a genome of linear, positive-strand RNA, approximately 7.5 kb in length, and a capsid containing multiple copies of three or four proteins (67,126,369). HAV RNA sediments at 33S and has a buoyant density of 1.64 g/cc (68,369). A 22S, double-stranded RNA form, closely resembling the replicative intermediate of other picornaviruses, also has been detected. Detailed genomic analysis has been accomplished with cloned or RT-PCR–amplified cDNA. Cultured cells can be infected with RNA transcribed from cloned HAV cDNA (62).

Genomic Organization

Like all picornaviral genomes, that of HAV can be divided into three parts: (a) a 5' noncoding region (NCR) that comprises approximately 10% of the genome, is uncapped, and is covalently linked at the 5' terminus to viral protein VPg; (b) a single open reading frame that appears to encode all of the viral proteins, with regions designated as *P1* for capsid proteins and *P2* and *P3* for nonstructural proteins; and (c) a short 3' NCR terminating in a poly(A) tract (Table 2 and Table 3; Fig. 4). Partial or complete sequences were determined for multiple HAV strains, including several mutants of strain HM175 (see GenBank listings). Complete sequence lengths range from 7,470 to 7,478 nucleotides, excluding a 3' poly(A) tail of 40 to 80 nucleotides. As noted earlier in the section on HAV strains, sequences for known human isolates are highly similar even when geographic and temporal origins are widely separated. This similarity suggests that genomic variation is constrained for HAV.

Region/Segment	Nucleotides	Comments
5' NCR	1–734	In other picornaviruses, this region stimulates synthesis of viral proteins and contains virulence and other phenotypic markers (6,186,307)
Stem-and-loop	1–40	Similar structures of different size predicted for picornaviruses; all have U(U) in first two nucleotide positions, required for HAV infectivity (163)
Pyrimidine-rich	88–138 (83%) 31–249 (68%)	Unique to HAV but may be structurally analogous to the poly(C) tract of cardio- and aphthoviruses; nucleotides 99–138 not required for growth in cell culture (3&63); highly variable in length and sequence between HAV strains
Hypervariable IRES	88–207 ~154–735	Mutations in this segment enhance HAV growth in cell culture Very inefficient for promoting translation; structure may be most similar to IRES of cardio- and aphthoviruses among picornavirus IRES
Conserved	88–95, 227–237, 483–731	Few mutations among HAV strains for which sequence is determined
Oligopyrimidine Translation initiation	712–728 720–744	Similar segment near initiation codons of other picornaviruses Approximates consensus sequence described for eukaryotic mRNAs (5,211)
3' NCR	7410–7478	High percentage of differences between strains, terminates in poly (A); poliovirus mutant in this region is temperature sensitive (246)

IRES, internal ribosome entry site; data from cultured cells; HAV, hepatitis A virus.

TABLE 2. *Genomic characteristics: noncoding regions (NCRs) of HAV strain HM175/Wild type*

Region encoding	Nucleotides	Length, in amino acids	C-terminal cleavage[a]	Proposed or known function	Comments (refs.)
Polyprotein	741–7413 or 735–7415	2226 or 2227			Most similar in length to PV polyprotein; downstream AUG codon at 741–743 preferred for translation initiation (402)
P1 (Capsid)					
1A	741–863 or 735–855	21 or 23	AG	Morphogenesis, VP4	Not myristylated (402); needed for capsid formation (316)
1B	864–1458	222	GAV	VP2	During maturation, precursor 1AB (VP2) may self-cleave to yield VP2 and VP4
1C	1470–2207	246	QG	VP3	Changes at Arg^{34} or Gln^{71} in neutralization-resistant mutants (316,318)
1D	2208–3029 or 3026 or 3023	274 or 275 or 277	SIM	VP1	Changes at Ser^{77}, Val^{111}, Ala^{170}, or Ser^{71} in neutralization-resistant mutants (318)
P2 (Nonstructural)					
2A	3030–3242	71	GA	Morphogenesis	Needed for particle formation (318); not a protease distinct from other 2A
2B	3243–3485	81	GS	?	Distinct from other 2B; mutations at nucleotide 3665 important for adaptation to cell culture (91)
2C	3536–5050	335	QG	RNA synthesis	Precedes NTP binding and RNA helicase activities (86,186); mutations important for adaptation to cell culture (34,318)
P3 (Nonstructural and VPg)					
3A	5051–5323	74	EG	Pre-VPg (ex 3AB)?	Distinct from other 3A
3B	5233–5291	23	QS	VPg	Tyr^{3} attached to 5′ termini of viral RNAs (109)
3C	5292–5948	219	QA	Protease	Cys^{172}-His^{44}, His^{172} or active site (3)
3D	5949–7413	488	—	RNA polymerase	Tyr^{273}-Gly^{274}-Asp^{275}-Asp^{276} in presumed active site (317)

[a] During polyprotein processing, cleavage is predicted to occur between the two residues indicated. The only known N-termini for native proteins are those of VP2 and VP1. All known cleavages in HAV except VP4/2A and VP4/VP1 are catalyzed by proteases 3C. The 2C/3A, 3B/3C, and 3C/3D cleavage sites have been determined by using in vitro synthesized proteins. Peptides representing VP2/VP3, VP3/VP1, and 2B/2C sites were efficiently processed by 3C produced in *E. coli* peptides representing 3B/3C and 3C/3D were inefficiently cleaved, and there was no detectable cleavage of a 2A/2B peptide. The C-terminus of VP1 is heterogeneous and could not be predicted by host proteases. Primary cleavage in P1 to yield 1B, at Pro-G four residues of 2A. Cleavage of P1/2A may be autocatalytic. See text for other details and references.

Source: (Revised from refs. 63, 236, and 406, with permission.)

TABLE 3. *Genomic characteristics: open reading frame of HAV strain HM 175/Wild type*

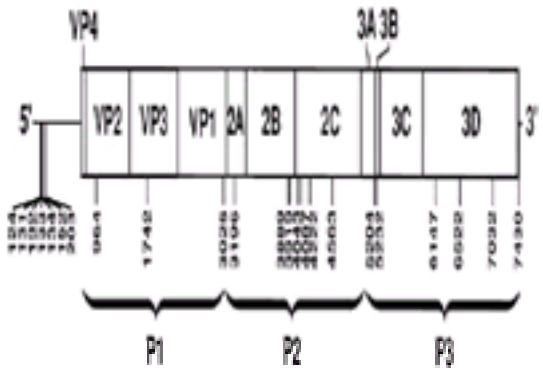

FIG. 4. Diagram of the hepatitis A virus (HAV) genome. Coding regions are in blocks. *Numbered vertical lines* indicate the nucleotide positions differentiating the virulent wild-type HM175 strain and the attenuated cell-culture–adapted variant HAV/7 (60).

Although the lengths of HAV genomic regions are most similar to those of enteroviruses (406), the HAV nucleotide sequence is not homologous with those of other picornaviruses (303). In addition, the low content of G + C (38%) in HAV is approximated only by that of rhinoviruses (39–40%). HAV and rhinoviruses have a preference for A or U in the third base position of codons (303).

Noncoding Regions

HAV NCRs have several notable features (236) (see Table 2). The 5' NCR appears to be the most conserved region of the genome (³89% nucleotide identity among seven strains representing genotypes I, II, and III) (45). A high proportion of C and U nucleotides is found near the 5' terminus (pyrimidines constitute 95% of nucleotides 99 to 138 and 68% of nucleotides 31 to 249; all numbering here refers to the HM175/wild-type sequence in ref. 63), as in cardiovirus and aphthovirus genomes. Deletion of the pyrimidine-rich tract spanning nucleotides 96 to 139 did not alter viral replication in cell culture (361) or decrease virulence for tamarins (362); however, deletions that also included nucleotides 140 to 144 yielded temperature-sensitive HAV, whereas deletion of nucleotides 93 to 134 was lethal (361). Nucleotides 99 to 207 are susceptible to naturally occurring mutations, particularly deletions (45). In contrast, nucleotides 66 to 95, 227 to 262, 681 to 721, and 712 to 720 are highly conserved among HAV strains. The latter is an oligopyrimidine tract near the translation initiation codons of HAV (nucleotides 735 to 737 or 741 to 743) and is similar to those in other picornaviral 5' NCRs (5,406). Some mutations in the 5' NCR enhance growth of HAV in cell culture (75,92,118) but do not appear to have a major role in determining virulence (60,119).

The 3' NCR has a high proportion of differences (up to 20%) between HAV strains and mutants. One study detected proteins in HAV-infected MRC5 or FRhK4 cells (47 kD and perhaps others £14 kD) that bound with nucleotides 7,430 to 7,452 or the corresponding bases from the 5' terminus of negative-strand HAV RNA (300). The onset of this interaction coincided with the establishment of persistent infection in culture. These data suggested that trans-acting cellular factors might participate in down-regulation of HAV RNA synthesis.

Little is known about the effects of 3' and 5' terminal sequences on picornaviral RNA synthesis, but they are thought to have important roles (5). All picornaviruses have putative stem-loops at the extreme 5' end, although these structures differ in size. The importance of this putative secondary structure was suggested by analysis of a poliovirus mutant with a nucleotide deletion that would disrupt such a structure; this mutant was deficient in RNA replication and translation (327). In addition, HAV RNAs were not infectious when they lacked nucleotides 1 to 2 or 2 to 3, which are thought to form part of a stem-and-loop (see Fig. 5, domain I) (159).

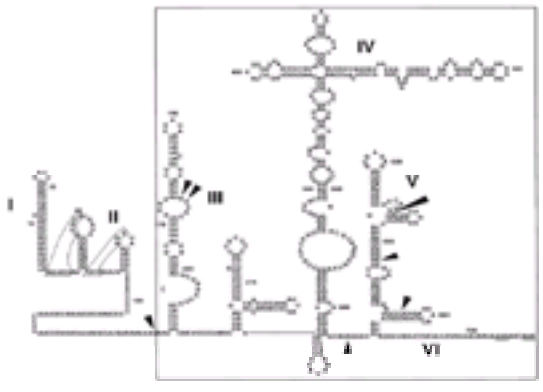

FIG. 5. Proposed secondary structure of the 5'8 noncoding region (NCR) of hepatitis A virus (HAV) RNA (strain HM175/wild-type). This model is based on a combination of phylogenetic comparisons, thermodynamic predictions, and nuclease digestions of synthetic RNA between nucleotides 300 and 735. Major structural domains are indicated by *roman numerals* beginning at the 5' terminus. Domains contributing to the HAV internal ribosome entry site (IRES; see text) are included in the box, although the boundaries are not precisely determined. Sites of mutations that appear to enhance HAV replication in cell culture are indicated (*arrows*). Two possible pseudoknots near the 5' terminus are outlined by *shaded interactions.* Two initiation codons for the open reading frame (nucleotides 735 to 737 and 741 to 743) are *underlined.* Several putative structures (pseudoknots, the single-stranded pyrimidine-rich tract between domains II and III, and the stem-and-loop near the top of domain V that also is predicted to form a pseudoknot) resemble structures predicted for the 5' NCR of encephalomyocarditis virus (EMCV). Modified from ref. 236, with permission.

Secondary structures and tertiary interactions in the complete HAV 5' NCR have been modeled by studying RNA digestion with single- or double-strand–specific RNases and by using computer programs to align sequences representing different strains and to predict thermodynamically stable base pairing (see Fig. 5) (45,219,361). These structures are most similar to those proposed for cardioviruses and aphthoviruses (5,185,219). In these models, pyrimidine-rich nucleotides 99 to 138 of HAV are not involved in stem-and-loop structures, but the presence of ordered structure in this region was indicated by the susceptibility to a double-strand–specific RNase (361). Nucleotides 705 to 726 of HAV RNA (overlapping with oligopyrimidine bases 712 to 720) may include sites of possible base pairing to 18S rRNA (219).

Translation

Significant advances have been made in understanding the role of 5' NCRs in translation. Structural models led to the recent identification of an internal ribosome entry site (IRES) in the HAV 5' NCR (46,48,133) that is somewhat similar to those previously recognized in other picornaviral 5' NCRs (5,185,312). Cis-acting picornaviral IRES are thought to interact with trans-acting cellular factors so that 40S ribosomal subunits bind and begin translation at the correct initiation codon and not at multiple AUG codons scattered throughout the 5' NCRs. This mechanism contrasts with typical eukaryotic translation, in which 40S subunits recognize the m7G-cap at the 5' mRNA terminus and then scan for the nearest AUG within an appropriate sequence motif (211). The HAV IRES was characterized *in vitro* and in cell culture by using bicistronic mRNAs in which translation of the second gene was dependent on the function of intact or altered versions of the HAV 5' NCR. *In vitro* studies suggested that the 5' IRES boundary was between nucleotides 151 and 257, and the 3' boundary was between nucleotides 695 and 735 (48), although more recent data suggested that it may extend an additional 66 to 114 nucleotides into the coding region (139). In HAV-permissive BS-C-1 cells, the boundaries, respectively, were downstream of nucleotide 45 and upstream of nucleotide 739 (133). Possible boundary discrepancies are not yet explained but could have resulted from differences between systems or 5' NCR alterations.

The HAV IRES was less efficient for directing translation than the EMCV IRES or nonpicornaviral leader sequences (capped or uncapped), in part because it competed poorly for cellular factors (48,133,432). Such competition almost certainly occurs in HAV-infected cells because the virus does not interfere with host protein synthesis. HAV replication may actually depend on continued synthesis of host factors. HAV IRES-directed translation in permissive cells was suppressed when cap-dependent translation of the cellular mRNAs was inhibited by poliovirus-encoded protease 2A (443). In contrast, poliovirus protease 2A stimulated EMCV IRES-directed translation (432). Picornaviral 5' NCRs appear to have negative-control elements for suppression of ribosomal scanning and an IRES for promotion of cap-independent translation (5,274); however, the HAV IRES appears to have been selected for low overall translational activity. The only report of increased HAV IRES activity in cells resulted from inserting four bases at position 633 (133).

Translation of HAV RNA in rabbit-reticulocyte lysates usually results in aberrant initiations within the open reading frame that can be corrected by replacing the HAV 5' NCR with that of EMCV, but not by adding extracts from HeLa cells (which improve translation of poliovirus RNA) or from BS-C-1 cells (which are permissive for HAV) (190). Recent efforts have attempted to identify cellular factors that could interact with the HAV IRES. Ribosome-associated proteins (30, 39, and 110 kD) from BS-C-1 and FRhK4 cells bound to several sites in the 5' NCRs of HAV and EMCV (54). *In vitro* the 57-kD protein, polypyrimidine tract-binding protein (PTB), and the 39-kD protein, glyceraldehyde 3-phosphate dehydrogenase (GAPDH), bind to overlapping sites in the 5' NCR. Local overexpression of GAPDH in transfected cells suppresses cap-independent translation directed by the HAV IRES, whereas local overexpression of PTB stimulates such translation. Thus it has been proposed that GAPDH suppresses translation by its ability to destabilize essential secondary RNA structures within the IRES (354), whereas PTB may reverse the inhibition by competing with GAPDH for binding or by stabilizing critical IRES structures (446). In support of this hypothesis, a naturally occurring mutation that enhances translation in African green monkey kidney cells (355) decreases the affinity of GAPDH for a stem-loop structure in the IRES (446). Another protein, identified as poly(rC) binding protein 2, is required *in vitro* for efficient translation of HAV (and of poliovirus) (138). Finally, an unidentified factor, presumably a protein, present in mouse liver cells but not BS-C-1 cells, also stimulates HAV translation *in vitro.* Evidence that accessory cellular proteins affect translation of HAV *in vivo* is indirect and is based on the discovery of host range mutations within the IRES (75,120,355). The HM175 strain of HAV

did not grow to detectable levels in MRC5 cells until serial passage resulted in the acquisition of a cluster of mutations in the region of the IRES preceding the initiation codon. These mutations selectively increased viral translation in MRC5 cells but were not required for growth or viral translation in FRhK4 cells (120).

There are two in-frame AUG codons at the beginning of the HAV open reading frame (nucleotides 735 to 737 and 741 to 743). Like other picornaviral initiation codons, both are within sequences that approximate, but do not match, the optimal context for scanning ribosomes (210). It has been suggested that suboptimal sequence context could contribute to poor translation and growth of HAV (5). Selective deletion demonstrated that the second AUG was preferentially used *in vitro* and in BS-C-1 cells, although either codon could function independently (403). It is not known why HAV has these two closely spaced initiation codons, but the simian AGM27 virus actually has a total of five in-frame AUG codons in this region (417).

The single open reading frame of picornaviruses is translated into one long precursor polypeptide, which is processed by a cascade of proteolytic cleavages to yield ultimately mature viral proteins (304). The primary cleavage, catalyzed by virally encoded protease 3C, takes place cotranslationally at the C-terminus of the P1/2A (capsid) precursor (see Table 3). All secondary cleavages, except VP1/2A and VP0, also are mediated by 3C (160,188,252,353). VP1 is apparently released from 2A by cellular proteases (142,260). Maturation cleavage involves the processing of VP0 (also designated 1AB) by an unknown, perhaps autocatalytic, mechanism to yield capsid proteins VP4 (or 1A) and VP2 (or 1B).

Detected Proteins

Nearly all of the viral proteins detected in HAV-infected cells are structural. Mature capsid proteins are the predominant form in virions: VP1 (or 1D; 30 to 33 kD by polyacrylamide gel electrophoresis), VP2 (or 1B; 24 to 30 kD), and VP3 (or 1C; 21 to 28 kD). Apparent molecular weights determined by electrophoresis often differ from those deduced by using amino acid sequences. A small protein, VPg (or 3B; predicted molecular mass of 2.5 kD), is covalently linked to the 5' genome terminus (428). Precursor polypeptides include VP0 (27 to 31 kD), which is a VP4/VP2 fusion protein; and PX (38 to 40 kD), which is a VP1/2A fusion protein. VPg and 2C have been detected among P2 and P3 proteins (418). Development of antibodies to P2 and P3 proteins was indirect evidence for their synthesis in HAV-infected primates (335,387), but concentrations of these nonstructural proteins appear to be very low in infected cells (418).

The 1A region of HAV encodes VP4, a polypeptide of 23 or fewer amino acids, with a maximal molecular mass of 2.5 kD (403). In other picornaviruses, VP4 is approximately 7 kD and is myristylated; myristyl-VP4 is found on the interior capsid surface of other picornaviruses and may contribute to particle assembly and stability or to binding and entry of virus into cells. Although the sequence of HAV VP4 includes a consensus sequence for myristylation (Gly-X-X-X-Ser/Thr), analysis of culture-adapted HAV and engineered mutants demonstrated that myristate was not incorporated (404). Efforts to detect mature VP4 in HAV particles have been unsuccessful.

Protein Processing

It was recently shown that the primary cleavage during HAV translation *in vitro* is mediated by viral protease 3C at a site approximately 40 amino acids beyond the P1-terminus, at the carboxy terminus of the 2A protein (192,261). This contrasts with primary cleavages for other picornaviruses (304). Enteroviruses and rhinoviruses produce a protease, encoded by the 2A region, that autocatalytically splits nascent 2A protein from the C-terminus of P1. An autocatalytic activity separates the 2A/2B junction of cardioviruses and aphthoviruses; 2A is subsequently cleaved from P1 by protease 3C. Protease activity has not been recognized for 2A of HAV, EMCV, or FMDV, or for HAV 2B (192,261). 2A/2B has virtually no amino acid similarity with the analogous portions of other picornaviral polyproteins. These observations are leading to revision of the VP1/2A/2B region of the HAV genomic map (see Table 3; Fig. 4). The C-terminus of 2A is now thought to be at amino acid 836 (from the first in-frame AUG codon), corresponding to the primary cleavage observed *in vitro.* Thus 2A is considerably smaller and 2B larger than previously predicted (see Table 3). Although dipeptide Gln/Ala (amino acids 836 to 837) is apparently the cleavage site (261), *in vitro* scission still occurred when this dipeptide was mutated to Asn/Ala or Ala/Ala. The capsid precursor would then be P1/2A, with a molecular mass of approximately 100 kD. A 100-kD protein that reacts with antibody to VP1 and VP2 has been identified in HAV-infected cells (39).

The C-terminus of VP1 is heterogeneous (142) and includes VP1-ser^{274} as the predominant form, along with minor forms that end at residue VP1 272 or 273. Similar proteins, including 42-kD (VP1/2A-like) and 98-kD (P1/2A-like) precursors, were produced in cells infected with a recombinant vaccinia virus that expressed the HAV polyprotein to yield virus-like particles (440).

The heterogeneity of the mature VP1 terminus and the multiple anti-VP1 reactive proteins in addition to PX (VP1/2A) in HAV-infected cells (192) contributed to the difficulty in identifying the terminus. Earlier, inaccurate predictions were based on comparisons with other picornaviruses and the apparently incorrect assumption that the 3C protease was involved. Generation of the majority species of VP1 requires a scission between Ser^{274}/Met^{275} that is not a known substrate for 3C protease (6). It has been proposed that, as for Mengo virus, cellular proteases probably trim 2A sequences from PX to generate intermediates and the mature VP1 protein (142,260).

HAV and Other Picornaviruses: Comparison of Proteins

Most HAV proteins are distinct from those of other picornaviruses, but regional amino acid sequence similarities exist. Proteins VP4, VP1, 2A, 2B, and 3A are particularly distinct. The predicted lengths of several HAV proteins are quite different from the analogous proteins of most other picornaviruses: VP4, VP2, and 3A are shorter, whereas VP3, 2B, 2C, 3C, and 3D are longer. Although the three major capsid proteins are thought to have the b-barrel structure common to other picornaviruses, alignment of amino acid sequences has been difficult because of minimal similarity. Unfortunately, it has not been possible to crystallize HAV for determination of its three-dimensional structure. Significant structural differences between HAV and other picornaviruses also are suggested by the resistance of HAV to acid, heat, and

disoxaril. However, studies of surface iodination (333), monoclonal antibody binding (179), peptide mapping (97,124), and digestion with trypsin and chymotrypsin (227) have identified HAV VP1 and VP2 as major surface proteins, as in other picornaviruses. The presence of VP1 and VP3 on the virion surface, or influencing surface structure, is implied by identification of important epitopes in these proteins (see Antigens and Antigenic Structure later). Overall, HAV has more sequence identity with encephalomyocarditis virus (EMCV) and rhinovirus type 2 than with other human picornaviruses. However, a recently characterized avian picornavirus, avian encephalomyelitis virus, is most similar to HAV (262). Similarity of HAV to human picornaviruses has been demonstrated in known or proposed functional regions of VP0, 2C, 3B, 3C, and 3D (see Table 3). Conserved sequences at the VP0 cleavage site and near the N-terminus of VP2 may participate in the maturation cleavage step (304). 2C probably functions in viral RNA replication (reviewed in 93), perhaps via RNA helicase activity (88), and has a putative guanosine triphosphate (GTP)-binding domain (136) and markers for guanidine resistance. Mutations in 2C and 2B were essential for adaptation of HAV HM175, and perhaps other strains, to growth in cell culture (see Propagation and Assay in Cell Culture later) (93,94,118). Mature 3B (VPg) is probably covalently linked through tyrosine residue 3 to the 5' terminus of viral RNA (428) (see Chapter 23). 3C has been crystallized and is a chymotrypsin-like serine protease (6) that generates secondary cleavages in cis and trans (125,160,252,353). The crystal structure indicates a strong preference for glutamine at the P1 cleavage site (6). 3D is assumed to be the viral RNA polymerase, although there are no direct data. Thus, although the function or specificity of certain HAV proteins (particularly VP4, 2A, 2B, 3A, and 3C) may differ from those of analogous proteins of other picornaviruses, other functions (capsid, genome-linked protein, major protease, and RNA polymerase) are conserved. The distinct characteristics of HAV proteins are contrasted by the high degree of sequence identity among HAV strains, particularly in the P1 region, which suggests a selective advantage for *in vivo* stability of HAV genotypes or constraints for replication in hepatocytes.

Capsid Assembly

A model for assembly of HAV capsids explains current observations of viral protein synthesis and processing in cells (12,35,39) and is consistent with data from other picornaviruses (see Chapter 23). In this model, primary cleavage of the HAV polyprotein by 3C yields a P1/2A precursor. The 2A portion of the precursor serves as the primary signal for five copies to assemble into a 13S pentamer (318), whereas this step is initiated by myristylated VP4 of poliovirus. Subsequently 3C catalyzes secondary VP2/VP3 and VP3/VP1 cleavages to produce 14S pentamers containing VP0, VP3, and VP1/2A (39). Twelve pentamers assemble to form empty capsids (procapsids) or incorporate virion RNA to form provirions. The assembly of HAV pentamers into empty capsids is very inefficient in the absence of VP4 (318). Processing of VP1/2A to yield VP1 begins after assembly of procapsids or provirions, but VP1/2A can be detected in provirions. Maturation cleavage of VP0 to release VP2 depends on RNA encapsidation, takes place at a linear rate over days, may be self-catalyzed, and is associated with an increase in sedimentation coefficient (implying conformational change) and infectivity (35). The fate of VP4 in this process is unknown.

Antigens and Antigenic Structure

As determined by neutralization assays, there is only one known serotype of HAV (96,229,232,451). The only naturally occurring antigenic variants are HAV strains collected from Old World monkeys (see Strains and Genotypes earlier).

Antigens of the intact virion appear to be conformational and generally different from those of isolated HAV proteins. Purified virus elicited neutralizing anti-HAV and antibodies to denatured capsid proteins in rabbits (not known to support HAV replication) (124). Mice developed neutralizing anti-HAV in response to 14S and 70S subviral particles that had been produced in recombinant vaccinia virus–infected cells (385). In contrast, antibodies to purified capsid proteins or to synthetic peptides have weak or no detectable neutralizing activity; only two peptides, representing sequences near the N-terminus of VP1 (residues 11 to 25) and amino acids 110 to 121 of VP3 (40), stimulated production of such antibody (97). Chimeric polioviruses that expressed HAV peptide sequences elicited weak neutralizing activity only when the peptide corresponded to VP1 residues 13 to 24 or 101 to 108 and only in a minority of the inoculated animals (228). It is unusual for neutralizing anti-HAV to develop in response to individual proteins, polypeptides, or uncleaved precursors produced by recombinant DNA techniques (23). Such proteins often react with rabbit anti-HAV or human convalescent-phase anti-HAV, and they sometimes elicit a priming response for anti-HAV (127,193,317). Priming amino acids appear to be located near the C-terminus of VP1 (158). It is likely that VP2 and perhaps VP3 also contain such helper T-cell epitopes (317). After inoculation with recombinant vaccinia virus expressing uncleaved HAV P1 precursor, two marmosets developed anti-HAV. The animals were not tested for neutralizing antibody, but were protected from challenge with virulent HAV (199).

Multiple epitopes have been detected on intact virions and on 14S pentameric subunits by using monoclonal anti-HAV. Most of these epitopes overlap as an immunodominant neutralization site, but there is evidence for a second, possibly independent, site (236,316,384). Monoclonal antibodies to HAV did not bind to isolated capsid proteins, but the Fab fragment of one such antibody was able to bind VP1 on intact virions (179). Later studies demonstrated three epitopes that overlap as part of an immunodominant site (224,384). Mutants selected by passage in the presence of a monoclonal antibody (291,316,384) became resistant to neutralization by that antibody. Among mutants selected against any one monoclonal, the number of amino acid positions with changes was limited: fewer than two positions for strain HM175 (at VP3 Asp^{70} and Gln^{74}, or at VP1 Ser^{102}, Val^{171}, Ala^{176}, and Lys^{221}), and only one amino acid was changed in each mutant (315,316). For strain HAS-15, there were only two patterns of change for mutants to each monoclonal, with mutations in one to three amino acid positions (at VP3 Pro^{65}, Asp^{70}, and Ser^{71} or at VP1 Asn^{104}, Lys^{105}, and Gln^{232}) (291). For both strains, all of the mutant residues appear to participate in the immunodominant site. Oligonucleotide-directed mutagenesis of VP1 Ser^{114}, and VP1 Lys^{221} also produced neutralization-escape mutants (69). Although most such studies have been performed with murine monoclonals, limited analysis of the human response to HAV infection has detected antibodies to VP3 Asp^{70} and to VP1 Ser^{102} and Ser^{114} (76). A recombinant human monoclonal anti-HAV appears to be directed against the immunodominant site (240). The immunogenic sites may be located within peptide loops that connect b-strands of the capsid proteins and project from the virion, analogous to immunogenic sites in other picornaviruses (236,316). Treatment of HAV with trypsin was reported to abolish reactivity and neutralization of the virus with a monoclonal antibody used to define the immunodominant site, without affecting infectivity (382). However, a similar study did not detect antigenic change after trypsin treatment but did observe cleavage of VP2 (which is not known to be

directly involved in this site) (227). This apparent discrepancy has not been resolved. A sequence near the N-terminus of VP1, which elicited weak neutralizing activity as a peptide or when inserted into a chimeric poliovirus (see earlier), is thought to be internally located in HAV capsids but externalized during virus attachment to cells (228). The finding of few amino acid changes among multiple neutralization-escape mutants suggests that there could be constraints to antigenic or structural variation of HAV. This notion is supported by the characteristics of HAV strains isolated from Old World monkeys (292,417). These HAV isolates have the antibody-binding phenotypes and amino acid changes of some of the neutralization-escape mutants. When one neutralization-escape mutant was inoculated into owl monkeys, revertant virus (with wild-type VP3 Asp^{70}) was selected, suggesting that there could be similar constraints *in vivo* (237) (see Strains and Genotypes earlier). Cytopathic mutants of HAV HM175 that spontaneously arise during persistent infection in cell culture also can develop, without selective pressure from antibody, a neutralization-escape phenotype with changes at some of the amino acid positions associated with the immunodominant site (235,316). It was suggested that a long period of persistent infection could reduce the pressure for maintaining structural features necessary for infecting new cells (235).

The nonstructural proteins of HAV also are immunogenic during natural and experimental infections. Antibodies were detected by immunoprecipitation of P2 and P3 proteins that had been synthesized *in vitro* (191,335) or by an ELISA specific for 3C protease expressed in *Escherichia coli* (387). However, chimpanzees did not develop anti-P2 or anti-3C antibodies after vaccination with inactivated HAV.

Stability

Unpurified, cell-adapted HAV retains most of its infectivity when subjected to pH 1.0 for 2 hours at room temperature, and is still infectious at 5 hours, whereas poliovirus type 1, coxsackieviruses types A9 and B1, and echovirus type 9 lose virtually all of their infectivity by 2 hours. Because it is a nonenveloped virus, HAV also is resistant to 20% ether, chloroform, dichlorodifluoromethane (Freon), and trichlorotrifluoroethane (Arklone, Genetron) (323,365,368).

HAV is relatively resistant to heat. Virus integrity or antigenicity is not affected when HAV is incubated at 60°C at neutral pH for 60 minutes and is only partially inactivated after 10 to 12 hours (306,372). The temperature at which 50% of HAV particles disintegrate and release their RNA in the absence of cations is 61°C after 10 minutes versus 43°C for poliovirus type 2 Lansing. When stabilized by 1 mol/L $MgCl_2$, disintegration occurs at 81°C and 61°C, respectively, with generation of empty capsids that retain antigenicity. Such reactivity is lost at 87°C for HAV and at 67°C for poliovirus. In human and animal experiments, HAV was inactivated within minutes at 98°C to 100°C (323).

Infectivity can be preserved for at least 1 month after drying and storage at 25°C with 42% humidity (271,380), or for years at –20°C or lower. HAV also was found to survive for days to months, and for longer periods than poliovirus, in experimentally contaminated fresh water, seawater, wastewater, soils, marine sediment, live oysters, and creme-filled cookies (380). Thermal treatment of oysters (60°C for 19 min), inoculated with HAV-containing feces and sealed in a can, failed to inactivate the virus totally (313).

Inactivation of HAV was reviewed by Coulepsis et al. (65). HAV is inactivated by autoclaving (121°C for 20 minutes), by ultraviolet radiation (1.1 W at a depth of 0.9 cm for 1 minute, or 197 µW/cm^2 for 4 minutes), by formalin (1:4,000 for 72 hours at 37°C, 3% for 5 minutes at 25°C, or 8% for 1 minute at 25°C), by b-propiolactone (0.03% for 72 hours at 4°C), by iodine (3 mg/L for 5 minutes), or by chlorine (10 to 15 ppm residual chlorine concentration after 30 minutes; free residual chlorine concentration of 2.0 to 2.5 mg/L for 15 minutes) or chlorine-containing compounds (sodium hypochlorite, 3 to 10 mg/L at 20°C for 5 to 15 minutes). Infectivity of HAV is substantially decreased by 70% ethanol at 25°C (2.25 and 5.5 log_{10} reductions in infectivity after exposure for 3 and 60 minutes, respectively).

Propagation and Assay in Cell Culture

Early attempts to isolate HAV in cell or organ cultures were generally unsuccessful. Five years passed between the discovery of HAV and the successful propagation by Provost and Hilleman (322) of marmoset-adapted HAV in primary explant cultures of marmoset (*Saguinus labiatus*) liver and in a fetal rhesus monkey kidney cell line (FRhK6). After incubation for weeks, cytopathic changes were not observed despite accumulation of viral antigen. Direct immunofluorescence analysis (264) was the key to demonstrating HAV replication. Many other laboratories have confirmed and extended these findings (for reviews, see refs. 146,236,339,406).

Various primary and continuous cells of primate origin will support HAV growth, although optimal replication often results from a particular combination of HAV strain, cell type, and temperature (24,34,366). Primary African green monkey kidney (AGMK) cells, primary human fibroblast cells, and continuous human diploid lung (MRC5) cells often have been used because of their suitability as potential vaccine substrates. Cell lines derived from AGMK (such as BS-C-1, Vero, and BGMK), fetal rhesus kidney (FRhK4, FRhK6, Frp/3), or human hepatoma (PLC/PRF/5) tissue also have been useful for studying replication and for propagating large amounts of virus (34,72,109,114,366). However, HAV is usually difficult to adapt and grow *in vitro.* Some wild-type strains have not been adapted to cell culture despite intensive efforts, and successful isolation from clinical specimens often requires several weeks and blind passages (34,72,322). Even when well adapted, HAV usually takes several days to weeks to reach maximal concentrations of propagated virus. Typical yields of 10^6 to 10^9 $TCID_{50}$/mL are usually recovered only by mechanical lysis because progeny of most HAV strains remain cell associated and spread to adjacent cells by an unknown mechanism.

HAV usually has few or no visible effects on the host cell, in contrast to many other picornaviruses (123; see Chapter 23). Some strains or mutants release progeny to the medium but usually without cytopathic effect. The only ultrastructural alterations observed in most infected cells are cytoplasmic accumulations of multilayered membranes as well as clusters of virus in vesicles (18,206,259). Cells infected with a cytopathic HAV mutant had similar changes; in addition, long polyribosomes were observed early in the infection, followed by degenerative changes in the rough endoplasmic reticulum. Thus detection of HAV multiplication usually requires an indirect method: (a) an immunologic assay for viral antigens (171,231,266,326), or (b) a hybridization assay for HAV RNA (186). A radioimmunofocus assay (231) can be

used for quantifying infectious HAV, selecting viral clones, and for measuring neutralizing anti-HAV. Cell culture is not useful for the diagnosis of typical hepatitis A.

Infectious cloned cDNA has made it possible to study the effects of mutations on the growth of HM175 in cell culture (60,93,94) and on virulence of HAV for primates (see Fig. 4) (60,91,92,95). As with other picornaviruses, particular phenotypes often represent contributions from multiple mutations. The different studies have used different assays (immunofluorescence microscopy for percentage of cells containing HAV antigens, immunoblot analysis of viral proteins, radioimmunofocus assay for number and size of viral growth foci, and hybridization for total HAV RNA), and these can alter the interpretation. However, several concepts have emerged about mutations selected during adaptation or passage: (a) those in gene 2B (particularly nucleotide 3889) or 2C (nucleotides 4043, 4087, 4222, and 4563) appear to be essential for the adaptation of HM175 (93), and perhaps other strains, to certain cells (140,141); (b) those in the 5' NCR (especially in the variable region near nucleotide 200) are not essential, but they enhance growth and alter the host range *in vitro* (75,118); and (c) other mutations throughout the genome also enhance growth (60,75,92,93 and 94,118,453).

Because HAV usually does not cause a cytopathic effect, persistent infection is readily established in many cell lines. Three phases of replicative events were identified in cells that became persistently infected with HAV under one-step growth conditions (77). From day 2 to day 8 after inoculation, negative- and positive-strand viral RNAs and the rate of infectious HAV synthesis reached their highest levels. During days 9 to 14, viral antigen production was maximal, and positive-strand HAV RNA and infectious HAV levels remained high, but negative-strand HAV RNA became undetectable. After day 14, there was minimal viral metabolic activity. Nearly all of the HAV remained cell associated.

Most HAV mutants attain maximal accumulation within 3 to 7 days but are not cytolytic. However, with some mutants, a cytopathic effect develops 2 to 14 days after inoculation (9,11,71,235,443). The development of a cytopathic effect depends on the interaction between particular HAV strains and cells at certain passage levels (71,235). In most cases, rapid cytolytic growth was selected by passaging HAV HM175 from persistently infected cells at intervals of 3 to 7 days. However, some strains of HAV were cytopathic within several passages after direct isolation from human feces (360,422,443).

Studies of chimeric infectious cDNAs indicate that multiple mutations outside of the P1 region, many of which are in 2C, are most likely to contribute to the cytopathic phenotype (235,282). Mutations in the P2 region of a cytopathogenic mutant enhanced replication, but additional mutations within the 5' NTR and P3 regions were required for the full cytopathogenic effect (453).

HAV RNA synthesis was detected 6 hours after inoculation of the cytopathic mutant HM175A (10,12,35,39) into BS-C-1 cells and reached maximal levels at approximately 12 to 27 hours during the logarithmic phase of HAV propagation. Cytopathic effect developed after 2 to 3 days, yielding 20 infectious doses of HAV per cell or approximately $10^{8.7}$ PFU/mL. Little negative-strand RNA was synthesized. Furthermore, nearly all positive-strand RNA was encapsidated at all times during the replication cycle.

Several mechanisms have been proposed to explain the typically slow cycle of HAV replication [reviewed by Lemon et al. (238)]. First, inefficient uncoating may limit the extent of replication. This may reflect incomplete maturation cleavage of VP0 (VP4/VP2) during the previous cycle (35,431). Second, replication is asynchronous in certain cells (reviewed in 57). Delayed uncoating could be a factor that contributes to asynchronous replication. Third, HAV does not shut off host-cell macromolecular synthesis, and HAV translation competes poorly with that of cellular mRNAs (133). Fourth, posttranslational processing may be inefficient, limited by low levels of protease 3C or perhaps activities responsible for maturation of VP1/2A (35,39). Fifth, viral RNA might be sequestered into nascent HAV capsids and thus be unavailable for translation or replication (10). Sixth, host-mediated HAV down-regulation may occur, as is suggested by binding of host proteins to the 3' NCR at the onset of persistent infection and the absence of such binding to the genome of a cytopathogenic mutant (300). Seventh, defective-interfering particles could inhibit HAV replication (122,299). It should be apparent that these mechanisms can be interdependent.

Receptor

Virtually nothing is known about the mechanism by which HAV enters susceptible cells. A study of virus attachment determined that HAS-15 was rapidly (within 20 minutes), but inefficiently (approximately 1% of the input virus), adsorbed to permissive FRhK4 cells (431). Attachment of an Italian strain to Frp/3 cells was abolished by treating the cells with trypsin, phospholipases, or b-galactosidase (360). More recently, HM175 attachment *in vitro* was shown to be calcium dependent. In one of these studies (452), attachment was maximal at 4°C, and HAV bound to both permissive and nonpermissive cells, whereas there was no temperature effect for permissive cells or binding to a nonpermissive cell type in the other study (381). Serum inhibited HAV attachment via high molecular-weight components perhaps related to b_2-macroglobulin (452). Arg-Gly-Asp (RGD) peptides did not interfere with attachment, even though that sequence corresponds to VP3 residues 112 to 114, and such peptides are involved in binding of FMDV and certain proteins to cells (381). Trypsin or chymotrypsin, respectively, digested HAV VP2 or VP1 and VP2 without affecting infectivity (see Genome and Proteins earlier) (227).

Recently a putative receptor for HAV was isolated from cultured cells by using recombinant technology in combination with an antibody that blocked binding of HAV to cells. A class I integral-membrane glycoprotein of unknown natural function was independently isolated by two groups from AGMK cells (197) and from a hybrid marmoset–Vero cell line (20), respectively. The receptor recovered from the two cultures differed slightly in size (451 versus 460 amino acids) and amino acid composition (95% similarity). Additional polymorphisms were found in different AGMK cell lines (106). Subsequently an even smaller receptor glycoprotein (359 amino acids) was isolated from human liver and kidney cells (107). The human homolog had only 79% identity with the AGMK receptor and was expressed in all organs analyzed (liver, small intestine, colon, spleen, kidney, testes), with higher levels in the kidneys and testes than in the liver. There

are no data on whether this molecule serves as the receptor for HAV *in vivo.*

Effects of Chemical Agents on Replication

Several agents that inhibit the growth of poliovirus and some other picornaviruses do not affect HAV, including arildone, disoxaril (formerly known as WIN 51711), 3'-methylquercetin, 2-amino-4,6-dichloropyrimidine, and 2-a-hydroxybenzyl) benzimidazole (78,367). Arildone and disoxaril represent a group of antivirals that, by filling the hydrophobic interior of VP1, stabilize the capsid and prevent uncoating of enteroviruses and rhinoviruses. The other agents interfere with intracellular replicative events: 3'-methylquercetin inhibits synthesis of poliovirus RNA, and 2-amino-4,6-dichloropyrimidine inhibits the morphogenesis of poliovirus (78).

Low concentrations (1 to 3 mmol/L) of guanidine reversibly inhibit replication of enteroviruses and rhinoviruses; effects vary with different combinations of virus strain and host cells (57). HAV strain MBB, adapted to growth in PLC/PRF/5 cells, was resistant to 2 mmol/L guanidine (366), whereas 3 mmol/L guanidine inhibited a cytolytic mutant of HM175 in FRhK4 cells (70). BS-C-1 cell-adapted HM175 was inhibited by 2 mmol/L guanidine, but replication was enhanced by 0.1 to 1 mmol/L guanidine.

Although there are no commercial antiviral drugs specifically licensed for treating HAV infection, ribavirin, amantadine, and 2-deoxy-D-glucose are among several antiviral substances known to interfere with HAV replication (the latter also inhibits poliovirus) (307,435). Amantadine (among several lipophilic amines) and monensin (an ionophore that raises intravesicular pH) are thought to prevent uncoating of HAV in endosomes or lysosomes. Monensin also may block HAV egress from cells by interfering with vesicular transport function; this is also observed with brefeldin A. The effects of 2-deoxy-D-glucose (an inhibitor of glycolysis) and dinitrophenol and sodium azide (inhibitors of oxidative phosphorylation) suggested that late, but not early, events of the HAV cycle are energy dependent. Similar results were obtained with colcemide, vinblastine, and cytocholasin D, which alter cytoskeletal structure.
Tricyclodecane-9-yl-xanthogenate, which inhibits a broad range of DNA and RNA viruses by an unknown mechanism, did not affect HAV or poliovirus (78). HAV propagation was enhanced, at least with particular combinations of virus and cells, by prostaglandin E_1, dexamethasone, or 5,6-dichloro-1-b-D-ribofuranosylbenzimidazole (123,434,435). The latter primarily inhibits transcription by cellular RNA polymerase II and does not enhance growth of poliovirus or echovirus type 6.

Host Range and Experimental Infection in Animals

Attempts to transmit HAV to experimental animals were generally unsuccessful until the 1960s. In 1962, Deinhardt et al. (80) described the development of biochemical and histopathologic changes of mild hepatitis in chimpanzees inoculated with acute-phase serum or feces. The significance of these results was minimized by expectations of jaundice (which rarely occurs in nonhuman primates) and by using assays for aspartate aminotransferase (AST) instead of more sensitive and specific assays for alanine aminotransferase (ALT). In 1967, Deinhardt et al. (81) serially transmitted agents of hepatitis in marmosets by using acute-phase sera from patients with disease that had the epidemiologic characteristics of hepatitis A. Interpretation was initially hampered by reactivation of a latent marmoset agent (or a human non-A, non-B hepatitis virus), which resulted in evidence of hepatitis (305). Transmission of HAV was confirmed when coded control and acute-phase sera from HAV-infected volunteers were correctly identified after inoculation of marmosets (175). Further evidence for transmission to marmosets soon followed, and HAV was subsequently transmitted to chimpanzees (84,269).

HAV is now known to produce disease in humans, chimpanzees (*Pan troglodytes*) (84,269), owl monkeys (*Aotus trivirgatus*) (221), stump-tailed monkeys (*Macaca speciosa*) (256), and several species of South American marmoset (tamarin), most notably *Saguinus mystax* and *S. labiatus* (24,81,175,246). Disease in nonhuman primates resembles that in humans but is usually milder (Fig. 6). After infecting these animals, HAV or viral antigen can usually be detected in serum, liver, bile, and feces. Other primate species are susceptible to infection but do not develop disease with human HAV strains; this limits their usefulness for studies of human strains. Cynomolgus monkeys (*Macaca fascicularis*) were found to have been infected with HAV in the wild (49). In the laboratory, hepatitis was induced in *M. fascicularis* and in *M. arctoides* by experimental inoculation with HAV YaM-55 (collected from cynomolgus monkeys) but not by human HAS-15 (16). The simian virus isolated from African green monkeys caused severe hepatitis in *Cereopithecus aethiops, S. mystax,* and *M. fascicularis,* but not in *P. troglodytes* (96). These data suggest that host species-specific variants may have been selected in certain nonhuman primates (232). This notion is supported by the demonstration of genomic and antigenic differences between HAV from Old World monkeys and humans (see Fig. 2, Strains and Genotypes, and Antigens and Antigenic Structure, earlier). In addition, it appears that a host-range alteration can be experimentally induced. After 20 passages in marmosets, HAV MS-1 was more virulent for marmosets but was attenuated for chimpanzees (45).

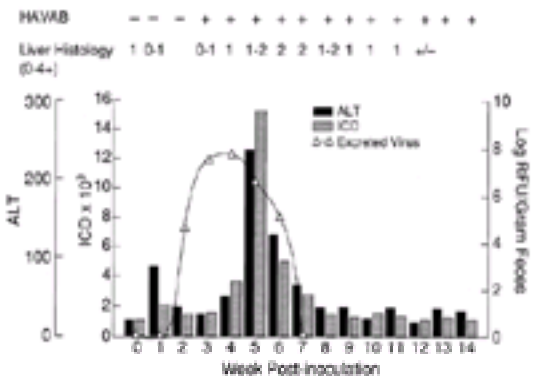

FIG. 6. Course of hepatitis A in an experimentally infected tamarin. A tamarin was inoculated intrahepatically with RNA transcribed from a cDNA clone encoding virulent HM175 virus. The isocitrate dehydrogenase (*ICD*) and alanine aminotransferase (*ALT*) levels in serum are shown, as are quantities of infectious virus [radioimmune focus units (RFUs)]

excreted in the feces. Results of the liver histology and serology tests are noted at the top of each graph. Modified from ref. 119, with permission.

PATHOGENESIS AND PATHOLOGY

Pathogenesis

Natural infection with HAV usually follows ingestion of virus from material contaminated with feces containing HAV. The sequence of events that begins with entry via the gastrointestinal tract and ultimately results in hepatitis has not been completely resolved. Although the primary site of replication for HAV is the hepatocyte, the factor that determines this tissue tropism has not been elucidated. The host-cell receptor used *in vivo* has not been identified, but *in vitro* studies suggest it may be a class I glycoprotein (20,107,197). During the incubation period, viremia is observed at about the same time that fecal shedding of HAV is occurring. It is believed that HAV reaches the intestinal tract in the bile (352,423). Viremia terminates shortly after hepatitis develops, whereas feces may remain infectious for another 1 to 2 weeks. In some situations, HAV may circulate in the blood enclosed in lipid-associated membrane fragments that may transiently protect the virus from neutralizing antibody (230). In this regard, vesicular structures containing virus have been observed in cell cultures (19) and in primate hepatocytes and Kupffer cells, where they appear to be released into the biliary system or the blood (363).

Although coproantibodies have been detected in acute hepatitis A (242,447), they apparently lack neutralizing activity (383). Nevertheless, immune complex formation between specific IgA antibodies and fecal HAV has been observed (198). This may explain why HAV can be detected by molecular hybridization long after HAV antigen becomes negative by immunoassays (399). Patients with relapsing type A hepatitis may continue to excrete detectable HAV, even though their serum remains positive for IgM anti-HAV (376). There is no evidence that defective secretory antibody production has anything to do with this phenomenon, which is simply not understood.

If a primary extrahepatic site of HAV replication exists, it has been difficult to demonstrate. In experimental infections in nonhuman primates, HAV viral antigen and/or genomic material have been observed in the spleen, kidneys, tonsils, and saliva, but have been inconsistently identified in the intestinal mucosa (59,198,212,264,267,401). A recent study in owl monkeys detected HAV antigen in the epithelial cells of the intestinal crypts and in cells of the lamina propria 3 days after inoculation and up to 5 weeks later, but RNA replication was not shown (18). Detection of HAV in tonsils and saliva, shortly after the appearance of virus in the blood, suggests that an early replicative event could occur in the oropharynx or salivary glands (59). In one human study, a saliva sample was about 10,000-fold less infectious than a serum sample, which was 100,000-fold less infectious than a stool sample collected on the same day (325).

Even though mature virions of other picornaviruses exit the cell by infection-mediated disintegration of the host cell, there is little evidence that HAV is directly cytotoxic to hepatocytes. Although some correlation has been seen between severity of disease and virus shedding in the stool (85,115), fecal excretion is, in most cases, greatest before the onset of liver injury, as manifested by an elevated ALT level (see Fig. 6). In addition, viral antigen is first detected in the liver parenchyma of experimentally infected primates 1 to 2 weeks after intravenous inoculation and may persist there throughout the acute phase (146,200,264). Cytopathology (by light microscopy) is unimpressive during this early phase of maximal viral replication, although organelle damage has been detected by electron microscopy (19). *In vitro,* cells persistently infected with HAV are not destroyed, and their metabolism is relatively unaffected (123). These findings suggest that virus-induced cytopathology may not be responsible for the pathologic changes seen in HAV infection and that liver disease may result primarily from immune mechanisms (reviewed in 371). *In vitro* studies have shown evidence of cell-mediated lysis of HAV-infected cells by natural killer cells and by human leukocyte antigen (HLA)-restricted, HAV-specific, $CD8^+$, cytotoxic T cells that were cloned from liver biopsies obtained during acute hepatitis A in patients with hepatocellular necrosis (217,419).

The juxtaposition of hepatitis with the appearance of IgM anti-HAV, immune complexes, and reduced levels of serum complement may be coincidental or relevant to the clinicopathologic changes (22,43,181,257,405). Analysis of the immune complexes has revealed IgM and IgG, HAV capsid proteins, and the C3d component of complement (257). However, patients with hepatitis A appear to lack complement-dependent cytotoxic antibodies (121,378). In addition to these host-mediated immunologic responses, increased levels of interferon have been detected in the serum of HAV-infected patients (74,239,450). Presumably, these biologic phenomena are responsible for the reduction in symptoms and in virus burden seen in patients after the onset of clinical disease.

Pathology

Needle biopsy of the liver is rarely indicated in acute viral hepatitis. In acute hepatitis A, both degenerative and regenerative parenchymal changes coexist with a diffuse and evenly distributed accumulation of mononuclear inflammatory cells, leading to lobular disarray (Fig. 7). Parenchymal cells undergo ballooning degeneration. These hepatocytes are swollen and have indistinct plasma membranes, enlarged nuclei, and a featureless cytoplasm, except for some cytoplasmic remnants condensed around the nuclei (Fig. 8). Disruption of bile canaliculi may lead to bile retention after liver cell enlargement or necrosis. Acidophilic degeneration (apoptosis) also occurs (Fig. 9). Cells become shriveled, angulated, and display increased eosinophilia. Both types of degeneration result in the migration of affected cells or cellular debris into the spaces of Disse and eventually into the sinusoids, where they are surrounded by reticuloendothelial cells (Kupffer cells) and undergo phagocytosis and digestion. Activation of Kupffer cells in the sinusoids and macrophages in the portal tracts results in marked hypertrophy and hyperplasia of these cells. The portal tracts are enlarged as a result of edema accompanied by a moderate-to-heavy infiltration of inflammatory cells, primarily lymphocytes, although neutrophils and eosinophils may be present (Fig. 10). Plasma cells are uncommon, however. Inflammatory cells may spill over into the adjacent parenchyma, a process called *piecemeal necrosis* or *interface*

hepatitis, but less than 25% of the limiting plate is involved. During the recovery stage, regeneration is prominent, as manifested by anisonucleosis, the presence of numerous bi- and trinucleated cells, and mitosis. The inflammatory response diminishes, but Kupffer cell hypertrophy persists for a few weeks. The damaged hepatic tissue is usually restored within 8 to 12 weeks.

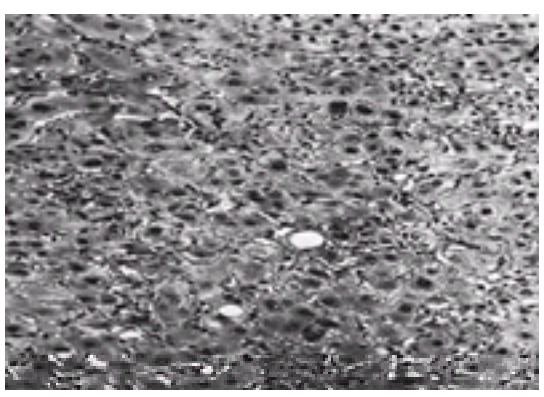

FIG. 7. Acute viral hepatitis. Lobular disarray characterized by anisocytosis, anisonucleosis, ballooning, and Kupffer cell hypertrophy (H&E, ×190). From ref. 183, with permission.

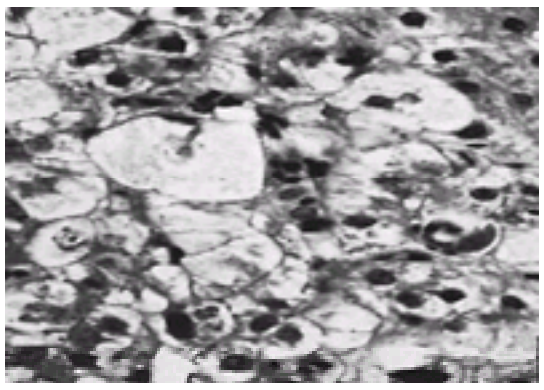

FIG. 8. Acute viral hepatitis. Ballooned hepatocytes show lysis of cell membranes and nuclei and an empty cytoplasm (H&E ×520). From ref. 183, with permission.

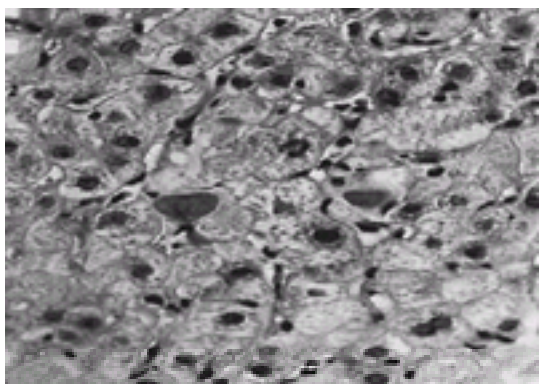

FIG. 9. Acute viral hepatitis. Two sinusoidal acidophilic (apoptotic) bodies can be seen in the center of the field (H&E ×490). From ref. 183, with permission.

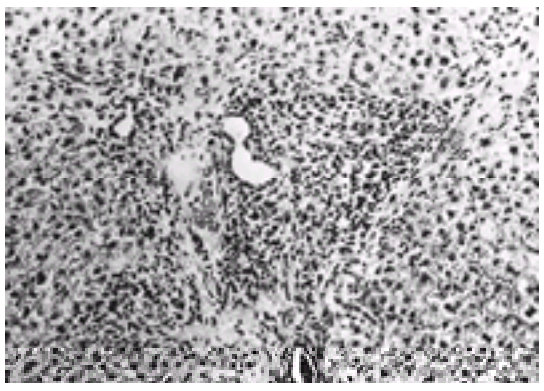

FIG. 10. Acute viral hepatitis. Portal area infiltrated with numerous inflammatory cells (H&E ×140). From ref. 183, with permission.

In 5% to 10% of patients, focal necrosis gives way to zones of necrosis that bridge portal tracts or join portal areas to

central veins (30,42). When this process of confluent (bridging) hepatic necrosis with impaired regeneration involves entire lobules or adjacent lobules, the terms *submassive* (involving the central and middle zones of lobules) or *massive* (involving entire lobules) are applied to the process (Fig. 11 and Fig. 12). The latter is characterized further by the proliferation of cholangioles in the periportal areas infiltrated by neutrophils. Confluent hepatic necrosis is potentially a progressive lesion that may lead to fulminant hepatitis and death in 30% to 60% of cases (42,426). Death appears to be inevitable when necrosis involves more than 65% to 80% of the total hepatocyte fraction (128,358). However, it is encouraging to note that neither functional nor pathologic sequelae are common in patients who survive an episode of acute fulminant hepatic failure despite previous widespread necrosis (176,201).

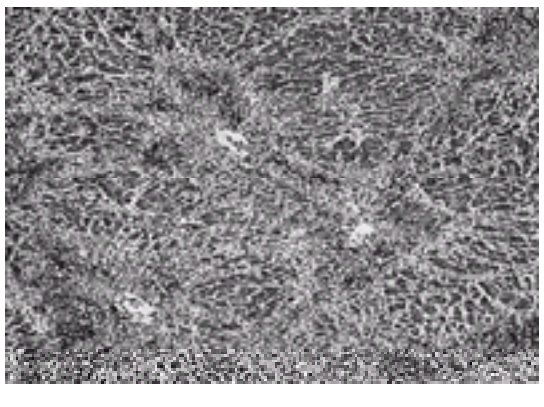

FIG. 11. Acute viral hepatitis with submassive necrosis. Zones of necrosis are linked together (bridging necrosis) (H&E ×43). From ref. 183, with permission.

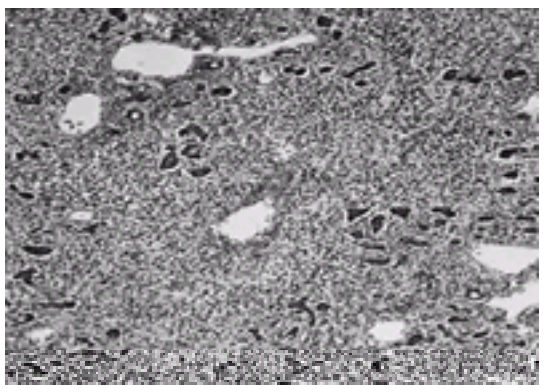

FIG. 12. Acute viral hepatitis with massive necrosis. All liver cells have “dropped out.” Note proliferating periportal cholangioles and infiltration of stroma and portal areas with inflammatory cells (H&E ×73). From ref. 183, with permission.

Rarely, patients with acute viral hepatitis A develop features of cholestasis (deep jaundice, acholic stools, pruritus) that may persist for 2 to 8 months (137). This appears to be more common in hepatitis A than in hepatitis B or C, but is less common than in acute hepatitis E (144,205,357,402). Histologically, there is severe centrilobular cholestasis associated with periportal inflammation that sometimes resembles chronic hepatitis, which never occurs in hepatitis A or E. Plasma cells are prominent throughout the inflammatory infiltrate.

Just as it is difficult to determine the etiology of acute hepatitis without serologic data, the pathologic changes in acute-phase liver tissue obtained from humans with different forms of viral hepatitis are qualitatively similar (30,31,183,250). In general, liver tissue from patients with acute hepatitis A has less conspicuous parenchymal changes (including focal necrosis, Kupffer cell proliferation, acidophilic bodies, and ballooning degeneration) and less occurrence of steatosis than does tissue from acute hepatitis C patients, but necrosis and mononuclear cell inflammation of the periportal region are more evident than in biopsies from patients with acute hepatitis B (2,216,314,402). In addition, the limiting plate is more likely to be disrupted in hepatitis A, and periportal inflammation often simulates chronic hepatitis.

CLINICAL FEATURES

The course of viral hepatitis may be extremely variable. Patients with inapparent or subclinical hepatitis have neither symptoms nor jaundice. These asymptomatic cases previously went unrecognized, but now can be identified by detecting biochemical or serologic alterations in the blood. In addition to the inapparent or subclinical cases, patients may develop anicteric or icteric hepatitis. The terms *inapparent* and *anicteric hepatitis* are often used interchangeably by physicians. However, anicteric hepatitis should be reserved for those patients who develop clinical symptoms but who are not jaundiced. Symptoms ranging from mild and transient to severe and prolonged may accompany anicteric or icteric hepatitis. Patients may recover completely or develop fulminant hepatitis and die (see Table 4).

Parameter	Predicted outcome	
	Children (<5 yr)	Adults
Inapparent infection	80–95%	10–25%
Anicteric or icteric disease	5–20%	75–90%
Complete recovery	99+%	98+%
Chronic disease	None	
Mortality rate		
≤14 yr	0.1%	
15–39 yr	0.3%	
≥40 yr	2.1%	

[a]See text regarding atypical manifestations of hepatitis A.

TABLE 4. *Predicted outcome after an infection with hepatitis A virus*[a]

Signs and Symptoms

Regardless of etiology, the course of acute viral hepatitis is similar and can be divided into four clinical phases: (a) incubation or preclinical period (time between exposure and the first day of symptoms or jaundice; (b) prodromal or preicteric stage; (c) icteric phase; and (d) convalescent period (reviewed in 210).

Although the median incubation period for each type of viral hepatitis may be distinctly different, there is considerable overlap, so that knowledge of the incubation period is rarely useful in determining etiology for individual cases. In addition, the patient may not know the time of exposure. The incubation period for hepatitis A ranges from 10 to 50 days, with a mode of approximately 1 month, regardless of the route of infection (294,309,310). However, the greater the dose of virus inoculated, the shorter the incubation period (310). When two cases occur less than 14 days apart, they are more likely to be co-primary, rather than secondary cases. Correspondingly, cases occurring more than 60 days after a point source epidemic are presumably due to secondary spread of the infection (214,425). During the incubation phase, the patient remains asymptomatic despite active replication of the virus. It is in this preclinical phase that transmissibility is of greatest concern (see Epidemiology).

A short prodromal or preicteric phase, varying from several days to more than a week, precedes the onset of jaundice. In more than half of the patients, the prodromal state typically is characterized by anorexia, fever (usually <103°F), fatigue, malaise, myalgia, nausea, and vomiting. Many of these symptoms may be mediated by interferon induction. In hepatitis A, the transition from well-being to acutely ill occurs abruptly (within a period of 24 hours) in more than 60% of the cases, whereas the onset is more insidious in hepatitis B. Fever above 100.5°F is more common in acute hepatitis A than in hepatitis B (425). Diarrhea, nausea, and vomiting are more frequent in children than in adults (223). In some cases, the diarrhea may be secondary to another enteric infection acquired from the same source (263). Weight loss of 2 to 10 pounds is common as a result of the anorexia that accompanies disorders of taste and smell (166,379). Older children and adults often complain of right-upper-quadrant pain or discomfort as a consequence of hepatomegaly, which usually precedes jaundice by 1 to 2 weeks (425). Severe abdominal pain and protracted vomiting are unusual manifestations and should lead the clinician either to question the diagnosis or to suspect a more severe variant of the disease.

The icteric phase of acute viral hepatitis is ushered in by the appearance of dark, golden-brown urine due to bilirubinuria, followed 1 to several days later by pale stools and yellowish discoloration of the mucous membranes, conjunctivae, sclerae, and skin. This icteric phase begins within 10 days of the initial symptoms in more than 85% of HAV cases (281,454). Jaundice becomes clinically apparent when the total bilirubin level exceeds 2.0 to 4.0 mg/dL (155). Patients often seek medical attention at this stage of their illness. Fever, if present, usually subsides after the first few days of jaundice.

Atypical manifestations of hepatitis A (reviewed in 349) include the development of cholestasis, relapsing hepatitis, extrahepatic manifestations, and the possible induction of type 1 autoimmune chronic hepatitis. Patients with cholestatic hepatitis A initially have pruritus, fever, diarrhea, and weight loss. The serum bilirubin usually remains above 10 mg/dL for more than 12 weeks, whereas the ALT level is often under 500 International Units (IU)/L. Resolution of the disease is slow, but patient recovery is complete. HAV also has been associated with gallbladder wall thickening (409) and acalculous cholecystitis on rare occasions (287). Detection of HAV antigen in bile epithelium and the gallbladder suggests a possible pathogenetic role in HAV-associated cholestasis and gallbladder disease.

Relapsing hepatitis occurs in 3% to 20% of the cases of acute hepatitis A (134,188,376). A recrudescence of the disease, which may be more or less severe than the original episode, occurs 4 to 15 weeks after the initial symptoms have resolved. In some patients, only biochemical changes are observed. IgM anti-HAV either reappears or increases in titer, and HAV has been detected in feces and serum. More than one relapse can occur, and enzyme elevations may persist for 5 to 12 months, although chronic sequelae are not observed.

Extrahepatic manifestations of hepatitis A are unusual, although transient rashes are observed infrequently, as is arthralgia. Clinically apparent arthritis and leukocytoclastic vasculitis have been reported, and mild, membranoproliferative glomerulonephritis associated with immune complex deposits has been seen in a postmortem specimen (174).

Physical examination of the patient with acute disease reveals tenderness to palpation or percussion of the liver that may be enlarged. Measurement by percussion is essential, because reduced size in a deteriorating patient often heralds massive necrosis. Approximately 5% to 15% of patients have splenomegaly. Generalized adenopathy is not a component of viral hepatitis A, B, or C, but often is present with cytomegalovirus- or Epstein–Barr virus–related hepatitis. Palmar

erythema and spider angiomata may be observed.

Occasionally, more extensive necrosis of the liver occurs during acute viral hepatitis, leading to severe impairment of hepatic synthetic processes, excretory functions, and detoxifying mechanisms. This entity, designated fulminant hepatitis if it occurs during the first 6 to 8 weeks of illness, is characterized by the sudden onset of high fever, marked abdominal pain, vomiting, and jaundice followed by the development of hepatic encephalopathy associated with deep coma and seizures (4,347,412). Ascites, a bleeding diathesis, and decerebrate rigidity lead to death in 70% to 90% of the patients. Mortality is highly correlated with age, survival being uncommon among hospitalized patients younger than 10 years or older than 40 years (28,285,301,412,413). Clinical signs portending liver failure include a rapid decrease in the size of the liver, increasing prolongation of the prothrombin time, and a decrease in the aminotransferase levels as the bilirubin level continues to increase. Fortunately, this clinical pattern is rare, occurring in less than 1.5% of the icteric patients hospitalized for acute viral hepatitis.

Table 4 provides a summary of the predicted outcomes that might occur after an infection with HAV. As noted, the disease is milder in children than in adults, complete recovery is the rule, and chronic disease has not been observed (220). Hospitalization and death due to hepatitis A is age dependent. Whereas two thirds of the clinical cases occur in children and young adults, more than 70% of the deaths are observed in patients older than 49 years. The mortality rate is considerably higher among patients with chronic hepatitis B or C who are superinfected with HAV (150,203,445), especially if the underlying liver disease is advanced.

Laboratory Findings

The clinical diagnosis of acute viral hepatitis is made by biochemical assessment of liver function. The initial laboratory evaluation of the jaundiced patient should include a biochemical liver panel, complete blood count (CBC), and prothrombin time. Typically, total serum bilirubin usually remains below 10 mg/dL, but levels of 20 mg/dL may occasionally be observed (393). After reaching a peak in 1 to 2 weeks, the rate of decline is more gradual, with resolution occurring within 6 weeks of onset in most patients (341). Discordant findings between serum and urine bilirubin may occur during convalescence when elevated levels of serum bilirubin may not be accompanied by bilirubinuria (129). This is due to the presence of a conjugated form of bilirubin (delta bilirubin) that is irreversibly (covalently) bound to albumin (427), thereby impairing its excretion in the urine.

Serum ALT and AST concentrations are highly sensitive indicators of hepatocellular injury. They provide a quantitative assessment of acute damage sustained by the liver, although the eventual outcome cannot be predicted from the level attained. ALT is primarily localized to the liver. Within hepatocytes, AST is found in mitochondria (80%) and the cytosol (20%), whereas ALT is limited to the cytosol. This compartmentalization of enzymes may partially explain the aminotransferase patterns seen in many forms of liver disease. For example, during “classic” acute viral hepatitis, liver biopsy sections reveal ballooning degeneration throughout the lobule. This is characterized by swelling of hepatocytes, indistinct or lysed cell membranes, and cytoplasmic rarefaction (183). Ultrastructural studies reveal marked dilatation of the cisternae of the endoplasmic reticulum, loss of glycogen, and condensation of the inner compartments of mitochondria (415). These relatively mild and reversible changes may be induced by factors that damage the plasma membrane (416), leading to ionic and water shifts followed by leakage of enzymes from the cytosol. Consequently, ALT levels are significantly higher than AST levels in uncomplicated acute viral hepatitis, resulting in a high ALT/AST ratio (>1.4) in almost all cases. Exceptions include those situations in which severe tissue necrosis develops, resulting in the release of mitochondrial AST into the blood. In these situations, the lactic dehydrogenase enzyme (LDH) is significantly elevated.

Certain serum proteins, such as albumin, prothrombin, fibrinogen, and vitamin K–dependent coagulation factors, are synthesized exclusively or predominantly by the liver. Because the intravascular half-lives of the various plasma-clotting factors range from 5 hours (factor VII) to 5 days (fibrinogen), measurement of both the extrinsic clotting system (prothrombin time) and the intrinsic pathways (partial thromboplastin time) may more accurately reflect early and potentially serious derangements in liver function than does the measurement of serum albumin, which has a half-life of 3 weeks. Conversely, because albumin is the major secretory protein produced by the liver and is important for osmotic regulation, transport of various metals, and binding of other substances, its concentration is a useful guide to prognosis and therapy.

Generally, the patient with typical acute viral hepatitis has a normal or slightly reduced number of segmented leukocytes and a relative lymphocytosis (164). When the white blood count is more than 12,000/mm^3, a more serious form of the disease is anticipated. Although mild to moderate reduction in red blood cell survival is frequent in acute viral hepatitis, the hematocrit and hemoglobin remain within normal limits (64,202). Occasionally, hemolytic anemia occurs, often in association with glucose-6-phosphate dehydrogenase deficiency (55,56,180,207,345). Rarely agranulocytosis (290), thrombocytopenia (99,251), pancytopenia (207), red cell aplasia (408), or aplastic anemia (152,251,439) accompanies acute viral hepatitis.

DIAGNOSIS

Because the agents of viral hepatitis often cannot be distinguished clinically and HAV superinfection of hepatitis B and C carriers can occur, serologic means are required for their identification (172,173). To accomplish this, a variety of diagnostic profiles are offered to the clinician to ensure that a misdiagnosis does not occur, because this may impinge on immunoprophylaxis. The most favored acute viral hepatitis profile includes assays for IgM anti-HAV, HBsAg, IgM anti-HBc, and anti-HCV. However, idiosyncrasies of the assays make it desirable to perform ancillary tests that will confirm a positive response. In detection of HAV infection, this would include a measurement for total anti-HAV (IgG and IgM), because the detection of IgM anti-HAV always should be accompanied by a positive test in the assay that measures total specific antibody. The reader is directed to the diagnosis section in Chapter 87 for an algorithm covering the serologic diagnosis of HAV, HBV, HCV, and HDV. HEV is discussed separately in Chapter 89.

Before the onset of clinical symptoms, HAV appears in the blood and stool (Fig. 13). Compared with the relatively high concentrations of virus shed in the feces (10^9 virions/g), virus concentrations found in blood are relatively low (10^5 virions/mL) (Fig. 14). Should preillness samples of blood or feces be available, HAV can be detected by experimental infection of nonhuman primates or cell cultures, immunoassays [enzyme imunoassay (EIA) and radioimmunoassay (RIA)], immune electron microscopy, hybridization assays, or the RT-PCR method (83,108,187,296,334,359,376,448) (see also Host Range and Experimental Infection in Animals , earlier). However, none of these methods is practical in a clinical setting; they are not necessary unless incontrovertible proof for the presence of infectious virus is needed.

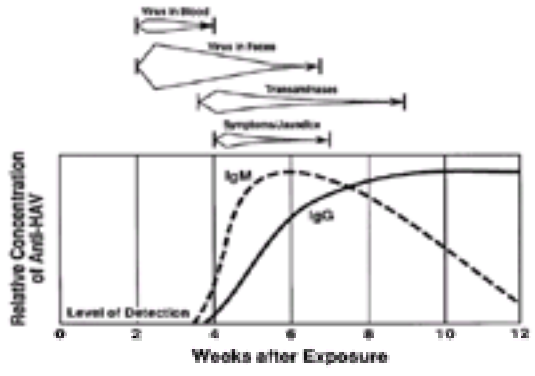

FIG. 13. Immunologic and clinically relevant biologic events associated with hepatitis A virus (HAV) infection in humans. Modified from ref. 172, with permission.

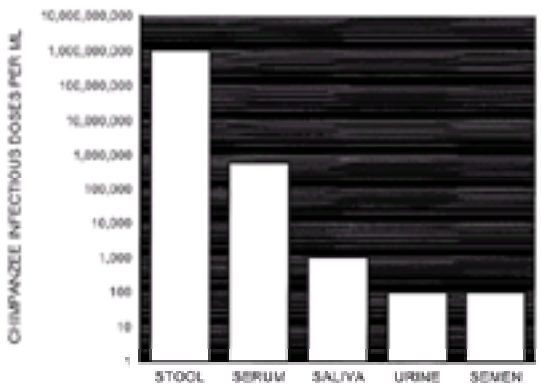

FIG. 14. Infectivity titers of body fluids and excretions.

To establish the diagnosis of acute or recent hepatitis A, blood samples are examined for the presence of IgM-specific anti-HAV (see Fig. 13) (43,79,243). This acute-phase antibody rapidly increases in titer over a period of 4 to 6 weeks, and then declines to nondetectable levels within 3 to 6 months in most patients (range of <30 to >420 days, with persistence for more than 200 days in 13.5%) (196). In more than 85% of the subjects, the liver enzymes will be normal before or at the time of disappearance of IgM anti-HAV. IgG anti-HAV can be detected simultaneously or within a week or 2 of the acute illness, and eventually replaces the IgM antibody. IgG antibody persists for years after infection (377).

The total anti-HAV assay is used primarily to determine the immune status of an individual after vaccination or infection or to assess risk in a person traveling to an endemic region or working in a high-risk area. In the absence of either IgM anti-HAV or an abnormal ALT level, clinicians can assume that the antibody being detected is of the IgG immunoglobulin type, indicating previous infection with HAV—or successful vaccination—and protection against future infection. These conclusions are valid only if passively acquired antibody from blood components, maternal antibodies crossing the placenta, or immune globulin immunoprophylaxis can be excluded. A more extensive review of the laboratory procedures and their interpretations can be found in reference 173.

Immunocytochemical staining has been used to identify HAV in the liver of infected primates from biopsies obtained early in the course of the disease (178,267,288,363). Viral antigen also has been localized to the cytosol–microsomal subcellular fraction (93%), with 4% in the nuclear fraction (204). Within the microsomal fraction, more than 85% was detected in the smooth microsomes. Studies of human liver have demonstrated HAV in cytoplasmic vesicles of hepatocytes and Kupffer cells and in the cytoplasm of epithelial cells of the biliary ducts (287).

MANAGEMENT OF ACUTE VIRAL HEPATITIS

There is no specific treatment for acute viral hepatitis, and liver biopsy is unnecessary. Therapy should be supportive and aimed at maintaining comfort and adequate nutritional balance. Although the disease is usually mild in children younger than 10 years, those who become clinically ill are more prone to develop fulminant hepatic failure compared with those who develop clinical HAV infection between the ages of 10 and 40 years. At older than 40 years, the severity of the liver disease intensifies. For most jaundiced patients, strict bed rest and prolonged confinement are probably not indicated (53). In one study, strenuous activity did not appear to have any adverse effect on the clinical course of acute viral hepatitis (330). Prudent therapy, however, seems to indicate a course of bed rest that is carefully tailored to the severity of the patient's illness and to his or her emotional well-being without insistence on prolonged inactivity.

Hospitalization is not ordinarily indicated.

The diet should conform to the patient's appetite and wishes; it must, however, supply adequate protein (1.0–1.5 g/kg) and calories (30 to 35 cal/kg). However, hospitalized patients may benefit from protein restriction, supplying all of the calories as an intravenous infusion of carbohydrate for a few days (see later). The use of oral, enteral, or intravenous amino acids in acute hepatitis has not been adequately evaluated. There is no good evidence that restriction of fats has any beneficial effect on the course of illness, and ingestion of fats in moderate amounts may help provide sufficient caloric intake. As a general rule, modest consumption of alcohol (<30 g/day for men and <15 g/day for women) during convalescence (6 to 24 weeks after symptoms develop) does not seem to be harmful when the ALT level has decreased to less than 1.5 times the upper limit of normal (410). Nevertheless, because of the direct hepatotoxic effect of ethanol, abstinence from alcoholic beverages during the acute phase of hepatitis seems prudent. Finally, women who are taking oral contraceptives need not discontinue their use during the course of hepatitis (356).

There is no indication for corticosteroids in the treatment of acute, uncomplicated hepatitis A. They have no effect on the rate of resolution of the underlying disease process and may increase the rate of relapse or result in death from fulminant hepatitis, especially if administered during the incubation or prodromal stage of infection (36,102,103,350). Conversely, a 4-week course of prednisone with tapering doses can hasten resolution in some patients who have developed prolonged, moderate-to-severe cholestasis, although complete recovery without therapy will eventually occur in all (349,438). Abruptly stopping the medication or shortening the interval of therapy can result in a relapse. Immune globulin or antibiotics also are of no value in acute viral hepatitis (101,131).

The treatment of confluent necrosis or fulminant hepatitis has been unsatisfactory, and mortality rates from these complications remain universally high. Nevertheless, intensive general supportive care and the management of complications are responsible for much of the recent improvement in survival rates. Although of no proven survival value in fulminant hepatitis, conventional treatment has included the reduction of protein intake and the use of cleansing (lactulose) enemas followed by the oral administration of neomycin or lactulose to decrease blood ammonia concentration, thereby alleviating hepatic encephalopathy. Clotting abnormalities should be corrected with the administration of fresh-frozen plasma. If the prothrombin time is prolonged, a single injection of vitamin K (10 mg) is justified. Seizures can be controlled with small, repeated doses of oxazepam (oral only) or lorazepam, because these drugs are conjugated by glucuronidation (which is not restricted to hepatic microsomes).

Because hypoglycemia is often prominent in critically ill, hospitalized patients, glucose levels should be monitored at least twice daily and caloric requirements maintained by the intravenous administration of glucose (16 cal/kg). It is recommended that 10% (rather than 5%) glucose be used, because patients with severe hepatitis sometimes have difficulties excreting a normal water load. Electrolyte disturbances also are common because of impaired sodium excretion and free water clearance, especially in patients receiving blood products for coagulation defects, osmotic diuretics for cerebral edema, or glucose in water for nutrition (437). Supplementation of the glucose with sodium and potassium chloride is recommended. In addition, vitamin B complex with thiamine is useful while intravenous therapy is being administered (340).

With regard to the treatment of fulminant and subfulminant liver failure with coma, a number of procedures designed to remove toxins temporarily (29,391) often improved the neurologic status of the patient, but survival was not improved, and complications often occurred. Massive doses of corticosteroids or adrenocorticotropic hormone (ACTH) also have not been effective and may be harmful (3,100,103,104). Recently the use of hybrid bioartificial liver systems to improve the metabolic status of patients with fulminant hepatitis until the liver tissue regenerates or orthotopic liver transplantation (OLT) can be arranged has yielded encouraging results. These systems use hollow-fiber membranes containing a human liver cell line or primary porcine hepatocytes (342,392). Primary hepatocytes are typically embedded in a collagen gel or attached to collagen-coated beads to maintain differentiated function and to allow cryopreservation (87). The addition of nonparenchymal cells to these support systems could stabilize the environment by contributing matrix protein for the control of morphogenesis and proliferation of the cells (390). They are being proposed for the treatment of liver failure in centers where the option of liver transplantation also exists. In the interim, acute liver failure is more successfully managed by the application of emergency OLT (total, auxiliary partial, or living-related partial), recognizing that the survival rate in fulminant hepatitis A ranges between 33% and 63% (29). Correspondingly, indications for emergency OLT in patients developing encephalopathy remain controversial. Selection is critical, because most patients with grade 4 encephalopathy (coma) will survive without a liver transplant and recover completely, whereas OLT results in survivors who require a lifetime of immunosuppression and medical management. By transplanting only those patients with a poor prognosis (reviewed in 301), 1-year survival rates of 80% for fulminant hepatitis A have been achieved.

EPIDEMIOLOGY

Hepatitis A is one of the most common causes of infectious jaundice in the world today. Recurrent epidemics are a prominent feature of the disease. In 1998, the total number of cases of viral hepatitis A reported to the National Notifiable Diseases Surveillance System operated by the Centers for Disease Control and Prevention was 23,229, yielding a rate of about 8.59 cases per 100,000 population (52). This preserves a downward trend seen in the hepatitis A rates since 1995.

Rates of disease are particularly high among children and young adults and in American Indians/Alaskan Natives and Hispanics (Fig. 15) (50). The rate in male subjects is approximately 1.6 times higher than that in female subjects, and the rate in the western United States is more than 2.5 times the average rate in the other regions of the country. The actual incidence of disease is much higher because many persons contract such a mild form of hepatitis that they do not seek treatment and because physicians report fewer than 12% of the hospitalized cases (209,258). It is estimated that 125,000 to 200,000 total infections occur annually, of which 84,000 to 134,000 are symptomatic. From 11% to 22% of those with overt hepatitis A are hospitalized, and approximately 100 die of fulminant hepatitis. Table 4 provides mortality data for HAV by age group. Based on the total number of viral hepatitis cases reported in 1998, 63% were due to hepatitis A, 28% to hepatitis B, and 9% to hepatitis C/non-A, non-B (Fig. 16) (52).

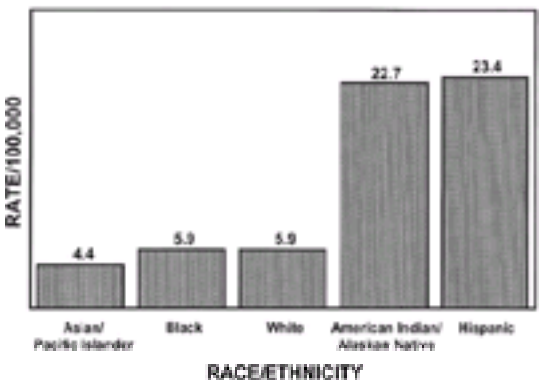

FIG. 15. Rates of reported hepatitis A cases (per 100,000 population) in the United States, by race/ethnicity, 1997. From ref. 50, with permission.

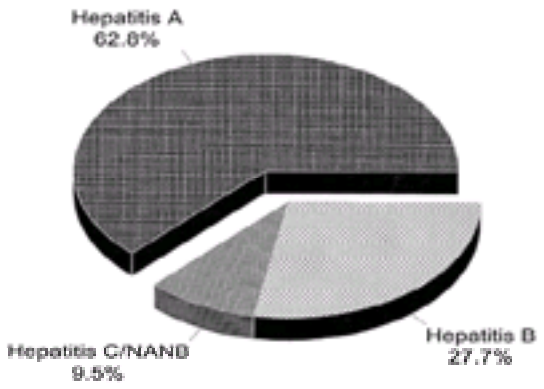

FIG. 16. Distribution of viral hepatitis cases in the United States reported to the Centers for Disease Control and Prevention in 1998. From ref. 52, with permission.

The level of sanitation or hygiene in the environment markedly influences the epidemiology of hepatitis A. In populations in which living conditions are crowded and sanitation is nonexistent or inadequate, prevalence studies reveal that infections occur at an early age and that close to 100% of children acquire immunity during the first decade of life (26,116,148,270,394,395). Transmission of HAV within crowded institutions, where the disease is hyperendemic, depends on length of institutionalization and is unrelated to the age of the individual at first admission (397). In contrast, in modern urban societies or in developing nations where improvements in sanitation and personal hygiene have delayed infection, a reduction in anti-HAV prevalence among younger persons has been found (149,184,270,348,395). Large segments of the population remain susceptible, and epidemics may occur after chance introduction of the virus to an area.

The principal mode of transmission for HAV is by the fecal–oral route, often resulting in community-wide outbreaks. Thus hepatitis A could be considered to be an enterically transmitted disease. Relatively high concentrations of HAV are shed in the feces 3 to 10 days before the onset of clinical symptoms or a few days before jaundice, near the time when the ALT level initially becomes elevated (see Fig. 13) (66,83,110,115,153,170,328). Communicability is apparently highest during this interval (27,82,328). Excretion of infectious HAV has been documented in stool specimens collected as early as 2 to 3 weeks before, and up to 8 days after, the onset of jaundice (Fig. 17) (161,215). Transmission is severely curtailed during the acute and early convalescent stages of the disease, requiring increasingly larger quantities of fecal material to elicit an infection (214,215,245,425). In adults, viral particles are more often detected in the stools of patients who become icteric, in contrast to patients who develop anicteric hepatitis or no clinical disease (85,115). In infants and young children, fecal excretion of HAV antigen and HAV RNA persist for a longer interval than is observed in adults (338,399). In one study (338), HAV RNA was detected by RT-PCR in three infants up to 4 to 5 months after they had been identified as being infected. Whether such excretion is biologically relevant has not been established.

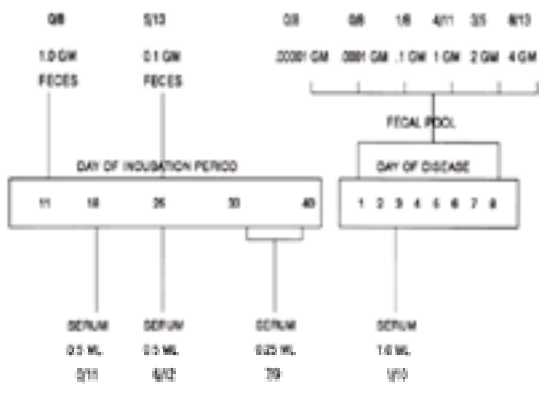

FIG. 17. Relative levels of infectivity observed for feces and serum samples collected during the incubation period and early stages of acute hepatitis A (215). The number of subjects tested versus the number developing clinical hepatitis A are provided for each quantity of sample evaluated. [In the absence of a diagnostic hepatitis A virus (HAV) assay in 1959 to determine susceptibility, the number tested is not necessarily equivalent to the number of susceptible subjects.]

Anti-HAV of the IgA class has been detected in stool specimens during the early stages of infection after the transaminase level has peaked and immunoglobulin-coated particles have been visualized (242,447). The appearance of immune complexes correlates with the abrupt reduction or absence of detectable particles of HAV seen in these preparations (44,83,85,110,170,328) and may account for the low rate of transmission to health care professionals exposed to hospitalized cases of hepatitis A (268).

The presence of virus in feces facilitates virus dissemination and accounts for the widespread outbreaks of hepatitis A that occur among household contacts or sexual contacts of a person who has hepatitis A (51). Other outbreaks have been observed in children and employees associated with day-care centers or with contacts of these individuals (151), in neonatal intensive care units (21,208,298,338), among the military, and in international travelers. Because clinical illness is uncommon in infants and young children, it is disease in their adult contacts that often calls attention to the problem in child-care centers and in neonatal intensive care units. In contrast to this person-to-person mode of spread, sudden, explosive epidemics of hepatitis A within communities or confined populations usually result from fecal contamination of a single source, such as drinking water, milk, or food prepared by persons in the incubation stage of their disease and served without further cooking (277,283,284,400,407).

Several outbreaks of hepatitis A have resulted from the consumption of raw or inadequately cooked oysters and clams obtained from water polluted with sewage (156,263,343). The largest outbreak in recent years occurred in Shanghai in 1988, when more than 300,000 reported cases of hepatitis A were identified, primarily in young adults who ingested uncooked clams from polluted water (445). A low case fatality rate of 0.015% was observed in this epidemic. The role of mollusks has been that of virus concentrators of water polluted by sewage rather than propagators of virus. During feeding, physiologically active bivalves (e.g., oysters or mussels) can filter up to 10 gallons of water per hour over a short period, during which HAV can be concentrated at least 100-fold and persist for about 7 days (98). The rate of uptake and depuration is slower without feeding. As expected, nonbivalve shellfish (e.g., lobsters, shrimp) do not impart the same risk.

Human transmission studies and molecular techniques have provided evidence that transient viremia occurs during infection from HAV (38,113,132,157,161,174,215,424,448). The viremia often precedes the development of symptoms by 7 to 14 days (see Fig. 13 and Fig. 17). By the time the patient seeks medical attention or the transaminases reach a peak, clinically relevant viremia has virtually disappeared. Rarely, viremia may be present during the early stages of jaundice (215). Studies using RT-PCR have shown that HAV RNA appears in the blood earlier than appreciated from the transmission studies, reaches its maximal concentration before peak ALT levels, and persists for 30 days or more from onset of symptoms (41,117,143,448). This discordance between detection of virus-specific nucleic acid and a capacity to transmit HAV is noteworthy, but not unexpected. However, the clinical relevance of these observations remains to be established.

HAV transmission through blood is a rare but potential cause of posttransfusion hepatitis (25,51,113,157,174,226). The problem is amplified in neonatal intensive care units where infants develop infection after receiving blood components from an infected donor, but do not develop symptoms of hepatitis. They subsequently transmit the virus to health care workers and family members, presumably by the fecal–oral route (21,208,298,338). The rarity of transfusion-associated hepatitis A probably can be attributed to several closely associated factors. First, the viremic stage is of short duration (see Fig. 13 and Fig. 17) (38,113,161,215), and the concentration of HAV in the blood is relatively low (171,174). The low incidence of HAV infection in the U.S. (52) and the absence of an HAV carrier state (214) also contribute to the rarity of HAV transmission through blood. For HAV transmission to occur, an infected donor would have to donate blood during a very restricted period. Even if this situation existed, the probability that the infectious unit would be given to an HAV-susceptible recipient diminishes with increasing age of the recipient (270,395). In addition, because most recipients are given more than one unit of blood, an anti–HAV-positive unit may be transfused with the HAV-positive unit, thereby neutralizing the virus. The last two scenarios often do not apply to neonates, which is why the risk is greater in this group. Current blood bank practices do not include specific screening of prospective donors for evidence of active HAV infection.

From 1989 to 1995, several outbreaks of hepatitis A occurred among hemophiliacs in Europe, South Africa, and the U.S. who received organic solvent- and detergent-treated factor VIII (253,332). Cases were dispersed geographically, were not associated with community outbreaks, and implicated lots contained HAV genomic material whose genetic sequence was identical to that obtained from patients. Subsequent experimental studies determined that 5.3 to 8.5 $\log_{10}$ $TCID_{50}$ of HAV would be inactivated during the processing of frozen plasma to cryoprecipitate (234). The solvent/detergent treatment had no effect on HAV infectivity, but could have been responsible for dispersing HAV in the product by releasing the virus from a nonneutralizable, lipid-associated environment. Older preparations of factor VIII contained substantial amounts of IgG that may have provided protection in this situation. However, the highly pure lots of factor VIII currently available were devoid of appreciable amounts of neutralizing anti-HAV. Thus the situation was favorable for factor-derived hepatitis A infections to occur when they did. Fortunately, effective heat-inactivation procedures now render these products safe.

There is little evidence for transmission of HAV by exposure to the urine or nasopharyngeal secretions of infected patients (163,248,295), although cases have been reported (1,132,194,248). Transmission of HAV by externally contaminated, coprophagous insects such as flies or cockroaches, or by biting insects, is conceivable but remains unconfirmed.

Other identified potential sources of exposure to HAV are nonhuman primates, usually chimpanzees, which have infected caretakers and other zoo personnel in close contact with them (73,167,168). In most instances, more than 3 months elapse between importation of the animals and appearance of the index case. Thus it is assumed that these animals

acquire the infection from humans or other primates shortly after arrival, and then transmit the disease to their caretakers.

PREVENTION AND CONTROL

Any decisions concerning the prevention of hepatitis A must consider the immunobiology of the disease and the principal routes of transmission (169). The most effective control measures must prevent fecal contamination of hands, food, water, or other sources by infected individuals before their clinical disease becomes apparent (51). Thus the most critical control measure is proper handwashing after bowel movements and before food preparation, and the avoidance of work practices that facilitate contamination of hands when taking care of children younger than 2 years. The importance of these measures cannot be overemphasized. Because the personal hygiene of young children is often difficult to control, they should be restrained from close contact with their susceptible playmates during the first 2 weeks of illness or for at least 1 week after the onset of jaundice.

Considering the low order of infectivity that is present during the icteric stage of disease in adults, many authorities have suggested that strict enteric precautions within the hospital are not necessary. Such precautions should be reserved for physicians and staff caring for newborn infants, young children, or fecally incontinent patients (105,298). Maynard (268) observed that physicians caring for most of the cases of hepatitis A had anti-HAV prevalence rates no greater than those in other specialties. In another study, Mosley (286) reported that no cases of hospital-acquired hepatitis A could be documented among several thousand patients admitted to the hepatitis service of a large hospital of indigent patients, despite continuing surveillance. It is noteworthy that toilet facilities were shared in this environment, and the personnel were not routinely gowned and gloved.

Although nosocomial infections appear to be rare, there have been situations in which transmission has occurred (21,90,208,298,338). In one outbreak, 55 cases of HAV (64% symptomatic) occurred among physicians, staff, parents, and relatives having direct contact with 11 neonates who were given blood from a single infected donor (298). The attack rate was 25% for susceptible relatives, 16% for nurses, and 4% for physicians. Transmission by aerosol or by contact with urine is highly improbable, regardless of the stage of infection. In general, universal precautions should be adequate when caring for patients with hepatitis A. Extraordinarily conservative measures, such as isolation of patients in private rooms and the use of gowns or masks, are unnecessary unless direct and excessive exposure to feces or fecally contaminated items is anticipated. More responsibility for preventing nosocomial hepatitis A can often be transferred to the patient unless that patient is a young child.

Although viremia also precedes clinical disease and could represent an additional potential source of infection (38,161,174,214,215), chronic viremia does not occur. Thus the potential for infection by inadvertent percutaneous transmission is very low, as corroborated by HAV prevalence studies in patients and staff of hemodialysis centers where parenteral exposure is common (396).

Immune Globulin Immunoprophylaxis

Current lots of conventional intramuscular immune globulin (IGIM) are sterile preparations of concentrated antibodies that have tested negative for HBsAg, anti-human immunodeficiency virus (HIV), and anti-HCV. No cases of HBV, HIV, or HCV have occurred after the administration of this product, in contradistinction to intravenous immune globulin (IGIV), which has been associated with the transmission of HCV in at least one IGIV preparation (135).

Prevention of clinical manifestations of hepatitis A through the administration of IGIM was documented initially by Stokes and Neefe (389) in 1944 during a common-source outbreak at a summer camp near Philadelphia. Among 53 persons who received IGIM, clinical hepatitis occurred only during the first 10 days in 11 (21%), and icterus was observed in only one of these early cases. In contrast, new cases of hepatitis continued to occur for 32 days in 67% of the 278 exposed controls, and 45% of these were icteric. This represents an 87% reduction in the attack rate observed among those receiving IGIM. Similar immunoprophylaxis studies were carried out simultaneously in military personnel and institutionalized children (130,165). In each instance, IGIM was capable of preventing anicteric or icteric hepatitis A in 80% to 90% of the participants. IGIM was effective when given up to 6 days before the onset of illness, and doses as low as 0.01 to 0.02 mL/kg were found to be protective. IG collected from any geographic region should be protective against HAV infection because only a single human HAV serotype exists.

Studies performed in institutions (Table 5) and those conducted during community outbreaks of hepatitis A indicate that passive immunization with IG can either completely suppress or modify the infection in HAV-susceptible persons, depending on the concentration of anti-HAV administered and the interval from exposure to treatment (89,154,388). As shown, larger doses of IG provide protection lasting a few more weeks, but passive–active responses are curtailed, leaving a larger percentage of susceptible persons available for infection after another exposure. IG is unlikely to be effective if given more than 3 weeks after exposure. The theoretic concern, that individuals might acquire HAV from persons who develop subclinical hepatitis after IG treatment, has not been borne out by epidemiologic investigations (302,388). Rigid isolation or compulsive cleanliness within a household is not necessary once the occupants have received adequate prophylactic treatment.

IG dose (mL/kg)	No. of susceptibles	No. with infection: Clinical	No. with infection: Subclinical	Seroconversion only	% Remaining susceptible
None	19	8	4	4	16
0.04	15	1	3	3	53
0.10	10	0	0	2	80

Source: Modified from ref. 154, with permission.

TABLE 5. *Effect of immune globulin (IG) on HAV infection among exposed residents of a custodial institution*

After the administration of IG, commercial assays for anti-HAV are unable to detect this antibody in the blood unless the procedure is optimized (8). However, low-level neutralizing antibody has been found to persist for at least 55 days when sera were evaluated with a sensitive neutralization assay. Because a direct correlation exists between antibodies detected by commercial immunoassay and the neutralization assay, it can be assumed that very low levels of anti-HAV are sufficient to prevent infection.

Vaccines

Until 1995, passive immunization with IG was the only option available for preventing HAV infection. However, IG is impractical when repeated exposure is anticipated. In 1978, Provost and Hilleman (321) produced the first prototype HAV vaccine, a formalin-treated homogenate of liver from an HAV-infected marmoset. The vaccine was used to inoculate susceptible marmosets, all of which developed anti-HAV and were protected against an intravenous dose of live HAV. These promising results, along with the successful propagation of HAV in cell culture (322), stimulated additional experimentation with HAV with approaches similar to those successful for poliovirus vaccines.

With the development and subsequent evaluation of hepatitis A vaccines, the potential for long-term immunity has become a reality. As nations develop public sanitation, the age at which individuals will become infected is delayed until adulthood, at which time the likelihood of developing symptomatic illness is considerably higher. The large outbreak of hepatitis A that occurred in Shanghai during 1988 was a recent example of this trend (445). HAV infection has become a public health problem elsewhere in Asia (Korea, Singapore), Europe (Greece, Italy, Portugal), and the Americas (Cuba, Chile) (145,195). HAV also causes up to 63% of the clinical hepatitis reported in industrialized nations (52,112,386). A review of the various products available for active immunization follows, concluding with strategies for immunoprophylaxis.

Inactivated Vaccines

Several laboratories have produced candidate killed virus vaccines by inactivating cell culture–derived HAV with formalin or b-propiolactone (14,225,324,370). Binn et al. (33) and Sjogren et al. (374) extensively characterized formalin-inactivated HAV HM175 that was propagated in human diploid MRC5 cells. Inoculated owl monkeys developed antibody that neutralized six HAV strains of wide geographic origin, and they were protected against challenge with live virus. Forty-two male volunteers were given three or four doses of vaccine over a 6-month interval; 86% developed neutralizing antibody 2 months after the last dose (374).

SmithKline Beecham (SKB) conducted large safety and efficacy trials using the HM175 strain adapted to MRC5 cells (147). HAV is extracted from the cells by freezing and thawing. This crude harvest is tested for the absence of extraneous agents, microbiologic sterility, and identity of the antigen. The harvest is then purified and concentrated by sterile filtration, ultrafiltration, and column chromatography (311). The purified virus is assayed for antigen, total protein, and bovine albumin concentration. Residual MRC5 cellular protein (<5 µg/adult dose) and traces of formalin (<0.1 mg/mL) are present. In addition, less than 8 µg of bovine albumin per adult dose remains in the vaccine. Virus particles with a buoyant density of 1.32 to 1.34 g/mL and a mean diameter of 27 nm are observed. Sterile-filtered virus is inactivated with formaldehyde for 15 days at 37°C. Separate sterile filtrations are done on day 6 and at the end of the inactivation period. Preclinical trials of this vaccine in chimpanzees have demonstrated safety and efficacy. There was no detectable excretion of HAV during the vaccination or challenge periods.

The licensed SKB adult vaccine (Havrix) contains 1,440 ELISA units (EL.U.) of viral antigen per milliliter (determined by a standardized ELISA), 0.5 mg aluminum as $Al(OH)_3$, and 5.0 mg 2-phenoxyethanol as a preservative. Primary immunization consists of a single dose of vaccine with a booster dose 6 to 12 months later to obtain a more durable immunity. Children (2 through 18 years of age) receive one half or one fourth of these amounts in 0.5 mL. The smaller amount is administered twice at 1-month intervals, whereas the 720 EL.U. is given once. For both pediatric schedules, a booster dose is recommended 6 to 12 months after the primary course. The vaccine should be administered by the intramuscular route, preferably in the deltoid region in adults. Suboptimal responses may result if the vaccine is administered in the gluteal region. Accelerated stability tests at 37°C for up to 3 weeks and after 15 months at 2°C to 8°C have revealed no loss of immunogenicity in mouse potency tests. Similar results were observed in humans. As has been discovered with the hepatitis B vaccine, freezing may be deleterious to the product.

Another licensed formalin-inactivated hepatitis A vaccine is VAQTA, distributed by Merck & Co., Inc. This vaccine also is prepared from MRC5 cell monolayers infected with an attenuated HAV, CR326F'. The original CR326 was isolated from a fecal sample that was inoculated directly into a FRhK6 culture (241,319). After 15 passages in these cells, it was adapted to MRC5 cells for an additional 16 passages, which led to its attenuation for marmosets and chimpanzees (319). Further

serial passage yielded highly productive replication of HAV sufficient for commercial development. Antigen is extracted by lysis of infected cells, and then concentrated and purified to greater than 90% protein purity. The purified preparation is free of detectable MRC5 protein and contains less than 0.1 µg of nonviral protein, less than 4×10^{-6} µg of DNA, 10^{-4} µg bovine albumin, and less than 0.8 µg of formaldehyde per 50-unit (U) dose. The antigen is composed of monodispersed, full and empty, HAV particles. Formalin inactivation proceeds for 5 days at 37°C, after which the product is adsorbed to aluminum hydroxide. The 50-U adult dose contains 450 µg aluminum, whereas a 25-U pediatric/adolescent dose, for ages 2 through 17 years, contains half as much. The vaccination regimen consists of one primary dose followed 6 to 18 months later with a booster dose. The vaccine was preclinically tested in chimpanzees, which developed neutralizing antibody within 2 weeks and were protected after intravenous challenge with infectious HAV.

Both of these vaccines have proven to be safe and immunogenic in humans (13,297). Reactions have been mild and clinically insignificant, with soreness at the injection site (generally mild and transient) being the most commonly elicited symptom. Hypersensitivity to components of the vaccine has not been observed. Of some 4 million doses of Havrix administered as of December 1993, only 353 adverse events (0.009%) have been reported to SKB, of which only six serious events (0.00015% or 1.5 per million doses) were believed by a physician to be related to the vaccine (13). These complications, and other postlicensure reports of serious adverse events received by the manufacturer or the Food and Drug Administration, without regard to causality, have included Guillain–Barré syndrome, neuropathy, myelitis, multiple sclerosis, encephalopathy, erythema multiforme, convulsions, anaphylaxis, anaphylactoid reactions, vasculitis, thrombocytopenia, diabetes mellitus, pancreatitis, rhabdomyolysis, allergic reactions (such as a rash), and paresthesia. In those situations in which background incidence data are available, rates in vaccine recipients are not higher than they are in an unvaccinated population.

Immunogenicity in adults, adolescents, and children aged 2 years or older is excellent, with protective levels of antibody (^{3}20 mIU/mL) being achieved in virtually all subjects after the basic immunization series. Development of IgM anti-HAV after vaccination is rare (364,374), and the ALT level remains normal, so confusion with recent viral hepatitis should not be an issue. Immunogenicity data for infants is sparse, but initial studies indicated excellent responses in seronegative infants without the development of serious adverse events (414). In contrast, responses in infants with preexisting maternal antibody have been impaired. Other factors associated with reduced immunogenicity include immunosuppression from disease or drugs and advanced age.

The administration of hepatitis A vaccine with other vaccines, such as diphtheria, poliovirus (oral or inactivated), tetanus, hepatitis B, yellow fever, typhoid, rabies, and Japanese encephalitis, has failed to modify the immune response to the hepatitis A vaccine or to increase the frequency of adverse events (7,32). Durability of immunity cannot be accurately assessed because the current vaccines have been under evaluation for only 7 to 8 years. However, estimates using kinetic models suggest that protective levels could persist in a majority of vaccinees for 20 years or more (420,436).

The simultaneous administration of inactivated hepatitis A vaccine with 3 to 5 mL IGIM should provide both immediate and long-term protection in adults (222). However, the anti-HAV titers are about twofold lower when compared with those observed when vaccine alone is administered, but more than 100-fold higher than levels considered to be protective. Although IG may not interfere with the immune response to oral poliovirus or yellow fever vaccine, or to inactivated vaccines, it may interfere with the response to live, attenuated vaccines such as measles, mumps, rubella, and varicella. Therefore IG should be delayed at least 2 weeks after such immunizations, or if IG is administered initially, at least 5 months should pass before these other vaccines are administered.

Two large efficacy trials have been conducted with the Merck and SKB vaccines. In the first, 505 evaluable children between the ages of 2 and 16 years received one injection of inactivated hepatitis A vaccine produced by Merck & Co., Inc. (430). An additional 508 children received a placebo. From day 21 after the initial injection, no clinical cases of hepatitis A occurred in the vaccine group compared with 34 cases among the placebo group for an estimated efficacy of 100% [95% confidence interval (CI) lower bound, 87%; $p < 0.0001$] (Fig. 18). Subclinical infection was not evaluated in this study. Because the only cases of hepatitis A that were observed in the vaccine group occurred during the first 16 days, these cases presumably represented persons who had been infected with HAV before, or shortly after, the initial injection of the vaccine. This assessment is based on results from previous immunogenicity trials conducted by Merck and SKB, in which a detectable level of anti-HAV is not observed until 1 to 2 weeks after injection in most vaccine recipients. During 6 years of follow-up, no vaccine recipient has developed hepatitis A despite continuing community exposure (429).

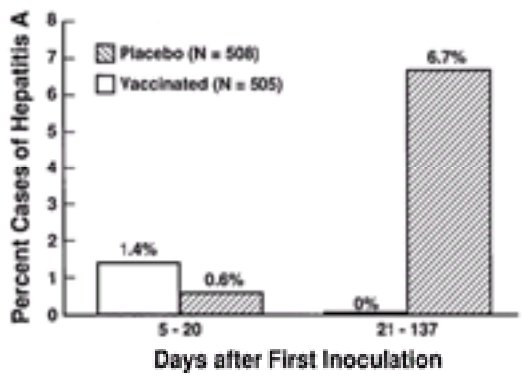

FIG. 18. Results of controlled efficacy trial of inactivated hepatitis A vaccine (VAQTA; Merck & Co., Inc., West Point, PA) in healthy children. From ref. 429, with permission.

A second clinical trial was conducted in Thailand, in which 19,037 children between the ages of 1 and 16 years received two injections of the SKB inactivated hepatitis A vaccine 1 month apart (182). An additional 19,120 children were given hepatitis B vaccine. Adverse reactions were mild and self-limited. During the surveillance period (days 138 to 386), two clinical cases of hepatitis A (based on clinical symptoms, an ALT >45 IU/L, and a positive IgM anti-HAV test) were documented in the vaccine group compared with 31 cases in the control group, for a vaccine efficacy of 94% (95% CI, 79–99%; $p < 0.0001$) (Fig. 19). A booster dose was administered at 12 months, and an additional 6 months of surveillance identified no new cases in the vaccine group compared with six cases in the control group. The cumulative efficacy rate for the entire study was 95% [two (0.01%) of 19,037 among vaccine recipients compared with 37 (0.19%) of 19,120 in the control group; 95% CI, 82–99%; $p < 0.0001$]. The two cases that occurred in the vaccine group were comparatively mild as manifested by (a) ALT levels <70 IU/L versus a mean of 827 IU/L in the HAV-infected control population (range, <8–3,310 IU/L); (b) no fever versus 42% prevalence among the controls; (c) a mean bilirubin of 1.8 versus 4.2 mg/dL; and (d) a delayed IgM anti-HAV response in the vaccinated cases.

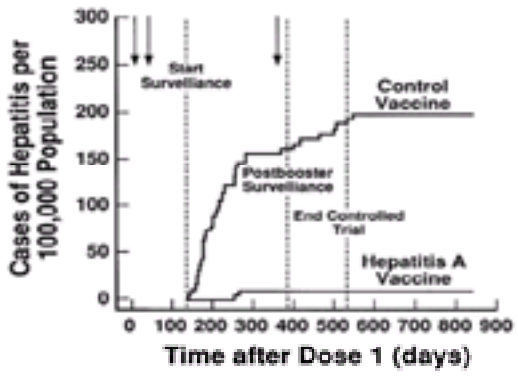

FIG. 19. Cumulative rates of symptomatic infection with hepatitis A virus (HAV) in healthy children receiving HBsAg vaccine (control group) and hepatitis A vaccine (Havrix; SmithKline Beecham, Philadelphia, PA). Timing of vaccine administrations is represented (*arrows*). From ref. 182, with permission.

Attenuated Vaccines

During the development of live poliovirus vaccines, some strains became attenuated during adaptation to growth in cell culture (344). Similarly, some HAV mutants induced an immune response in certain primates without causing hepatitis. These vaccine strains have been propagated in cells certified for vaccine production (AGMK, MRC5, or other human diploid fibroblasts) after direct isolation from human feces or after multiple passages in marmosets. They were shown to be free of, or to have diminished, residual virulence for marmosets and chimpanzees and to prevent infection after challenge with wild-type HAV.

Provost et al. (319,320) evaluated many mutants of HAV strain CR326. Initially, these were derived from virus passaged 31 times in marmosets and then in cell culture at 32°C or 35°C. Despite attenuation for chimpanzees, these mutants remained virulent for marmosets. Passage at 32°C appeared to result in highly attenuated variants that lacked immunogenicity or protective properties. Several mutants that were avirulent in nonhuman primates were not immunogenic when administered subcutaneously to humans. Emphasis then shifted to the evaluation of CR326 mutants that were directly isolated in cell culture from human feces because these appeared to be less virulent for marmosets. The most promising mutants were designated F and F'. Mutant F was immunogenic in 17 of 20 subcutaneously inoculated humans, but five individuals had biochemical evidence of mild hepatitis. Mutant F' was even more attenuated. At the highest dose used in a dose-escalating trial ($10^{7.3}$, $TCID_{50}$), neutralizing antibody was detected at 3 and 6 months after vaccination in all 10 recipients (278). The vaccine was well tolerated, and HAV was not recovered from stool or blood specimens.

Purcell et al. (60,324,375) obtained analogous results with HAV strain HM175 isolated directly from human feces into AGMK cells and then propagated at 35°C. Selection of HAV that was attenuated for chimpanzees occurred after only 10 passages in AGMK cells. However, the virus remained virulent for marmosets through 21 passages (200) but was substantially attenuated after 32 passages. All animals inoculated with passage 21 or passage 32 virus developed high levels of neutralizing anti-HAV. After challenge with live HAV, nearly all of the chimpanzees and marmosets were protected. Comparison of wild-type and cell-culture–adapted HM175 virus indicates that multiple mutations in the 2A/2B/2C region and the 5' non-coding region are essential for cell-culture adaptation and for attenuation of virulence for marmosets (60,91,92,95,119).

Mao et al. (254,255) developed an attenuated hepatitis A vaccine that has been reported to be safe, immunogenic, and efficacious. The H2 strain of HAV was serially passaged in newborn monkey kidney cells and adapted for growth in human lung fibroblast (KMB17) cell culture. More than 20 million people in China have received this vaccine. In one study, 6,298 children in 11 schools were immunized over a 4-year interval. No cases of clinical hepatitis A were reported in that group. Among a nonvaccine control group from the same schools and from other primary schools where immunization was not offered, more than 495 cases of hepatitis A occurred for a morbidity rate of 204/100,000 person-years of observation ($p < 0.001$). In another city in China, the mean rate of HAV infection averaged 32 cases/year (morbidity of 214/100,000). After 5 years of a successful immunization program that reached almost 90% of the population, hepatitis A declined to six cases, none of whom were vaccine recipients. No cases have occurred in the last 2 years of the project. There is no evidence that the vaccine virus is shed in the feces or that it causes vaccine-associated infections. Correspondingly, reversion to a more virulent strain has not occurred. Unfortunately, oral inoculation has been

unsuccessful, even when higher doses of vaccine were administered.

Strategies for the Use of Hepatitis A Vaccine and Intramuscular Immune Globulin

Anti-HAV Responses After Passive and Active Immunization

Figure 20 compares anti-HAV responses in the following situations (223):

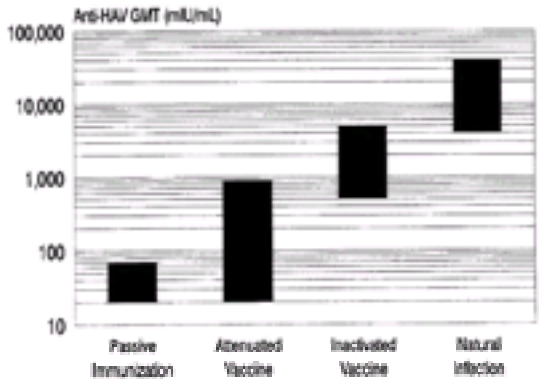

FIG. 20. Hepatitis A virus (HAV) antibody concentrations (mIU/mL) observed in persons after passive and active immunization and after natural HAV infection. Adapted from ref. 224, with permission.

1. At 3 to 5 days after receiving 5 mL of IGIM (passive immunization);
2. At 6 to 12 months after one injection of live-attenuated HAV vaccine;
3. At 6 to 12 months after two injections of inactivated hepatitis A vaccine; and
4. At 10 to 20 years after a natural infection.

Preexposure Prophylaxis

Although universal childhood immunization for hepatitis A is a worthy goal, it is unlikely that such a recommendation will be issued by policymakers in developing countries or regions with transitional economies, given the economic and political realities of global immunization programs. Even in developed countries, the integration of hepatitis A vaccination into routine childhood immunization schedules is unlikely to be recommended in the near future. In the interim, several high- and intermediate-risk groups will be targeted to receive the vaccine (see Table 6), with IGIM being relegated to more specific indications. Nevertheless, the greatest impact on the incidence of hepatitis A will be realized only when the vaccine is included in the routine childhood immunization schedule, and successive cohorts of children are vaccinated.

• Travelers to or workers in foreign countries (including the military) where the risk of acquiring hepatitis A is enhanced because of living conditions, the prevalence of HAV in the area, and the length of stay
• American Indians/Alaskan natives
• Men who have sex with men
• Illegal drug users, injecting or noninjecting
• Persons who work with HAV or with nonhuman primates
• Persons with chronic liver disease
• Persons who have clotting factor disorders
• Persons residing in areas where extended community outbreaks exist or children living in states that have high and intermediate rates of disease (e.g., approximately twice the national average rate per 100,000 population)
• Refugees residing in temporary camps after catastrophes

HAV, hepatitis A virus.

TABLE 6. *High-risk groups potentially targeted for preexposure prophylaxis*

As shown in Table 6, immunization should be offered to persons traveling from industrialized countries to regions of the world where HAV is endemic (Asia, Africa, Latin America, eastern Europe, the Middle East, and the Commonwealth of Independent States). For most travelers, vaccine alone will suffice if administered at least 4 weeks before travel. When time is a factor, both IGIM and the initial dose of vaccine should be administered at different anatomic sites. At-risk individuals who are allergic to components in the vaccine or who do not elect to receive the vaccine can be given small doses of IG (0.02 to 0.04 mL/kg or 2.0 mL for adults) if the anticipated period of exposure is 2 to 3 months (Table 7). Larger doses (0.05 to 0.06 mL/kg or 5.0 mL for adults) should be administered every 4 to 6 months for more prolonged exposure. The incidence of symptomatic hepatitis A for a 1-month trip to a developing country is three to six cases per 1,000 susceptible travelers (386). The incidence rate is sixfold higher (20 cases per 1,000) in vagabonds or other persons whose travel arrangements or personal hygiene place them in jeopardy of acquiring an infection. In making a recommendation for vaccination, it is important to recognize that hepatitis A is contracted at least 100 times more frequently than is typhoid fever or cholera (386).

Person's weight (lb)	IG dose (mL)	
	Routine	High risk[a] (prolonged exposure)
<50	0.5	1.0
50–100	1.0	2.5
>100	2.0	5.0

[a]Within limits, larger doses of IG provide longer-lasting, but not necessarily greater, protection. Therefore, a higher dose of IG is prescribed in high-risk situations where continuous exposure is anticipated (institutional contacts; traveling to foreign countries where HAV is endemic). Hepatitis A vaccination is recommended for most of these situations.

TABLE 7. *Guidelines for immune globulin (IG) prophylaxis against hepatitis A*

Because HAV infection is uncommon among U.S. military personnel except during peace-keeping missions or military campaigns, routine immunization could be reserved for those who regularly travel to endemic areas, such as shipboard personnel, or those in rapid-deployment units. For other soldiers, typical reassignment or deployment times of several weeks would be sufficient for generating a protective response to vaccination. Vaccines should be offered to staff and residents of institutions for the mentally disabled or to members of other relatively closed communities, such as American Indians or Alaskan Natives, who are at high risk of acquiring hepatitis A. It also should be offered to preschool children attending day-care centers, the staff, parents, and siblings, and to any other group whose life style, occupation, or behavior places them in the high-risk category (e.g., illegal drug users and men who have sex with other men). Other groups that have been targeted for possible immunization for hepatitis A are listed in Table 6.

One of the more controversial recommendations concerns the administration of hepatitis A vaccine to patients with chronic liver disease, especially those with hepatitis B or C. Several reports have suggested that the occurrence of hepatitis A in these patients is associated with a higher frequency of severe illness or fulminant hepatic failure, leading to substantially higher case fatality rates (203,421). Other investigators, however, reported conflicting data on the clinical course and outcome of hepatitis A in chronic liver disease (275,398). In the interim, it seems prudent to offer vaccination to all patients with chronic liver disease as recommended by the Advisory Committee on Immunization Practices (ACIP).

Postexposure Prophylaxis

The need for immediate protection after exposure mandates that IG be administered as soon as possible, but not more than 2 weeks after an incident. The recommended dose of IG for postexposure prophylaxis is 0.02 to 0.04 mL/kg or use “Routine” dosing schedule from Table 7.

Postexposure prophylaxis with IGIM has been recommended for household and sexual contacts of hepatitis A cases, for staff and attendees of day-care centers where a case has been identified, for residential facilities experiencing an outbreak, or for the military where one or more cases are recognized. Similarly, postexposure prophylaxis should be considered for potential common-source exposure (e.g., a foodhandler with hepatitis A) if exposure is recognized before cases have begun to occur or if consumers can be identified and treated within 2 weeks of exposure. Day-care centers with children in diapers present the greatest risk to employees. Within the hospital, prophylaxis should be reserved for those situations in which health-care personnel are unknowingly exposed to a case of hepatitis A. Examples of this type of exposure include late recognition of a case in a newborn nursery or pediatric intensive care unit. Accidental needle-sticks should be managed as described in the section, Guidelines to Postexposure Immunoprophylaxis, in Chapter 87. IG is not routinely recommended for casual school or work contacts; however, playmates of children with hepatitis A should receive IGIM (Table 8).

Close personal contact: all household and sexual contacts of persons with hepatitis A
Day-care centers: staff, attendees, and all members of households whose diapered children attend
Schools, preschools, and institutions for custodial care: not generally warranted unless an institutional or classroom-centered outbreak is identified
Hospitals: not routinely indicated for hospital personnel. Instead, sound hygienic practices should be emphasized
Offices and factories: not indicated under usual working conditions for persons exposed to a fellow worker with hepatitis A
Common-source exposure: not warranted once cases have begun to appear. When a food handler with hepatitis A is identified, IGIM should be given to other kitchen employees and may be considered for patrons if (a) the infected person is directly involved in handling foods that are not to be cooked or with cooked foods before they are eaten; (b) the hygienic practices of the worker are deficient; and (c) consumers can be identified and treated within 2 weeks of exposure

HAV, hepatitis A virus

TABLE 8. *Recommendations for the use of postexposure prophylaxis with IGIM after exposure to HAV*

Data are limited regarding hepatitis A vaccine for postexposure prophylaxis. Efficacy may depend on the interval between exposure and vaccination—a longer delay would favor the use of IG (or simultaneous administration of the two products). An exception may be in the management of extended community outbreaks in which vaccination holds greater promise for permanent success. In these situations, at least 70% coverage is recommended and should be achieved as rapidly as possible.

Perspectives

To provide higher antigen mass and possibly reduce the number of doses and costs for immunization in the future, it may be advantageous to use specialized equipment such as cell factories or microcarriers and to identify cell-culture parameters that would increase virus yields (336,366,433). A better understanding of HAV virulence mechanisms and identification of possible extrahepatic sites of HAV replication will be important for the development of safe and effective live vaccines.

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CHAPTER 27 Human Caliciviruses

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The family *Caliciviridae* is composed of small (27 to 40 nm), nonenveloped, icosahedral viruses that possess a linear, positive-sense, single-stranded RNA genome. Caliciviruses have a broad host range and can cause various disease syndromes, including respiratory disease in cats caused by feline calicivirus (FCV), an often-fatal hemorrhagic disease in rabbits associated with rabbit hemorrhagic disease virus (RHDV), and epidemic gastroenteritis in humans caused by the Norwalk group of viruses. This chapter will focus predominantly on the caliciviruses associated with epidemic gastroenteritis in humans, but several features of the caliciviruses associated with disease in animals will also be addressed because important information has come from the study of animal caliciviruses that grow in cell culture.

Norwalk virus is the prototype strain of a group of related, noncultivatable human caliciviruses associated with epidemic gastroenteritis. This “Norwalk group” of human caliciviruses has recently been assigned to a new genus (tentatively named the “Norwalk-like viruses”) in the *Caliciviridae.* The “Norwalk-like viruses” are the major cause of nonbacterial epidemic gastroenteritis, a disease that usually occurs in family or community-wide outbreaks (11,98,173). In a recent study, “Norwalk-like viruses” were detected in 86 (96%) of 90 nonbacterial gastroenteritis outbreaks that occurred over an 18-month period in the United States; an item of food was implicated in at least 37% of the outbreaks (89). In addition to the “Norwalk-like viruses,” a second distinct group of human caliciviruses is associated with diarrheal disease. These viruses, previously called the classical caliciviruses because their distinct morphologic appearance is similar to that of known animal caliciviruses, belong to a new genus in the *Caliciviridae*, tentatively designated the “Sapporo-like viruses.” The impact of the “Sapporo-like viruses” has not been fully established, but they do not appear to share the predominant role of the “Norwalk-like viruses” in epidemic gastroenteritis. It should be noted that neither group of human caliciviruses has been commonly associated with severe gastroenteritis in infants and young children (33,188). The predominant etiologic agent of severe diarrheal illness in this young age group is the 70-nm rotavirus in the family *Reoviridae* (described in Chapter 55).

HISTORY

Volunteer Studies

Bacteriologic and parasitologic discoveries made during the past century identified the etiology of only a small proportion of diarrheal illnesses, leaving a major segment that could not be associated with any known agent (138,296). During the 1940s and 1950s, it was assumed by exclusion that viruses were responsible for the major portion of these illnesses of unknown cause. Because these presumed viral etiologic agents could not be recovered and propagated *in vitro*, volunteer studies were carried out in the 1940s and 1950s in the United States and Japan (92,99,100,101,162,186,297,372). These studies showed that bacteria-free stool filtrates derived from outbreaks of gastroenteritis were capable of reproducing the illness, demonstrating that viruses were the likely cause. Many of these fecal specimens were analyzed intensively in the laboratory for an etiologic agent, but without success. A second generation of human volunteer studies was initiated in the 1970s, and specimens collected during a gastroenteritis

outbreak in Norwalk, Ohio, proved to be important (50,76,77,241,369).

The Norwalk outbreak, which was investigated by the Centers for Disease Control (CDC), began in October, 1968 (1). During a 2-day period, acute gastrointestinal illness developed in 50% of 232 students and teachers of an elementary school, and a secondary attack rate of 32% was observed among family contacts of primary cases. The disease lasted about 24 hours, with an incubation period of approximately 48 hours, and was designated winter vomiting disease because it resembled this syndrome first described by Zahorsky in 1929 (375). Although some of the patients had diarrhea, the predominant clinical manifestations were vomiting and nausea. An intensive laboratory study of clinical specimens from the Norwalk outbreak failed to yield an etiologic agent (1). Subsequently, a bacteria-free filtrate prepared from a rectal swab specimen obtained from a secondary case of gastroenteritis in the Norwalk outbreak was fed to three volunteers (76). Two of these individuals developed acute gastroenteritis. The agent was passaged serially in other volunteers and induced an acute gastroenteric illness in approximately 50% of these individuals (77). Although stool filtrates that contained an infectious agent were now identified, attempts to cultivate the agent in cell or organ culture or to transmit it to various experimental animals were unsuccessful (29,76,77).

Discovery of Norwalk Virus

In 1972, Kapikian et al. discovered virus particles in human-passaged fecal material derived from the Norwalk outbreak (170). These particles, described as 27 nm in their shortest diameter and 32 nm in their longest diameter (Fig. 1A), were the first viruses associated with gastroenteritis to be visualized and identified. This was accomplished by the technique of immune electron microscopy (IEM), first used in studies of tobacco mosaic virus in 1941, which involves the direct observation of antigen–antibody complexes by electron microscopy (EM) (3,5,13). Specific antibody molecules aggregate virus particles, concentrating them for easier detection, and in addition, they form a characteristic “fuzzy” layer by binding to the surface of the virion (167). The technique of IEM would prove important in the characterization of Norwalk virus, and other fastidious viruses as well, by an approach that became known as “particle” or “direct” virology (reviewed in ref. 165).

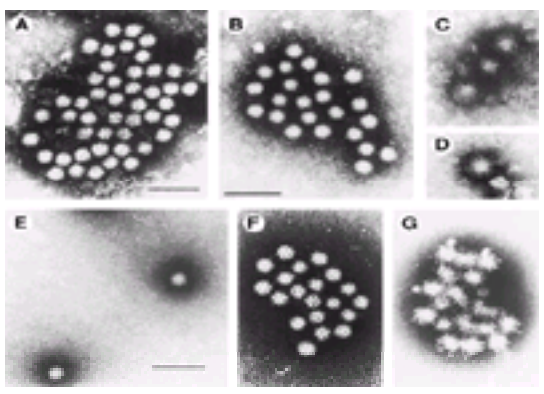

FIG. 1. Norwalk virus and Sapporo virus in stool material. **A:** Norwalk virus particles in a stool filtrate visualized by immune electron microscopy (IEM). (From ref. 170, with permission.) **B, C,** and **D** demonstrate an antibody response to Norwalk virus by IEM (from ref. 167, with permission). **B:** An aggregate observed after incubation of Norwalk virus stool filtrate with a 1:5 dilution of a volunteer's prechallenge serum. Particles have a light coating of antibody molecules. The amount of antibody present was given an overall rating of 1–2 to 2+, and the serum was given an overall rating of 1–2+ on a scale of 0 to 4+. **C,D:** Three single particles **(C)** and one individual particle **(D)** observed after incubation of the Norwalk virus stool filtrate with a 1:5 dilution of the same volunteer's postchallenge convalescent serum. Particles are heavily coated with antibody molecules. The amount of antibody was given a rating of 4+ and the serum was given an overall rating of 4+. **E, F,** and **G** demonstrate an antibody response to Sapporo virus by IEM (from ref. 252 and S. Nakata, personal communication). **E:** Sapporo virus particles partially purified from stool material and incubated with guinea pig preimmune serum that was given a rating of 0 on a scale of 0 to 4+. The distinct, hollow cup-like structures are apparent. **F** and **G** show Sapporo virus particles following incubation with a low **(F)** or high **(G)** concentration of hyperimmune serum from the same guinea pig. **F:** The amount of antibody coating the aggregate of particles was given a rating of 1+. **G:** An aggregate of particles heavily coated with antibody. The amount of antibody was given a rating of 4+.

The spherical 27-nm Norwalk virus particles were visualized in a fecal filtrate that had been shown to induce acute gastroenteritis in volunteers, but additional evidence was needed to establish the association of these particles with infection and disease (170). The IEM technique was adapted for this purpose. The fecal filtrate was incubated with prechallenge or convalescent-phase serum from a volunteer who had become ill following ingestion of the filtrate. After centrifugation of the mixtures, the reconstituted sediments were examined by EM. Figure 1 shows the striking difference between the appearance of the particles after incubation with the prechallenge serum (part B) and after incubation with convalescent-phase serum (parts C and D). In contrast to the virus particles that were exposed to the prechallenge serum, the particles incubated with convalescent serum were coated with a thick layer of antibody molecules, indicating that the volunteer had developed a specific serologic response. Four volunteers who ingested the virus, as well as naturally infected individuals who became ill during the original Norwalk outbreak, also developed a serologic response to the 27-nm particle by IEM. From this and other evidence, it was suggested that the 27-nm particle was the etiologic agent of the Norwalk outbreak (170).

The discovery of Norwalk virus in stool material, and soon after, the human rotavirus in duodenal mucosa by Bishop et al. (23), stimulated the search for new enteric viruses associated with gastroenteritis. In 1976, Madeley and Cosgrove (217) reported the presence of “typical” caliciviruses in the stools of infants and young children (2 to 18 months of age), and these viruses showed a striking morphologic similarity to previously characterized animal caliciviruses that were known to exhibit “classical” distinct cuplike depressions on the surface of the virion. That same year, Flewett and Davies (90)

observed similar calicivirus particles in a sample from the intestinal lumen of a child at autopsy, and in the feces of a child with gastroenteritis. Chiba et al. (48) described a typical calicivirus, associated with gastroenteritis, in infants and young children living in an infant home in Sapporo, Japan, in 1977. Another virus (later designated Sapporo/82/Japan) that exhibited classical calicivirus morphology was subsequently detected from this same infant home (see Fig. 1E) and was found to be antigenically distinct from Norwalk virus by IEM (252). Hyperimmune serum was raised in guinea pigs against Sapporo/82/Japan virus particles purified from stool material, and the technique of IEM was used to establish its antigenic characteristics (see Fig. 1F and Fig. 1G, respectively).

Numerous descriptions of small, round, predominantly noncultivatable viruses in feces in the 1970s and 1980s led to the development of an interim system to classify these agents on a morphologic and physicochemical basis (42). Two main morphologic groups were discerned: the “featureless” and the “structured.” The featureless group included those viruses with a smooth outer edge and without a defined surface structure. Representative members included two picornaviruses (Coxsackie virus B5 and hepatitis A virus), as well as several parvoviruses, including mink enteritis virus and certain agents detected in various gastroenteritis outbreaks (Ditchling and cockle agents). The structured group was further subdivided into three groups: astrovirus (now classified in a distinct family, *Astroviridae*, described in Chapter 28); classical caliciviruses (represented by Sapporo virus); and small round structured viruses, or SRSVs (represented by Norwalk virus), which consisted of those viruses with a defined surface structure and/or a ragged edge.

Virus-positive stools provided a source of antigen for the development of diagnostic tests such as IEM, immune adherence hemagglutination (IAHA), and immunoassays for the detection of Norwalk virus, Sapporo virus, and a number of emerging reference strains such as Hawaii virus (from a family outbreak of gastroenteritis that occurred in Honolulu in 1971) (346) and Snow Mountain virus (from an outbreak in a Colorado resort camp in 1976) (80,81,117,137,169,241,247,307,349). These assays enabled the first epidemiologic studies that established the importance of Norwalk virus and other SRSVs in epidemic gastroenteritis, as well as limited characterization of the Norwalk virus in regard to its density, antigenic characterization, and tentative classification as a calicivirus (119,120,168). However, the limited availability of virus-positive stool specimens for these assays limited such studies primarily to only a few laboratories. Progress continued to be hampered by the absence of a cell culture system, and by the late 1980s, the relationships, epidemiology, and classification of many of these viruses remained uncertain.

Molecular Advances

In the late 1980s, the stool filtrate derived from the Norwalk, Ohio, outbreak was fed to adults in volunteer studies to obtain adequate quantities of virus particles for characterization of the viral genome (148). Using Norwalk virions partially purified from human stool material in this study, Jiang et al. (148) accomplished the cloning and initial characterization of the RNA genome of Norwalk virus in 1990, which firmly established its identity as a member of the *Caliciviridae.* A second, independent report followed, describing the isolation of a cDNA clone of the Norwalk virus genome encoding an immunoreactive region (227). Subsequently, the genomes of other “Norwalk-like” human caliciviruses associated with infectious gastroenteritis were successfully cloned using reverse transcriptase-polymerase chain reaction (RT-PCR) and primers designed from the Norwalk virus sequence (7,59,107,113,194,195,204,205 and 206,226,238). It soon became apparent that Norwalk virus was representative of a large, genetically and antigenically diverse group of viruses associated with epidemic gastroenteritis. These “Norwalk-like viruses” segregated into two major phylogenetic groups that were designated genogroups I (GI) and II (GII), with Norwalk virus belonging to GI and the Hawaii and Snow Mountain viruses belonging to GII (107,205,356). Nearly full-length genomic sequences were first reported independently in 1993 for Norwalk virus and the Southampton virus, an SRSV that was obtained from a 2-year-old child during a family outbreak of gastroenteritis that occurred in Southampton, United Kingdom, in 1991 (155,194). Subsequent studies reported the presence of 12 additional nucleotides at the 5´-end of the Southampton and Norwalk viruses (130,196).

As described in detail later, the RNA genomes of the Norwalk and Southampton viruses are organized into three major open reading frames (ORF1, -2, and -3) with a polyadenylated 3´-end, similar to that found in FCV (38). Furthermore, the ORF1 encodes a large polyprotein, with three regions containing nonstructural protein motifs similar to those of the picornavirus 2C helicase, 3C proteinase, and 3D polymerase that had previously been identified in the ORF1 of FCV (254). The ORF2 encodes the major capsid protein and ORF3 encodes a predicted basic protein with no homology to other known proteins. Recently, the cloning and characterization of Norwalk virus–related caliciviruses associated with infection in large animals such as swine (SW43 virus) and cattle (Jena virus) have been described, indicating a broader host range for this group of viruses (66,213,336,350).

The cloning and initial genetic characterization of a genome of a classical human calicivirus (the Plymouth virus, associated with acute gastroenteritis in a 16-month-old child in the United Kingdom in 1992), followed by the cloning of Sapporo/82/Japan, confirmed that viruses in this group were members of the *Caliciviridae* (195,226). Comparison of the classical human calicivirus sequence with others available at that time for the family *Caliciviridae* indicated that these viruses shared higher sequence identity to known animal caliciviruses than to Norwalk virus. The striking genetic diversity between Norwalk virus and the human caliciviruses with classical morphology validated the previous division of these viruses into two distinct groups (SRSVs and classical caliciviruses) that had been based primarily on differences in virion morphology (42). The initial cloning of the classical group was based on low temperature amplification using RT-PCR primers derived from the Norwalk virus sequence; the availability of sequence information allowed the design of more efficient primers (146,226,260). “Sapporo-like viruses” have subsequently been detected in sporadic and outbreak cases of gastroenteritis that occur predominantly in younger age groups (47,146,252,352). As was seen for the “Norwalk-like viruses,” there is evidence for genetic diversity within the Sapporo-like group (146,260,352). The genomic sequence of Manchester virus, which is closely related to Sapporo virus, and which was obtained from an infant with acute vomiting and diarrhea in Manchester, England, in 1993, was reported by Liu et al. in 1995 (212). The organization of the RNA genome of Manchester virus shows a striking difference from the Norwalk and Southampton viruses in that the predicted capsid-encoding sequence is in frame with the large nonstructural polyprotein, making the genome organization of this virus more similar to that of RHDV than to FCV (235). Subsequently, the molecular characterization of porcine enteric calicivirus (PEC), an agent associated with diarrheal disease in pigs, showed that viruses related to the classical or Sapporo-like group are found also in large animals (126).

INFECTIOUS AGENT

Classification

The two major groups of human caliciviruses have been classified into distinct genera within the family *Caliciviridae*, and they have been provisionally named the “Norwalk-like viruses” and the “Sapporo-like viruses” (reviewed in ref. 110). Although all caliciviruses share a common ancestor in phylogenetic analyses, the “Norwalk-like viruses” and “Sapporo-like viruses” form distinct genetic clades within the *Caliciviridae* (22,260). In addition, certain features of their RNA genome organization distinguish them from each other and from other genera in the *Caliciviridae* (Fig. 2). The family has two additional genera, *Lagovirus* and *Vesivirus*, each of which includes caliciviruses of veterinary importance such as RHDV and FCV, respectively. The taxonomic structure of the *Caliciviridae* and representative viruses in each of the four genera are shown in Table 1. A cryptogram is useful for identification of calicivirus strains and is organized as follows: host species from which the virus was obtained/genus abbreviation/species abbreviation/strain name/year of occurrence/country of origin. A comparison of the deduced amino acid sequences from the capsid proteins of selected viruses from each of the four genera illustrates the considerable genetic diversity among strains within and between calicivirus genera (Table 2).

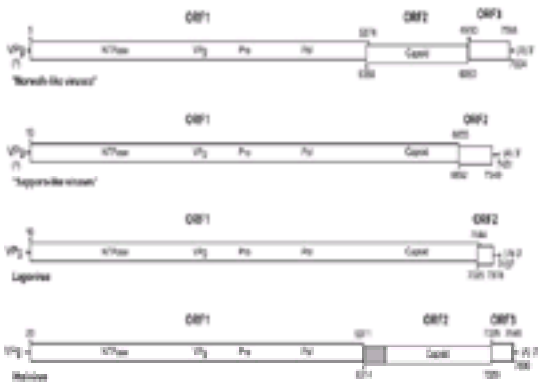

FIG. 2. Calicivirus genome organizations. The genomic organization and reading frame usage (numbers indicate nucleotide position) are shown for representative strains in the genera “Norwalk-like viruses” (Hu/NLV/I/Norwalk/1968/US, GenBank Accession Number M87661), “Sapporo-like viruses” (Hu/SLV/Manchester/1993/UK, GenBank Accession Number X86560), *Lagovirus* (Ra/LV/RHDV/GH/1988/GE, GenBank Accession Number M67473), and *Vesivirus* (Fe/VV/FCV/F9/1958/US, GenBank Accession Number M86379). *Arrows* denote the location of the first AUG in each predicted open reading frame (ORF) and the termination codon is included in the nucleotide numbering for the ORF. The predicted “2C-like” nucleoside triphosphatase (NTPase), VPg, proteinase (Pro), and polymerase (Pol) regions present in all calicivirus genomes are indicated. The *shaded region* of the ORF2 of the representative member of the genus *Vesivirus* illustrates the leader sequence (approximately 125 amino acids in length) of the capsid precursor protein.

TABLE 1. *Taxonomic structure of the Caliciviridae*

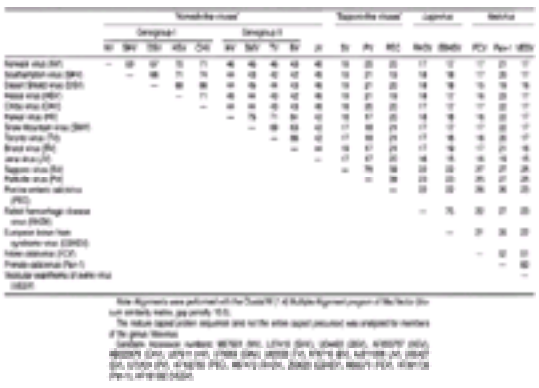

TABLE 2. *Overall amino acid identities between capsid proteins from viruses representing the four genera of the Caliciviridae*

Propagation in Cell Culture

Although some animal caliciviruses such as FCV and other members of the genus *Vesivirus* grow efficiently in cell culture, the human caliciviruses have not been propagated in cell culture or in human embryonic intestinal organ culture (56,77,171). PEC, a member of the genus "Sapporo-like viruses," can be propagated in cell culture, but optimal replication requires the use of a growth medium that contains an intestinal content preparation derived from an uninfected, germ-free pig (281).

Biologic Characteristics

The human "Norwalk-like viruses" associated with epidemic gastroenteritis share certain characteristics. These viruses (a) are shed into feces of patients with gastroenteritis, (b) have a polyadenylated positive-sense single-stranded RNA genome with three major ORFs (155,194), (c) have a buoyant density of 1.33 to 1.41 g/cm^3 in cesium chloride (CsCl) (42,168,219,346), (d) usually lack distinctive calicivirus cuplike morphology when viewed by EM (170), and (e) form a distinct phylogenetic clade within the *Caliciviridae* (22,260).

The Norwalk virus retains infectivity for volunteers following (a) exposure of the stool filtrate to pH 2.7 for 3 hours at room temperature, (b) treatment with 20% ether at 4°C for 18 hours, or (c) incubation at 60°C for 30 minutes (77). Norwalk virus is resistant to inactivation following treatment with 3.75 to 6.25 mg/L of chlorine (free residual chlorine of 0.5 to 1.0 mg/L), a chlorine concentration consistent with that found in a drinking water distribution system (177). However, Norwalk virus is inactivated following treatment with 10 mg/L of chlorine, a concentration that is used to treat water supply systems after contamination has been detected. Norwalk virus is more resistant to inactivation by chlorine than poliovirus type 1, human rotavirus (Wa), simian rotavirus (SA11), or f2 bacteriophage (177).

The human "Sapporo-like viruses" associated with gastroenteritis (a) are shed into feces of patients with gastroenteritis, (b) have a polyadenylated positive-sense single-stranded RNA genome with two (or three) major ORFs (212), (c) have a buoyant density of 1.37 to 1.38 g/cm^3 in CsCl (343), (d) often possess distinctive calicivirus cuplike morphology when viewed by EM (217), and (e) form a distinct phylogenetic clade within the *Caliciviridae* (22,260).

Description of Key Antigens

Norwalk virus capsid protein and soluble antigen were initially identified in fecal specimens obtained from infected volunteers (120). Convalescent-phase serum antibodies immunoprecipitated the major virion-associated capsid protein and a soluble protein with estimated molecular masses of 59,000 and 30,000 daltons, respectively (120). A 32,000-dalton soluble protein observed in Norwalk virus stool material was subsequently shown to be a proteolytic cleavage product derived from the C-terminal part of the capsid protein (133). The recognized cleavage site (K227/T) was consistent with cleavage by trypsin or a trypsin-like enzyme.

Further evidence that the Norwalk virus capsid protein contains major antigenic sites has been found in recombinant protein expression studies. When the genes encoding the Norwalk virus capsid protein (ORF2) and the terminal ORF3 product are transferred into a recombinant baculovirus and the proteins are expressed in insect cells, the capsid protein spontaneously self-assembles into recombinant virus-like particles (rVLPs) (154). When studied by negative-stain EM, Norwalk rVLPs exhibit a morphology similar to that of the native Norwalk virus, although they appear empty because the RNA genome is not present (Fig. 3A).

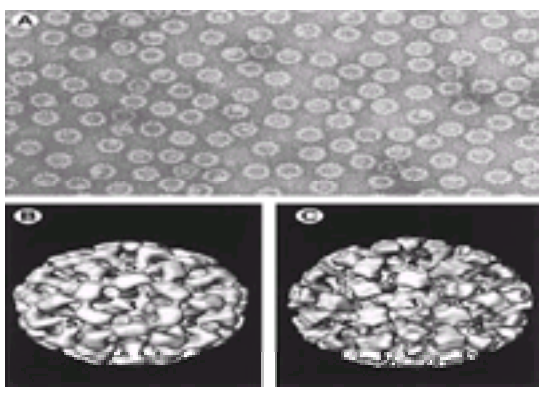

FIG. 3. The structure of Norwalk virus particles. **A:** Norwalk virus recombinant virus-like particles (rVLPs) prepared as described by Jiang et al. (154) and visualized by direct EM. **B:** Three-dimensional structure of Norwalk virus rVLPs viewed along the icosahedral threefold axis at 22Å (291). The Norwalk virus rVLPs have a distinct architecture, and they exhibit T=3 icosahedral symmetry; the twofold, threefold, and fivefold axes of symmetry are shown, and the cuplike depressions are evident at the threefold and fivefold axes. **C:** Three-dimensional structure of primate calicivirus Pan-1, viewed along the icosahedral threefold axis at 22Å (290). The architecture and symmetry are similar to that of Norwalk virus, but the edges of the cuplike depressions are more defined. (**A**) provided courtesy of M. K. Estes; (**B,C**) provided courtesy of B. V. V. Prasad.

The rVLPs are similar in antigenic and biochemical properties to native virus and can be used as an antigen in enzyme-linked immunosorbent assays (ELISAs) and IEM (105,112,154,239,276). Volunteers infected with Norwalk virus develop specific antibodies to this protein (112,276). Furthermore, the rVLPs are immunogenic when used to prepare hyperimmune sera in laboratory animals (154) or when given orally to mice or adult volunteers (17,18). Virus-like

particles have been generated for several other “Norwalk-like viruses” (with and without the ORF3 protein), and these reagents have been important tools in epidemiologic and antigenic studies of the “Norwalk-like viruses” (73,111,150,156,182,183,201,286).

Structural Studies

The Norwalk rVLPs have been important also for structural studies of the viral capsid (289,291). The Norwalk virus capsid is formed predominantly from a single major capsid protein that does not require packaging of the RNA genome for assembly into VLPs. Analysis of Norwalk rVLPs by electron cryomicroscopy and computer image processing shows that the particles (38 nm in diameter by this technique) have a distinct architecture and exhibit T=3 icosahedral symmetry (see Fig. 3B). The capsid contains 90 dimers of the capsid protein that form a shell from which 90 archlike capsomeres protrude at the local and strict twofold axes. These arches are arranged in such a way that there are large hollows at the icosahedral five- and threefold positions, and these hollows are seen as cuplike structures on the surface of caliciviruses (*calici* is derived from the Latin word *calyx*, or cup). The three-dimensional structure of a morphologically classical animal calicivirus (primate calicivirus, strain Pan-1 in the genus *Vesivirus*) has been determined (see Fig. 3C); it differs from that of Norwalk virus in that its protruding, archlike domains are longer in length and also differ in shape, consistent with the difference observed by negative-stain EM described previously (290). The shape of the top of the arch also differs in such a way that the Norwalk virus exhibits a feathery appearance by negative-stain EM, whereas the primate calicivirus has sharply defined edges (290). The Norwalk virus recombinant capsid protein can also self-assemble into smaller VLPs (23 nm) with suspected T=1 symmetry that is thought to be composed of 60 copies of the capsid protein (361). Smaller corelike particles of 25 to 27 nm have been observed in liver tissue of rabbits infected with RHDV, and these are composed of an approximately 30,000-dalton protein derived from the capsid protein (103).

The structure of the Norwalk rVLPs has been resolved to the atomic level by x-ray crystallography, so that the precise location of the primary amino acid sequence is now known (289). The electron cryomicroscopy studies had defined two major domains in the capsid—the shell (S) and the protruding arm (P) (Fig. 4A). The S domain forms the inner part of the capsid that surrounds the RNA genome, and the P domain forms the archlike capsomeres that protrude from the virion. The amino acid residues that correspond to these domains are diagrammed in Figure 4C. A ribbon model of the capsomere derived from the crystallography data (see Fig. 4B) shows a more detailed view of these domains. The NH_2-terminal (N) arm, located within the S domain, consists of residues 10 to 49 and faces the interior of the capsid. The part of the S domain that forms a beta-barrel consists of amino acids 50 to 225. The entire S domain (amino acids 1 to 225) corresponds to the N-terminal region of the capsid protein that is relatively conserved among “Norwalk-like viruses” in sequence comparisons (region 1, residues 1 to 280 for Norwalk virus) (diagrammed in Fig. 4D). In addition, the N-terminal region of the capsid protein is highly conserved among other caliciviruses within a related group, such as the B region described in a comparison of selected animal caliciviruses in the genus *Vesivirus* (255) (diagrammed in Fig. 4E). The P domain corresponds to the C-terminal half of the capsid protein, which is more variable in sequence. The P domain is divided into the P1 subdomain, encompassing amino acids 226 to 278 and 406 to 520, and the P2 subdomain, encompassing amino acids 279 to 405. The P2 subdomain corresponds to a region (region 2, residues 281 to 404 for Norwalk virus) of the capsid protein that shows the highest sequence variability among “Norwalk-like viruses.” The P1 subdomains form the sides of the arch of the capsomeres and position the P2 subdomain at the top of the arch. The exposure of the capsid variable region on the surface of the virion is consistent with its suspected role in the formation of a major antigenic site and in receptor binding (132,289,360). This observation may also explain the observed specificity of IEM, in which antibody molecules bind efficiently to the surface of the infecting virion (166). The variable region 2 among the capsid proteins of the “Norwalk-like viruses” corresponds to the E variable region of the *Vesivirus* capsid proteins, which was also proposed to be involved in the antigenic structure (255). Sequence analysis of monoclonal antibody neutralization-resistant variants has identified the E region of FCV as part of a major neutralization antigenic site (347). Additional evidence that the E region of FCV is involved in the formation of the antigenic structure of the virus has been found in studies of chimeric viruses in which the exchange of this region between FCV strains can alter neutralization specificity (257).

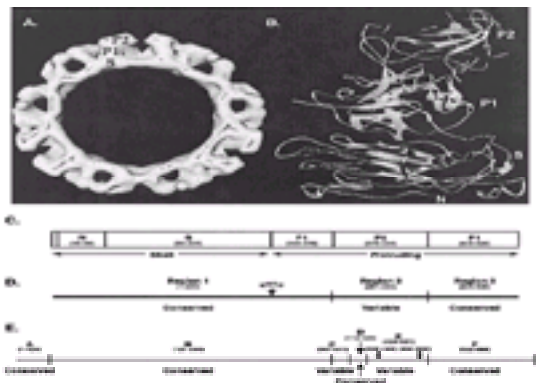

FIG. 4. Detailed view of Norwalk virus capsid protein. The Norwalk virus capsid structure is made up of 90 dimers of a single protein, which form a shell domain from which archlike capsomeres protrude (291). **A:** A central section of the Norwalk virus rVLPs perpendicular to the icosahedral threefold axis. The arch is composed of the protruding (*P*) domain (divided into subdomains P1 and P2) and the shell (*S*) domain (291). **B:** Three-dimensional ribbon representation of an individual subunit from the rNV capsomere derived from x-ray crystallography studies of Norwalk rVLPs at 3.4Å (289). **C:** Regions 1, 2, and 3 of the Norwalk virus capsid protein primary amino acid sequence (amino acid residue boundaries shown in *parentheses*), which correspond to the P1, P2, and S domains (291), respectively, are indicated. **D:** Regions of conserved and variable amino acid sequence (amino acid boundaries shown in *parentheses*) defined in a comparative analysis of human “Norwalk-like viruses” (M. K. Estes, personal communication). The location of the trypsin cleavage site between amino acid residues lysine and threonine (K227/T) that was mapped in *in vitro* studies of Norwalk rVLPs is shown (133). **E:** Regions of conserved and variable capsid precursor protein amino acid sequence in the genus *Vesivirus* (255). Amino acid changes in monoclonal antibody neutralization-resistant variants of FCV are located in two discrete regions of variable sequence (residues 426 to 460 and 490 to 520) within the “E” region ( ); they include amino acid

substitutions at residues 441, 448, 449, 455, 493, and 494 (347).

Antigenic Relationships of "Norwalk-like Viruses"

Antigenic relationships among the "Norwalk-like viruses" have been evaluated primarily by (a) cross-challenge studies in volunteers, (b) IEM or solid phase IEM (SPIEM), employing sera from infected individuals and antigens present in fecal suspensions, and (c) ELISAs or IEM using rVLPs (112,170,208,209,258,268). Initially, IEM was used to establish evidence for at least four distinct antigenic serotypes (proposed as serotypes 1 to 4), of which the prototype strains were the Norwalk, Hawaii, Snow Mountain, and Taunton viruses, respectively (80,167,170,174,208,209,369). Norwalk virus and Hawaii virus were shown further to be antigenically distinct in cross-challenge studies (369). An IEM analysis of SRSVs derived from outbreaks of acute gastroenteritis in Japan identified nine distinct antigenic types (designated S1 to S9) (268). Several of these viruses were similar by IEM to Norwalk virus, Hawaii virus, or the Otofuke virus (associated with a mild gastroenteritis outbreak in adolescents and adults in Japan) (267,268,341). A third typing system based on SPIEM was proposed in England in which three antigenic types and a possible fourth antigenic type of SRSVs (designated SRSV UK1 to UK4) were defined (208). The prototype strains for this system were Taunton (SRSV UK1), Norwalk virus (SRSV UK2), Hawaii virus (SRSV UK3), and Snow Mountain virus (SRSV UK4?). Comparative sequence analyses of the capsid protein from the Norwalk, Hawaii, and Snow Mountain viruses (with Taunton virus not available for this analysis) found marked genetic diversity (75% amino acid identity or less in pair-wise comparisons) that was consistent with the antigenic differences observed by IEM and SPIEM (131,155,179,205) (see Table 2). Comparison of the Norwalk, Hawaii, and Snow Mountain virus capsid sequences with those from other human "Norwalk-like viruses" indicates that several genetic clusters (or genotypes) of capsid proteins are circulating worldwide (8,258,353) (Table 3).

TABLE 3. *Grouping of human "Norwalk-like viruses" (NLVs) by genetic relatedness in the complete major capsid protein*

A unified system for serotyping based on neutralization is not available because of the fastidious nature of these viruses, so it is not yet clear whether each genetic cluster within a genogroup represents a distinct serotype. However, it is likely that distinct serotypes of the "Norwalk-like viruses" do exist, in view of the evidence for antigenic differences in the cross-challenge studies, IEM and SPIEM, and rVLP-based ELISAs described earlier. The continued generation of rVLPs and hyperimmune sera specific for viruses from the different genetic groups will be needed to develop a unifying interim antigenic grouping system.

Antigenic Relationships of "Sapporo-like Viruses"

The key antigens of the "Sapporo-like viruses" are associated also with the major capsid protein. Five distinct antigenic types of human caliciviruses (designated independently from the SRSV system described previously, as UK1 to UK4, and a fifth from Japan, also called human calicivirus Japan) were initially identified by IEM (57). These morphologically classical caliciviruses appeared to be related to Norwalk virus when studied by radioimmunoassay (RIA); however, these relationships were not evident by IEM (57). Sequence analyses later revealed that certain caliciviruses with classical morphology in these studies may have been "Norwalk-like viruses" (59). As found in the "Norwalk-like viruses," there is evidence for several distinct genetic groups of "Sapporo-like viruses" capsid protein sequences (Table 4). Expression of the capsid protein from Sapporo virus and "Sapporo-like viruses" in the baculovirus system yields rVLPs that will be useful for the study of antigenic relationships, until a neutralization assay is available (157,261).

Reference virus[a]	GenBank Accession number	Viruses with >80% amino acid identity with corresponding reference virus (GenBank Accession number)
Manchester/1993/UK	X86560	Sapporo/1982/JP (U65427); Plymouth/1995/UK (L23828); Houston/1998/US (J04940)
Parkville/1994/US	U73124	Houston 27/1998/US (J88644); Pelindaba/2000/GE (AF354738)
London 28845/1992/UK	U95645	
Stockholm 318/1997/SE	AF194182	

[a]Reference viruses share 79% amino acid identity or less in pair-wise comparisons of the capsid protein sequence with those of other reference viruses and are listed in order of historical precedence based on date of deposition of the full-length capsid gene sequence into GenBank.

References for the original description and the genetic characterization of the viruses shown are Wallchester virus (212), Sapporo virus (248,261), Plymouth virus (185), Houston/86 virus (148), Parkville virus (280), Houston/90 virus (148), Pelindaba virus (unpublished GenBank submission), London virus (148), Stockholm virus (352).

Country abbreviations: GE, Germany; JP, Japan; SE, Sweden; UK, United Kingdom; US, United States.

TABLE 4. *Grouping of human "Sapporo-like viruses" (SLVs) by genetic relatedness in the complete major capsid protein*

Infection of Experimental Animals, and Host Range

Numerous attempts have been made to induce illness in experimental animals. The Norwalk virus failed to induce illness following introduction into the alimentary tract of mice, guinea pigs, rabbits, kittens, calves, baboons, rhesus monkeys, marmosets, owl monkeys, patas monkeys, and cebus monkeys (29,76,77,370,371) (R. G. Wyatt et al., unpublished studies). Chimpanzees inoculated with Norwalk virus developed a serologic response as detected by IEM and RIA, but they did not develop illness (123,370). In addition, inoculated chimpanzees shed a soluble Norwalk virus antigen in their stools (123).

Caliciviruses infect a broad range of animal species (34,56,305,367). As noted previously, viruses in the genera "Norwalk-like viruses" and "Sapporo-like viruses" have recently been found in both humans and animals and are predominantly associated with infection and disease of the enteric tract. Viruses in the genus *Vesivirus* have been associated with infection in a wide variety of hosts, including cats, dogs, cattle, swine, marine mammals, skunks, mink, reptiles, fish, amphibians, birds, and nonhuman primates. A disease of veterinary importance in this genus is FCV, which causes an acute respiratory illness in kittens (reviewed in ref. 141). Thus far, members of the genus *Lagovirus* have been found only in rabbits and hares and are associated with a generalized viremic infection in which massive liver necrosis can trigger disseminated intravascular coagulation and rapid death (265).

Information concerning the frequency of interspecies transmission among caliciviruses is limited. The recent finding of genetically related human and animal enteric caliciviruses in the same phylogenetic clade suggests that such transmission might have occurred at some point in the evolution of these viruses (reviewed in ref. 350). However, the human and animal enteric caliciviruses are sufficiently divergent to suggest that they have undergone host species adaptation for some time. There are reported cases of calicivirus interspecies transmission in settings where the ecologic niche of the virus has been altered. These include the transmission of marine caliciviruses in the genus *Vesivirus* to domesticated swine in the United States in the 1930s in uncooked garbage (reviewed in ref. 325) and the infection of a laboratory worker handling San Miguel sea lion virus (324).

Molecular Biology of the Caliciviruses

The inability to grow human caliciviruses in cultured cells has hampered many aspects of research. However, considerable progress has been made by the analysis of cDNA clones generated from the genomic RNA of virions in stool material. In addition, information gained by the study of caliciviruses that grow efficiently in cell culture [such as FCV and vesicular exanthema of swine virus (VESV)] or that have an animal model and a limited cell culture system (such as RHDV), has been important for the identification of features that are likely to be shared among members of the *Caliciviridae*.

All caliciviruses have a linear, single-stranded, positive-sense RNA genome. Two major themes are observed in the organization of their major ORFs. Members of the genera "Norwalk-like viruses" and *Vesivirus* encode the capsid structural protein in a separate ORF (ORF2), whereas viruses in the genera "Sapporo-like viruses" and *Lagovirus* encode the capsid protein contiguous with the large nonstructural polyprotein (ORF1) (see Fig. 2). As a result, viruses in the genera "Sapporo-like viruses" and *Lagovirus* have at least two major ORFs (ORF1 and ORF2). In the genus "Sapporo-like viruses," the ORF1/2 junction consists of a one- or four-nucleotide (nt) overlap between the stop codon of ORF1 and the first AUG codon of ORF2. This creates a –1 frameshift. A third ORF has been described in certain strains that "overlaps" the capsid protein gene in a +1 frameshift (49,212). In the genus *Lagovirus*, a longer overlap [20 nt in RHDV and 8 nt in European brown hare syndrome virus (EBHSV)] is present at the ORF1/2 junction that includes the stop codon of ORF1 and the first AUG of ORF2 that also creates a –1 frameshift. Viruses in the genera "Norwalk-like viruses" and *Vesivirus* have three major ORFs (ORF1, ORF2, and ORF3). In the "Norwalk-like viruses," the first and third ORFs are in the same reading frame. Two independent changes are responsible for this. First, there is an overlap of ORF1/2 (a 17-, 20-, and 14-nucleotide overlap in GI, GII, and the bovine "Norwalk-like" enteric strain Jena virus, respectively) that includes the stop codon of ORF1 and the first AUG of ORF2. This creates a –2 frameshift. Second, there is a 1-nt overlap between the ORF2 stop codon and the ORF 3 initiation codon that restores the original reading frame. The *Vesivirus* genome is organized so that ORF1 and ORF2 do not overlap. There is either a 2- or 5-nt spacer sequence that results in a –1 frameshift for FCV and VESV, respectively. However, canine calicivirus strain No. 48 has a 3 nt spacer region between the stop codon of ORF1 and the initiation codon of ORF2, making the capsid gene coding sequence in-frame with that of the nonstructural polyprotein (301).

Calicivirus RNA genomes range from 7.3 to 8.3 kilobases in length, excluding the poly(A) tract (Table 5). All genomes begin with a 5´-end terminal GU. This 5´-end sequence is repeated internally in the genome and most likely corresponds to the beginning of a subgenomic-sized RNA transcript that is co-terminal with the 3´ end of the genome and that has been observed in FCV- and RHDV-infected cells as well as packaged into virions (135,236,256). A comparison of the 5´-end sequences of representative viruses within each of the four genera and the corresponding repeated internal sequence suggests that this is a feature that is characteristic of the caliciviruses (Fig. 5). The first in-frame AUG of ORF1 is relatively close to the 5´ end of the genome (ranging from 4 to 21 nucleotides), and the first in-frame AUG of the subgenomic RNA is also relatively close to the 5´ end. The synthesis of a subgenomic RNA in calicivirus-infected cells is a major difference between the replication strategy of caliciviruses and picornaviruses (85), although several of the replicative enzymes share distant homology (189). It is not known whether caliciviruses have a picornavirus-like internal ribosomal entry site (IRES) (145) for the initiation of translation, but it has been proposed as unlikely (at least for the calicivirus subgenomic RNA) because translation of the FCV capsid precursor protein begins at the first AUG of the subgenomic RNA (329).

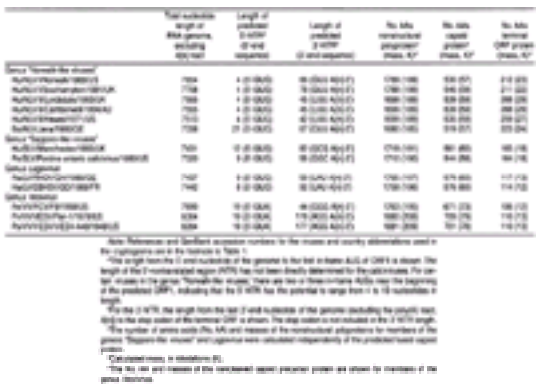

TABLE 5. *Comparison of the full-length genomes of representative caliciviruses*

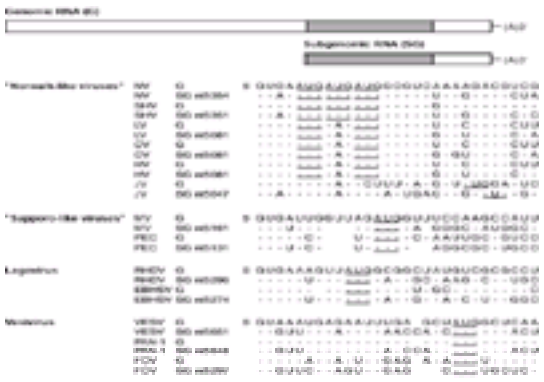

FIG. 5. Conserved sequences at the 5' ends of genomic and subgenomic RNA transcripts from viruses representing the four genera of the *Caliciviridae*. The genomic (*G*) sequence for each virus begins at the precise 5' end of the genomic RNA, and the subgenomic (*SG*) sequence begins at the internal nucleotide (*nt*) position, where similarity is shared with the 5'-end. The 5' end of the FCV and RHDV SG RNA has been mapped directly (135,236). The in-frame AUGs in the G and SG RNA that could be potential initiation codons are *underlined.* Strain abbreviations are NV, Norwalk virus; SHV, Southampton virus; LV, Lordsdale virus; CV, Camberwell virus; HV, Hawaii virus; JV, Jena virus; MV, Manchester virus; PEC, porcine enteric calicivirus; RHDV, rabbit hemorrhagic disease virus; EBHSV, European brown hare syndrome virus; VESV, vesicular exanthema of swine virus; PAN-1, primate calicivirus; FCV, feline calicivirus. The GenBank accession numbers for these viruses are shown in the footnote to Table 1. Identical nucleotides between the “reference” 5'-end genomic sequence in each genus and the selected viruses within that genus are indicated (–). Alignments were performed with the Clustal W (1.4) Multiple Alignment program of MacVector (blosum similarity matrix, gap penalty 10.0), and gaps are indicated by an *open space.*

The length of the 3´-end nontranslated region (NTR) of the representative full-length calicivirus genomes in Table 5 ranges from 42 to 178 nucleotides and is followed by a poly(A) tract. A longer 3´ NTR (235 nt) has been reported for the canine calicivirus (301). In contrast to the globally conserved pGpU of the 5´-end, the precise 3´ end just upstream of the poly(A) tract varies in sequence. A stable stem-loop structure has been predicted for the 3´ NTR of FCV and RHDV, and three stem-loops have been predicted for the longer primate calicivirus (Pan-1) 3´ NTR (300,319).

The predicted numbers of amino acids and calculated molecular masses of the proteins encoded in the different reading frames of representative viruses from each of the four genera are summarized in Table 5. Members of the genus *Vesivirus* are apparently unique among the caliciviruses in that the ORF2 encodes a capsid precursor protein (ranging from 73,000 to 78,000 daltons) that is proteolytically processed by the viral proteinase to yield the mature capsid protein of approximately 60,000 daltons that is found in virions (39,329). The protein encoded by the terminal ORF is most variable in size among caliciviruses (12,000 to 29,000 daltons) and is also divergent in sequence among viruses within certain groups (40,317).

Caliciviruses derive their mature nonstructural proteins by proteolytic cleavage from a large polyprotein encoded in ORF1. The length of the polyprotein encoding calicivirus nonstructural proteins ranges from 1680 (Jena virus) to 1881 amino acids (VESV), and the predicted noncleaved precursor of the nonstructural proteins is approximately 200,000 daltons (excluding the in-frame capsid protein sequences for the genera *Lagovirus* and “Sapporo-like viruses”). This large precursor has never been observed in *in vivo* or *in vitro* studies (24,329), most likely because proteolytic processing is rapid and cotranslational (211,330). The proteolytic cleavages are mediated by a virus-encoded “3C-like” cysteine proteinase that maps just upstream of the RNA-dependent RNA polymerase in the genome (211,330,364,365). Studies with RHDV have given insight into the likely proteolytic processing strategy of caliciviruses in infected cells (Fig. 6). A genomic-sized transcript (7.4 kilobases) serves as a template for translation of the polyprotein encoded in ORF1. Intermediate-sized proteins of 16,000, 60,000, 41,000, and 72,000 daltons that correspond to the nonstructural region of ORF1 have been detected both *in vitro* and in infected cells (187,237). Evidence for additional proteolytic processing into the putative “mature” nonstructural proteins has been found. The functions of these cleavage products were first inferred based on analogous sequences in the picornaviruses and included a “2C-like” helicase [with a predicted nucleoside triphosphate (NTP)-binding domain], a “3C-like” proteinase, and a “3D-like” RNA dependent RNA polymerase (254). Recent studies have confirmed that the RHDV “2C-like” protein binds ATP and has ATPase activity (221). Mutagenesis studies have confirmed the identity of the RHDV proteinase (364), and expression of the putative mature RHDV polymerase region in bacteria has verified that it is an RNA-dependent RNA polymerase (351). The genomic location of the protein covalently linked to the viral RNA (designated VPg, for virus protein, genome) of RHDV was determined using region-specific antisera (365). The functions of the N-terminal ORF1 cleavage product and the protein encoded just

upstream of the VPg are not known for the caliciviruses, and these proteins do not share significant homology with other known proteins. Several cleavage sites utilized by the virus-encoded “3C-like” cysteine proteinase have been identified for RHDV in bacterial expression studies (364,365). The cleavage sites are E/G and E/T dipeptides, similar to those recognized by the picornavirus 3C proteinase (reviewed in ref. 198). Recently, E/D and Q/G cleavage sites have also been identified in the RHDV polyprotein (237).

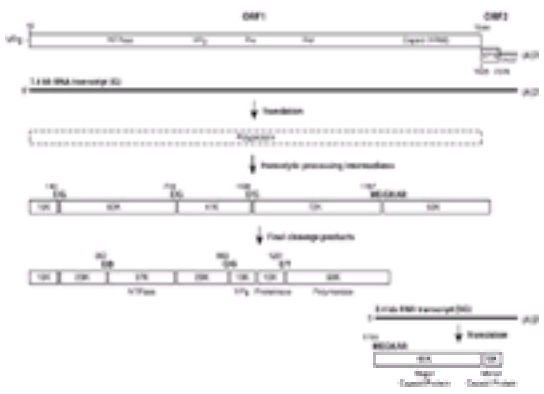

FIG. 6. Schematic diagram of the proposed replication strategy of rabbit hemorrhagic disease virus (RHDV) relating to protein translation and proteolytic processing. Two major RNA transcripts have been detected in RHDV-infected liver cells (236). The 7.4-kb genomic-length (*G*) RNA serves as a template for translation of the nonstructural polyprotein that is processed into intermediate and mature forms by the virus-encoded cysteine “3C-like” proteinase. The 2.4-kb subgenomic-length (*SG*) RNA serves as a template for translation of the major structural capsid protein (VP60) and the minor structural protein, VP10. (The RHDV ORF1 proteolytic cleavage map was modified from ref. 187,237.)

A cleavage map has been generated for the Southampton virus using cDNA clones derived from the large ORF (ORF1) encoding the nonstructural polyprotein (Fig. 7) (211,214). The cleavage map shares several similarities with RHDV. At least five cleavage sites (Q399/G, Q762/G, E961/G, E1099/A, and E1280/G) have been identified that would generate six proteins with masses of 48,000, 41,000, 22,000, 16,000, 19,000, and 57,000 daltons. The 41,000-, 16,000-, 19,000-, and 57,000-dalton proteins would correspond to the “2C-like” helicase (or NTPase), VPg, “3C-like” proteinase, and “3D-like” RNA dependent–RNA polymerase, respectively. Mutagenesis of the cysteine in the predicted active site of the Southampton virus proteinase region abrogates *in vitro* cleavage, confirming its identity as the proteinase (211). The cleavage sites identified in the Southampton virus polyprotein are conserved in the sequences of Norwalk virus, Lordsdale virus, Camberwell virus, Hawaii virus, and Chiba virus (287a,318,326a).

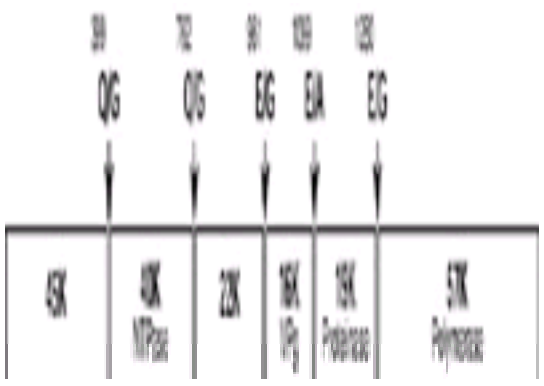

FIG. 7. Predicted proteolytic cleavage map of the nonstructural polyprotein of Southampton virus. Cleavage at the indicated dipeptide cleavage sites by the viral cysteine proteinase would result in the generation of six mature nonstructural proteins with the calculated mass (expressed in kilodaltons, K) (211,214). The predicted function (if known) is indicated.

A common characteristic among caliciviruses is the presence of the VPg covalently linked to the 5´-end region of the positive-strand RNA genome (36,309). The VPg of RHDV is linked also to the 5´-end region of the subgenomic RNA that is packaged into virions (236). The VPg of the caliciviruses (approximately 15,000 daltons) is larger than that of the picornaviruses (approximately 8,000 daltons) and has been reported as essential for the infectivity of RNA purified from virions (36). The function of the calicivirus VPg is not yet known, but it has been proposed that it plays a role in translation because proteolytic removal of the FCV VPg from RNA transcripts purified from FCV-infected cells leads to a decrease in translation efficiency *in vitro* (136). RNA transcripts derived from a full-length cDNA clone of FCV are infectious without the physical association of VPg, and it has been proposed that the 5´ terminal cap structure on the transcripts might compensate for the absence of the VPg in the initiation of translation (327).

Another general theme among caliciviruses is the presence of a 3´-end terminal ORF that encodes a basic (pI >8.0) protein. This protein has recently been identified as a minor structural protein in RHDV (designated as VP10), Norwalk virus (designated as the ORF3 protein), and FCV (designated as VP2) (97,328,365). The subgenomic RNA serves as a template for translation of both the capsid and terminal ORF proteins (see Fig. 6) (135,236,256). It has been suggested that the generation of a subgenomic RNA facilitates efficient synthesis of the major structural capsid protein during infection, similar to the replication strategy used by the alphaviruses (256). The ratio of the calicivirus terminal ORF protein to the capsid protein is much lower in virions, and the frameshift between the terminal ORF protein gene and the

capsid gene may serve as a mechanism to regulate translation of the terminal ORF product (135,256,365). The presence of the terminal ORF protein is not required for the self-assembly of the capsid protein, because recombinant capsid protein will self-assemble into VLPs without inclusion of the terminal ORF in the expression vector (197,201). One proposed function for this protein is that it interacts with RNA (because of its highly basic properties) and may be involved in packaging the genome into virions.

PATHOGENESIS AND PATHOLOGY

Incubation Period

Based on volunteer studies with the Norwalk virus, the incubation period ranges from 10 to 51 hours, with a mean of 24 hours (29,76,77,332,369). Illness usually lasts about 24 to 48 hours. The incubation period recorded in 22 naturally occurring outbreaks of Norwalk virus gastroenteritis was between 24 and 48 hours in 20 of the outbreaks, and the range was from 4 to 77 hours (173). During experimental infection of volunteers, virus shedding as detected by IEM coincided with the onset of illness and usually did not extend more than 72 hours after the first symptoms (345). However, the shedding has been detected for up to at least 7 days when analyzed by RT-PCR (102). The incubation period of experimentally induced Snow Mountain virus illness ranged from 19 to 41 hours, with a mean of 27 hours (80).

Entry and Site of Primary Replication

Human caliciviruses enter the body predominantly via the oral route. Virions are acid stable, consistent with an ability to survive passage through the stomach. Indirect evidence from epidemiologic studies suggests that viruses may enter also via aerosols, such as in those generated from the explosive vomiting that often occurs during illness (41,44).

The site of primary replication for the human caliciviruses has not been established, but it is assumed that they replicate in the upper intestinal tract. Biopsies of the jejunum of volunteers who develop gastrointestinal illness following oral administration of the Norwalk or Hawaii virus exhibit histopathologic lesions (2,78,311,312). There is broadening and blunting of the villi of the proximal small intestine, although the mucosa itself remains histologically intact (Fig. 8).

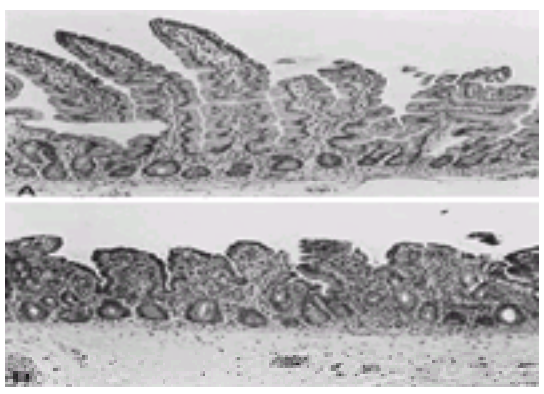

FIG. 8. A: Normal-appearing jejunal tissue from biopsy of a volunteer prior to challenge with Norwalk agent (H&E, ×390). **B:** Broadened and flattened villi in jejunal biopsy tissue from same volunteer during illness with Norwalk-induced gastroenteritis (H&E, ×390). (From ref. 2, with permission.)

Infiltration with mononuclear cells and cytoplasmic vacuolization is also observed. When viewed by transmission EM, the epithelial cells are intact, but there is shortening of the microvilli. Biopsies obtained during the convalescent phase of illness are normal. Virus has not been detected by EM in epithelial cells of the mucosa. It is of interest that the characteristic jejunal lesion has also been observed in volunteers who were fed Norwalk or Hawaii virus but who did not become ill (234,311,312). Histologic lesions are not observed in the gastric fundus, antrum, or rectal mucosa of volunteers with Norwalk virus–induced illness (362).

A transient malabsorption of fat, D-xylose, and lactose is observed during experimentally induced Norwalk virus illness (29,311). Levels of small intestinal brush-border enzymes (trehalase and alkaline phosphatase) are significantly decreased when compared to baseline and convalescent-phase values, whereas adenylate cyclase activity in the jejunum is not elevated following Norwalk or Hawaii virus-induced illness (2,203). In addition, interferon was not detected in serum, jejunal aspirates, or jejunal biopsies of Norwalk or Hawaii virus-infected volunteers (75). Gastric secretion of hydrochloric acid, pepsin, and intrinsic factor did not appear to be altered during Norwalk virus illness (234).

A marked delay in gastric emptying was observed in infected volunteers who became ill and developed the typical jejunal mucosal lesion (234). It has been proposed that abnormal gastric motor function is responsible for the nausea and vomiting associated with these viral agents (234).

Cell Tropism and Release

Information about the cell and tissue tropisms of human caliciviruses is limited. Radiolabeled Norwalk rVLPs bind to a variety of cell types, and in certain cells, they are apparently internalized with a low efficiency (360).

Human caliciviruses are released from the enteric tract of the host in feces. Norwalk virus and “Norwalk-like viruses” have been detected also in vomitus by IEM and RT-PCR (109,122,178,231).

Animal Models

Animal models for calicivirus diarrheal disease are in development with the recent discovery that enteric caliciviruses genetically related to the “Norwalk-like” and “Sapporo-like” viruses infect swine and cattle (66,126,213,336,350). Pathologic studies of the intestinal mucosa of pigs infected with PEC (a Sapporo-like virus) showed blunting of the small intestinal villi, similar to that observed during Norwalk virus infection (91). Immunofluorescence staining in this study indicated that villous epithelial cells throughout the small intestine were infected, with the greatest number of infected cells in the duodenum (upper small intestine).

Virulence

Little is known about human calicivirus virulence. The factors (host or viral) responsible for the emergence of an “epidemic” strain are not known. There are descriptions of strains that vary in pathogenicity among other groups in the *Caliciviridae*. Rabbit hemorrhagic disease virus generally causes a highly fatal hemorrhagic disease in rabbits, but a nonpathogenic variant has been identified in wild rabbits (37). Feline calicivirus strains have been described that appear to vary in virulence and disease manifestations (140), but the reproducibility of these observations could not be demonstrated in a challenge study (344), and sequence analysis of the capsid gene could not identify potential genetic determinants in this protein (95).

Persistence

Analysis of stool specimens from adult volunteers who were administered Norwalk virus by the oral route has shown that shedding of virus can continue for up to 2 weeks after the symptomatic phase of illness and that some volunteers undergo an asymptomatic infection (102,269). This observation suggests that infected individuals recovering from a “Norwalk-like” viral illness may continue to shed virus beyond the symptomatic period, a finding that has implications in the management of outbreaks caused by ill infected food-handlers (65,275,295,359). The “Sapporo-like viruses” have been associated with asymptomatic infection and prolonged shedding in children attending day care centers (124,226).

There is little evidence that the “Norwalk-like viruses” and “Sapporo-like viruses” cause chronic infection in a normal host. However, one study reported evidence for chronic infection in immunocompromised infants and young children, with one patient shedding the “Norwalk-like” virus GII strain for at least 8 months (202). Certain viruses in the genus *Vesivirus* have been associated with long-term, persistent infection in animals (70,161,285,294,326,357).

EPIDEMIOLOGY

Prevalence

Following the development of IAHA and RIA-blocking (BL) tests for detection of antibodies to the Norwalk virus, seroepidemiologic surveys were conducted for the prevalence of these antibodies in different populations and age groups (123). The prevalence of Norwalk virus antibodies measured by IAHA was compared with that of rotavirus antibodies in infants, children, and adults (169). The pattern of acquisition of antibodies in various groups in the United States differs for these two viruses. Norwalk virus antibodies are acquired gradually in childhood, and acquisition accelerates during the adult years so that 50% of individuals have such antibodies by the fifth decade. In contrast, antibodies to rotavirus are acquired rapidly during infancy and early childhood so that over 90% of individuals have such antibodies by 36 months of age. This pattern of acquisition of Norwalk virus antibodies was similar to that described for hepatitis A and certain rhinovirus serotypes, which suggested that the Norwalk virus was not an important cause of illness in infants and young children, at least in the United States (127,339).

The acquisition of antibodies to Norwalk virus in pediatric populations in various parts of the world was also evaluated by RIA-BL (26,62,83,118,164,304). The pattern deviates from the U.S. experience, as antibodies to Norwalk virus are acquired more rapidly in developing countries than in the United States, Taiwan, or Yugoslavia. For example, in two Matlab villages in Bangladesh, 43% of infants and children 2 to 49 months of age possessed Norwalk virus serum antibodies. The prevalence of antibodies was only 7% in the 2- to 7-month-old age group but increased to 100% in 4-year-old children (26).

When studied by RIA-BL, a majority of adults in both developed and developing countries were found to possess Norwalk virus antibodies (118). Male and female homosexuals in the United States had a prevalence of Norwalk virus antibody comparable to nonhomosexual adults (118).

Many, but not all, of the observations in these early studies of Norwalk virus in developing countries have been confirmed in recent studies that used more sensitive diagnostic ELISAs based on Norwalk rVLPs (93,105,262,283,293,322,342). For example, in Ga-Rankuwa, South Africa, antibodies to Norwalk virus were acquired early, reaching 100% by 2 years of age (323). However, in contrast to the early studies, in a developed country such as Finland, 73% of 154 infants and young children examined developed antibodies to Norwalk virus by approximately 2 years of age, indicating that these viruses had a wider distribution than considered earlier (207). As shown in Figure 9, recent studies enabled by the development of more sensitive assays using rVLPs for the detection of antibodies to not only Norwalk virus (GI) but also one or two GII viruses, demonstrate that the prevalence of antibodies to either genogroup characteristically increased more gradually by age in developed than in developing countries (156). However, a majority of individuals in both developed and developing countries are infected before adulthood. Antibodies to GI and GII “Norwalk-like viruses” have been detected on every continent (26,58,62,83,93,113,118,119,121,123,158,169,175,264,278,286,304,322,323). The prevalence of antibody to the GII viruses (Mexico, Hawaii, or Lordsdale) appears to be greater than that of the GI viruses in most studies (58,71,156,264,277,322). For example, in Italy, the acquisition of antibodies occurred earlier in life to the Lordsdale virus (GII) than to the Southampton virus (GI) and, in addition, the antibody prevalence in adults was 91.2% and 50.5% for the two viruses, respectively (286). This is not a consistent pattern, as in Japan and Southeast Asia,

antibodies to the Mexico virus (GII) were low in the younger age groups and increased gradually, a pattern similar to that described previously for the Norwalk virus in earlier studies in the United States (139). It is not known whether the overall higher infection rate with the GII viruses reflects a consistently higher prevalence or is merely a cyclical occurrence.

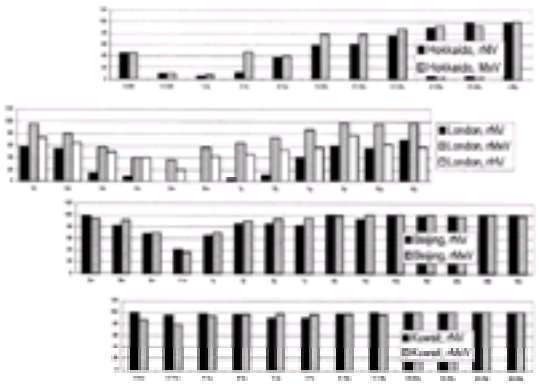

FIG. 9. Prevalence of antibody by age (m, month; y, year) to “Norwalk-like viruses” in Japan, United Kingdom, China, and Kuwait as measured by ELISAs that employed rVLPs from Norwalk virus (*NV*), Mexico virus (*Mx*), or Hawaii virus (*HV*) as antigen. (From ref. 156, with permission.)

In an attempt to determine if Norwalk virus is associated with serious diseases of unknown etiology with a gastroenteritic component, the prevalence of antibodies was evaluated in selected patients with such illness. Significant differences in the prevalence of Norwalk virus antibody or in Norwalk virus antibody titers were not demonstrated between patients with Crohn's disease and controls (116). Furthermore, exacerbations of either Crohn's disease or ulcerative colitis were only rarely associated with Norwalk virus infection (94). The prevalence of Norwalk virus antibody was also similar in patients with cystic fibrosis and controls (61). In additional studies aimed at determining if Norwalk virus was associated with gastroenteritis in human immunodeficiency virus (HIV)-positive patients, infection with Norwalk virus was comparable in HIV-infected and control patients (64,163). The role, if any, of other “Norwalk-like viruses” in such diseases has not been examined.

The “Sapporo-like viruses” have a worldwide distribution (47,252). The Sapporo virus antibody prevalence rates for adults in Japan, Canada, United States, China, Singapore, Indonesia, and Papua New Guinea ranged from 70% to 100% (252). The acquisition of antibodies to Sapporo virus begins early in life (250,252). In the developing country setting, such as Kenya, antibodies to the Sapporo virus were acquired by 1 to 2 years of age, as observed for the “Norwalk-like viruses” in such areas (250).

Incidence

Specific incidence data for illness caused by the “Norwalk-like” and “Sapporo-like” viruses are not available in the United States. However, the importance of viral agents is suggested from data on the incidence of enteric illnesses of any etiology in family studies, which indicate, that on average, each family member experiences more than one such illness per year (72,240). With the advent of sensitive detection systems, in the Netherlands it was possible to establish that 18% of the community cases of gastroenteritis over a single winter season and at least 5% of gastroenteritis cases that resulted in a visit to a physician were associated with the “Norwalk-like viruses” (191).

Predominant Role of the “Norwalk-like Viruses” in Epidemic Gastroenteritis

Although the prevalence of antibodies to the “Norwalk-like viruses” establishes that these viruses are distributed globally and in all age groups, it does not shed light on the prominent disease-causing potential of the “Norwalk-like viruses” in epidemic gastroenteritis. It is clear that the “Norwalk-like viruses” are the single most important cause of nonbacterial gastroenteritis outbreaks that occur in communities, schools, institutions, camps, cruise ships, nursing homes, universities, and families (119). This might have been anticipated, as viruses of this group were detected initially in specimens derived from gastroenteritis outbreaks in a school (Norwalk), in families (Hawaii, Montgomery County in Maryland, and Southampton), in a resort camp (Snow Mountain), and in a hospital (Taunton) (1,241,287,346). Outbreaks can vary in size, involving small family groups to hundreds of individuals (144). It is striking that one recent outbreak involved an estimated 5 million school children in Japan (228).

The important role of the Norwalk virus in the etiology of gastroenteritis outbreaks was established in early studies, when by RIA-BL it was found that 24 (34%) of 70 nonbacterial gastroenteritis outbreaks examined were associated with Norwalk virus infection (119). These outbreaks occurred in camps, recreational areas, elementary schools, cruise ships, nursing homes, colleges, restaurants, small families, and community settings. It is striking that more recently, with the availability of more sensitive diagnostic techniques, “Norwalk-like viruses” were detected by RT-PCR in 86 (96%) of 90 nonbacterial gastroenteritis outbreaks reported to the CDC from January 1996 to June 1997 (89) (Fig. 10). The major role of the “Norwalk-like viruses” as agents of epidemic gastroenteritis has now been reported in numerous locations throughout the world (67,144,354). Especially noteworthy is the frequent association of the “Norwalk-like viruses” with food-related outbreaks of gastroenteritis. It is estimated that food-borne diseases cause approximately 76 million illnesses, 325,000 hospitalizations, and 5,000 deaths in the United States each year the Norwalk-like viruses and bacterial agents assuming special importance in the overall etiology of food-borne illnesses, most of which still defy an etiologic agent (233), with bacteria such as *Salmonella* cited as the major cause (at least 75%) of food-borne disease outbreaks (270). However, improved detection methods for the “Norwalk-like viruses” suggest that their role in the etiology of food-borne illnesses has been grossly underestimated (51,270,334). The various common-source food-borne vehicles that have been implicated in “Norwalk-like viruses” outbreaks include bakery products (frosting), salad, celery,

melon, potato salad, vermicelli consommé, fruit salad, cole slaw, tossed salad, cold foods, sandwiches, lettuce, cold cooked ham, commercial ice, and water (reviewed in ref. 164). In addition, the “Norwalk-like viruses” are the most frequent cause of outbreaks of acute gastroenteritis following ingestion of raw shellfish (9,21,82,96,125,185,200,242,244,245,266,271,337). A relatively high rate of acute gastroenteritis was also observed following ingestion of cooked shellfish (10,180,230,242). The impact of these viruses on food-related disease is highlighted by the observation that in the United Kingdom, SRSVs were reported as the major cause (90%) of food-related gastroenteritis outbreaks (292). In Japan, 26 of 38 nonbacterial gastroenteritis outbreaks in which an SRSV was found were considered to be food-borne; it was striking that in 92% of the food-borne outbreaks, the vehicle of transmission was considered to be oysters (268). Finally, in Minnesota, food-borne outbreaks that are associated with the Norwalk and related viruses occurred more frequently (41%) than those associated with any single bacterial agent (69,193).

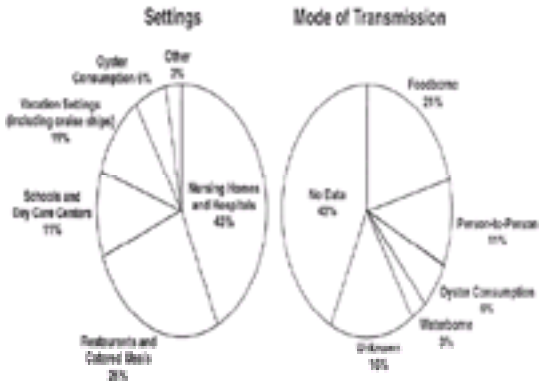

FIG. 10. Settings and presumptive modes of transmission for 90 outbreaks of nonbacterial gastroenteritis in the United States from January 1996 to June 1997. “Norwalk-like viruses” were detected in 86 (96%) of the 90 outbreaks by RT-PCR (range of detection within outbreaks 11% to 100%). (From ref. 89,98, with permission.)

“Norwalk-like viruses” have also been documented as important agents of gastroenteritis in military populations in several different areas of the world (12,14,31,52,142,143,229). Among U.S. military personnel deployed to South America or West Africa, Norwalk virus infection was detected in 10% of ill personnel, second in importance to enterotoxigenic *Escherichia coli*, which was the most frequently encountered pathogen (17%). In addition, large-scale outbreaks of gastroenteritis have been attributed to the “Norwalk-like viruses” on ships such as aircraft carriers on which hundreds of crew members became ill (53,272,320).

Norwalk virus does not appear to be an important cause of traveler's diarrhea. Its estimated role in different settings ranged from 0% to 15% (25,84,176,253,303). However, it is possible that the role of human caliciviruses in traveler's diarrhea has been underestimated because few studies have examined this possibility (98).

Importance of Human Caliciviruses in Different Age Groups

The “Norwalk-like viruses” have been associated with infection and disease in all age groups, although the majority of gastroenteritis outbreaks occur in settings that involve older, school-aged children and adults. The precise role of human caliciviruses in pediatric populations is not known, but several studies have indicated that the role of the “Norwalk-like viruses” in pediatric gastroenteritis deserves further evaluation. An early study in Bangladesh (26) found that children who had a serologic response to Norwalk virus (measured by RIA) experienced an excess incidence of 0.3 diarrheal episodes per 100 days. Thus, 1% to 2% of the diarrheal episodes in these children (who averaged 5.6 episodes per year) were estimated to be caused by Norwalk virus. This study provided the first evidence that the Norwalk virus might cause diarrhea in infants and young children by comparing the incidence of diarrhea not associated with other pathogens during periods when Norwalk virus infection did or did not occur. However, it should be noted that Norwalk virus infection was not identified as an important cause of dehydrating diarrheal illness in Bangladesh. For example, only 1 of 31 children under 10 years of age who were treated for dehydrating diarrhea in a hospital and who did not have rotavirus, enterotoxigenic *E. coli*, *Salmonella*, *Shigella*, or *Vibrio cholerae* in their stools had a serologic response to Norwalk virus (26).

Several EM studies have shown that viruses morphologically similar to Norwalk virus are observed only infrequently in stool specimens of infants and young children with gastroenteritis (33,169,190,284,335). For example, in an 8-year study conducted at the Children's Hospital National Medical Center in Washington, D.C., particles that were Norwalk virus–like in morphology were detected in stools by IEM in only 1.6% of 1,537 patients hospitalized with gastroenteritis (33,169). In addition, in Japan, in a cross-sectional study of pediatric patients hospitalized with gastroenteritis that spanned more than 6 years, caliciviruses or small round viruses were detected by EM in only 0.9% of 1,910 patients (188).

With the development of newer, more sensitive diagnostic techniques, studies in pediatric patients indicate that the “Norwalk-like viruses” may indeed be associated with an as yet undetermined portion of gastroenteric illness. For example, in a recent survey of 214 cases of gastroenteritis associated with SRSVs that occurred over the 1997 to 1998 winter season in Japan, 50% of the patients were 1 year of age or under (144). In Finland, 49% of 154 infants and young children exhibited evidence of Norwalk virus infection during a period of almost 2 years when studied by an ELISA using rVLPs as antigen, indicating that infections with “Norwalk-like viruses” may occur frequently in this age group (207). In this regard, “Norwalk-like” caliciviruses are considered to be the second most common cause of diarrhea in Finnish children, after rotavirus (273,274). The “Norwalk-like viruses” have been detected in the stools of pediatric patients with gastroenteritis in several different settings (30,202,206,222,251,263,279).

The “Norwalk-like viruses” have frequently been associated with gastroenteritis in the elderly, especially those living in institutional settings such as nursing homes (16,67,134,151,302,333). An estimated 43% of 90 nonbacterial gastroenteritis outbreaks reported to the CDC from January 1996 to June 1997 occurred in the hospital or nursing home setting (see Fig. 10).

Caliciviruses that are putatively in the genus “Sapporo-like viruses” have been considered to be primarily associated with pediatric gastroenteritis, but only infrequently with severe infantile gastroenteritis requiring hospitalization (48,55,90,217,232,246,249,250,252,338). In contrast to the “Norwalk-like viruses,” these viruses are not characteristically associated with outbreaks in adults and older children. Analysis of a collection of 59 stool specimens that were positive for caliciviruses with classical morphology and that were obtained over a 10-year period (1988 to 1998) in Europe and Scandinavia, demonstrated that 55 (93%) were “Sapporo-like viruses” by RT-PCR, and 51 of these were obtained from children less than 5 years of age (352). However, in a large study that included 1,273 sporadic cases of acute gastroenteritis in children in Sapporo, Japan, over a 3-year period, only 23 (1.8%) stool specimens reacted with a cDNA probe derived from Sapporo/82/Japan (184). The contribution of these agents to pediatric gastroenteritis was studied further in Finland using “Sapporo-like viruses” RT-PCR primers, and 44 (5.7%) of 775 cases of pediatric gastroenteritis were positive for “Sapporo-like viruses” (273).

The “Sapporo-like viruses” do not appear to be important agents of food-borne gastroenteritis outbreaks, although one such outbreak has been documented in adults (260).

Transmission

Studies in volunteers demonstrated that the Norwalk, Hawaii, Montgomery County, and Snow Mountain viruses induced gastroenteritis when administered by the oral route, with an average incubation period of 24 hours (76,77,160,203,241,332,369). Transmission via the respiratory route has not been established for this group of agents, although it has been suggested from epidemiologic observations in selected settings (45,308). The virus was detected by IEM in vomitus obtained from infected volunteers; vomitus has been considered to be a source of transmission in certain settings (122). Nasopharyngeal washings from a volunteer with experimentally induced Norwalk gastroenteritis did not induce illness in three volunteers (77). There is evidence that the human caliciviruses are stable in the environment and that contamination of water, food, and surfaces by infected individuals can lead to inadvertent exposure (46,109,138,202,215,282,316).

The explosive nature of some Norwalk virus outbreaks, in which a large number of persons become ill within 24 to 48 hours, suggests that infection is often acquired from a common source. This was suggested in the original Norwalk virus outbreak, but a common-source exposure could not be identified (1). Later, a review of 38 Norwalk virus–associated outbreaks suggested that a common source of infection was likely in 31 (82%) (173). The vehicle of transmission could be identified in 17 of the 31 outbreaks, including water in 13 instances and food in four others. Waterborne infection was attributed to a municipal water system (twice), semipublic water supply (seven times), stored water on a cruise ship (twice), and recreational swimming (twice). The food-borne outbreaks were associated with the ingestion of oysters or salad. A large outbreak of gastroenteritis in Minnesota was traced to contamination of cake frosting by Norwalk virus (192). Primary person-to-person transmission occurred in 7 of the 38 outbreaks (173). Sufficient data were available from 26 of the outbreaks to permit estimation of secondary attack rates (173). In 20 of 23 common-source outbreaks and in each of three person-to-person outbreaks, secondary transmission was observed, with attack rates ranging from 4% to 32%. In a large outbreak for which age data were available, the secondary attack rate was highest in children under 10 years of age. The median duration of the 38 outbreaks was 7 days (ranging from 1 day to 3 months). The number of individuals who became ill ranged from 2 to 2,000, with the attack rate being higher in common-source outbreaks (median 60%, range 23% to 93%) than in primary person-to-person outbreaks (median 39%, range 31% to 42%) (173). Recent studies have confirmed, for the most part, the various means of transmission of the “Norwalk-like viruses” (see Fig. 10) (89).

In the Colorado outbreak caused by the Snow Mountain virus, 61% of the 418 illnesses began on a single day (80,241). A waterborne source was suggested as the etiologic agent because the attack rate was directly related to the amount of water or ice-containing beverages consumed. In addition, the water supply was contaminated by a leaking septic tank and was inadequately chlorinated.

The efficient transmission of human caliciviruses by contaminated food and water vehicles has raised concerns about public safety in a global market economy (310). A recent example of an international outbreak of gastroenteritis occurred with the widespread distribution of contaminated raspberries from a single source in eastern Europe (288).

Molecular Epidemiology

Molecular epidemiologic studies have demonstrated a marked genetic diversity among circulating “Norwalk-like viruses.” As described earlier, two major genogroups within the “Norwalk-like viruses” have been identified as GI and GII. Genogroup I strains include Norwalk virus, Southampton virus, Desert Shield virus, Chiba 407 virus, Musgrove virus, Hesse virus, and Winchester virus, whereas genogroup II strains include Hawaii virus, Melksham virus (and the closely related Snow Mountain virus), Toronto virus (and the closely related Mexico virus), Bristol virus (and the closely related Lordsdale and Camberwell viruses), Hillingdon virus, Seacroft virus, Leeds virus, and Amsterdam virus (see Table 3). These viruses represent branches, or genetic clusters, of closely related viruses within their corresponding genogroups that have been designated in some studies as genotypes or subtypes (7,8,107,113,195,204,205 and 206,226,238,353). A numbering system has recently been proposed for the identification of “Norwalk-like viruses” in which the capsid sequence of a candidate virus would be compared to those of reference viruses (8), and the new virus would then be assigned both a genogroup and genetic cluster number (see Table 3). Most viruses in the genus analyzed thus far fall within the two major genogroups, although it has recently been proposed that the Jena bovine enteric calicivirus represents a third genogroup (GIII) within the “Norwalk-like viruses” (8). There is evidence also for genetic diversity

within the “Sapporo-like viruses” (see Table 4), but a numbering system for genetic groups has not been established. The biologic and epidemiologic significance of genetic diversity among the caliciviruses will require further study.

Large-scale molecular epidemiologic studies have given insights into important genetic features of the “Norwalk-like viruses.” First, viruses appear to be genetically stable over time (152,356). For example, the KY-89 virus, associated with illness in Japan in 1989, showed an overall amino acid identity of 96% in its capsid protein with Norwalk virus, which was associated with disease in the United States in 1968. Second, genogroup II viruses, particularly those related to the Bristol and Lordsdale viruses, have been the predominant viruses circulating globally in the last few years (8,20,30,106,114,178,202,220,238,274,282,306,314,331,354,366,368,373). Third, there is evidence for the widespread distribution of a global “common” strain during certain time periods, illustrating an efficient transmission of these viruses in the human population (89,259). Finally, evidence for mixed infections within the same individual or within the same outbreak has been reported (6,35,104,128,215,337), and such infections may allow recombination between RNA genomes, a possibility recently suggested from sequence analyses of naturally occurring viruses (131,147).

Geographic and Temporal Distribution

Human caliciviruses have a worldwide distribution (see preceding section, Prevalence). In a survey of outbreaks from eight countries, sporadic cases and outbreaks of “Norwalk-like viruses” gastroenteritis occurred in all age groups throughout the year, with a seasonal peak during the cooler months in 11 of the 12 studies (243). During a 1-year prospective study in Bangladesh, a serologic response to Norwalk virus was most frequent in the cool, dry periods of the year (26). In Australia, SRSVs were detected throughout the year, but most were detected from late winter to early summer (368). Moreover, in Japan, cases of gastroenteritis in pediatric patients that were associated with SRSVs peaked during the cooler months but somewhat earlier than the cool-weather rotavirus peak (144).

CLINICAL FEATURES

Viral gastroenteritis is generally considered to be mild and self-limiting, although the illness can be incapacitating during the symptomatic phase that usually lasts 24 to 48 hours. Illness induced by “Norwalk-like viruses” has occasionally been severe enough to require medical intervention (12,14,173,274,320).

Clinical manifestations observed in 38 outbreaks associated with Norwalk virus included the following (expressed as the median percentage of patients): nausea (79%), vomiting (69%), diarrhea (66%), abdominal cramps (30%), headache (22%), fever (subjective) (37%), chills (32%), myalgias (26%), and sore throat (18%) (173). Bloody stools were not reported. Vomiting occurred more frequently than diarrhea in children, whereas in adults the reverse was observed. The duration of illness in 28 outbreaks ranged from 2 hours to several days, with a mean or median of between 12 and 60 hours in 26 of the 28 outbreaks. In six outbreaks, illness lasted more than 3 days in up to 15% of the affected individuals. The attack rates did not differ significantly with age or sex in six outbreaks in which this was analyzed (173).

The spectrum of clinical manifestations observed in two volunteers who developed illness following Norwalk virus challenge is shown in Figure 11 (76). Although both individuals received the same inoculum, one volunteer developed an illness characterized by vomiting without diarrhea, whereas the other had diarrhea without vomiting. Clinical manifestations observed in 31 experimentally infected volunteers who became ill included the following: fever above 99.4°F (45%), diarrhea (81%), vomiting (65%), abdominal discomfort (68%), anorexia (90%), headache (81%), and myalgias (58%) (369). The illnesses were characteristically mild and usually lasted 24 to 48 hours; however, one volunteer was given parenteral fluid because he vomited 20 times within a 24-hour period. Fourteen of 16 volunteers who developed illness following Norwalk or Hawaii virus infection developed transient lymphopenia which affected T-, B-, and null-cell lymphocyte subpopulations (79). This was attributed to a redistribution of circulating lymphocytes to the site of viral infection in the small intestine. The lymphocytes remaining in the circulation responded normally or exhibited an exaggerated response to mitogenic stimuli.

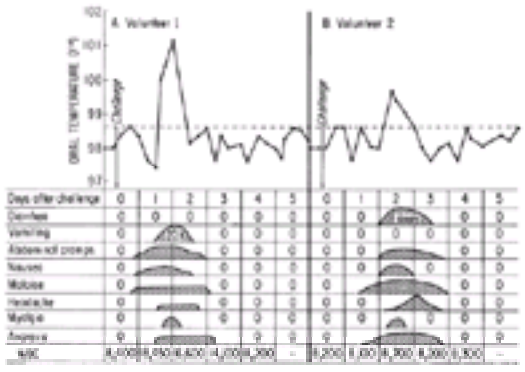

FIG. 11. Response of two volunteers to oral administration of stool filtrate derived from volunteer who received original Norwalk rectal swab specimen. The height of each curve is directly proportional to the severity of the sign or symptom. Volunteer 1 had severe vomiting without diarrhea, whereas volunteer 2 had diarrhea without vomiting, although both received the same inoculum. (From ref. 76, with permission.)

Illnesses induced by the Hawaii, Montgomery County, and Snow Mountain viruses in volunteers cannot be distinguished clinically from those caused by the Norwalk virus (50,80,369). Subclinical infections with Norwalk or Hawaii virus have been observed under experimental as well as natural conditions. Of interest, the characteristic jejunal lesions can develop in the absence of symptoms (173,234,311,312,332).

A comparison of the clinical features of gastroenteritis caused by the “Norwalk-like viruses” or “Sapporo-like viruses” in Finnish infants and young children showed that the “Norwalk-like viruses” induced vomiting as the predominant clinical feature, whereas the “Sapporo-like viruses” usually caused diarrhea (273).

IMMUNITY

Immunity to Norwalk virus is poorly understood but it does not appear to resemble the pattern observed with most other viruses. Because serum or intestinal secretory neutralizing antibodies cannot be measured in tissue culture, most of the information on immunity comes from volunteer studies. Adults consistently demonstrate a high degree of susceptibility to both naturally occurring and experimentally induced Norwalk illness. In some outbreaks, more than 80% of adults became ill (173). In addition, approximately 50% of unselected adult volunteers consistently developed illness following challenge with Norwalk virus (29,77,332,369).

Volunteer challenge studies have established that there are two forms of resistance to Norwalk virus: One is short term and the other is long-term (77,280,369). Short-term immunity, which follows the traditional pattern, is apparently serotype specific. Thus, volunteers who become ill following Norwalk virus challenge are usually resistant to rechallenge with this virus 6 to 14 weeks later. However, challenge of such volunteers with the heterotypic Hawaii virus induces illness. Similarly, volunteers who recently became ill following infection with Hawaii virus are susceptible to challenge with the Norwalk virus (369). Long-term immunity, however, deviates from the traditional pattern. Twelve volunteers who were challenged with the Norwalk virus on two occasions, 27 to 42 months apart, exhibited two different patterns of resistance to sequential challenge (280). Six volunteers developed gastrointestinal illness following the initial challenge, and they developed it again following rechallenge 27 to 42 months later. In contrast, the other six individuals failed to become ill following the initial challenge and were also resistant after rechallenge 31 to 34 months later. Serologic studies in which prechallenge serum antibodies to Norwalk virus were measured by IEM and RIA failed to provide an explanation for the difference in susceptibility (63,121,280). Paradoxically, volunteers who did not become ill had little, if any, antibody to Norwalk virus measurable by IEM in either prechallenge serum specimen (280). Also, they failed to develop a significant serologic response following each challenge. However, volunteers who became ill following each challenge, and who were evaluated serologically, developed a serologic response after each challenge. The observation that serum antibody to Norwalk virus does not correlate with resistance to illness has been reproduced in more recent volunteer studies (102,160).

Additional evidence for nonimmunologic factors in resistance was obtained when prechallenge serum and local jejunal antibody levels were determined in 23 additional volunteers who were challenged with Norwalk virus (121). The geometric mean Norwalk antibody titer (by RIA-BL) in serum or jejunal fluid did not correlate directly with resistance to illness. On the contrary, individuals who developed illness had a mean prechallenge jejunal fluid antibody titer that was significantly higher than that of volunteers who did not become ill. They also tended to have a serum antibody titer that was higher than that of volunteers who did not develop illness. These findings were confirmed in subsequent volunteer studies and during natural outbreaks of disease (19,28,159,160,218).

Several explanations for these observations have been suggested (27,60,280). For example, it has been proposed that a genetically determined variation in virus receptors in the intestinal tract is responsible for long-term resistance in the absence of appreciable Norwalk virus antibodies (19,27,60,280). Clustering of susceptibility to Norwalk virus illness in families exposed to contaminated water supports this explanation and suggests that genetic susceptibility operates under natural as well as experimental conditions (190). On the other hand, persons who become infected with the virus do develop specific antibodies and exhibit short-term homologous immunity. However, it is not clear why adults with various levels of Norwalk virus antibodies do not exhibit long-term homologous resistance to experimental challenge. In studies performed in a natural setting in developing countries such as Bangladesh or Panama, the presence of serum antibodies to the Norwalk virus was found to correlate with resistance to Norwalk virus infection (26,304). Perhaps a clearer understanding of immune mechanisms involved in protection will emerge when assays become available for examining the role of neutralizing antibodies to individual serotypes.

The “Sapporo-like” human caliciviruses have not been studied in volunteers, so even less is known about immunity to this group of viruses. There are suggestive data that antibodies may be important in immunity because antibodies measured by RIA to the Sapporo virus exhibited a correlation with resistance to illness in infants and young children (247,248).

DIAGNOSIS

Differentiation of illness caused by Norwalk virus from that caused by other members of the genus “Norwalk-like viruses” cannot be made on clinical grounds. However, an analysis of the common features of 38 Norwalk virus outbreaks (173) indicate that a provisional diagnosis of illness by this group can be made during an outbreak if the following criteria are met: (a) bacterial or parasitic pathogens are not detected; (b) vomiting occurs in more than 50% of the cases; (c) the mean or median duration of illness ranges from 12 to 60 hours; and (d) the incubation period is 24 to 48 hours (172).

Electron Microscopy

Electron microscopy continues to be a valuable diagnostic tool for viral gastroenteritis because it is rapid and requires minimal sample preparation. For acute gastroenteritis that occurs in an infant or young child from 6 months to 2 years of age, rotavirus should be the primary etiologic suspect and it can often be readily diagnosed by EM because the virus is shed in high titer in stool material (32). However, direct examination of unconcentrated stool material from gastroenteritis outbreaks (which usually occur in older children and adults) is of limited value as a routine diagnostic tool for the “Norwalk-like viruses,” because particles are usually present in low concentration (32,43,166,171,287,299). Furthermore, the significance of particles that are visualized directly cannot be established without other tests because stools contain many small, round objects of a size similar to that of viruses of this group. For this reason, putative “Norwalk-like viruses”

should be identified in stools by the technique of IEM or SPIEM (72,166,170,171,181,210,299). The time of stool collection is critical to success in virus detection. For example, Norwalk virus was detected in the stools of 11 of 23 volunteers who developed illness following challenge (345). Virus was detected in 26 of 54 specimens collected during the first 72 hours after onset of illness but in only 2 of 11 specimens collected after this interval.

Detection of the “Sapporo-like viruses” in stool material by EM may be less difficult because of their distinct morphology. The virions have been described as having a feathery edge, a six-pointed star with a dark hollow in the center (a Star-of-David) appearance, and distinct surface hollows that appear round or oval (216). However, caliciviruses can exhibit variable appearances (56) and morphology alone should not be used to conclusively distinguish between the “Sapporo-like viruses” and “Norwalk-like viruses” (59,184,206).

Reverse Transcriptase-Polymerase Chain Reaction

RT-PCR is currently the most widely used technique for detection of human caliciviruses. Several primer pairs have been described that were deduced from highly conserved regions of the RNA genome (usually the polymerase region) (reviewed in ref. 8). With this method, human caliciviruses can be detected in clinical specimens (feces or vomitus) and contaminated food, water, or fomites (15,68,128,153,200,315,316,321,363). RT-PCR has been used extensively to detect and characterize “Norwalk-like viruses” in various outbreaks (see preceding section, Molecular Epidemiology). One requirement for successful application of RT-PCR for outbreak investigation is the choice of primers, because considerable genetic diversity exists among circulating strains. Recently, a primer pair has been described that detects both “Norwalk-like” and “Sapporo-like” viruses (149).

Immunoassays

The development of rVLPs has had a major impact on the development of antibody-based diagnostic assays such as ELISAs (reviewed in ref. 156). Human calicivirus rVLPs are antigenically similar to native virions in stool and can be produced in large quantity. Immunoassays for the detection of several viruses from both the “Norwalk-like viruses” and “Sapporo-like viruses” groups have been developed that employ hyperimmune antisera prepared against recombinant VLPs. However, although sensitive, these assays are often highly specific for the immunizing VLP (102,152). The expression of calicivirus capsid proteins in bacteria has been explored as an approach to generate cross-reactive antigens for the development of diagnostic reagents (374). Efforts are in progress to develop broadly reactive polyclonal or monoclonal antibodies (129,132) for use in ELISAs that can detect viral antigen in clinical specimens.

ELISAs that use rVLPs as antigen are specific, sensitive, and efficient for detecting infection with the human caliciviruses and have been used in several large-scale sero-epidemiologic studies (see preceding section, Prevalence). The Norwalk rVLP ELISA has been shown to detect broadly reactive antibody responses in volunteers given Norwalk virus, Hawaii virus, or Snow Mountain virus, although the maximal response was observed in volunteers challenged with the Norwalk virus (102,239,348). It is impossible to identify the antigenic type of the infecting strain by serologic analysis because of the cross-reactive antibodies detected by ELISA (258). However, the demonstration of an antibody response in 50% or more of the individuals involved (or examined) in an outbreak to a calicivirus antigen is strong evidence linking the virus with the outbreak (173).

TREATMENT

As noted earlier, the Norwalk group of viruses characteristically induces a mild, self-limited gastroenteritis that normally resolves without complications (29,74,76,77,160,332,369). Oral fluid and electrolyte replacement therapy is usually sufficient to replace fluid loss (4,54). However, parenteral administration of fluids may be necessary if severe vomiting or diarrhea occurs. As noted earlier, hospitalization for severe dehydration, although rare, can occur with Norwalk virus gastroenteritis. In addition, deaths from Norwalk virus gastroenteritis in debilitated elderly patients and in immunocompromised children infected with human caliciviruses have been reported (88,173); however, these fatalities were considered to be caused, in large part, by an underlying disease. Oral rehydration therapy should not be administered to patients with depressed consciousness because of the possibility of fluid aspiration.

Oral administration of bismuth subsalicylate after onset of symptoms significantly reduced the severity and duration of abdominal cramps during experimentally induced Norwalk virus illness in adults (332). In addition, the median duration of gastrointestinal symptoms was reduced from 20 to 14 hours. The number, weight, and water content of stools, as well as the extent of virus excretion, were not significantly affected by treatment. The use of various medications for symptomatic treatment of acute diarrhea in infants and young children in the 1 month to 5 years age group was reviewed: bismuth subsalicylate, loperamide, anticholinergic agents, adsorbents, or *Lactobacillus*-containing compounds, was not recommended by the American Academy of Pediatrics, and in addition, the use of opiates as well as opiate and atropine combination drugs was contraindicated (4).

CONTROL AND PREVENTION

Specific methods are not available for the prevention or control of human calicivirus infection or illness. Because these viruses are highly infectious, effective hand washing and disposal or disinfection of contaminated material may decrease transmission within a family or an institution. Special care must also be given to the hygienic processing of food in view of the frequent occurrence of food-borne outbreaks. Depuration of oysters does not adequately clear tissues of Norwalk virus (9,125,315), so precaution must be taken to prevent contamination of oyster beds with feces or vomitus. Measures that increase the purity of drinking water or swimming pool water should also decrease the frequency of outbreaks (215).

One of the major obstacles to the formulation of a strategy for immunization against Norwalk virus and its related viruses is that the basis for immunity is not understood (reviewed in ref. 87). The inability to cultivate these viruses in cell culture has prevented an analysis of the role of neutralizing antibodies. Furthermore, the viruses themselves cannot be propagated as a source for live or inactivated vaccine. However, this latter problem may be circumvented by the present

availability of rVLPs, which are a replenishable and abundant source of capsid antigen (86). In phase I studies of Norwalk rVLPs as vaccines, they were safe and immunogenic when administered to adult volunteers by the oral route (17). Furthermore, expression of the Norwalk virus capsid in plants yielded VLPs that were immunogenic in laboratory animals and volunteers (224,298,340). If the mechanism of immunity is elucidated and antibodies are found to play a relevant role in prevention, the rVLPs may prove to have potential as a subunit vaccine. A safe and effective vaccine could reduce the incidence of epidemic viral gastroenteritis. An effective vaccine, if feasible, would be of special importance to college students, military personnel, nursing home residents, and individuals in various institutional settings. In addition, it might reduce the number of episodes of gastroenteritis in infants and children, especially those who live in developing countries. Although Norwalk virus gastroenteritis tends to be a mild illness, a reduction in diarrheal episodes may be important in the debilitated, malnourished infant, because it has been suggested that repeated diarrheal episodes may be a precipitating factor in the development of malnutrition through sequential damage to the intestinal mucosa (224).

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CHAPTER 28 Astroviruses

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The family *Astroviridae* includes human and animal astroviruses that share a characteristic starlike surface structure when viewed by electron microscopy (EM). These small icosahedral viruses comprise a third family of nonenveloped human and animal viruses whose genome is composed of plus-sense, single-stranded RNA. As described later, they have several important features that distinguish them from the two other families of such viruses, the *Picornaviridae* and the *Caliciviridae.*

Astroviruses have been isolated from humans as well as numerous animal species. In most species, astroviruses are found in association with gastroenteritis. In avian species, however, both intestinal and extraintestinal manifestations of disease have been observed. The medical importance of astrovirus in humans was established initially in studies conducted in Thailand and Guatemala, where astroviruses were found to be the second most common cause (after rotavirus) of viral diarrhea in young children. Improved detection techniques and increased awareness of astrovirus infection have helped to define the epidemiology of this illness.

HISTORY

The term *astrovirus* was coined by Madeley and Cosgrove in 1975 (106) to describe small round viruses (SRVs) with a distinctive five- or six-pointed starlike appearance (astron, “star” in Greek) by direct EM (Fig. 1). They observed these small, 28-nm-diameter, round viral particles in the stools of infants hospitalized with diarrhea and in outbreaks of gastroenteritis in newborn nurseries (105). Ironically, the first astroviruses described in the literature did not display the characteristic astrovirus surface features. A few months prior to the naming of astroviruses, Appleton and Higgins (4) reported an outbreak of mild diarrhea and vomiting among infants in a maternity ward. SRVs that were 29 to 30 nm in diameter and without defining features were shed in large numbers in fecal samples from 8 of the 14 infants in this outbreak. By EM evaluation, these viruses differed in size and morphology from the previously identified Norwalk virus and rotavirus, but it was not until years later when specific immunologic reagents became available that these viruses were demonstrated to be astroviruses (26).

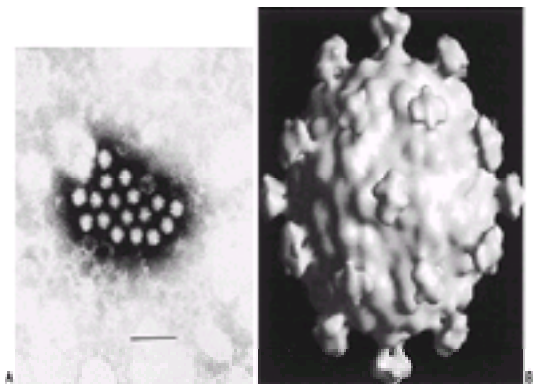

FIG. 1. A: Electron micrograph of human astrovirus in a fecal specimen. Bar = 100 nm. (Courtesy of T. W. Lee and J. B. Kurtz.) **B:** Surface-shaded views of the three dimensional reconstruction of human astrovirus, along the twofold axis of symmetry. (Courtesy of M. Yeager.) Cell culture–adapted human astrovirus serotype 1 (Oxford strain) was purified by isopycnic centrifugation and imaged by cryoelectron microscopy (193). External diameter (including spikes) is approximately 430 Å.

Subsequently, viral particles that were of similar size and had characteristic starlike surface features were identified in association with gastroenteritis in a wide variety of young mammals and birds, including lambs (162), calves (189), deer (171), piglets (23,159), kittens (61), mice (76), dogs (112,184), and turkey poults (118,150). Ducklings infected with astrovirus-like particles developed a rapidly fatal hepatitis (46), and viral particles were found in liver as well as feces. Astrovirus appears to cause infection in a species-specific manner (88), and at present there is no animal model to study human astrovirus infection.

An important milestone was achieved in 1981 when Lee and Kurtz (94) reported the isolation and passage of human astrovirus in cell culture. That astroviruses could be propagated in cell culture clearly distinguished them from Norwalk virus and other human caliciviruses that remain refractory to growth in such systems. This achievement also led to the recognition of five serotypes of human astrovirus in 1984 (87), development in the late 1980s of an enzyme immunoassay (EIA) that detects viral antigen (58,59), confirmation of its medical importance in 1991 (60), and cloning and sequencing of the astrovirus genome (67,99,176).

INFECTIOUS AGENT

Propagation of Human Astroviruses

Two years after astroviruses were identified by direct EM of human feces, Lee and Kurtz (93) demonstrated by immunofluorescence the infection of primary human embryonic kidney (HEK) cells with fecal extracts containing human astrovirus. It was not until 1981, however, that serial passage of astrovirus in primary HEK cells was made possible by incorporating trypsin in the serum-free growth media (94). The optimal trypsin concentration of 10 µg/mL yielded viral titers of 10^4 to 10^7/mL at approximately 48 hr after infection. The concentration of trypsin required in the medium was significantly higher than that required for propagation of rotavirus. After six passages in HEK cells, primary baboon kidney (PBK) cells and a continuous line of rhesus monkey kidney epithelial (LLCMK2) cells could be infected. The requirement for trypsin in the media remained unchanged. The first five serotypes of human astrovirus described (87) were adapted to growth in LLCMK2 cells. Attempts to establish serial passage by direct inoculation of PBK or LLCMK2 cells with fecally derived astrovirus were not successful. Although infected LLCMK2 cells do not display distinguishable cytopathic effects (CPEs), a plaque assay for at least three serotypes was developed to investigate viral neutralization and determine viral titer (63).

Willcocks et al. (179) directly infected a continuous line of human colon carcinoma (CaCo-2) cells with a fecal extract containing astrovirus serotype 1 from a 1988 outbreak of gastroenteritis in the United Kingdom. The optimal trypsin concentration in this system was determined to be 5 µg/mL, and CPE was apparent beginning 2 days after infection. Astrovirus serotypes 6, 7, and 8, the types identified most recently, have been successfully propagated in CaCo-2 cells (16,96). This is a promising technique that may allow more rapid isolation and subsequent propagation of wild-type astroviruses (148). Growth in CaCo-2 cells may be a way to distinguish astrovirus from calicivirus infection. In addition, the ability to achieve higher viral titers in CaCo-2 cells facilitates studies of astroviruses in cell culture.

Human astroviruses isolated in HEK cells, but not in Caco-2 cells, undergo a deletion of 15 partially repetitive amino acids, approximately 120 amino acids upstream of the carboxyl terminus of the open reading frame (ORF) 1a product (174). It was suggested that this deletion may improve the efficiency of adaptation to certain cells, but its significance is not well understood.

A hepatoma cell line (PLC/PRF/5) was found to support propagation of cell culture–adapted reference strains of human astrovirus (167). This cell line can also be used to isolate human astrovirus directly from fecal samples.

Propagation of Animal Astroviruses

Adaptation to growth in cell culture has been attempted for nearly every animal astrovirus identified by EM. Bovine (6) and porcine (159) astroviruses have been propagated in cell culture. Two factors appear to be important in achieving serial passage of animal astroviruses: (a) the use of host cells from the animal species in which the astrovirus infection was originally detected, and (b) the use of trypsin in the media of infected cells.

In the original description of bovine astrovirus, experiments aimed at adapting and maintaining these viruses in cell

culture were described (189). Primary calf kidney and testicular cell cultures were infected with a bacteria-free fecal preparation or the intestinal contents of infected gnotobiotic calves. Immunofluorescence could be demonstrated in the initial infection but was lost after a single passage. Several years later, passage of bovine astrovirus was attempted in primary, secondary, and tertiary bovine embryo kidney (BEK) cells (191) and this, too, was unsuccessful. It was speculated that either relatively fewer susceptible cells were present in secondary and tertiary BEK cells or that trypsin may be required for successful passage. In 1989, Aroonprasert et al. (6) demonstrated passage of bovine astrovirus in primary neonatal bovine kidney (NBK) cells in medium containing 50 µg/mL trypsin. Trypsin was necessary both for serial passage of virus and the release of infectious virus. Both NBK and primary BEK cells were infectable with bovine astrovirus derived from feces and cell culture. This system was used subsequently to study viral infection *in vitro* (see Pathogenesis section, later).

A porcine astrovirus was isolated from an outbreak of acute gastroenteritis at a pig farm in Japan (159). A fecal filtrate containing porcine astrovirus was used to infect embryonic swine kidney cells in media with 0.5 µg/mL trypsin. Maximal CPE was evident 4 to 5 days after inoculation of cell monolayers in trypsin-containing media. CPE was not produced if trypsin was removed from the medium or if the virus inoculum was mixed with convalescent phase serum.

Astroviruses from lambs (162), red deer (171), kittens (61), dogs (112,184), turkeys (118), and ducks (46) also were inoculated into cell cultures, but serial passage has not been established. Trypsin was not used in any of these experiments and in some cases (112,118,171), cells from the same species as the host were not used.

Physical Features

In 1982, Caul and Appleton (26) proposed an interim scheme to classify the SRVs based on their physical and EM features. The viruses considered in this scheme were first differentiated by whether the virion surface was featureless or structured. The featureless viruses included the enterovirus, parvovirus, and parvovirus-like particles that tended to be smaller in diameter than the structured viruses. Astroviruses were one of three groups of SRVs with surface structure. Astroviruses, shown in Fig. 1A, were described as 28- to 30-nm particles with a smooth margin and a characteristic five- to six-pointed star on the surface. They differed from the two other categories of structured SRVs: calicivirus, with its “Star of David” pattern with cuplike surface hollows and a ragged outline; and the small, round-structured viruses (SRSVs), including Norwalk and Norwalk-like viruses, that had an amorphous surface and a ragged outline. Although the surface star of astrovirus is its most striking feature, it is important to note that this may be discernible on only 10% of the viruses in a given preparation (106).

Antibody coating of viral aggregates in stool or in preparations of immune EM (IEM) may further obscure some of the surface features (8,85,88). In some preparations, “bridging structures” that may be surface extensions of the virus have been observed between adjacent astrovirus particles (61,104,106). The significance of these structures remains to be elucidated.

Astroviruses were described generally to have a diameter of 28 nm, based on the systematic measurement of over 1,000 negatively stained (phosphotungstic acid) astroviruses from infected infant stools by Madeley (104). The diameter of astrovirus particles may vary, however, depending on the source of virus and the method of preparation for EM. For example, bovine astrovirus serotype 2 (US2) propagated in primary NBK cells had an average diameter of 34 nm (range, 30 to 37 nm) after fixation in glutaraldehyde plus 1% osmium tetroxide and staining with uranyl acetate (6). Similarly fixed and stained sections of infected gnotobiotic calf ileum revealed bovine astroviruses that had an average diameter of 30 nm (range, 27 to 35 nm) (191).

More detailed ultrastructural analysis of human astrovirus serotype 2 grown in LLCMK2 cells in the presence of trypsin revealed particles with icosahedral symmetry and an array of spikes emanating from the surface (152). These particles had an external diameter on negatively stained preparations of 41 nm (including spikes). They did not display the starlike surface appearance characteristically found on fecally shed virus. The starlike morphology was inducible after alkaline treatment (pH 10), and the particles were found to disassemble rapidly when exposed to more alkaline solutions (pH 10.5).

Purified preparations of cell culture–adapted human astrovirus serotype 1 [Oxford strain from Kurtz and Lee (87)] particles evaluated by electron cryomicroscopy and image analysis demonstrated a rippled solid capsid shell (330 Å in diameter) decorated with 30 dimeric spikes extending 50 Å from the surface (Fig. 1B) (193).

Astrovirus particles were shown to be stable at pH 3 and resistant to chloroform, a variety of detergents (nonionic, anionic, and zwitterionic) and lipid solvents (88). Human astrovirus retains activity after 5, but not 10, minutes at 60°C. Viral particles appear to be stable when stored at ultralow temperatures (–70°C to –85°C) for 6 to 10 years (185), but they may be disrupted with repeated freezing and thawing.

A range of buoyant densities in cesium chloride (CsCl) was reported for human and animal astroviruses. Intact human astrovirus particles typically band at densities of 1.35 to 1.37 g/mL (26,88,114,179). Buoyant densities of 1.39 to 1.40 were observed for astrovirus particles from an outbreak of gastroenteritis at a school in Japan (78) and from a volunteer inoculated with astrovirus derived from an outbreak of gastroenteritis at a convalescent home in California (121).

Human astrovirus propagated in CaCo-2 cells produced two visible light-scattering bands at buoyant densities of 1.35 and 1.32 g/mL (179). The polypeptide profiles of both bands were identical, suggesting that the higher-density band very likely contained complete viral particles and the lower-density band empty virions. This speculation was not proven, as the RNA content of each of these fractions was not assessed. Evaluation of another human astrovirus serotype 1, propagated in LLCMK2 cells, yielded similar results (114). Fractions from the CsCl gradient were tested for the presence of astrovirus antigen by EIA (58,59), and two peaks were identified. The major peak at density 1.37 g/mL was demonstrated to contain RNA that hybridized with probes from two different regions of the astrovirus genome. Nucleic acid extracts from the minor peak at density 1.33 g/mL did not hybridize with either probe, suggesting that astrovirus

particles devoid of viral nucleic acid were present in this fraction. In addition, these results suggest that the lower-density band was not composed of encapsidated subgenomic RNA (see later), as has been described for the calicivirus rabbit hemorrhagic disease virus (RHDV) (119).

The buoyant density of canine (184) and porcine (159) astroviruses was found to be in the range of 1.34 to 1.35 g/mL. The buoyant density of ovine astrovirus from two sources, intraluminal contents and infected intestinal tissues, was determined as well (55). Viral aggregates in intraluminal contents banded at 1.39 g/mL, whereas single particles banded at 1.365 g/mL. Most of the viral RNA was observed in the higher-density fraction. Intracytoplasmic ovine astrovirus obtained from infected intestinal tissues was found in fractions with densities of 1.38 to 1.40 g/mL.

Although most of the data regarding the buoyant density of astrovirus particles were collected using CsCl density gradients, it was observed that separation of fecal SRVs (including astrovirus) in this medium and further concentration by pelleting disrupts virus morphology (7). Highly purified viral particles, requiring a CsCl purification step, are needed for evaluation by electron cryomicroscopy. The inclusion of divalent cations (Ca^{2+} and Mg^{2+}) in the purification solutions was found to stabilize viral particles undergoing isopycnic centrifugation in CsCl (193). An alternative gradient used to purify enveloped viruses and subviral structures (27,139) was proposed by Ashley and Caul (7). Potassium tartrate progressively diluted with glycerol yielded a positive-density, negative-viscosity gradient in which virus morphology was maintained and from which viral particles could be pelleted without deleterious effects. Peak density for astroviruses in this medium was observed to be 1.32 g/mL.

Genome Composition, Molecular Biology, and Viral Proteins

Astroviruses have a plus-sense, single-stranded RNA genome that is approximately 6,800 nucleotides (nt) in length, excluding the poly(A) tail at the 3'-end. RNA extracted from virions is infectious (41). Infectious RNA also can be transcribed from a full-length cDNA clone of human astrovirus serotype 1 (AVIC) (41). BHK cells, previously thought not infectable with astrovirus (94), were shown to support astrovirus replication and maturation when transfected with RNA extracted from virions or transcribed from AVIC. Astrovirus produced in these BHK cells can infect monolayers of CaCo-2 cells in trypsin-containing media.

Complete nucleotide sequences of five strains of human astrovirus are currently available: (a) the serotype 1 Oxford reference strain propagated in LLCMK2 cells (99), (b) a serotype 1 Newcastle strain isolated and maintained in CaCo-2 cells (176,177 and 178), (c) the serotype 2 Oxford reference strain propagated in LLCMK2 cells (67), (d) a serotype 3 strain (GenBank accession AF141381), and (e) a serotype 8 strain (GenBank accession AF260508). Genomic sequences also are available for avian astroviruses [avian nephritis virus (64) and turkey astrovirus (77) and sheep astrovirus (GenBank accession NC002469)].

During infection of susceptible cells, it was noted that both the full genomic (6.8 kb) and a subgenomic (2.4 kb) RNA are produced (114,132). The viral genome comprises three ORFs. The two ORFs at the 5'-end of the genome, designated ORF-1a and ORF-1b by the International Committee on the Taxonomy of Viruses (129), encode nonstructural proteins. The third ORF, designated ORF-2, encodes a structural protein and is found at the 3'-end of the genome (99,131,178). This ORF is common to both the genomic and the subgenomic RNA. Sequences of the capsid-encoding ORF-2 are available in GenBank for human serotypes 1 through 6 and 8, and for feline, porcine, chicken, and turkey astroviruses.

Sequence Analysis

A schematic representation of the astrovirus genome is shown in Figure 2. A 5'-untranslated region of 80 (serotype 8), 82 (serotype 2), or 85 (serotypes 1 and 3) nt precedes ORF-1a. ORF-1a is 2,763 nt in length for the Oxford reference serotypes 1 and 2 that were first isolated in HEK cells, and it is 45 nt longer in the Newcastle serotype 1 strain that was directly isolated in CaCo-2 cells. ORF-1a of the human astrovirus serotype 3 is 2,784 nt (GenBank accession AF141381) and that of serotype 8 is 2,766 nt in length. A 73-nt overlap exists between ORF-1a and ORF-1b in astrovirus serotype 1 and 2 strains, but it is 61 nt for serotype 3, and 70 for serotype 8. ORF-1b is 1,560 nt in length for serotypes 1 and 2 strains, 1,557 nt for serotype 8, and 1,548 nt for serotype 3. The overlap between the stop codon of ORF-1b and the start codon of ORF-2 is 8 nt for serotypes 1 through 3 and 8. ORF-2 varies in length among human astroviruses from 2,316 nt for a serotype 4 strain, to 2,391 nt in the Oxford serotype 2 strain (67). An 80-nt (serotype 1 strains), 82-nt (serotypes 2 and 3), or 85-nt (serotype 8) 3'-untranslated region is found between ORF-2 and the poly(A) tail at the extreme 3'-end of the genome.

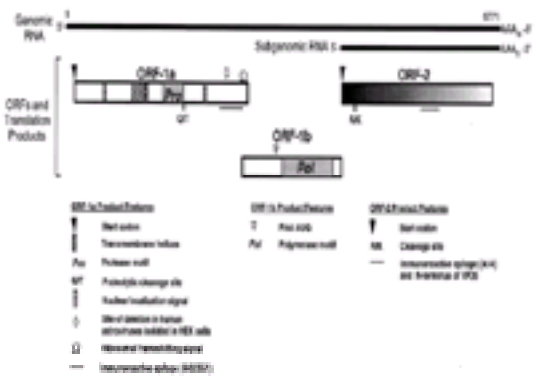

FIG. 2. Genome organization of human astrovirus serotype 1. Genomic and subgenomic RNA are indicated in the upper panel. ORFs and translation products are depicted in the middle panel. Features shown for each ORF are summarized in the lower panel. The ORF-1a product features transmembrane helices (*diagonally shaded boxes*), protease motif (*PRO, shaded*), a putative protease-dependent cleavage site (*Q/T*) (73), a nuclear localization signal (*box with horizontal lines*), a ribosomal frameshifting signal (W), and an immunoreactive epitope [A43/35/1 (114), *thin horizontal line*). The site at which a 45-nt (15-amino-acid) deletion occurs in human astroviruses isolated in HEK cells is indicated (à) (174). In

ORF-1b, the first in-frame AUG is found at the *open arrowhead.* ORF-1b encodes an RNA-dependent RNA polymerase motif (POL, shaded). ORF-2 encodes a capsid protein precursor that appears to be cleaved at amino acid 70/71 (*R/K*) before capsid assembly (10). The N-terminal half of the ORF-2 product is highly conserved (*darker shading*) in contrast to the majority of the C-terminal half, which is quite variable (*lighter shading*) among human astrovirus strains and serotypes. Immunoreactive epitope A14 (114) overlaps the N-terminus of capsid protein VP26 as shown (*thin horizontal line*). VP26 is recognized by a neutralizing monoclonal antibody (154).

The length of the astrovirus 3'-untranslated region and its predicted secondary structure are reminiscent of certain picornaviruses (177), but direct sequence comparisons disclose minimal sequence identity between these two viral groups. In a separate study, the terminal 19 nt of ORF-2 and the adjacent 3'-untranslated region were found to be highly conserved among all 8 serotypes (128). Of note, similarities in sequence and folding of the 3'-untranslated region of human, sheep, pig, and turkey astroviruses, avian infectious bronchitis virus (a coronavirus), and equine rhinovirus serotype 2 (a picornavirus) were observed (68); the investigators suggested that this homology arose from recombination events.

The polypeptide sequences of ORFs 1a and 1b indicate that nonstructural proteins are likely to be encoded by these ORFs. The polypeptide encoded by ORF-1a is 920 (67,99), 921, 927, and 935 (176) amino acids in length for serotypes 1, 8, 3, and 2, respectively, and it contains a viral serine protease motif. The protease motif of astrovirus has features consistent with chymotrypsin-like proteases of other plus-stranded RNA viruses, including RHDV and feline calicivirus (67). An important difference found when comparing the astrovirus protease motif with that of the caliciviruses is the substitution of a serine for a cysteine at the third catalytic amino acid residue. This substitution is seen in several viruses, sobemoviruses, luteoviruses, and arteriviruses, but the putative astrovirus protease bears stronger overall resemblance to the calicivirus protease.

Sequence analysis also demonstrates a bipartite nuclear localization signal encoded downstream of the viral protease motif in ORF-1a (67,176). Recent studies of human astrovirus serotype 1 have indicated that this signal is functional and directs ORF-1a-encoded proteins to the nucleus (175). Of note, bovine astrovirus proteins were found previously in the nucleus of infected cells by immunofluorescence (6). Further investigation is required to determine the significance of nuclear localization in the astrovirus life cycle.

Analysis of ORF-1a sequences of reference serotypes 1 to 7 indicates two distinct genogroups that correlate with serotype (13). Types 1 to 5 (13) and 8 (16) are found in one genogroup (A), and types 6 and 7 fall into a separate genogroup (B). These phylogenetic associations for ORF-1a sequence are different from that observed for ORF-1b or ORF-2, in which nearly equidistant clustering of serotypes is found with unrooted phylogenetic trees. Whether these differences are attributable to recombination remains to be studied.

ORF-1b encodes a polypeptide of 515 (serotype 3), 518 (serotype 8), or 519 amino acids (serotypes 1 and 2) (67,99,176) that contains motifs suggestive of an RNA-dependent RNA polymerase. The polymerase motifs are highly conserved among astrovirus serotypes 1 to 8 (13,99). In one analysis, serotypes 1 and 2 appear to be the most closely related, and serotypes 4 and 5 are the least related in this region (99). The astrovirus polymerase motifs are most similar to those of plant viruses Bymovirus and Potyvirus, and they can be aligned with other polymerases of Koonin's supergroup I (79), which includes picornaviruses, caliciviruses, and certain plant viruses (67). It has been suggested that the similarities between astrovirus and plant viruses in this region do not necessarily indicate a common origin (67).

Regions that may encode an RNA helicase, a methyltransferase, or a papain-like protease have not been identified in the astrovirus sequences that are currently available. It is unusual for a plus-strand RNA virus with a genome length of more than 6,000 nt to lack a helicase domain (67,71). It has been suggested that astrovirus encodes a VPg, based on the absence of a methyltransferase-encoding region and the similarity of its RNA-dependent RNA polymerase with primarily VPg-containing viruses. A convincing VPg domain has not been identified, but it may be located between the putative protease motif and upstream transmembrane alpha-helices, where a serine at amino acid position 420 that could link the VPg to genomic RNA is found. ORF-1a also encodes an immunoreactive epitope (amino acids 757 to 899) identified by antisera produced to purified viral particles, but its significance is not known.

The greatest sequence variability is found in ORF-2, the ORF that encodes a viral structural protein that varies in length from 782 amino acids for serotype 8 (assuming translation begins at the third in-frame methionine, as for serotype 1), to 786 amino acids (176) to 787 amino acids (99) for serotype 1, to 796 amino acids (67) for serotype 2, to 794 amino acids for serotype 3 (see later). Complete capsid sequences are also available for feline (GenBank accession AF056197; 816 amino acids), porcine (AB037272; 776 amino acids), sheep (NC002469; 762 amino acids), turkey (724 amino acids) (77), and chicken (683 amino acids) (64) astroviruses. Alignments of capsid polypeptide sequences indicates a high degree of conservation of the N-terminal 415 (or so) amino acids among the human serotypes (182). In this region, the amino acid sequence for the feline, porcine, and sheep strains share certain conserved stretches of amino acid residues with human strains and can be aligned with minimal gaps, whereas the avian strains vary considerably from the human and other mammalian strains. Beyond this region, there is considerable variability in sequence among the serotypes, requiring gaps to be introduced for optimal alignment. The C-terminal eight amino acids are highly conserved, particularly among the human strains.

Translation Strategies

The mechanism of ORF-1b translation is not readily apparent by routine sequence analysis. The first AUG codon occurs over 400 nt into ORF-1b (shown in Fig. 2) and is in a suboptimal context for initiation according to Kozak's rules (82). The overlap region between ORF-1a and ORF-1b is highly conserved among serotypes and displays a heptameric shift sequence (A AAA AAC) and the potential for the formation of a downstream stem-loop structure with possible pseudoknot

that strongly suggests that a (–1) ribosomal frameshifting mechanism is involved in ORF-1b translation (67,99,176). These elements resemble those required for frameshifting events that are used to translate the retrovirus protease and polymerase (28,65,66,187), and coronavirus polymerase (25). The astrovirus heptameric shift sequence and stem-loop structure were shown to be critical for ribosomal frameshifting to occur (101,102,110). However, unlike retroviruses and coronavirus, the potential pseudoknot-forming sequences were not required for frameshifting (101,102). Nucleotide sequences that can be aligned with the shifty heptamer and stem-loop motifs of human astrovirus also were identified in avian astroviruses (64,77).

As noted, ORF-2 is found in both the genomic and the subgenomic RNA. It has been suggested that the role of the subgenomic RNA is the production of viral structural proteins (131,132). Other viruses, such as the enveloped alphaviruses, use their subgenomic RNA to synthesize large amounts of structural proteins that are used to assemble intact progeny viruses efficiently (155). Proof to support such a role of the astrovirus subgenomic RNA was provided by expression of ORF-2 in an *in vitro* transcription and translation system, followed by immunoprecipitation of the 87-kd product by antibodies produced to purified astrovirus particles (99). This protein is the precursor to the smaller, 20- to 40-kd, structural proteins that have been identified in human and animal astroviruses (10,15,99,132). Production of monoclonal antibodies directed at a neutralization epitope in the 26-kd fragment of the 87-kd protein corroborates these data (11,154). The exact length of the 5' noncoding region of the subgenomic RNA has not been resolved. The length of this region in astrovirus serotype 1 [Newcastle (178)] is reported to be 3 to 19 nt longer than the comparable region in astrovirus serotype 2 [Oxford (131)].

In summary, the general organization of the astrovirus genome places the ORFs encoding the nonstructural proteins (ORF-1a and ORF-1b) at the 5' end and the ORF encoding a structural protein (ORF-2) at the 3' end. This arrangement resembles the genome organization of caliciviruses (165). However, several features serve to distinguish astroviruses from caliciviruses. These include differences in the size, number, and possible processing of structural proteins, the lack of a helicase domain in astroviruses, the use of ribosomal frameshifting to translate the RNA-dependent RNA polymerase, and distinctive morphologic features described previously. Basic features that distinguish astroviruses from picornaviruses include differences in genome organization, lack of a helicase domain, and use of translational strategies such as ribosomal frameshifting and subgenomic RNA. Astroviruses appear to incorporate specific features of a number of different viruses without strictly resembling any one group and therefore are assigned to a separate viral family, the *Astroviridae* (129).

Nonstructural Proteins

Information regarding processing of the nonstructural proteins is limited. A preliminary report by Gibson et al. (42) noted a “lack of processing” *in vitro* of ORF-1a and ORF-1a/1b translation products containing the 3C-like serine protease motif. By contrast, Willcocks et al. (175) found no full-length (101 kd) ORF-1a product, but they detected proteins of 74, 34, 20, 6.5, and 5.5 kd in astrovirus-infected CaCo-2 cells by Western blot analysis with antisera specific to the C-terminal 298 amino acids of the ORF-1a product. Using antisera to a recombinant ORF-1b-Baculovirus protein, products of 160 kd (most likely corresponding to the ORF-1a/1b fusion product) and 59 kd (ORF-1b product) were detected, suggesting that cleavage occurs near the boundary between ORF-1a and ORF-1b.

In other studies (73), ORF-1a was transcribed and translated in a cell-free system and products were immunoprecipitated by antibodies specific to N- and C-terminal epitopes. ORF-1a products identified were the full-length 101-kd protein (p101), an N-terminal cleavage product (p64), and a C-terminal cleavage product (p38). Mutation and deletion analyses indicated the important role of the catalytic triad of the viral 3C-like serine protease (Ser-551, Asp-489, and His-461) in processing of p101.

Structural Proteins

The polypeptide profiles of ovine, porcine, and several serotypes of human astrovirus have been examined. These studies did not fully concur on the size and number of structural proteins that comprise astrovirus particles. Examination of astrovirus particles shed by experimentally infected lambs revealed two polypeptides that migrated at about 33 kd (55). For porcine astrovirus, five proteins that range in mass from 13 to 39 kd have been described (159). Most of the studies of human astrovirus polypeptide composition were performed on cell culture–adapted viruses. Four proteins (36.5 kd, 34 kd, 33 kd, and 32 kd) were identified in astrovirus serotype 4 that was propagated in cell culture in the presence of trypsin (88). It was postulated that the faint 36.5-kd band may represent a precursor protein that undergoes subsequent processing, in a manner similar to the VP0 protein of enterovirus (157), to yield one of the more abundant, smaller proteins. Two additional proteins of 24 kd and 5.2 kd were found by Kurtz (84) in cells infected with astrovirus serotype 1. In contrast, CaCo-2 cells infected with astrovirus serotype 1 yielded three astrovirus-specific polypeptides (33.5 kd, 31.5 kd, and 24 kd), as determined by reactivity with astrovirus serotype 1–specific antiserum (179). In some preparations, a 27-kd polypeptide was also noted, and it was hypothesized that this may be derived from one of the larger proteins by proteolytic digestion. In studies of human serotype 5 isolated from volunteer fecal specimens, a single 30-kd structural protein was immunoprecipitated (121).

When protein synthesis was studied in astrovirus-infected LLCMK2 cells in the absence of trypsin, a single, approximately 90-kd protein was immunoprecipitated from infected cell lysates with hyperimmune antiserum to purified human astrovirus serotype 2 (132). Pretreatment of the lysates with trypsin resulted in immunoprecipitation of three proteins, a predominant 29-kd protein as well as a 31-kd and a 20-kd protein. These results suggested that the (about) 90-kd protein may be proteolytically cleaved to yield at least three smaller proteins. In cells infected with human astrovirus serotype 2, grown in trypsin-containing media, Sanchez-Fauquier et al. (154) used a neutralizing monoclonal antibody (PL-2) to determine that an 86-kd protein (P86) is the precursor to VP26, a major component of viral particles. VP29, a minor component of virions and the product of alternative processing of P86, and intermediate proteins ranging from P74 to P35 also were identified in infected cells by PL-2. Although these results suggest that processing of P86 to P26 and/or P29 occurs within infected cells, the presence of trypsin in the medium impairs optimal interpretation of the

data.

Belliot et al. (15) examined the capsid protein composition of partially purified preparations of astrovirus reference strains (serotypes 1 to 7) grown in cell culture. Human astrovirus reference types 1 to 4 consisted of three proteins, P1 (about 33 kd), P2 (about 33 kd), and P3 (25 to 28 kd, depending on serotype), that were immunoprecipitated with homologous rabbit antisera. For reference types 5 to 7, three infection-specific proteins were observed in radiolabeled samples, but only P2 and P3 reacted with type-specific rabbit serum. N-terminal sequencing of the P3 protein of serotype 1 virus indicated it is equivalent to the VP26 protein of serotype 2 (154). The P2 protein appears to contain at least one group-reactive epitope.

Recent studies by Bass and Qiu (10) suggest that assembly of astrovirus particles requires cleavage of 70 N-terminal amino acids of the 87-kd capsid precursor protein. The resultant 79-kd protein forms the viral capsid within astrovirus-infected CaCo-2 cells grown in the absence of trypsin. These particles are virtually noninfectious, but they can be made infectious with trypsin treatment, which also cleaves the 79-kd protein to three smaller proteins (34 kd, 29 kd, and 26 kd).

Serotypes of Human Astrovirus

Initially, five serotypes of human astrovirus were identified by immunofluorescence, neutralization, and IEM in the 1980s (63,87,95). Each of these reference strains was isolated from natural infections in the United Kingdom and adapted to growth in cell culture (94). Serotypes 6 and 7 originally were identified in the United Kingdom in 1989 and 1991 to 1992, respectively (33,96). Astrovirus serotypes 6 and 7 were indistinguishable from serotypes 1 to 5 by EM, cultivated in CaCo-2 cells in media supplemented with trypsin, and recognized by the group-reactive monoclonal antibody (see later). An eighth serotype was identified by immunofluorescence assay (16). Capsid sequence for the ORF-2 product of a serotype 8 virus is available (GenBank accession no. Z66541).

A limited number of serotyping studies using various methods were done, but the reference reagents used in these studies were all provided by the Oxford group (87,95). In general, astrovirus serotype 1 is the most common type found (40,70,87,88,96,133,135,137,138,143,153,186), but the predominant serotype may vary depending on location (52). The community-acquired astroviruses were monitored in Oxford, United Kingdom, between 1975 and 1987 (88). In this population, 72% of the astroviruses encountered were serotype 1. Serotypes 2, 3, 4, and 5 each accounted for 6% to 8% of the astrovirus strains. A more recent survey by Lee and Kurtz (96) examined the astrovirus serotypes found in the Oxford region between 1976 and 1992. Once again, serotype 1 was found most frequently and accounted for an average of 65% of the cases. If the astrovirus-positive cases were divided between those from pediatric wards and those submitted by community physicians, serotype 1 strains were found in 77% and 60%, respectively. Serotypes 2 to 7 accounted for 11%, 9%, 11%, 2%, 0.3%, and 0.7%, respectively. In all the years studied, there was only one year, 1991, in which the number of serotype 2 cases exceeded serotype 1 cases. A biennial peak of serotype 1 prevalence was noted over the last 5 years of the survey. A third study from the United Kingdom found a similar predominance of astrovirus serotype 1 (186). Serotype 1 was found by IEM in 55% of 40 astrovirus-containing stools. By contrast, a longitudinal study of diarrhea in a cohort of Mexican children found that astrovirus serotype 2 was most common (35%) and serotypes 1 and 5 were least common (4% each) (52).

Antiserum derived from animals hyperimmunized with each of the human astrovirus serotypes 1 to 7 appears to behave in a serotype-specific manner in immunofluorescence tests and IEM (58,88,96). In addition, rabbit polyclonal antiserum neutralizes astrovirus in a serotype-specific manner in a plaque assay (58). All eight serotypes of human astrovirus are recognized by a single monoclonal antibody produced by Herrmann et al. (59,96). This monoclonal antibody, which does not neutralize virus, is directed at a viral structural protein (70), most likely an epitope of the highly conserved N-terminal half of the ORF-2 product (16, and Geigenmüller and Matsui, unpublished data, 2000). An EIA in which the monoclonal antibody serves as a capture antibody and polyclonal antiserum is used as a detector antibody has been developed (59) and employed in large-scale seroepidemiologic studies (described later). A typing enzyme immunoassay (TYPE-EIA) that uses serotype-specific reference antisera for capture and the group-reactive monoclonal antibody as detector has been developed to assess the antigenic types of astroviruses found in clinical samples (138). Alternatively, molecular methods for genotyping clinical isolates by reverse transcriptase polymerase chain reaction (RT-PCR) with primers from the more conserved N- or C-terminal regions of ORF-2 may be used (113,138). A high concordance between results of genotyping and serotyping was observed. A rapid latex agglutination test to detect astrovirus serotypes 1 to 4 also was developed, but it has not yet been extensively tested (5).

Serotypes of Animal Astrovirus

Human, lamb, piglet, red deer, kitten, and calf astroviruses were shown to be antigenically unrelated to one another (54,95,171,191). Bovine astroviruses are the only nonhuman astroviruses that have been analyzed for serotypic variation. Three strains of bovine astrovirus have been described. The first reported (called UK) was found in the feces of calves with diarrhea in the United Kingdom in 1978 (189). Two U.S. strains of bovine astrovirus (US1 and US2) were described as well. The three isolates shared common antigens that were detectable by immunofluorescence (190). Antisera to each of the three bovine astroviruses did not cross-neutralize either the US1 or the US2 astrovirus, indicating that there are at least two serotypes of bovine astrovirus. Whether a third serotype exists can be determined only if the antisera are tested for cross-neutralization against the UK isolate.

Species Specificity of Infection

In all cases where antigenic relationships among astroviruses of different animal species have been examined, there has been no evidence for cross-reactivity between species (23,54,95,171,191). Thus, as an example, convalescent sera from lambs infected with astroviruses react only with lamb astrovirus and will not recognize calf or piglet astrovirus. In general, infection of animals and cell culture is also species specific. Astrovirus isolated from red deer has been shown to infect BEK cells, but serial propagation was not achievable (171). Furthermore, convalescent sera from red deer did not

recognize lamb astrovirus antigens in infected lamb intestinal tissue or bovine astrovirus propagated in bovine cell culture.

PATHOGENESIS AND PATHOLOGY

Transmission

Astroviruses can be transmitted through the fecal–oral route, as shown by human volunteer studies (89,121). In these studies, however, few of the adult volunteers developed a diarrheal illness with fecal shedding of virus and/or production of antibody to the virus in the inoculum. Person-to-person spread was deemed responsible for a protracted outbreak of gastroenteritis in Marin County, California, in 1978 (142). Although astrovirus diarrhea does not usually develop in adults, it was observed that adult caregivers of infected children, including parents, teachers, and medical personnel, did become ill (3,85,88,140). An outbreak among military troops was also described (14). This suggested that the ill adults were exposed to a larger dose of astrovirus or through a variable route (e.g., fomites, contaminated food or water). This pattern also was found with rotavirus illness (62). In addition, astrovirus was detected in water from an area where an outbreak of astrovirus gastroenteritis occurred (147), and 93% of surfers in the United Kingdom had antibodies to human astrovirus serotype 4, compared to 22% of age-matched controls (134). A study of sewage and environmental samples from a water treatment facility in the United Kingdom failed to detect astrovirus RNA by RT-PCR (37). A case of astrovirus gastroenteritis was diagnosed at the end of the sampling period, but it occurred outside the surveyed area. Using an integrated cell culture RT-PCR method coupled with nested PCR to detect enteric viruses in surface water samples, astrovirus was identified in 15 of 29 samples and eight samples contained infectious virus (29). In Japan, astrovirus was found in naturally grown oysters (15 of 112 samples) most often during winter months. For comparison, SRSVs (genogroup II) were detected in 28 and hepatitis A virus in two of the samples (74).

Viral Pathogenesis

Pathogenesis of astrovirus infections in humans has not been well studied. A brief report of two children with diarrhea correlated fecal shedding of astrovirus with identification of astrovirus particles in intestinal epithelial cells, suggesting that virus replication occurs in intestinal tissue in humans (146). Astrovirus particles in the biopsy specimen were localized to the epithelial cells in the lower regions of the villus in a patient with sucrase isomaltase deficiency and in the “exposed surface epithelium” of a second patient who had a severe enteropathy caused by sensitivity to cow's milk formula. The significance of astrovirus infection was difficult to assess in these patients because of their underlying gastrointestinal problems and fecal shedding of *E. coli* 086 or rotavirus prior to astrovirus. Studies on human volunteers have not examined the histologic effects of human astrovirus–related diarrhea (89,121).

Viral pathogenesis has been studied more extensively in animals. Classic experiments in which gnotobiotic lambs were administered an outbreak strain of ovine astrovirus revealed that mild transient diarrhea was caused by infection of mature enterocytes in the apical two thirds of villi and subepithelial macrophages, followed by transient villus atrophy and crypt hypertrophy. Infected lambs developed loose stools, then diarrhea on days 2 to 4, and they shed astrovirus on days 2 to 9 after inoculation (161,162). Infected intestinal cells were detected by immunofluorescence between 14 and 70 hours after inoculation. The largest number of infected cells was found in the incubation period from 14 to 38 hours after inoculation (161). During this time, virus particles were observed by EM in aggregates along the microvilli or in lysosomes and autophagic vacuoles. In some enterocytes, indirect evidence of infection, including intracytoplasmic inclusions, vacuoles, and degenerate nuclei, was seen (47). Histologic features of astrovirus infection resolved by the fifth day after inoculation (161).

In contrast to lamb astroviruses that cause symptomatic infection, bovine astroviruses are considered nonpathogenic, as inoculation of gnotobiotic calves with bovine astrovirus alone does not result in diarrhea (24,191). Bovine astrovirus appeared to cause infection by a different mechanism—namely, the infection of M cells and absorptive enterocytes overlying the dome villi of Peyer's patches in the small bowel (53,191). Infected cells were sloughed and replaced by cuboidal cells, and they formed an exudate with inflammatory mononuclear and eosinophilic cells above the dome villi. The dome epithelium contained similar inflammatory cells and was observed to fuse at times with the epithelium of an adjacent villus. The lamina propria was infiltrated with neutrophils and contained cells with degenerate nuclei. Lymphoid cell depletion was noted in the central region of germinal centers beneath infected dome villi.

Turkey poults inoculated orally with turkey astrovirus developed postinfection diarrhea on day 2 that persisted through day 10 (169). In the intestines, mild crypt hyperplasia was observed by light microscopy in the proximal jejunum on postinfection day 1, in the distal jejunum and ileum on day 3, and in the duodenum on day 5. Intracytoplasmic aggregates of astrovirus were found by EM in enterocytes on the sides and base of villi in the ileum and distal jejunum on day 3. Specific maltase activity, a reflection of intestinal disaccharidase activity, was monitored in other studies (170). During acute astrovirus infection (days 3 to 7 after infection), maltase activity decreased transiently and significantly, compared to controls, and was associated with disaccharide malabsorption, maldigestion, and osmotic diarrhea. Maltase activity returned to normal with resolution of symptoms.

A strain of turkey astrovirus (TAstV) also was isolated from birds with poult enteritis mortality syndrome (PEMS), an emerging infectious disease of young turkeys in which enteritis, growth depression, lymphoid atrophy and immunosuppression, and high rates of mortality are prominent features (77,156). TAstV was observed in the intestines, thymus, and bursa of affected turkeys. PEMS-like illness was induced in naive turkey poults who were given independently filtrates of intestine and thymus from infected poults (156).

Studies of Viral Pathogenesis in Cell Culture

These observations suggest that astroviruses are likely to enter cells through the apical surface. In polarized CaCo-2 cells, however, human astroviruses isolated from wild-type infections appear to enter through the basolateral surface (179). This result may be an artifact of the cell culture system and may not reflect the arrangement of astrovirus receptors

in differentiated intestinal epithelial cells *in vivo* (180).

The mechanism by which astroviruses enter cells was studied using Graham 293 cells, a transformed line of primary HEK cells (36). The effects on astrovirus infection of lysosomotropic agents (ammonium chloride, methylamine, and dansylcadaverine) and the ionophore monensin were investigated. All of the chemicals, used at nontoxic levels, inhibited viral infection. These results imply that a functional endocytic pathway is necessary for delivery of infectious astrovirus into the cytoplasm. This impression was supported by ultrastructural analysis, which showed viral particles being internalized by invagination of the cell membrane at coated pits approximately 10 minutes after attachment to the cell surface. Other studies have shown that flavanoids also inhibit astrovirus antigen synthesis in LLCMK2 cells in a dose-dependent fashion (166). Flavanoids have been shown to inhibit early steps in picornavirus replication, but their precise mechanism of action is not known.

A system to study bovine astrovirus infection in cell culture was developed as well. Bovine astrovirus was propagated in primary NBK cells in serum-free media containing 50 µg/mL of trypsin (6), and the expression of viral antigens was followed by fluorescent antibody probes. In a typical infection, less than 2% of the cells were infected, but occasionally infection could be detected in 10% to 20% of the cells. Immunofluorescence was first detected at 7 hours after infection in the cytoplasm of infected cells. Shortly thereafter, several brilliant immunofluorescent granules were identified in the nucleus, in a pattern suggesting nucleolar involvement. This was followed by dense immunofluorescent granules appearing in the perinuclear region and diffuse staining of the cytoplasm. The role of the nuclei and/or nucleoli in astrovirus replication is not known, but actinomycin D, an inhibitor of DNA transcription, did not inhibit astrovirus infection in this system.

CLINICAL FEATURES

Characteristics of Human Illness

Gastroenteritis caused by human astrovirus infection primarily affects young children, many under 6 months of age (135), throughout the world. Although its medical importance has been questioned in the past, studies demonstrated that it is the second most common viral agent found in young children with diarrhea evaluated in outpatient settings (17,37). A population-based cohort study from Egypt indicated that in children under the age of 3 years, the overall incidence of diarrhea caused by astrovirus was the same as for that caused by rotavirus (135). Astrovirus serotype 1 appears to be the most prevalent strain (88,96,143,186). Severe infections have been noted in young adults infected with astrovirus serotype 4 (33). Astrovirus infections have been recognized in elderly, institutionalized patients (48,100,115,186) and immunocompromised individuals (see later).

Adult human volunteer studies (see later) established the incubation period to be 3 to 4 days (89,121). A shorter incubation period of 24 to 36 hours was calculated from the secondary spread characteristics during an outbreak of gastroenteritis in a Japanese kindergarten (78). Human astrovirus infection induces a mild, watery diarrhea that typically lasts for 2 to 3 days, associated with vomiting, fever, anorexia, abdominal pain, and various constitutional symptoms that last for less than 4 days (21,49,85,88,92,144). Protracted diarrhea and viral shedding was observed in some studies (20,30,88). In children, it may be difficult to distinguish diarrhea caused by astrovirus from that caused by rotavirus on clinical grounds alone (32,60,88). In general, astrovirus diarrhea is milder and does not lead to significant dehydration or hospitalization (52,60,91,98,116,125,136,144). However, studies of hospitalized children found astrovirus to be the second most common viral pathogen, after rotavirus (40,143, and R. I. Glass, personal communication, 2000). Severe dehydration was an associated symptom in 17% of rural Egyptian children with astrovirus gastroenteritis (135). Improved assays for astrovirus detection have helped to identify the small subgroup of patients with astrovirus infection severe enough to require hospitalization (143,149,158). The role of astrovirus in persistent diarrhea remains to be established (172). Prolonged lactose intolerance and, less commonly, sensitivity to cow's milk have been a problem for some patients (38,91,136). Deaths associated with astrovirus infection are extremely rare but have been reported (115,160).

Volunteer Studies

Adult volunteer studies facilitated assessment of astrovirus pathogenicity. In one study, fecal extracts containing astrovirus from an infected child were administered to eight adult volunteers in the United Kingdom (89). It was established that these volunteers had little or no detectable astrovirus-specific antibody at entry. Only one of the volunteers developed overt illness, characterized by vomiting on days 3 and 4, constitutional symptoms on days 4 to 6, and copious, watery diarrhea on day 6 with shedding of large numbers of viral particles. Symptoms resolved rapidly, and the volunteer was completely well on day 7. One other volunteer had constitutional symptoms and mild abdominal discomfort but no vomiting or diarrhea. An inoculum was prepared from the fecal specimen of the overtly ill volunteer and administered to nine others, five of whom developed mild symptoms without vomiting or diarrhea. Of 17 volunteers in this two-phase study, viral shedding was observed in four and appeared to be proportional to the severity of the illness. Seroconversion was demonstrated in 13 of 16 by immunofluorescence testing, and in 10 of 16 by IEM.

Astrovirus serotype 5 from an outbreak of gastroenteritis at a nursing home in Marin County, California, was administered to 19 adult volunteers in the United States (121). Because none of the first 17 volunteers became ill after receiving a 1-mL inoculum, a 20-mL inoculum was given to the last two volunteers. One of the two developed nausea, vomiting, diarrhea, and malaise, beginning 5 days after infection, and symptoms lasted for 36 to 48 hours. Large numbers of 27-nm virus particles were detected in the feces of this individual by IEM. This volunteer developed an antibody response to not only the Marin County virus, but to all of the five available reference strains of astrovirus (serotypes 1 to 5). A total of nine volunteers developed a serologic response, as documented by radioimmunoassay and/or biotin–avidin immunoassay. In general, those who had negligible preinfection antibody were more likely to develop an immune response to the virus challenge. These studies indicate that astrovirus, unlike Norwalk virus, is of relatively low pathogenicity in adults.

Disease Spectrum in Animals

Astrovirus has been identified in association with diarrhea in several animal species. Direct correlation of astrovirus identified in stool to disease in animals was complicated by the fact that multiple viruses may be found concurrently in diarrheic feces. In most of the animals listed previously, astroviruses were separated from other viruses and shown to cause illness. However, attenuated illness was observed when astrovirus alone was administered to pigs (159) and cats (54), and bovine astrovirus (189,190 and 191) does not appear to cause illness in gnotobiotic calves inoculated with this virus alone, as noted before. On the other hand, coinfection of gnotobiotic calves with bovine astrovirus and either Breda virus or rotavirus resulted in severe diarrhea and more severe infection of dome epithelial cells overlying jejunal and ileal Peyer's patches (191).

Astrovirus infection caused a rapidly fatal hepatitis in ducklings (46). In 1983, an epidemic of hepatitis among ducks less than 6 weeks of age occurred at a commercial farm. Mortality rates of up to 50% were observed. Histologic examination of the liver revealed widespread hepatitis with hepatocellular necrosis and bile duct hyperplasia. Viral particles with characteristic astrovirus features were found by EM in the liver and feces of these animals. In addition, a reovirus-like agent was isolated in embryonating duck eggs and tissue cultures, but inoculation of ducklings with this agent did not result in disease. By contrast, the subcutaneous injection of a 20% (w/v) suspension of infected livers into twenty 2- to 3-day-old ducklings recapitulated the hepatitis with all of the histologic features of the original outbreak and death within 2 to 4 days in 25% of the ducklings. Serial passage of this agent resulted in mortality rates of 25% to 55%. This agent was shown to be serologically distinct from duck virus hepatitis type I virus. These results strongly suggest that the astrovirus-like agent was responsible for disease in ducklings. This conclusion could not be confirmed, however, because the astrovirus-like agent could not be propagated in tissue culture.

Intestinal and extraintestinal illness was noted in other avian species. Cloning and sequencing of avian nephritis virus (64) confirmed that it is an astrovirus that infects chickens and causes growth retardation and acute interstitial nephritis, as its name suggests. TAstV (see the previous section, Pathogenesis) was found in the intestines, thymus, and bursa of turkey poults with PEMS, a highly pathogenic multisystem disease (77,156).

Immunity

The determinants of immunity to astrovirus are not well understood. Symptomatic astrovirus infection is found in primarily two age groups: young children and elderly, institutionalized patients. This biphasic distribution of symptomatic infection suggests that antibody acquired early in life provides protection from illness through most of one's adult life and that immunity to astrovirus wanes late in life. Volunteer studies indicated that those with detectable serum antibody to astrovirus did not develop diarrhea after virus challenge (89). In other studies, a volunteer infected with the Marin County virus, a serotype 5 astrovirus, developed both IgM and IgG responses to serotype 1 astrovirus. The anamnestic response observed in this volunteer was postulated to be the result of earlier infection with astrovirus serotype 1 and antigenic relatedness between these two serotypes. Studies of a birth cohort in Egypt provide evidence for homotypic, but not heterotypic, immunity (135). Of note, subsequent astrovirus infections were observed to be milder.

The normal mucosal immune system plays an important role in protecting individuals from repeated infections with human astrovirus (127). T cells that recognize astrovirus antigens in a human leukocyte antigen (HLA)-restricted manner were found to reside in the intestinal lamina propria of adults. These human astrovirus–specific CD4+ T cells produced helper T-cell subtype 1 (T_H1)-type cytokines, interferon gamma and tumor necrosis factor, when activated.

Role in Immunocompromised Hosts

It has been suggested that astrovirus plays an important role in causing diarrhea in immunocompromised patients. Astrovirus was found in 13 of 109 (12%) fecal specimens from 65 human immunodeficiency virus (HIV)-infected individuals with diarrhea, whereas only 2 of 113 (2%) fecal specimens from 65 HIV-infected patients without diarrhea (controls) were astrovirus positive (51). In this study, seven different types of viruses were identified in 35% of the patients with diarrhea. In most cases, single agents were isolated, and detection of bacteria and parasites were less common. A study from Argentina failed to demonstrate an association between astrovirus and diarrhea in HIV-infected patients (43). One study from Venezuela found viruses in 10% of patients with acquired immunodeficiency syndrome, and in 14% of those with symptoms. However, no astrovirus, Norwalk virus, or rotavirus was detected (45). In contrast, another study from Venezuela found that infection with astrovirus and enterovirus occurs frequently in HIV-infected children, but in general, enteric viruses do not cause the type of diarrhea that often plagues these children (103).

A study to evaluate the role of enteric pathogens in the diarrhea that develops among bone marrow transplant recipients found an enteric pathogen in 13% of cases (31). Among these, astrovirus was most frequently implicated, accounting for 7 of 150 episodes (4.7%) of diarrhea. Diarrhea due to astrovirus infection developed between hospital days 21 and 65 and had a duration of 2 to 31 days. In a separate case (88), a child who had undergone bone marrow transplantation for combined immunodeficiency developed chronic astrovirus diarrhea with viral shedding, which persisted until his death. No antibodies to astrovirus were detected in the patient's serum. These findings were confirmed by a larger investigation of an outbreak caused by human astrovirus serotype 1 in a pediatric bone marrow transplantation unit in the United Kingdom (34). When EM, EIA, and RT-PCR were compared, RT-PCR was found capable of detecting astrovirus shedding earlier in the course of infection and for a prolonged period of time after EM and EIA studies no longer detected virus. In a region of the capsid sequence that is markedly variable among serotypes, the outbreak virus shared 92.5% homology with a serotype 1 strain that was isolated in the United Kingdom 9 years prior (174). Persistent diarrhea with shedding of rotavirus for more than 450 days and concurrent infection with astrovirus for at least 225 days also has been reported in a child with congenital T-cell immunodeficiency (188).

The important role of CD4+ T cells is further suggested by case reports in which patients developed prolonged diarrhea caused by astrovirus after treatment with fludarabine, which is known to deplete CD4+ T cells (18). In one case (30), a middle-aged patient with chronic lymphocytic leukemia developed protracted diarrhea after the fifth course of chemotherapy with fludarabine monophosphate. On hospital admission, after 10 days of profuse diarrhea (20 stools per

day), the patient's CD4+ lymphocytes were noted to be disproportionately depressed (0.28×10^9/L) compared to the neutrophil count (1.48×10^9/L). The patient's diarrhea resolved after 6 days of supportive care in the hospital. Four weeks later, astrovirus antigen was detected in stool by EIA, but viral shedding was not detected by EM, and the patient was without symptoms. In another report (20), an elderly patient with Waldenstrom's macroglobulinemia and chronic peptic ulcer disease, intermittently treated with acid-suppressing medications, developed watery diarrhea containing large numbers of astrovirus particles, nausea, and vomiting 3 weeks after the third course of fludarabine. This patient's condition worsened while on symptomatic treatment for 3 weeks. The patient then received high-dose intravenous immunoglobulin (0.4 g/kg) for 4 days, with resolution of the diarrhea within 24 hours. Astrovirus remained detectable by EM in the stool 2 weeks (but not 4 weeks) later. The integrity of other T cells may also be important in modulating infection. Cubitt et al. (34) observed that production of CD16+ and CD8+ T cells in a patient, who shed virus for a long period of time, was correlated with decreased levels of viral shedding (detectable only by RT-PCR).

The role of immunoglobulin in treating immunocompromised patients requires further study. Timing, route of administration, patient age, and other factors may be important variables. Limited numbers of cases receiving this treatment have been reported and outcomes have varied. The elderly patient with Waldenstrom's macroglobulinemia (described previously) had a rapid and dramatic clinical response after receiving intravenous immunoglobulin (20). Similarly, a young adult bone marrow transplant recipient, who developed astrovirus diarrhea 7 days after transplantation, was successfully treated with intravenous and oral gammaglobulin (194). By contrast, oral administration of high-titer hyperimmune gammaglobulin to pediatric bone marrow transplant recipients with diarrhea caused by astrovirus serotype 1 resulted in the shedding of large viral aggregates in the stool, but it did not prevent viral replication (34).

DIAGNOSIS

Electron Microscopy

Viruses traditionally were detected in fecal specimens by direct EM with negative staining. In contrast to patients with Norwalk and Norwalk-like virus infections, those with diarrhea caused by astrovirus frequently shed large numbers of viral particles (£1×10^{10}/mL, or 10^8 viable particles/mL) (88). In those patients who may be excreting fewer viral particles or in situations where immune response to astrovirus necessitates measurement, IEM techniques are useful (19,75,78). The sensitivity of EM has been estimated to be 10^6 to 10^7 virus particles per gram of stool.

In spite of the characteristic surface features described for astroviruses, accurate interpretation of electron micrographs may require an experienced microscopist (108). Generally, only 10% of the particles in a given specimen will display the distinctive astrovirus surface star (106). Oliver and Phillips conducted a retrospective analysis of fecal viruses that were originally identified as small, round, featureless viruses by EM alone (141). Of note, they found that many of these viruses, particularly astroviruses, had been misclassified. When complementary immunologic techniques were applied to careful reexamination of the specimens by direct EM, 14 of the 53 samples (26%) first classified as small, round, featureless viruses were shown to be astrovirus. Two caliciviruses and a Norwalk-like virus were also found to be misclassified. Another example of how difficult it can be to classify these viruses solely based on morphology by EM is the case of the Marin County virus, isolated in 1978 from an outbreak of gastroenteritis in a California convalescent home. The initial impression was that this was a Norwalk-like virus (142). Subsequent analyses, however, proved that the Marin County virus was an astrovirus serotype 5 (56,57).

Enzyme Immunoassay

Herrmann et al. (58,59) developed an EIA that relies on a group-reactive monoclonal antibody (8E7) to capture viral antigen and polyclonal antiserum as the detector antibody. A modification of this EIA uses a biotinylated detector antibody (126). Both EIAs are of comparable sensitivity (91%) and specificity (98%) to IEM. EIAs have been useful for rapid detection of astrovirus antigen in studies where a large number of samples must be assayed (32,60,98,126,158). EIA- and RT-PCR-based methods to type human astroviruses found in clinical samples have been described recently (138; see preceding). An astrovirus antigen EIA that uses a group-reactive monoclonal antibody [8G4 (158)] for both capture and detection (biotinylated) has also been developed. The sensitivity of EIA is estimated to be 10^5 to 10^6 viral particles per gram of stool (59,126).

Although not currently available in the United States, a commercial EIA based on group-reactive monoclonal antibody 8E7 (59) is available elsewhere for the detection of astrovirus antigen in clinical specimens (IDEIA Astrovirus EIA, DAKO Diagnostics, U.K.). In one survey from Australia, this qualitative assay was found to have a sensitivity of 100% and a specificity of 98.6% (116).

Reverse Transcriptase Polymerase Chain Reaction

As more information regarding the sequence of human astrovirus genomes became available, detection techniques that employ molecular probes and RT-PCR were developed (69,126,181,183). The sensitivity of RNA probes is similar to EIA (126,181). By contrast, the sensitivity of RT-PCR is estimated to be 10 to 100 particles per gram of stool (153). Investigation of an outbreak of astrovirus gastroenteritis at a day care center (124) found that the prevalence of astroviruses, determined by EIA, varied from 50% in affected rooms to 20% in rooms with older children. Analysis by RT-PCR indicated that nearly all children were infected and viral shedding was detectable for weeks despite resolution of symptoms. In addition, RT-PCR detected astrovirus in 32% of the samples tested, compared to EIA which had a detection rate of 10%.

At present, the complete sequence of five strains of astrovirus (two serotype 1, one serotype 2, one serotype 3, and one serotype 8) are available (see previous section, Molecular Biology). It was reported that oligonucleotide primers selected from sequences at the 3' end of the genome can successfully amplify astrovirus-specific products from all five reference serotypes (131). Primers derived from the highly conserved RNA-dependent RNA polymerase motif may also be suitable

candidates for amplification of different human astrovirus serotypes by RT-PCR (99). RT-PCR also was used to confirm astrovirus-positive samples detected by EIA (31,51,109,125). Good correlation was observed between these two detection assays (44).

Regions that encode immunoreactive viral epitopes may play an important role in the development of recombinant antigens and more specifically targeted antibodies that can be produced on a large scale in the future. Although tests that detect astrovirus antibody are useful for epidemiologic investigations (80,83,121,122,134), tests that rapidly and accurately identify astrovirus antigen are likely to be preferable in the clinical setting.

TREATMENT

Gastroenteritis caused by astrovirus is generally a mild, self-limiting illness that may disrupt an individual's activities for a few days but does not require specific therapy. In the young child or rare adult patient who becomes dehydrated, oral or intravenous fluid resuscitation may be necessary. Complications such as dehydration may develop in patients with underlying gastrointestinal disease, poor nutritional status, severe mixed infection, immunodeficiency, or protracted illness (32,88). Intravenous immunoglobulin may be a useful adjunct in severely immunodeficient patients who fail to respond to conservative measures (20,194), but larger studies are needed to determine efficacy and establish indications.

EPIDEMIOLOGY

Astrovirus infections have been found worldwide, primarily, but not exclusively, in young children with diarrhea (4,8,9,12,31,32,34,35,38,39,44,48,50,51,60,78,81,91,97,98,100,105,111,117,120,125,123,126,130,135,136,141,142,145,146,160,163,164,172,192). Sporadic outbreaks of gastroenteritis caused by astrovirus have been reported among elderly patients (48,100,115,142) and military recruits (14). Several studies associate astroviruses with diarrhea in immunocompromised adults (20,30,31,51). Large, food-borne outbreaks, affecting thousands of individuals in Japan, have occurred among otherwise normal school-age children and adults as well (140,173).

Most astrovirus infections are detected in the winter months in temperate regions and in the rainy season in more tropical climates, a pattern that resembles rotavirus infections (32,88,92). As noted, infection is transmitted by the fecal–oral route. Both community-acquired (60,81,107,117,136,158,168) and nosocomial (4,8,17,38,39,81,91,105,120,136,151,158,172) infections have been described.

Role in Disease

It is difficult to ascertain the true incidence of astrovirus gastroenteritis and its role in disease. Gastroenteritis caused by astrovirus is usually a mild, self-limiting illness that does not require hospitalization, extensive outpatient evaluation, or clinic visits. The virus may be difficult to detect by direct EM, as only 10% of the particles display the distinctive surface star, and this may lead to misclassification of astroviruses that are present in fecal samples. Even in humans, astrovirus may be found together with other potential gastroenteritis agents in diarrheic feces (136). Determining which of these viruses is the etiologic agent is a clinical challenge. Detection methods such as those described previously helped to define the epidemiology of human astroviruses, but the true disease burden caused by these viruses is not known (44). Prevalence of astrovirus among children with sporadic diarrhea was found by EIA to range between 2.5% and 10% (22,32,44,52,60,81,98,164,173).

Herrmann et al. (58,59) developed an EIA that is capable of detecting human astrovirus serotypes 1 to 8 in fecal specimens. They used this EIA in two separate studies (32,60) to address the medical importance of astroviruses. Controlled studies done in Thailand (60) examined the incidence of astrovirus gastroenteritis in pediatric outpatients compared to illness due to rotavirus and enteric adenovirus. As expected, rotavirus was the most frequently detected viral pathogen, found in 19% of patients with gastroenteritis. An unexpected result was that astrovirus was second to rotavirus, accounting for diarrhea in 8.6% of patients, and enteric adenovirus was a distant third, found in only 2.6% of the patients with diarrhea. A similar study was conducted on rural Guatemalan children (32). In this population, astrovirus was found in 7.3% of those with diarrhea and was again second to rotavirus. These findings were unexpected because hospital-based EM studies indicated that enteric adenovirus is the second most common cause of viral diarrhea in young children, and astrovirus incidence rates did not exceed 4% (72).

In Australia, astrovirus was found to be the second most common cause of gastroenteritis in children, after rotavirus (143). Infection with serotype 1 predominated, particularly in the winter months when gastroenteritis is common (40,116,143). A biennial winter peak of astrovirus infection among hospitalized children was observed (133). Serotype 4 strains were associated with infection in summer and autumn months (116). Among hospitalized patients, the incidence of astrovirus infection was 4.2%, far lower than rotavirus (63.2%), equivalent to all bacterial infections, and slightly higher than adenovirus (3.7%), suggesting that severe infection with astrovirus is seen in a subgroup of infected patients.

In a population of Mayan infants, astrovirus was the most common enteric pathogen identified in stool samples collected during a prospective study of oral poliovirus immunogenicity (109). The prevalence of astrovirus was compared with that of rotavirus, adenovirus 40/41, *Salmonella*, *Shigella*, and *Campylobacter* species. In 61% of the infants, astrovirus was detected by EIA from at least one stool sample over the 18-week period each infant was followed. Twenty-six percent of 305 diarrheal episodes reported at study visits were associated with astrovirus. The EIA results were confirmed by RT-PCR in a subset of EIA-positive and -negative samples. The reason for the high prevalence of astrovirus infection and the low rate of rotavirus infection (4% detected by a commercial EIA) in this semiclosed community is not known, but it may be related to temperature, rainfall, humidity, or other environmental factors.

In the group child care setting, astroviruses were shown to be an important cause of outbreaks of diarrhea. Children up to 36 months old attending child care centers were at greatest risk of developing diarrhea (98,125). An investigation of eight outbreaks of astrovirus diarrhea (five human astrovirus serotype 1 and three serotype 2) in six child care centers showed

that 20% of the children with diarrhea shed astrovirus (123). Although each outbreak was associated with a single astrovirus serotype, two distinct serotypes were identified in sequential outbreaks during one winter diarrhea season.

Antibody Acquisition and Prevalence

Human infection is common, as evidenced by the presence of antibody to human astrovirus in gammaglobulin pools from the United States (92), Japan (78), and other areas of the world (44,186). In U.S. gammaglobulin pools, antibodies to all five of the originally identified serotypes of astrovirus are detectable (92). It is not known at this time whether antibodies to the more recently described serotypes 6, 7, and 8 (16,96) are present in these preparations. Using an EIA based on recombinant Baculovirus-expressed capsid protein (83), Cubitt et al. (34) determined the IgG titer to astrovirus serotype 1 to be 1:128,000 in a gammaglobulin preparation given to a patient.

Antibodies to astrovirus tend to be acquired in early childhood. A survey of 87 children less than 10 years old in the Oxford region of the United Kingdom revealed that antibody prevalence rises rapidly from 7% in 6- to 12-month-old infants, to 70% by school age (86). Astrovirus antibodies could be detected in 75% of the 10-year-old children studied. As a point of reference, young adults (nursing students) were also surveyed, and 77% were found to have such antibodies.

One study of seroprevalence among children treated at a hospital in the United Kingdom found rates of 86% for serotype 1, 1% for serotype 2, 8% for serotype 3, and 6% for serotype 4 (137). In an age-stratified sample from Utrecht Province in The Netherlands, seroprevalence of neutralizing antibodies was found to be 91% for serotype 1, 31% for serotype 2, 69% for serotype 3, 56% for serotype 4, 36% for serotype 5, 16% for serotype 6, and 10% for serotype 7 (80). Of those that were seropositive for types 1 to 4, serotype 5 antibody acquisition was observed to be more gradual and suggested a different epidemiologic niche for astrovirus serotype 5. A separate study examined the prevalence of antibodies to astrovirus types 1 and 3 in children and adolescents in the United States (122). Peak prevalence of antibody to human astrovirus was found in children 6 to 9 years old: 94% for serotype 1 and 42% for serotype 3.

PREVENTION AND CONTROL

Interruption of transmission is the key factor in preventing astrovirus infection. This is especially important in hospitals and other institutions, day care centers, and families, where person-to-person transmission is likely to occur. Universal hygienic procedures must be enforced in these settings. Astrovirus is quite resistant to alcohol disinfection (90). Methanol has been shown to be more effective in reducing astrovirus infectivity than equivalent concentrations of isopropanol or ethanol. Whereas 70% methanol was found to decrease astrovirus infectivity by 3 $\log_{10}$, 90% methanol limited viral titers to less than 10 infective units per milliliter.

Astrovirus survival in dechlorinated drinking water was comparable to rotavirus serotype 3 and enteric adenovirus 40/41, both of which can be transmitted through the waterborne route (2). Astrovirus and adenovirus were more resistant than poliovirus to inactivation by the addition of free chlorine to the water, but less resistant than human rotavirus or hepatitis A virus (1).

Those in the business of handling or preparing food should be mindful that shedding of astrovirus in the feces may continue for several days after diarrhea resolves. In immunocompromised individuals, viral shedding may last for weeks after resolution of symptoms (20,30). In addition, foods such as shellfish that have been implicated in outbreaks of astrovirus gastroenteritis should be carefully selected and prepared.

Potential for Vaccines

A better understanding of the fundamentals of immunity to astrovirus and estimates of the disease burden caused by astrovirus are required before vaccine development can be initiated. Evidence that astrovirus is a medically important pathogen (32,60), coupled with the fact that astroviruses primarily cause infection in the same age group as rotaviruses, makes it tempting to consider designing a vaccine that will protect young children against both viruses. Because astroviral diarrhea develops largely in infants less than a year old (with an age-specific incidence of 0.38 for infants less than 6 months old and 0.40 for infants 6 to 11 months old), a basic requirement of any vaccine candidate would be the ability to induce immunity in very young children (135).

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CHAPTER 29 *Togaviridae:* The Viruses and Their Replication

Sondra Schlesinger and Milton J. Schlesinger

TOGAVIRUSES

The *Togaviridae* were among the first well-characterized viruses containing a lipid envelope. Initially this family included a wide spectrum of viruses associated with different diseases (166). The relatively early recognition that they had a “coat” led to their being classified as Togaviruses (from the Latin *toga,* a Roman mantle or cloak). Many of the original members are now in different families, and the *Togaviridae* consists of only two genera: alphaviruses and rubivirus. Both are small, lipid-enveloped, spherical particles with a single-stranded positive RNA genome ranging in size from 9.7 for rubivirus to 11.8 kilobases for alphaviruses. Members of the alphavirus genus are grouped on the basis of genome homologies. In amino acid sequence, they share a minimum identity of about 60% in the nonstructural proteins and 40% in the structural proteins. From recent taxonomic data, the alphaviruses consist of 22 separate species, based on a combination of antigenic relationships and on divergence in genome sequence of greater than 23% and amino acid sequence of greater than 10% in the E1 gene (240). A phylogenetic relationship has been estimated based on the nsP1, nsp4, and E1 genes (240), and seven antigenic complexes can be grouped by serologic cross-reactivity (240). This number may increase, as salmon pancreas disease virus also appears to be a member of this genus (252).

The type-specific member of the alphaviruses is Sindbis virus, whose structure and replication have been studied in great detail. In addition, both Sindbis and Semliki Forest viruses have provided valuable models for examining the synthesis, posttranslational modifications, and localization of membrane glycoproteins. Most of the information presented in this chapter was obtained by studies with these two viruses, although other alphaviruses are beginning to contribute to our knowledge of the replication strategy and diversity of this genus.

Rubella virus, the sole member thus far of the rubivirus genus, is well known for its ability to cause disease in humans. Alphaviruses and rubella virus share many features, which originally suggested that they evolved from a common ancestor. A more detailed scrutiny of homologies, however, makes it difficult to propose a straightforward evolutionary relatedness.

Virion Structure

The dominant feature of alphavirus structure is a T=4 icosahedral nucleocapsid surrounded by a lipid envelope embedded with glycoprotein components also arranged in a T=4 icosahedral lattice. A clear picture of the organization of protein and lipid in the virion emerged from low-angle x-ray scattering (74), from negatively stained electron micrographs, and most recently from images obtained by cryoelectron microscopy (see later). Virions are about 70 nm in diameter, and the core or nucleocapsid is 40 nm in diameter. The molecular weight of the virion is about 52×10^6, with a density of 1.22 g/cc. The RNA genome is encapsidated in an icosahedral protein shell composed of a single species of protein (the capsid protein of 30–33 kd) and enveloped by a lipid bilayer derived from the host-cell plasma membrane. Lipid accounts for 30% of the dry weight of virions and is enriched in cholesterol and sphingolipid. Image reconstruction from cryoelectron micrographs has led to low-resolution structures for Semliki Forest, Sindbis, and Ross River viruses (25,45,58,157,158,213). These images (Fig. 1) reveal an arrangement of glycoprotein spikes distributed as 80 trimers, which project about 100 Å from the outer surface and are located at the local and strict three-fold axis of the icosahedral lattice. Each trimer contains three heterodimers consisting of the two viral glycoproteins E1 and E2. The heterodimers appear intertwined but flare out in a triangular, flower-like shape to form distinct bilobed petals. The most distal portion of the lobes contains domains of the E2 glycoprotein, which have epitopes recognized by monoclonal antibodies, and images with Fab fragments attached to either Sindbis or Ross River virus E2 have been obtained from cryoelectron micrographs (213). Each spike has a hollow cavity in its center, and the glycoproteins splay out to form a protein shell or skirt covering most of the outer surface of the membrane bilayer. Small holes in this shell appear at the two-fold and five-fold axis and at the base of each spike. Each spike is anchored in the membrane by three pairs of transmembrane domains, and each third of a trimeric spike interacts at the inner leaflet of the membrane with a different hexamer or pentamer of the nucleocapsid. Protein mass that is found immediately underlying the membrane is believed to represent the 33-amino-acid cytoplasmic domain of the E2 glycoprotein, which forms a binding site for nucleocapsids during the assembly process. A cryoelectron micrograph image of a mutant of Sindbis virus defective in cleavage of the precursor (PE2) to E2 and a small peptide, E3, revealed that the amino-terminus of PE2 is positioned as a bulge in the heterodimer localized midway between the center of the trimer and the tip of the spike (158). Analysis of a similar type of mutant of

Semliki Forest virus indicated that the PE2 form of the trimer was a more open arrangement of spikes when compared with the wild-type virus, and the E3, which is retained in the virion, is positioned at the distal portion of the mature spike (45).

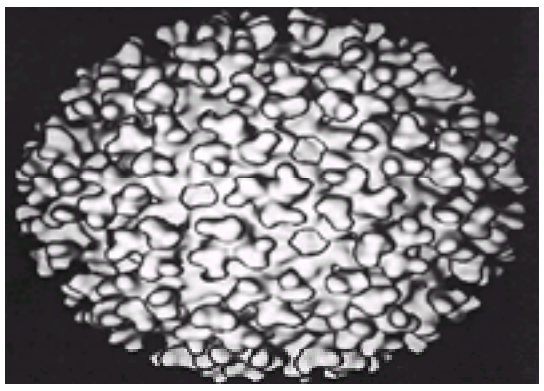

FIG. 1. Image reconstruction from cryoelectron micrographs of Ross River virus. The view is down the three-fold axis and shows the outer surface composed of flower-like projections of the E1/E2 heterodimers arranged as trimers and the interconnective portions that form a shell of protein. Very little of the lipid envelope is visible through three small openings of this shell. The resolution is about 20 Å. Courtesy of R.J. Kuhn, Purdue University, West Lafayette, IN.

The alphavirus nucleocapsid consists of 240 copies of the capsid protein (25,26,58,157,159). The Sindbis virus capsid protein was crystallized, and the atomic structure of the C-terminal half determined by x-ray diffraction to a resolution of 3 Å (Fig. 2) (26,224). Amino acids from positions 114 of the capsid sequence to the carboxy terminus at amino acid 264 were traced. The monomer fold was like mammalian serine proteases of the chymotrypsin family and closely related to the bacterial serine proteinases such as a-lytic proteinase, a result consistent with previous observations that alphavirus capsid proteins are autoproteases (5,68,132). The chain is folded into two Greek key b-barrel domains with the C-terminal tryptophan located between the domains and occupying the active proteinase site. The structure of the latter reveals the catalytic triad of serine, histidine, and aspartate (conserved among the serine proteinases) surrounding a hydrophobic pocket occupied by the tryptophan, which is the substrate for cleavage of the capsid from the nascent polypeptide (26,224). After cleavage, further catalytic activity is prevented by the tryptophan in the substrate-binding pocket. A structure of the Semliki Forest virus capsid protein, purified from virions, has been determined from crystals that diffracted to a resolution of 3 Å (28). The carboxyl-terminal 149 residues are folded like those of the Sindbis virus capsid in a chymotrypsin-like serine proteinase; however, a hydrophobic surface pocket that is occupied by amino acids from a neighboring molecule in the Sindbis virus capsid structure and may function as a binding site during assembly is empty. Several alphavirus capsid proteins have been produced as recombinant proteins in *Escherichia coli,* and the structure of the Sindbis virus capsid protein has been extended to residue 106, based on crystals that diffracted to a resolution of 2 Å (99).

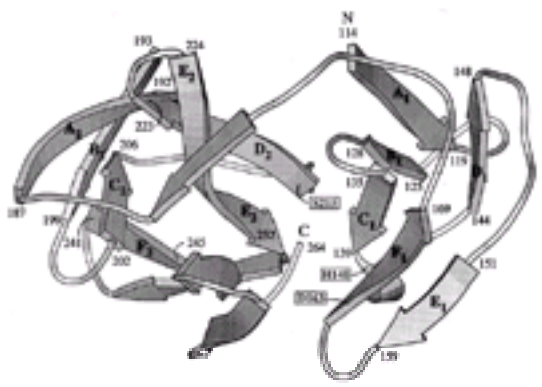

FIG. 2. Schematic drawing of the polypeptide chain of Sindbis virus capsid protein with secondary structure nomenclature based on x-ray diffraction analysis. The structure has since been extended toward the amino terminus to amino acid 106 (27). The catalytic triad of the active site serine-proteinase are in boxes. This picture was taken from Choi et al. (26), with permission, and was provided by M.G. Rossmann, Purdue University, West Lafayette, IN.

Genome Structure and Organization

The overall organization of the genomes of alphaviruses and rubella virus is shown in Fig. 3, where similarities and distinctions between them are noted. The genomes are arranged in two modules: the 5' two thirds codes for the nonstructural proteins (nsPs) required for transcription and replication of the RNA, and the 3' one third codes for the structural proteins. The 5' terminus is capped with a 7-methylguanosine, and the 3' terminus is polyadenylated. During replication, a discrete subgenomic mRNA species, identical in sequence to the 3' terminal one third of the genomic RNA, is synthesized (see also Fig. 4). This RNA also is capped and polyadenylated and serves as the mRNA for the synthesis of the viral structural proteins [reviewed in more detail in (219)]. An additional virus-specific RNA, detected in Sindbis virus–infected cells, contains the 5' two thirds of the genome; the 3' end maps to four nucleotides upstream of the start of the subgenomic RNA sequence. Its role in replication is not known; it is a minor species and may be the single-stranded version of one of the double-stranded replicative forms first identified almost 30 years ago in Sindbis virus–infected cells

after treatment of the RNA with RNase (257).

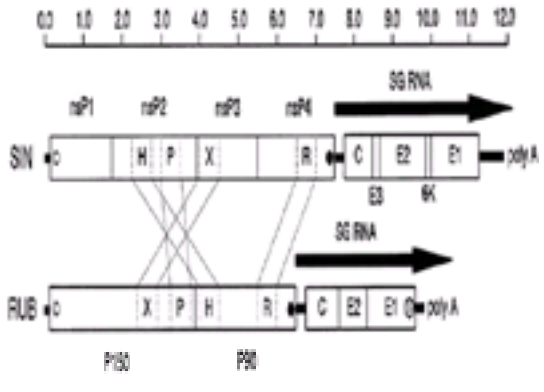

FIG. 3. Diagrams of the genomes of the alphavirus, Sindbis virus, and the rubivirus, rubella virus. The 5' two thirds of these genomes codes for the nonstructural proteins (nsPs), and the 3' one third codes for the structural proteins, translated from subgenomic (SG) RNAs. Untranslated regions are designated by *black lines*, and open reading frames, by *open boxes*. Designations are for individual proteins, which are processed from precursors. The scale at the top of the figure is in kilobases. The *open circles* near the 5' termini and the *closed circles* at the start of the subgenomic RNAs indicate conserved sequences in the alphavirus and rubella virus genomes. The location of the amino acid motifs for helicases (*H*), replicases (*R*) and cysteine proteases (*P*) are indicated. See text for more details. *X*, A small region of homology between the deduced amino acid sequences in the Sindbis virus nsP3 and the nsP of rubella virus. This figure was adapted from ref. 35 and 49, with permission, and was provided by T. Frey, Georgia State University, Atlanta, GA.

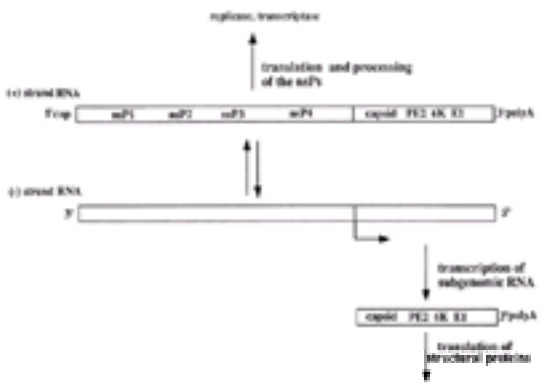

FIG. 4. Replication and transcription of alphavirus RNA. The first two thirds of the (+) strand genomic RNA serves as the mRNA for translation of the nsPs, which are eventually processed into four proteins (see text and Fig. 5). The (–) strand complementary strand is the template for the synthesis of both new genomic RNAs and the subgenomic 26S RNA, which codes for the structural proteins. The 5' terminus of the (–) strand has a polyU stretch that is assumed to be the template for the polyA tract at the 3' terminus of the genomic and subgenomic RNAs.

Sequence comparisons of different alphaviruses identified four regions of the genome that are highly conserved (149,150 and 151). These include (a) the 19 nucleotides at the 3' terminus, (b) 21 nucleotides that span the junction between the nonstructural and structural genes and include the start of the subgenomic RNA (the junction region), (c) 51 nucleotides near the 5' terminus, and (d) the 5' terminus, although this region appears to be conserved more in potential secondary structure than in sequence. The latter three regions also are conserved in rubella virus (35), and all four regions appear to be involved in the regulation of viral RNA synthesis. Sequence analyses also revealed similarities in the genome organization and in the sequences of the nonstructural proteins of alphaviruses and a number of plant viruses, some of which are quite distinct from alphaviruses in their overall structure [reviewed in (219)]. This suggested an evolutionary relatedness between the plant and alphaviruses and led to the definition of a Sindbis-like superfamily. It was this type of comparison that suggested that rubiviruses may be more distantly related to alphaviruses than are some of the plant viruses (35,49).

ALPHAVIRUSES

Attachment, Entry, and Uncoating

Alphaviruses have a wide host range and replicate in a variety of different species, as well as in many different cell types. Because attachment of a virion to a cell surface is one of the steps that can define host range, it seemed that these viruses must use a variety of different molecules for attachment or else use a ubiquitous surface molecule as a receptor. The high-affinity 67-kd laminin receptor is a receptor for Sindbis virus in baby hamster kidney (BHK) and several other mammalian cells (236) but not in chicken embryo fibroblasts (CEF), in which a 63-kd protein acts as the receptor (237). Mouse neuronal cells have other receptors: a 74-kd protein was detected on neuroblastoma cells that are loosely adherent, and a 110-kd protein was identified on firmly adherent cells (227). Recent studies have shown that heparan sulfate is an effective cell-surface ligand for binding of Sindbis virus to mammalian cells (19,90). A Chinese hamster ovary cell line selected for resistance to Sindbis virus infection is deficient in expression of sulfated glycosaminoglycans, heparan sulfate, and chondroitin sulfate (83). A strain of Sindbis virus composed of a consensus sequence derived from natural isolates does not bind to heparan sulfate; variants that do bind are rapidly selected by growth on cultured BHK cells (90). Thus heparan sulfate–like molecules may not function as natural receptors for Sindbis viruses, and

high-affinity binding of laboratory strains to this ligand appears to be a consequence of cultured-cell adaptation. Non–heparan-binding strains of Sindbis virus are more virulent in animals (90).

The viral glycoprotein E2 is the protein that interacts with these receptors. Antibodies directed primarily against the E2 protein neutralize viral infectivity, and amino acid changes in the E2 protein can affect both the efficiency of binding of the virus to neural cells and virulence in mice (81,105,199,226). Convincing evidence for the importance of E2 in binding virus to the cell surface came from experiments in which Sindbis virus mutants unable to bind to CEF were altered in the E2 protein (39).

Attachment of Sindbis virus to cells leads to the exposure on both glycoproteins of new transitional epitopes, which are indicative of conformational changes (46,135). These epitopes were detected also when purified virus was treated with heat, reducing agents, or low pH (134). Some of these changes may involve reduction of critical disulfide bonds in the glycoproteins, which may disrupt protein–protein associations in the envelope and be important for disassembly of the particle (1,7,8). Disulfide bond exchange had been proposed as a mechanism for allowing entry of virus at neutral pH at the cell surface, but further studies with both Semliki Forest and Sindbis virus failed to show significant inhibition of virus infection in the presence of thiol-blocking reagents (65).

In most cells, alphaviruses gain entry to the cell via the endocytic pathway, which normally functions for uptake of receptor–ligand complexes (33,128). Bound virus accumulates in coated pits, which are endocytosed to form coated vesicles. These vesicles are subsequently uncoated to form acidified endosomes, providing conditions that trigger fusion of the virus membrane with the vesicle membrane. The mechanism of receptor-mediated endocytosis for alphavirus penetration into cultured cells is supported by many studies, which include (a) the inability of bound Sindbis virus to effect antibody-mediated, complement-dependent cell lysis, indicating a rapid loss of virus glycoprotein from the cell surface (44); (b) the ability of lysosomotropic amines, which raise the pH of endocytic vesicles, to inhibit initiation of virus replication, demonstrating that acidified vesicles are required for early virus-replication events; (c) a requirement for a low pH (5–6) for fusogenic activity by Sindbis and Semliki Forest virus glycoproteins (230,254,255 and 256); (d) a block in fusion by concanamycin, a selective inhibitor of vacuolar proton–adenosine triphosphatase (ATPase) (80); and (e) the detection of virus in coated vesicles immediately after uptake from the cell surface, as revealed by electron micrographs of virus entry (256). Alternative pathways not involving acidified endosomes may exist, as a Chinese hamster ovary cell mutant defective in the endosome acidification was able to replicate Sindbis virus (41).

Studies with Semliki Forest and Sindbis viruses have demonstrated that the low-pH environment of the endosome leads to a dissociation of the E1/E2 heterodimer and a concomitant trimerization of the E1 subunits, which acquire new epitopes and become trypsin resistant (212,233,234). These alterations suggest that the E1 trimer is the fusion-active form of the protein, with a structure possibly analogous to the low pH–induced structure of the influenza virus HA2 protein (22,209). A highly conserved, hydrophobic sequence in the Semliki Forest virus E1 protein about 80 amino acids from the amino terminus is postulated to function as a fusion peptide, and mutations in this sequence shift the pH threshold for fusion to lower pH values (87), or block E1 homotrimer formation (89). Mutations in PE2 that prevent its cleavage to E2 also can affect fusion by controlling the formation and dissociation of the E1/E2 dimer (65,87).

Fusion of Semliki Forest and Sindbis viruses also requires the presence of cholesterol and sphingolipid in the target membrane (121,144,163,212). Dependence of Semliki Forest and Sindbis viruses on membrane cholesterol could be relieved by mutations in the E1 glycoprotein (121,231). One of these mutants, noted as srf-3, in which Pro^{226} in E1 was changed to Ser, was less dependent on cholesterol for trimer formation of E1 but retained the requirements for low-pH activation and sphingolipid exhibited by the wild-type virus (88).

Fusion leads to release of the nucleocapsid into the cytoplasm and must be followed by an uncoating event to permit the RNA to become accessible to ribosomes for initiation of translation. Several reports, based on *in vivo* and *in vitro* experiments, suggested that the binding of the nucleocapsid to ribosomes triggers the uncoating process (205,229,245,247,249,250). The capsid protein itself appears to have a ribosome-binding site between amino acids 94 and 106 of the capsid sequence (251), which is in the same region of the protein that interacts with viral RNA during encapsidation (64,152).

Transcription, Translation, and Replication of the Genome

The genomic RNA serves as the messenger RNA for translation of the viral nonstructural proteins and as a template for the synthesis of the complementary minus strand. The minus strand, in turn, provides the template for the synthesis of both new genomic RNA and subgenomic RNA [Fig. 4; for a more detailed review, see (219)]. Translation of the virion RNA is initiated at a single AUG near the 5' terminus of the RNA and proceeds uninterrupted for two thirds of the mRNA until encountering three termination codons located just downstream of the start of the sequences corresponding to the subgenomic RNA. The polyprotein is cleaved to four distinct polypeptides designated nsP1, nsP2, nsP3, and nsP4, according to their order in the genome. The protease responsible for these cleavages is encoded in sequences at the C-terminal domain of nsP2 (34,73,216). The genomes of Sindbis virus and several other alphaviruses have an opal termination codon a few codons before the nsP4 gene, and nsP4 is produced by read-through of the opal codon followed by proteolytic cleavage (218). Mutagenesis of the opal codon to one that encodes an amino acid or to the other two translation-termination codons adversely affects viral replication at low multiplicities of infection (109). An opal codon at this position in the genome of alphaviruses is common but not universal, and no termination codon exists at this position in Semliki Forest or O'Nyong-nyong virus genomes (217).

A number of activities must be carried out by the nsPs in their role as replicative enzymes. The isolation, characterization, and mapping of temperature-sensitive (*ts*) mutants defective in viral RNA synthesis at the nonpermissive temperature provided initial clues to identify the functions of each of the polypeptides (17,18,69,71). The RNA^{-} *ts* mutants fell into four complementation groups, which have been correlated with specific nsPs. The individual functions were predicted by gene-sequence homologies with known proteins [reviewed in (219)]. These include domains that are conserved among the alphavirus-like superfamily in three of the four nsPs. The nsP1 protein contains a

methyltransferase domain; the amino terminal region of nsP2 contains nucleotide-triphosphate binding motifs homologous to those found in bacterial helicases (see Fig. 3); and nsP4 has a GDD motif present in a number of viral RNA polymerases.

The predictions based on sequence homologies are being confirmed by functional tests. nsP1 has both guanine-7-methyltransferase and guanyltransferase activities (4,136,193). Mutagenic studies have defined amino acids essential for these activities (3,235). nsP1 also has been implicated in the synthesis of minus strand RNA (71,238). The protein is palmitoylated, and mutagenesis of the conserved cysteine residues, Cys ^{418}, Cys^{419}, and Cys^{420}, to alanine prevented the addition of palmitate and decreased the ability of nsP1 to bind to membranes (93). This protein appears to be involved in the association of the alphavirus replicase to membranes (4). Expression of nsP1 in cultured cells using a vaccinia virus expression system induced filopodia and rearrangement of actin filaments (94). These effects, also observed in virus-infected cells, indicate that nsP1 can affect the cytoskeleton.

nsP2 is involved in the regulation of minus strand RNA synthesis (189) and in the initiation of subgenomic RNA synthesis (222). As mentioned earlier, it also functions in proteolytic processing of the nsPs (34,73,216). The Semliki Forest virus nsp2, expressed in and purified from *E. coli,* is an RNA helicase (66). About half of the Sindbis and Semliki Forest virus nsP2 is associated with the nuclear fraction in virus-infected BHK cells, although its function there is not known (12,160). A pentapeptide in the carboxy-terminal region of the Semliki Forest virus nsP2 is required for nuclear localization (177). A mutant of Semliki Forest virus, in which the Arg ^{649} of the nuclear localization signal (P ^{648}RRV) in nsP2 was converted to Asp, was distinguished from the parental virus by two criteria: inhibition of host DNA synthesis was less than that seen in cells infected with the parental virus, and even more striking, its pathogenicity in mice was greatly diminished (176). Studies with Sindbis virus and Sindbis virus replicons (self-replicating genomes that lack the structural protein genes) have shown that mutations in nsP2, and specifically in Pro ^{726}, can lead to a significant reduction in viral RNA synthesis and permit the establishment of persistent infections in cultured BHK cells (38,50).

nsP3 does not have sequence motifs found in other RNA virus genomes, and its function remains unknown. It is phosphorylated (108,161), and mutants in this gene define an additional complementation group (96). nsP4 has been identified as the viral polymerase. Mutations in this gene affect the synthesis of all viral RNAs, and *in vitro* studies of Sindbis virus–infected cells establish nsP4 as the viral polymerase (13,69,100,188). All of the sequenced alphaviruses have a tyrosine at the amino terminus of nsP4. Shirako and Strauss (204) made a series of mutations to alter this amino acid and found that viable viruses were obtained only when the N-terminus was an aromatic amino acid or histidine.

Replication and transcription of alphavirus RNAs occur on cellular membranes (12,57). Studies with Sindbis virus have defined the proteolytic processing scheme for the nsPs and have led to an elegant model for the regulation of viral RNA synthesis. Proteolytic processing has been analyzed by translation of mRNAs *in vitro* (32,73), by expression of nsP genes in a vaccinia virus transient expression system (101,102 and 103), and by expression in cultured cells of Sindbis virus genomic RNAs containing site-directed mutations in the nsP genes (202,203). Each of these has provided important information for the proposed scheme illustrated in Fig. 5. The Sindbis virus nsPs are initially translated as two polyproteins, P123 and P1234, depending on whether the in-frame opal termination codon is suppressed. Cleavage of the latter at the 3/4 site occurs rapidly to form the complex P123 and nsP4, which is postulated to initiate minus-strand synthesis. Evidence for this is based on the result that nsP4 plus a P123, which is cleavage defective, but not a cleavage-defective P1234, can function efficiently in the synthesis of minus-strand RNAs (101,102 and 103,203). In the vaccinia expression system that leads to high levels of the nsPs, both plus- and minus-strand RNAs were synthesized, although minus stands were synthesized preferentially. In addition, in the vaccinia virus system, functional replication complexes were not formed when the nsPs were expressed as individual polypeptides or as different combinations of individual nsPs (101,102). The most convincing argument for a role for P123 in the synthesis of minus strands is based on studies in which the Asn^{614} of nsP2 was changed to Asp (103,216). This mutation leads to more efficient processing of P123 *in vitro,* but RNAs containing this mutation do not give rise to viable Sindbis virus in transfection assays (216). With the vaccinia virus expression system, minus-strand RNA synthesis could not be detected when this mutation was present in P123, but it was restored when the mutation was inserted into a P123 protein that was also cleavage defective (103).

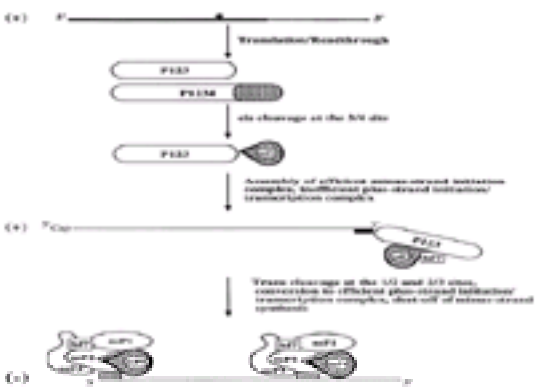

FIG. 5. A model for the temporal regulation of minus- and plus-strand RNA synthesis. The protein complex that initiates (–) strand RNA synthesis is composed of P123 and nsP4. This complex is also able to synthesize (+) strands, but inefficiently. Efficient synthesis of (+) strand genomic and subgenomic RNAs occurs after trans-cleavage at the 1/2 and 2/3 sites of the polyprotein; the latter cleavage results in the shut-off of (–) strand synthesis. Host factors (hf) are most likely involved in these reactions. From ref. 103, with permission, and provided by Drs. J. Lemm and C.M. Rice, Washington University, St. Louis, MO.

This model explains some aspects of the regulation of alphavirus RNA synthesis, in particular, the low level of minus strands synthesized by the replication complexes, the continued requirement for protein synthesis for production of minus

strands, and the cessation of minus-strand synthesis several hours after infection (190,191). *In vitro* translation studies show that processing of P123 occurs in trans and, in infected cells, the accumulation of P123 should accelerate its cleavage, leading to cessation of minus-strand synthesis. The partially and fully processed complexes carry out plus-strand RNA synthesis, which continues throughout the infection cycle. Not all of the earlier studies can be explained by this model, and there are mutations in both nsP4 and in nsP2 that permit continued synthesis of minus strands, even after cleavage of the proteins has occurred (188,189).

Attempts to study alphavirus RNA replication in cell-free extracts had been hindered by an inability to initiate viral RNA synthesis. Extracts prepared from alphavirus-infected cells would elongate endogenous RNA, but added templates were not functional (13). Based on the studies described earlier (and see Fig. 5), Lemm et al. (100) established an *in vitro* system that uses a Sindbis virus RNA template to initiate and elongate minus-strand RNAs. Extracts were prepared from cells infected with vaccinia virus recombinants: one expressing an nsP123 that had a defective protease so that the polyprotein would not be cleaved, and the other expressing nsP4. The addition of a plus-strand Sindbis virus RNA template led to the synthesis of the complementary minus stand. Furthermore, membrane fractions from extracts that expressed only the 123 polyprotein could synthesize genome-length minus-strand RNAs on addition of the appropriate template and solubilized nsP4, indicating that this system can be used to purify and characterize the nsP4 polymerase. In these studies, nsP4 by itself had no detectable polymerase activity. None of the extracts tested was able to synthesize plus strands.

The four conserved sequences, noted earlier, in the alphavirus genome were presumed to be replicase-binding sites and to function as promoter elements. Strauss et al. carried out extensive mutagenesis on three of these regions in the Sindbis virus genome: the 3' nontranslated region (91), the 51 nucleotides located between nucleotides 155 and 205 (143), and the 5' nontranslated 44 nucleotides (142). They show an important role for these sequences, as most of the mutations affect viral growth, frequently in a host cell–dependent manner, with strikingly different effects in chicken embryo fibroblasts and mosquito cells. These results support the contention that host proteins are involved in alphavirus RNA replication.

The fourth conserved region of the alphavirus genome surrounds the start of the subgenomic RNA. The minimal promoter region contains 19 nucleotides upstream and 5 nucleotides downstream of the start site for transcription of the subgenomic RNA, which coincides with the conserved region (107). Promoter activity is enhanced, however, when additional sequences in this region are included in the RNA (107,173). Additional studies of this promoter were carried out by the construction of Sindbis virus derivatives with two subgenomic RNA promoters: the wild-type promoter which was used for expression of the structural protein genes, and a second promoter, which was placed upstream of the chloramphenicol acetyltransferase gene and was subjected to modifications (173). Minimal promoters from other alphaviruses, but not from rubella virus, could be used by the Sindbis virus nonstructural proteins (77).

A role for host-cell proteins in alphavirus RNA synthesis was inferred from observations made in both vertebrate and invertebrate cells [reviewed in (219)]. Gel-retardation assays have been used to identify potential candidates (154,155) and led to the isolation from mosquito cells of a 50-kd protein that has a high affinity for the 3' terminus of the Sindbis virus minus-RNA strand and the identification of this protein as the mosquito homolog of the La autoantigen (156). The human La protein has been shown to interact with several other viral RNAs, suggesting that it may have a central role in viral RNA replication.

Expression of Virus Structural Protein Genes

Alphavirus structural proteins are translated from the subgenomic RNA early (2–3 hours after infection) in the replication cycle, probably as soon as the subgenomic mRNA is formed. Ribosomes initiate translation at a single site near the 5' end of this RNA and proceed uninterrupted for some 3,000 nucleotides to a termination site about 300 nucleotides from the 3' poly A terminus [reviewed in (219)]. The first sequences to be translated encode the viral capsid polypeptide, which folds to form a serine–protease catalytic site (see Fig. 2) that cleaves the capsid from the nascent polyprotein at a tryptophan–serine bond. Substitution of the C-terminal tryptophan by alanine, arginine, or phenylalanine showed that only tryptophan is an effective autoproteolytic substrate (210). Continued translation of the mRNA produces a signal sequence that facilitates translocation of the remaining polypeptide into the lumen of the endoplasmic reticulum (Fig. 6). As the protein emerges into this vesicle, it is modified by covalent attachment of oligosaccharides (201) and, later, by proteolytic cleavages carried out by host-cell signalase (110). The final products of translation are the capsid, two large (~45 kd) type I transmembrane glycoproteins, noted p62 or PE2 and E1, and a small (6 kd) membrane-embedded protein; the topology of these three membrane-bound proteins is shown in Fig. 7. Much of the data supporting this model derive from (a) assignments of domains with 22 hydrophobic amino acids in the sequences of the p62, 6K, and E1 as either signal- or stop-transfer sequences that span the membrane (see Fig. 6); (b) expression of cDNA constructs of Semliki Forest virus subgenomic RNA under the control of a heterologous promoter or expression of the Semliki Forest virus RNA genome, which show that the carboxy termini of either p62 or the 6-kd protein can function as a signal sequence for E1 (110,131); and (c) a change in the localization of the E2 carboxyl terminus to the inner leaflet of the bilayer during its transport to the cell surface (113). The p62, E1, and 6K proteins appear to move as a cohort through the secretory vesicles from their site of synthesis on the endoplasmic reticulum to their ultimate location at the plasma membrane of the cell (see Fig. 6). This process takes about 30 minutes at physiologic temperatures and leads to several posttranslational modifications. Among these are the trimming of and additions to the three oligosaccharides on Sindbis virus p62 and to the two on E1. Cellular enzymes localized to various compartments of the secretory system carry out these modifications in a manner resembling that found for cellular proteins destined for the plasma membrane or secretion to the extracellular fluid. Thus the precise composition of the oligosaccharides on the virus is dependent on the host cell in which the virus replicates (86).

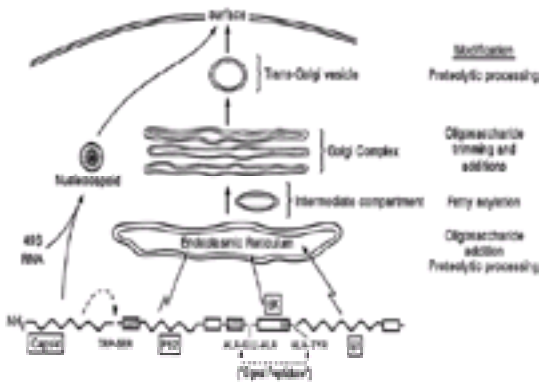

FIG. 6. Processing and modifications of the Sindbis virus structural proteins. Capsid is released co-translationally by autoproteolysis and assembles to encapsidate the 49S genomic RNA. The p62, 6K, and E1 proteins are modified during transport to the cell surface. Signal sequence, *shaded rectangle*; hydrophobic sequences, *open rectangle*.

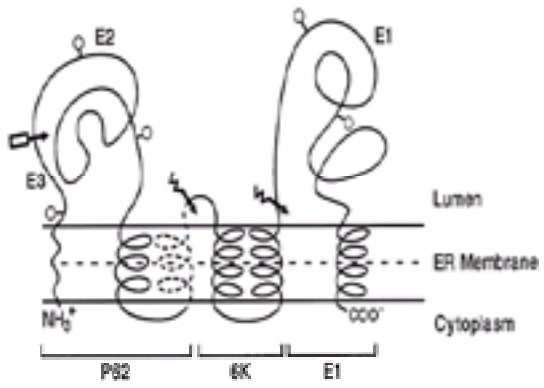

FIG. 7. Topology of the Sindbis virus p62 and E1 glycoproteins and the 6K protein in the endoplasmic reticulum membrane. The *dashed* part of p62 is removed from its transmembrane configuration and binds to the inner face of the lipid bilayer during transport to the cell surface. Host cell signalase activity, ; host cell furin-type protease, . Refer to text for detail.

A heterodimer of p62 and E1 forms very shortly after synthesis of the latter, and association is more effective when both proteins are expressed from the same genome instead of different RNAs (11). The folding of these proteins requires the formation of intrachain disulfide bonds and has been shown to involve a transient covalent interaction between the nascent and newly synthesized E1 and PE2 and the host-cell chaperones, protein disulfide isomerase and Erp57 (139). These disulfide-bonded intermediates could be detected by radioactive labeling, as the viral proteins are the only ones being synthesized in the infected cells. The folding pathway also includes noncovalent interactions with calnexin and calreticulin. In the absence of p62, E1 folds into disulfide-linked aggregates and fails to be exported from the endoplasmic reticulum (6,21), but p62 alone is capable of proper folding and transit to the plasma membrane. The newly synthesized E1 is not folded into its thermodynamically stable state but becomes trapped in a metastable conformation through the chaperone-like activity of p62 (6,36). The heterodimer complex trimerizes while still in the endoplasmic reticulum and is relatively stable to low-pH exposure. Continuous chaperoning by p62 and its proteolytically modified E2 maintains E1 in this metastable state in the virion spikes until low pH dissociates the dimers and leads to trimers of thermodynamically stable E1. The latter are distinguishable by new epitopes and rearrangement of disulfide bonds (140,197).

Palmitate acylation occurs at three sites on the Sindbis virus p62, four sites on the 6K protein, and one site on E1 during transport of the proteins after they have exited from the endoplasmic reticulum and before entrance into the Golgi complex [see Fig. 6 and (14,124,198)]. Cysteines oriented to the cytoplasmic face of the lipid bilayer are the sites of fatty acylation. The role of these fatty acids in glycoprotein function is unknown, but they probably affect interactions of the proteins with the lipid bilayer. Blocking their attachment by cerulenin (194) or by mutationally altering cysteine attachment sites (59,82) interferes with virus assembly and, in some cases, virus stability to heat and detergents (182).

A critical step in glycoprotein maturation is a proteolytic cleavage in p62 to form E3, which contains for Sindbis virus 64 amino acids of the amino terminus of p62, and the membrane-anchored E2. This cleavage occurs at a sequence enriched in arginines and lysines by a host cell furin-type enzyme localized to a trans-Golgi vesicle (138,186,239). Failure of the proteolytic cleavage does not interfere with assembly and budding of particles in vertebrate cells (167,181), but conversion of p62 to E2 is essential for particle formation in insect cells and in most cases is required for infectivity of the newly replicated particles in vertebrate cells (39,75,168,181,184). The failure of an alphavirus containing p62–E1 spikes to replicate in a cell may be due to the inability of the E1 to undergo the low pH–induced conformation required for its fusogenic activity (116,117). One variant of Sindbis virus, which has an uncleaved p62, contains an additional suppressor mutation in the E2 protein and is as infectious as the wild-type virus (76). A strain of Venezuelan equine encephalitis virus that was blocked in P62 cleavage gave rise to viable revertants containing suppressor mutations in either E2 or E1 (31). The E1 revertant grew normally in BHK cells but poorly in insect cells and was attenuated in adult mice.

Another important change occurs in the topology of the carboxy-terminal 33 amino acids of p62 as it transits from the endoplasmic reticulum to the Golgi vesicles. This domain is shifted from a transmembrane orientation (see Fig. 7) to one in which most of these amino acids are at the cytoplasmic face of the membrane (113). In its initial topology, p62 would

be unable to bind nucleocapsid, thus precluding premature assembly of particles at the endoplasmic reticulum membranes.

Assembly of Nucleocapsids and Enveloped Virions

The assembly of nucleocapsids includes both oligomerization of the capsid protein and selection of the genomic RNA for encapsidation. Although the subgenomic RNA is present in molar excess, it is the genomic RNA that is packaged into nucleocapsids and virions. The first evidence that selectivity was due to a packaging or encapsidation signal in the 5' region of the genome was the identification in the Sindbis virus genome of a 132-nucleotide fragment extending from nucleotide 945 to 1,076 in the nsP1 gene that interacts specifically with the capsid protein in binding assays and increases the encapsidation of an RNA in infected cells (56,241,242). Specificity appears to lie in the structure rather than in the sequence of the oligonucleotide (241). In contrast, the encapsidation signals for both Ross River and Semliki Forest virus are in the nsP2 gene (56,253). These two viruses are more closely related to each other than they are to Sindbis virus. It seems that as alphaviruses diverged from each other, the location and sequence of the encapsidation signal also diverged. Furthermore, although there could be similarities in structure, the Ross River virus capsid does not appear to recognize the Sindbis virus encapsidation signal (51,56).

The region of the Sindbis virus capsid protein that binds to the encapsidation signal is embedded in a 32-amino-acid region extending from amino acids 76 to 107 (64). This segment of the alphavirus capsid protein also interacts with ribosomal RNA and may be involved in uncoating of the RNA (251). Residues 97 to 106 are highly conserved among alphaviruses. Deletion of residues 97 through 106 from the capsid protein of Sindbis virus did not significantly inhibit particle assembly but reduced infectious titers more than 100-fold (152). ost of the particles contained the subgenomic RNA instead of the virus genome supporting the conclusion that these amino acids are important in packaging specificity.

Loss of discrimination in encapsidation also was observed with a Sindbis virus recombinant in which the capsid gene was replaced with the Ross River capsid gene (51). Furthermore, a deletion of amino acids 65 to 109 from this capsid protein did not reduce particle production, but they were almost completely devoid of viral genome RNA (51). This deletion eliminated most of the basic residues in the peptide chain and indicated that capsid–capsid interactions can occur in the absence of nucleic acid binding. The ability to form empty particles by removal of positively charged residues in the protein was first observed with icosahedral plant viruses (43,215).

Another exception to the strong selection of genomic over subgenomic RNA for encapsidation was described with Auravirus. This alphavirus packages both RNAs, although the ratio of genomic to subgenomic RNA is three- to 10-fold higher in virions than in infected cells (180). This result originally suggested that Auravirus subgenomic RNA contained an encapsidation signal; however, it is more likely that an encapsidation signal in the genomic RNA is not so efficient as that in other alphaviruses, resulting in less specificity in packaging. It appears that specific interactions between an RNA and an alphavirus capsid protein will favor encapsidation of that RNA, but in their absence, the capsid protein will interact with and package those RNAs that are most abundant.

In vitro assembly of nucleocapsids was carried out initially with capsid protein purified from virus (246,250), but recombinant capsid proteins expressed in *E. coli* have been used more recently (223). Both studies demonstrated that assembly can occur with a variety of nucleic acids, but in competition experiments, Sindbis virus RNA is preferentially packaged (196,223). Sindbis capsid proteins that lack the first 19 amino acids can assemble into nucleocapsids, but those deleted in the first 31 amino acids do not assemble by themselves (223). These and those missing the first 80 amino acids can be incorporated into nucleocapsids in the presence of competent capsid proteins. In mixed reactions with Sindbis and Ross River virus capsid proteins, only homologous nucleocapsids were detected (223).

The Sindbis capsid protein starting at amino acid 106 was crystallized, and the x-ray structure analysis showed that the amino terminal residues 108–111 of one chain associate with an adjacent molecule and that these residues bind into a hydrophobic pocket of the neighboring molecule (27). This finding, taken together with their mutational studies, led Lee et al. (99) to propose that residues 108 to 110 are important in the interactions between capsid monomers and also in the binding between the nucleocapsid and the C-terminal tail of the E2 glycoprotein (99).

The final stages in the replication of alphaviruses occur when nucleocapsids interact with the host-cell plasma membrane at sites occupied by viral transmembrane glycoproteins. This nucleation event leads to binding of additional virus glycoproteins and a bending of the membrane around the nucleocapsid until the bilayers meet and fuse to release the enveloped particle (20,62). Electron micrographs graphically illustrate this process (Fig. 8). Budding of alphaviruses requires the presence of both nucleocapsids and the heterodimeric complex of virus-encoded glycoproteins (10,220,263); however, mutations in the capsid protein that prevent the accumulation of stable nucleocapsids in the cytoplasm do not decrease the efficiency of Semliki Forest virus budding (48,211). This suggests that capsid–capsid interactions are stabilized by binding of spikes to the capsid protein, and in some cases, nucleocapsid assembly may occur concomitant with virus budding. The specific molecular interactions between alphavirus nucleocapsids and spike glycoproteins have not been identified; however, a motif at the carboxy terminus of E2 that is within a hydrophobic sequence localized to the cytoplasmic face of the membrane bilayer is essential for assembly. A tyrosine and a leucine residue near the amino-terminal portion of this sequence are highly conserved among the alphaviruses and are postulated to bind in a hydrophobic pocket containing a tyrosine and a tryptophan on the surface of the nucleocapsid (98,99,211). On association of E2 with the nucleocapsid, this pocket is hypothesized to undergo a conformational change that destabilizes and activates the core for eventual disassembly after virus entry into a host cell (98).

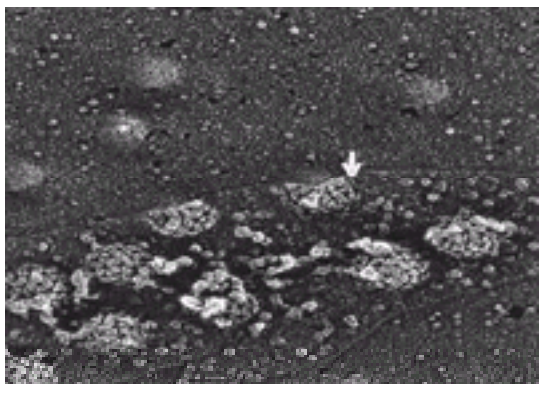

FIG. 8. Budding of Sindbis virions from infected chicken embryo fibroblasts. Cells were quick frozen and freeze fractured. The electron micrograph shows the fracture across the surface membrane of the cell (*white arrow*). On the bottom is the outer surface of the cell with clusters of virus glycoproteins at sites of budding. On the top is the inner leaflet of the surface lipid bilayer with viral nucleocapsids bound to the inner cytoplasmic side of the membrane. Sample preparation for electron microscopy was carried out by Dr. J. Heuser, Washington University School of Medicine, St. Louis, MO.

A number of studies using site-directed mutations in the E2 cytoplasmic domain and the capsid protein (60,82,97,153,211) provided further evidence for an interaction between the E2 cytoplasmic tail and the nucleocapsid, and mutations in the Sindbis virus capsid gene can partially suppress mutations in the E2 cytoplasmic domain that inhibit assembly (183). Capsid–E2 binding specificity also was demonstrated by experiments in which the gene for the Sindbis virus capsid protein was embedded in the Ross River virus genome (120). Virion particle production was greatly reduced with this chimera, but could be increased when seven amino acids of the Sindbis virus E2 cytoplasmic sequence were substituted for Ross River virus sequences. These results supported earlier studies in which a very hydrophobic six-amino-acid peptide corresponding to a conserved sequence within the E2 carboxy-terminal domain were shown to inhibit selectively Sindbis and Semliki Forest virus assembly and budding (30). Similar kinds of peptides with sequences identical to those in the E2 carboxyl terminus of Semliki Forest virus were reported to bind capsids (133). Not all chimeras, however, are defective; those in which the capsid protein gene was derived from either Semliki Forest virus or Ross River virus with the remaining genes from Sindbis virus assembled infectious virus at normal levels (51,214).

Effective budding also requires lateral interactions among the glycoprotein heterodimers, leading to trimeric and higher oligomeric forms such as the pentameric and hexameric structures that are positioned around the two-fold and five-fold axes of the virion. Hexagonal arrays of spikes were detected when membranes from Sindbis virions were examined by electron microscopy (232). These larger forms of the heterodimer would lead to polyvalent binding of the nucleocapsid to the E2 cytoplasmic ligands and could provide the force for membrane bending (62). Their formation also precludes insertion of host-cell proteins into the virus envelope. Defects in one of the glycoproteins (42) or its substitution by a chimeric form (i.e., E2 of Sindbis virus and E1 of Ross River virus) can interfere with the oligomerization process and decrease the amounts of secreted virus (262). In the latter case, heterodimers were formed, but no binding of nucleocapsids occurred at the plasma membrane. A series of suppressor mutations (five in Sindbis virus E2 and two in Ross River virus E1) increase progeny virus formation (260). The two adaptive changes in E1 were located adjacent to the membrane anchor domain of this protein. Based on cryoelectron microscopic reconstructions of Ross River virus (25), this region is part of the stalk that forms the spikes on the surface of the virion and consists of three entwined heterodimers; thus the mutations could affect trimer formation. Abnormal, multicored particles are released from cells infected with a Sindbis virus mutant containing the substitution of valine for alanine at position 344 in the E2 protein, and this phenotype can be partially corrected by a second, compensatory mutation in E1 (219).

The small, hydrophobic, fatty acylated membrane-associated 6K protein plays an important but still undefined role in alphavirus assembly and budding. Mutations in the 6K gene affect virus formation both quantitatively and qualitatively. Single-site mutations can lead to enhanced production of aberrant particles that contain multiple nucleocapsid cores, and a Semliki Forest virus mutant that totally lacks the 6K gene is highly defective in virus budding in BHK cells but much less so in insect cells (112,118). In the deletion mutant grown in BHK cells, virus cores align along the plasma membrane, and glycoproteins are not altered in their maturation and translocation. The 6K protein also interacts specifically with the E1/PE2 heterodimer, as shown by studies with a chimeric virus genome containing Sindbis virus glycoprotein sequences but a Ross River virus 6K virus sequence. This chimera was highly defective both in virus formation and in conformational changes occurring during maturation of the E1 glycoprotein (262). Mutations in the 6K Sindbis virus gene can be suppressed by mutations in the E2 glycoprotein, indicating a role for this protein in glycoprotein oligomerization during virus assembly (81). Because of its lipophilic properties, the 6K protein has been postulated to influence the selection of lipids that interact with the transmembrane domains of the glycoproteins, which, in turn, affect the deformability of the bilayer required for the extreme curvature that occurs as budding proceeds (20).

In most infected vertebrate cells, the plasma membrane is the site of alphavirus assembly; however, budding of Semliki Forest virus is selective in polarized epithelial cells, occurring only at the basal lateral surface (178,179) and, in cultured rat hippocampal neurons, this virus is secreted exclusively from membranes of the dendrites (37). In insect cells, budding occurs intracellularly, and virus-loaded vesicles are later disgorged to the extracellular fluid (137). In general, the lipid composition of alphaviruses closely resembles that of the membrane of the host cell (104). The ratio of cholesterol to phospholipid, however, is much higher in the membrane of alphaviruses than in the cellular membranes (175), and this may explain why Semliki Forest and Sindbis virus budding requires the presence of cholesterol in exiting the host-cell membrane (121,126). Virus membranes also are much more densely packed with protein and have a greater curvature than do the corresponding cellular membranes; thus their membranes are less fluid than are cellular membranes (200).

Alphaviruses as Vectors for the Expression of Heterologous Proteins

Sindbis virus, Semliki Forest virus, and Venezuelan equine encephalitis virus have all been genetically engineered to express heterologous proteins. Several different types of vectors have been described; the most versatile are those in which a heterologous gene replaces the structural protein genes (111,172,258). These vectors are self-replicating (replicons) but are not packaged and released from cells as virion particles. They can be complemented by defective "helper" RNAs that provide the structural proteins and allow replicons to be assembled into particles under conditions in which the helper itself is not packaged (15,111,172). Such particles are infectious but self-limiting, as they produce nsPs as well as genomic and subgenomic RNAs but, in the absence of structural proteins, do not form new particles.

Studies with replicons lacking the structural proteins led to the discovery of a translational enhancer in the viral subgenomic mRNA within the coding region of the capsid (54,208). The sequences that function as an enhancer are located about 25 nucleotides downstream of the AUG start codon and form a hairpin-like structure (55). Translation is stimulated about 10-fold, but only in infected cells (54,207). The existence and importance of this enhancer was clearly demonstrated for replicons derived from Sindbis and Semliki Forest virus but not for those from Venezuelan equine encephalitis virus (55,172).

A second type of expression vector contains two subgenomic RNA promoters; one controls the synthesis of the subgenomic mRNA that codes for the viral structural proteins, and the other controls the synthesis of the subgenomic RNA that codes for the heterologous protein (52). These vectors produce infectious virus particles able to spread throughout a culture.

Alphavirus vectors are being used for production of proteins in a variety of eukaryotic cells and have provided a useful tool for studies of protein expression and modification (52,63,122,195). They also are being developed as vaccines; for example, replicons expressing heterologous viral antigens, packaged into infectious particles or as DNA plasmids, have been introduced into animals. Initial studies in mice with DNA plasmids have been promising, as they induce an immune response at much lower doses than do conventional DNA plasmids.

A completely different approach to the use of alphaviruses as vectors had been to insert heterologous amino acid sequences into the viral structural proteins in such a way that the virus is still viable, but the heterologous epitope is expressed and can function as an immunogen. London et al. (119) made random insertions of an epitope from Rift Valley Fever virus into the structural protein genes of Sindbis virus. They then selected viable viruses containing the insert and found not only that the insertions were tolerated but also that the chimeric Sindbis virus could be used as a vaccine to make mice resistant to infection by Rift Valley Fever virus. Those initial studies and the subsequent isolation of Sindbis virus mutants that were defective in their ability to bind to vertebrate cells (39) served as a foundation for Ohno et al. (145), who devised a strategy to target Sindbis vector particles to specific cell types. They inserted the immunoglobulin G (IgG)-binding domain of protein A into the PE2 glycoprotein. This allowed them to target these Sindbis virus replicon particles to a particular cell type by adding monoclonal antibodies against a surface marker on those cells.

Recombination

Based on sequence comparisons, at least one alphavirus, western equine encephalitis virus, arose by recombination between eastern equine encephalitis virus and a Sindbis-like virus (67). Recombination also has been observed between replicons and defective helper RNAs. The latter events may arise more frequently than those between two virus genomes because crossovers do not have to be precise. Sequence analysis of these recombinant RNAs demonstrated that crossovers occurred within the defective modules (244). Recombinational events giving rise to infectious virion RNAs could create deletions, rearrangements, or insertions, as long as they occurred outside of each functional module (172,244).

More-detailed studies have been undertaken in efforts to define the mechanism and requirements for recombination (72,78,174). Surprisingly, recombination was detected with Sindbis virus RNA molecules that were unable to replicate because of deletions at the 5' or 3' terminus. Recombination events observed in laboratory studies usually take place under conditions in which high concentrations of RNAs are mixed together. Although these events are probably less likely to occur under conditions of natural infection, they do indicate that exchange between RNA molecules may play a role in the evolution of RNA viruses.

Effects of Alphavirus Infection on the Host Cell

Infection of vertebrate cells by most alphaviruses leads to inhibition of host-cell protein synthesis and cell death. Mechanisms that have been proposed for shut-off of host-cell protein synthesis include inhibition of host factors required for protein synthesis, with preferential translation of viral mRNAs, changes in the ionic environment of infected cells, which favors translation of viral RNAs, and direct inhibition by capsid protein [reviewed in (219)]. Infection of BHK cells with Sindbis virus replicons showed that inhibition of host-cell protein synthesis occurs at essentially the same rate under conditions in which no structural proteins or no viral subgenomic mRNA is synthesized (53). These data provide strong evidence that the structural proteins are not involved in mechanisms that inhibit host protein synthesis. Synthesis of the viral glycoproteins, however, leads to a much more rapid appearance of cytopathic effects than is seen in their absence (53). Furthermore, as mentioned later and in more detail (in Chapter 30), differences in the viral glycoprotein E2 can greatly affect how the cell responds to infection.

Cell death in alphavirus-infected vertebrate cells occurs by apoptosis (106,192), but in mosquito cells, the mechanism appears to be different and may more closely resemble necrosis (85). Infection of many *Aedes albopictus* (mosquito) cell clones leads to persistent infection (16), although it also may be cytopathic (84,187,225). In those cell cultures in which a persistent infection is established, the production of virus is comparable to that observed in most vertebrate cells during the first 24 hours, but then decreases several orders of magnitude during the persistent infection phase. The mechanism leading to persistence in mosquito cell lines is not known, although there have been reports that cells infected with

Sindbis virus produce an antiviral factor, and intracellular factors appear to restrict virus production (84).

It has been possible to establish persistent infections in some vertebrate cells. Two different types have been described. In one, essentially all of the cells are infected, but the synthesis of virus-specific RNA and protein in each cell is greatly reduced. This type of persistent infection was established in BHK cells by using defective interfering particles (243). The defective RNAs interfere with the replication of the genomic RNAs, leading to inhibition of viral RNA replication and virus production. The virus itself underwent a genetic change, converting it to a much less cytopathic mutant that was itself able to cause persistent infections in BHK cells. Several mutations were identified, but only one that converted the Pro ^{726} to Ser in the nsP2 protein was able to confer this phenotype on the virus (38). The decrease in cytopathic effects also was correlated with an eight- to 10-fold decrease in viral RNA synthesis.

A completely different approach to establishing this type of persistent infection in BHK cells was undertaken by using Sindbis virus replicons that express a gene (the *pac* gene) that codes for an enzyme that destroys puromycin. In the presence of puromycin, only cells that could eliminate this compound would survive, and only those cells that contained a Sindbis virus replicon that was not cytopathic could survive its replication and transcription, which would be required to express the *pac* gene. This selection procedure led to the isolation of several noncytopathic replicons (50). The one that has been analyzed in most detail has a single mutation, which led to the change of a single amino acid (Pro ^{726} to Leu) in nsP2, the same amino acid that was changed in the Sindbis virus mutant isolated from the persistent infection. The identification of several mutations in the nsP2 gene that lead to persistent infections and the localization of nsP2 in the nucleus suggest that this protein may have some as yet undefined functions affecting host-cell survival (176). These noncytopathic replicons are now being used as expression vectors in BHK cells (2).

A second type of persistent infection that has been described is one in which most of the cells are not infected, but the virus continues to persist because of a small fraction of cells that are susceptible and maintain the virus. In this case, the titer per culture is low, but those cells that do get infected may produce normal levels of virus and do not survive. An early example was the establishment of a persistent infection of mouse L929 cells by Semliki Forest virus. Only 1% to 20% of these cells expressed virus-specific antigens, and induction of interferon was the major factor in permitting the L cells to survive infection (130).

More recently the expression of other cellular genes has been shown to cause a significant decrease in virus replication and may lead to a persistent infection. The human MxA protein is a member of a family of guanosine triphosphatases (GTPases). When it was expressed in the human cell line Hep-2, the cells became almost completely resistant to infection by Semliki Forest virus: only 1% of the cells showed evidence of infection, but the virus was eventually cleared from the culture (95). Persistent infections by Sindbis virus and Semliki Forest virus could be established in a rat prostate carcinoma cell line (AT-3 cells) expressing the human *bcl-2* gene (106,192). The bcl-2 protein is a well-established antiapoptotic agent. In these cells, virus replication is severely restricted, with titers 10- to 100-fold lower in the AT-3 *bcl-2*–expressing cells than in the controls (192,228). As in the previous examples, only a small fraction of the AT-3 cells produced virus-specific antigens, indicating that the low titers were due to only a small number of the cells being productively infected. Further studies with Sindbis virus led to the discovery that the ability to establish this type of persistent infection was dependent on the strain used and was correlated with a single amino acid in the E2 glycoprotein (228) [and see Chapter 30].

Another restriction on Sindbis virus replication has been observed in mouse cells and is dependent on the class I major histocompatibility complex (MHC) haplotype. Sindbis virus replication was more than 100-fold higher in mouse cells that express the H-2^{k} haplotype than in cells expressing the H-2^{d} haplotype. The difference does not appear to be at binding or entry, but at the level of viral RNA synthesis (70). This difference also depends on the strain of Sindbis virus and specifically on the E2 glycoprotein.

RUBIVIRUSES

Rubella virus has long existed in the shadow of the alphaviruses. In the early studies of structure and replication of Togaviruses, it seemed that rubella virus was very similar to alphaviruses, but perhaps its replication was not so robust. This picture is gradually changing as we learn more about rubella virus—the properties of this virus and its interactions with its host cell have some special features. One major difference is the limited host range of rubella virus: it is found only in humans and does not grow in insect cells. Furthermore rubella virus grows slowly and to low titers in cultured vertebrate cells. Infection is much less cytopathic than that observed with alphaviruses, and persistent infections are readily established [reviewed in (49)]. It has been established that cell death in infected Vero cells occurs by apoptosis and involves a p53-dependent pathway (129,171). Chapter 31 describes these viruses in more detail.

Virion Structure and Entry

Rubella virions closely resemble alphaviruses in general morphology and physical characteristics; however, virion preparations are much more pleomorphic, and little is known about the icosahedral lattices and distribution of the glycoproteins on the virion surface. Two type 1 membrane glycoproteins form spikes projecting from the lipid membrane envelope; they are noted as E1 (282 amino acids) and E2 (481 amino acids) and are postulated to form heterodimers [reviewed in (49)]. Virion cores are similar to but somewhat smaller than alphavirus nucleocapsids and contain multiple copies of a single capsid protein about 290 to 300 amino acids in length that are present in virions as disulfide-linked dimers. The amino-terminal portion of the capsid is like that of the alphaviruses, enriched in arginines and containing a binding site for the genomic 40S RNA (115). Binding assays identified a 29-nucleotide region (nucleotides 347–375) in the rubella virus genome as the sequence required for specific interaction of RNA with the capsid protein. Studies to determine if this sequence is essential for or enhances encapsidation of the RNA have not yet been done.

In cultured Vero cells, rubella virus infection but not transfection of RNA was inhibited by lysosomotropic agents (162); thus this virus appears to enter the cell by receptor-mediated endocytosis and after membrane fusion in an acidified

endosome delivers its RNA to the infected cell cytoplasm.

Transcription, Translation, and Replication of the Genome

The replication of rubella virus does follow the same strategy as that of alphaviruses: the genomic RNA is first translated to produce the nonstructural proteins that are required for the synthesis of the complementary minus-strand RNA, the genomic RNA, and the subgenomic RNA [reviewed in (49)]. The subgenomic RNA is the mRNA for the viral structural proteins. Amino acid sequence homologies between rubella virus and the alphaviruses exist only within the nsPs (see Fig. 3), but proteolytic processing of the rubella virus nsP is distinct from that of alphaviruses. The 5' two thirds of the rubella virus genome is translated to a polyprotein of 240 kd that is posttranslationally cleaved into two polypeptides, one 150 and the other 90 kd. Region-specific antibodies located the 150-kd protein at the amino terminus, and the 90-kd protein at the carboxyl terminus of the polyprotein (47). Mutation of the cysteine at residue 1151 led to the accumulation of the largest protein at the expense of the two smaller proteins, providing evidence that the larger protein was the precursor of the smaller and that the cysteine was essential for protease activity (127). The protease responsible for cleavage is located at the C-terminus of p150 and is a papain-type cysteine protease. It requires divalent cations, contains zinc binding domains required for activity, and functions both in cis and trans (114,261). A histidine at position 1273 also is critical for activity. The site of cleavage is a gly-gly-gly sequence at positions 1299 through 1301 (23).

The first cDNA clone of rubella virus able to be transcribed into infectious RNA was derived from a strain (w-Therien) that forms opaque plaques on Vero cells. cDNA fragments obtained from a different strain (f-Therien) of rubella virus that gives clear plaques were used to replace equivalent segments in the clone (169). A swap of the 3' half of the genome led to an increase in specific infectivity, but the exchange of the 5' fragments resulted in clear plaques. The exchange of a region near the most 5' terminal fragment had the strongest effect on cytopathogenicity. The more cytopathic viruses produced more nonstructural proteins, and because the levels of the viral RNA species were similar, this result suggests that the amounts of the rubella virus nonstructural proteins can affect cell killing.

The 3'-terminal 305 nucleotides of rubella RNA has four stem–loop structures, two of which (SL1 and SL2) are located in the coding region of the E1 glycoprotein gene and two in the 59-nucleotide untranslated region. Nakhashi et al. (141) first showed by UV light–induced covalent cross-linking and gel-retardation assays that the 3' terminus of the genomic plus-strand RNA bound three cellular proteins of molecular masses 61, 63, and 68 kd. The 61-kd protein was identified as calreticulin, a calcium-binding protein usually found in the endoplasmic reticulum of eukaryotic cells (206). SL2 is the binding site for calreticulin, and binding is localized to the N-terminal domain of the protein (9). Site-directed mutagenesis of SL2 led to loss of binding of calreticulin, in particular for those mutations that destabilized the stem structure (24). Decreased binding of calreticulin did not correlate with inhibition of virus growth. Although this suggests that calreticulin binding is not required for virus viability, it was proposed that local intracellular concentrations of the protein might be high enough to allow binding to occur. Alternatively, the binding of calreticulin may have some role in human infections in which innate and immune responses can affect virus replication.

Extensive mutagenesis of SL3 and SL4 indicated that most of these regions were required for virus replication. In addition, further studies demonstrated the formation of three RNA–protein complexes with the 3' untranslated region, and UV cross-linking detected host proteins with molecular masses of 120, 80, 66, 55, 48, and 36 kd bound to this region of the RNA (24).

A very detailed mutagenesis analysis also was carried out for the 5' terminus of rubella virus RNA (170). Although many of these mutations had an effect on virus growth, most were not lethal. Mutations that were lethal included all of those that removed the two As at nucleotides 2 and 3, either by mutation or by deletion. Two cellular proteins, one 59 and the other 52 kd, when bound to the 5' terminus, were immunoprecipitated with a Ro-type human polyclonal serum obtained from patients with autoimmune disorders (164,165). A subset of sera recognized the La autoantigen or both the La and Ro autoantigens. The La autoantigen binds to the 5' stem–loop of rubella virus genomic RNA with relatively high specificity both *in vivo* and *in vitro* and affects the expression of the RNA (40). It is not yet possible to interpret the significance of the binding of these proteins to rubella virus RNA. The finding that these proteins are autoantigens has led to the speculations that their involvement in infection may lead to the arthritic symptoms associated with rubella virus infections. As mentioned previously, the La autoantigen is now implicated in the replication of a number of different RNA viruses, but how it is involved remains to be discovered.

Some headway on rubella viral RNA replication is being made. Virus replication complexes in Vero-infected cells have been localized to vacuoles of endolysosomal origin and tubular membranes with antibodies directed against the p150 replicase (92). Colocalization of lysosomal markers with double-stranded RNA antibodies was found by confocal microscopy, supporting the proposal that replication occurred on virus-modified lysosomes (125).

Synthesis of Structural Proteins and Virus Assembly

Translation of the rubella subgenomic mRNA produces a polyprotein that is proteolytically processed by a pathway different from that of the alphaviruses (29,146,147 and 148). The rubella virus capsid protein lacks an autoprotease activity. Stretches of 23 and 20 hydrophobic amino acids precede the amino termini of the E2 and E1 glycoproteins and act as signals for the translocation of the glycoproteins into the lumen of the endoplasmic reticulum. Cleavages between the capsid and E2 and between E2 and E1 are catalyzed by the host-cell signalase. After cleavage, the E2 signal sequence remains attached to the capsid protein, which becomes anchored to the membrane (79,221). The latter interaction is probably important in targeting the assembly of the virus nucleocapsid to intracellular membranes. There is no small hydrophobic “linker” protein between the two glycoproteins nor is there a precursor form of E2.

The two rubella virus glycoproteins resemble those of the alphaviruses in name, but their structure and synthesis are distinct [(29,146,147 and 148) and reviewed in (49)]. Both rubella virus glycoproteins contain N-linked oligosaccharides, and E2 also contains O-linked glycans (123,185). These proteins are retained in the Golgi organelle, and only a small fraction reach the plasma membrane. The transmembrane domain of E2 has a Golgi retention signal in contrast to E1,

which contains a retention signal for the endoplasmic reticulum in its transmembrane and cytoplasmic domain. The latter is overridden in the heterodimer, but both are required for virus particles to be secreted from infected cells (61). Site-directed mutagenesis studies indicate that a 29-amino-acid sequence in E1 may be the fusion peptide for the heterodimer complex of this virus (259). The accumulation of the viral glycoproteins in the Golgi complex may explain why virus assembly appears to occur mainly at this site. Nucleocapsids of rubella virus become visible only during the budding process and apparently do not assemble independent of an association with the glycoproteins.

PERSPECTIVES

Togaviruses and, in particular, alphaviruses are among the best-characterized RNA enveloped viruses. This chapter has documented much of our knowledge of the structure and replication of these viruses, but many questions remain unanswered. Progress on determining the three-dimensional structure of the virus continues to be made by using cryoelectron microscopy, and a structure for the E1 glycoprotein is being determined (248). These and other structural studies should provide a clearer picture of assembly and disassembly of these viruses. In the last few years, several functions of the individual alphavirus nonstructural proteins have been identified, but little progress has been made on how these proteins work in concert to replicate and transcribe viral RNA. Proteolytic cleavage of the nsP complex plays an essential role in the regulation of viral RNA synthesis, but the details have not yet been worked out. It will be important to learn if this type of regulation extends to the synthesis of rubella virus RNAs. Several different host-cell proteins that bind to viral RNAs have been identified, but there are no models for how these proteins might be involved in viral RNA synthesis. The ability to initiate alphavirus RNA synthesis in cellular extracts represents an important first step in uncovering the details of viral RNA synthesis (100).

There is at least one unsolved puzzle that stems from studies on the requirements for alphavirus RNA synthesis in cultured cells. Studies with both Sindbis and Semliki Forest virus replicons have demonstrated that viral RNA replication and the shut-off of host-cell protein synthesis occur in the absence of any of the viral structural proteins. Based on those results, it would seem that neither the viral glycoproteins nor the capsid protein plays a role in viral RNA replication nor in the inhibition of host-cell protein synthesis. In contrast, several publications proposed that the Sindbis virus glycoproteins are involved in regulating viral RNA synthesis. Relatively few changes in these viral glycoproteins can lead to extensive differences in the outcome of infection, as was reported for a rat prostate carcinoma cell line expressing *bcl-2* (228) and for mouse cells depending on their class I MHC molecules (70). In these examples, as for Semliki Forest virus in Hep-2 cells expressing the human MxA protein (95), the block in replication occurs at a very early step, but it has not yet been established to be a direct inhibition of viral RNA synthesis. One explanation to resolve this apparent discrepancy would be that the interaction of certain alphavirus glycoproteins with cellular membranes can interfere with the binding of the nsP complex to these membranes; this and other alternatives should be explored. An important future goal will be to correlate these observations in cultured cells with pathogenesis in humans and animals.

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CHAPTER 30 Alphaviruses

Diane E. Griffin

The genus Alphavirus includes 25 viruses (Table 1). All are transmitted by arthropods, share a common replication strategy, and are geographically restricted in their distributions. Alphaviruses have been found on all continents except Antarctica and on many islands. In general, the pathogenic alphaviruses are divided into the viruses that cause human diseases characterized by rash and arthritis, primarily found in the Old World, and viruses that cause encephalitis, primarily found in the New World. For many alphaviruses, no human or veterinary disease has been recognized. Alphaviruses cycle primarily between mosquitoes and small mammals or birds. Larger mammals, such as humans and horses, that tend to develop severe or fatal disease are often dead-end hosts unimportant to the endemic virus-transmission cycles, but can be important for sustaining epidemics.

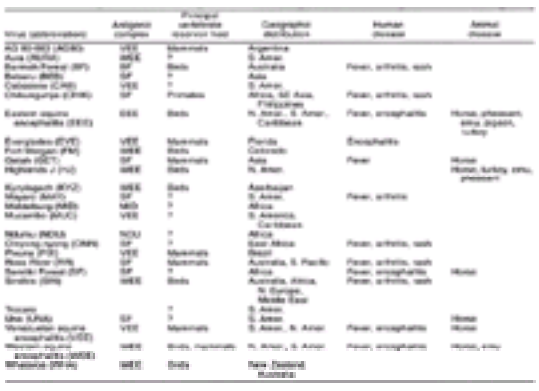

TABLE 1. *Alphaviruses, abbreviations, biologic features, and association with disease*

HISTORY

Records of diseases almost certainly due to alphaviruses date to the 18th and 19th centuries when epidemics of fatal encephalitis in horses in the northeastern United States and outbreaks of arthritis in Southeast Asia were recognized and recorded (71,208,557). The first clear report of epidemic encephalitis comes from the summer of 1831 when 75 horses died in Massachusetts (208). Over the next 100 years several local outbreaks of encephalitis in horses were noted along the Atlantic seaboard of the United States and in the pampas regions of South America (481). However, the first alphavirus to be cultured was western equine encephalitis virus (WEEV). This virus was isolated in 1930 from the central nervous system (CNS) tissues of two horses involved in an epidemic of equine encephalitis that occurred in the San Joaquin Valley of California (376). The virus of eastern equine encephalitis (EEEV) was isolated from the brains of affected horses in New Jersey and Virginia in 1933 (556). Both diseases occurred in summertime epidemics, suggesting an arthropod vector, and in 1933, Kelser showed that WEEV could be transmitted by mosquitoes (275). In 1936, an epizootic of equine encephalitis occurred in the Guajira region of Venezuela, and the virus isolated was not neutralized by antisera against EEEV or WEEV and was designated Venezuelan equine encephalitis (VEE) virus (184,300).

Summertime epidemics of polyarthritis were recognized in Australia and New Guinea in 1928 (119,407), and subsequent outbreaks of polyarthritis were reported in Northern Europe, Africa, and Southeast Asia (516). It is likely, of course, that the alphavirus-induced arthritic diseases are much older than these dates but were not clearly described or differentiated from more prevalent infections in these regions, such as dengue. This is considered to be particularly true of outbreaks of chikungunya (CHIK) that have occurred in India and Southeast Asia over the last 200 years (71,557). Viruses associated with epidemic polyarthritis were eventually isolated, both from mosquitoes collected in the areas of human disease, and later from humans. The first of these viruses was isolated in 1952 from a pool of *Culex* spp. mosquitoes collected near Sindbis, Egypt (551,552). However, it was many years before Sindbis virus (SINV) was linked to human disease (352).

The first clear association of an alphavirus with arthritic disease came in 1953 when CHIKV was isolated from the blood of individuals in Tanzania with severe arthritis (477). During the next several years, a number of viruses causing arthritis, often accompanied by a rash, were isolated in Africa, Australia, and South America (79,388,624). These viruses were added to the growing list of arthropod-borne (arbo) viruses, defined by the World Health Organization in 1967 as

> viruses which are maintained in nature principally, or to an important extent, through biological transmission between susceptible vertebrate hosts by hematophagous arthropods; they multiply and produce viremia in the vertebrates, multiply in the tissues of arthropods, and are passed on to new vertebrates by the bites of arthropods after a period of extrinsic incubation (440).

In 1954 arboviruses were divided by Casals and Brown (440) into three serologic groups, A, B, and C, based on cross-reactivity in hemagglutination inhibition (HI) tests. EEE, WEE, and VEE viruses constituted the group A arboviruses. A second cross-reacting set, including dengue, St. Louis encephalitis, and yellow fever viruses, constituted the group B arboviruses, and the nonreactive viruses were designated group C. As viruses became classified on the fundamental properties of the virion and the genome, the group A viruses became the Alphavirus genus within the *Togaviridae* family of enveloped RNA viruses.

INFECTIOUS AGENTS

Alphaviruses are enveloped plus-strand RNA viruses with icosahedral symmetry. The virions are 60 to 70 nm in diameter and sensitive to ether and detergent. The RNA is contained within a capsid formed by a single protein arranged as an icosahedron with T=4 symmetry. The nucleocapsid is enclosed in a lipid envelope derived from the host-cell plasma membrane that contains the viral-encoded glycoproteins, E1 and E2. These proteins form heterodimers that are grouped as trimers to form 80 knobs on the virion surface. Glycoproteins are arranged such that 240 copies of each interact with 240 copies of capsid protein.

The 49S genome is composed of a single-strand, nonsegmented, capped, and polyadenylated message-sense RNA that is infectious. Complete sequence information is available for representative members of most alphavirus complexes (87,101,140,285,303,306,316,372,538). The genomes are 11 to 12 kb in size and are organized with the nonstructural proteins (nsPs) at the 5'-end and the structural proteins at the 3'-end. The nsPs are translated from genomic RNA, and the structural proteins, from a subgenomic RNA (540).

Five potential structural proteins (C, E3, E2, 6K, and E1) are encoded as a polyprotein. The N-terminal portion of C is conserved, basic, and is presumed to bind the viral genomic RNA, whereas the C-terminal portion interacts with the cytoplasmic tail of E2 and with other copies of the C protein to form the nucleocapsid (540). The E3 protein serves as a signal sequence for E2, is cleaved from E2 in the trans-Golgi, and is often shed from the surface of the infected cell (617). The E2 glycoprotein is a transmembrane protein that has two or three N-linked glycosylation sites and contains the most important epitopes for neutralizing antibody. The intracytoplasmic portion has a second stretch of hydrophobic amino acids that probably tethers it to the inner surface of the membrane. The 6K protein serves as a signal peptide for E1, is important for budding, and small amounts are incorporated into the virion (51,167,324). The E1 protein has one or two N-linked glycosylation sites, a short (one or two residue) intracytoplasmic tail, and a positionally conserved internal hydrophobic stretch of amino acids in the N-terminal portion that serves as the fusion peptide for virion entry into the cell. Lipid content and glycosylation patterns are determined by the host cell (274,460). The 5' portion of the genome encodes four nsPs that function in replication of the viral RNA and production of the subgenomic RNA (540) (covered in Chapter 29).

Propagation and Assay in Tissue Culture

Initial isolations of alphaviruses have classically been by intracerebral inoculation into suckling mice, a host very susceptible to infection with most alphaviruses (237,477,485,551,552,613,624). Many alphaviruses also can be isolated

and propagated efficiently in primary chick embryo fibroblasts (CEFs) and in a wide variety of continuous mammalian cell lines such as human epithelial (HeLa, MRC5) cells, baby hamster kidney (BHK) cells, monkey kidney (Vero) cells, and mouse fibroblast and neuroblastoma cells (Fig. 1) (59,303,413).

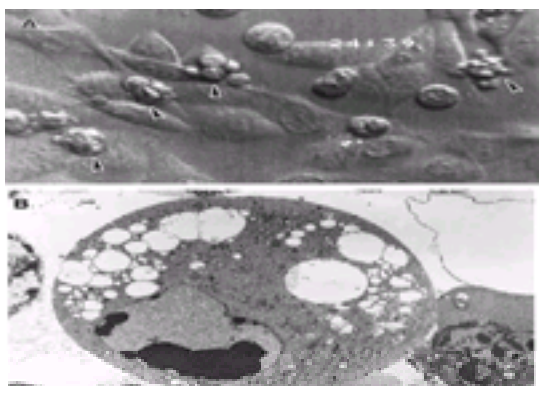

FIG. 1. Effects of Sindbis virus infection on vertebrate and invertebrate cells. **A:** Cytopathic effects (CPEs) in baby hamster kidney (BHK) cells infected at a low multiplicity. Infected cells show rounding, shrinkage, and cytoplasmic blebbing. **B:** Electron micrograph of an infected BHK cell, showing chromosomal condensation and cytopathic vacuoles.

Most alphaviruses will form plaques on susceptible mammalian or avian cells under an agar overlay. Mosquito cell lines also support replication, but often without cytopathic effect (CPE) (447,535,537,565). The first plaque assay of an animal virus was performed by using WEEV on CEF cells (131), and plaque assay remains a convenient and sensitive way to quantitate infectious virus. Plaque size has been used to differentiate strains and is determined by the type of overlay used, by relative virus binding to negatively charged sulfated polysaccharides present in the overlay, and by replication efficiency (52,56,494,549).

Biologic Characteristics

Hemagglutination

Alphaviruses can hemagglutinate avian (e.g., goose, chicken) erythrocytes (439,482), and this has been used as a method for quantitating virus and for measuring antiviral antibody by HI (93). Hemagglutination requires prior exposure of the virus to acidic pH, is dependent primarily on the E1 glycoprotein, and probably reflects binding of the fusion domain of the E1 glycoprotein to lipids in the erythrocyte membrane (88,93,102,585,619). E2 also participates in hemagglutination because some monoclonal antibodies (MAbs) specific for E2 also have HI activity (38,451). The HI test has been useful for determining antigenic relationships among alphaviruses (69).

Cellular Receptors

The nature of alphavirus receptors and the role of receptors in tissue and cellular tropism is an area of active investigation. Binding of virus to the cell surface and entry into the cell is a multistep process that is dependent on virus glycoproteins E1 and E2, cell-surface molecules, low pH in the endosome, and fusion of membrane lipids. Variations in any of these components will affect the efficiency of infection and the likelihood that any particular cell will become infected *in vivo.* Virus-specific attachment to cells *in vivo* occurs at neutral pH and is primarily a function of the E2 glycoprotein. The important role for E2 in initiating virus–cell interaction is evidenced by the ability of anti-E2 MAbs to inhibit binding to cells (56,335), of antiidiotypic antibodies to E2-specific MAbs to recognize putative virus receptors on cells (580,601), and of amino acid changes in E2 to alter virus binding to cells of different types (130,567).

Identification of specific alphavirus receptors has been difficult and may be complicated by experimental use of virus strains that are adapted to replicate in tissue culture (293). Because each alphavirus infects a wide range of hosts, often including birds, mammals, and mosquitoes, they must use either an evolutionarily well-conserved cell-surface molecule or multiple molecules as receptors for initiation of infection. None of the alphavirus receptors identified to date has been shown to be used exclusively, suggesting the possibility of several receptors. Alternatively, alphaviruses may use receptor–coreceptor combinations to achieve wide host range and the specific tropisms observed *in vivo.* The first specific alphavirus receptor to be identified was the major histocompatibility complex (MHC) class I molecule receptor for Semliki Forest virus (SFV) on mouse and human cells (223). This molecule is not absolutely required because cells lacking MHC molecules can still be infected with SFV (415). The high-affinity laminin receptor has been identified as a receptor for SINV on BHK cells (599) and as a likely receptor for SINV and VEEV on C6/36 mosquito cells (335,599). Again, this molecule appears to account for only a portion of the total virus interaction with the cells studied and does not, for instance, contribute to SINV binding to avian cells (599).

Heparan sulfate, a ubiquitously expressed glycosaminoglycan, has recently been identified as an important initial binding molecule for SINV, but not for RRV (56,293). Addition of heparin, treatment of cells with heparinase, or use of cells deficient in heparan sulfate decreases SINV binding to cells and virus plaque formation. Interaction with heparan sulfate, a highly sulfated, negatively charged molecule, probably explains the effects of ionic strength and charge on the attachment of virus to cells (356,358,435). The E2 glycoprotein of SINV contains a heparin-binding domain that overlaps the E2c neutralizing epitope (Fig. 2), and amino acid changes in this region toward more positively charged residues increase the efficiency of attachment to cells in tissue culture (57,293). However, these changes generally decrease virulence when tested in mice, suggesting that the changes represent adaptations of the virus to replication *in vitro* (57,293).

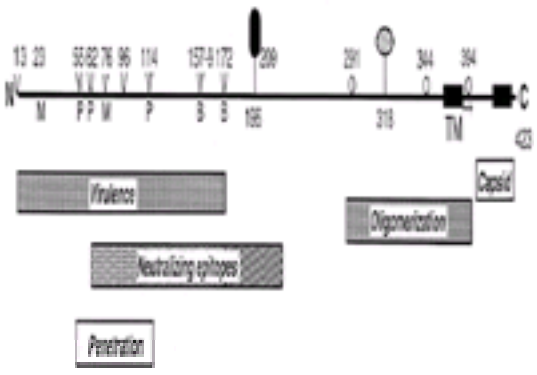

FIG. 2. Linear map of the E2 glycoprotein of Sindbis virus showing glycosylation sites, hydrophobic domains, and amino acid residues involved in induction of neutralizing antibody, oligomerization with E1 (*0*), virion binding (*B*), and penetration (*P*), capsid interaction, virion maturation (*M*), and virulence (*V*).

Entry into the cell after initial binding requires endocytosis followed by a conformational change in the trimer of E1–E2 heterodimers that is induced by exposure to low pH in the endosome (221,622). E1 proteins dissociate from E2 and form E1 trimers. During this change, the internal fusion peptide in E1 is exposed (48,166,220). Amino acid changes in E1 affect the requirement for cholesterol in the target cell membrane and the optimal pH for fusion (39,282,334,433). Fusion with the cell membrane to initiate infection is dependent on presence of a membrane potential (222).

Alphaviruses can interact and fuse with liposomal membranes that do not contain a receptor protein, and this *in vitro* system has been studied extensively to define the fusion process. Binding to these artificial membranes requires exposure of the virion to low pH (387,521,622), the E1 glycoprotein (422), and cholesterol in the target cell membrane (282,334), and probably involves a mechanism similar to hemagglutination of avian erythrocytes (387). Subsequent fusion with liposomes is dependent on the low pH-induced trimerization of E1 and on the presence of sphingomyelin in the target membrane (99,282,334,404,521,628).

Effects on Vertebrate Cells

Alphaviruses replicate rapidly in most vertebrate cell lines with the release of progeny virus typically within 4 to 6 hours after infection. At the time of virus entry, there is a transient increase in permeability (397). Insertion of newly synthesized glycoproteins into the plasma membrane renders infected cells capable of adenosine triphosphate (ATP)-dependent polykaryocyte formation on exposure to acid pH (276). Infection causes extensive CPE, characterized by cell rounding, shrinkage, and cytoplasmic blebbing (see Fig. 1A), with the death of infected cells within 24 to 48 hours (315,447). Many alphaviruses kill cells by inducing apoptosis, a process initiated by infection and carried out by the targeted cell (11,183,315). This process is associated with blebbing of the plasma membrane, condensation of nuclear chromatin, and formation of apoptotic bodies (see Fig. 1B) (315). Viral proteins are concentrated in the surface blebs from which budding continues to occur (474). This process does not hamper, and may enhance, virus replication because inhibition of apoptosis usually decreases virus yield (311,323,490).

The mechanism(s) by which alphaviruses induce apoptosis is not completely understood. Apoptosis of cultured cells can be initiated at the membrane during SINV fusion (259). Membrane-bound sphingomyelinases are activated, perhaps by the glycoprotein transmembrane domains, and sphingomyelin, a lipid necessary for alphavirus fusion, is degraded, releasing ceramide, an efficient inducer of cellular apoptosis (258,261). Once the apoptotic process is initiated, cellular caspases are activated with cleavage of caspase-3 substrates such as poly(ADP ribose) polymerase and fragmentation of chromosomal DNA (582). Alphavirus-induced apoptosis can be slowed or prevented, often in virus strain- and cell type–dependent ways, by expression of ceramidase (258), expression of a dominant inhibitory form of Ras (260), inhibition of constitutive expression of NFkB (325,326), overexpression of Bcl-2 family member and interacting proteins (183,315,318,323,490,583), and inhibition of caspase activity (403).

Alphavirus-induced vertebrate cell death also can occur by nonapoptotic mechanisms. Alphaviruses efficiently shut down protein synthesis in infected cells (402), deplete nicotinamide adenine dinucleotide (NAD) and energy stores (582), and induce dysfunction of Na^+K^+ATPase, causing loss of membrane potential and change in intracellular cation concentrations (27,172,584).

Persistent infection can occasionally be established in mammalian cell cultures *in vitro.* Mouse fibroblasts producing interferon (IFN), or BHK cells with high concentrations of defective interfering particles, can be persistently infected with SINV (243,616). Infection with SINV mutated at position 726 of the nsP2 protein (Pro ® Ser) leads to reduced RNA synthesis and persistent infection (128). Persistent infection also can be established if the cell infected is resistant to virus-induced apoptosis. This occurs in primary cultures of mature neurons (315,581) and in AT3 rat prostatic carcinoma cells overexpressing the cellular antiapoptotic protein Bcl-2 (315).

Effects on Invertebrate Cells

Studies of alphavirus infection of cell lines derived from *Aedes albopictus* and *Ae. aegypti* larvae demonstrate differences in alphavirus replication between vertebrate and invertebrate cells. Kinetics of virus replication are similar, but frequently virus matures within vesicular structures in the cell, and virions are released by exocytosis rather than at the plasma membrane (178,377,537). In mosquito cell lines, alphaviruses are much more likely to cause persistent noncytopathic infection (105,178,242,429,430,447,535,537) or death by a nonapoptotic process (271) than in vertebrate cells. The uncloned lines derived from *Ae. albopictus* larvae contain many types of cells with properties representative of different

mosquito tissues. Several cloned cell lines have been derived that vary in their responses to alphavirus infection. Lytic infection occurs in clones that support high levels of virus replication, as is seen with SINV infection of the C7-10 clone and SFV infection of HVP clones (377,565). Persistent infection is associated with a short period of relatively high virus replication followed by a decrease in virus production and in the numbers of cells in the culture that are producing virus, as is seen with SINV infection of the C6/36 clone (105).

Superinfection Exclusion

Vertebrate and invertebrate cells infected with one alphavirus cannot be productively infected with the same, or a closely related, alphavirus at a later time. Exclusion is established after translation of the nsP genes of the first virus to enter. The superinfecting genome can be translated, but not replicated (536). Recent studies suggest that this is due to the presence of the trans-acting protease nsP2, which prematurely cleaves the replicase polyprotein required for minus-strand synthesis, thus blocking replication of the superinfecting virus (272).

Antigenic Composition

All alphaviruses are related and share common antigenic sites, as revealed by HI and complement-fixation (CF) tests with polyclonal immune sera (69) and by cytotoxic T-lymphocyte lysis of infected cells (330,395). Antigenic cross-reactivities probably reflect sequence conservation in the C protein and E1 glycoprotein (185,330) and may confer some cross-protection and also interfere with sequential alphavirus immunization (116,363,539). These cross-reactivities formed the basis for the original classification into the group A arbovirus serogroup (440) and continue to be a valuable means for initial identification and classification of alphaviruses (69). Closely related viruses within a serogroup form a complex. Seven broad antigenic complexes have been identified within the alphavirus serogroup: Barmah Forest (BF), EEE, Middelburg (MID), Ndumu (NDU), SF, VEE, and WEE (see Table 1), and an eighth, Trocara, has recently been described (566). The BF, EEE, MID, and NDU complexes each contain only a single virus, whereas the SF, VEE, and WEE complexes include several viruses. Viruses within each complex can be subtyped by using reactivity with MAbs, kinetic HI, or neutralization assays (60).

Antibodies to E1 are more likely to cross-react with other alphaviruses than are antibodies to E2 (38,241,629,634). This is consistent with the documented sequence conservation in the E1 protein. Competitive binding assays using MAbs have identified approximately seven epitopes on the E1 glycoproteins of SINV, SFV, WEEV, and VEEV (38,241,373,470,495). Most E1 epitopes are not exposed on the virion surface, but are present on the surface of infected cells or on acid-exposed virions (9,193,373,495,531,532,629). he *in vitro* biologic activities of antibodies to E1 include HI, neutralization of virus infectivity, and inhibition of fusion. Neutralization of virus infectivity and HI activity often map to separate epitopes on E1, indicating that these are functionally distinct domains (38,86,241,495,531). Mapping of MAb binding sites to specific E1 sequences has been limited. One acid-exposed epitope involves E1-157 of SFV (9). A neutralizing MAb distinguishes strains of SINV differing only at residues 72 and 313 of E1, and an MAb escape mutant for a different epitope has a change at E1-132, suggesting the involvement of these regions in neutralization (193,531,539).

Antibodies to E2 are usually virus specific. Competitive binding assays using MAbs have identified four to five epitopes on the E2 glycoproteins of SINV, SFV, RRV, and VEEV (38,279,373,419,470,472,592). *In vitro* biologic activities of antibodies to E2 include HI, neutralization of virus infectivity, and blocking of virus binding to the cell surface (102,427,470). Many anti-E2 MAbs have both neutralizing and HI activity, suggesting that these functions overlap and that the conformational change in E2 that occurs after exposure to acidic pH is less drastic than that of E1 (193). Neutralization escape mutants, naturally occurring variants, lgt11 expression libraries, site-directed mutagenesis, and recombinant viruses have been used to map the various epitopes on E2 and have identified two major neutralizing sites (107,431,497,533,539,600). Overlapping epitopes in the middle of the E2 linear sequence encompass residues 181 to 216 in SINV, 180 to 216 in VEEV, and 216 to 251 in RRV, and identify one neutralizing site (7,107,193,262,539,592) (see Fig. 2). This is a hydrophilic region that for many alphaviruses includes an N-linked carbohydrate, suggesting that this portion of the protein forms a loop exposed on the virion surface. There are probably linear determinants in this region because these MAbs frequently react in Western blots and recognize l fusion proteins, and antibodies to peptides from this region are protective against challenge (531,600).

The second neutralizing epitope on E2 appears to be more complicated and is primarily conformational. Amino acid changes over a large linear range (e.g., residues 62–159 of SINV and 4–234 of RRV) affect MAb binding, and MAbs to this epitope do not react with denatured proteins in Western blots (see Fig. 2). The region defined by this epitope is responsible for SINV binding to heparan sulfate and is obscured if pE2 is not cleaved (56,480). Visualization by cryoelectron microscopy of the binding of MAbs to this epitope on SINV and RRV identifies one of the two knobs on the glycoprotein spikes as this region of E2 (522).

Evolution and Phylogeny

Alphaviruses, which replicate in arthropods, birds, and mammals, derive from a single unknown protoalphavirus and are part of the alphavirus superfamily of viruses. Viruses in this superfamily, including many RNA plant viruses, have similar genetic organization and replicase proteins, but diverse coat proteins (297,541). Among alphaviruses, amino acids important in secondary structure (e.g., cysteines) have been conserved for the glycoproteins E1 and E2, suggesting that the three-dimensional structure of the virion is similar for all. The capsid protein and E1 are the most conserved of the structural proteins, whereas E2 is more divergent. Sequence analysis, which includes information from the entire genome, generally groups the viruses similarly to that derived by antigenic analysis and has detected at least one recombination event (Fig. 3). Current criteria for species demarcation of alphaviruses combine genetic, ecologic, and antigenic information. Species generally have distinct transmission cycles and differ by more than 23% at the nucleotide level and 10% in amino acid sequence when E1 protein genes are compared. Based on E1 sequence, alphaviruses are currently grouped into six clades that generally correspond to recognized antigenic complexes: BFV, NDUV, SFV,

WEEV, EEEV, and VEEV (see Fig. 3).

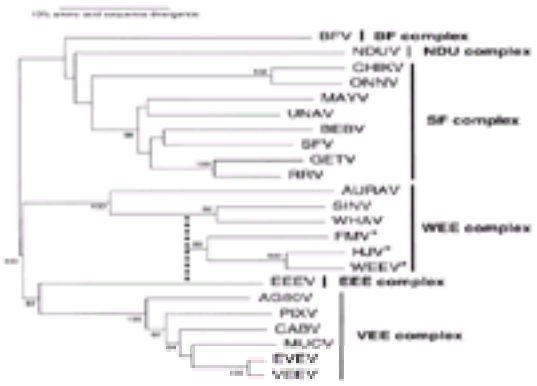

FIG. 3. Unrooted phylogenetic tree of representative alphaviruses generated from E1 envelope glycoprotein amino acid sequences using neighbor-joining analysis. Numbers adjacent to nodes indicate bootstrap support values exceeding 80 for groups to the right. *, Recombinant viruses. *Dashed line,* the presumed recombination event that generated an ancestral recombinant virus from eastern equine encephalitis (EEEV)- and SINV-like ancestors. Courtesy of Scott Weaver.

Like other RNA viruses, alphaviruses undergo genetic change primarily by accumulation of point mutations in the genomic RNA. However, this occurs at a rate that is much slower ($1–7 \times 10^{-4}$ substitutions/nucleotide/year) than is estimated for other RNA viruses (5×10^{-3} substitutions/nucleotide/year) (53,95,444). Recombination between alphaviruses can be demonstrated *in vitro* but is infrequent and usually puts the chimeric virus at a replicative disadvantage (301,541). However, successful recombination has occurred at least occasionally in nature because WEEV resulted from recombination between EEEV and a Sindbis-like virus, an event that is estimated to have occurred thousands of years ago (205,605) (see Fig. 3). Because there are New World WEE complex viruses that do [WEE, Fort Morgan (FM), Highlands J (HJ)] and do not (Aura) show evidence of this recombination and Old World members of the WEE complex (SINV, Whataroa) do not have recombinant genomes (606), alphaviruses most likely originated in the Americas and later spread to the rest of the world (316,606). Other data that support this view of alphavirus evolution are phylogenetic reconstructions, the greater sequence divergence within the New World viruses than the Old World viruses, and the antigenic relationship between Mayaro virus in South America and SFV in Africa (69).

Alphaviruses replicate in and are transmitted horizontally by a wide range of mosquito species. However, each virus usually has a principal or preferred mosquito vector for the enzootic cycle. Most alphaviruses can infect a variety of vertebrates, but use either birds or mammals as their primary amplifying host (see Table 1). The specific mosquito and amplifying host used by an alphavirus will contribute significantly to determining the geographic distribution of that virus. Experiments modeling evolution *in vitro* by passaging virus in vertebrate (BHK) cells, invertebrate (C6/36) cells, or alternating vertebrate and invertebrate cells show that fitness increases with passage in all instances, but fewer mutations accumulate if replication alternates, as it does in nature, between vertebrate and invertebrate cells (603). It is hypothesized that a short transmission season and host mobility influence alphavirus genetic diversity and evolution in a geographic region (95,604). Viruses using avian enzootic hosts (e.g., EEEV, WEEV, SINV) extend over wide geographic regions and evolve as a few highly conserved genotypes, whereas viruses using mammalian enzootic hosts with a more limited range of dispersal (e.g., RRV, VEEV) evolve within multiple geographically restricted genotypes (348,484,630).

Some strains of alphaviruses associated with epidemics or epizootics are antigenically and biologically distinguishable from enzootic strains. Recent phylogenetic evidence indicates that, at least for VEEV, the virulent epizootic strains evolve by mutation from avirulent viruses being maintained in the enzootic cycle (444,461).

PATHOGENESIS AND PATHOLOGY IN VERTEBRATES

Excellent and well-studied model systems exist for the study of several alphaviruses, and much of our detailed knowledge about alphavirus pathogenesis comes from studying mice infected by various routes of inoculation to induce infection. Information from these models is combined where appropriate with information from studies of humans with alphavirus-induced disease to deduce the pathogenesis of infection. Specifics are covered in sections on the individual viruses.

Entry

The primary mode of alphavirus transmission to vertebrates is through the bite of an infected mosquito. Mosquitoes salivate during feeding and deposit virus-infected saliva extravascularly (574). Saliva virus titers are highest early after the mosquito is infected and decline, along with transmission rates, after 1 to 2 weeks, but mosquitoes remain infected for life (379).

Sites of Primary Replication

The initial sites of virus replication probably vary with the virus and host. Mice have received most extensive study. After subcutaneous inoculation, viruses may infect skeletal muscle at the local site (e.g., EEEV, WEEV, SFV, RRV, SINV, and Getah virus) or be taken up by and infect Langerhans cells in the skin (e.g., VEEV) (Fig. 4) (197,230,265,331,398). Langerhans cells transport virus to lymph nodes draining the site of inoculation, which also may become infected (346).

Infection of Langerhans cells may be the most common mode of initiating natural infection.

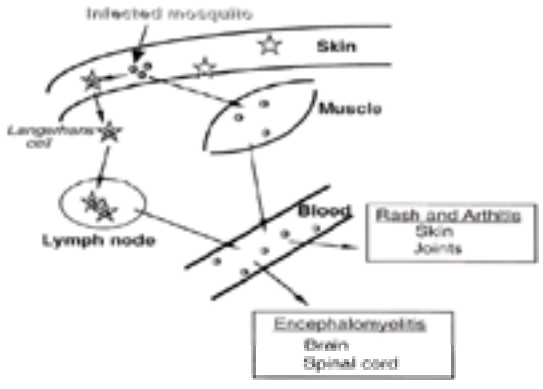

FIG. 4. Basic steps in alphavirus infection of vertebrates. Virus is delivered extravascularly by an infected mosquito and infects local muscle cells or Langerhans cells in the skin. Langerhans cells can carry virus to local lymph nodes. Virus is delivered to the blood and spreads to target tissues such as skin, joints, and the central nervous system, in addition to distant muscle and lymphatic tissue.

Spread

Alphaviruses induce a substantial plasma viremia in their amplifying hosts and in hosts susceptible to disease (see Fig. 4 and Fig. 5). The ability to mount and sustain a viremia is dependent on the continued efficient production of virus at a primary site of replication, delivery of virus into the vascular system, and slow clearance from the blood. Animal studies have shown that small plaque viruses are generally less virulent because they are cleared more rapidly from the circulation than are large plaque viruses (249,256,441). This phenomenon is related to the ability of small plaque viruses to bind heparan sulfate, and thus to be rapidly removed from the circulation by the highly sulfated glycosaminoglycans in the liver (57). Ability to invade target organs is dependent in part on the duration and height of the viremia, but also on other invasive characteristics of the virus (343).

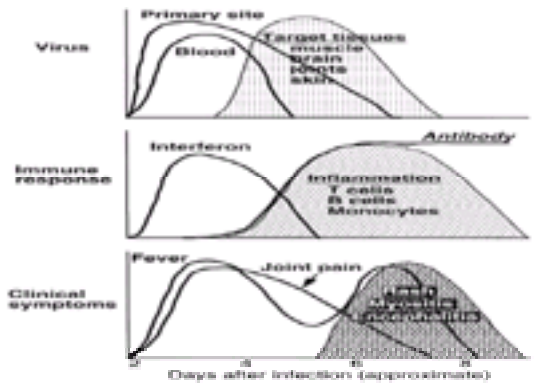

FIG. 5. Schematic diagram of the pathogenesis of alphavirus-induced disease. Viremia may be accompanied by production of interferon, other proinflammatory cytokines, and fever. Virus then spreads through the blood to other target tissues. As the immune response is induced, the viremia is terminated, but fever is renewed with appearance of a mononuclear inflammatory response in the infected tissue. In infections that lead to rash and arthritis, joint pain usually appears early after infection and before the appearance of the rash.

Cell and Tissue Tropism

Viruses that replicate initially in skeletal muscle and lymph nodes near the site of inoculation often spread through the bloodstream to more distant skeletal muscles and other lymphatic tissues. In mice, cardiac myocytes, brain and spinal cord neurons, and brown fat cells are frequent secondary sites of replication for many alphaviruses (see Fig. 5) (8,265,331,386,399). RRV and Getah virus cause polymyositis (230,399,503). EEEV, WEEV, SFV, and SINV cause encephalitis (23,125,247,331,400), and VEEV causes lymphoid depletion and encephalitis (177,180,255,587,597).

In humans, the skin is a target for alphaviruses that cause a rash (160,352); the joints, for those alphaviruses that cause arthritis (157); and the nervous system, for alphaviruses that cause encephalitis (171). RRV and SINV have been recovered from skin biopsies (234,352). The target cells for RRV replication in skin are basal epidermal and eccrine duct epithelial cells (160). The specific target cells within joints for infections leading to arthritis are not clear. In mice, RRV and WEEV infect chondrocytes and periosteal cells (8,399), and human synovial cells support RRV infection *in vitro* (100,268). Joint fluid taken from humans with acute arthritis has not yielded infectious virus, but viral antigen can be detected in macrophages, and macrophages support RRV replication *in vitro* (157,329).

The mechanism by which encephalitic alphaviruses enter the CNS is not entirely clear. Murine studies have shown infection or transport by cerebrovascular endothelial cells (265,281,426,525), infection of choroid plexus epithelial cells (331), and infection of olfactory neurons (90,590). Tissue culture–adapted strains of RRV and SFV can productively infect mouse brain endothelial cells *in vitro* (126), and SINV, SFV, and EEEV have been detected in mouse brain endothelium *in vivo* (265,281,426,525). EEEV also may initiate CNS infection in experimental animals by infecting choroid plexus

epithelial cells (331). For VEEV, initial infection is of olfactory epithelium with spread to olfactory neurons and subsequently along neural tracts to the CNS (90,590). Neuroinvasiveness is a component of virulence that varies between viruses and virus strains (129,343). Once within the CNS, virus can be spread cell to cell or through the cerebrospinal fluid (CSF) (246,417). For most encephalitic alphaviruses, the targeted cell within the CNS is the neuron (135,247,331,417), and damage to this cell can be severe and irreversible. SFV, RRV, and VEEV also can cause persistent CNS infection associated with infection of microglial and oligodendroglial cells, leading to demyelination (54,505).

Immune Responses

Innate Responses

Type I (a/b) IFN is induced after alphavirus infection of experimental animals (44,174,231,254,510,588) and presumably of humans (see Fig. 5). Study of *ts* mutants suggests that formation of dsRNA is the necessary step in replication for IFN induction because viruses with mutations in the protease domain of nsP2, which cannot process the nonstructural polyprotein and thus cannot initiate plus-strand RNA synthesis, do not induce IFN (206,354,595). Alphaviruses and strains of alphaviruses vary in their ability to induce IFN (441,494,510), and the amount of IFN produced is usually linked to the amount of virus produced (203,254,588). IFN appears rapidly, may not require virus replication, and is diminished by splenectomy (25,254,296). The primary source of IFN after peripheral inoculation may be macrophages or dendritic cells in lymphatic tissue (35,518). *In vitro* production of IFN follows the initial release of virus from infected cells by 2 to 3 hours (232).

IFN is an important part of the host response to alphavirus infection. Animals can be protected from lethal infection if treated with IFN or IFN-inducers before or soon after infection (150,254,504,554,633). Animals unable to respond to IFN because of deletions of the IFN receptor or of crucial IFN signaling molecules develop fatal infections even when infected with normally avirulent strains of virus (55,188,530). IFN appears to act primarily to limit virus replication early, during the time the specific immune response is being induced (Fig. 6). Treatment of cells with IFN inhibits alphavirus replication (113,254,391,396), but the mechanism by which this occurs, and therefore the IFN-induced host responses important for control of replication, are not known. Attachment and entry are not affected, and input mRNA is translated (113,452). However, later replication steps, including formation of replication complexes, structural protein synthesis, and morphogenesis, are inhibited (163,396,452).

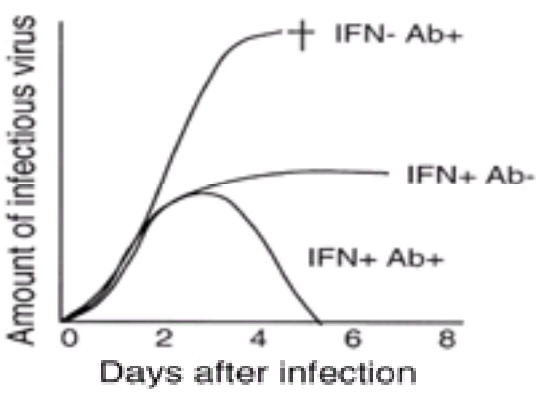

FIG. 6. Independent effects of type 1 interferon (*IFN*) and antiviral antibody (*Ab*) on alphavirus replication in tissue. Early control of replication is dependent on IFN, and in the absence of IFN, infection is often fatal. Clearance of virus is dependent on virus-specific immune responses, and antibody plays a prominent role.

The only IFN-induced protein shown to have an effect on alphavirus replication is human MxA, a large cytoplasmic guanosine triphosphatase (GTPase) that prevents accumulation of genomic and subgenomic RNA by an unknown mechanism (304). Transgenic expression of the human MxA protein in IFN-ab receptor knock-out mice decreases SFV replication and provides some protection against the otherwise fatal disease that occurs in these mice (218). The roles of other IFN-induced host proteins have not been reported. Virus strains vary in their sensitivity to the antiviral activities of IFN, and this generally correlates with virulence (49,530). Mutations associated with altered sensitivity to IFN have been mapped to nsP1 (476).

IFN also may contribute to alphavirus-induced disease. Fever during the viremic phase of infection, as is seen with CHIKV and RRV, is probably a response to the IFN induced early after infection (see Fig. 5). It has been postulated that the rapidly fatal disease induced by alphaviruses in newborn mice may be due to the production of large amounts of IFN and proinflammatory cytokines (292). Acute-phase responses induced by alphaviruses before the virus-specific immune response include increases in tumor necrosis factor (TNF)-a, interleukin (IL)-1, and IL-6, and levels generally correlate with the extent of virus replication (186,292,620). Adult mice deficient in IL-1b have reduced mortality after CNS infection with a neurovirulent strain of SINV, again suggesting the possibility that cytokine effects may contribute to mortality (322).

Virus-Specific Adaptive Responses

Both cellular and humoral immune responses are induced by infection (see Fig. 5). In experimentally infected adult mice, antiviral antibody is usually detected in serum within 3 to 4 days after infection (190,503). The cellular immune response, manifested by the presence of virus-reactive lymphocytes in draining lymph nodes and blood and the infiltration of mononuclear cells into infected tissues, also appears within 3 to 4 days after infection (118,194,364). These responses appear later (7–10 days after infection) in neonatal mice (510). The relative roles of each in recovery from infection and

protection against reinfection is an area of ongoing investigation.

Humoral Immunity

Virus-specific IgM antibody is detectable very early in human disease (61,72) and often provides a means for rapid diagnosis of infection (61,65,74). Virus-specific immunoglobulin A (IgA) also appears early in infection, but declines rapidly (73). IgG antibody appears in serum after 7 to 14 days and is maintained at relatively high levels for years (63,119,281). Rapidity of host antibody synthesis is predictive of outcome from encephalitis, because patients without evidence of antibody at the time of illness are most likely to die (61). Many lines of evidence support the hypothesis that recovery from alphavirus infection is dependent primarily on the antibody response (195,416,637,638). Antibody can neutralize virus infectivity and promotes virus clearance by the reticuloendothelial system in conjunction with complement (252). Appearance of antibody correlates with cessation of viremia (see Fig. 5).

The most extensive experimental studies to define the antibody specificity and the mechanisms of antibody-mediated recovery and protection have been done using VEEV, SFV, and SINV for infection of mice. Protection can be demonstrated by passive transfer of antibody before or after infection. Multiple epitopes on the E1 and E2 glycoproteins induce protective antibody. Both neutralizing MAbs, mostly anti-E2, and nonneutralizing MAbs, both anti-E1 and anti-E2, can protect against alphavirus challenge and promote recovery (36,37 and 38,198,241,359,373,472,496,532,634), suggesting that virus neutralization *per se* is not the only mechanism of protection. There is a correlation of protection with the ability of the MAb to bind to the surface of infected cells, but this is not absolute (532). Protection requires intact bivalent antibody, but complement is not required, suggesting that complement-mediated lysis of infected cells is not the mechanism of protection (227,360). However, complement may play an auxiliary role because virus clearance from blood is delayed in complement-deficient mice (228,229).

Antiviral antibody also can alter intracellular alphavirus replication and therefore promote clearance and recovery. Treatment of immunodeficient mice persistently infected with SINV or SFV with antiviral antibody clears infectious virus from the CNS without causing death of infected neurons or neurologic damage (12,314). SINV clearance from tissue is dependent on antibodies to the E2 glycoprotein (314), and MAbs specific for either of the neutralizing epitopes on SINV E2 can down-regulate intracellular virus replication *in vitro* by a nonlytic mechanism (314). Antibody against an N-terminal peptide of VEEV E2 that is not neutralizing can limit virus replication *in vivo* (240), and a nonneutralizing MAb to SFV E2 can limit virus replication *in vitro* (37). Anti E1 MAbs also may be able to alter intracellular virus replication, but this has been less extensively studied (85).

The process by which antibody alters intracellular virus replication requires bivalent antibody, but does not require the Fc portion of the MAb, complement, or other cells (314,581). Soon after antibody binding, virion budding from the plasma membrane is inhibited, perhaps by restoring cellular Na^+K^+ATPase function and K^+ flux or host protein synthesis (112). The effects of antibody on control of virus production *in vitro* can be amplified by treatment of infected cells with IFN-a (113). *In vivo* studies also suggest that IFN and antibody act synergistically to promote recovery from alphavirus infection (see Fig. 6), but the mechanisms by which these systems interact have not been identified (97,520).

Mechanisms of virus clearance that depend on antibody may be particularly important in the nervous system, where limited expression of MHC antigens may restrict the role of T lymphocytes (283,314). The disappearance of infectious virus is rapid, whereas the decline in viral RNA occurs more slowly (314). After recovery from encephalitis, viral RNA remains detectable in the CNS for life. Therefore one consequence of a nonlytic mechanism for clearance of virus from tissue is that the virus genome is not completely eliminated if the originally infected cells survive (312,313). This leads to a need for long-term control of virus replication that is accomplished in part by infiltration of antibody-producing B cells into the CNS that remain present for the life of the animal (189,191,192,576,577).

Antibody also is important for protection from infection (390). Inactivated vaccines protect against EEE, VEE, and WEE (448,449). Passive transfer of antibody before infection can protect from fatal disease (195,502). Mammals retain seropositivity for long periods after infection (72) and thus are protected from reinfection, but antiviral antibody may be more transient in birds (351).

Cellular Immunity

Alphavirus infection induces virus-specific lymphoproliferative, cytokine, and cytotoxic T-lymphocyte responses (3,68,194,330,355,383). The importance of these cellular immune responses for recovery or their potential contribution to fatal disease has not been established. After epidermal virus inoculation, Langerhans cells increase expression of MHC class II antigens, as well as accessory and costimulatory molecules that enhance activation of naive T cells (267). The mononuclear inflammatory process in alphavirus encephalitis is immunologically specific (364) and includes infiltration of natural killer (NK) cells, CD4 and CD8 T lymphocytes, B cells, and macrophages (118,244,382,393,478). Relative proportions of these mononuclear cells vary with the time after infection (244,382). T cells probably play some role in virus clearance *in vivo* because viral RNA decreases more rapidly when CD8 T cells are present (77). VEEV-specific T lymphocytes can inhibit virus replication *in vitro,* and RRV-specific CD8 T cells can clear virus from persistently infected macrophages (281,330).

Pathologic Changes

Encephalomyelitis

Pathologic changes in the CNS of humans with fatal neurologic disease and mice with experimentally induced encephalomyelitis begin with infiltration of mononuclear and occasional polymorphonuclear inflammatory cells into perivascular regions (196,364,382,409). This phase often includes perivascular extravasation of red blood cells and endothelial cell swelling and hyperplasia (409). Lymphocytes and monocytes then move from the perivascular regions to infiltrate areas of the parenchyma that contain virus-infected neurons. This inflammatory process is accompanied by

gliosis and evidence of inflammatory and glial cell apoptosis (171). Neonatal mice and human infants may die with widespread virus-induced neuronal cell death before the inflammatory process, a manifestation of the cellular immune response, can be initiated (319,400).

Neuronal cell death may be apoptotic, particularly in young animals (319), or may be characterized by cytoplasmic swelling, vacuolation, membrane breakdown, and cellular degeneration, suggesting necrosis (171,212,400). Demyelination has been described as a consequence of EEEV and WEEV infection in humans (28,409,410) and of WEEV, RRV, and SFV infection of mice, probably as a result of infection of oligodendrocytes (54,505).

Reticuloendothelial Infection

The pathology of VEE in horses includes cellular depletion of bone marrow, spleen, and lymph node tissue, and pancreatic necrosis (289). Small mammals also develop widespread necrosis of reticuloendothelial system tissues and may develop ileal necrosis (20,181,587,597). Leukopenia is commonly observed during infection (385).

Arthritis

Synovial fluid from patients with chronic RRV-induced arthritis contains increased protein, CD4 T lymphocytes, and abundant activated macrophages (94,157). Complement levels are normal, and immune complexes are not present (158). Little information is available on the pathologic changes in affected joints because the disease is not fatal in humans, and animal models for alphavirus-induced arthritis have not been developed.

Rash

Skin biopsies taken from patients with RRV-induced rash show perivascular infiltration of lymphocytes (primarily CD8 T cells) and monocytes. There is no evidence of immune complex deposition (160).

Release and Transmission

A common feature of alphaviruses is their transmission by mosquitoes and maintenance in a natural cycle of replication in vertebrate and invertebrate hosts. Mosquitoes become infected by feeding on a viremic host, are able to transmit the virus 4 to 10 days later (external incubation), and remain persistently infected. Maintenance of this cycle requires an amplifying host that develops a viremia of sufficient magnitude to infect feeding mosquitoes. For many alphaviruses, humans are dead-end hosts, unable to infect mosquitoes efficiently (289). However, there is evidence that human–mosquito–human transmission has been important in epidemics of RRV, ONNV, and CHIKV polyarthritis (337,485,560), and that horse–mosquito–horse transmission is important in epizootics of VEE (466,547).

Other modes of transmission occasionally are important. Horses infected with VEEV may shed virus in nasal, eye, and mouth secretions as well as in urine and milk (76,288). In this situation, VEEV also can be transmitted by the respiratory route. Aerosol transmission of VEEV and CHIKV has occurred in laboratory settings (76,307,557,564). EEEV persists in the feather follicles of infected pheasants, and transmission among penned pheasants has been documented to occur through feather picking and cannibalism (488). Person-to-person transmission has not been documented (307,466).

Veterinary Correlates and Animal Models

The first alphaviruses to be cultured, WEEV, EEEV, and VEEV, came to the attention of virologists because they caused fatal disease in horses; these viruses remain important equine pathogens (117,289,376,442,556). EEEV and WEEV cause encephalitis in horses, whereas VEEV causes severe respiratory disease associated with leukopenia; encephalitis may or may not be present. Getah virus causes an urticarial rash and hind-leg edema in horses (508). WEEV, EEEV, and HJV cause disease in domesticated birds such as chickens, pigeons, pheasants, turkeys, and emus (a species exotic to the Americas) (21,110,146,152,200,336,578,594,614). The alphaviruses associated with arthritis in humans have not been recognized as important causes of disease in domestic animals, although isolated cases are occasionally reported (170).

Good small-animal models exist for the encephalitogenic alphaviruses but are less satisfactory for study of the arthritogenic alphaviruses. In mice, alphaviruses generally infect lymphatic tissue, muscle, brown fat, brain, and spinal cord, but the extent and relative importance of infection at these sites differs among these viruses. For instance, RRV and Getah cause primarily myositis, VEEV causes reticuloendothelial infection, and WEEV and EEEV cause encephalitis (8,380,399). In mice infected with relatively avirulent strains of SFV, RRV, and VEEV, the acute encephalitic phase is accompanied by infection of oligodendroglial cells and demyelination (54,505). For all alphaviruses, fatal infection in mice is usually associated with CNS infection, even if encephalitis is not a manifestation of the human infection. For instance, SINV infection of mice is studied as a model for acute viral encephalitis, although SINV causes arthritis and rash, not encephalitis in humans (246,347,575). Specifics of these animal model systems are discussed with the individual viruses.

Virulence

Virulence is a measure of the ability of the virus to cause fatal disease, and for alphaviruses, this usually reflects the severity of neurologic disease (370). Outcome is influenced by the host, with most showing an age-dependent susceptibility to disease (Fig. 7) (8,45,203,389,399,417,503). Resistance increases with maturation and is associated with decreased virus replication in tissues at the site of virus inoculation and in target tissues (e.g., brain) and not with changes in the ability of infected mice to mount a virus-specific immune response (12,24,190,197,389,399). Avirulent alphavirus strains may replicate poorly even in newborn animals, whereas virulent strains can usually cause disease in adult as well as newborn animals. The ability of a virus strain to cause fatal disease or a particular complication of

infection also is often dependent on the genetic background of the host (123,534,562,568).

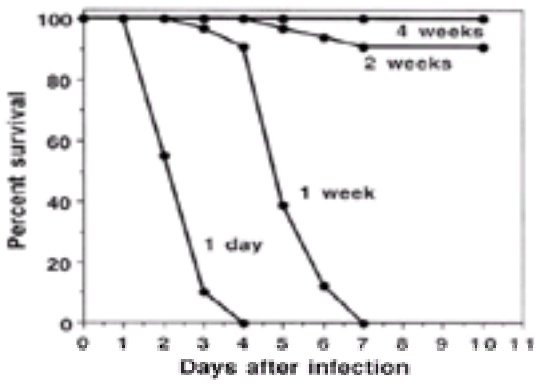

FIG. 7. Age-dependent susceptibility of mice to fatal disease after infection with the AR339 strain of Sindbis virus (SINV). Reprinted from ref. 196, with permission.

For encephalitic alphaviruses, another component of virulence is their ability to enter the CNS efficiently (neuroinvasiveness). Many alphavirus strains can cause fatal disease after intracerebral or intranasal inoculation, but not after subcutaneous or intraperitoneal inoculation. The time course of virus clearance from the blood often correlates with virulence, virulent strains being cleared more slowly than avirulent strains (250,251,253,256). The determinants of peripheral replication, viremia, neuroinvasiveness, and neurotropism (ability to replicate in CNS cells) all contribute to virulence and are likely to be influenced by different molecular characteristics of the virus.

The viral determinants of virulence have been most extensively studied in murine models for SINV, SFV, RRV, and VEEV infections. Viruses with altered virulence have been selected after chemical mutagenesis (26,50,423), by passage in tissue culture (32,108,263,280,553), by passage in mice (196,370,553), by isolation of MAb escape mutants (593) or plaque variants (249), and by manipulation of cDNA virus clones (108,344,550,567,570). Nucleotide and amino acid changes affecting virulence have been mapped to the 5' nontranslated region (NTR), nsP2, nsP3, E1, and E2. Specifics are covered in the sections dealing with each of these viruses.

Persistence

There is substantial evidence that alphaviruses can persist after appearance of an immune response, clearance of virus from the circulation, and apparent clearance of virus from tissue (313). Several progressive cases of WEEV in humans have been reported, and pathologic examination of CNS tissue months to years after resolution of acute encephalitis has shown an active inflammatory process (408,409). Viral RNA and proteins can be detected in the nervous system long after recovery of mice from SINV- or SFV-induced encephalitis (123,281,312,313,577). It is postulated that this persistence of RNA is due to failure of the virus or the immune system to eliminate the infected cells. Interestingly, passive antibody protection against fatal encephalitis predisposes to persistent infection and the late onset of progressive disease (502).

Congenital Infection

Alphaviruses can be transmitted transplacentally. This has been documented in mice for RRV, SFV, VEEV, and Getah virus (2,16,18,302,378,529) and in humans for RRV, WEEV, and VEEV (5,98,369,512). In mice the virus infects the placenta, where it is able to persist and spread to the fetus despite the development of maternal antibody. The outcome of fetal infection is dependent on the timing of infection relative to transfer of maternal antiviral IgG to the fetus. Fetuses are protected if transfer occurs before infection, but transfer of antibody after infection does not mediate recovery (378). In monkeys, congenital infection with VEEV induces malformations of the brain and eye (332). In humans, no abnormalities were observed in infants infected with RRV at 11 to 19 weeks of gestation; however, earlier infection may lead to fetal death (5). Epidemics of VEE are associated with increases in spontaneous abortion (466,609).

PATHOGENESIS AND PATHOLOGY IN MOSQUITOES

The ability of alphaviruses to infect mosquitoes efficiently with spread to and replication in the salivary glands is essential for maintaining the natural cycle of transmission. Not all mosquitoes taking a blood meal from a viremic host will become infected, and not all infected mosquitoes develop salivary gland infection and the ability to transmit the virus. Many alphaviruses preferentially infect a narrow range of mosquito species, and this host specificity plays an important role in determining the geographic distribution of the virus. Even within a species, strains of mosquitoes may vary in susceptibility to alphavirus infection. For instance, strains of *Ae. albopictus* from different geographic regions differ in susceptibility to infection with CHIKV and in the amount of virus produced after infection (559), and field and laboratory populations of *Culex tarsalis* differ in susceptibility to WEEV (235). Strains of virus also differ in their abilities to infect mosquitoes, and laboratory-adapted strains may establish infection relatively inefficiently (500,501). The extrinsic incubation period, or time between taking an infected blood meal and ability to transmit infection, is dependent on the rapidity of virus replication and dissemination to the mosquito salivary glands. This period is relatively short (2–7 days) for alphaviruses compared with other arboviruses (500). Studies of the pathogenesis of alphavirus infection of invertebrates are few compared with studies in vertebrates. The most extensive studies are of EEEV infection of its enzootic vector *Culiseta melanura* and of WEEV infection of its vector *Cx. tarsalis.*

Entry and Sites of Primary Replication

Posterior midgut epithelial cells are the initial site of infection (498,610), and infection is facilitated when virus in the serum is concentrated next to these cells as the blood meal clots (Fig. 8) (612). Susceptibility of mosquitoes to alphavirus infection is probably determined in large part by the ability of the virus to bind and infect these epithelial cells (235,500). Changes in both the virus and the vector can affect this interaction (632). Infection is often established more efficiently when mosquitoes are reared at low temperature (372,456). WEEV rapidly fuses with microvillar membrane preparations from *Cx. tarsalis* mosquitoes, and both WEEV and CHIKV bind better to membranes from susceptible mosquitoes than to membranes from refractory mosquitoes (235,394). A receptor for VEEV and SINV on the surface of the C6/36 line of larval *Ae. albopictus* cells is the high-affinity laminin receptor (335,599), and proteins of 60 and 38 kd have been identified as putative receptors for CHIKV on brush-border membranes from *Ae. aegypti* (394).

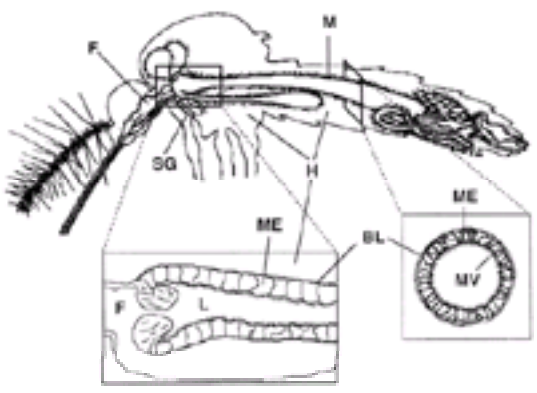

FIG. 8. Diagram of mosquito internal anatomy showing the essential sites for alphavirus infection. *BL*, basal lamina; *L*, lumen; *F*, foregut; *M*, midgut; *ME*, midgut epithelium; *MV*, microvilli or brush border; *SG*, salivary gland. Adapted from ref. 611, with permission.

The efficiency of alphavirus entry is affected by the levels of cell-membrane cholesterol (334,357). Mosquitoes, like other insects, obtain sterol needed for reproduction and development from dietary blood. Cholesterol-independent mutants of SFV replicate better than do parental SFVs in cholesterol-depleted C6/36 cells and in adult *Ae. albopictus* mosquitoes (10).

Spread

For the mosquito to become capable of transmission, virus must reach and replicate in the salivary glands (see Fig. 8). For most alphaviruses, spread beyond the midgut to other tissues is through the hemolymph (500). Virus buds primarily from the basolateral surface of the infected midgut epithelial cells and accumulates next to the basal lamina (500), a layered structure composed of mucopolysaccharide, which acts as a barrier to hemocoel entry. Infected midgut epithelial cells often degenerate and slough 36 to 48 hours after infection, and this process may facilitate penetration of the virus into the hemocoel (500,611).

Infection of salivary gland acinar cells from the hemolymph requires that the virus again traverse a basal lamina, a process that may depend primarily on hemolymph titer. The fat body is an important site for virus amplification (602,610). EEEV infects the midgut, fat body, muscle, and salivary glands without involving the nervous system or the ovarioles (498,610), whereas VEEV infects the nervous system (602), and SINV infects respiratory tissue (42).

Once salivary gland infection is established, virus matures by budding into apical cavities or randomly into vesicles, basolaterally and apically. This process may be associated with cytopathic changes in the salivary glands (379). The rapidity of virus growth and dissemination to the salivary glands is dependent on the ambient temperature. Higher temperatures accelerate the transmission cycle in warm months (458,571,572). This can be as short as 2 to 3 days (500). In general, virus content of an individual mosquito reaches peak values within 4 to 7 days after infection (379,500).

For transovarial transmission to occur, virus must be able to infect oocytes early in development. Failure to infect ovarioles regularly precludes efficient transovarial transmission of most alphaviruses (42,114,245,498,602). Eggs also can be infected after they have been fertilized. Low levels of vertical transmission have been documented in the laboratory for RRV in *Ae. vigilax* and for SINV in *Ae. australis* (424). Vertical transmission in nature has been reported for RRV, SINV, and WEEV (114,165,327,455).

Pathology, Persistence, and Host Response

Although generally considered to be benign for the invertebrate vector, infection may reduce survival and reproductive capacity (499,500). Mosquitoes remain infected and infectious for life (379,500). In the fat body and salivary glands, infection is persistent, although titers decline after the acute phase of infection, decreasing efficiency of transmission (498). Cultures of cells that become persistently infected also produce large amounts of virus initially, and then 1,000-fold lower amounts coincident with the production of an antiviral protein (105,463). The antiviral protein is a small (3 kd) hydrophobic peptide that inhibits cell proliferation and induces an antiviral state (96,341,463). Cultures can be cured by frequent media changes or by addition of antiviral antibody (242,462). Clones of mosquito cells differ with respect to virus replication and production of and response to the antiviral protein. It has been suggested that these biologically different clones are representative of cells from different tissues in the mosquito. Some tissues (e.g., fat bodies) produce large amounts of virus, whereas others (e.g., head ganglia) produce small amounts, and others (e.g., ovarioles and malpighian tubules) remain virus negative (377).

ALPHAVIRUSES ASSOCIATED PRIMARILY WITH ENCEPHALITIS

Eastern Equine Encephalitis

EEE was first recognized as a disease of horses in the northeastern U.S. In the summer of 1831, more than 75 horses died in three coastal counties of Massachusetts (208). Between 1845 and 1912, epizootics were recorded on Long Island and in North Carolina, New Jersey, Florida, Maryland, New Jersey, and Virginia (208,500). The virus responsible for EEE was first isolated in 1933 from the brains of affected horses in New Jersey and Virginia during a widespread outbreak that also involved coastal areas of Delaware and Maryland (556). Although human cases were suspected earlier, it was not until 1938 that an outbreak in the northeastern U.S. resulted in 30 cases of fatal encephalitis in children living in the same areas as equine cases. At that time EEEV was isolated from the CNS of humans (153,613), as well as from pigeons (152) and pheasants (578a).

Based on seasonality, location of cases near salt marsh areas, lack of evidence of transmission from horse to horse through contact, short period of equine viremia, and geographic distribution of cases, Ten Broeck et al. (555) postulated that insects transmitted infection and that birds were likely to be the reservoir host. The first arthropod isolates were actually from chicken mites and lice, which can transmit infection only inefficiently (132,500). Transmission of EEEV by *Ae. sollicitans* in the laboratory was accomplished in 1934 (374), and subsequently multiple *Aedes* species were shown to be competent vectors (109), but recovery of EEEV from naturally infected mosquitoes did not occur until 1949, with isolates from *Mansonia perturbans* in Georgia (239) and *Culiseta melanura* in Louisiana (83). Subsequent work showing the competence of *Cs. melanura* and a consistent association between infected birds and the isolation of virus from this vector has led to the current understanding that *Cs. melanura* is the primary enzootic vector for EEEV in North America (103,236,305,489,500).

Epidemiology

EEEV causes localized outbreaks of equine, pheasant, and human encephalitis in the summer. EEEV is enzootic in North America from New Hampshire southward along the Atlantic seaboard and Gulf Coast to Texas, in the Caribbean, and in Central America. Inland foci exist in the Great Lakes region and South Dakota (392). EEEV also is enzootic along the north and east coasts of South America and in the Amazon Basin, but human infections in these regions cause mild or subclinical disease (Fig. 9) (77). In North America, the primary enzootic cycle is maintained in shaded swamps, where the vector is the ornithophilic mosquito *Cs. melanura* (Fig. 10) (489,500). The enzootic vector in the Caribbean is probably *Cx. taeniopus* (147,596). In South and Central America, the enzootic cycle is maintained in moist forests where *Culex* (*Melanoconion*) spp. appear to be the primary vectors (500).

FIG. 9. Geographic distribution of eastern equine encephalitis (EEEV). The map shows the locations of the isolation of viruses from the four recognized EEEV lineages. Reprinted from ref. 608, with permission.

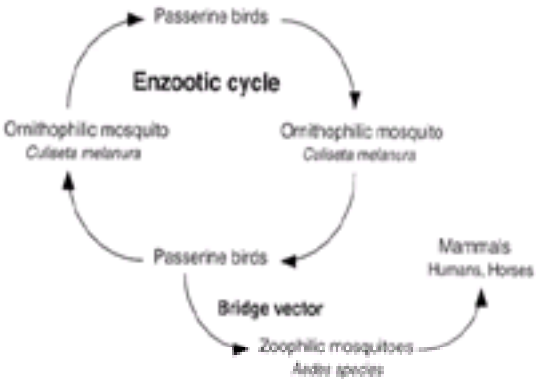

FIG. 10. Enzootic and epidemic/epizootic cycles of eastern equine encephalitis (EEEV) as an example of an alphavirus that uses a species of mosquito with different host feeding preferences to bridge from the enzootic avian–ornithophilic mosquito cycle to infect mammals such as humans and horses. Similar mechanisms are operative in transmission of Sindbis virus to humans.

Birds are the primary reservoir host, and many species are susceptible to infection, but often remain asymptomatic despite prolonged viremia (289). The amplifying species for EEEV in North America are wading birds, migratory

passerine songbirds, and starlings (103,295,368). Young birds in general are probably important for virus amplification because they are more susceptible to infection, have a prolonged viremia, and are less defensive toward mosquitoes (103). In Central and South America, forest-dwelling rodents, bats, and marsupials are frequently infected and may provide an additional reservoir, but these transmission cycles are not well characterized (500,579). Infection also has been reported in reptiles and amphibians (392).

The mechanism of virus overwintering in temperate areas is not known. The virus may be maintained by resident birds or reintroduced annually by migratory viremic birds or wind-borne infected mosquitoes coming from subtropical areas of year-round transmission (507,608). There is no convincing evidence for overwintering in mosquitoes, although laboratory evidence of transovarial transmission by *Cq. perturbans* has been obtained (392). Ovarioles are not involved during experimental infection of *Cs. melanura* (498).

Morbidity and Mortality

EEEV is the most virulent of the encephalitic alphaviruses, causing a high mortality due to encephalitis. Most cases are associated with exposure to wooded areas adjacent to swamps and marshes (60,392), and approximately equal numbers occur in female and male subjects (144). Children younger than 10 years are most susceptible (144), with one in eight infections in children resulting in encephalitis compared to one encephalitis case in 23 infections in adults (179). The case fatality rate was 60% to 70% in earlier studies and 30% to 40% in more recent studies, with the highest rates in children and the elderly (60,111,141,144,445).

Origin and Spread of Epidemics

In the northern part of the North American range, human and equine cases of EEE occur between July and October, whereas in the southern region, cases can occur throughout the year (445). In the Caribbean, outbreaks appear to be linked to virus introduction by southbound migratory songbirds (500). In North America, human and equine cases usually occur within 5 miles of swamps maintaining enzootic transmission. Outbreaks are initiated when the virus spreads from the enzootic cycle involving ornithophilic mosquitoes into mosquito populations that feed on a variety of hosts (see Fig. 10). Multiple species of mosquitoes with catholic feeding habits such as *Cq. perturbans, Ae. sollicitans, Cx. salinarius, Ae. canadensis, Ae. vexans, Anopheles quadrimaculatus,* and potentially *Ae. albopictus* can act as bridge vectors in different regions (381,500,586).

Cases of equine encephalitis are usually the first indication of an outbreak. Epizootics appear approximately every 5 to 10 years and are associated with heavy rainfall and warmer water temperatures that increase the populations of enzootic and epizootic mosquito vectors (182,310,350,392). Most of the epidemics have occurred in Massachusetts and surrounding states (144), but the largest outbreak on record occurred in 1947 in Louisiana and Texas, with 14,344 cases of equine encephalitis and 11,722 horse deaths (87). Since 1955, an average of seven cases of EEE have been diagnosed in humans in the U.S. each year (Fig. 11) (60). There is no evidence that horses or humans are important in the transmission cycle during epizootics (290).

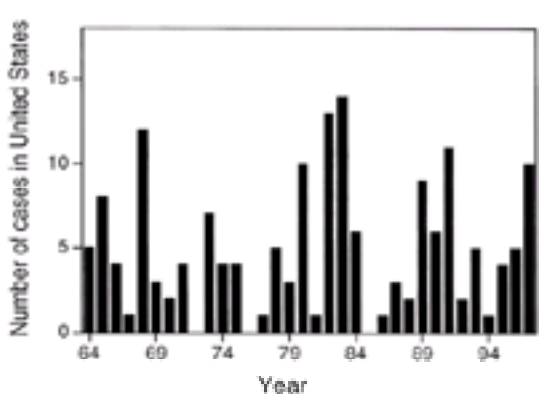

FIG. 11. Numbers of human cases of eastern equine encephalitis reported annually in the United States since 1964. Data from the Centers for Disease Control and Prevention, with permission.

Molecular Epidemiology

Antigenic differences between North and South American strains of EEEV have long been recognized (58,75), and the strains can be distinguished by reactivity with MAbs to the E1 glycoprotein (471). Sequence comparisons indicate that EEEV has evolved independently in North and South America over the last 1,000-year period. Currently one variety of EEEV is recognized in North America and the Caribbean, and three varieties are recognized in South America: one in the Amazon basin, one on the coasts of South and Central America, and a third in Brazil (46,608) (see Fig. 9). North American isolates are highly conserved, differing over a large geographic range by less than 2% in nucleotide sequence over a 63-year period, providing a calculated yearly nucleotide substitution rate of 1.6×10^{-4} (46,608). South American viruses comprise several genotypes, which differ by up to 25% in nucleotide sequences and are evolving at a faster rate (4.3×10^{-4} substitutions/nucleotide/year) (604). The South American groups are estimated to have diverged about 450 years ago and may be evolving locally within different vector–host relationships (604,608). Restriction digestion patterns of PCR products from the E1 gene and 3' NTR can distinguish these EEEV strains and provide a rapid tool for screening specimens (543).

Clinical Disease and Pathology

Illness may consist only of 1 to 2 weeks of fever, chills, malaise, and myalgias, followed by recovery (392). In cases of encephalitis, prodromal symptoms of fever, headache, and myalgias are common. The onset of encephalitis tends to be fulminant and is associated with continued fever, increased headache, vomiting, restlessness, irritability, seizures, obtundation, and coma (111,141,144,211,392). Meningismus is frequent, and focal signs including cranial nerve palsies and hemipareses are not uncommon (111,445). Hyponatremia due to inappropriate secretion of antidiuretic hormone is a common complication, and edema of the face and extremities a few days after onset has been noted (111,141). Death typically occurs within 2 to 10 days after onset of encephalitis (392).

CSF examination is almost always abnormal. Pressure is increased, and white cell counts range from 10 to 2,000/µL. Polymorphonuclear leukocytes are often abundant early in disease, with a shift to mononuclear cells over the first few days of illness (111,392). The presence of red blood cells or xanthochromia is not uncommon (445). CSF protein levels are increased, whereas glucose is low to normal (141,445). Electroencephalographic (EEG) findings are relatively nonspecific, usually showing slowing (111,445). Computed tomography (CT) scans may be normal or show only edema (111,445). Magnetic resonance imaging scans are more often abnormal, with focal lesions most commonly observed in the thalamus, basal ganglia, and brainstem (111).

Poor outcome is predicted by high CSF white cell count or severe hyponatremia, but not by the size of the radiographic lesions (111). Recovery is more likely in those individuals with a long (5- to 7-day) prodrome and without coma (445). Sequelae, including paralysis, seizures, and mental retardation, are common, with 35% to 80% of survivors, particularly children, having significant long-term neurologic impairment (111,141,144).

Histopathology on fatal cases demonstrates a diffuse meningoencephalitis, with widespread neuronal destruction, perivascular cuffing with polymorphonuclear as well as mononuclear leukocytes, and vasculitis with vessel occlusion in the cortex, basal ganglia, and brainstem. The spinal cord is frequently spared (141,153). Virus antigen is localized to neurons, and neuronal death is marked by cytoplasmic swelling and nuclear pyknosis (171). Apoptotic glial and inflammatory cells are frequently found in the regions of affected neurons (141,171,392).

Veterinary Correlates, Host Range, and Animal Models

EEEV is an important cause of disease in horses, pheasants, emus, and turkeys in North America and has been reported to cause encephalitis in dogs and pigs as well (110,141,152,201,336). Horses develop encephalitis with signs of depression, progressive incoordination, seizures, and prostration (392). The case fatality rate is 80% to 90%. Most survivors are left with neurologic sequelae (500).

Birds vary in their susceptibility, with some birds developing disease, while many others show no morbidity or mortality despite a prolonged viremia (289,336). In general, birds, even with fatal disease, do not develop encephalitis (289). Chickens, turkeys, emus, and whooping cranes have viscerotropic involvement with multifocal necrosis in the heart, kidney, and pancreas and lymphoid depletion in thymus, spleen, and bursae (110,201,392,578). Pheasants are an exception and develop encephalitis with a 50% to 70% mortality (336).

Laboratory studies of monkeys, mice, guinea pigs, and hamsters confirm the neurovirulence of EEEV for mammals (125,153,331,389,556,635). Newborn mice show extensive neuronal damage and rapid death (400). At age 3 to 4 weeks, mice become relatively resistant to peripheral, but not intracerebral, inoculation of virus (331,389). Hamsters develop encephalitis, hepatitis, and lymphatic organ infection (125).

Virulence

EEEV strains vary in their pathogenicity, with the South American strains being less virulent than the North American strains in experimental animals (500). Human encephalitis has not been recognized in South America (77), but occurs regularly in North America. Viruses with a temperature-sensitive, small-plaque phenotype and decreased virulence for mice and hamsters have been selected after chemical mutagenesis (49,50,125). The molecular bases of these differences in virulence between strains of EEEV have not yet been defined.

Diagnosis and Prevention

Diagnosis is based on virus isolation or detection of antibody. Virus can be isolated from CSF, blood, or CNS tissue by inoculation into newborn mice or onto a variety of tissue-culture cells, most commonly CEF or Vero cells (528). Antibody is usually measured by enzyme immunoassay (EIA) (500), with detection of IgM in serum and CSF particularly useful (61,62).

Infection in mosquito populations can be monitored by virus isolation, by reverse transcription–polymerase chain reaction (RT-PCR), or by seroconversion of sentinel pheasants or chickens. This information can be used to guide insecticide spraying to reduce adult and larval mosquito populations and prevent human cases (15,182). A formalin-inactivated vaccine derived from a North American strain of EEEV (PE-6) is available for horses and emus and for investigational use in humans to protect laboratory workers. This vaccine does not induce significant neutralizing or anti-E2 antibody to South American strains of EEEV (542).

Western Equine Encephalitis

Epizootics of viral encephalitis in horses were described in 1908 in Argentina, and in 1912, 25,000 horses were estimated to have died in the central plains of the U.S. (481). In the summer of 1930, a similar epizootic occurred in the San Joaquin Valley of California, causing an estimated 6,000 cases of equine encephalitis. During this outbreak, WEEV was isolated from the brains of two affected horses by intraocular inoculation of another horse, and this virus was subsequently used to infect a variety of experimental animals (376). WEEV was suspected at that time to be a cause of

human as well as equine encephalitis (375), and in 1938, WEEV was recovered from the brain of a child with fatal encephalitis (237).

Mosquitoes were implicated in disease transmission when it was demonstrated that horses developed a viremia (238) and that experimentally infected *Ae. aegypti* mosquitoes were capable of transmitting the virus to horses (275). However, it was not until the summer of 1941 during a widespread epidemic in the northern plains of the U.S. and Canada that the virus was isolated from naturally infected *Cx. tarsalis* mosquitoes (207). Subsequently, WEEV was isolated from *Cx. tarsalis* in other epizootic sites (104) and recognized to be the principal vector.

Other New World WEEV Complex Viruses

The WEE complex in the New World includes three viruses in addition to WEEV: Highlands J (HJ), Fort Morgan (FM), and Aura viruses (64). These viruses have different ecologic niches and vary in virulence. Only WEEV is recognized to cause human disease (60). Viruses in the WEE complex found in the Old World (e.g., Sindbis, Whataroa, and Kyzylagach viruses) are discussed in the section on Sindbis virus. WEEV, HJV, and FMV are all of the lineage that diverged since recombination between a Sindbis-like virus and EEEV (205). Aura virus is a “prerecombinant” virus (see Fig. 3).

Highlands J Virus

WEEV-like viruses were first isolated in the eastern part of the U.S. in 1952, and the prototype HJV was isolated in 1960 from a blue jay in Florida (225). HJV is enzootic on the East Coast of the U.S. and is maintained in a cycle similar to that of EEEV, with *Cs. melanura* the primary vector (489) and migrating birds the primary reservoir (103). HJV was initially thought to be a variant of WEEV, and it is now recognized that all alphaviruses in the WEEV complex isolated in the eastern U.S. are strains of HJV (216). Rates of divergence of WEEV and HJV of 0.1% to 0.2% per year have been estimated (95). HJV can occasionally cause encephalitis in horses (270), but is a recognized pathogen for turkeys, pheasants, partridges, ducks, emus, and whooping cranes (146,200,201,594,608).

Fort Morgan Virus

FMV and its close relative, Buggy Creek virus, were isolated from swallows, sparrows, and swallow nest bugs in eastern Colorado (67) and Oklahoma (233). FMV is not recognized as a pathogen.

Aura Virus

Aura virus was isolated in 1959 in Brazil from *Culex* (*Melanoconion*) spp. collected near the Aura River and later from *Ae. serratus* in Brazil and Argentina (78). This virus is likely to be related to the “Sindbis-like” virus that recombined with EEEV to produce WEEV (479). It has not been linked to human disease and is relatively nonpathogenic for mice (78,479).

Epidemiology

WEEV is widely distributed in the western plains and valleys of the U.S. and Canada and in South America. In North America, WEEV is maintained in an endemic cycle involving domestic and passerine birds (particularly finches and sparrows) and *Cx. tarsalis,* a mosquito well adapted to irrigated agricultural areas (60). Occasional isolations have been made from *Ae. melanimon* and *Ae. dorsalis,* also competent vectors. There is substantial evidence for a transmission cycle involving *Ae. melanimon* and the blacktail jackrabbit (60,209) in addition to the *Cx. tarsalis* avian cycle. Serosurveys and virus isolations provide evidence of natural infection in chickens and other domestic birds, pheasants, rodents, rabbits, ungulates, tortoises, and snakes (60,209). In some areas of South America, most mosquitoes from which WEEV has been isolated feed primarily on mammals, and antibodies are common in small mammals including rats and rabbits, whereas in other areas, antibodies are found primarily in birds (514,608). The mechanism of overwintering of WEEV in endemic areas is unclear. Interseasonal persistence can occur in salt-water marshes, where vertical transmission of WEEV in *Cx. tarsalis* has been demonstrated. These mechanisms would allow intermittent virus dissemination into regions without evidence of WEEV activity for substantial periods (455,457).

Morbidity and Mortality

WEEV in the western U.S. has caused epidemics of encephalitis in humans, horses, and emus, but the case fatality rate of 10% for humans, 20% to 40% for horses, and 10% for emus is lower than that for EEE (21). Increased risk is associated with residence in a rural environment (308,333). In North America, cases are seasonal, with the largest numbers of cases in July and August (333). In older children and adults, male subjects are 2 to 3 times more likely to develop disease than are female subjects (333). The estimated case-to-infection ratio is 1:58 in children younger than 5 years and 1:1,150 in adults (60). Clinically apparent disease is most common in the very young and those older than 50 years (333). Severe disease, seizures, fatal encephalitis, and significant sequelae are more likely to occur in infants and in young children (133,149,294). The overall case fatality rate is 3%, but increases to 8% in those older than 50 years (333).

Seroprevalence in humans was 34% in rural areas of California endemic for WEEV in 1960 (164) and 1.3% to 2.6% in similar areas in 1993 through 1995 (453,454). An average of 34 human cases of WEE occurred per year in the U.S. from 1955 to 1984, but have steadily declined in frequency since that time (Fig. 12). No human cases of WEE have been reported in the U.S. since 1994.

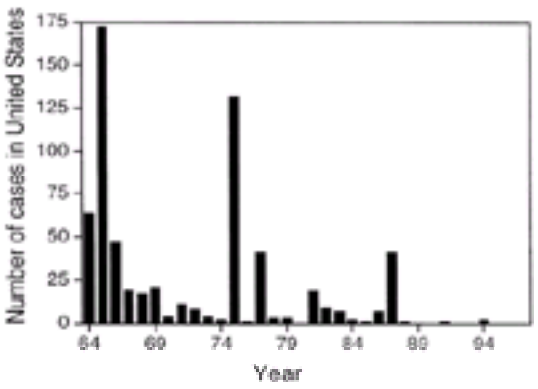

FIG. 12. Numbers of human cases of western equine encephalitis reported annually in the United States since 1964. Data from the Centers for Disease Control and Prevention, with permission.

The lack of significant human disease during equine outbreaks of WEE in South America may be related to the feeding habits of the vector or to a difference in virulence for humans and horses of South American strains of WEEV (481).

Origin and Spread of Epidemics

Periodically, large, often widespread, epidemics of equine encephalitis have occurred from mid-June to late-September in North America, with significant spillover into the human population. Typically, in an epidemic season, equine cases appear before cases in humans (442). Major epizootics occurred in the U.S. in 1931, 1933 through 1935, 1937, 1938, 1941, 1944, 1947, 1950, and 1952. In 1941, more than 3,400 human WEE cases were reported in the plains of the western U.S. and Canada, with attack rates up to 167 cases/100,000 population (60). During the 1952 epidemic in California's Central Valley, attack rates were 36 cases/100,000 humans and 1,120/100,000 equids (60).

These outbreaks correlated with increases in the population densities of *Cx. tarsalis* in the region (420). Large equine WEEV epizootics are separated by silent periods without apparent WEEV activity. Studies of differential virulence of epizootic and enzootic strains first suggested that epizootic strains arise from nonpathogenic enzootic strains (33). Sequence analysis of the viruses found at the initial focus of the 1982 WEE epizootic in Argentina indicated that the enzootic virus was the source of a virulent variant, which emerged by mutation or selection to cause the epizootic. The epizootic vector in South America is *Ae. albifasciatus,* and rabbits may serve as an amplifying host (608).

Molecular Epidemiology

WEEV has four major lineages, two in South America and two widely distributed in North and South America and the Caribbean (606,608). One was isolated during the 1982 to 1983 epizootic in Argentina; a second is related to the McMillan strain found in North and South America and Cuba from 1930 to 1972; a third was found in the same regions from 1964 to 1993; and a fourth was found in Brazil and Argentina (608). Within California, separately evolving lineages have been identified in the Central Valley and the southern part of the state (299).

Clinical Disease and Pathology

WEEV causes encephalitis with signs and symptoms similar to those of EEE. There is a 3- to 5-day prodrome of fever and headache that may progress to restlessness, tremor, irritability, nuchal rigidity, photophobia, and altered mental status (149,294,308). Paralysis is not uncommon (294,369). Infants often present with rigidity, seizures, and a bulging fontanel (149,308,369). Transplacental transmission results in perinatal infection manifesting within the first week of life as fever, failure to feed, and seizures (98,369,512). CSF pleocytosis is typical, with 100 to 1,500 cells/µL. Neutrophils are present early in disease, and mononuclear cells later (308,369).

In infants younger than 1 year, approximately 60% of survivors have brain damage, and in some, the disease is progressive (369,410). Common problems are mental retardation associated with quadriplegia, spasticity, recurring seizures, cortical atrophy, ventricular dilatation, and intracranial calcification (133,149,410,527). In older individuals, recovery is typically rapid, with remission of signs and symptoms within 5 to 10 days, and sequelae are less common (~5%) (149).

Pathology of acute cases of WEE shows leptomeningitis and perivascular cuffing with polymorphonuclear leukocytes in the earliest cases, and lymphocytes, plasma cells, and macrophages at later times. This inflammatory process is accompanied by endothelial hyperplasia, petechial hemorrhages, and glial nodules in the areas of neuronal degeneration. Lesions are found primarily in the basal ganglia, brainstem, cerebellum, cerebral cortex, and spinal cord (149,409). In addition, there are areas of focal necrosis and demyelination, particularly in the subcortical white matter and basal ganglia (409,410).

Occasionally in infants and children, there is pathologic evidence of progressive disease consistent with persistent infection (226,369,409,410). Individuals surviving months to years after onset of encephalitis (often with progressive disease) may have cystic lesions, gliosis, and demyelination, with areas of active mononuclear inflammation (226,409,410).

Veterinary Correlates, Host Range, and Animal Models

WEE was first recognized as a neurologic disease of horses, mules, and ponies characterized by fever, incoordination,

drowsiness, and anorexia, leading to prostration, coma, and death in approximately 40% of the affected animals (117,375). Emus also develop symptomatic, often fatal, disease characterized by ataxia, paralysis, and tremors (21). As a part of its enzootic cycle, WEEV infects sparrows, finches, blackbirds, mourning doves, pheasants, cowbirds, swallows, and chickens. The virus can cause fatal disease in sparrows, whereas chickens, which are often used as sentinels, develop asymptomatic infection (459). Mammals are less frequently involved, but bats, blacktail jackrabbits, gray squirrels, and ground squirrels become infected (579).

Experimentally infected newborn mice die within 48 hours after infection, with involvement of skeletal muscle, cartilage, and bone marrow. In weanling mice, brain, heart, lung, and brown fat appear to be the primary target tissues (8). After intracerebral inoculation, there is infection of the choroid plexus and ependyma, with subsequent spread to neurons and glial cells in the brain, cerebellum, and brainstem and to motor neurons in the spinal cord (331). After peripheral inoculation, WEEV replicates in skeletal muscles, causes a necrotizing myocarditis, and occasionally spreads to the CNS (331,386). Infection of hamsters with relatively avirulent WEEV strains leads to progressive neuropathologic changes consisting of perivascular inflammation, microcavitation, and astrocytic hypertrophy (641).

Virulence

Variants of WEEV with decreased pathogenicity for mice and hamsters (e.g., B628 clone 15) have been selected by passage on CEF cells (263,641). Large-plaque strains tend to be more virulent than do small-plaque strains (249). Epizootic strains appear to be optimized for viremia and neuroinvasiveness and are generally more virulent for mice and guinea pigs than are enzootic strains, and North American strains are more virulent than are South American strains (33,34,210). The molecular bases for any of these differences in virulence have not been defined.

Diagnosis and Prevention

Diagnosis can be made by detecting WEEV-specific IgM in serum (61), by isolating virus in mice or cultured cells, or potentially by RT-PCR with patient serum or CSF (432). An inactivated vaccine is available for horses and as an experimental preparation for laboratory workers (449).

Venezuelan Equine Encephalitis

In 1934 during an outbreak of equine encephalitis in the Guajira region of Venezuela, a virus (VEEV) was isolated from the brains of encephalitic horses. This virus was antigenically distinct from the viruses causing equine encephalitis in the eastern and western portions of North America (EEEV and WEEV) and became the third encephalitic alphavirus identified in the Americas (30,300). Between 1943 and 1963, VEE-related viruses were isolated and identified in many locations in South America, Central America, the Caribbean islands, and the southern regions of the U.S. (84,493). The first reported human cases of VEE were in laboratory employees working with these isolates (76,307).

Studies in Central America during this period indicated an enzootic cycle involving *Culex* (*Melanoconion*) spp. mosquitos and rodents (168,184,493). In 1969 an epizootic/epidemic of VEE appeared and spread over the next few years from Peru through Central America, Mexico, and into Texas, causing human disease and a high mortality among horses (287). Virus isolations during the epizootic were primarily from *Psorophora confinnis* and *Ae. sollicitans* mosquitoes and from horses, suggesting that the epizootic and enzootic transmission cycles differed (547).

The VEEV Complex Viruses

Using a short-incubation HI test, isolates of viruses related to VEEV were originally classified into subtypes I through IV (636). When Cabassou (CAB) and AG80-663 viruses were isolated and shown to be within the VEEV antigenic complex, they became subtypes V and VI, respectively (66,116). Currently the VEE complex includes six subtype viruses: VEE (subtype I), Everglades (EVE, subtype II), Mucambo (MUC, subtype III), Pixuna (PIX, subtype IV), CAB (subtype V), and AG80-663 (subtype VI). The VEE subtype I viruses have been further subdivided serologically into IAB, IC, ID, IE, and IF (66,636), and the MUC subtype III viruses, into IIIA, IIIB (Tonate), and IIIC (286). However, analysis of the phylogenetic relationships of the VEEV complex viruses gained from sequencing has led to a suggested reclassification of EVE (II) with IAB, IC, ID, and IE, and of IF with AG80-663 (VI) (Fig. 13) (285,372,444).

FIG. 13. Evolutionary relationships of all the antigenic subtypes and varieties of Venezuela equine encephalitis virus (VEEV) complex viruses based on E3-E2 nucleotide sequences. The dose relationships of subtypes IAB and IC epizootic prototype strains to the prototype enzootic subtype ID strain are apparent. Virus strains are listed by subtype and virus name or strain. Numbers indicate bootstrap values for groups to the right. Reprinted from ref. 444, with permission.

Epidemiology

Enzootic VEE complex viruses are involved in perennially active transmission cycles in subtropical and tropical areas of the Americas (e.g., EVE in Florida; VEE ID and IE in Central America; VEE IF, MUC, PIX, CAB, and AG 80-663 in South America). In endemic areas, mosquito isolates are made primarily from *Culex* (*Melanoconion*) spp. mosquitoes that live in tropical and subtropical swamps throughout the Americas and breed near aquatic plants (168,184,493). These mosquitoes feed at dawn and dusk on a wide variety of rodents, birds, and other vertebrates. Wild birds are susceptible to infection (82), but mammals, such as cotton rats and opossums, have been implicated as the most likely reservoir hosts by virus isolation and serology (184,493,596). Only VEEV causes significant disease in horses or humans, although there have been isolated case reports of clinically evident human infection with other VEE complex viruses (136,137).

Morbidity and Mortality

Humans living in areas of enzootic transmission have a high prevalence of antibody, but there has been little recognized disease in these populations (184,492). During epidemics, attack rates vary widely between regions [for instance, from 13% to 93% in different communities during the 1995 outbreak in La Guajira, Colombia (466)]. All ages and both sexes are equally susceptible to infection; however, disease manifestations vary with age (466). Individuals younger than 15 years are more likely to develop fulminant disease with reticuloendothelial infection, lymphoid depletion, and encephalitis. In older children and young adults, a relatively benign influenzal illness is most common (137). Individuals older than 50 years also are prone to develop encephalitis, but most recover (137). The incidence of encephalitis in clinically ill humans is generally less than 5%, and the mortality, less than 1% (466). Essentially all deaths occur in children.

Origin and Spread of Epidemics

VEE epizootics/epidemics have occurred at approximately 10- to 20-year intervals in cattle-ranching areas of Venezuela, Colombia, Peru, and Ecuador, when heavy rainfall leads to increased populations of the epizootic vectors *Ae. taeniorhynchus* and *Ps. confinnis* (466). Formalinized vaccines containing residual live virus were probably responsible for initiating the IAB outbreaks in South America that spread to Central America and Texas between 1969 and 1972 (287) and in Peru in 1973 (444,607). Until recently, viruses causing epizootics were always subtype IAB or IC, whereas enzootic viruses were ID, IE, or IF, and the epizootic and enzootic strains were thought to be maintained in separate transmission cycles. During 1995, a major VEE outbreak occurred in coastal areas of Venezuela and Colombia, causing disease in 75,000 to 100,000 people. This region had experienced a similar outbreak in 1962 through 1964. Viruses isolated on both occasions and during a smaller 1992/1993 outbreak in Venezuela were serotype IC. A similar virus had been obtained from a mosquito pool collected in Venezuela in 1983, when there was no epidemic activity. Analysis of these viruses indicated that the epizootic IC virus was not maintained in a separate cycle, but rather evolved from an enzootic ID virus in northern South America (Fig. 14) (444,461,609). Further evidence for enzootic viruses as a source of epizootic strains is the association of subtype IE viruses with two recent equine epizootics in Mexico (411).

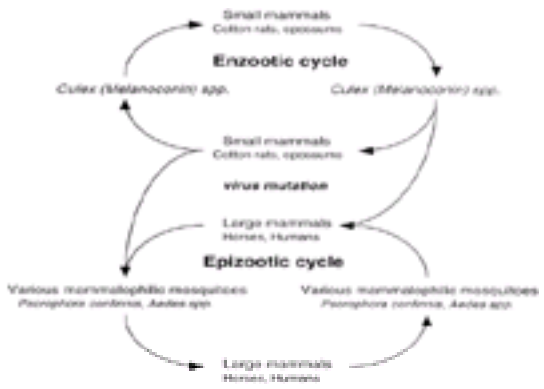

FIG. 14. Enzootic and epidemic/epizootic cycles of Venezuela equine encephalitis virus (VEEV) as an example of an alphavirus for which the epizootic strain arises by mutation from the enzootic virus. Similar mechanisms may be operative in transmission of epidemic western EEV (WEEV).

During outbreaks, virus has been isolated from several species of mosquitoes including *Ae. taeniorhynchus, Ae. aegypti, Mansonia dubitans,* and *Ps. confinnis* (547). During epizootics, horses are an important amplifying species, and availability of susceptible equines provides a means for virus spread (547).

Molecular Epidemiology

The classification and analysis of distribution of the VEEV complex viruses is an area of active investigation. Sequence analysis shows the greatest divergence for the E2 glycoprotein and the C-terminal region of nsP3 (372,444). Four enzootic subtype IAB/IC/ID/II lineages have been identified: ID isolates from Colombia and coastal Ecuador; the Everglades (EVE, II) viruses from Florida; ID strains from Panama; and ID strains from Colombia, Venezuela, and Peru. All epidemic/epizootic strains are related to the latter lineage (444). Three distinct geographically separate IE lineages also have been recognized: northwestern Panama, Pacific coast (Mexico and Guatemala), and Gulf/Caribbean coast (Mexico and Belize) (412).

Clinical Disease and Pathology

Enzootic strains produce mild disease in humans. Infection with epizootic strains of VEEV can cause significant disease, and transmission can occur by the respiratory route, as well as by mosquitoes (264,307,609). Accidental laboratory aerosol infections of young adults with epizootic strains of VEEV cause a febrile illness with the abrupt onset of chills, headache, myalgia, somnolence, vomiting, diarrhea, and pharyngitis 2 to 5 days after exposure, without evidence of encephalitis (76,137,307). Natural epidemic human infection was first described in 1952 in Colombia (137) and subsequently in Venezuela and Panama during the 1962 through 1964 outbreak that resulted in 32,000 human cases and 190 deaths (137,264). The 1995 outbreak in Colombia caused an estimated 75,000 human cases, 3,000 with neurologic complications, and 300 deaths (466,609). Neurologic symptoms tend to appear 4 to 10 days after onset of illness, with headache and vomiting the most common initial symptoms. Specific neurologic abnormalities include focal or generalized seizures, paresis, behavioral changes, and stupor or coma (466,609). Children recovering from encephalitis may be left with neurologic deficits, particularly seizure disorders (309).

Laboratory studies often show lymphopenia (264). Pathology on fatal cases has shown myocarditis, focal centrilobular hepatic necrosis and inflammation, and generalized lymphoid depletion (264). Fetal abnormalities, spontaneous abortions, and stillbirths may occur with infection during pregnancy (466,609). Congenitally infected infants show severe neurologic damage, with widespread necrosis, hemorrhage, and hypoplasia resulting, in the most severe cases, in hydranencephaly (618).

Veterinary Correlates, Host Range, and Animal Models

Enzootic strains of the VEE complex can infect horses, but these infections are asymptomatic or cause short-term fever, low-level viremia, and little clinical illness, and may immunize horses against infection with epizootic strains (148,224). Disease induced by epizootic strains is characterized by fever, depression, and diarrhea leading to death 6 to 8 days after infection. Viremia persists until death, with titers up to 10^8 infectious units/mL blood. Virus also can be recovered from eye washings, nasal washings, and urine. Leukopenia coincides with the viremia and is progressive in fatal cases. In animals that recover, antibody appears approximately 7 days after infection (288). Pathology on fatal cases shows pancreatic necrosis and cellular depletion in bone marrow, lymph nodes, and spleen. The brains of horses with signs of neurologic disease show swollen cerebrovascular endothelial cells, edema, extravasation of blood, and leukocytic infiltration into the perivenular spaces (177,288).

Infection of rhesus macaques with epizootic strains of virus elicits a biphasic febrile response: the first phase is coincident with the viremia, and the second phase with termination of viremia [i.e., the appearance of the immune response (see Fig. 5) (177,385)]. Symptoms are usually mild, consisting of anorexia, loose stools, irritability, and occasionally tremor or myoclonus at the time of fever (385). Examination of tissues shows lymphocyte depletion early, mild hepatitis, myocarditis, and encephalitis. Lesions in the brain are found primarily in the olfactory cortex and basal ganglia and consist of lymphocytic perivascular cuffing and glial nodules (177,587). Leukopenia is common. Abnormalities in glucose metabolism can be detected in young monkeys for several months after infection (41). Infection with enzootic strains causes little disease (385).

Experimental infection of small laboratory animals with VEEV produces a variety of disease patterns. After subcutaneous inoculation of guinea pigs, rabbits, or hamsters with virulent strains of VEEV, there is a viremia, and virus replicates in bone marrow, lymph nodes, spleen, and brain. There is rapid destruction of myeloid and lymphoid cells in lymph nodes, spleen, thymus, intestinal and conjunctival lymphoid tissue, liver, and bone marrow, and damage to the intestinal wall and pancreas. Hamsters also develop cerebral hemorrhage and neuronal cell death (177,180,255,587,597). Death occurs 2 to 4 days after infection and may be associated with ileal necrosis, bacteremia, and endotoxemia (181). EVEV, MUCV, and PIXV are progressively less virulent (255).

Mice are increasingly used for studies of VEEV pathogenesis. In addition to myeloid and lymphoid necrosis, mice develop encephalomyelitis, leading to death in 6 to 7 days (177,587). After subcutaneous inoculation, virus replicates first in Langerhans cells, which migrate to the draining lymph node (see Fig. 4) (346). Virus enters the CNS by the olfactory route after respiratory or peripheral inoculation and then spreads caudally to all regions of the brain, causing encephalitis and neuronal apoptosis (90,248,534). There is transplacental transmission of infection (529).

Virulence

Comparative studies of the virulent TRD and avirulent TC-83 strains of the IAB serotype and construction of recombinant viruses have led to identification of the 5' NTR (nt 3) and the E2 glycoprotein (particularly residues 76, 120, and 209) as important determinants of VEEV virulence for mice (108,284). Attenuated viruses infect dendritic cells less efficiently and replicate less well in lymphoid tissue and in the CNS than do virulent viruses (187,346,534). Analysis of the E2 sequences of endemic and epidemic VEE viruses suggests that determinants of equine virulence are different from determinants of murine virulence (444). Changes most frequently associated with acquisition of equine virulence are a Thr to Met change at position 360 of nsP3 and replacement of uncharged residues with Arg at positions 193 and 213 of the E2 glycoprotein (598).

Diagnosis and Prevention

Diagnosis can be made by virus isolation from blood or pharynx (76,307,609) or by documenting the presence of anti-VEEV IgM or an increase in IgG antibody. HI, CF, or neutralization or EIA tests can be used for serologic diagnosis (473), and RT-PCR, for detection of viral RNA (47).

The earliest vaccines to be developed for horses and laboratory workers were formalin-inactivated preparations (448,449). These vaccines had repeated problems with residual live virus producing disease and with poor

immunogenicity and are no longer in use (287,444,548,607). A live attenuated vaccine (TC-83), developed by serial passage of the Trinidad donkey (TRD) strain in guinea-pig heart tissue culture cells (32), is protective for horses and laboratory workers, but 15% to 30% of recipients develop fever and pharyngeal viral shedding (134,436). Therefore a formalin-inactivated TC-83 vaccine (C-84) was produced (134). Both the live and inactivated vaccines are immunogenic, but live TC-83 provides better protection against aerosol challenge in hamsters than does C-84 and is therefore preferred despite the reactogenicity (257). To produce an optimal vaccine, several experimental VEEV vaccines are currently under development (31,89).

A number of measures have been shown to be effective in controlling an outbreak. These include immunizing equines, limiting equine movements from regions of infection, applying larvicides to mosquito-breeding sites, and spraying insecticides to control adult mosquitoes (466,547).

ALPHAVIRUSES ASSOCIATED PRIMARILY WITH POLYARTHRITIS AND RASH

Chikungunya

An outbreak of a crippling arthritic disease of sudden onset was first recorded in the Newala District of Tanzania in 1952 and initially thought to be dengue (468). Because of the severe arthritic symptoms, the disease was given the Swahili name, *chikungunya*, meaning “that which bends up” (468). The virus, isolated in 1953 from serum and from *Aedes* spp. and *Culex* spp. mosquitoes, was distinct from dengue virus and related to the group A arboviruses (477). Subsequent large epidemics were recognized in the Transvaal of South Africa (173), Zambia (469), India (72), Southeast Asia (278), and the Philippines (80). CHIKV is a member of the SFV complex.

Epidemiology

Chikungunya is endemic in India, Southeast Asia, the Philippines, and most of sub-Saharan Africa (Fig. 15) (80,278,557,558). In Africa the virus is maintained in cycles similar to that of yellow fever virus. There is a rural cycle involving *Ae. africanus, Ae. furcifer,* and wild primates, and an urban cycle involving *Ae. aegypti* and humans (115). In rural areas, the disease is endemic, with small numbers of cases occurring most years (115). In urban areas, outbreaks are sporadic and explosive, with infection of a large proportion of the susceptible population within a few weeks (468). Outbreaks typically occur during the rainy season and are associated with increased population densities of *Ae. aegypti* (72,278,337). All ages are susceptible. After an epidemic, the disease typically vanishes from an affected region for years, possibly because large portions of the population have become immune. In Asia, where there is no evidence for a rural cycle, the virus may be maintained in an urban cycle with *Ae. aegypti* or require reintroduction before the onset of epidemics (443). Laboratory-acquired infections also have been reported (564).

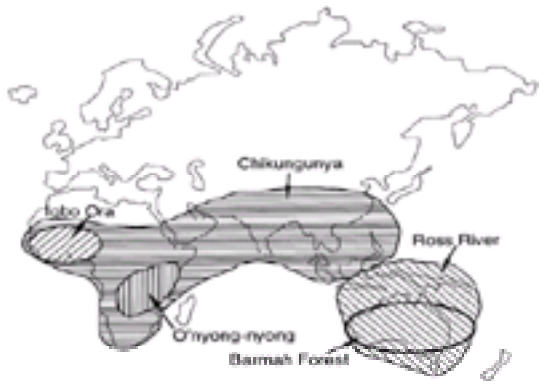

FIG. 15. Geographic distribution of several alphaviruses that cause epidemic polyarthritis and rash: chikungunya, o'nyong-nyong, Igbo Ora, Barmah Forest, and Ross River viruses.

CHIKV is thought to have originated in Africa and then spread to other parts of the world. Two distinct lineages exist, one in western Africa, and another in southern and eastern Africa and Asia (443). In the latter lineage, the Asian strains group as a genotype distinct from the African strains.

Clinical Features

The disease is of sudden onset, with an incubation period estimated at 3 to 12 days (468). No prodrome is recognized (72). Fever increases rapidly to 103°F to 104°F and may be accompanied by a rigor. The onset of fever corresponds to the period of viremia and may be related to the ability of this virus to induce large amounts of IFN (see Fig. 5) (174). Virus titers in blood reach greater than 10^6 pfu/mL (72). Joint pain appears suddenly and is often incapacitating in its severity. Patients typically lie on their sides with all joints flexed. Essentially any joint can be involved, and pain is rarely accompanied by redness or swelling (277). Headache is typically present, and in 80%, a maculopapular rash appears 4 to 8 days after the initial illness. The rash may be associated with a second increase in fever (see Fig. 5), lasts approximately 2 days, and is described as “irritating” or “itchy” (468,563). Leukopenia is typical.

In the majority of patients, joint pains continue to recur in a milder form without fever for many months after the original illness (477). Joint radiographs are normal or show soft-tissue swelling without evidence of bone or joint damage (277). Fatalities are rare, and when they occur, are associated either with young age or with a thrombocytopenic, hemorrhagic version of the illness that can lead to shock (72,487,563). In India, but not Africa, inguinal lymphadenopathy and red

swollen ears also are observed as part of the clinical picture (72,563).

Diagnosis and Prevention

The primary differential for CHIK is between dengue and o'nyong-nyong (ONN) fevers. Dengue overlaps the CHIK geographic distribution extensively, but is characterized more by myalgia than by arthralgia (277). ONN is clinically similar, but has geographic overlap only in east Africa (see Fig. 15) (72,173). CHIKV can be isolated from serum during the initial fever (173,477). Detection of IgM antibody provides a specific and reliable means of early diagnosis (62).

An experimental live attenuated vaccine has been developed by passage of an isolate from Thailand in MRC-5 cells (317). This vaccine induces long-term production of neutralizing antibody and can be used to protect laboratory workers from infection (363).

O'nyong-nyong and Igbo Ora

In 1959 an outbreak of a new disease, originally mistaken for dengue, was reported from northwestern Uganda (516). It is likely that an epidemic of ONN occurred in the same region in 1904 through 1906 (516). The name *o'nyong-nyong* originated from one of the first tribes to be affected, the Acholi, and refers to the painful joints characteristic of the disease (204). Virus was first isolated from the serum of a patient with acute arthritis (624) and subsequently from anopheline mosquitoes (626). ONNV is a member of the SFV complex and antigenically most closely related to CHIKV, from which it is estimated to have diverged thousands of years ago (443,624). The virus reemerged in southern Uganda in 1996 to 1997 to cause the most recent epidemic (303), suggesting a 30- to 50-year epidemic cycle.

In 1967 a related virus, Igbo Ora, was isolated from humans in western Nigeria (388). It has subsequently been recovered from humans and anopheline mosquitoes in the Central African Republic, Nigeria, and the Ivory Coast (320).

Epidemiology

ONNV is a virus of East Africa (see Fig. 15) that causes sporadic, widespread outbreaks of fever, rash, and arthritis with high attack rates. The first epidemic recognized to be ONN originated in northwestern Uganda in 1959 and spread south and east to Kenya, Tanzania, Zaire, Malawi, Mozambique, and Zambia to affect more than 2 million people (625). Serologic studies suggesting that the virus also occurs in the West African countries of Nigeria, Ghana, and Sierra Leone (631) may reflect infection with the closely related Igbo Ora virus (320).

The enzootic vector and vertebrate reservoir host for ONNV are unknown. Interepidemic seroconversions and mosquito isolations indicate continuous sporadic transmission in East Africa, and it is possible that humans or nonhuman primates are the primary reservoir (303). ONNV is transmitted principally by *Anopheles funastus* and *An. gambiae* (345,626). Human–mosquito–human transmission occurs during epidemics, and spread from one region to another occurs through the movement of viremic humans (485). The most recent outbreak began near swamps and lakes in the rural Rakai district of south-central Uganda. Serosurveys showed infection rates of 45% to 96% in areas of epidemic transmission, with the ratio of apparent to inapparent infection ranging from 1:4 to 1:24 (485,627). All ages and both sexes are equally susceptible (291). The seroprevalence for Igbo Ora in Nigeria is approximately 25%. Among domestic animals in the same region, cattle have the highest seroprevalence (40%) (414), but the enzootic reservoir for this virus also is not known.

Clinical Features

The onset of fever is sudden and often accompanied by a rigor. The characteristic syndrome includes joint pains, rash, lymphadenitis, and conjunctivitis. Fever is typically moderate (100°F to 101°F) and lasts approximately 5 days. Joint pain most often occurs in the knees, but ankles, elbows, wrists, and fingers also can be affected. The pain usually lasts 6 to 7 days and is severe enough to be immobilizing in 80% to 90% of patients. Pain can persist for up to 3 months, but this is uncommon. The generalized morbilliform maculopapular rash erupts 4 to 7 days after the onset of symptoms and is similar to that of CHIK. It begins on the face and extends to the trunk and extremities, occasionally affecting the palms. Cervical lymphadenopathy distinguishes the disease from CHIK, occurs in approximately half of the patients, and most frequently involves posterior cervical lymph nodes. Leukopenia is common. Fatalities have not been described, but morbidity is substantial (291,516). The disease caused by Igbo Ora virus infection is similar, but lymphadenitis is uncommon (320,388).

Diagnosis

The disease is often clinically confused with malaria, measles, denque, and rubella (516), but also resembles CHIK. Virus can be isolated from serum early in the illness (625). Diagnostic serology includes a positive IgM EIA or neutralizing antibody to ONNV that is more than two-fold greater than that to CHIKV (485). IgM persists for about 60 days after infection (291).

Ross River

Epidemics of polyarthritis in Australia were first described in towns on the Murrumbidgee River in New South Wales in 1928 and then in troops stationed in the Northern Territory and Queensland during World War II (124,347,407,517). Serologic studies subsequently suggested an alphavirus as the causative agent (515), and RRV (strain T48) was first isolated from *Aedes vigilax* mosquitoes trapped near the Ross River in Queensland in 1959 (347). The first human isolate was made in 1971 from the blood of a child with fever (121). In 1979 RRV spread from Australia to Fiji, Samoa, and the Cook Islands, causing an explosive outbreak of tens of thousands of cases of polyarthritis on these Pacific islands. Virus

was first isolated from the serum of patients with polyarthritis during this epidemic (4,475,560).

Epidemiology

RRV is endemic throughout the coastal regions of much of northern and central Australia and epidemic in the rest of Australia, in Papua New Guinea, and in the Solomon Islands (6,475,560). In general, the Wallace and Weber hypothetical lines in the Indo-Australian archipelago that separate the fauna of the Asian and Australian regions appear also to separate the geographic distributions of CHIKV and RRV (see Fig. 15) (347,558). RRV is the most common cause of human arboviral disease in Australia, and cases occur primarily in late summer and early autumn (215,347). Two salt marsh–breeding mosquitoes, *Ae. camptorhynchus* and *Ae. vigilax,* are probably the major vectors in coastal regions of Australia (22,70,169). In suburban areas, fresh water–breeding mosquitoes, *Cx. annulinostris* and *Ae. notoscriptus,* are important vectors (465). A wide variety of mammals, primarily macropods, appear to serve as vertebrate hosts for the enzootic cycle (122,170,475).

Morbidity and Mortality

Infection rates can be as high as 1:30 during outbreaks (347). Estimates of ratios of apparent-to-inapparent infection vary from 1:3 to 1:50 (6). Seroprevalence is similar in both sexes and, in populations living in epidemic areas, ranges between 6% and 15%, whereas in endemic areas, it reaches 5% to 72%, with estimated infection rates of 0.4% to 2.4% per year (156,215). Clinically apparent infections are rare in children, and the highest incidence of disease is in the 30- to 40-year age group (4,214). Clinical disease rates in coastal regions of Queensland have been estimated at 0.37/1,000 adults/year (6). Polyarthritis may be more common in female subjects, but not all studies show this trend (4,6,119,214). The MHC class II antigen HLA-DR7 may predispose to clinical disease (161), but no association with MHC class I molecules HLA-A or HLA-B has been identified (6).

Origin and Spread of Epidemics

In Australia, outbreaks are seasonal and have been associated with prolonged inundation of salt marshes due to increased tidal heights or heavy rains with localized flooding during the summer (347). In arid regions, outbreaks often occur within 2 or 3 weeks of heavy rains, suggesting that RRV survives in desiccation-resistant mosquito eggs with vertical transmission (327). This is further suggested by isolation of RRV from field-caught immatures and male *Ae. vigilax* and *Ae. tremulus* mosquitoes (114,327). Epidemic polyarthritis, initiated by a viremic traveler from Australia to Fiji, occurred on several South Pacific Islands between 1979 and 1980 in explosive outbreaks similar to those of urban CHIK with a human–mosquito–human transmission cycle and *Ae. polynesiensis* as the vector (475,560). Human viremias of more than 10^6 mosquito ID_{50}/mL have been documented (560). RRV has not been found in the region since, suggesting that an endemic cycle was not established.

Molecular Epidemiology

Strains of RRV from the northeastern (Queensland) and southeastern (New South Wales) regions of Australia are antigenically distinguishable. Genotyping reveals a third distinct group in western Australia (483,630). Pacific Island isolates all belong to the southeastern genotype. The data suggest genetic divergence and independent evolution of RRV within geographically isolated enzootic foci, consistent with mammals as a vertebrate reservoir (483).

Clinical Features

RRV infection most often induces fever, arthralgia, and rash (see Fig. 5), but not all patients develop all three symptoms (214,557). The incubation period for onset of disease can be as short as 2 days or as long as 21 days, with an average of 7 to 9 days (162,475). Usually the illness has a sudden onset. Fever is the initial symptom, is low grade, and is typically followed by the onset of pain, swelling, and tenderness in multiple joints. Joint involvement is usually symmetric, and both small and large joints can be affected. Characteristically, the rash appears on the third or fourth day of illness, is maculopapular, nonpruritic, and present on the face, trunk, and extremities, with occasional involvement of the palms and soles. The rash generally lasts 3 to 4 days, but can be prolonged (155). Frequent accompanying signs and symptoms include lymphadenopathy, lethargy, headache, myalgias, and photophobia. Glomerulonephritis also has been reported (159). Duration of illness varies considerably. The average period of incapacity is 6 weeks (214). Joint symptoms may be protracted, with almost half of affected individuals still having chronic generalized malaise and/or joint stiffness a year after infection (506). Essentially all experience gradual improvement without destructive joint changes or long-term problems.

Examination of synovial fluid shows increased protein and the presence of lymphocytes, monocytes, and activated macrophages (94,157). Cell counts are moderate, ranging from 1,500 to 15,000 cells/mm^3 (157). RRV replicates in and causes persistent infection of synovial cells and macrophages *in vitro,* but this has not been documented to occur *in vivo* (100,268,329). There is no evidence of a role for immune complex formation in the pathogenesis of prolonged and recurrent arthritis (94,158).

The skin lesions show mononuclear perivascular inflammation without evidence of immune complex deposition. Most of the infiltrating cells are $CD8^+$ T lymphocytes. RRV antigen is present in the basal epidermal and eccrine duct epithelial cells (160).

Animal Models and Host Range

Despite its extensive host range, RRV is known to induce clinically evident disease only in humans, mice, and possibly horses (169,553). The most commonly studied animal model of RRV infection is the mouse. The prototype T48 strain has been extensively passaged in mice and is virulent (380,399). In newborn mice, T48 infects cardiac muscle, causing

myocardial necrosis and death 3 to 4 days after infection (399). The mechanism of virus entry into muscle is not clear because there is no evidence of infection of vascular endothelial cells (399,505). By 1 week of age, infection is no longer fatal, and infection of the CNS leads to demyelination and destruction of the internal granule cell layer of the cerebellum (505). The ability of the virus to replicate in muscle and CNS continues to decline with increasing age. Sites of myocardial and skeletal muscle infection become calcified (399,503). Necrosis and calcification are results of virus-induced cellular damage, not of immune-mediated damage (503). Replication also occurs in perichondrium, periosteum, and skin (399). In pregnant mice, virus can replicate in the placenta and spread to the fetus 1 to 2 days later (378).

The less virulent NB5092 strain induces severe myositis and muscle cell death in newborn mice after subcutaneous inoculation (399). Mice become stiff and unable to move, and then gradually regenerate muscle and recover function after virus is cleared (399). CNS disease is minimal, with patchy infection of ependymal cells and ependymitis, leading to hydrocephalus. Neurons are only occasionally infected (380). Brown fat is another important site of virus replication (399). By age 1 week, mice no longer develop signs of disease (399).

Virulence

Two prototype strains of RRV isolated from *Ae. vigilax* are recognized. T48, isolated in Queensland, is virulent for newborn mice, whereas NB5092, isolated in New South Wales, is not (170). These viruses have amino acid differences at 36 residues in the nsPs and at 12 residues in the structural proteins (140). Virulence has been associated primarily with amino acid changes in E2. Viruses with changes in epitope E2-b2 show more rapid penetration kinetics for BHK cells and decreased virulence for mice (593). A small-plaque mutant of T48 with a deletion of 21 nucleotides encoding E2 residues 55 to 61 grows normally in BHK cells, but replicates less well in muscle and brain of newborn mice, causing less paralysis than does the parental T48 strain (591).

Passage of the avirulent NB5092 strain in newborn mice selected for a more virulent virus with amino acid changes in E2 (370,553). Passage of NB5092 on CEF cells led to the isolation of less virulent variants with antigenic and amino acid changes in E2. Viruses with an Asn to Lys change at E2-218 produce lower viremia and more rapid clearance than does the parental virus (280). Of unproven but possible significance is the fact that the epidemic strain of RRV in the Pacific differed from the parent Australian strain by a change from Thr to Ala at E2-219 (53).

Diagnosis and Prevention

Diagnosis is aided by geographic location and travel history. The differential diagnosis includes rubella, other alphavirus-induced arthritides, Henoch–Schönlein syndrome, and, in the chronic form, rheumatoid arthritis and other chronic rheumatic diseases (155). Virus can be recovered from blood early in disease (4,475,560), but most diagnoses are made by using serology. The most sensitive and commonly used serologic test is an IgM capture EIA, which can remain positive for 1 to 2 years after the onset of disease (74).

Vector control remains the primary means of prevention. A candidate inactivated vaccine has been developed and tested in animals (1).

Sindbis

The prototype strain of Sindbis virus, AR339, was initially isolated from *Cx. univittatus* collected near Sindbis, Egypt, in 1952 (552). Because the virus caused fatal disease in mice, it was originally thought to be a coxsackievirus (551). Humans living in the Nile Delta at that time had a seroprevalence of 27%, but no disease was recognized to be associated with infection (552). Over the next 25-year period, SINV was isolated in Europe, the Middle East, Africa, India, Asia, Australia, and the Philippines from a variety of mosquito and vertebrate species (120,339,352,405,615). SINV was first isolated from the blood of febrile humans in Uganda in 1961 and recognized in South Africa as a cause of rash and arthritis in 1963 (352).

Epidemiology

SINV is the most widely distributed of the alphaviruses causing arthritis in humans (Fig 16). The primary sites of recognized SINV-mediated human disease are in Northern Europe (Ockelbo in Sweden, Pogosta Fever in Finland, and Karelian Fever in Russia) (139) and South Africa (see Fig. 16) (269). In the many other regions where the virus exists, human infection occurs, but results only in subclinical disease fever or mild arthralgias. The basic maintenance cycle of SINV is between *Culex* spp. and *Culiseta* spp. mosquitoes and wild birds (347). In Sweden the enzootic cycle involves *Cx. torrentium, Cs. morsitans,* and passerine birds (154,338). In Australia isolates are primarily from *Cx. annulirostris* (603). Vertical transmission in mosquitoes can occur (114).

FIG. 16. Geographic distribution of Sindbis virus. Northern Europe and South Africa, regions with significant outbreaks of

arthritis and rash disease in humans, are designated in bold. Mild disease also has been recognized in Australia.

Morbidity and Mortality

The age-adjusted prevalence for SINV is 2% to 8% in endemic regions of northern Europe and 0.1% to 0.2% in neighboring nonendemic areas. Risk is highest between parallels 60 and 64 (139,339).

Origin and Spread of Epidemics

Spread of SINV from its enzootic cycle between birds and ornithophilic mosquitoes to humans involves introduction of the virus into *Aedes* spp. mosquitoes with less specialized feeding habits, as bridge vectors (see Fig. 10) (573). In Northern Europe the primary bridge vector is *Ae. cinereus,* and availability of this species may determine the frequency of human infection. Cases begin to appear in late July and continue into the fall. Large outbreaks occur approximately every 7 years (339).

Molecular Epidemiology

Antigenic and genetic analyses of strains from different locations indicate that viruses from Africa and Europe form one major lineage, and those from Asia and Australia, another lineage, suggesting that the ancestral SINV has diverged into two distinct groups (340,421,484). A third lineage has recently been identified in southwestern Australia (484). Strains isolated in Northern Europe and South Africa, where SINV-induced disease occurs, are more closely related to each other than to strains isolated in south and central Europe and the Middle East, where disease is rare (339,513). Within a region, strain divergence is temporal rather than geographic, consistent with migratory birds serving as the major vertebrate host (484).

Clinical Features

When clinical disease is apparent, the primary manifestations of infection are rash, joint pain, and mild fever, often occurring concurrently (139,575). SINV can be recovered from skin lesions, which have a macular base with central vesicle formation and are occasionally hemorrhagic (139,199,352). The rash is distributed diffusely over the trunk and limbs and affects the palms and soles. Joint pain preferentially affects large joints and may be severe enough to be immobilizing. Most patients recover within 14 days, but joint pain and stiffness may persist for months to years (139,366,575). In Australia, SINV-induced arthritis and rash is milder and less frequent than RRV-induced disease or SINV-induced disease in northern Europe and Africa (347).

Animal Models and Host Range

SINV can infect a wide variety of vertebrates and has been extensively studied in mice as a model for acute encephalitis. The virus has been most frequently isolated from domestic and wild birds, its major host, but on occasion from small mammals and amphibians. Seroprevalence is 11% in central Sweden in passerine birds without recognized disease.

In mice there is an age-dependent susceptibility to fatal encephalitis (see Fig. 7) (266). In young mice, virus replicates to high titer and spreads rapidly, causing death in 3 to 5 days. In older mice, virus replication is more restricted, and mice often recover. After peripheral inoculation, virus replicates in muscle, produces a viremia, and then spreads to the brain and spinal cord, where the primary target cells are neurons (246). Ability of SINV to spread to the CNS and cause fatal disease is dependent on the strain of virus and the genetic background, as well as the age of the mouse (247,298,342,343,357,510,568). Within 3 to 4 days, neutralizing antibody and a SINV-specific perivascular mononuclear inflammatory response appears, consisting of T cells and monocytes (382). Spread of infection is limited initially by IFN, and infectious virus is cleared within 7 to 8 days after infection, primarily through the effects of antiviral antibody (see Fig. 6) (55,189,314,450). Even after clearance of infectious virus, viral RNA persists in the CNS, and reactivation of infection appears to be prevented by continued production of antiviral antibody by B cells resident within the CNS (312,313,577).

Virulence

Strains of SINV differing in virulence have been derived from independent isolates from Egypt (AR339), South Africa (SR86), and Israel (SV-Peleg). Variants of AR339 and SV-Peleg differing in virulence have been derived by passage in mice (194,342) and in tissue culture (373). Virulence is determined primarily by the 5' NTR and the E2 glycoprotein, but can be influenced by changes in E1 and nsP2 (106,344,367,497). Changes in nucleotide 5 or 8 from A to G increase neurovirulence by unknown mechanisms (129,367). A number of amino acid changes in the E2 glycoprotein affect virulence by altering efficiency of virus entry into the CNS and of neuronal infection (see Fig. 2) (106,219,438,497,567,570). Neuroinvasion is affected by changes at residues 55 and 190 of E2 (129). A Gln-to-His change at E2-55 increases efficiency of infection of neurons and is a major determinant of increased virulence in older mice (127,569,570).

Diagnosis

Diagnosis is most often made by serology, with the major differential being with other causes of acute rash and arthritis such as parvovirus B19 and rubella. SINV-specific IgM increases during the acute phase of the disease and then tends to decrease slowly for 3 to 4 years, independent of persistent symptoms (406).

Barmah Forest

Barmah Forest virus (BFV) is an Australian alphavirus first isolated in 1974 from *Cx. annulirostris* mosquitoes collected in the Barmah Forest in the Murray River Valley region of southeastern Australia and soon thereafter in southwest Queensland (14). It is the only recognized member of the BFV complex and has a unique E2 protein without N-linked glycosylation (306). Human disease was first reported in 1986 with an epidemic of polyarthritis in New South Wales (40), and BFV was first isolated from the blood of a patient in 1988 (434). In 1989 BFV, previously restricted to eastern portions of Australia, was isolated in Western Australia. Human cases of BFV-induced arthritis in this region were documented in 1992, and an outbreak occurred during the summer of 1993 to 1994, suggesting recent introduction of the virus into this region (328).

The vertebrate reservoir host is unknown, but serosurveys suggest that marsupials may play a role (328). Conversely, sequence analysis shows a high degree of homology over the geographic distribution, consistent with an avian vertebrate host (437). The main mosquito vectors appear to be *Ae. vigilax* and *Ae. camptorhynchus,* salt marsh–breeding mosquitoes that also are the primary vectors of RRV in the same regions (43,328).

Serosurveys show higher rates of infection in men than in women (1.5:1) The highest seroprevalence is in the north coastal areas of New South Wales, where 13% of the population tested was positive (14,213). Infection rates of 0.23% per year occur in the population of Queensland (434).

Clinically evident disease is most common in the 30- to 50-year age group, with both sexes equally affected (29). The most common clinical features are fever, lethargy, polyarthritis, and myalgia, which are often accompanied by a vesicular rash (14,29,434). Diagnosis may not be made because the illness is frequently mild and overlaps the clinical spectrum and geographic distribution of RRV and SINV infections (see Fig. 15 and Fig. 16). A single case of glomerulonephritis has been reported (273). In more than half of infected individuals, recovery takes several weeks, with lethargy the most prominent persisting symptom (29). Rash is more prominent in BFV, whereas arthritis is more prominent in RRV (151). The serology is distinct (434).

Mayaro

Mayaro virus was first isolated in 1954 from the blood of febrile forest workers in Trinidad and then from several individuals with a febrile illness associated with frontal headache in the Guama River area of Brazil (13,79). Subsequently the virus has been documented to be widely distributed in northern South America and the Amazon Basin. Human cases are sporadic and occur primarily in persons with recent contact with humid tropical forests. The principal mosquito vectors are in the forest-dwelling genus *Haemagogus,* but the virus also can infect and be transmitted by *Ae. aegypti,* and the possibility of urban outbreaks has been raised. The diagnosis can be made by isolation of virus from blood in Vero or C6/36 cells or by serology with an IgM capture EIA (62,561).

OTHER ALPHAVIRUSES

Semliki Forest

SFV was first isolated in 1942 from *Ae. abnormalis* collected in the Semliki Forest of western Uganda (523). It is widely distributed in Africa with mosquito (e.g., *Ae. africanus, Ae. argenteopunctatus*) isolates documented from Mozambique, Nigeria, and the Central African Republic (349,361,365). Although SFV is one of the most extensively studied of the alphaviruses, and serosurveys indicate that human infection is relatively common (339,361,523), SFV has been linked to human disease on only two occasions (361,623). In the first case, reported in 1979, a 26-year-old laboratory worker in Germany with a 1-year history of “purulent bronchitis,” working with the Osterrieth strain of SFV, developed fever and headache followed by seizures, coma, and died of encephalitis. SFV was isolated from CSF and from brain. No antiviral antibody was detectable at the time the CNS symptoms began, but was detected at the time of death 1 week later (623). The history of chronic pulmonary infections and failure to produce antiviral antibody rapidly suggest that this individual had an immunodeficiency disorder involving antibody production. In 1987 SFV was isolated from serum samples of individuals in the Central African Republic with fever, persistent headache, myalgias, and arthralgias (361).

Animal Models

SFV can cause encephalitis in horses, mice, rats, hamsters, rabbits, and guinea-pigs (19,45,467,523,640). Severity and type of disease are dependent on the age of the animal at the time of infection, on the route of inoculation, and on the virulence of the strain of SFV used for infection (45,197). Mice have been most extensively studied, and the strain of inbred mouse infected also can influence outcome (545).

In newborn and suckling mice inoculated peripherally, virulent and avirulent strains of SFV replicate rapidly and extensively in muscle, elicit a high-titered viremia, spread to the CNS, and cause death within 2 to 4 days (197,398). SFV replicates in cerebrovascular endothelial cells *in vitro* (126), and viral antigen also can be detected in endothelial cells *in vivo* (281,525), but it has been suggested that endothelial cells transport virus from blood to brain without replicating virus (426). Once within the CNS, the virus replicates primarily in neurons and spreads rapidly along neural pathways, producing neuronal cell death (417).

In weanling (3- to 5-week-old) mice, SFV replicates rapidly, but reaches lower peak titers in muscle, blood, and brain than in younger animals. There is a continued age-dependent decrease in virus replication to age 12 to 15 months (197). Virus enters perivascular regions of the brain and initiates foci of infection within the CNS. After intranasal inoculation, virus infects olfactory neurons first and then spreads within the CNS (417). The primary target cells in the brain are neurons, but glial cells also are infected (19,143,281). A mononuclear inflammatory response consisting of T lymphocytes, B lymphocytes, and monocytes is apparent 3 to 4 days after infection, peaks at 2 to 3 weeks, and is mostly

resolved by 6 weeks (393). Inflammation is accompanied by microglial and astrocyte activation (91,118,393,546).

Mice that survive infection develop demyelination, accompanied by mild paralysis, 2 to 4 weeks after infection (92,425,546). Clearance of infectious virus is complete 7 to 10 days after infection, and this clearance is mediated by antibody (12). Viral RNA and protein persist for months (123,281), and hydrocephalus can be a late consequence of infection (639). Mice infected with virulent strains can be passively protected from fatal encephalitis with immune serum, but then develop a delayed disease associated with persistent infection, inflammation, and neuronal degeneration (502). Focal areas of demyelination are found in the optic nerve and cerebellum 14 to 21 days after SFV infection and are characterized initially by swelling and vacuolation of oligodendrocytes and loss of myelin sheaths, followed by apparent oligodendrocyte dedifferentiation and then remyelination (54). Demyelination is macrophage mediated and appears to be the result of oligodendrocyte infection, the cellular immune response to infection, and induction of an autoimmune response to myelin basic protein (142,509,544). SJL mice have prolonged inflammatory responses and demyelination after infection compared with other strains of mice (123,524).

SFV infection of the CNS also can increase the susceptibility of mice to induction of experimental autoimmune encephalomyelitis (384), apparently by damaging the blood–brain barrier, increasing adhesion molecule expression on endothelial cells, and facilitating entry of autoimmune T lymphocytes into the CNS (138,525,526).

Virulence

Isolates from mosquitoes collected in 1942 in Bwamba, Uganda (523), in 1948 in Kumba, Nigeria (349), and in 1959 in Namancurra, Mozambique (365), have given rise to a variety of strains of SFV with differing levels of virulence. The most commonly studied are virulent strains V12, V13, and L10 [Uganda strain independently passaged in mice in Bethesda (V) and London (L)], and avirulent strain A7 (Mozambique strain AR2066 passaged in mice) and its less virulent derivative A7-74 (45). In addition, avirulent strains of L10 have been derived by chemical mutagenesis (e.g., m9) (26). Virulent and avirulent strains differ in their ability to invade and replicate in the CNS of weanling mice and rats after peripheral inoculation (19,44,446), but all strains cause fatal disease in newborn or suckling mice (197,398). Resistance to fatal disease develops between ages 1 and 3 weeks (45,418). In 3- to 4-week-old mice, avirulent SFV is restricted in replication and spread in the CNS compared with virulent strains of virus and compared with avirulent strains in younger mice (143,418). This difference in replication is associated with decreased budding of infectious virus and is independent of host immune response (12,143). Mature neurons and pancreatic and myocardial cells can be made more susceptible to replication of avirulent virus by treatment with aurothiolate compounds, which induce intracellular membrane proliferation (371,491). In general, reduced virulence correlates best with reduced ability to replicate in neurons (23).

In vitro studies of the differences between virulent and avirulent strains of SFV have shown differential replication in mouse neuroblastoma cells and primary cultures of rat neurons (17). Efforts to identify specific nucleotide and amino acid changes important for virulence have used comparative sequence analysis and an infectious SFV cDNA clone pSP6-SFV4 derived from the prototype virulent L10 strain (324). However, SFV4, the virus derived from this cDNA, is less virulent than L10 (176). Construction of SFV4/A7 chimeric viruses has shown that determinants of virulence reside in both the structural and nonstructural regions of the genome (550). NsP2, nsP3, and E2 are important determinants of virulence (175,464,486). The 3' NTRs differ significantly in length, but this is not important for virulence (486).

Other SFV Complex Viruses

The SFV complex includes nine viruses and has representatives in both the Old World (BEB, CHIK, GET, ONN, RR, and SF) and the New World (MAY and Una). Human disease, when present, is generally characterized by fever that may be accompanied by arthritis and rash. CHIKV, ONNV, RRV, MAYV, and SFV were discussed previously.

Una Virus

Una virus was first isolated from *Psorophora ferox* mosquitoes collected in the Amazonian region of Brazil in 1959 (78). This virus is pathogenic for mice, relatively unstudied, and is not recognized to cause human disease. Una virus also has been isolated in Trinidad (78).

Getah Virus

Getah virus was first isolated from *Culex* spp. mosquitoes collected in Malaysia in 1955 and causes myositis when inoculated into mice (230). It is maintained in a cycle similar to that of Japanese encephalitis virus with transmission by *Cx. tritaeniorhynchus* and amplification in domestic pigs (519). Getah virus is widespread in Asia, with documented presence in India, Sri Lanka, China, Southeast Asia, Malaysia, and Japan (353,428,558). Disease in humans is limited to fever (321), but it causes abortion in pigs and is an important pathogen of horses (508,511). The equine disease is characterized by fever, an urticarial rash, and hind-leg edema (508).

Me Tri Virus

Me Tri virus was isolated from *Cx. tritaeniorhynchus* mosquitoes collected in the Hanoi province of Vietnam. Serologic surveys suggest that infection of humans and domestic animals is common. It may be a significant human pathogen because a retrospective study identified nine cases of encephalitis in children associated with seroconversion to this virus (202).

Alphaviruses of Fish

Atypical alphaviruses, sleeping disease virus and salmon pancreas disease virus, have recently been isolated from rainbow trout and Atlantic salmon. These viruses cause significant disease on fish farms. Both have unusually large E1

and E2 structural glycoproteins and relatively low (30–34%) homology to other alphaviruses (589,621).

DIAGNOSIS

The differential diagnosis of alphavirus-induced diseases often includes more than one alphavirus in addition to other mosquito-borne viral diseases such as dengue and West Nile fevers, other rash diseases such as rubella and parvovirus B19, and other causes of encephalitis. IgM capture EIAs can be used for diagnosis early in disease (65). The IgM response is relatively specific for each antigenic complex and is useful even at later times, because IgM persists for at least 2 to 3 weeks after onset of disease (62). RT-PCR primers can amplify the conserved region of all alphaviruses (432) and should be useful for rapid diagnosis.

PREVENTION AND CONTROL

Treatment

There is currently no available specific antiviral treatment for any alphavirus-induced disease. Supportive treatment for those with encephalitis can be lifesaving, because individuals can make remarkable recoveries from coma. Symptomatic treatment for arthritis is beneficial.

Vaccines

Formalin-inactivated vaccines against EEE, WEE, and VEE are available for horses and against EEE for birds. Experimental inactivated human vaccines against EEE, WEE, and VEE also are available for laboratory workers exposed to these agents, with yearly booster doses required for EEE and WEE. PE-6, the investigational inactivated EEE vaccine for humans, induces good immunoreactivity against the North American, but not the South American, varieties of EEEV (542). Live attenuated vaccines have been developed for VEEV (TC-83) and CHIKV. Side effects are common after immunization with TC-83 (134,317,436). In both horses and humans, prior immunization against one alphavirus interferes with development of neutralizing antibody to subsequent alphavirus vaccines (116,363).

Other

Prevention of infection with most alphaviruses relies primarily on efforts to control mosquito populations by spraying and reducing breeding places. A variety of means can be used for assessing the need for mosquito abatement. These include monitoring mosquito population densities, seroconversion of sentinel animals, and presence of virus in populations of mosquitoes capable of transmitting virus to humans or domestic animals. Use by individuals of protective measures such as mosquito repellents and protective clothing is important.

PERSPECTIVE

The ability to construct full-length cDNA alphavirus clones that can be transcribed into infectious RNA provides tremendous potential for understanding the functions of various genes and their importance for replication and virulence in the multiple hosts necessary for maintenance of these viruses in their natural cycles. An understanding of the folding and three-dimensional structures of the structural glycoproteins is needed for interpretation of much of the sequence and virulence data that have been acquired. Further sequence information on virulent and avirulent strains and functional analysis of the nonstructural proteins is likely to provide the next level of understanding of virus–host relationships.

In addition, there is a need for improved approaches to prevention and treatment. There is a particular need for a vaccine for RRV and an improved VEEV vaccine. In addition, there is a need for effective antialphaviral drugs. Continued advances in understanding of the structure of viral proteins is likely to offer new approaches to therapeutics. Both of these areas have implications for defense against biologic warfare and bioterrorism, because VEEV, EEEV, and WEEV are all on the Centers for Disease Control (CDC) category B list of critical biologic agents (81).

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CHAPTER 31 Rubella Virus

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Rubella virus (RV) is the etiologic agent of a mild exanthematous disease associated with low-grade fever, lymphadenopathy, and a short-lived morbilliform rash. Originally described as a distinct disease in the early 1800s (195), it was named Rõtheln by the German physician de Bergen (cited in reference 103), leading to the common name of *German measles,* a milder form of the much more serious exanthem caused by the paramyxovirus, measles virus. The physician H. Veale proposed the name *rubella* in 1866 (345) as a term more “euphonious” to English ears.

Rubella is predominantly a childhood disease, endemic throughout the world, although a comprehensive vaccination program in most industrialized regions, including North America, Europe, Japan, and Australia, has reduced the incidence of disease to low levels in these areas. Vaccination is not carried out in many of the developing countries, however, and therefore most of the world's population is still infected naturally and is susceptible to epidemics of rubella that occur in irregular cycles at about 6- to 10-year intervals.

Rubella was considered a relatively benign infection, associated with considerably less morbidity than measles, until an observation by an astute ophthalmologist, Dr. Norman Gregg, uncovered a sting in the tail of this underestimated virus. In 1941, Gregg encountered a large number of children with cataracts, many of whom had additional serious congenital defects. He noted that this apparent epidemic of congenital cataracts was directly preceded by a large rubella outbreak. He proposed that the cataracts and the often associated congenital cardiac abnormalities were the consequence of maternal infection during pregnancy (128). Although initially met with considerable skepticism, further studies by other investigators made it clear that RV could have devastating effects on a developing fetus when acquired by the mother in early pregnancy (358). This important finding introduced the concept of viruses as teratogenic agents and spurred the efforts to develop an attenuated vaccine.

Isolation and growth of the virus in cell culture was achieved in 1962 (254,357), and this opened the way for vaccine development. Finally in 1969, rubella vaccination was introduced in the United States, 5 years after the last major epidemic in this country and shortly before the next significant outbreak was predicted. It never occurred, and the vaccine strategy adopted in the United States has been remarkably successful in controlling natural rubella and its devastating teratogenic effects (306). Recently, recommendations for a drive to eradicate rubella have been issued by the World Health Organization by addition of rubella vaccine to the measles vaccine already being administered worldwide.

INFECTIOUS AGENT

RV is the only member of the genus *Rubivirus* in the family Togaviridae. This family of viruses is characterized as small enveloped particles that contain a positive-stranded RNA genome of 10 to 12 kb. The other genus in this family comprises the alphaviruses, a large group of arthropod-transmitted viruses that share a similar genetic organization and replication strategy. There is, however, no serologic cross-reactivity between the alphaviruses and RV, and only limited regions of genetic homology in the non-structural proteins representing the functional domains for enzymes such as the replicase and protease (see Structure and Organization of the Genome).

An unusual feature of RV is that there are no related animal viruses (c.f., measles and canine distemper viruses), and only one serotype exists, although some strain variation in virulence, teratogenicity, and antigenicity has been reported, particularly between wild-type and attenuated vaccine strains (see Phenotypic Variation). Although the virus shares many aspects of its genomic structure and replication strategy with the alphaviruses, it does not have an arthropod vector, and humans are believed to be the only natural host and reservoir for the virus.

Morphology and Physicochemical Properties of the Virion

Electron microscopy of purified preparations of RV or thin sections of infected cultured cells, stained with

phosphotungstic acid, have shown the virion to be a spherical particle measuring 60 to 70 nm in diameter (222) (Fig. 1). Each virion consists of a 30-to 35-nm electron-dense core surrounded by a lipid envelope derived from the host cell. Embedded in this are two glycoproteins (E1 and E2), which form a “fringe” 5 to 8 nm in diameter that can occasionally be resolved as surface spikes with knobbed ends, containing both envelope proteins. Between the core and the envelope is an electron-lucent zone that is wider and more distinctive than that of the other togaviruses (222). The core has been suggested to have a T=3 icosahedral symmetry and contain 32 capsomers (196), further distinguishing RV from the alphaviruses, which are reported to have a T=4 symmetry. The core consists of a single strand of 40S RNA comprising slightly less than 10 kb complexed with multiple copies of a single capsid protein, C, in the form of disulfide-linked homodimers (16). The RNA in the core structure is susceptible to degradation with ribonuclease, a treatment that causes disintegration of the core, indicating that the RNA is an integral component of the core structure (157).

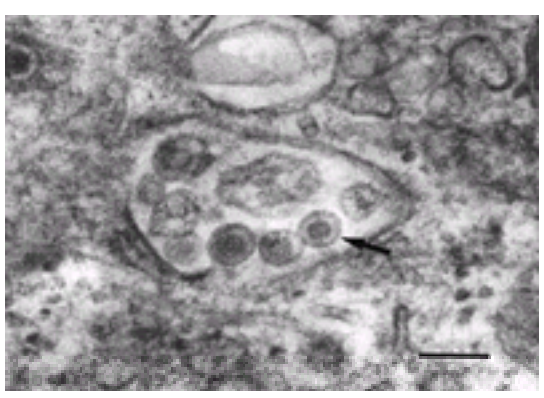

FIG. 1. Mature rubella virus (RV) virions in a cytoplasmic vacuole. A typical RV virion (*arrow*) consisting of a spherical core surrounded by a host-derived lipid bilayer. The bar represents 100 nm. (Electron micrograph courtesy of Dr. Jia-Yee Lee.)

The buoyant density of rubella virions in sucrose gradients has been reported to be between 1.17 and 1.19 g/mL (reviewed in 155). Sucrose-gradient purified virus observed by electron microscopy is contaminated with membranous material, derived from the cellular membranes from which the virus buds during assembly. This may explain the wide range of sedimentation coefficients that have been reported for this virus, ranging between 240S and 350S (13,297).

Rubella virions are heat labile and rapidly inactivated at 56°C (half-life, 5 to 20 minutes) and also at 37°C with a slower decay curve (half-life, 48 hours) associated with some infectivity remaining even after 7 days (210). The virus is stable for prolonged periods if stored in the presence of protein stabilizers at –60°C or below, but infectivity is rapidly lost at –20°C. The virus is also sensitive to hydrogen ion concentrations outside the pH range of 6.8 to 8.0. It can be lyophilized and is then stable at 4°C for years and at room temperature for months, a property that has been exploited for vaccine distribution (199).

RV can be inactivated by lipid solvents, such as ether, chloroform, and detergents like sodium dodecyl sulfate (SDS), sodium deoxycholate, and saponin (255). Protein denaturing agents like formaldehyde, ethylene oxide, and b-propriolactone, as well as ultraviolet light or photodynamic dyes that can cause RNA breaks, also abrogate infectivity.

Biologic Characteristics

RV has a restricted host range (only humans) *in vivo* but replicates in a wide variety of mammalian cell types *in vitro,* including primary AGMK cells and BHK21, RK13, and Vero cell lines. All strains of the virus (including wild-type strains) are slightly temperature sensitive, giving higher yields of virus at 35°C than at 37°C, and the vaccine strains RA27/3 and Cendehill are completely growth restricted at 39°C, whereas some wild-type isolates replicate to some degree at 41°C (54). In comparison to the rapid lytic infection of mammalian cells by the alphaviruses, RV replicates more slowly with an eclipse phase of 10 to 12 hours (4 hours for Sindbis or Semliki Forest viruses) and with maximal titers of virus released between 30 and 48 hours (about 8 hours for alphaviruses). In addition, viral yields are low, typically in the range of 0.1 to 10 infectious progeny per cell (about 10^4 plaque-forming units per cell [pfu/cell] for Sindbis virus). The reasons for the poor replicative ability are not well understood but may relate to low uptake of virus, to inefficiency of the replicase-transcriptase, or to the assembly process. One factor that may play a role is the high guanosine + cytosine (G+C) content of the virus (69.5%), which is associated with a high degree of secondary and tertiary structures in the genomic RNA that may impede replication, transcription or translation. Also related to the G+C content, codon use by RV is significantly different from that of normal eukaryotic cells (reviewed in 110), requiring use of transfer RNA that may be of low abundance in the cell, a factor that may limit the rate of protein synthesis (327).

Effects on Host Cell

At high multiplicities of infection (MOIs), RV causes rounding and detachment of cells commencing 24 hours after infection and increasing over the next 48 to 72 hours. At low (less than 1 pfu/cell) or moderate MOIs (1 to 3 pfu/cell), however, there is little visible cytopathic effect in many cell lines (174), and chronic infections can readily be established (see Persistence). Cellular macromolecular synthetic pathways appear not to be inhibited to a substantial degree even after high MOIs (5 to 10 pfu/cell). For example, cell RNA synthesis occurs for 72 hours after infection (135), and protein synthesis continues at normal or only mildly reduced levels for 48 to 72 hours in most cell lines (48,135). In mitogen-stimulated peripheral blood mononuclear cells, however, cell protein synthesis is inhibited by more than 90% by 48 hours after infection (55). In a number of specialized cell types, RV infection has been reported to affect “luxury functions” while allowing cell survival (360,373), although the growth rate of such chronically infected cells is reduced (373). Growth inhibition is particularly notable in primary diploid cells derived from human fetal organs (284), providing an

explanation for underdevelopment of organs in congenitally infected children that results in microcephaly and in autocrine and bone abnormalities (see Congenital Rubella Syndrome).

Although RV is not considered a highly cytopathic virus, infection of cultured cells disturbs the organization of the cytoskeletal elements, in particular actin filaments (32). Recently, apoptosis or programmed cell death has been shown to play a role in the cytopathic response of several cells (98,280). Most detached cells showed evidence of chromatin fragmentation and cell death that could be prevented by the caspase inhibitor z-VAD-fmk but not by bcl-2 or bcl-XL, at least in RK13 cells (98). However, cell survival rather than induction of apoptosis is clearly the dominant response to RV infection at MOIs of 1 pfu/cell or less, with many cells surviving in a chronically infected state.

Stimulation of membrane biosynthesis may also occur in RV-infected cells. Both proliferation and distention of endoplasmic reticulum (ER) and Golgi membranes, often associated with budding virus, have been identified by electron microscopy (223). The proliferation of membranes and formation of vesicular structures is believed to be associated with viral replication complexes and identifies sites of both viral RNA synthesis and assembly (181) (see Life Cycle). Other effects on host cells include the presence of chromosomal breakages in both human diploid cells infected *in vitro* (156) and cell lines derived from congenitally infected fetuses (7,46).

Phenotypic Differences Among Rubella Virus Strains

Although there is only one serotype of RV, strains of the virus exist that differ in properties such as hemagglutination (184), plaque morphology (174), temperature sensitivity, virus yield, and cell tropisms (54). Phenotypic variation also occurs *in vivo,* particularly in the reduced virulence of the vaccine strains, which are associated with milder acute symptoms and a lower incidence of complications than natural infection (21,331). This includes joint and neurologic symptoms (105,331) as well as teratogenic effects (17) (see Adverse Reactions).

All RV strains cross-react serologically, and the major neutralization epitopes appear to be conserved, although differences have been noted in the kinetics of neutralization (125) and in reactivity to certain monoclonal antibodies (54). Both infection and immunization, however, are believed to give protection against all other strains for the duration of the immune response elicited, except in cases in which incomplete immunity is induced (332). The vaccine strains RA27/3 and Cendehill display different tissue tropisms from wild-type–positive strains, including growth restriction in both peripheral blood mononuclear cells (PBMC) and the B-cell lines, Raji and Cess cells (54). These two strains also show limited growth in cells derived from human joint tissue (210), with the Cendehill strain completely inhibited in synovial organ cultures. In comparison, wild-type strains grow to high titer (54) in all of these cells. The determinants of tropism for joint tissue have recently been mapped to the 5' end of the genome, not to the structural proteins, and therefore are thought to involve intracellular replication and not binding and entry (187).

Structure and Organization of the Genome

The genome of RV is a single molecule of positive-stranded RNA that is about 10 kb in length. The 5' end of the RNA has a 7-methyl-guanosine cap (239), and at the 3' end, there is a poly(A) tract with a mean length of 53 nucleotides (348). The genome has a G+C content of 69%, the highest of any RNA virus identified to date. Early studies on RV in the 1970s identified two species of virus-induced RNA in infected cells of 38S to 40S and 24S to 26S (158,308). The larger species represents the full-length genome and is infectious when transfected into cells (158). The 24S species was initially shown in the alphaviruses to represent a subgenomic messenger RNA (mRNA) that encodes the structural viral proteins; a similar strategy for expression of RV structural proteins was confirmed in 1984 (239).

The genome comprises two long polycistronic open reading frames (ORFs) separated by a nontranslated region of 123 nucleotides (278,372) (Fig. 2). The 5' ORF encodes the two nonstructural proteins p150 and p90, whereas the 3' ORF, which is also represented in the 24S subgenomic RNA species, encodes the three proteins, E1, E2, and C, that form the virion structure.

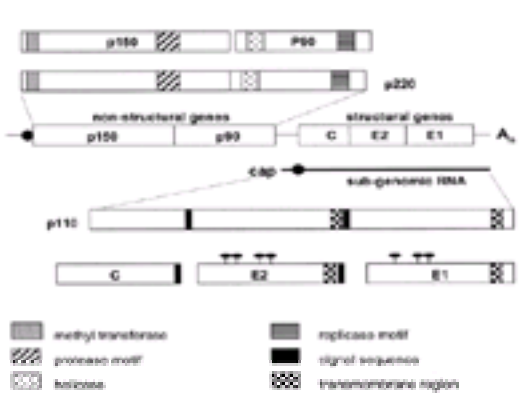

FIG. 2. Schematic diagram showing the organization of the rubella virus (RV) genome and the synthesis and processing of the nonstructural and structural proteins. Transmembrane regions and signal sequences of E1 and E2 are indicated. Regions of the nonstructural proteins believed to encode enzymatic functions, including the putative replicase and protease, are marked.

Cis-Acting Elements

Preceding each ORF is a short nontranslated region that is predicted to form a stem-loop structure believed to be important in viral RNA translation or initiation of RNA synthesis (229,272). The predicted 5' stem-loop encompasses

nucleotides 15 to 65 and has a terminal loop as well as a bulge in the stem and a hinge region (Fig. 3). It is thought to form a pseudoknot (279), a structure that has been shown in a number of plant and animal viruses to enhance protein binding and have a role in RNA replication (80,94). Within the single-stranded leader sequence (nucleotides 1 to 14) and the stem-loop, there are three AUG start codons at nucleotides 3, 41, and 57. Translation of the long ORF encoding the nonstructural genes is initiated at AUG_{41}, whereas the first and third AUGs are in a different reading frame and are terminated at nucleotides 54 and 90, respectively. Site-specific mutagenesis studies have indicated that AUG_3 is not essential for viral replication (278), and it is not known whether a short peptide synthesized from either AUG_3 or AUG_{57} plays any role in replication, but it is notable that the three AUGs are conserved in all strains sequenced to date (187,279). Analysis of the 5' end sequences of a number of RV strains has shown that the stem-loop is largely conserved, with mutations restricted to the terminal loop or the hinge region (161,279). Mutations in the 5' stem-loop are associated with differences in viral yield and plaque morphology (279) as well as tropism for joint tissue (187). Several proteins are known to bind to the 5' stem-loop, including the autoantigen La (lupus antigen) (272), a known RNA-binding protein that complexes with several viral RNAs, including the poliovirus 5' non-translated region (NTR) (203) and the human immunodeficiency virus TAR element (47). La affects translation efficiency of HIV (326), whereas in Sindbis virus, La binds the 3' positive-strand stem-loop and has been suggested to have a role in initiation of negative-stranded synthesis (253). In RV, calreticulin, not La, binds to the 3'positive-strand stem-loop with a potential role in negative-strand synthesis (229).

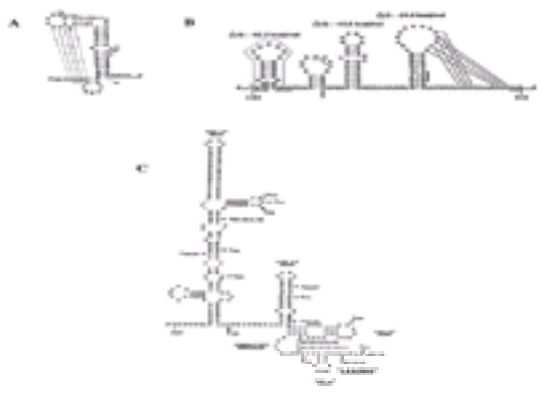

FIG. 3. Predicted stem-loop structures in the 5'end sequence (**A**), the 5' subgenomic end sequence (**B**), and the 3' end sequence (**C**). Potential pairing of bases to form pseudoknots in structures A and B are shown with *dotted lines.* (**A** and **B** from ref. 110, with permission; **C** from ref. 61 with permission.)

The 123-nucleotide region between the nonstructural and structural genes also contains a region that is predicted to form a series of stem-loops (see Fig. 3). This region has 58% homology with the equivalent sequence in Sindbis RNA and is thought to be involved in regulating synthesis of the subgenomic RNA. These stem-loops are also present in the 78-nucleotide 5' NTR of the subgenomic RNA, where they may play a role in translation of the structural genes. At the 3' end, there is a short NTR of 59 nucleotides following the stop signal at nucleotide 9701. A complex secondary structure involving three major stem-loop structures has been predicted for the 3'-terminal 240 nucleotides (61) (see Fig. 3). This is the region that binds the phosphorylated form of calreticulin (229), a calcium-binding protein that has been identified in both the nucleus and cytosol, in the lumen of the ER in plasma membrane fractions, and in the extracellular space (315). In addition to calreticulin's originally identified function in regulating calcium metabolism in muscle, it interacts with integrins and modulates expression of hormonally regulated genes (88). Possible roles for calreticulin in RV replication have been suggested to include promotion of viral RNA replication or translation, targeting of RV RNA to certain cell membranes where RNA synthesis occurs, and sequestering viral RNA during persistence (10).

Encapsidation Signal

The region on the RNA involved in binding to the capsid protein is a 29-nucleotide sequence located in the 5' nonstructural gene region between nucleotides 347 and 375 (183).

Nonstructural Gene Open Reading Frame (ORF)

The nonstructural genes are clustered at the 5' end of the genome within a long open reading frame commencing at AUG_{41}. This is translated into a single polyprotein, greater than 200 kd, that is cleaved into two products, p150 and p90 (192) (c.f., the four nonstructural genes found in the alphaviruses).

P150. The 5' proximal protein is a 150-kd protein comprising 1300 amino acids (106). It encodes a cysteine protease with catalytic activity shown by site-specific mutagenesis to be associated with the catalytic dyad, cys1151 and His1272 (62). This cleaves the polyprotein between Gly1300 and Gly1301 in a process that can occur both in cis and trans (372). A predicted methyl transferase (MT), with sequence homology to an MT domain in the a-like supergroup of positive-strand RNA viruses (295), is found at the 5' end of p150. It comprises a glycine-rich loop preceded by a hydrophobic segment implicated in binding S-adenosyl methionine. MT is believed to have a role in capping the viral RNA (173). In addition, p150 contains a region, X, with sequence homology to the alphavirus protein NSP3. The function of this protein is not known, but it appears to be essential for alphavirus replication (130).

P90. The second nonstructural protein, p90, comprises 905 amino acids and encodes the replicase and helicase motifs. The GDD tripeptide at amino acids 1965 to 1967 is identified as the core component of the replicase by comparison with global RNA-dependent RNA polymerase consensus sequences (162). An RNA-stimulated nucleoside triphosphalase (NTPase), involved in promoting unwinding of the template during RNA replication has been found associated with the

helicase motif (124).

The Structural Gene ORF

The structural genes are clustered within the 3' ORF and are translated only from the subgenomic 24S RNA. There are two in frame AUG initiation codons in the subgenomic RNA separated by 21 nucleotides. The downstream AUG is in the more favorable context for translation initiation (175), but the results of *in vitro* mutagenesis experiments suggest that both codons can be used indiscriminately (66). The gene order was determined by synchronized translation to be *NH2-C-E2-E1-COOH* (236), several years before the subgenomic RNA had been sequenced (65,114,347). As with the nonstructural genes, the subgenomic ORF is translated into a precursor polyprotein of 110 kd that is subsequently cleaved to give the individual structural proteins.

C. The 32-kd capsid protein exists as a disulfide-linked dimer (16,350) that interacts with the genomic RNA to form the electron-dense core of the virus particle. The amino-terminus is rich in arginine and proline residues, and the net basic charge helps to neutralize the negative charge on the phosphate backbone of the RNA, allowing its condensation into the nucleocapsid structure. The peptide sequence involved in interaction with the RNA lies within this region, encompassing 29 residues between amino acids 28 and 56 (183). Unlike the capsid protein of the alphaviruses, C protein of RV does not possess autoprotease activity, and its release from the polyprotein requires cellular signal peptidase (65). The hydrophobic recognition signal required by this enzyme is located in the carboxylterminus of C, and cleavage occurs at the same time as translocation of E2 into the lumen of the ER (149,237). The retention of the signal sequence by C is believed to promote association of this protein with membrane fractions, which in turn affects capsid morphogenesis, a process that occurs in association with membranes (15,325) (see Life Cycle).

E1. The 58-kd envelope glycoprotein E1 normally exists as a heterodimer with E2 to form the viral spike that protrudes from the surface of the virion. E1 consists of 481 amino acids and has three potential N-linked glycosylation sites (asn-X-Ser/Thr), all of which are used (148). The molecular weight of the unglycosylated E1 computed from the amino acid composition (predicted from sequence data) is 53 kd (65,114), and a molecule of this size has been identified in tunicamycin-treated cells (238,299). E1 is a class 1 membrane protein, and its single transmembrane sequence comprises 22 amino acids near the carboxylterminus (65). A region between amino acids 81 and 109 is thought to be involved in interaction with E2 and also in the membrane fusion induced by E1 at low pH (371). Palmitylation of residues within the transmembrane region, on the cytoplasmic side of the lipid bilayer, is also thought to occur (350).

E2. The second component of the spike, E2, is a heavily glycosylated polypeptide that exists in infected cells as a heterogeneous population of species varying in molecular weight from 42 to 54 kd (238; reviewed in 110). E2 consists of 282 amino acids, and its unglycosylated precursor is 30 kd (238,299). Thus, 15 to 25 kd of its molecular mass is carbohydrate, comprising both N- and O-linked glycans (188). There are four potential N-linked glycosylation sites (Asn-X-Ser/Thre) in most RV strains (the M33 strain has only three), and all appear to be used (282). On analysis by SDS-PAGE, E2 migrates as a broad smear that can sometimes be resolved into two to four discrete bands (54,238) and by isoelectric focusing (pH, 5.0 to 8.6) into more than 15 species (350). E1/E2 dimers are frequently seen in extracts of infected cells and are the dominant species in mature virions (16). E2 is believed to be largely buried beneath E1 in the virus particle because it is protected from the activity of glycosidase (141), neuraminidase, and trypsin (259) as well from monoclonal antibodies specific for the aminoterminal portion (351). Like E1, E2 is a class 1 membrane protein with a 39 residue transmembrane region located in the carboxylterminus between amino acid 517 and 555 (113). This region is in juxtaposition to a stretch rich in arginine residues that is located on the inside of the envelope and is thought to form a hydrophilic anchor. It is followed by a further 20 hydrophobic residues that serve as the signal sequence of E1 (15). The arginine-rich sequence is a potential cleavage site for trypsin-like proteases but appears not to be cleaved, so that the E1 signal sequence is retained on E2 (15,191,237). Electrostatic binding between these positively charged arginines and clusters of aspartic acid and glutamic acid residues in the capsid protein are thought to mediate interaction between the nucleocapsid and E2, and thereby play a role in viral assembly (118).

Life Cycle

The replicative cycle of RV is similar in many respects to that of other Togaviridae (see Chapter 29 and Chapter 30) and will only be summarized here with emphasis on differences that have been found; it has been reviewed in detail in (110). The virus growth cycle is much longer than that described for alphaviruses, with an eclipse phase of 10 to 12 hours and maximal viral titers released between 36 and 48 hours after infection of permissive cell lines such as BHK/21 cells and Vero cells (135). Infection of cultured cells is an asynchronous process even when high MOIs are used (5 to 10 pfu/cell); foci of infection are first seen at 24 hours, and the infection then spreads to other cells over the next 24 hours (308). The reason for this may relate to poor uptake of the virus or to host factors affected by the cell cycle, although partially synchronized RK13 cells infected 24 hours after subculture of a stationary monolayer exhibit the same effect (48).

The cellular receptor for RV has not yet been identified. There is some evidence that it may be a lipid because treatment of Vero cells with phospholipases A2 and C abrogated RV infection, whereas proteases and glycosidases had only a limited effect (194). Entry of the virus is believed to be through receptor-mediated endocytosis, and in agreement with this, exposure of the envelope glycoproteins to low pH (less than 6.0) makes them fusogenic (166). In addition, there is evidence that capsid protein C undergoes a conformational change at low pH that renders it hydrophobic (197). This may allow the nucleocapsid to adhere to the endosomal membrane and facilitate uptake of the viral RNA into the cytoplasm.

The genomic RNA provides the mRNA template for translation of the nonstructural genes as a greater than 200-kd precursor that is proteolytically cleaved to yield the two nonstructural genes *p150* and *p90* (see Fig. 2). The replicase encoded by *p90* then uses the input RNA as a template for negative-strand complementary RNA in a process that has not been well characterized for RV but that is thought to resemble that described for the alphaviruses (outlined in Chapter 29). Only full-length negative-strand RNA has been detected in infected cells; therefore, this must act as template for both the full-length progeny positive-strand RNA and the subgenomic mRNA that encodes the structural proteins (135). Stem-loop structures at the 3' ends of both the positive- and negative-strand RNAs bind several host

proteins that may have a role in RNA replication, including the phosphorylated form of calreticulin (227). The negative-strand RNAs are found solely in double-stranded complexes with positive-strand RNA (19S to 20S replicative intermediates) and have been localized to vesicles intimately associated with rough ER by 8 hours after infection. These replication complexes (Fig. 4) form a striking feature of RV morphogenesis and are characterized as virus-modified lysosomes lined internally with vesicles containing irregular internal structures (180,189). The localization of double-stranded replicative intermediates and RV nonstructural proteins within the replication complexes indicates that they represent sites of viral RNA synthesis (178,181). In addition, the detection of RV core particles at the cytoplasmic side of the vesicles lining the lysosomal vacuole suggests that the complexes are also the sites of nucleocapsid assembly (182).

FIG. 4. A typical rubella virus (RV) replication complex. The replication complex is characterized as a virus-modified lysosome containing vesicles (*closed arrows*) that line the inner membrane of a vacuole (*v*). Each vesicle has a double membrane and contains an irregular internal structure. These vesicles have a distinct morphology and should not be mistaken for RV virions. The rough endoplasmic reticulum (*open arrows*) surrounds the replication complex. The bar represents 200 nm. (Electron micrograph courtesy of Dr. Jia-Yee Lee.)

Both full-length and subgenomic positive-strand RNAs are synthesized from the full-length negative-strand templates in a molar ratio of 1.6:1 (135). Stem-loop structures at the 3' end of the negative-strand RNA and in the region surrounding the subgenomic promoter, which are believed to act as cis-regulatory sequences, are described under Structure and Organization of the Genome (see Fig. 3). The progeny full-length positive-strand RNA has three possible fates. It may be sequestered in the replication complexes as template for negative-strand synthesis, undergo translation to yield the nonstructural protein precursor, or at later stages of infection, be encapsidated as part of the assembly process. The mechanism whereby these events are regulated is not yet understood.

The subgenomic RNA has only one purpose: to act as template for the structural viral protein 110-kd precursor (65,239). Its translation occurs in hypertonic salt conditions that greatly reduce cellular mRNA translation, indicating that it has a higher affinity for ribosomes or translation factors than cell mRNAs, a factor that may be important in view of the inability of RV to inhibit host translation (48). The processing of the 110-kd precursor into the three virion polypeptides requires cellular signal peptidase and occurs during translocation of E1 and E2 into the lumen of the ER (149). This is a marked difference from the alphaviruses, for which C is cotranslationally cleaved from the structural protein precursor by an autoprotease located within the capsid protein (205). Moreover, in RV, the E2 signal sequence is retained by C, giving it a hydrophobic “tail” that allows it to remain membrane-associated, a factor thought to have important implications in virus assembly (15).

Posttranslational processing of the envelope glycoproteins has been studied in considerable detail (15). The translocation of the E1/E2 precursor into the lumen of the ER occurs at the same time as its cleavage by signalase into polypeptides of about 30 and 53 kd. Within the ER, E1 and E2 form disulfide-linked heterodimers that are subsequently transported to the Golgi stack. Transport to the Golgi occurs much more slowly (60 to 90 minutes) than for the alphaviruses, a finding thought to be due to slow maturation and folding of E1 in the ER. When expressed separately in COS cells from plasmid expression vectors, E2 is transported alone to the Golgi complex, although less efficiently than in the presence of E1 (15). E1 is not transported alone, however, because of an ER location signal located in a region encompassing the transmembrane domain and the cytoplasmic tail (146). Instead, it aggregates and accumulates in cytoplasmic vesicles derived from an intermediate compartment between the ER and Golgi apparatus (150).

The E1/E2 heterodimers are targeted to the medial and *trans*-Golgi apparatus, where glycosylation occurs. N-linked glycosylation of proteins is thought to be important in the initiation and maintenance of folding into the correct biologically active conformation as well as in protecting proteins from proteolytic attack (261). It also affects the immunogenicity and antigenicity of proteins (120). Mutants in which the glycosylation sites in either E1 or E2 have been eliminated by site-directed mutagenesis were found to be processed more slowly and transported less efficiently to the cell surface (282). They were also found to be less stable and undergo aberrant disulfide bonding. Both E1 and E2 are N-glycosylated, a process that is heterogeneous and involves the addition of high-mannose, complex-type, and hybrid-type glycans (188,299). In addition, E2 is O-glycosylated (188,299) at sites that are less well defined but have been suggested to include a region rich in hydroxy amino acids and proline (amino acids 97 to 123) (110). O-glycosylation involves the initial addition of *N*-acetyl galactosamine, which can occur in the ER or the Golgi apparatus, followed by the addition of galactose and sialic acid in the *trans*-Golgi complex.

Some of the processed glycoproteins are transported to the plasma membrane, but most are retained in the Golgi stack or are found in the ER or in membranes surrounding intracellular vesicles (15,145). Virus budding occurs at all of these membranes (14,243), with the majority of virion assembly occurring at intracellular membranes. Nucleocapsid formation also occurs in association with membranes, in part due to the hydrophobic region at the carboxylterminus of C that acts

as the signal sequence for E2 in the polyprotein (325). Thus, unlike the alphaviruses, RV RNA encapsidation does not occur in the cytoplasm forming crystalline arrays of capsids visible under EM; instead, capsids are first apparent in association with partially budded membrane (see Fig. 4). Interaction of C with the cytoplasmic tail of membrane-bound E1 is believed to play a role in virion assembly (147). The ability of RV to mature within cytoplasmic vesicles, rather than at the cell surface, may help the virus to avoid elimination by the immune system and to produce persistent infections.

RV Persistence

Although cell death by apoptosis occurs in various highly susceptible cell lines infected at high MOI, infection of a wide variety of cells *in vitro* with low MOIs of RV results in viral persistence (361). Cell lines derived from congenitally infected infants have also been shown to be chronically infected (284,285). Both temperature-sensitive mutants (28,361) and defective interfering (DI) particles are generated during RV persistence and are thought to play a role in controlling the degree of viral replication (2), but neither is required to establish persistent infection. During long-term persistence of Vero cells, DI RNA becomes the dominant species of viral RNA, with genomic RNA decreasing to low levels (2,112). In summary, RV persistence generally occurs as a chronic infection, with most cells expressing viral antigen and RNA, much of which is DI RNA. These cultures release low levels of temperature-sensitive progeny virus and DI particles.

The interferons may play a role in viral persistence and are induced in a number of chronically infected cell lines (322) as well as in human PBMC, both *in vitro* (37) and *in vivo* (258). Both interferon-a and interferon-g restrict RV replication, reducing the levels of viral RNA in the cell as well as inhibiting viral translation (228). This could promote both establishment and maintenance of the carrier state. Interferon, however, is clearly not essential as persistent infections can be established in Vero and BHK/21 cells, which are unable to produce interferon, and exogenous interferon was not found to have an effect on RV persistence in these cells (322). Antiviral antibody also inhibits RV replication while allowing persistence of the viral genome (51). Incubation of human lymphoid cells with a polyclonal anti-RV rabbit antibody after viral adsorption prevents expression of viral proteins within the cells while allowing maintenance of the viral genome for at least 14 days. Subsequent removal of the antibody is associated with enhanced synthesis of viral proteins and release of progeny virus. A similar phenomenon described with measles virus has also been proposed to play a role in viral persistence (116). PBMCs are a known site of RV persistence *in vivo* both in congenitally infected infants (284) and in adults who develop rubella-associated arthritis after natural infection or immunization (53). Viral persistence *in vivo* occurs in the presence of high levels of neutralizing antibody, which may limit viral spread but actually promote viral persistence. *In vitro,* persistent infection of many cell lines occurs in the absence of exogenous antibody (361), and addition of antibody to these persistently infected cultures reduces the level of virus produced but does not cure the cells of the infection (2,361).

Another site of RV persistence *in vivo* is joint tissue (57,108) (see Complication). Both wild-type and vaccine strains of the virus have been found to infect and persist in a chondrocyte cell line (54) as well as in primary cultures of human joint tissue (210). Only the Cendehill vaccine strain showed no growth in cell and organ cultures derived from human joints; all other strains tested replicated readily in joint tissue and persisted for more than 3 months. Virus derived from these chronically infected cultures was temperature sensitive, but DI particles were not detected.

Animal Models

There are no reliable animal models for the study of clinically symptomatic rubella infection. However, a variety of laboratory animals (including subhuman primates) can be asymptomatically infected with RV. Rhesus and African green monkeys, as well as baboons and chimpanzees, develop viremia, shed virus in respiratory secretions, and produce humoral immune responses in a manner that parallels events in humans (152). Marmosets are also susceptible to infection but do not show clinical disease and do not transmit virus to their offspring (256). Conflicting results have been reported from experiments in rats demonstrating subclinical infections in which the virus either does (11) or does not (301) invade the fetus to cause growth retardation and ocular abnormalities. Rabbits also provide a model for the growth retardation and ocular malformations of congenital rubella infection (171). In this system, a concentration of virus occurs in chondrocytes, indicating a proclivity of RV for joint tissue (185). Ferrets develop viremia after intranasal inoculation of RV (83), and ferret pups reportedly develop central nervous system (CNS) vascular lesions similar to those reported in the congenital rubella syndrome (293). Finally, mice do not develop overt disease, but both humoral and cell-mediated responses to infection can be detected after intravenous or intrasplenic injection. Even highly immunodeficient SCID mice do not develop significant symptoms after injection of 10^7 pfu of Therien strain (J.K. Chantler, unpublished observation, 2000). Unfortunately, none of the animal systems has proved sufficiently reliable to study details of the pathogenesis of either acquired or congenital rubella.

Antigenic Composition and Determinants

Complement-Fixing and Hemagglutination Antigens

Early serologic investigations of rubella used crude complement fixation (CF) antigens extracted from virus-infected cells. These particulate CF antigens could be separated into two fractions on density gradients composed of mixtures of mature and relatively immature forms of the two virion glycopolypeptides (307). The relative proportions of E1 and E2 differed in the two fractions, and only the rapidly sedimenting fraction contained detectable amounts of C (143). This fraction lacked hemagglutination activity but had some residual infectivity, probably because of the small amount of nucleocapsid found in it. Both fractions were contaminated by host cellular components (143).

Antigen for hemagglutination inhibition (HAI) assays has generally been prepared from free virus or the slowly sedimenting CF antigen. Tween 80 and ether extraction of the crude preparation provides a potent and noninfectious hemagglutinin (HA). The minimal structure that retains HA activity of RV remains uncertain, however. The classic HA antigen is composed of nearly equal proportions of the E1 and E2 glycoproteins embedded in a slowly sedimenting particle with a density of 1.08 to 1.10 in sucrose (342). This compares with a density of 1.18 to 1.20 for the intact virion or nucleocapsid core. The extracted HA is a 26S particle that retains biologic activity. By electron microscopy, it appears as

a 15-nm rosette with a hollow core (341). Whether molecular interactions between the E1 and E2 molecules must be preserved to retain the biologic functions associated with specific regions of either glycoprotein has not been determined.

B-Cell Epitopes

The mature E1 molecule contains monoclonal antibody-defined domains that are involved in virus attachment to the surface of red blood cells (hemagglutination) and the initiation of infection (368). Less is known about the epitopes of E2 because few monoclonal antibodies have been produced that react with this protein. Polyclonal human serum reacts well with E2, however, indicating that this molecule is antigenic; in addition, several strain-specific antigens are thought to reside on this molecule (95). Current evidence indicates that E1 is the dominant surface molecule of the virus particle and is probably the main target for the detection and subsequent elimination of RV by the host's humoral immune system. During the last 10 years, the fine antigenic structure of E1 has been characterized, using expressed proteins, proteolytic peptide fragments, and synthetic peptides to deduce functional sites on E1 (59,215). HAI and neutralization sites have been localized to $E1_{245}$–$E1_{285}$, with three critical regions for antibody binding ($E1_{245}$–$E1_{251}$, $E1_{260}$–$E1_{266}$, and $E1_{275}$–$E1_{284}$) (142). A peptide-($E1_{213}$–$E1_{239}$)–based enzyme-linked immunoassay (ELISA) has shown good correlation between conventional ELISA, HAI, and neutralizing titers (218).

T-Cell Epitopes

Lymphocyte proliferation, cytotoxicity, and lymphokine secretion have been measured using T cells from individuals with diverse major histocompatibility complex (MHC) backgrounds who have either been infected naturally or have been vaccinated. At least 17 immunodominant T-cell epitopes stimulated with peptide antigens have been identified by lymphoproliferation. These include four major regions on E1 (amino acids 38 to 74, 190 to 280, 324 to 377, and 389 to 422) (215,246), two on E2 (amino acids 150 to 170 and 244 to 263) (215), and four on C (amino acids 1 to 29, 119 to 152, 205 to 233, and 255 to 280) (200,246). A further set of T-cell epitopes capable of inducing cytotoxic class I or II MHC-restricted T-cell clones are identified in Table 1. However, the functional significance of immunologic recognition of lymphoproliferative or cytotoxic T-cell epitopes in protective immunity remains poorly defined.

Protein	a.a. Sequence	T-cell clone	MHC restriction	References
C	9-22	$CD8^+$	A2	186
	11-29	$CD8^+$	A23 or B57 or B62	186
	125-139	$CD4^+$	DR9	245
	205-216	$CD4^+$	DR4	245
	264-272	$CD8^+$	A11, A3	245
	265-273	$CD4^+$	DR4 (DRB1 0403, 0406, 0407, 0901)	245, 248
E2	54-65	$CD4^+$	DR7	245
E1	273-284	$CD4^+$	DR4 (DRB1 0403, 0406, 0407)	249

a.a., amino acid; MHC, major histocompatibility complex.

TABLE 1. *Defined T-cell epitopes of rubella virus*

Recent studies have identified potential antigenic mimicry epitopes of RV using techniques of monoclonal antibody cross-reactivity, *in vivo* immunization, and identification of sequence homology (Table 2). The significance of these epitopes in the induction of autoimmune disorders associated with rubella infection *in utero* (see Late-Onset Sequelae) remains unclear.

Rubella protein	a.a. Sequence	Tissue specificity	Methodology employed	Reference
C	64-87	Pancreatic islet cell la-2 protein	Monoclonal antibody cross-reactivity	162
C	21-33	Collagen type I (1169-1180)	Sequence homology	362
E2	244-252	Myelin P.P (156-166)	Sequence homology	236
E2	Recombinant protein	Pituitary gland	In vivo immunization	375
E1	482-471	Myelin P.P (1-10)	Sequence homology	9
E1	Recombinant protein	Pituitary gland	In vivo immunization	375

a.a., amino acid; P.P, myelin proteolipid protein.

TABLE 2. *Antigenic mimicry epitopes of rubella virus*

EPIDEMIOLOGY

Incidence

Rubella is endemic worldwide, with regular seasonal peaks occurring in the spring months in temperate climates. Before the introduction of vaccines, epidemics of rubella occurred at intervals of 6 to 9 years in the United States and England; somewhat more frequent epidemics have been reported in other European countries (8). In tropical countries, epidemics

probably also occur, but the lack of significant clinical symptoms in affected children allows them to pass unrecognized (67).

Age

The age at which rubella is naturally acquired in the industrialized world has shifted since the introduction of universal vaccination at 12 to 18 months of age. Before this, peak infection occurred in the 5- to 9-year-old group, with the disease being uncommon among preschool-aged children (153,172). The burden of disease in the United States, however, has in large part shifted from children to young adults (43). In developing countries, however, where rubella vaccine is not consistently given (more than 70% of countries of low economic status do not have a national policy of rubella vaccination, including much of Africa and India) (290), infection at an early age is still the norm, with high seroconversion rates found in both preschool populations and in the 5- to 9-year-old group (123). Despite this, a substantial number of women of childbearing age (10% to 25%) were found to be rubella susceptible in a survey in India (24), and the incidence of congenital rubella syndrome (CRS) during rubella outbreaks in many developing countries is high: 1 to 2 per 1000 live births (84).

Mortality

Death attributed to acquired rubella is exceedingly uncommon. Less than 0.1% of all cases of rubella reported to the Centers for Disease Control and Prevention between 1966 and 1994 were fatal (42,45).

Origin and Spread of Epidemics

Because humans are the only known natural host of RV, continued cycling of the virus within the human population is required for perpetuation of the disease between seasons of maximal endemic occurrence. Congenitally infected infants who shed high levels of virus for many months of postnatal life represent a potential source of maintenance of the virus in nature for relatively short intervals. The development of pools of susceptible individuals generally explains the appearance of epidemics at intervals of 3 to 9 years in different geographic locations, with major epidemics of worldwide scope every 10 to 30 years. Much of the regional variation in the age of onset of rubella, the incidence of the disease, and the appearance and spread of epidemics can be explained on the basis of population densities, socioeconomic factors, and levels of medical sophistication in a given community. Infection rates during the course of an epidemic are highest among closely housed susceptible individuals, including adolescents and young adults (e.g., student populations or military establishments) (275).

Molecular Epidemiology

Studies of the genetic variation in RV strains isolated from different locations worldwide have recently been carried out by sequence comparison of the E1 gene (111,165). Two genotypes were identified: genotype 1 comprised 60 viral isolates from North America, Europe, and Japan, whereas genotype 2 represented 3 isolates from China and India. The two genotypes were found to differ by 8% to 10% at the nucleotide level, as opposed to about 2% within each genotype. This translated into only 1% to 3% amino acid substitutions in the E1 protein, attesting to a high degree of genetic conservation between rubella strains. Moreover, only one strain from genotype 2 contained a mutation in one of the known epitopes defined by monoclonal antibodies, confirming the antigenic similarity between strains previously noted (see Phenotypic Variation).

CLINICAL FEATURES

Acute Rubella

The infection caused by RV in early childhood or adult life is usually mild, with most cases passing as subclinical or unrecognized events (127,309). Clinically apparent rubella is characterized by any combination of symptoms that include maculopapular rash, lymphadenopathy, low-grade fever, conjunctivitis, sore throat, and arthralgia (377). The rash is the initial manifestation of the disease in about 50% of all cases (Fig. 5). It is first seen on the face and spreads to the trunk and limbs (153). Lesions appear as distinct pink maculopapules that later coalesce and then fade rapidly over several days. An associated posterior cervical and suboccipital adenopathy is characteristic. The entire clinical syndrome clears rapidly in days and is seldom attended by more significant symptoms, but arthropathy, thrombocytopenia, and encephalopathy can occur. The course of the acquired infection and the accompanying immune response is shown in Figure 6.

FIG. 5. Child with rubella showing rash with delicate pink macules. Occasional maculopapules and hemorrhagic elements may be seen. Individual macules often coalesce to form a pinkish flush. The rash is often absent in mild cases.

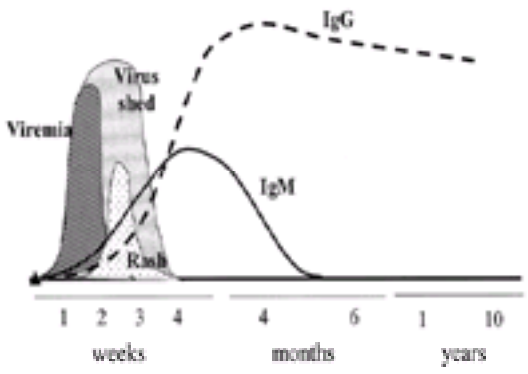

FIG. 6. Schematic diagram representing the course of acute rubella infection from the time of initiation of the infection by droplet spray. Note that the rash, which is often the first overt sign of infection, appears after 2 weeks and lasts 2 to 10 days. The patient is infectious and shedding virus from the time of infection until 1 to 2 weeks after the appearance of rash.

RV appears to be spread principally by aerosols. The mucosa of the upper respiratory tract and the nasopharyngeal lymphoid tissue serve as portals of virus entry and are the initial sites of virus replication. Spread of virus through lymphatics or a transient viremia then seeds regional lymph nodes. Local replication of virus in these nodes accounts for the posterior cervical and occipital nodal enlargement that may begin 5 to 10 days before the onset of the rash (127). The incubation period of 7 to 9 days is followed by the appearance of virus in serum, and the onset of viral shedding into the nasopharynx and stool, providing a source of spread to susceptible individuals. This viremic phase may be marked by mild prodromal symptoms and malaise. The maculopapular rash begins 14 to 21 days after natural exposure, but somewhat earlier after experimental infection or vaccination. The presence of virus in serum ceases shortly after the rash appears (305), with the onset of detectable circulating antibodies. Mononuclear cell-borne viremia (34,56) and virus in nasopharyngeal secretions, however, can be detected for a week or more after the rash subsides. Other sites of virus isolation include the conjunctival sac (134), urine (357), synovial fluid (139), lung (303), and cerebrospinal fluid (CSF) (321). Most of these isolations have been made at the time of the rash.

Immune Response

A serologic response is measurable at the onset of the rash and continues to evolve over the next few weeks. Immunoglobulin M (IgM) antibodies are present at the onset of clinical symptoms (300) and, depending on the sensitivity of the assay used, may be detectable for more than a year after acute infection (5). IgG antibodies can also be detected early in some patients, and by 2 weeks, anti-RV antibodies are present belonging to all immunoglobulin classes, including IgA, IgD, and IgE (300). Low levels of IgF antibody persist indefinitely after natural infection. IgG detected at early stages after primary infection is of low avidity (31), maturing gradually during the next 3 months. Detection of this low-avidity IgG together with antiviral IgA has been promoted as an alternative to the measurement of rubella-specific IgM, to distinguish reinfection or viral persistence from primary exposure (70,129). This is of particular importance when infection is suspected in the first 3 months of pregnancy when therapeutic abortion may be under consideration.

When measured by immunoprecipitation or immunoblot techniques, most of the immunoglobulin response appears to be directed at the E1 glycoprotein, with proportionally lesser amounts of the response directed at E2 or C (60,230). Male patients may have a more rapid and robust antibody response than female patients, but the latter have a stronger anti-E2 response (219). A peptide-based IgG ELISA ($E1_{213}$–$E1_{239}$) that uses a molecule that simulates the putative disulfide-bonded neutralization epitope of E1 shows good concordance with both conventional whole RV-based ELISA and neutralization titers (218).

Immune complexes containing RV-specific antigen are frequently found up to 3 months after either natural or vaccine-induced rubella infection (76). Notably, they are detected for longer periods in patients with chronic joint symptoms, indicating that virus persists in these patients (77). Deposition of such viral immune complexes on articular surfaces may form part of the pathogenic mechanism of rubella-associated arthritis. In addition, complexes of rubella antigen with antibody have been shown to cause release of superoxide anion from human polymorphonuclear cells *in vitro,* a reaction that would promote an inflammatory reaction *in vivo* (170). High levels of rubella-specific immune complexes have also been detected in children with CRS and may play a role in the pathogenesis of late-onset manifestations in this syndrome (77).

Transient depression of lymphocyte responsiveness to mitogenic stimulation follows natural or vaccine infections in children and adults (36,190). Similarly, delayed-type hypersensitivity to irrelevant antigens, such as purified protein derivative, is suppressed for several weeks after natural rubella (220). During this period of generalized immune suppression, RV-specific cell-mediated immune responses develop and can be measured *in vitro* within 1 or 2 weeks of onset of the clinical illness (35). The cellular responses wane over the next few years but persist at low levels indefinitely after natural rubella. Recent studies have defined the epitope specificity of defined CD4+ and CD8 + T-cell clones (186,246) (see Table 1).

Complications

Joint Symptoms

Natural rubella can be complicated by acute arthralgia or arthritis, especially when it occurs in adolescent and adult women (105,317). Incidence rates for arthralgia and arthritis exceed 60% in some outbreaks and may be considered a manifestation of rubella rather than a complication of the infection (133). For example, in one documented outbreak of rubella, 52% of adult women and 9% of adult men developed objective evidence of arthritis, with symptoms being more severe among the women (331). Manifestations of rubella arthropathies vary from joint pain alone to joint swelling, effusions, and loss of joint motion with local heat and erythema. Joint symptoms usually begin within 1 week of the appearance of the rash and may involve any joint, with the fingers and knees being most commonly affected (53,331). Paresthesia may accompany the arthropathy, especially if there is an associated synovitis and compression of the median nerve below inflamed carpal ligaments (75,302). Symptoms usually resolve within several weeks but may persist for years, are sometimes episodic, and, rarely, can be disabling (53,217,331). A similar set of symptoms occurs 9 to 27 days after vaccination but is encountered at a reduced frequency compared with that after natural rubella (105,331) (see Vaccine Adverse Reactions). Factors that affect the incidence of joint symptoms include age (355), gender (331), and MHC gerotype (216) of the infected patient, with adult women forming the most susceptible population. Hormonal influences also affect the incidence of joint symptoms. This includes an increased incidence after puberty in girls and women (105), a higher incidence in women immunized postpartum (333), and variation in incidence relating to the time of infection relative to the menstrual cycle (226). Reinfection with RV, although usually asymptomatic, can also result in arthropathy (177).

The pathogenesis of rubella arthritis is unknown, but the mechanism may involve local viral replication. Several groups have reported the isolation of RV from synovial fluids of symptomatic joints for up to 1 month after acute infection (108,139,235). Virus has also been isolated from PBMC of patients with symptoms (52,53) and has been detected by amplification of RNA extracted from PBMC of several women with chronic arthropathy after vaccination (217). Replication and persistence of virus in an extraarticular site and deposition of immune complexes on articular surfaces may therefore play a role in pathogenesis.

A current controversy is whether RV can be causally implicated in some forms of chronic degenerative arthritis, particularly juvenile and adult forms of rheumatoid arthritis (260,262,318). It is known that different strains of RV can infect and persist in joint tissue for prolonged periods both *in vitro* (210) and *in vivo* (235,320), and joints may be a preferred site of persistence because of their lower temperature (81). Evidence in favor of a role for RV in juvenile or adult rheumatoid arthritis includes the established arthrotropism of RV, with a number of case studies showing a temporal association of rubella infection with onset of arthropathy, the progression of joint symptoms in patients with documented rubella arthritis to a disease indistinguishable from adult or juvenile rheumatoid arthritis (193,201), and several studies that have detected rubella antigen in rheumatoid synovial tissue (juvenile or adult) (126,234) or have isolated the virus from this site (57). Indirect evidence implicating RV includes the detection of higher levels of antirubella antibody in synovial fluid relative to serum of some patients with arthritis (213), a finding of spontaneous secretion of RV-specific immunoglobulin by synovial cells cultured *in vitro* (58), the presence of proliferative responses to RV antigen by synovial lymphocytes (50,104), and unusual *in vitro* responsiveness of peripheral blood mononuclear cells from a patient with rubella-associated arthritis to synthetic peptides (214). However, more recent studies using the reverse transcription–polymerase chain reaction (RT-PCR) technique have failed to confirm the previous results showing the presence of RV in joint tissue of patients with adult or juvenile rheumatoid arthritis (30,109). Whether this is an issue of sensitivity of the RT-PCR technique remains to be determined. Moreover, an inability to detect RV in synovial fluid does not discount the possibility that the virus triggers an immune reaction that may damage joint tissue. Overall, the body of evidence indicates that RV is still a plausible candidate as one of several viral triggers for chronic degenerative forms of arthritis.

Thrombocytopenia

Transient asymptomatic depression of thrombocyte counts is common with rubella (73), but symptomatic thrombocytopenic purpura follows rubella in only 1 of 1,500 cases (344). The latter condition is usually self-limiting and may occur in the absence of rash as a plausible cause of some cases of idiopathic thrombocytopenic purpura (251). Very rarely, epidemic rubella has been associated with hemolytic anemia (344). There are sporadic reports of cardiac arrhythmia with acute childhood rubella (117) and of thyroiditis (225) and hepatic dysfunction (240) with rubella in adults.

Encephalopathy

The most serious complication of postnatal rubella is postinfectious encephalopathy or encephalomyelitis (18). Estimated to occur in 1 of 6,000 cases of natural rubella, the symptoms of postinfectious rubella encephalopathy appear abruptly 1 to 6 days after the onset of rash in an otherwise typical case of rubella. The most frequently encountered symptoms include headache, vomiting, stiff neck, lethargy, and generalized convulsions. An isolated myelitis or polyradiculitis may also occur (25). Physical examination often reveals nystagmus, diminished superficial skin reflexes, and variable deep tendon reflexes. The CSF is clear, with cell counts averaging 50/mm^3, comprising largely lymphocytes (349). Whereas the electroencephalogram is characteristically normal in uncomplicated rubella, it is pathologically altered in rubella encephalitis (252). Evidence for the direct involvement of RV in the pathogenesis of rubella postinfectious encephalopathy is not consistently found. However, RV antigens have been detected *in situ* within brain tissue (71), and virus has been isolated from one CSF sample (321) indicating that RV has the capacity to invade the mature CNS.

Rubella postinfectious encephalopathy usually requires only supportive treatment, and the course of the disease is generally concluded within a few days. Treatment of rubella encephalopathy with corticosteroids has been reported (231). The necessity for routine intervention is questionable, however, because 80% of all patients recover spontaneously (168). In the 20% of patients who die, the disease course typically includes coma, respiratory distress, apnea, and then death, usually within a few days of onset of symptoms.

Congenital Rubella Syndrome

Although postnatal rubella is rarely associated with severe complications, infection of a developing fetus after transplacental transmission of virus from the mother has dire consequences for fetal development (73,285,354). Maternal infection shortly before conception does not appear to lead to intrauterine infection (101). After conception, however, infection occurs in 90% of cases during the first 8 weeks of gestation, falling to a low of 25% to 35% during the second trimester and rising again near term (78,212,354) (Fig. 7).

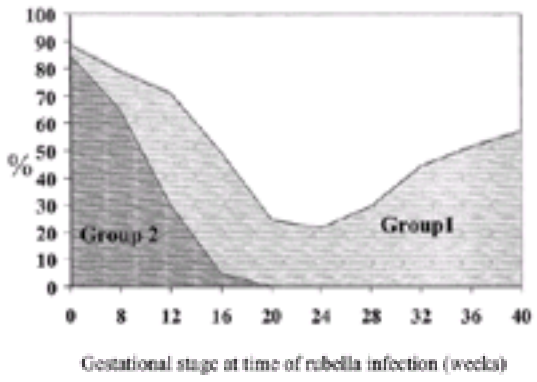

FIG. 7. Relationship between the time of maternal rubella infection and the consequences to the fetus. In group 1, the fetus is infected but shows no clinical manifestations at birth. Those in group 2 are born with congenital rubella syndrome (CRS).

Effects on Placenta

RV infection of the placenta in early gestation produces scattered foci of necrotic syncytiotrophoblast and cytotrophoblast cells and damages the vascular endothelium (338). Infection at later stages induces chronic multifocal mononuclear cell infiltrates in the placental membranes, cord, and decidua, along with vasculitis. These culminate in placental hypoplasia and macroscopic placentitis (119). After placental infection is established, there can be subsequent dissemination of virus to the fetus. This is not invariant, however, and virus is more often recovered from placental products than from fetal products of conception (4). After entry, virus is capable of spreading widely throughout the developing fetus, and almost any organ may be infected (19). A chronic and generally nonlytic infection is then established in the fetus.

Clinical Consequences

The effects of RV invasion of fetal tissue are varied. Very early infection may result in resorption of the embryo. Whether placental infection alone can lead to spontaneous abortion or abnormalities of fetal development is conjectural. Premature delivery and stillbirths are other potential outcomes of the fetal infection (212), but in most cases, the infected fetus survives, and the pregnancy continues to term (97). Increasingly, in many countries, clinically recognized maternal rubella now terminates in therapeutic abortion, particularly when infection occurs during the first 8 weeks of gestation when the occurrence of birth defects is extremely high (67% to 85% in different studies) (Fig. 7) (212,221). Fetal damage, therefore, is not universal even when there is serologic (221) or virologic evidence of fetal infection (263). The current rubella vaccine RA27/3 has extremely low teratogenicity, and inadvertent vaccination in early pregnancy is no longer considered an indication for therapeutic abortion (276). Viral transmission to the fetus resulting in persistent infection has, however, been described (150a).

The most common clinical manifestations of congenital rubella (present in at least 10% of patients) include hearing loss, congenital heart disease usually manifested as patent ductus arteriosus or pulmonary artery or valvular stenosis, psychomotor retardation, cataract or glaucoma, retinopathy, neonatal thrombocytopenia purpura, hepatomegaly and splenomegaly, and intrauterine growth retardation (75). Less common features (present in 5% to 10% of patients) include bony radiolucencies, hepatitis (usually with jaundice), and hemolytic anemia (3,72,97) (Table 3). Many of the clinical manifestations of congenital rubella are evident at birth or shortly thereafter (Fig. 8). Some, including hepatosplenomegaly and hepatitis, are transient and resolve within weeks in surviving infants (74). Intrauterine growth retardation resolves more slowly, if at all (277,337). Other defects remain as permanent and generally nonprogressive sequelae of the *in utero* effects of RV. Still other defects may not be recognized at birth. Delayed appearance can be explained by either the late onset of postnatal active multisystem disease, the dependence of these conditions on the normal development of function in the child before they are manifest, or the very delayed development of organ-specific progressive dysfunction.

Group A	Group B
1. Cataracts or congenital glaucoma	1. Thrombocytopenia Purpura
2. Congenital heart defects	2. Hepatosplenomegaly
3. Sensorineural hearing loss	3. Jaundice
4. Pigmentary neuropathy (retinopathy)	4. Microcephaly
	5. Meningoencephalitis
	6. Radiolucent bone disease
	7. Progressive or late-onset manifestations
	a. Mental retardation
	b. Diabetes mellitus
	c. Progressive Panencephalitis

TABLE 3. *Manifestations of congenital rubella syndrome*

FIG. 8. Child with congenital rubella syndrome.

Most children with CRS (80%) show some type of neural involvement (92). Clinical manifestations expressed in infancy include bulging anterior fontanel, lethargy, irritability, and motor tone abnormalities. Residual signs in survivors include mental retardation, motor disabilities, abnormal posture and movements, and neurosensory hearing loss. Laboratory findings may include increased CSF protein and abnormal electroencephalogram. Virus has been isolated from about 30% of CSF samples (93). Neuropathologic findings at autopsy of symptomatic newborns and infants differ from those found in early products of gestation (reviewed in detail in reference 364). Inflammatory changes in the brain are variably observed (93,292).

RV can be isolated from almost any organ at birth and from selected tissues for 1 year or more in surviving infants (206). More than 80% of congenitally infected newborns excrete substantial amounts of RV into their nasopharyngeal secretions and urine at birth, and 3% continue to shed virus for as long as 20 months (72). This chronic shedding of RV by neonates is an indicator of early gestational infection (78).

Mechanisms of Teratogenesis

The pathogenic mechanisms underlying RV teratogenesis remain unknown but are likely to be multifactorial (97,266). Direct effects of virus replication on focal clones of cells and their progeny during critical stages of the ontogeny of specific fetal organs are thought to give rise to the wide range of abnormalities that together comprise CRS (275). Chronic infection of fetal cells from many different tissues results in slowed growth rates and reduced survival. Infection of epithelial cells *in vitro* is associated with a marked depolymerization of actin filaments, and disruption of these cytoskeletal structures could disturb mitosis (32). Human embryonic cells persistently infected *in vitro* with RV display an altered responsiveness to the growth-promoting properties of epidermal growth factor as well as a decreased capacity for collagen synthesis (373). Thus, it is probable that noncytopathic RV infection of selected embryonic cell types *in utero* upsets the normal delicate balance of cellular growth and differentiation and has profound effects on organogenesis. Any of these effects, alone or in concert, could explain the observation that the small, but otherwise grossly normal, organs of congenital rubella infants contain reduced numbers of cells (224).

Direct cytolytic effects of RV also contribute to the observed damage. For example, the cataracts of congenital rubella contain highly characteristic pyknotic cells in the lens nucleus, and noninflammatory necrosis of the pigmented cells of the retinal epithelium produces focal areas of decreased and increased pigmentation of the retina (176). Focal cellular necrosis has also been noted in the myocardium, skeletal muscle, and inner ear (338). Necrosis of vascular endothelium is also prominent early in the *in utero* infection (338), and the evolution of these vascular lesions could readily explain many of the vascular abnormalities observed at term (208). In the CNS, focal areas of noninflammatory parenchymal and perivascular necrosis are common, and damage of large vessels is sometimes accompanied by ischemic necrosis of adjacent neural tissue (292).

In addition to these direct effects of RV replication in host tissue, there is considerable evidence that perinatal and also postnatal damage is, at least in part, immune mediated. Pathologic signs in infants who die at some interval after birth usually include mononuclear inflammatory infiltrates in one or more organs, particularly the lungs and brain (314,328). Circulating immune complexes containing RV antigens may involved in the immunopathogens of late-onset disease (328). They are found in the serum of nearly half of all infants with the congenital rubella syndrome, and there is an increased likelihood of finding rubella-specific immune complexes in the serum of CRS children with active clinical problems (77).

The persistence of RV in fetal tissue throughout gestation, and in infants with CRS for prolonged periods after birth, raises the question of how the virus avoids immune elimination. Clearly, neither transferred maternal IgG nor fetal IgM can eliminate virus *in utero,* although both have neutralizing capacity *in vitro* (75). Antibody may in fact promote persistence, as it has been shown to do *in vitro* (2,51). Moreover, fetal IgM is not synthesized until about 20 weeks of gestation, and cell-mediated immune responses are not detectable until 27 weeks, leaving the infant highly vulnerable before this time (211).

Immunologic Responses

Maturation of the immune response is abnormally slow in CRS with regard to both isotype switching and the development of high-avidity IgG (136,330). In addition, the humoral immune response of CRS infants differs qualitatively from that of naturally infected or immunized children. Whereas the latter groups possess some IgG class antibodies against each of the three structural proteins of the virus, CRS infants often lack antibodies to the C protein and demonstrate weak reactivity to the E2 protein (91,281). Perhaps more telling, these children show selective tolerance to the E1 protein (198) and, more specifically, little or no antibody to the putative E1 neutralization domain (218,281).

Infants with CRS also demonstrate prolonged impairment of RV-specific cell-mediated immune responses (35), including cytotoxicity (115) and lymphokine secretion *in vitro* (35). RV-responsive T-cell lines derived from several congenital rubella children and adults failed to respond to RV peptides that commonly stimulate lymphocyte proliferative responses in normal immune adult donor cells (246). These data suggest that early fetal infection results in selective immune tolerance to a limited but critical number of RV epitopes that must be recognized to clear the virus successfully.

Late-Onset Sequelae

Although many of the effects of fetal rubella are manifest at birth and may be transient, some defects, such as retinopathy, mental retardation, hearing loss, and endocrine abnormalities, may not become clinically apparent for several years (202,310). One study reported that 37% of preschool CRS children were mentally retarded, 7.4% were autistic, and 3.3% had behavioral disorders consistent with neural damage (64). Hearing loss is also a frequent manifestation in about 60% of CRS children reaching school age (221,359). In addition, asymptomatic congenital rubella (with positive IgM serology at birth) accounted for a substantial proportion of all cases of sensorineural hearing loss in early childhood before the widespread use of rubella vaccines (257). Eyesight may also be affected in up to 80% of young adults born with CRS (122). In these patients, bilateral pigmentary retinopathy (57%), bilateral or monocular cataracts (27%), and microphthalmia (23%) were the most common manifestations of ocular disease. Psychomotor retardation (62%), cardiac abnormalities (58%), and mental retardation (42%) are also frequently associated with CRS, and multiorgan disease is found in 88% of patients (122).

Delayed Endocrine Disease

Congenital rubella infection is also associated with a variety of endocrine abnormalities, including polyglandular autoimmunity (283,310). Type 1 diabetes mellitus occurs as overt or latent disease in as many as 20% of young adults with the congenital rubella syndrome (121,311). One follow-up study over 36 years of a series of 50 individuals with congenital rubella determined that 9 had developed clinical diabetes mellitus at a mean age of 28 years (207). In a younger cohort composed primarily of congenital rubella cases from the 1964 to 1966 epidemic, 20% had islet-cell surface antibodies, and 12% had developed diabetes by 17 years of age (121). An increased frequency of the class 2 antigens DR3 and, to a lesser extent, DR4 has been found in CRS patients who develop diabetes (121,296). This is the same MHC antigen pattern found in type I diabetes mellitus and in autoimmune polyglandular syndrome type II (283). Overall, children with congenital rubella appear to have a 50-fold increased risk for developing type I diabetes as compared with age-matched controls (232). In contrast, no clear association has been found between acute postnatal rubella and type I diabetes, although infection occasionally results in the transient induction of insulin autoantibodies or in the appearance of islet cell antibodies (26). A study of two children with acute-onset, fatal type I diabetes failed to show the presence of the RV genome in pancreatic tissues by RT-PCR amplification or *in situ* hybridization (33).

Several mechanisms to explain the induction of type I diabetes by viruses have been proposed, with virus-induced autoimmune events being central (376). It is of interest that the pancreas is probably a frequent target for RV replication *in utero* (72) and that human fetal islet cells support rubella replication *in vitro* (233). The lack of a suitable animal model has limited studies on pathogenesis. In one study, however, rubella RA27/3 vaccine virus adapted to growth in neonatal hamster pancreatic b-cells was found to induce both hypoinsulinemia and hyperglycemia in infected hamster pups (287). In these animals, cell-free RV was recoverable from pancreas, viral antigens were present in islet cells, and increasing mononuclear infiltration of the islets occurred over the first 3 weeks of the infection. Islet cell antibodies developed in 40% of infected pups (287).

The target antigens of the islet cell autoantibodies found in type I diabetes sera include antibodies to insulin, islet cell antigen (ICA) 69, glutamic acid decarboxylase (GAD) 65 and 69, and heat-shock protein (hsp) 65 (12,250,346). It has been suggested that cross-reactivity of antiviral antibodies or T-cell–mediated immune responses with islet antigens may trigger an autoimmune reaction against islet tissue (374). Evidence in favor of this includes the identification of a monoclonal antibody to RV capsid protein that reacts with pancreatic islet cell Ia2 protein (163), and CD4 and CD8 T-cell clones isolated from CRS patients have been shown to recognize GAD 65 peptides in an HLA-restricted manner (247).

Antibodies reactive with microsomal or thyroglobulin fractions of thyroid tissue have also been frequently found in congenital rubella sera (in 20% to 40% of cases in selected series [68,121,311]). In a study of children with congenital rubella enrolled in residential schools for the deaf (11 to 12 years of age), thyroid function studies were abnormal in 5% and present in 20% of those with thyroid autoantibodies (311). The relation of congenital rubella infection to the thyroid dysfunction is unclear, although in one case, RV antigen was detected in thyroid tissue obtained at surgery from a 5-year-old boy with CRS and Hashimoto's thyroiditis (378). Pituitary involvement may also occur as some children with CRS are growth hormone deficient (277). Hamsters infected with the M33 strain of RV or immunized with recombinant RV E1 or E2, develop pituitary autoantibodies and lymphocytic hypophysitis (375). These observations suggest that virus-induced autoimmune mechanisms may partially account for the late appearance of polyendocrine diseases that frequently complicate congenital rubella.

Delayed Neurologic Disease

An unusual late-onset rubella encephalitis has been described, referred to as *progressive rubella panencephalitis* (PRP) (339,356). Like other slow virus diseases of the CNS, PRP is characterized by a prolonged asymptomatic period,

followed by the onset of symptoms of neural deterioration during the second decade of life. These include behavioral changes, intellectual decline, ataxia, spasticity, and sometimes seizures (365). Ingravescent neurologic dysfunction follows, ultimately leading to death within 8 years. Although PRP has most often been associated with congenital rubella in children and young adults, it may also be a very rare, late complication of natural childhood rubella (1,87,366). Several lines of evidence link RV to the etiology of PRP. Sera and CSF from affected children contain antibodies that react with the structural proteins of RV (369). Rubella-specific immune complexes have been demonstrated in sera (76), and RV antigen has been visualized *in situ* within CSF cells (1). RV has been isolated from PBMC (367) and from brain by co-cultivation techniques (79).

Neuropathologic changes are found throughout the brain parenchyma and include microscopic evidence of subacute to chronic inflammatory change in the leptomeninges and in perivascular cuffs composed of mononuclear lymphocytes and plasma cells (339,340,356). There is extensive destruction of white matter, accompanied by loss of myelin, relative preservation of axons, and severe gliosis. No intranuclear or intracytoplasmic inclusions are seen, and viral particles have not been identified (365).

The pathogenesis of PRP is unclear. However, the continued or renewed generation of RV proteins, if not the complete virus, is likely during symptomatic persistent infection to provide the viral components of the circulating immune complexes found in PRP (76). The organ-specific vascular lesions of PRP strongly suggest that immunoglobulins are deposited in response to antigens produced locally. The nearly complete destruction of Purkinje cells indicates either some degree of selective tropism for RV within the CNS or a more profound and lethal effect of infection on the metabolism of these cells. This could also account for the selective loss of myelin and oligodendroglia, although immunopathologic mechanisms could also be active.

DIAGNOSIS

Differential

The most common symptoms of rubella (lymphadenopathy, erythematous rash, and low-grade fever) can readily be confused with similar illnesses associated with maculopapular rash caused by other common viral and nonviral pathogens or drug-induced eruptions (27). The basic differential diagnosis includes measles, scarlet fever, roseola, erythema infectiosum, and rash-associated enterovirus or adenovirus infections (74). In one series of patients with rubelliform rash and arthritis or arthralgia, rubella was confirmed in only 36% of the cases. Of the remainder, 7% were attributable to human parvovirus infections, and 54% were of unknown etiology (312). Parvovirus B19 infections typically cause a rubelliform rash and arthralgias in young female patients (343), and dengue can be confused with rubella in endemic areas (38). Therefore, a definitive diagnosis of rubella can be made only by specific laboratory studies.

Similarly, confirmation of a diagnosis of congenital rubella cannot be established solely on the basis of clinical findings. It requires either the direct isolation of RV or serologic evidence of acute infection in the infant. In the absence of confirmatory laboratory data, a clinical diagnosis compatible with CRS requires the presence of any two of the following: cataracts or congenital glaucoma, congenital heart disease, hearing loss, or pigmentary retinopathy. In the presence of only one of the preceding manifestations, the additional finding of purpura, hepatosplenomegaly, jaundice, microcephaly, mental retardation, meningoencephalitis, or radiolucent bone disease can suffice (41) (see Table 3).

Laboratory

Diagnosis of postnatal rubella and congenital infection (following birth) is normally carried out serologically by detection of rubella-specific IgM by ELISA (63). After postnatal rubella, IgM is usually detectable from the time of presentation until 5 to 6 weeks after the symptoms subside (209). Infants infected *in utero* produce rubella-specific IgM for many months after birth (330). Although tests for rubella-specific IgM are highly sensitive, they may be compromised by cross-reactivity with other viruses (especially parvovirus B19 and Epstein-Barr virus) (352) or by the presence of rheumatoid factor, which can give rise to false-positive results in assays that measure IgM indirectly (298). Confirmation of rubella infection has normally been carried out by a standard fourfold rise in antibody titer between an acute and convalescent serum sample (63). More recently, measurement of low-avidity rubella-specific IgG has been proposed as an alternative test for recent infection (31,129). This test is based on the finding that the avidity of antibodies for their antigenic target increases with time, reflecting maturation of the immune response. By inclusion of a mild detergent, such as 35 mM diethylamine, or a chaotropic agent, such as 8 M urea, in the wash buffer of a standard IgG ELISA test, the avidity of the IgG can be measured (31).

Other diagnostic serologic tests include measurement of HAI (167,323) or neutralization antibodies (291), but these are difficult to perform and are now used infrequently. ELISA tests, however, do not distinguish antibodies that are of functional significance, and patients may possess detectable ELISA antibodies in the absence of neutralizing or HAI antibodies (332). In addition, analysis of the antigen specificity of ELISA-positive sera on immunoblots has shown that some patients have a skewed response to the major viral antigens E1 and E2. This is particularly notable in CRS patients but has also been shown in patients undergoing reimmunization (91,198,204).

Viral isolation may occasionally be warranted, particularly to confirm infection during pregnancy or in a neonate. In acute infection, the virus is readily isolated from nasopharyngeal secretions for about 6 days before and after the appearance of rash (86). Although virus can be isolated from circulating lymphocytes and affected skin, these sites do not yield virus frequently enough for clinical diagnostic purposes. Cord blood or placental tissue may be used to confirm congenital infection at time of birth. In addition, virus can readily be isolated from nasopharyngeal secretions, feces, and urine of the neonate. Later, the throat and CSF become the best sources of viral isolation because the infants with CRS shed less virus (264).

Nucleic acid amplification techniques have also been developed for detection of RV RNA in clinical samples (29,49,99,164,289). This involves reverse transcription of RNA isolated from a tissue source, followed by amplification of

the complimentary DNA produced by polymerase PCR using primers specific for a selected region of the RV genome (usually the E1 gene). The technique appears to be as sensitive as viral isolation although not much more sensitive, owing to limitations of the RT step (29,49). Sequencing of the amplified product provides confirmation that it is rubella specific and not due to mispriming. A restriction endonuclease digestion step has been successfully used to eliminate false-positive results caused by carryover from a previous reaction (96). In the case of suspected congenital infection, *in utero* diagnosis using RT-PCR has been applied to samples obtained by amniocentesis, cordocentesis, or chorionic villus sampling (29,144,289). The technique is sensitive and specific in this setting and provides results much earlier than viral isolation (288).

PREVENTION AND CONTROL

Treatment

Most cases of acquired rubella are subclinical or mild and require no specific treatment. The complications of rubella, when they occur, are treated symptomatically. Under highly controlled experimental conditions, the administration of pooled human gammaglobulin containing high-titer, neutralizing, antirubella antibody abrogated clinical symptoms and prevented detectable viremia (304). However, nasally infected volunteers receiving gammaglobulin containing low-titer, RV-neutralizing antibody were variably protected from developing either rash or viremia (304). When the gammaglobulin was protective, it was administered within 24 hours of mucosal virus challenge. Because patients with rubella shed virus before the onset of the rash, and many never develop either a rash or symptoms, the use of gammaglobulin to modify the course of rubella or to attempt to prevent congenital infection in pregnant contacts of rubella cases has little merit. The successful use of antiviral chemotherapy to limit virus replication in congenitally infected infants has not been reported.

Vaccines

Development

After isolation of RV in cell culture in 1962 (254,357), attenuation of the virus was carried out by serial passage in a variety of cell lines, giving rise to several vaccine strains (reviewed in 20). These included the original vaccine used widely in North America, HPV77/DE5 (39); the Cendehill strain, used more extensively in Europe (265); and several Japanese strains, Takahashi, To336, and Matsuba (313). An early variant of the HPV77 strain passaged in dog kidney cells, HPV77/DK12, was found to be highly reactogenic, even in children, and was soon removed from distribution (319,320). The Cendehill and To336 strains have been found to be the best tolerated by adult women, who are particularly susceptible to side effects (21). The HPV77/DE5 strain was the predominant strain used in North America until 1979, when it was replaced by the RA27/3 vaccine because of concerns about waning immunity in HPV77/DE5 vaccine recipients (154). The RA27/3 strain was derived from an isolate from the kidney of a rubella-infected fetus and was attenuated by passaging 4 times in human embryonic kidney followed by 17 to 25 times in WI-38 fibroblasts (269). This strain was reported to induce a more vigorous immune response than HPV77/DE5 and had the added advantage that it had been attenuated in human cells and was therefore not subject to possible side effects associated with vaccines grown in nonhuman cells (267,268). RA27/3 was licensed in 1979 and is now the only rubella vaccine available in North America. After subcutaneous administration, it induces an antibody response in about 95% of recipients older than 12 months of age (154). The vaccinated individuals show only mild symptoms, and transmission to susceptible bystanders is thought not to occur (131). In addition, although the antibody titers are lower than those following natural infection, protection is believed to endure for more than 12 years in most of those immunized (137,241).

Adverse Reactions

Unfortunately, the current RA27/3 vaccine is not problem free (159,160). The incidence of acute adverse reactions is lower than that after natural rubella (331) but higher than that reported for the Cendehill and To336 strains (21,105). This includes fever, lymphadenopathy, arthralgia and arthritis, paresthesia, and carpal tunnel syndrome (105). Between 10% and 40% of adult rubella seronegative women vaccinated with the RA27/3 vaccine develop acute, usually transient, arthritis or arthralgia (273,331,334). In a few subjects, the joint reactions can be recurrent or persistent, similar to symptoms seen when they follow natural rubella (333,334). In patients immunized with HPV77/DE5 vaccine, the virus was shown to persist in PBMC for up to 6 years after immunization (52,53). In addition, rubella-specific immune complexes persist for extended intervals after vaccination and are found more frequently in patients who experience complications (77). In a recent randomized double-blinded study of adverse effects of rubella vaccine in 546 rubella seronegative women immunized postpartum with vaccine or placebo, acute arthritis or arthralgia was recorded in 30% of women receiving vaccine, and persistent or recurrent joint manifestations lasting up to 12 months in 58 patients (22%) (334). Acute joint symptoms, however, were also found in 20% of women receiving placebo, and 15% of these women experienced persistent joint reactions (41 patients), raising the question of the role of the vaccine in inducing chronic joint symptoms. Two other studies have not found a significant increase in the incidence of joint symptoms in women receiving rubella vaccine over controls (286,316). It seems clear that the incidence of arthritis due to rubella vaccination is lower than previously thought (331,335). Natural rubella, however, is well-known to be frequently complicated by arthritis (151,244,331), and the various vaccines have different levels of association with joint symptoms that parallel their ability to infect joint tissue *in vitro* (210). Therefore, it would seem likely that a proportion of the joint symptoms seen after rubella immunization are vaccine induced. The finding of an association of HLA-DR1 and HLA-DR4 with an eightfold higher incidence of joint symptoms after RA27/3 rubella vaccination (216) suggests that RV causes joint symptoms selectively in genetically predisposed individuals. This factor should be taken into account in any future studies based on statistical analysis of populations.

Vaccination Policy

Rubella vaccine is probably the only vaccine that is administered to protect a population other than the one that is immunized. More specifically, young children are vaccinated to provide herd immunity and to protect the developing fetuses of susceptible pregnant women. In view of this, divergent vaccination policies have been developed in different

countries, with vaccine delivery targeted at different age groups and sexes (89). Consequently, there have been markedly varied initial results in achievement of the vaccine's key goal (90). The United States and Finland chose to disrupt the natural spread of infection by mandatory vaccination of all preschool-aged children, supplemented by vaccine administration to susceptible postpubertal women (6). In the United Kingdom and other European countries, however, a decision was made to immunize adolescent girls and allow natural rubella to continue to circulate, owing to concerns that vaccination would not provide long-lasting protection (140,336). After a rubella epidemic in 1986 (23), however, the vaccination program in the United Kingdom was expanded to include preschool-aged children, with booster doses provided to adolescent girls. Several European countries (138) and Australia have also switched to universal vaccination of infants, together with selective vaccination of seronegative adult women (324).

Risk in Pregnancy

A serious concern of rubella vaccination programs has been the potential risk to the developing fetus in mothers who are immunized during early pregnancy. In such instances, the placenta and fetal tissue may become infected (132). None of the 712 children born of mothers inadvertently immunized within 3 months of conception, however, were found to have malformations compatible with CRS (17,40). This included vaccination with the earlier Cendehill and HPV-77/DES vaccines as well as with the current RA27/3 vaccine. Thus, vaccine strains of RV appear to lack the teratogenic capacity of natural (wild-type) RV. Because a potential risk to the fetus still exists, pregnancy remains a contraindication to rubella vaccination. Inadvertent vaccination of a seronegative pregnant woman, however, is not generally considered to be sufficient reason for routine termination of pregnancy (276).

Reinfection

Reinfection occurs rarely after natural infection but has been reported in a small number of individuals after rubella vaccination (82,267). Subclinical reinfection, defined by a fourfold or greater rise in preexisting antibody titer, was found in about 10% of girls immunized 5 years previously with the RA27/3 vaccine after reexposure to the virus during an epidemic (82). Lack of an early vaccine-induced anti-E1 response was correlated with reinfection and resulted in an anamnestic antibody response, with RV-specific, high-avidity IgG produced (100,329). Failure to detect specific IgG in sequential sera collected after rubella exposure also suggests reinfection (329). Whereas limited replication of RV in the nasopharynx is seen during reinfection, viremia and systemic manifestations appear to be uncommon (242). As a consequence, reinfection during gestation rarely results in viral spread to the fetus and CRS (102,107). Unfortunately, there are several dozen rare but well-documented exceptions (22,85,353).

New Vaccine Strategies

Despite the efficacy of the current rubella vaccine program in the United States, problems still exist. These include potential dangers associated with vaccination of pregnant women or immunocompromised patients and also lack of immunity of some women of childbearing age due either to “missed opportunity” for vaccination (179), vaccine failure, or durability of the immune response elicited (169). A further problem is the refusal of some women to be vaccinated either because of concerns about side effects or for religious reasons.

New rubella vaccine strategies under assessment include the development of subunit vaccines and the use of recombinant DNA technology to engineer a vaccine strain that combines desirable properties of strains currently available. A subunit vaccine comprising the E1 protein, which contains the major neutralizing epitopes, has been proposed (370). In addition, DNA vaccines incorporating all three structural proteins, or just E1 and E2, have been constructed and have shown some promise in mice (274). Although the immune response induced may not be long lasting, such nonreplicating vaccines can be given safely to pregnant women to boost immunity during the critical stage of fetal development. Similarly, in immunocompromised individuals, a subunit vaccine would not be associated with the risk for dissemination and disease that accompanies immunization with a live vaccine. With regard to the general population, use of a live attenuated vaccine that will promote the strongest and most enduring immune response is still advisable. With current knowledge on the genetic determinants of virulence and joint tropism, the potential exists for development of a recombinant vaccine designed to be highly immunogenic but not associated with unwanted side effects (187).

PERSPECTIVES

In North America and much of Europe, where universal rubella vaccination has been carried out for some time, rubella and CRS have been largely controlled (69). Notably, there were 20,000 infants born with CRS after the 1964 epidemic in the United States, whereas the total number of cases reported in the 30 years since introduction of the vaccine has been less than 1,000 (306). Regrettably, RV is still endemic in many developing countries, although accurate information on the incidence of rubella and, more importantly, of congenital rubella, is difficult to obtain (84). Recently, the World Health Organization and the Pan American Health Organization have issued recommendations for a drive to eradicate rubella by addition of rubella vaccine to the measles vaccine already being administered worldwide. Implementation of a policy to distribute a combined measles–rubella vaccine to the less advantaged populations of the world has the potential to reduce greatly the burden (both economic and social) of CRS at little extra cost than that of the univalent measles vaccine. Because RV infects only humans, the potential to eradicate rubella and, more importantly, the devastating consequences of congenital infection is clear (44,270).

Eradication of rubella is a laudable goal but will not happen quickly. In the interim, improvements in the vaccine and the policy for its administration need to be addressed. Recent recommendations by the Advisory Committee on Immunization Practices in the United States have included the introduction of a second dose of MMR (measles–mumps–rubella) vaccine to be administered 1 month after the first (294). In some other countries, a second dose of vaccine given before school entry or in early adolescence has been introduced, whereas in others, a single dose in infancy followed by selective vaccination of seronegative women is recommended. Careful monitoring of the success of each of these strategies will need to be done to determine which is the most effective. With regard to the vaccine itself, the RA27/3

vaccine has a good safety record and provides long-lasting immunity (271). It is not, however, problem free (160,334), and production of recombinant or subunit vaccines for selected populations, including pregnant women and the immunocompromised, should be considered. In addition, the recent finding of an association of HLA genotype with incidence of joint reactions (216) may allow the prediction of which individuals have the genetic susceptibility to develop complications and opens the way to selective immunization of this target group with a more attenuated vaccine.

In conclusion, it is clear that RV is still a significant human pathogen that takes an enormous toll on the health of the world's population through the widespread manifestations of CRS, most notably in relation to blindness and hearing defects. Although the virus has now been cloned and sequenced, there is still remarkably little known about its pathogenesis, particularly regarding its teratogenicity, its relationship to autoimmune diseases like diabetes and arthritis, and its mode of persistence at the molecular level.

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CHAPTER 32 *Flaviviridae*: The Viruses and Their Replication

Brett D. Lindenbach and Charles M. Rice

At the dawn of human virus research, nearly one century ago, Walter Reed demonstrated that the disease known as yellow fever could be experimentally transferred via the filtered serum of an infected individual (565) and that this infectious agent was transmitted to man by mosquitoes (566). The discovery of this viral pathogen eventually led to the derivation of a live-attenuated strain that has been effectively used for human vaccination for over 60 years (452,664,701). It is now appreciated that yellow fever virus (YF) is but one representative of a large family of related positive-strand RNA viruses, the *Flaviviridae* (from the Latin *flavus*, yellow). This family currently consists of three genera: the flaviviruses, the pestiviruses (from the Latin *pestis*, plague), and the hepaciviruses (from the Greek *hepar*, *hepatos*, liver) (177,761). In addition to these genera, a group of unassigned viruses, the GB agents, are awaiting formal classification within the family. As detailed later, members of this family exhibit diverse biologic properties and a lack of serologic cross-reactivity, although they share similarities in virion morphology, genome organization, and presumed RNA replication strategy. The increasing significance of *Flaviviridae* as human and animal pathogens (Chapter 33 and Chapter 34) emphasizes that their study remains no less pertinent than in Reed's time.

The flaviviruses and pestiviruses were formerly considered to be members of the family *Togaviridae*, based on traditional methods of virus classification. However, through the study of viral genes, virus structure, and the viral replication cycle, it became apparent that these viruses represented an evolutionarily distinct group, and they have been subsequently reclassified as a separate virus family, the *Flaviviridae* (761). The diversity of viruses within the *Flaviviridae* is partially illustrated in Figure 1 and Table 1. Comparative sequence analysis has permitted classification of positive-stranded RNA viruses based on inferred evolutionary relationships. The *Flaviviridae* are members of the positive-strand virus supergroup 2, bearing distant similarity in their RNA-dependent RNA polymerases (RdRps) to coliphages and the plant-infecting carmo-, tombus-, diantho-, and subgroup I luteoviruses (345). The *Flaviviridae* are the only member of polymerase supergroup 2 to also encode RNA helicases, although members of polymerase supergroup 1 contain similar helicases. Thus this family of viruses is likely to exhibit a unique mode of genome replication.

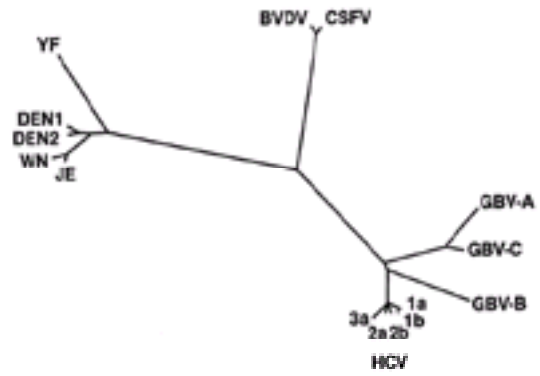

FIG. 1. The *Flaviviridae*. Phylogenetic tree based on parsimony analysis of NS3 helicase regions. Shown are members of the *Flavivirus* genus: yellow fever virus (*YF*), dengue-1 (*DEN-1*), dengue-2 (*DEN-2*), West Nile virus (*WN*), and Japanese encephalitis (*JE*); the *Pestivirus* genus: bovine viral diarrhea virus (*BVDV*) and classical swine fever (*CSFV*); several hepacivirus (*HCV*) isolates; and the unclassified viruses GBV-A, GBV-B, and GBV-C. (Figure adapted from ref. 640, with permission.)

TABLE 1. *Members of the Flaviviridae*

FAMILY CHARACTERISTICS AND REPLICATION CYCLE

Common features believed to be shared by the three genera and highlights of the replication cycle are diagrammed in Figure 2. Our understanding of these steps is far from complete and the current view is based largely on studies with flaviviruses. Modifications are likely as more information becomes available, particularly for hepatitis C virus (HCV) and the pestiviruses. Enveloped virions are composed of a lipid bilayer with two or more species of envelope (E) proteins surrounding a nucleocapsid that consists of a single-stranded positive-sense genome RNA complexed with multiple copies of a small, basic capsid (C) protein.

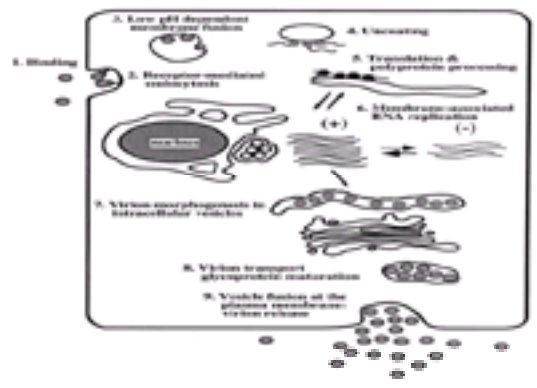

FIG. 2. Life cycle of the *Flaviviridae*. See the text for further details.

Binding and uptake are believed to involve receptor-mediated endocytosis via cellular receptors specific for viral envelope proteins. Fusion of the virion envelope with cellular membranes delivers the nucleocapsid to the cytoplasm, where translation of the genome RNA occurs. The organization of the genome RNA is similar for all genera. All known viral proteins are produced as part of a single long polyprotein of more than 3,000 amino acids that is cleaved by a combination of host and viral proteases. The structural proteins are encoded in the N-terminal portion of the polyprotein, with the nonstructural proteins in the remainder. Sequence motifs characteristic of a serine protease, RNA helicase, and an RdRp are found in similar locations in the polyproteins of all three genera (444). The cleavage products containing these regions are believed to form the enzymatic components of the RNA replicase. RNA replication occurs in cytoplasmic replication complexes that are associated with perinuclear membranes, and it occurs via synthesis of a genome-length minus-strand RNA intermediate. RNA synthesis is resistant to actinomycin D, an inhibitor of DNA-dependent RNA polymerases. Progeny virions are thought to assemble by budding through intracellular membranes into cytoplasmic vesicles. These vesicles follow the host secretory pathway, fuse with the plasma membrane,

and release mature virions into the extracellular compartment.

THE FLAVIVIRUSES

Background and Classification

The flavivirus genus consists of nearly 80 viruses, many of which are arthropod-borne human pathogens. Flaviviruses cause a variety of diseases including fevers, encephalitis, and hemorrhagic fevers (Chapter 33). Entities of major global concern include dengue fever with its associated dengue hemorrhagic fever (DHF) and shock syndrome (DSS) (reviewed in refs. 219,234, and 584), Japanese encephalitis (451), and yellow fever (reviewed in ref. 709). Other flaviviruses of regional or endemic concern include Kyasanur Forest disease, Murray Valley encephalitis (MVE), St. Louis encephalitis, tick-borne encephalitis (TBE), and West Nile (WN) viruses. Decreases in mosquito control efforts during the latter part of the 20th century, coupled with societal factors such as increased transportation and dense urbanization, have contributed to the reemergence of flaviviruses such as dengue (DEN) in South and Central America (453, 480). In 1999, WN virus was isolated for the first time in the western hemisphere during an outbreak in New York City that was responsible for several human cases of encephalitis, including four deaths (17,22,358).

Vaccination is available for YF, using the live-attenuated 17D strain, and for TBE and Japanese encephalitis (JE) using inactivated virus (253). Efforts to derive live-attenuated strains of other flaviviruses have met with only limited success. Development of effective DEN vaccines that exhibit cross-protection, thought to be important for preventing subsequent dengue-associated immunopathogenesis (see later), are proving to be particularly challenging. The ability to genetically manipulate flaviviruses, described later, is being used to develop novel approaches to vaccine design (65,95,223,224,408,526). One promising candidate live-attenuated vaccine is a chimeric flavivirus created by replacing the structural glycoproteins of YF-17D with those of JE (95,224,454,455). A similar approach to create a vaccine against DEN-2 is under investigation (223).

Viruses within the genus are categorized into antigenic complexes and subcomplexes based on classical serologic criteria (85,86) or into clusters, clades, and species, according to molecular phylogenetics (349,807). These latter methods have permitted the classification of viruses such as YF, which lacks close relatives. Mosquito-borne and tick-borne flaviviruses, although quite distinct, appear to have evolved via a common ancestral line that diverged from non-vector-borne viruses (i.e., those for which no arthropod vectors are known) (349,807). The salient features of flavivirus taxonomy are illustrated in Figure 3, with emphasis given to viruses that are mentioned in this chapter. Two additional points should be clarified with regard to this genus organization. First, the trivial name tick-borne encephalitis virus is commonly applied to both Central European encephalitis virus and Far Eastern encephalitis virus, although they clearly represent distinct viruses with differences in vector species, geographical distribution, and sequence relatedness (153). In addition, because of the high mutation rate of RNA viruses, it is expected that each flavivirus exists as a complex, interrelated population of quasi-species (Chapter 13). Recent evidence for intertypic recombination among DEN viruses hints at the unrealized diversity potentially lurking within flavivirus populations (774).

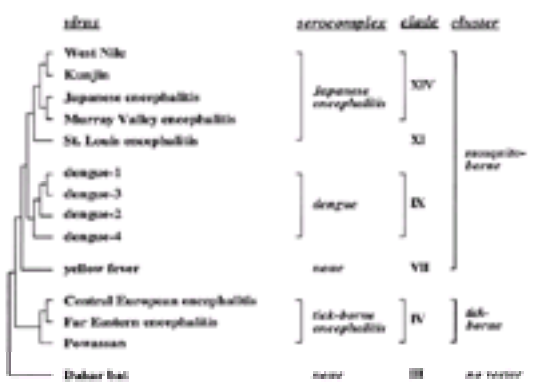

FIG. 3. Organization of the *Flavivirus* genus. The dendrogram on the *left* shows the relationships of selected flaviviruses based on a recent phylogenetic analysis (349). Evolutionary distance is not represented in this figure. The serologic and phylogenetic classifications of these viruses are indicated to the *right*.

Experimental Systems

Flaviviruses can be cultured in whole animals such as chick embryos, suckling mouse brain, and mosquitoes, as well as in primary or established cell lines of mammalian, avian, or insect origin. In vertebrate cells, dramatic cytopathic and ultrastructural effects can occur, including vacuolation and proliferation of intracellular membranes (472); infection is commonly cytocidal, although some vertebrate cell types do not show these effects and become chronically infected. Even during the peak of virus production, a major inhibition of host macromolecular synthesis is not observed (68,713,759,760). Arthropod cells in culture may demonstrate cytopathic effects, most frequently manifested as cell fusion and syncytia formation (reviewed in refs. 68,663, and 712). However, infection of mosquito cells is often noncytopathic, and persistent infections can be established (476). Mosquitoes remain chronically infected for life and produce extremely high levels of infectious virus particles in the salivary glands.

The life cycle of flaviviruses is increasingly understood at the molecular genetic level, and complete genome sequences exist for at least one member of each taxonomic group (see, for example, refs. 232,248,322,412, and 573). Furthermore, flaviviruses can be recovered from cells containing RNA transcripts of viral cDNAs, allowing flavivirus biology to be probed with reverse genetics (213,218,281,310,322,331,354,366,410,527,572,665). Such reverse genetic systems are also being exploited to express foreign genes (323,726), to provide stable genetic stocks for live-attenuated flavivirus

vaccines (331,572), and to assist in the design of novel vaccine strategies (65,95,223,224,408,526).

Structure and Physical Properties of the Virion

Flavivirus particles appear to be spherical, 40 to 60 nm in diameter, containing an electron dense core (about 30 nm diameter) surrounded by a lipid bilayer (Fig. 4A) (472). Mature virions sediment between 170 and 210S, have a buoyant density of 1.19 to 1.23 g/mL, and are composed of 6% RNA, 66% protein, 9% carbohydrate, and 17% lipid (592,712). Because of the lipid envelope, flaviviruses are readily inactivated by organic solvents and detergents (592). Three viral proteins are associated with virions: the E (envelope), M (membrane), and C (capsid) proteins. The E protein is the major surface protein of the viral particle, probably interacts with viral receptors, and mediates virus–cell membrane fusion. Antibodies that neutralize virus infectivity usually recognize this protein (reviewed in ref. 253), and mutations in E can affect virulence (reviewed in Chapter 33). M protein is a small proteolytic fragment of prM protein, which is important for maturation of the virus into an infectious form, as described later. Discrete nucleocapsids, composed of C protein and genomic RNA (120 to 140S, buoyant density 1.30 to 1.31 g/mL), can be isolated after solubilization of the envelope with nonionic detergents (592).

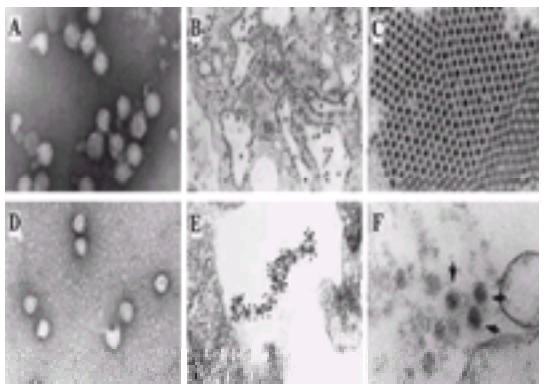

FIG. 4. Electron micrographs of virions and infected cells. **A:** Purified St. Louis encephalitis (SLE) virus negatively stained with ammonium molybdate (472). Surface projections appear as a very thin, indistinct layer. (Courtesy of Dr. Frederick A. Murphy.) **B:** Thin section of a BHK-21 cell at 48 hours after infection showing SLE virions in the cisternae of the endoplasmic reticulum (765). (Courtesy of Frederick A. Murphy, Sylvia G. Whitfield, and A. K. Harrison.) **C:** Para-crystalline array of SLE virus in a *Culex pipiens* mosquito salivary gland cell 25 days after blood-meal-feeding on an infected suckling mouse. (Courtesy of Sylvia G. Whitfield, Frederick A. Murphy, and W. Daniel Sudia.) **D:** Classical swine fever virus (CSFV) virions negatively stained with uranyl acetate. (Courtesy of Dr. Frank Weiland.) **E:** Ultrathin section of STE cells infected with CSFV and immunostained with E^{rns}-specific monoclonal antibody (MAb) 24/16 and colloidal gold. Bar = 100 nm. (From ref. 746, with permission.) **F:** Thin section showing virus-like particles (*arrows*) in HPBALL cells harvested 14 days after infection with HCV (632). Particles measured approximately 50 nm in diameter. (Courtesy of Dr. Yokho Shimizu.)

Binding and Entry

It is thought that flaviviruses attach to the surface of host cells through an interaction of E protein with one or more receptors, and many E-reactive antibodies have been shown to neutralize virus infectivity by interfering with virus binding (251,280). The patterning of receptor expression in animal tissues is likely to contribute to flavivirus tropism *in vivo*, although such receptors have not been specifically identified. Several cell surface proteins have been described as candidate flavivirus receptors (47,329,346,416,470,548,596). In addition, recombinant E protein from DEN-2 virus has been shown to interact with highly sulfated glycosaminoglycans, and cell surface expression of heparan sulfate was required for efficient infection of mammalian cells by a laboratory-passaged strain of DEN-2 virus (107). Consistent with a role for glycosaminoglycans in flavivirus entry, the infectivity of DEN viruses can be partially neutralized by incubation with heparin or highly sulfated heparan (107,280). An additional mechanism of flavivirus binding, typically referred to as antibody-dependent enhancement (ADE), has been demonstrated for a number of flaviviruses cultured *in vitro* (236,237 and 238,388,509,510 and 511,520,616). ADE involves the increased binding of virus opsinized with subneutralizing concentrations of flavivirus-reactive antibodies to cells expressing immunoglobulin Fc receptors (509,616). It has been hypothesized that this mechanism might occur *in vivo* and, by altering tissue tropism, could contribute to the enhanced pathogenesis frequently observed in secondary infections by other DEN types (reviewed in refs. 234,235, and 584).

After binding, it is generally believed that virions are taken up by receptor-mediated endocytosis (192,292,481,487), although direct fusion at the plasma membrane has also been observed (244,245). Ultrastructural studies have localized single virions and virion aggregates to clathrin-coated pits on the cell surface, and uptake of virus particles into coated vesicles rapidly follows attachment (192,194). Virions are later found in uncoated pre-lysosomal vesicles, where an acid-catalyzed membrane fusion is thought to release the nucleocapsid into the cytoplasm (192,194,254,481). Consistent with this, a conformational change in the viral E protein, which probably exposes a fusogenic domain (567; and see later), occurs at low pH (11,221,254,330,583,611,659). Acid pH can promote fusion of virions with liposomal membranes *in vitro* or at the plasma membrane of cultured cells (193,194,222,330,550,667), although in the latter case this mode of entry does not lead to productive infection (194,330). Following entry and fusion, nucleocapsids are presumably disassembled, genomic RNA is translated, and RNA is initiated.

Genome Structure

The genome of flaviviruses consists of a single-stranded RNA about 11 kilobases (kb) in length. This RNA contains a 5' cap (m^7G5'ppp5'A) at the 5' end and lacks a polyadenylate tail (756). Genomic RNA is the messenger RNA for translation

of a single long open reading frame (ORF) as a large polyprotein, as described later. Surrounding the ORF are 5' and 3' noncoding regions (NCRs) of around 100 nucleotides (nt) and 400 to 700 nt, respectively. These regions contain conserved sequences and predicted RNA structures that are likely to serve as cis-acting elements directing the processes of genome amplification, translation, or packaging. Although it is not yet feasible to directly study the structures of flavivirus RNAs *in vivo*, the ability of RNA to adapt alternative foldings could regulate these competing processes (231,538).

The 5' NCR sequence is poorly conserved between flaviviruses, but it appears to contain common secondary structural elements that influence the translation of flavivirus genomes (69,84,231). However, the most significant function of the 5' NCR probably resides in its reverse complement, the 3' NCR of viral minus strands, which forms the site for initiation of plus-strand synthesis. Deletions engineered into the 5' NCR of DEN-4 were shown to have dramatic effects on the ability to recover live viruses, but they do not correlate with the translational efficiency of the mutant genomes (84). Interestingly, one of the resultant mutants exhibited a limited host-range growth phenotype, suggesting potential interactions of this region (or its reverse complement) with host-specific factors. In keeping with this, cellular proteins were shown to bind specifically to a terminal stem-loop in the 3' NCR of WN virus minus strand (627).

The 3' NCR of the flavivirus genome, which presumably functions as a promoter for minus-strand synthesis, exhibits a great deal of sequence divergence and size heterogeneity. Nevertheless, computational analyses have revealed features that are common to all flaviviruses or that are conserved within specific taxonomic groups, and that tend to cluster in a region proximal to the 3' end (538,552,735,739). RNA folding predictions based on thermodynamic considerations, covariation analyses, and biochemical probing support the existence of a 90- to 120-nt stem-loop structure at the 3' terminus of all flavivirus genomes, with the potential to form a pseudoknot involving an adjacent stem-loop (70,206,231,411,412,538,552,573,627,666,677,751,811). Several lines of evidence suggest a functional role for this structure in viral RNA replication. Mutations engineered within the 3' terminal stem-loop of the genome of DEN-2 virus greatly affected the ability to recover live viruses, and one of the recovered viruses exhibited a host restricted-growth phenotype (808). Several cellular proteins bind to this region of WN virus RNA, including the phosphorylated form of translation elongation factor 1a (49,50). Furthermore, viral replicase proteins NS3 and NS5 have been shown to bind to the terminal stem-loop RNA *in vitro* (103,133). The conserved portion of the 3' NCR also contains consensus sequences (CS1 and CS2) that are retained among all mosquito-borne flaviviruses, one of which has the potential to base-pair with a conserved region in the 5' region of the ORF, suggesting that cyclization of flavivirus genomes is possible (231). A DEN-4 genome engineered to contain a deletion in CS1 indicates that this element is required for virus viability (429). Complementarity in the proposed cyclization sequences was recently shown to be required for efficient RdRP activity *in vitro*, although the upstream element could be supplied in trans (803). Similarly, stretches of conserved RNA sequence and potential cyclization sequences are also found in the genome RNAs of tick-borne flaviviruses (412,413,415). Other regions in the 3' NCR, which may be dispensable for virus replication in culture, nevertheless appear to be important determinants for growth in mammalian hosts, and further analysis of such regions is likely to contribute greater insight into flavivirus pathogenesis and vaccine development (414,429,537).

Translation and Proteolytic Processing

The flavivirus genome is translated as a large polyprotein that is processed co- and posttranslationally by cellular proteases and a virally encoded serine protease (described later) into at least 10 discrete products. As depicted in Figure 5, the N-terminal one quarter of the polyprotein encodes the structural proteins, and the remainder contains the nonstructural (NS) proteins, in the following order: C-prM-E-NS1-NS2A-NS3-NS4A-NS4B-NS5 (573). Based on the deduced hydrophobicity of the viral proteins, their predicted membrane topology is illustrated in Figure 6. This model is supported by experimental data (89,757), although the transmembrane regions have not been well defined.

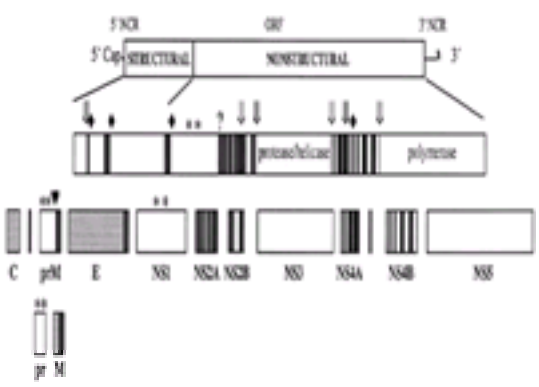

FIG. 5. Translation and processing of the flavivirus polyprotein. At the top is depicted the viral genome with the structural and nonstructural protein coding regions, the 5&cap, and the 5' and 3' NCRs indicated. Boxes below the genome indicate precursors and mature proteins generated by the proteolytic processing cascade. Mature structural proteins are indicated by *shaded boxes* and the nonstructural proteins and structural protein precursors by *open boxes.* Contiguous stretches of uncharged amino acids are shown by *black bars. Asterisks* denote proteins with N-linked glycans but do not necessarily indicate the position or number of sites utilized. Cleavage sites for host signalase (w), the viral serine protease (‾), furin or other Golgi-localized protease (©), or unknown proteases (**?**) are indicated.

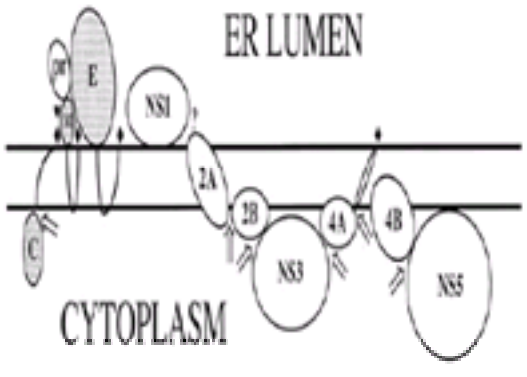

FIG. 6. Membrane topology of flavivirus proteins. The proposed orientation of the flavivirus polyprotein cleavage products with respect to the ER membrane is shown. The proteins are drawn to scale (areas are proportional to the number of amino acids) and arranged in order (left to right) of their appearance in the polyprotein. Mature structural proteins are *shaded* and C-terminal membrane-spanning segments of M and E are indicated. Cleavage sites for host signalase (w), the viral serine protease (‿), furin or other Golgi-localized protease (©), or unknown proteases (**?**) are indicated.

Features of the Structural Proteins

The C Protein

C protein (about 11 kd) is highly basic (55,573,711), consistent with its proposed role in forming a ribonucleoprotein complex with packaged genomic RNA. Basic residues are concentrated at the N- and C-termini of C, and they probably act cooperatively to specifically bind genomic RNA (323). The central portion of C contains a hydrophobic domain that interacts with cellular membranes and may play a role in virion assembly (418). The nascent C protein (anchC) contains a C-terminal hydrophobic domain (491) that acts as a signal sequence for translocation of prM into the lumen of the endoplasmic reticulum (ER). Mature C protein is generated by viral serine protease cleavage at a site upstream of this hydrophobic domain (16,395,785).

The prM Protein

The N-terminus of prM (about 26 kd) is generated in the ER by host signal peptidase. This processing event appears to require prior processing of anchC by the cytoplasmic viral serine protease (16,395,788), although the temporal order of these cleavages has been questioned (787,788). However, in support of this model, signalase cleavage at this site can be made viral protease-independent by cleaving anchC with another protease (660) or by improving the context of the signalase cleavage site (366,661). Interestingly, improvement of the prM signalase cleavage context was shown to be lethal for YF virion production (366). Thus, the availability of viral protease activity may regulate structural protein processing, and therefore virion assembly, over the course of an infection. During the egress of virions through the secretory pathway, prM is cleaved by the trans-Golgi resident enzyme furin (653), to form the structural protein M (about 8 kd) and the N-terminal “pr” segment, which is secreted into the extracellular medium (474). The N-terminal “pr” segment is predominantly hydrophilic and contains one to three N-linked glycosylation sites (93) and six conserved cysteine residues, all of which participate in intramolecular disulfide bridges (492). The structural protein M, located in the C-terminal portion of prM, is present in mature virions and contains a shortened ectodomain (about 41 amino acids) followed by two potential membrane-spanning domains. Antibodies to prM can mediate protective immunity (316), perhaps by neutralization of released virions that contain some uncleaved prM (see later).

The E Protein

E protein (about 50 kd) is a type I membrane protein, containing adjacent transmembrane domains in the C-terminus that serve to anchor this protein to the membrane and as the signal sequence for NS1 translocation (573). E protein contains 12 highly conserved Cys residues that form intramolecular disulfide bonds (492), and it is often glycosylated (93,768).

The three-dimensional structure of TBE E protein was determined by x-ray crystallography of a soluble tryptic fragment obtained from purified virions (Fig. 7) (567). These data indicate that E protein is divided into three structural domains and forms head-to-tail homodimeric rods that are thought to lie parallel to the virion surface in a meshlike network. This structural model provides a framework for understanding the molecular basis of E protein interactions. Structural regions of the ectodomain contain putative receptor binding sites (107, 567), a potential fusogenic domain (567), contact sites for E homodimer formation (567), a region involved in prM–E interaction (12), and the trigger for low-pH-induced rearrangement (659). E protein homodimers disassociate at low pH, and each monomer reassociates with two adjacent E proteins, perhaps around a three-fold axis of symmetry at the stalk, to form trimeric complexes (11,254,659). E trimers are thought to extend outward from the virion surface, presumably exposing the hydrophobic fusogenic domains. The stem-anchor region of E, for which structure was not determined, contains determinants for E trimer formation and for stabilization of prM-E heterodimers (12,741). Mutations in the putative receptor binding site in the TBE E protein gave rise to viruses with attenuated growth in culture and reduced virulence in mice (409). For some mosquito-borne flaviviruses, a similar region of E protein contains a putative integrin-binding motif Arg-Gly-Asp. Mutation of this sequence in E of MVE virus produced viruses with decreased entry kinetics, increased reliance on glycosaminoglycans for entry, and attenuated virulence (366).

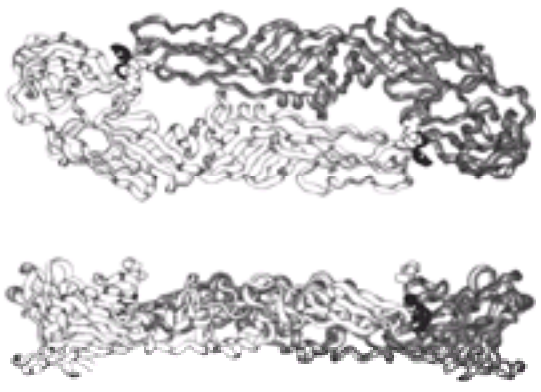

FIG. 7. Envelope protein structure. The structure of a tick-borne encephalitis (TBE) E glycoprotein dimer is represented in this ribbon diagram, as viewed perpendicularly (*top*) or laterally (*bottom*) with respect to the lipid bilayer. Individual E monomers are colored *gray* or *white*, and the proposed fusogenic domains are in *black.* (Kindly provided by Steve Allison and F. X. Heinz. Adapted from ref. 567, with permission.)

Features of the Nonstructural Proteins

The NS1 Protein

The NS1 glycoprotein (M_r of about 46 kd) exists in cell-associated, cell-surface, or extracellular nonvirion forms. NS1 is translocated into the ER lumen and released from the C-terminus of E by signal peptidase (161,571,757). It contains 12 highly conserved cysteines that form intramolecular disulfide bonds, and it is rapidly glycosylated on two or three Asn residues (367,419,646). NS1 is cleaved from its downstream neighbor, NS2A, around 10 minutes after synthesis by an unknown, membrane-bound, ER-resident host protease (93,160,161). The eight C-terminal hydrophobic residues of NS1, and more than 140 amino acids of NS2A, are necessary determinants of this cleavage (93,160,272,517). Truncated and elongated forms of NS1, which presumably contain alternate C-terminal cleavage sites, have also been described for some viruses (53,419). In addition, murine cells persistently infected with JE virus have been found to produce truncated forms of NS1, although this appears to be caused by an uncharacterized adaptation of the host cells rather than by alterations within the viral genome (104). Twenty to 40 minutes after synthesis, NS1 forms homodimers that are resistant to denaturation with 6M urea or 5M guanidinium-HCl, but that are unstable at high temperatures or low pH (769). Dimers of NS1 exhibit a partially hydrophobic nature, pelleting with membranes and fractionating equally into the aqueous and detergent phases upon extraction with Triton X-114 (769). A point mutation has been identified in Kunjin NS1 that destabilizes NS1 dimers and confers a replication defect on the virus (233), indicating a functional role for NS1 dimerization. NS1 appears to be peripherally associated with membranes, as it does not contain any putative transmembrane domains or known lipid modifications, and it can be released from membranes by sodium carbonate (pH 11.5), 8M urea, or 5M guandinium-HCl (161,757).

NS1 is slowly secreted from mammalian cells and is not secreted from mosquito cells (419,530,770). During secretion, one of the N-linked glycans is modified to contain complex sugars (367,419,477,540), and three NS1 dimers come together into a soluble hexameric form (132,170).

NS1 was first characterized as the soluble (non-virion-associated) complement-fixing (SCF) antigen present in the sera and tissues of experimentally infected animals (63). It is now understood that the extracellular forms of NS1 strongly elicit humoral immune responses, and immunization with purified or recombinant NS1 can be protective (159,297,383,546,617,706). Furthermore, protective immunity can be passively transferred with antibodies against NS1, apparently by their ability to direct complement-mediated lysis of infected cells via interaction with the cell-surface-associated form of NS1 (255,618,619). The secretion of a viral NS protein that elicits protective immune responses is one of the more curious aspects of flavivirus biology that await further inquiry.

Several lines of evidence implicate NS1 in the process of RNA replication. NS1 has been found to co-localize with markers of RNA replication in association with membrane structures that are presumed sites of replication (404,764). Mutations at the first or both N-linked glycosylation sites of NS1 dramatically impair virus replication (477,539) and demonstrate a decrease in viral RNA accumulation (477). Furthermore, a YF mutant containing a temperature-sensitive lesion in NS1 showed a profound decrease in RNA accumulation under nonpermissive conditions (478). NS1 can be supplied in trans to a YF genome lacking a functional NS1 gene, and RNA replication is blocked at a very early stage in the absence of complementing NS1 (384). Further genetic analysis has revealed that DEN NS1 does not productively interact with the YF replicase in trans (385). This block in replication can be suppressed by a mutation in NS4A, indicating that an interaction between NS1 and NS4A is critical for replicase function and suggesting a potential mechanism whereby NS1 participates in the cytoplasmic process of RNA replication (385).

The NS2A and NS2B Proteins

NS2A is a relatively small (about 22 kd), hydrophobic protein of unknown function. Cleavage of NS1-2A occurs in the ER, as previously mentioned, whereas the C-terminus is generated via cleavage at the NS2A/2B junction by the cytoplasmic serine protease, indicating that this protein must be membrane spanning. An alternative cleavage within YF NS2A can also be utilized by the viral protease, leading to a C-terminally truncated form of this protein that is about 2 kd smaller in mass (92,483). Mutations that block cleavage at either site are lethal for some aspect of YF replication (483). NS2A has been localized to presumed sites of RNA replication, and *in vitro* studies with a recombinant glutathione-S-transferase-KUN NS2A fusion protein suggest that this protein binds to NS3 and NS5, as well as to RNA transcripts of the 3' NCR (405). Thus, this protein might function in recruitment of RNA templates to the membrane-bound

replicase.

NS2B is a small (about 14 kd) membrane-associated protein containing two hydrophobic domains surrounding a conserved hydrophilic region (573,757). It forms a complex with NS3 and is a required cofactor for the serine protease function of NS3 (20,90,94,162,299). Deletion analysis indicates that a 40-amino-acid region in the central conserved domain of NS2B is required for NS2B-3 protease activity, and mutations in the central conserved domain that destabilize interaction with NS3 abolish protease activity (94,121,146,162,299).

The NS3 Protein

NS3 is a large (about 70 kd) cytoplasmic protein that associates with membranes via its interaction with NS2B (20,94,121). NS3 contains several enzymatic activities that implicate this protein in polyprotein processing and RNA replication.

The N-terminal one third of NS3 contains homology to trypsin-like serine proteases (34,35,198), an enzymatic activity that has been confirmed by deletion analysis (97,163,534,752) and by site-directed mutagenesis of the residues comprising the proposed catalytic triad (YF NS3 residues His-53, Asp-77, and Ser-138) (97,541,722,752,810) or the substrate-binding pocket (533,722). Mutations in NS3 that abolish protease activity also prevent the recovery of viable mutant viruses, substantiating a crucial role for this enzyme in the virus life cycle (94). Expression of the N-terminal 167 to 181 residues of NS3, together with NS2B, is sufficient to form the active protease (90,373). The NS2B-3 protease cleaves in both cis and trans configurations, and it mediates cleavages at the NS2A/NS2B, NS2B/NS3, NS3/NS4A, and NS4B/NS5 junctions, as well as cleavages that generate the C-termini of mature C (16,786) and NS4A (377), and minor cleavages within NS2A (483) and NS3 (20,700). Alignment of known cleavage sites and mutation of these residues have been used to characterize the substrate specificity of this enzyme. These data indicate that the NS2B-3 protease preferentially cleaves after pairs of basic residues and before an amino acid with a small side chain (94,96,378,483). However, some nonconservative mutations at these sites are tolerated, and several canonical dibasic cleavage motifs within the polyprotein are apparently not utilized, suggesting that contextual information contributes to cleavage site selection. The structure of a recombinant DEN-2 NS3 protease domain, in the absence of cofactor NS2B, has recently been resolved to 2.1 Å by using x-ray crystallography (475). This model exhibits structural similarities to other serine proteases including the NS3-4A protease of HCV (described later), provides a framework for the interpretation of NS3 mutagenesis studies, and suggests that NS2B could contribute to substrate binding specificity (475).

The C-terminal three quarters of NS3, which slightly overlaps the serine protease domain (373), has been implicated in RNA replication. This region has significant homology to RNA helicases containing the motif Asp-Glu-Ala-(Asp/His) (199). These enzymes utilize the energy of nucleoside triphosphate (NTP) hydrolysis to power RNA unwinding (302). RNA-stimulated NTPase activity has been demonstrated for full-length and N-terminally truncated NS3 (373,744,754). Recently, DEN-2 NS3 has been shown to contain an NTP-dependent RNA unwinding activity, consistent with the function of NS3 as an RNA helicase (373). The precise role of an RNA helicase is not known for flaviviruses (302), but it may help to dissociate nascent RNA strands from their template during RNA replication, or perhaps unwind secondary structures involved in template recognition or initiation of RNA synthesis. In this regard, NS3 was shown to bind to a region of the 3' NCR containing the terminal stem-loop, in association with NS5 (103,133). A minor truncated form of NS3 has been described that apparently results from recognition of a cryptic NS2B-3 protease cleavage site within the helicase domain of NS3 (20,541,700). The biologic consequences of this alternative processing event are not well understood, although it may serve to disrupt the helicase function of NS3. In addition to the NTPase/RNA helicase activity of NS3, the C-terminal region of this protein also contains an RNA triphosphatase activity, which is distinct from the NTPase activity (755). This enzyme is likely to be involved in modifying the 5' end of the genome in preparation for 5' cap addition by a guanylyltransferase activity.

NS4A and NS4B

NS4A and NS4B are relatively small (about 16 kd and 27 kd, respectively) hydrophobic proteins that are membrane associated (377,405,762). Based on its subcellular distribution (405) and interaction with NS1 (385), NS4A appears to function in RNA replication, perhaps by anchoring replicase components to cellular membranes. NS4B also localizes to presumed sites of RNA replication, but it also appears to be dispersed throughout cytoplasmic membranes, and possibly the nucleus (762).

The N-terminus of NS4A is generated by the NS2B-3 protease (394), whereas the C-terminus of NS4A contains a transmembrane domain that serves to translocate NS4B into the ER. The NS2B-3 protease cleaves at a site within NS4A, just upstream of this signal sequence, and mutations that block this cleavage also block subsequent signal peptidase cleavage in the ER lumen to generate the N-terminus of NS4B (377,533). This unusual coordinated processing scheme, which is strikingly similar to processing at the C–prM junction, might serve to regulate replicase function. Furthermore, unprocessed NS3-4A and NS4A-4B have been detected in flavivirus-infected cells (93,394,533), suggesting that polyprotein cleavage in this region may be inefficient or controlled in additional ways. NS4B is posttranslationally modified to a form that appears to be about 2 kd smaller than the nascent protein, although the nature of this modification is unknown (93,533).

NS5

NS5, the largest (about 103 kd) and most conserved flavivirus protein, contains sequence homology to RdRPs of other positive-strand RNA viruses, including the invariant Gly-Asp-Asp (GDD) motif common to these enzymes (573). Purified recombinant NS5 exhibits RdRP activity in primer extension reactions (681), and NS5 protein has been found to fractionate with RdRP activity in infected cell extracts (116). NS5 also shares homology with methyltransferase enzymes involved in RNA cap formation and is thus probably involved in methylation of the 5' RNA cap structure (344). Site-directed mutagenesis has confirmed that motifs implicated in polymerase and methyltransferase activities are essential for virus replication, and that the functions of NS5 can be supplied in trans (318,319,320 and 321). NS5 can be

phosphorylated by an unknown cellular Ser/Thr kinase (311,466,561), a feature that is also conserved in the NS5A proteins of the pestiviruses and HCVs (see later). The role of NS5 phosphorylation remains obscure, but it may regulate the interaction of NS5 and NS3 (311), or a subcellular redistribution of NS5 into the cell nucleus (176,311).

RNA Replication

Following translation and processing of the viral proteins, a viral replicase is assembled from NS proteins, the viral RNA, and presumably some host factors. The replicase associates with membranes, probably through interactions involving the small hydrophobic NS proteins. Replication begins with the synthesis of a genome-length minus-strand RNA, which then serves as a template for the synthesis of additional plus-strand RNAs. The first round of minus-strand accumulation has been detected just over 3 hours after infection (384). Viral RNA synthesis appears to be asymmetric *in vivo*, with a plus-strand accumulation more than 10 times greater than that of minus strands (120,477). Viral minus strands appear to accumulate even late after infection and have been isolated exclusively in double-stranded forms (120,756). Flavivirus replication can be followed *in vivo* by metabolic labeling of virus-specific RNAs in the presence of actinomycin D, an inhibitor of DNA-dependent RNA polymerases. Three major species of labeled flavivirus RNAs have been described, one type sedimenting at 40S, another at 20S, and a heterogeneous population at 20S to 28S (120,756). 40S RNA is sensitive to RNase treatment and is identical to virion-associated RNA, consistent with its being genomic RNA (756). 20S RNA, frequently termed the replicative form (RF), is likely to be a transiently stable duplex of viral plus- and minus-strand RNAs based on RNase resistance and conversion to an RNase sensitive form that comigrates with 40S RNA by heat denaturation (756). The 20S to 28S RNAs are partially sensitive to RNase treatment and are described as replicative intermediate (RI) RNAs that most likely contain duplex regions and recently synthesized plus-strand RNAs displaced by nascent strands undergoing elongation (117,120). Pulse-chase analyses indicate that RF and RI RNAs are precursors to the 40S (genomic) RNA (117,120). This mode of replication, with minus strands serving as templates for the production of multiple plus strands, can be described as semiconservative and asymmetric (117).

RdRP activity has been studied *in vitro* by using crude cytoplasmic preparations of infected cells, and similar RNA forms have been demonstrated in these reactions (31,115,117,214,215 and 216,803). However, these *in vitro* systems appear to involve chain elongation rather than *de novo* initiation, and the complete process of flavivirus RNA replication has not yet been fully reconstructed in an *in vitro* model. Physical characterization of these preparations indicates that flavivirus RdRP activity co-sediments with dense membrane fractions enriched for several NS proteins and membrane structures morphologically similar to those found in virus-infected cells (116). RdRP activity has also been retained after solubilization in various detergents (116,214), although an intact flavivirus replicase has not yet been purified.

Membrane Reorganization and the Compartmentalization of Flavivirus Replication

Several studies have described ultrastructural changes in membranes of flavivirus-infected cells, predominately in the perinuclear region. In general, the earliest event is the proliferation of ER membranes (473,486), followed by the appearance of smooth membrane structures around the time of early logarithmic virus production. Smooth membrane structures are small clusters of vesicles containing electron-dense material within the lumen of smooth ER (87,363,473,486,488). These structures continue to accumulate during later times of infection, when they become adjacent to newly formed convoluted membranes. Convoluted membranes appear to be contiguous with the ER as randomly folded membranes or highly ordered structures that are sometimes described as paracrystalline arrays (363,473). All of these membrane alterations are concentrated in densely sedimenting membrane fractions from Kunjin-infected cells that are enriched for RdRP activity along with viral proteins NS2A, NS2B, NS3, NS4A, NS4B, and possibly NS1 (116). With the improved preservation of cellular membranes afforded by cryosectioning, Mackenzie et al. (404) demonstrated the appearance of vesicle packets, clusters of vesicles (each 100 to 200 nm in diameter) bound by a smooth membrane, during late times of infection. It seems likely that vesicle packets are related to or identical with smooth membrane structures, and they are often associated with smooth ER or Golgi-like membranes undergoing a morphologic process of wrapping. Such structures are also enriched for markers of the trans-Golgi (403).

The subcellular sites of RNA replication have been probed by metabolic labeling of nascent RNAs (64,662,763), by immunolabeling with sera reactive to double-stranded RNA, which presumably recognizes RF and RI RNAs (404,405,763,764), and by *in situ* hybridization (212). Apart from one report of nuclear replication (64), all investigations concur that viral RNAs accumulate in association with cytoplasmic membranes in the perinuclear region of mammalian cells and, in particular, with vesicle packets (404). NS1, NS2A, NS3, and NS4A have all been shown to localize to vesicle packets (404,405,764). Thus, it appears that along with double-stranded RNA, proteins implicated in RNA replication associate with vesicle packets. In contrast, the components of the viral serine protease, NS2B and NS3, co-localize with convoluted membranes (764). The membrane reorganization that occurs in infected cells might therefore give rise to adjacent, but distinct subcellular structures where viral polyprotein processing or RNA replication take place. It should however be emphasized that vesicle packets have been described only at late times after infection compared to the onset of RNA replication, and the sites of early RNA synthesis have not been defined. Furthermore, much remains to be learned about how interactions among NS proteins (103,133,311,385,405), between NS proteins and viral RNA (103,133,405), and between viral RNA and host factors (49,50,627) combine to form an active replicase.

Assembly and Release of Virus Particles

Ultrastructural studies indicate that virion morphogenesis occurs in association with intracellular membranes (reviewed in ref. 472). Electron microscopic studies of flavivirus-infected cells have consistently demonstrated morphologically mature virions within the lumen of a compartment believed to be the ER (142,246,247,292,333,363,404,424,473,485,497,650,651,740). In many studies, virions appear to accumulate within disorderly arrays of membrane-bound vesicles (see Fig. 4B). Budding intermediates and clearly distinguishable cytoplasmic nucleocapsids have not been frequently observed, suggesting that the process of assembly is rapid. Nascent virions are believed to be transported by bulk flow through the secretory pathway to the cell surface, where exocytosis occurs. Budding of virions at the plasma membrane has been occasionally observed (246,424,497,650), and it does not appear to be a major mechanism for virion formation. These ultrastructural observations, together with studies on structural protein biosynthesis, oligomer formation, and the properties of intracellular and released virions, suggest the

following model for virion assembly and maturation: The highly basic C protein interacts with viral genomic RNA in the cytoplasm to form a nucleocapsid precursor. The orientation of C, prM, and E with respect to the ER membrane would suggest that nucleocapsids acquire an envelope by budding into the ER lumen. Cosynthesis of E and prM is necessary for proper folding of E (341). These proteins have been shown to be associated as detergent-stable heterodimers (11,12,254,741,753) that can form higher-order structures, which may represent virion-associated lattices (741).

Later stages in virion maturation include glycan modification of E (for some viruses) and prM by trimming and terminal addition (93,419,491), implying that virions move through an exocytosis pathway similar to that used for synthesis of host plasma membrane glycoproteins. Although differences in the efficiency of prM cleavage have been noted (474,741), this cleavage generally distinguishes released virus from intracellular virus particles (626). Intracellular M-containing virions have not been detected, suggesting that prM cleavage occurs just prior to release of mature virions. This cleavage can be inhibited by elevating the pH in intracellular compartments (254,550,626), consistent with catalysis by furin (254,550). Although inhibition of prM cleavage does not impair virus release, studies on prM-containing particles suggest that this cleavage is required to generate highly infectious virus (11,220,254,551,626,653,753). As seen for the alphavirus structural proteins (729,730 and 731, and see Chapter 29), experimental data suggest that flaviviruses use oligomerization and prM cleavage to regulate the activation of E-protein-mediated fusion activity. The current hypothesis is that the function of prM in the prM–E heterodimer is to prevent E from undergoing an acid-catalyzed conformational change during transit of immature virions through an acidic intracellular compartment (220,222,254,550,753). Upon cleavage of prM and release of mature virus, the E–M interaction is destabilized (753). The differences in intracellular and extracellular virions are illustrated in Figure 8. The hemagglutination activity exhibited by flaviviruses, which depends on low pH, probably results from activation of the fusogenic activity of E protein.

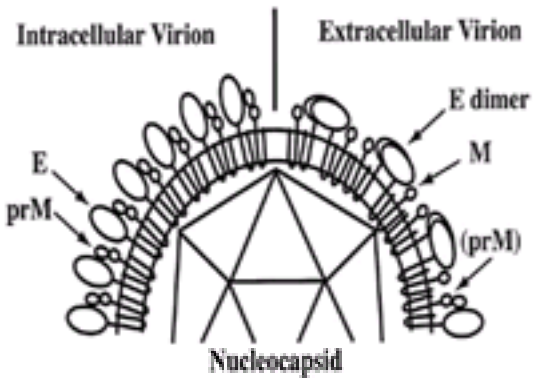

FIG. 8. Envelope proteins of intracellular and extracellular flavivirus virions. (Adapted from ref. 91, with permission.)

In addition to mature virions, slowly sedimenting (70S) noninfectious particles, which are also capable of agglutinating red blood cells at acid pH (called SHA, for slowly sedimenting hemagglutinin; for review, see ref. 592), are released from flavivirus-infected cells. SHA particles appear as 14-nm doughnut-like structures and are composed of E and M, with variable amounts of prM (592). Recent studies have shown that expression of prM and E is sufficient for release of SHA-like particles (11,341,420). These particles, which are fusogenic, provide an excellent model system for examining the functions of E and prM (12,611), and they show promise as immunogens that elicit protective immunity (128,342,343,420,524).

Generation of Defective Flaviviruses and the Involvement of Host Resistance Genes

Defective-interfering (DI) particles have been valuable tools for the study of RNA virus replication, and DIs can contribute to viral pathogenesis *in vivo*. For some virus families, strongly interfering DI particles, containing truncated and rearranged genomic RNAs, are easily generated by high-multiplicity passage. These RNAs contain cis-acting sequences necessary for replication and packaging but do not encode a complete or functional set of viral proteins, and therefore they need a helper virus to supply these functions in trans. In the case of flaviviruses, although DI particles have been observed in persistently infected vertebrate cell cultures, strongly interfering DIs are not readily obtained under these conditions or during serial high-multiplicity passaging (68). Several potential explanations exist for this observation, including the possibility that, under the conditions tested, most of the virus-specified components of the replication machinery are required in cis. In this regard, some viral replicase components can be supplied in trans (151,266,318,319,320 and 321,384), whereas several Kunjin virus nonstructural genes or their gene products are apparently required in cis (321). It is unclear whether this latter observation is the result of defects at the protein or RNA level. One group has recently described DI genomes in Vero cells persistently infected with MVE virus (357). Characterization of these RNA species indicated that they contain in-frame deletions of 2.3 to 2.6 kb in the prM, E, and NS1 genes. It is unlikely that the truncated NS1 is functional, and it is most likely complemented in trans, although it may contribute to interference. Of the few flavivirus DIs that have been characterized, all contain in-frame deletions, suggesting that selective pressure maintains the downstream ORF for the translation of one or more NS proteins in cis.

A system in which DIs appear to be readily generated has been studied in some detail (reviewed in ref. 68). Investigations into the heritable susceptibility of mice to YF infection (608) led to the discovery that multiple dominant alleles at a single locus on chromosome 5 can confer resistance to flaviviruses (67,598,599,600 and 601,719,720). Flaviviruses can replicate in resistant mice, but the spread of infection is slower, with significantly lower peak viremias (10^3- to 10^4-fold) than in susceptible mice. In primary fibroblasts from resistant mice, viral RNA synthesis was reduced, lower titers of infectious virions were released, and a high proportion of DIs were found even after a single growth cycle (67). Analyses of viral RNAs in the brains of infected mice suggest a block in RNA replication but do not necessarily support the existence of DI particles in this system (721). Nevertheless, these results indicate that a specific, but as yet unidentified, host gene can have dramatic effects on flavivirus RNA synthesis. Interestingly, RNA–protein complexes

involving the 3' NCR of the viral minus strand were found to be less stable in cell extracts prepared from a flavivirus-resistant mouse strain (627).

THE HEPATITIS C VIRUSES

Background and Classification

Following the development of diagnostic tests for hepatitis A and B viruses, an additional agent, which could be experimentally transmitted to chimpanzees (14,265,671), became recognized as the major cause of transfusion-acquired hepatitis. The causative agent, previously designated non-A, non-B hepatitis, and now referred to as hepatitis C virus, was identified in 1989 through expression cloning of cDNAs derived from the serum of an experimentally infected animal (113). Recognition of this pathogen led to the development of diagnostic tests for screening blood supplies, which dramatically decreased the incidence of posttransfusion hepatitis. Nevertheless, about 170 million people, roughly 3% of the human population, are infected with HCV (19), and virus transmission remains a significant public health concern. The most notable feature of HCV infections is that they typically persist, often lasting for decades, with more than 70% of cases progressing to chronic hepatitis, a condition that predisposes patients to developing chronic active hepatitis, cirrhosis of the liver, and hepatocellular carcinoma (Chapter 34). In addition to the sequelae resulting from chronic liver infection, HCV has been associated with other human diseases. Of nearly 30 such associations, which include a number of autoimmune diseases, the linkage between HCV infection and mixed type II cryoglobulinemia is the most striking. This disease is characterized by symptoms ranging from palpable purpura and fatigue, to life-threatening vasculitis of vital organs, such as the kidneys (2). Treatment for HCV typically involves the use of interferon-a, with or without ribavirin, although poor response to these treatments is not uncommon. The considerable diversity among HCV isolates, the emergence of genetic variants in chronically infected individuals, and the low level of protective immunity elicited after HCV infection present major challenges to the development of more effective therapies and vaccines. HCV is extremely refractile to growth in culture and there are currently no good animal models of this disease outside the chimpanzee. Despite these obstacles, intense scientific scrutiny over the past decade has yielded an astonishing wealth of information about this virus and its components. Much has been learned about the enzymology of viral replication proteins, and high-resolution crystallographic analyses have provided structural information for approximately 40% of the HCV genome and more than half of the nonstructural region. Understanding the biologic roles of these proteins is likely to come from recent progress in efficient initiation of HCV RNA replication in culture.

HCV is the sole member of the *Hepacivirus* genus (582). Hepaciviruses share some common features with the pestiviruses including genome organization, limited sequence relatedness, and a similar mechanism of translational control. HCV exhibits even greater similarity to the recently described GB agents, but there are also some important differences such as the potential to encode an obvious capsid protein. Within the *Hepacivirus* genus, numerous isolates of HCV are currently grouped into six major genotypes and several subtypes based on phylogenetic analysis of complete genome sequences or subgenomic fragments (81,636), although this taxonomic scheme appears to be inadequate for fully describing the diversity of recent HCV isolates, particularly those from Southeast Asia (582). It remains unclear whether there is a correlation between HCV genotype and disease severity or clinical outcome, although patients with genotype 1 viruses are clearly less responsive to treatment with antivirals (see ref. 457 and references therein).

Experimental Systems

Progress toward an understanding of HCV molecular biology has been hampered by its limited replication in cell culture and the lack of small animal models. Most of the early work, aimed at defining the physical properties of the virus and pathogenesis induced during acute and chronic infection, involved clinical samples from patients with posttransfusion hepatitis and chronic liver disease, or from experimental infection of chimpanzees (734). In chimpanzees, HCV RNA is detected in the serum as early as 3 days after inoculation and typically increases to 10^5 to 10^7 HCV RNA molecules per milliliter during the acute phase. Little evidence of hepatocellular damage is seen despite these high levels of viremia. Two to 3 months after infection, inflammatory infiltrates are seen in the liver, with areas of focal necrosis and release of enzymes such as alanine amino transferase (ALT) into circulation. This acute hepatitis coincides with the emergence of HCV-specific cellular immune responses and the appearance of HCV-specific antibodies in many (but not all) cases. In humans, acute hepatitis with overt clinical disease is seen in only about 25% of those infected. Circulating virus becomes undetectable and is apparently spontaneously cleared in 20% to 30% of cases (15). In chimpanzees, the rate of acute resolved infection may be somewhat higher (32). Chronic infections predominate, with only rare instances of clearance without treatment with interferon alone or in combination with ribavirin. Although HCV was originally thought to replicate poorly *in vivo*, recent models based on measuring viral loads after interferon therapy (356,484) or plasmapheresis (549) suggest a production rate of about 10^{12} virions per day and a virion half-life of 2 to 3 hours.

Since 1997, the availability of functional cDNA clones and infectious transcribed RNAs have allowed studies in the chimpanzee model to define the importance of viral replication determinants and to follow the evolution of the virus and host immune responses in acute-resolving versus chronic infections. For example, it has been shown that the HCV-encoded enzymes and conserved RNA elements in the 3' NCR are essential (340,792) and that extensive variation in the glycoproteins or elsewhere are not required to establish chronic infection (406). Subunit vaccination studies (112) have provided evidence for protective immunity and encourage efforts to develop HCV vaccines for health care workers and other high-risk groups.

In contrast to the chimpanzee model, efforts to develop small-animal models for HCV infection have met with only limited success (202). One model, an irradiated beige/nude/X-linked immunodeficient (BNX) mouse reconstituted with bone marrow from mice with severe combined immunodeficiency disease (SCID), and engrafted with human liver tissue, supported limited HBV and HCV replication (189). Similarly, immunodeficient mice engrafted with human hepatocytes under the kidney capsule and maintained long term by stimulation of the hepatocyte growth factor receptor allow HBV and HDV replication but have not been shown to be permissive for HCV (496). Other murine models for HBV (73) or woodchuck hepatitis virus (516) may ultimately succeed for HCV, but further work is needed. A single report indicates that a Chinese subspecies of the tupaia or tree shrew, *Tupaia belangeri chinensis*, is susceptible to HCV infection (778).

These animals have not achieved widespread use, as they are outbred, caught in the wild, and difficult to maintain and breed in captivity, and HCV infection in them is sporadic even in immunocompromised animals.

Because of their remarkable contribution to the study of HBV pathogenesis (109), transgenic mice with liver-directed expression of HCV proteins have also been created. Thus far, the establishment of a transgenic mouse line that can launch replication-competent HCV RNAs or produce infectious virus particles has not been reported. Mice expressing the HCV capsid protein (also referred to as core or C protein), the structural region or even the entire HCV polyprotein have been derived. Interestingly, at least one line expressing the HCV C protein develops lipid droplets in hepatocytes (i.e., steatosis) and progresses to hepatocellular carcinoma (464,465). This could be related to HCV-associated disease in humans and C-protein-induced lipid droplet formation (25) and transformation (98,300,554,714) in cell cultures. However, studies with other transgenic mice have not confirmed these C-protein-associated effects (317,503,733). Transgenic mice expressing the HCV structural region have been reported to develop spontaneous focal infiltration of lymphocytes and hepatocyte necrosis, and they were more sensitive to liver cell damage induced by injection of anti-Fas antibody (267). In other cases, no pathology was noted (317,336). The Cre/loxP system can be used for activation of HCV transgenes in hepatocytes; it provides an elegant approach for studying the effects of HCV proteins on hepatocyte function and the role of HCV-specific immune responses in pathogenesis (732,733).

Most cell culture studies of HCV have utilized transient transfection protocols or infection with recombinant viruses designed to express HCV proteins. Although such experiments have provided useful information, they may not mimic the situation in HCV-infected hepatocytes. For more than two decades, attempts have been made to propagate the non-A, non-B agent, and later HCV, in various cell cultures. In the postgenomic era of HCV research, when viral RNA can be measured qualitatively and quantitatively, much more has been published on this subject. Continuous hepatoma, B-cell and T-cell lines, primary hepatocytes from humans and chimpanzees, and peripheral blood mononuclear cells (PBMC) have all been reported to support HCV replication (see ref. 29 for review). HCV replication is usually defined by strand-specific reverse transcriptase polymerase chain reaction (RT-PCR) detection of minus-strand RNA. Unfortunately, this technique has been generally unreliable because of false priming during the RT step (225,359,597). Furthermore, accumulation of minus-strand RNA does not necessarily reflect complete replication as opposed to arrest after a single round of minus-strand synthesis. Immunofluorescent detection of HCV antigens, *in situ* hybridization, selection of variant sequences during culture, infection of naive cells or a chimpanzee with cell culture supernate, electron microscopy, inhibition by interferon-a, antisense oligonucleotides, and HCV-specific ribozymes have all been used as indicators of HCV replication in cell culture. Unfortunately, none of the culture systems reported to date have been robust enough to permit classical virologic, biochemical, or genetic dissection of the HCV replication cycle.

One problem that has plagued (and perhaps confused) cell culture infection studies is the limited availability of standardized inocula. As described later, the physical properties of circulating HCV RNA and the specific infectivity of different isolates (RNA molecules per chimpanzee infectious dose, or CID) are highly variable. The best sources of infectious material are acute-phase human or chimpanzee sera that have been titrated for infectivity in chimpanzees. Recently, several laboratories have been making such inocula (often derived from infectious cDNA clones) for each of the major HCV genotypes and subtypes (see ref. 79, and later).

Human hepatitis viruses have been notoriously difficult to grow in cell culture. This presumably results from defects at one or multiple steps of the replication cycle. For positive-strand RNA viruses such as HCV, transfection with infectious RNA can circumvent the entry steps, allowing translation and initiation of RNA replication in permissive cells. Full-length cDNA clones of HCV have been constructed for genotypes 1a, 1b, and 2a and the infectivity of transcribed RNAs validated by intrahepatic inoculation of chimpanzees (36,337,790,791,793). Despite this, attempts to initiate replication by transfection of the same RNAs into cell cultures have not met with success. Such experiments are complicated by a high background of transfected RNA that can persist for months, depending on cell type. Two reports have claimed productive replication after transfection with transcribed RNAs (134,799). These results remain unconfirmed, and it is worth noting that neither study used transcripts containing the correct 3' NCR. This sequence is the most highly conserved RNA element in the HCV genome, and it was later shown to be required for replication *in vivo* (see later).

A breakthrough for the field was reported in 1999 by Lohmann et al. (397). Based on results for the pestiviruses (43), these investigators engineered bicistronic subgenomic HCV replicons in which the neomycin resistance gene replaced the majority of the HCV structural region and the internal ribosome entry site (IRES) from the encephalomyocarditis virus (EMCV) was used to drive translation of the HCV replicase (beginning with either NS2 or NS3). At low frequency, G418-resistant colonies could be selected that harbored persistently replicating HCV RNAs at a copy number of 500 to 5,000 plus-strand RNAs per cell. More recently, adaptive mutations in the NS5A protein have been identified that allow efficient initiation of HCV RNA replication in as many as 10% of transfected hepatoma cells (51). Thus far, this system has been established for only a single genotype 1b HCV isolate and is restricted to Huh7 cells, a human hepatoma line. Despite this limited host range, we now have a powerful genetic system that should also enhance efforts to identify specific inhibitors of HCV RNA replication. A major gap in our experimental arsenal continues to be the lack of systems that support efficient replication of full-length genome RNAs, particle assembly, and release of infectious virus.

Structure and Physical Properties of the Virion

The size of the infectious virus, based on filtration experiments, is between 30 and 80 nm (62,250,806). HCV particles isolated from pooled human plasma (675), present in hepatocytes from infected chimpanzees, and produced in cell culture (303,535,625,631) have been tentatively visualized by electron microscopy (see Fig. 4F). Initial measurements of the buoyant density of infectious material in sucrose yielded a range of values, with the majority present in a low density pool of less than 1.1 g/mL (60). Subsequent studies have used RT-PCR to detect HCV-specific RNA as an indirect measure of potentially infectious virus present in sera from chronically infected humans or experimentally infected chimpanzees. From these studies, it has become clear that considerable heterogeneity exists between different clinical samples, and that many factors can affect the behavior of particles containing HCV RNA (261,704,745). Such factors include association with immunoglobulins (261,745) or low-density lipoprotein (704,705,745) that may be influenced by genotype (479). In highly infectious acute-phase chimpanzee serum, HCV-specific RNA is usually detected in fractions of low buoyant density (1.03 to 1.1 g/mL) (88,261). In other samples, the presence of HCV antibodies and formation of

immune complexes correlate with particles of higher density and lower infectivity (261). Treatment of particles with chloroform, which inactivates infectivity (61,166), or with nonionic detergents, produces RNA-containing particles of higher density (1.17 to 1.25 g/mL) believed to represent HCV nucleocapsids (261,307,446) of about 33 nm (675). The virion protein composition has not been determined, but putative HCV structural proteins include a basic C protein and two membrane glycoproteins, E1 and E2 (see later).

Binding and Entry

For the reasons just stated (lack of standardized infectious stocks, heterogeneous physical properties of virions, and poor infection in cell culture), these steps in the HCV replication cycle have been difficult to study. Even in the infection systems reported, there has been no systematic determination of basic parameters such as optimal adsorption conditions, or requirement for internalization or acidification. Interestingly, it has been shown that bovine and human lactoferrin can block infectivity of HCV in cell culture (288), presumably by binding to virus particles via the E2 glycoprotein (798).

Candidate receptors include the tetraspanin CD81 and the low-density lipoprotein receptor (LDLR). CD81 binds the ectodomain of the E2 glycoprotein and, to a lesser extent, HCV RNA-containing material from infectious plasma (523). The determinants in CD81 responsible for E2 binding reside in the large extracellular loop (LEL), a domain that exhibits species-specific heterogeneity. Specific amino acids in the LEL, important for binding to human and chimpanzee CD81 but not to that of African green monkey, have been mapped (256). However, this initial match between E2–CD81 binding and HCV species tropism is not absolute, as HCV E2 binds even more avidly to tamarin CD81 even though these animals are probably not permissive hosts (10,431). CD81 is expressed on most cell types, including B and T cells, making it unlikely that it is the sole determinant of HCV hepatotropism. Interaction between HCV E2 and CD81-containing signalling complexes on B cells has been postulated to play a role in modulating B-cell activation. This is of interest given the association of HCV with extrahepatic B-cell disorders such as mixed type II cryoglobulinemia and possibly non-Hodgkins B-cell lymphoma (for review, see ref. 570). LDLR has been attractive as a candidate HCV receptor since the discovery of the association between infectious HCV and low-density lipoprotein (LDL) or very low density lipoprotein (VLDL) (3,456,704,705). Coating of virions with serum lipoproteins during their secretion from hepatocytes or after release into circulation could shield the virus from neutralizing antibodies and provide a mechanism for binding and entry that is independent of the HCV glycoproteins. Recent studies indicate that HCV RNA-containing particles can be endocytosed via the LDLR in cell culture (3). What is not clear from the experiments thus far is whether either of these interactions (with CD81 or LDLR) actually leads to productive infection.

In lieu of an efficient infectivity assay, surrogate approaches have been employed. One report utilized vesicular stomatitis virus pseudotypes incorporating cell surface expressed chimeric forms of the HCV glycoproteins (353). Particles expressing either E1 or E2 demonstrated low levels of infectivity that could be neutralized by polyclonal antibodies produced in chimpanzees that had been immunized with candidate glycoprotein subunit vaccines. Because the two HCV glycoproteins are believed to assemble as heterodimers and mature in the ER (see later), it is unclear if the infectivity being measured in this assay reflects the function of these proteins in the native HCV particle. More recently, a cell–cell fusion assay has been described that requires cell surface expression of both HCV glycoproteins, exhibits low-pH enhanced fusion, and shows some selectivity for certain hepatoma lines (678). Experiments using this system suggest that expression of human CD81 is not sufficient for fusion and that other cell surface proteins and glycosaminoglycans are important.

Genome Structure

The HCV genome is about 9.6 kb in length, considerably shorter than that of flaviviruses. Based on characterization of the HCV translation strategy, and the absence of sequences corresponding to the presumed methyltransferase and RNA triphosphatase enzymes of flaviviruses, it is expected that the HCV genome does not include a 5' cap structure. The 5' NCR is a highly conserved RNA sequence element, about 341 nt in length, that biochemical probing and computer modeling indicate is folded into a complex structure consisting of four major domains and a pseudoknot (Fig. 9) (72,270,645,738). The 5' NCR probably contains the reverse complement of the information that is read by the replication machinery (i.e., from the 3' end of viral minus strands) to direct plus-strand RNA initiation. As described later, the 5' NCR also functions as an IRES to direct cap-independent translation of a single large ORF of about 3,011 codons. The 3' NCR was initially thought to terminate in polyadenosine (239) or polyuridine (101,106,249,312,498,499 and 500,676,685,742,782). However, the previously mentioned studies could not exclude false-priming as a source of these findings. Improved methods for cloning 3' ends of RNAs later revealed that the HCV 3' NCR actually consists of a short (about 40-nt) variable domain and a polyuridine/polypyrimidine tract, followed by a highly conserved 98-nt sequence (339,683,684,783). The latter two regions were shown to be essential for recovery of infectious HCV in chimpanzees (340,792). Biochemical probing has not fully resolved the secondary structure of the conserved 98-nt element, although the 3'-most 45-nt sequence has been shown to form a stable stem-loop (52). The HCV 3' NCR has been shown to interact with several cellular proteins including polypyrimidine tract binding (PTB) protein (197,295,715), which may contribute to the enhanced translational efficiency observed for RNAs containing a 5' IRES element and the HCV 3' NCR (296), as well as glyceraldehyde-3-phosphate dehydrogenase (519), and ubiquitous cellular proteins of 87 and 130 kd (289).

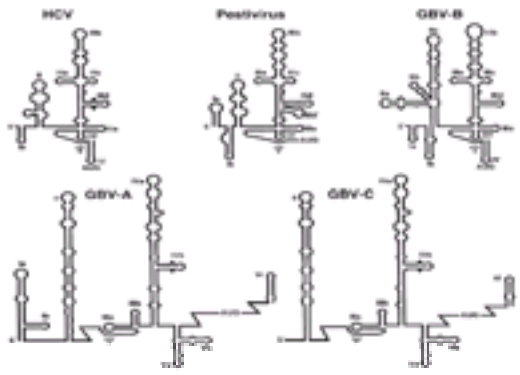

FIG. 9. Predicted secondary structures of pestivirus, hepatitis C virus (HCV), and GB virus internal ribosome entry site (IRES) elements. Model for the secondary and tertiary structures within the 5' NCRs of HCV, pestiviruses, GB virus A (*GBV-A*), B (*GBV-B*), and C (*GBV-C*). The polyprotein initiation site is indicated by AUG. Major structural domains are labeled *I to VI*. Predicted hairpin loops are indicated by *letters*, and pseudoknots are emphasized with a Y symbol. The models are based on computer-assisted folding and mutational analysis. (Courtesy of R. Rijnbrand and S. Lemon. For reviews see ref. 581.)

Translation and Proteolytic Processing

As mentioned before, the 5' NCR of HCV has been shown to have IRES activity, directing cap-independent initiation in a number of translation systems (182,270,287,401,569,578,716,737). The secondary structure of the HCV 5' NCR (see Fig. 9) provides a framework for understanding the function of the HCV IRES. Although it is unclear whether the approximately 109 5'-proximal nucleotides are necessary for IRES function (568,716), deletion of stem-loop I seems to have a positive effect on translation (270,578,800). The AUG codon used for initiation is precisely defined at a position within stem-loop IV (568,580), the stability of which affects IRES activity (268). The 3' boundary of the IRES is unclear, with conflicting reports concluding that sequences downstream of the initiation AUG are necessary (270,401,569) or that sequences lacking a complete stem-loop IV are sufficient (716,737). A pseudoknot formed by base-pairing of stem-loop IIIf to a region just upstream of stem-loop IV has been shown to be critical for IRES function (736, and see ref. 581 for further discussion). Interestingly, biochemical reconstitution of internal ribosomal entry for both hepacivirus and pestivirus IRES elements indicated that ribosomal 40S subunits and translational initiation factor eIF3 directly bind to discrete IRES regions, thereby bypassing the need for canonical translation initiation factors eIF4A, eIF4B, and eIF4F (514,515,642). Additional cellular factors that have been shown to bind the HCV IRES, typically enhancing translation, include PTB (8,304), La antigen (9), heterogeneous nuclear ribonucleoprotein L (230), and proteins of 25, 87, and 120 kd (184,797). Furthermore, IRES activity may also involve interaction with one or more cell cycle–specific factors (269).

The organization and processing of the HCV polyprotein is shown in Figure 10. Ten major polypeptides are produced by co- or posttranslational cleavage of the polyprotein and are arranged in the order NH_2-C-E1-E2-p7-NS2-NS3-NS4A-NS4B-NS5A-NS5B-COOH (203,204 and 205,379). Cleavages within the structural region (C/E1, E1/E2, and E2/p7) and at the p7/NS2 junction are thought to be mediated by the ER resident cellular signal peptidase, based on the hydrophobicity of regions just upstream of these cleavage sites (258,379,448,449,623), the dependence of cleavage on the presence of membranes (258,282,379,448,449,602,603) and signal-recognition particles (602,603), and mutagenesis of cleavage sites into suboptimal substrates for signal peptidase (448,449). An additional cleavage event probably removes the E1 signal sequence to produce mature C protein (282,602), although the responsible enzyme has not been definitively identified. Processing at the E2/p7 junction is incomplete, leading to accumulation of E2–p7 (379,448,623). Cleavages in the nonstructural region of the polyprotein are mediated by two virus encoded proteases: the zinc-stimulated NS2-3 autoprotease, which cleaves at the NS2/3 junction (204,259), or the NS3 serine protease (26,152,203,259,407,707), which utilizes NS4A as a cofactor for efficient processing at the 3/4A, 4A/4B, 4B/5A, and 5A/5B sites (27,157,380,688).

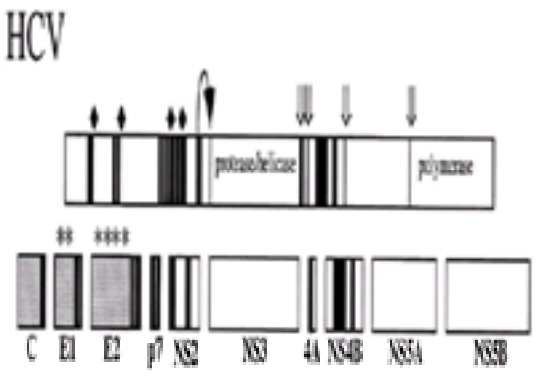

FIG. 10. Organization and processing of the HCV polyprotein. *Shading* and *symbols* identifying proteolytic cleavages are the same as described in Figure 3, except that the *curved arrow* indicates the autocatalytic cleavage at the NS2-3 site catalyzed by the NS2-3 autoprotease.

Features of the Structural Proteins

The C Protein

C is a highly conserved basic protein, appearing as 19- and 21-kd forms (82,205,241,282,390,391,461,553,602,670,796). The slower-migrating minor form is believed to result from signalase cleavage at only the C/E1 junction, whereas the faster-migrating “mature” form results from cleavage at a second site near residue 173, as discussed before. C protein associates with membranes, particularly the cytoplasmic surface of the ER (99,241,258,326,461,553,602,670). A minor 16-kd truncated form of C has also been reported by one group (391,392). In terms of HCV particle assembly, binding of C to the HCV 5' NCR (284,686) has been demonstrated, although nonspecific binding of RNA to C has also been reported (284,602). Multimerization of C, another likely step in the assembly process, has also been observed and requires the N-terminal 115 amino acids of C (423,490). Interaction of C with E1, but not with E2, has been suggested by coprecipitation experiments (393).

Nuclear localization of C, particularly truncated forms lacking C-terminal hydrophobic sequences, has also been reported (99,390,391,553,629,670,796). However, nuclear localization of the intact C protein remains controversial (25,82,461,462,602). Several functions have been proposed for nuclear forms of C protein, including the modulation of cellular gene transcription (555,558,559), repression of transcription from the human immunodeficiency virus 1 (HIV-1) long terminal repeat sequences (649), and the suppression of hepatitis B virus (HBV) transcription and replication in Huh-7 cells (629). This latter effect may be regulated by phosphorylation of C protein at Ser-99 and Ser-116 (628). However, expression of C protein in mice does not appear to inhibit transgene-initiated HBV replication (503). Several additional functions have been proposed for cytoplasmic forms of capsid protein. C protein has been shown to associate with 60S ribosomal subunits (602), which could be involved in the process of virion uncoating. C protein has also been found to associate with lipid droplets and to co-localize with apolipoprotein II (25), which may be related to the observation that the capsid protein has been shown to induce steatosis in transgenic mice (465) or to the reported association of HCV virions with lipids in the bloodstream (704,705). C protein also interacts with the lymphotoxin b receptor (102,422) and tumor necrosis factor receptor 1 (TNFR-1) (815), two members of the TNFR family of cytokine receptors. Interaction with these signalling pathways could be involved in modulating the effectiveness of host antiviral immune responses. In addition, these observations may be related to correlations between C protein expression and cellular sensitivity to apoptosis, although conflicting effects have been reported (102,556,557,585,815). The C protein has also been reported to transform primary rat embryo fibroblasts (REF) in cooperation with Ras (554) or an immortalized REF cell line called Rat-1 (98). In one study, capsid protein expression in transgenic mice induced hepatocellular carcinoma (464), although such tumors were not reported in other studies of transgenic mice expressing C protein (317,503,733). Thus, although the capsid protein has been the subject of much research, additional work is needed to determine the significance of these observations with respect to specific steps of the viral life cycle and HCV pathogenesis.

Envelope Glycoproteins

HCV E1 and E2, glycoproteins containing type I C-terminal transmembrane anchors, are heavily modified by N-linked glycosylation (205,258,275,283,290,335,336,360,372,425,426,443,489,547,593,623,648). Based on the extent and type of glycosylation (148,257,360,547,593,623,648), protection from protease digestion (258,393), and association with the ER-resident proteins calnexin (114,149), calreticulin (114), immunoglobulin heavy-chain binding protein (BiP) (114), and protein disulfide isomerase (138), E1 and E2 have been shown to be retained within the lumen of the ER. Signals for ER retention have been mapped to the transmembrane domains of both glycoproteins (122,123,150,172). E1 folds slowly and noncovalently interacts with the membrane-proximal domain of E2 to form what are believed to be native heterodimers (123,138,148,149,205,360,426,443,547). In addition, disulfide-linked aggregates of E1 and E2 have been described (148,205). A valuable tool for studying HCV glycoprotein maturation has been a conformation-sensitive monoclonal antibody, H2, with specificity for noncovalently associated E1E2 heterodimers, which are protease resistant and have been released from the ER chaperone calnexin (138). Kinetic studies with this reagent indicated that stable noncovalent E1E2 complexes form slowly, with a $T_{1/2}$ of about 2 hours, and that formation of native heterodimers is inefficient, accounting for about 5% of the E1E2 complexes (138). The limiting events in complex assembly appear to be formation of intramolecular disulfide bonds in E1 and glycosylation of E1, steps that require coexpression of E2 (147,149,443), as well as productive interaction of the glycoproteins with ER chaperones (114,149,443). Interestingly, calreticulin and BiP preferentially associate with aggregates of misfolded proteins, whereas calnexin is associated with newly synthesized glycoproteins, oxidized monomeric forms, and noncovalent heterodimers (114,149). Overexpression of these three chaperones (using vaccinia virus), either individually or in combination, does not increase the efficiency of productive E1E2 folding (114), suggesting that other unidentified chaperones and folding enzymes may be limiting for proper HCV glycoprotein folding.

As viral glycoproteins, E1 and E2 must have critical interactions with host molecules. It has been noted that E1 residues 264 to 290 bear similarity to suspected or known fusion peptides from flavivirus and paramyxovirus glycoproteins and may perform an analogous function during HCV entry (173). As mentioned, the ectodomain of E2 was shown to bind to the human tetraspanin-family cell surface membrane protein CD81 (523), an interaction that appears to involve a conformationally sensitive region of E2 (171,173) and an extracellular subdomain of CD81 (171,256). This interaction may play a key role in HCV binding and entry, but direct demonstration of this is lacking. In addition, interaction of CD81 with soluble forms of E2 was shown to have antiproliferative effects on lymphocytes (171). The N-terminal portion of E2 contains a hypervariable region (HVR1) (351,493) that is likely to reflect adaptation of the virus to the host immune response and selection of immune escape variants (164,165,315,622,633,750). Indeed, E2 HVR1 has been shown to be a target for neutralizing antibodies (257,314,749,750). Nonetheless, recent work has shown that HVR1 is not essential for replication or chronic infection in chimpanzees, although deletion of this sequence debilitates the virus (175). In addition, expression of E2 has been shown to inhibit the function of the interferon-inducible double-stranded-RNA-activated protein kinase PKR (699), although it is unclear how the proposed interaction involving the E2 ectodomain with cytoplasmic PKR actually occurs.

Features of the Nonstructural Proteins

p7 and NS2

No function is known for the small hydrophobic p7 protein, which is thought to be inefficiently released from the C terminus of E2 by signal peptidase (379,448,449,623). By analogy with pestivirus p7 (154), HCV p7 and E2–p7 are probably not associated with virions, although an efficient cell culture system is needed to test this.

NS2 (about 23 kd) contains a predicted cysteine protease domain (200), which interacts with the immediately downstream 180 amino acids of NS3 to form the NS2/3 autoprotease that cleaves at the NS2/3 junction (204,259). NS2/3 protease activity is necessary for the *in vivo* infectivity of full-length HCV genomes (340). However, NS2 is dispensable for RNA replication of subgenomic HCV replicons in culture (51,397), and no function is known for NS2 other than in NS2–3 cleavage. NS2/3 activity, which is distinct from the serine protease activity of NS3, is stimulated *in vitro* by addition of microsomal membranes (204,603), various detergents (522), and Zn^{2+} (259). The former two reagents probably allow for proper conformation of the moderately hydrophobic NS2. It is unclear whether Zn^{2+} plays a catalytic or structural role in 2–3 processing. Interestingly, crystallographic (328,400,789) and biochemical (135,658) analyses have identified a site for tetrahedral coordination of Zn^{2+} (HCV polyprotein residues Cys-1123, Cys-1125, Cys-1171, and His-1175) in the region of NS3 required for NS2–3 protease activity, and suggested that this Zn^{2+} ion plays an essential structural role in the NS3 serine protease domain. Consistent with a role for this Zn^{2+} ion in both types of processing, mutations at Cys-1123, Cys-1125, and Cys-1171 inhibit NS2–3 cleavage as well as downstream cleavages catalyzed by the serine protease (259). Mutation of His-1175 has a less dramatic effect (204,259,658), perhaps because this residue indirectly interacts with Zn^{2+} via an H_2O molecule. Although cleavage at the 2/3 site is thought to occur by an autocatalytic mechanism, bimolecular cleavage has been shown to occur, albeit inefficiently, with functional NS2 and/or NS3 protease subunits when coexpressed with substrates containing mutations in either NS2 or NS3 (but not both) that inactivate the NS2–3 protease (204,562). Requirement for a functional NS2 or NS3 region in cis suggests that the overall conformation of NS2/3 may play a role in orienting the NS2/3 cleavage site. In support of this model, mutagenesis of the P5 to P3' positions at the NS2/3 cleavage site indicated that conformation is a more important determinant for cleavage than primary sequence (263,562).

The NS3 Protein

Like the flavivirus NS3 protein, HCV NS3 (about 70 kd) encodes a serine protease domain in the N-terminal one third of the protein, and an NTPase/helicase domain in the C-terminal two thirds. The serine protease is responsible for cleavage at the NS3/4A, NS4A/4B, NS4B/5A, and NS5A/5B sites (26,152,203,205,259,407,707), and it is required for infectivity of HCV genomes *in vivo* (340). Cleavage at the NS3/4A site occurs in cis, whereas trans cleavage can occur at downstream sites (27,380,689,707). Analysis of cleavage products has revealed that these cleavage sites are highly conserved and conform to the sequence (Asp/Glu)XXXX(Cys/Thr)(Ser/Ala) (152,203,525). As shown by site-directed mutagenesis and molecular modeling, the P1 residue appears to be an important determinant for cleavage efficiency except at the NS3/4A autocleavage site (28,338,370,525). NS4A is a cofactor for serine protease activity, critical for all serine protease-dependent cleavages except NS5A/5B, although this cleavage can be stimulated by NS4A (27,157,380,690). Cofactor activity requires stable complex formation between NS3 and NS4A (30,158,381,607), an interaction that also serves to stabilize NS3 and anchor it to cellular membranes (260,690,773).

The structure of the NS3 serine protease domain alone (400), or in complex with an NS4A-derived cofactor (328,789) has been determined by x-ray crystallography. The HCV serine protease structure is similar to other members of the trypsin superfamily, with active site residues and a substrate binding pocket located in a cleft separating two b-barrel domains. Unique to the HCV enzyme, the NS4A cofactor forms an integral part of this structure, interacting with the extreme N-terminal residues of NS3 to form two antiparallel b strands not found in trypsin and altering the geometry of the catalytic site (328,789). These data also reveal an important structural role of the coordinated Zn^{2+} ion for proper folding of the serine protease domain (328,400), a result that is supported by biochemical studies (135,658). Within the predicted substrate-binding pocket, Phe-1180 probably interacts with the conserved Cys residues present in the P1 position of the 4A/4B, 4B/5A, and 5A/5B sites (328,400,525,789). Based on the interaction of NS3 and NS4A, protein engineering studies have created single-chain NS3–NS4A fusion proteins (scNS3–NS4A) with full serine protease activity (144,274,504,692). The structure of full-length NS3 with NS4A cofactor was determined by x-ray crystallography of one scNS3–NS4A (Fig. 11) (795). Folding of the protease and helicase domains of this molecule resembles that of each individual domain (the helicase domain is described later), separated by a single flexible loop (795). Interestingly, the C-terminus of NS3 interacts with the protease active site in a manner consistent with a protease:product (795). Since NS3/4A cleavage occurs autocatalytically, this model provides a unique glimpse at polyprotein cis processing and suggests structural rearrangements that are needed for subsequent trans processing events (795).

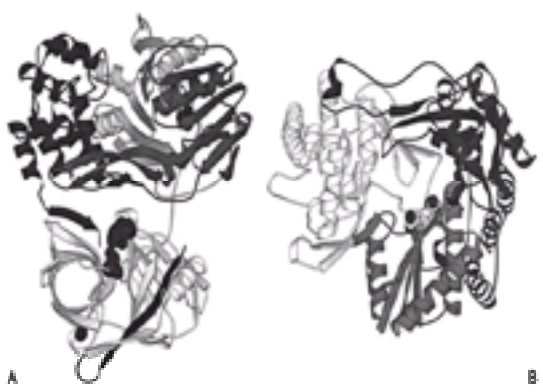

FIG. 11. Structures of enzymatic components of the HCV replicase. **A:** The b-barrels of the HCV protease and three subdomains of the helicase are colored from *white to black* along the polypeptide chain. The NS3 protease catalytic triad,

the solvent-exposed protease-associated zinc, and a phosphate ion in the helicase NTP binding site are indicated by *dark spheres*, and NS4A is represented by a *black strand.* **B:** The HCV NS5 RNA-dependent RNA (RdRP) polymerase exhibits a globular shape, unique among known polymerase structures. Nevertheless, this viral enzyme retains the typical polymerase subdomain organization consisting of fingers, palm, and thumb subdomains shown in *black*, *gray*, and *white*, respectively. Catalytic residues within the canonical RdRP GDD motif are shown as space-filling *spheres.* In both diagrams, ribbons follow the polypeptide fold and reveal locations of sheets and helices. [Courtesy of N. Yao, C. Lesburg, and P. Weber (see refs. 371 and 795).]

HCV NS3 is a member of the Asp-Glu-Cys-His (DECH) subfamily of DEAD-box helicases. The NTPase/ helicase activities of NS3 have been demonstrated for recombinant full-length NS3 (188,271,352,463) or the C-terminal two thirds of NS3 (252,301,325,531,669,674). Like the flavivirus enzyme, HCV NS3 ATPase activity is stimulated by single-stranded nucleic acids (227,228,271,301,305,463,531,669,674). NS3 unwinds RNA and DNA homo- and heteroduplexes in a 3' to 5' direction (674), and this requires Mg^{2+} or Mn^{2+} and ATP, suggesting that helicase activity is coupled to ATP hydrolysis (301,463, 531). However, other NTPs and dNTPs can substitute for ATP in this reaction (301,464), and ATP is not required for RNA binding (188). The crystal structure of the HCV NS3 helicase domain has been determined to a resolution of 2.1 to 2.3 Å in the presence (327) or absence (110,794) of a bound oligonucleotide. These data all reveal three well-defined structural domains surrounding a central axis and separated by distinct clefts (110,327,794). The first two domains, which contain all of the conserved NTPase/helicase motifs, each contain a parallel, six-stranded b sheet surrounded by a number of a helices. However, domain 1 contains a seventh, antiparallel b strand, and domain 2 contains an additional pair of antiparallel b strands that extend into the vicinity of domain 3, which is completely a helical. The catalytic site is located in domain 1 near the cleft that separates it from domain 2. RNA substrate binds in the cleft that separates domains 1 and 2 from domain 3, with the 5' end near domain 2 (327). Although the precise role of RNA helicases in replication is unknown, mutations in NS3 that disrupt helicase activity ablate HCV infectivity *in vivo* (340).

In addition to its roles in HCV polyprotein processing and RNA replication, several other functions have been proposed for NS3. Cyclic AMP–dependent protein kinase (PKA)-dependent phosphorylation was inhibited by synthetic peptides corresponding to a region of HCV NS3 that exhibits similarity to a PKA inhibitor protein and a PKA autophosphorylation site (56,57 and 58). Furthermore, a truncated NS3 protein containing this region was able to interact with the catalytic subunit of PKA and inhibited its forskolin-stimulated nuclear translocation and PKA-catalyzed phosphorylation (57). The serine protease domain of NS3 has been found to have weak transforming activity in NIH-3T3 cells (595) and can suppress actinomycin D–induced apoptosis (180). It is unclear whether these effects may be related to an observed subcellular co-localization of NS3 and the cellular tumor suppressor gene product p53 (294,471).

NS4A and NS4B

The 54-residue NS4A protein (about 8 kd) contains a hydrophobic N-terminal domain followed by a highly charged region. The serine protease cofactor activity of NS4A (see preceding) is contained within a 12-amino-acid region in the central portion of NS4A (30,381,630,690,708). NS4A also associates with membranes (690,773), probably through the hydrophobic N-terminal region, and interacts with other replicase components (293,382). NS4B (about 30 kd) is a hydrophobic protein of unknown function.

NS5A

NS5A is a hydrophilic but membrane-associated protein that exists in at least two forms with apparent molecular masses of 56 and 58 kd. Although these forms were originally thought to be the product of alternative proteolysis (260), it is now clear that they result from differential phosphorylation (306,691). NS5A phosphorylation occurs mostly on serine residues and, to a lesser extent, on threonine (306,564). The “basal” phosphorylation site (or sites) of HCV-J NS5A, which is thought to occur for both p56 and p58, has been mapped by serial deletion to the region downstream of polyprotein residue 2350 (690). p58 is “hyperphosphorylated” at additional sites that remain unmodified in the p56 form. Deletion analyses suggest that these hyperphosphorylation sites reside in a conserved, central region of the polyprotein that extends from residue 2200 to residue 2250 (690). Site-directed mutagenesis of the nine conserved serines in this region tentatively identified these sites of p58 phosphorylation as Ser-2197, Ser-2201, and Ser-2204 (690). For an HCV genotype 1a isolate, a major site of *in vitro* and *in vivo* phosphorylation has been mapped to NS5A residue Ser-2321. However, this serine residue is not conserved among the different HCV genotypes (563). For the HCV-J isolate, NS4A associates with NS5A (21) and enhances p58 production and, by inference, hyperphosphorylation at the upstream sites (306,691). However, this effect of NS4A seems to be isolate specific (262) and more recent studies suggest that multiple determinants, including all of the upstream NS proteins, influence NS5A phosphorylation when the protein is expressed in the context of an HCV NS polyprotein (334,389,482).

The kinase responsible for NS5A phosphorylation is thought to be of cellular origin, since (a) NS5A contains no recognizable kinase motifs, (b) phosphorylation of NS5A expressed transiently in cultured cells occurs in the absence of other viral proteins (564,691), and (c) phosphorylation of NS5A expressed in *E. coli* depends on the addition of eukaryotic cell extracts (285,563). Although the identity of the NS5A kinase is not known, biochemical properties of an NS5A-associated kinase activity with similarities to the kinase responsible for NS5A phosphorylation *in vivo* have already been characterized. This NS5A-associated kinase is active *in vitro* over a broad pH range, has an apparent preference for $MnCl_2$ over $MgCl_2$, and is inhibited strongly by $CaCl_2$ at concentrations over 0.5 mM (564). Furthermore, specific inhibitors of PKA and protein kinase C have little or no effect on NS5A phosphorylation *in vitro* or *in vivo*. However, both types of NS5A phosphorylation are inhibited by olomoucine, an inhibitor of certain proline-directed kinases. The resistance of NS5A phosphorylation to treatment with a specific PKA inhibitor casts some doubt on the suggestion that PKA is the major effector of NS5A phosphorylation (285). Although the preference of the NS5A-associated kinase for Mn^{2+} over Mg^{2+} is somewhat unusual among serine/threonine kinases, as is its inhibition by Ca^{2+}, the NS5A and NS5 proteins of bovine viral diarrhea virus (BVDV) and YF, respectively, associate with a kinase that has similar properties

(561). More work is needed to determine the role of NS5A and NS5A phosphorylation in viral replication.

Analysis of full-length genome sequences for three HCV genotype 1b isolates obtained from different Japanese patients before and after interferon therapy found mutations clustered primarily in E2 HVR1 and the C-terminal half of NS5A (155). Further analysis suggested that the amino acid sequence from polyprotein position 2209 to 2248 in NS5A correlated with the effectiveness of interferon treatment in these and other Japanese patients infected with genotype 1b. Consequently, this stretch of amino acids was designated the interferon sensitivity–determining region (ISDR) (155,156). Although confirmed by most other groups working with Japanese patients infected with genotype 1b, 2a, or 2b HCV strains, this correlation was substantially weaker or lacking in patients infected with genotype 1a strains or European patients infected with strains of genotype 1b, 2b, or 3a (see ref. 508 for review).

In any case, following up on this possible link between NS5A and response to interferon, NS5A was found to interact with and inhibit the interferon-stimulated, double-stranded-RNA-dependent kinase PKR (187). PKR is a major effector of the host antiviral defense pathway that represses translation by phosphorylating the a subunit of the translation initiation factor eIF2. Evidence suggests that HCV NS5A interacts with PKR and inhibits dimerization that is required for PKR activation (185). The region of NS5A implicated in this interaction with PKR includes the ISDR as well as downstream sequences. Several studies have shown that cells expressing NS5A can partially resist the antiviral effects of interferon (186,505,528,647). Somewhat surprisingly, such effects can be observed in the absence of the ISDR (528) and occur independently of the PKR-eIF2 pathway in the context of the complete HCV polyprotein (178). Interestingly, mutations that allow more efficient initiation of HCV subgenomic replicon replication in cell culture cluster in NS5A in the region just upstream of the ISDR and include a 47-amino-acid deletion that encompasses the ISDR (51). These data suggest that NS5A plays an active role in RNA replication and that the ISDR is not essential in cell culture. In addition, deletion of the ISDR does not appear to increase the sensitivity of HCV RNA replication to interferon a in this system (51), consistent with previous observations (528).

N-terminally truncated forms of NS5A fused to the DNA-binding domain of the *Saccharomyces cerevisiae* Gal4 protein activate transcription of reporter genes under the control of promoters containing Gal4 binding sites (119,313,687). This trans-activating ability has been linked to ISDR sequences that may be associated with increased interferon sensitivity (181). The physiologic relevance of these findings is questionable, because the full-length NS5A protein lacks this trans-activating ability and has a cytoplasmic localization. However, NS5A does contain a sequence in its C-terminal region that has the potential to function as a nuclear localization signal (286), raising the possibility that proteolytic removal of the N-terminal region of NS5A (417,463) could result in its transport to the nucleus and activation of the transcription of certain cellular genes. Indeed, recent work has provided evidence for caspase-mediated cleavages in NS5A that liberate a nuclear form whose ability to function as a transcriptional activator is regulated by PKA (606).

In addition to these features, NS5A interacts with several cellular proteins. These include growth-factor-receptor-bound protein-2 adaptor protein, an interaction that can perturb mitogenic signalling (682); a SNARE-like protein, hVAP-33, that is ER and Golgi associated and may participate in intracellular membrane trafficking (717); a novel cellular transcription factor SRCAP (190); and human karyopherin beta3, a protein that may participate in nuclear trafficking of RNAs and proteins (118). The role of these interactions in HCV replication or in virus-induced modulation of host cell function remains to be established.

NS5B

NS5B (about 68 kd) is a hydrophilic protein containing the GDD motif common to RdRPs. As expected, mutation of the polymerase active sites destroy the *in vivo* infectivity of HCV genomes (340) and replication of HCV subgenomes in culture (51,397). RdRP activity has been demonstrated *in vitro* for recombinant NS5B (7,44,396,784,805), although these reactions do not show specificity for HCV templates. DNA or RNA oligonucleotide primers can be extended by the NS5B RdRP on homopolymeric templates or cellular RNAs (6,7,44,396,784). RNAs terminating with the HCV 3' NCR, which contains a stable 3' hairpin (see preceding), can also be utilized as self-priming templates for RNA extension by NS5B (44,396). *De novo*, primer-independent initiation of RNA synthesis has recently been demonstrated in RdRP assays, usually by increasing concentrations of the initiating nucleotide (402,494,814). The more reliable of these studies (402,814) included templates that were chemically blocked at the 3' end and therefore unable to function as primers. Biochemical characterization of RdRP activity reveals a requirement for the divalent cations Mn^{2+} or Mg^{2+}, but not Zn^{2+} (6,44,167,396,402,784,805), near neutral pH (7,44,396,784), and low (i.e., 100-mM) concentrations of salt (399,784). The rate of elongation on a genome-length HCV RNA template was estimated to be 150 to 200 nucleotides per minute at 22°C (399,494), and it was independent of NS5B concentration (399), indicating that NS5B is highly processive. Terminal nucleotidyl transferase activity was reported in one preparation of NS5B purified from insect cells (44). However, several other purified NS5B preparations were apparently free of this activity (7,784,805), suggesting that it is more likely to be due to a copurifying cellular enzyme than an inherent property of NS5B (396).

The hydrophobic C-terminal 21 amino acids can be deleted from NS5B to produce soluble, fully active RdRP (167,396,402,784). This finding greatly enabled several high-resolution structural determinations of NS5B by x-ray crystallography (4,66,371). NS5B bears structural similarity to other polymerases, adopting a “right hand” conformation with a palm subdomain containing active site residues, and discernible fingers and thumb subdomains (see Fig. 11). However, extensive interactions between the fingers and thumb subdomains serve to fully encircle the NS5B active site. Despite the absence of substrate in these experiments, this resembles the closed conformation described for other polymerases (66), suggesting that NS5B may not undergo the typical conformational rotation upon template–primer binding (298,374). Residues thought to be involved in metal ion coordination, substrate binding, and nucleotide discrimination are spatially organized as for HIV-1 reverse transcriptase (4,66,371). Another notable feature of NS5B is a unique b-loop projecting from the thumb subdomain that probably interacts with the template–primer and could affect NS5B fidelity and/or processivity (4,66,371). The thumb subdomain also contains structural similarity to “armadillo” repeats, which could be involved in mediating protein–protein interactions within the replicase complex (66).

The C-terminal hydrophobic domain of NS5B mediates association with perinuclear membranes, the presumed site of

HCV RNA replication (784), and includes determinants for interaction with NS5A (784). Although NS5B binds RNAs nonspecifically, it does show some preference for RNAs containing the HCV 3' NCR (108,495). NS5B has also been shown to directly interact with NS3 and NS4A (293), which in turn have been shown to form complexes with NS4B and NS5A (21,382). It is likely, yet unproven, that these interactions are important for assembly of a functional HCV RNA replication complex.

RNA Replication

As in other *Flaviviridae*, RNA replication in HCV is likely to occur in association with perinuclear membranes and involve the combined actions of the viral polymerase, helicase, other viral nonstructural proteins, and presumably some host factors (260,293,334,382,482). Because of the lack of an efficient cell culture system, the events of HCV RNA replication are only poorly understood. Detection of viral plus- and minus-strand RNAs in infectious culture systems or clinical samples have been limited to RT-PCR amplification. The most reliable of these methods are designed to eliminate false priming and ensure strand specificity during reverse transcription (225,359). Characterization of HCV subgenomes efficiently replicating in culture by RT-PCR or Northern blot reveals that subgenomes accumulate to 50 to 5,000 copies per cell in these systems (51,397), and that minus strands are 5- to 10-fold less abundant than plus strands (397), which is in agreement with the plus-to-minus-strand ratio observed in infected hepatocytes (359). It has not yet been determined whether viral minus strands can be isolated as RIs or in duplex RFs.

Assembly and Release of Virus Particles

In the absence of an efficient system for culturing HCV, much of what we know about HCV virion assembly comes from heterologous expression studies. Although packaging signals of the HCV genome are not yet characterized, C protein has been shown to interact with stem-loop IIId of the 5' NCR (634,686). Furthermore, homotypic interactions of C protein (423,490) could be relevant to the formation of viral nucleocapsids. Based on the ER retention of viral glycoproteins E1 and E2, it seems likely that nucleocapsid precursors bud into the ER to acquire a lipid envelope and native heterodimers of the viral glycoproteins, then pass through the host secretory pathway and are released at the cell surface. Formation of virus-like particles (VLPs) by expression of the HCV structural proteins has met with limited success. Presumably nonenveloped (about 30 nm) and enveloped (about 45 nm) VLPs containing C protein were observed in HeLa G cells expressing the full-length HCV coding region via the vaccinia virus-T7 system (447). Enveloped VLPs containing HCV RNA were also observed in insect cells expressing the HCV 5' NCR and structural region via a recombinant baculovirus (33). These VLPs were observed in intracellular vesicles late in infection (at 72 to 96 hr) but were not secreted and could be released only after mild detergent treatment and sonication. One study indicated that extracellular HCV particles partially purified from human plasma do contain complex N-linked glycans, suggestive of transit through the secretory pathway, although these carbohydrate moieties were not shown to be specifically associated with E1 or E2 (604).

Association of Hepatitis C Virus with HCC

A significant fraction of chronically infected patients slowly progress from chronic active hepatitis to cirrhosis and then to hepato-cellular carcinoma (HCC) (see refs. 54 and 635 and references therein). The mean onset for development of primary HCC has been estimated to be 20 to 30 years (332,594). Studies have identified both positive- and negative-strand HCV RNA in tumorous tissue from some but not all patients. The slow onset and the apparent association with preexisting cirrhosis suggest that HCV may not directly cause HCC but rather predisposes the organ to carcinogenic events. However, these observations do not exclude the possibility that expression of particular HCV gene products in chronically infected cells might contribute to carcinogenesis. In this regard, expression of C (98,300,554,714), NS3 (595), NS4B (501), and NS5A (186,191) in certain cell cultures can lead to anchorage-independent growth, enhanced formation of colonies in soft agar, and induction of tumor formation in immunodeficient mice. As mentioned earlier, some transgenic mouse lines expressing the C protein develop hepatocellular carcinoma similar in appearance to the human cancer (464). However, the relevance of these observations to the association between HCV and HCC in humans remains to be established.

THE PESTIVIRUSES

Background and Classification

Pestiviruses are animal pathogens of major economic importance, particularly in the livestock industry. They include the type member, bovine viral diarrhea virus, as well as classical swine fever virus (CSFV), formerly known as hog cholera virus, and border disease virus (BDV) of sheep (536). Based on comparative studies, two distinct species of BVDV have recently been identified, BVDV-1 and BVDV-2 (39,42,513,576). Newly described pestiviruses further indicate that additional diversity exists within this genus (39,724). Within the *Flaviviridae*, pestiviruses show greater similarity in genome structure and translation to the hepaciviruses than do the flaviviruses. For this reason, pestivirus research has received increased attention in the search for surrogate model systems to understand the less tractable hepaciviruses.

Pestiviruses are responsible for a spectrum of diseases within their natural hosts (reviewed in ref. 702). CSFV, typically transmitted oronasally, leads to acute or chronic hemorrhagic syndromes with significant mortality. Ruminant pestiviruses, on the other hand, usually cause inapparent or mild symptoms in adult animals. A notable exception is BVDV-2, which is associated with a severe acute hemorrhagic condition in calves (130,513,560,576). In addition, congenital transmission of pestiviruses can cause fetal death and acute syndromes of the newborn, or it can lead to persistent infection of the offspring in a carrier state. Persistently infected ruminants can be susceptible to a rare but fatal mucosal disease (MD) later in life. Interestingly, development of MD correlates with viral genome alterations that affect the outcome of viral infection on host cells in culture. Recent efforts in pestivirus research have revealed interesting and novel features of these virus–host interactions. Although live attenuated strains and inactivated virus preparations are available for vaccination against CSFV and BVDV (450), there is a need for improved pestivirus vaccines. Insights into pestivirus biology are also being applied to the design of such tools (18,59,77,131,243,588,725,766).

Experimental Systems

In infected animal hosts, viral antigens and infectious virus can be detected in a variety of tissue types including epithelial cells at the site of entry, endothelial cells, lymphoreticular cells, and macrophages. In persistently infected animals, BVDV can be detected in most tissues including PBMC, the gastrointestinal tract, and neurons. Primary and continuous cell lines from natural host species are usually permissive for pestivirus replication in cell culture, although considerable differences in replication efficiencies have been noted (273,588). Highly permissive cell lines have been described for propagation of CSFV (458,588). Infection of permissive tissue culture cells is usually noncytopathic; however, variants of the ruminant pestiviruses capable of causing cytopathic effects can be isolated from animals with mucosal disease. Based on this cell culture phenotype, pestivirus isolates are referred to as either noncytopathogenic (ncp) or cytopathogenic (cp) biotypes.

Complete genome sequences have been determined for a number of pestiviruses (40,71,126,137,139,432,437,459,460,574,575,586). This has led to the construction of reverse genetic systems (347,430,437,442,459,586,727) that are being used to probe mechanisms of pestivirus replication and the molecular basis of cytopathogenesis and virulence.

Structure and Physical Properties of the Virion

Pestiviruses have been difficult to purify because of modest growth in cell culture, inefficient release from infected cells, and association with cellular debris (362). Identification of efficient culture systems has facilitated visualization of virus particles by electron microscopy (450,746) (see Fig. 4D,Fig. 4E) and the characterization of the structural components of the virion (703). The spherical particles are 40 to 60 nm in diameter and enveloped, and they contain an electron-dense inner core with a diameter of about 30 nm (273). Pestivirus virions band at a buoyant density of 1.134 g/mL in sucrose and are inactivated by heat, organic solvents, and detergents (588). Unlike flaviviruses, which are rapidly inactivated by low pH, pestiviruses can survive over a relatively broad pH range (376). The chemical composition of highly purified preparations of pestivirus particles has not been determined, but in addition to the genome RNA and lipid bilayer, four structural proteins are present. These proteins include capsid protein, C, and three envelope glycoproteins E^{rns} (for ribonuclease, soluble), E1, and E2 (591,703).

Binding and Entry

Based on examples from other viruses, the binding and entry of pestiviruses is likely to be a multistep process involving initial attachment of virions, interaction with specific receptor(s), internalization, and membrane fusion. Specific cell surface receptors for pestiviruses have not been fully characterized. One candidate receptor is a pair of proteins, with apparent relative masses of 60 kd and 93 kd, which are recognized by three monoclonal antibodies capable of blocking infection of several BVDV-1 strains (613). Another potential receptor is a 50-kd cell surface protein identified by using an antiidiotypic antiserum (directed against E2-specific antibodies) that can block binding of BVDV-1 to bovine cells (445,780,781). It has been shown that recombinant E2 and E^{rns} can bind independently to cell surfaces (277). E2 adsorption competitively inhibits infection with homotypic and heterotypic pestiviruses, whereas inhibition by E^{rns} demonstrates virus specificity and requires large amounts of E^{rns} (277). This latter point may be explained by a high receptor density, which correlates with the observed binding of E^{rns} to cell surface glycosaminoglycans (291). Additional information regarding the entry process may come from further characterization of a mutant bovine kidney cell line that is resistant to infection by several pestiviruses but is capable of supporting their replication following RNA transfection or chemically induced virus–cell fusion (174). This cell line was also found to be deficient in expression of the LDLR, a factor putatively involved in hepacivirus binding and entry, and antibodies against this receptor inhibited infection of bovine cells with BVDV-1 (3).

Genome Structure

The pestivirus genome consists of a single-stranded RNA of about 12.3 kb in length (71,126,137,139,432,460). As discussed later, larger and smaller genome RNAs containing duplications, deletions, and other rearrangements have been found for most cytopathic pestiviruses. The long ORF of about 4,000 codons is flanked by a 5' NCR of 372 to 385 nucleotides, and a 3' NCR of 229 to 273 nucleotides (71,126). The 5' terminus does not appear to contain a cap structure (71,140). Construction of chimeric BVDV genomes containing 5' NCRs of HCV or EMCV revealed that two 5'-terminal stem-loop structures in the BVDV genome (domains Ia and Ib in Fig. 9) are important for efficient RNA replication, and that the minimal 5' cis-acting replication element consists of only the terminal tetranucleotide, 5'-GUAU (179). Pestivirus genome RNAs do not contain 3' poly(A) (126,432,458) but appear to terminate with a short poly(C) tract. The 3' NCR consists of a variable region near the end of the ORF, followed by a conserved region (140,804). Structural probing revealed that the conserved region forms two hairpins separated by a single-stranded region, and mutational analysis indicated that the terminal hairpin and single-stranded region contain important primary and secondary structural elements that probably function in cis to direct minus-strand initiation (804).

Translation and Proteolytic Processing

Cap-independent translational initiation of the pestivirus genome is mediated by a 5' IRES element that bears structural and functional similarity to the HCV IRES (see Fig. 9) (72,140,529,579). Structure–function studies have demonstrated that domain I is dispensable for IRES activity, that domains II and III contain structures critical for function, that the 3' end of the IRES may extend into the ORF, and that a pseudoknot formed from base-pairing of loop IIIf to a region just upstream of the translational start site is necessary for IRES activity (111,529,579). As for hepaciviruses, the pestivirus IRES has been shown to bind ribosomal 40S subunits independent from translation initiation factors eIF4A, eIF4B, and eIF4F (514,515,642). Pestivirus proteins are translated from genomic RNA as a single large polyprotein, which is processed into individual viral proteins (458,543,587). The current model of pestivirus polyprotein processing comes mainly from analysis of virus-infected cells (5,125,127,591) and expression of pestivirus polyproteins using the vaccinia

or baculovirus systems (348,518,590,591,693,771,779). Cleavage products have been localized in the polyprotein using region-specific antisera (125,656) and proteolytic processing sites within the polyprotein have been determined by protein sequencing (154,348,591,655,693,779). The order of the cleavage products in the BVDV-NADL polyprotein is NH_2-N^{pro}-C-E^{rns}-E1-E2-p7-NS2-NS3-NS4A-NS4B-NS5A-NS5B-COOH (Fig. 12) (125,127,439).

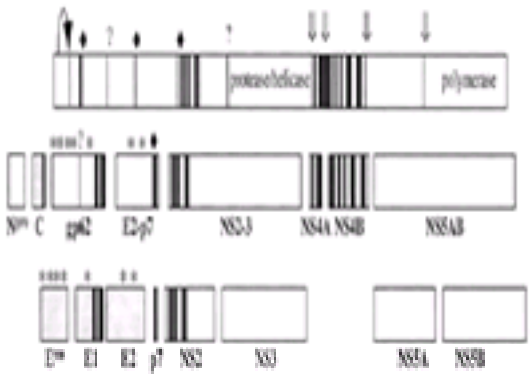

FIG. 12. Processing of the pestivirus polyprotein. *Shading* and *symbols* identifying proteolytic cleavages for the cpBVDV NADL strain are the same as those described in Figure 3, except that the proposed autocatalytic cleavage releasing the N-terminal nonstructural protein N^{pro} from the pestivirus polyprotein (see refs. 655 and 771) is indicated by a *curved arrow*.

Unlike other *Flaviviridae*, the first pestivirus protein encoded in the long ORF is a nonstructural protein, N^{pro}. This autoprotease is responsible for cleavage at the N^{pro}/C site (655,703,771). Processing in the pestivirus structural region appears to be mediated by at least two additional proteases. Although some of the cleavages are slightly delayed, host signal peptidase is believed to cleave at the C/E^{rns} and E1/E2 sites and the site generating the C terminus of E2 (591). The E^{rns}-E1 polyprotein (gp62) is converted slowly to the mature products by an unknown mechanism (591). E1 and E2 are believed to be anchored in the lipid bilayer via C-terminal membrane segments, and some E^{rns} remains associated with the virion via noncovalent interactions that have not been defined (591). A small hydrophobic protein, p7, is produced by signal peptidase, although incomplete processing at the E2/p7 site is observed leading to accumulation of uncleaved E2/p7 (154,242). The pestivirus NS3 protein contains the viral serine protease domain and is responsible for processing at four downstream nonstructural cleavage sites to generate NS4A, NS4B, NS5A, and NS5B (see Fig. 12) (693,771,779). Differences in NS2–3 processing are observed between pestivirus isolates (see ref. 42 and citations therein). Isolates of ncpBVDV do not process NS2-3, whereas those of BDV are able to cleave NS2–3 inefficiently, producing NS3. For CSFV and cpBVDV biotypes, NS2–3 cleavage is efficient but incomplete so that both NS2–3 and NS3 are observed. In the case of the cpBVDV biotypes, the cleavage generating the NS3 N-terminus is produced via several different mechanisms involving RNA recombinational events (detailed later). Enzymes responsible for cleavage at the NS2/3 site in the CSFV and BDV polyproteins have not been elucidated.

Features of Pestivirus Proteins

The N^{pro} Autoprotease

As mentioned earlier, the first protein in the pestivirus polyprotein, the N^{pro} autoprotease (655,771), is a nonstructural protein (703). This enzyme cleaves at its C-terminal sequence Cys¯Ser, which is conserved among pestiviruses (655). Site-directed mutagenesis has identified residues Glu-22, His-49, and Cys-69 as being important for catalysis, and it has been suggested that N^{pro} may be an unusual subtilisin-like cysteine protease (589). Autoproteolysis is the only known function of N^{pro}, and cellular ubiquitin, which directs appropriate C-terminal cleavage by ubiquitin C-terminal hydrolase, can functionally substitute for N^{pro} in replication-competent genomes (43,694,710). Furthermore, N^{pro} is dispensable for autonomous RNA replication in engineered and spontaneously derived subgenomes (43,467,694,710).

Pestivirus Structural Proteins

N^{pro} is followed by the virion nucleocapsid protein C, a conserved, highly basic, 14-kd polypeptide consisting of 21% Lys with a net charge of +12. The C terminus of the virion C protein has not been defined, and it is unknown if it retains the hydrophobic segment postulated to serve as the signal sequence initiating translocation of E^{rns} into the ER lumen.

The E^{rns} glycoprotein (gp44/48, formerly known as E0), is heavily glycosylated at seven to nine potential N-linked glycosylation sites, and it forms disulfide-linked homodimers (703). This protein does not contain a potential membrane-spanning domain and is found noncovalently associated with released virus particles or secreted into the culture medium (591,746,748). Recombinant E^{rns} binds strongly to the surface of cells, probably via interaction with glycosaminoglycans, and it can inhibit infection in a virus-specific manner (291,748). Interestingly, E^{rns} has been shown to possess an unusual ribonuclease activity with specificity for uridine residues (276,620,767). Glycosylation and dimer formation are not required for this activity (767). Although the function of E^{rns} ribonuclease activity is not yet clear, it appears to be important for some aspect of the virus life cycle. Antibodies that inhibit ribonuclease activity also tend to neutralize virus infectivity (767), and mutations in E^{rns} that destroy ribonuclease activity give rise to viruses that may be more cytopathic in culture but are attenuated *in vivo* (278,435). In addition, recombinant E^{rns} appears to be toxic to lymphocytes *in vitro* (76), perhaps contributing to the marked leukopenia seen in natural infections (668). Although cytotoxicity is a feature of other soluble ribonucleases (reviewed in ref. 612), it is unclear whether the ribonuclease

activity of E^{rns} is related to its toxicity.

Both E1 (gp33) and E2 (gp55) are predicted to be integral membrane proteins and contain two to three, and four to six N-linked glycosylation sites, respectively (see ref. 747). E1 and E2 are associated as disulfide-linked heterodimers that form slowly (591); E2 is also present in homodimers (703,747). As mentioned before, recombinant CSFV E2 can bind to cells and block infection of CSFV and BVDV, suggesting that a common E2 receptor is utilized by these pestiviruses for binding and entry (277). Although the precise roles of the viral glycoproteins in virus assembly and entry remain to be defined, monoclonal antibodies to E^{rns} (746) or E2 (145,506,723,747,758) can neutralize virus infectivity, and both antigens can elicit protective immunity (279,590,725).

Pestivirus Nonstructural Proteins

Following the virion structural proteins, the first nonstructural protein is p7, and it consists of a central charged region separating hydrophobic termini (154). The role of this small protein is unknown, but it appears to be required for production of infectious virus (242) but not RNA replication (43). Like the hepacivirus protein, pestivirus p7 is inefficiently cleaved from E2, probably via signal peptidase (154,379). Uncleaved E2–p7 is not required for replication in cell culture (242) and both E2–p7 and p7 appear to remain cell associated and do not copurify with virus particles (154).

The NS2 protein (about 54 kd) is present as the N-terminal portion of NS2–3 (about 125 kd) and is found as a mature cleavage product only for some cpBVDV strains (ref. 441 and citations therein). As detailed later, NS2/3 cleavage in cpBVDV can be regulated by several alterations in the NS2 coding region, including genome rearrangements and insertion of cellular sequences. The precise role of NS2 in NS2–3 processing is unclear, and the function of NS2 is largely unknown. Reconstruction of DI-like subgenomes that lack NS2 indicate that it is dispensable for autonomous RNA replication (43,467,694). However, a correlation between the efficiency of NS2–3 cleavage and the levels of RNA replication has been noted (347,430). NS2 also appears to contain a segment with homology to zinc-finger motifs present in some DNA binding proteins (136).

As for all members of the *Flaviviridae*, the pestivirus NS3 contains an N-terminal serine protease domain (34,91,198,772) followed by motifs characteristic of RNA helicases (199). Uncleaved NS2–3 must be capable of functioning in pestivirus replication, as this protein is not processed in cells infected with ncpBVDV strains. NS3 protease activity (and probably NS2–3 protease activity) requires NS4A as a protein cofactor (779). From cleavage site mapping and comparative analysis, the serine protease was shown to cleave between Leu and small uncharged amino acids L^{-}(S/A/N) (693,779). Most substitutions of the serine nucleophile eliminate processing at these sites (771,779) and destroy virus infectivity (779). Surprisingly, however, enzymatic activity was retained in mutants containing threonine at this position (695). Additional mutations that disable serine protease activity confirm its essential role in virus viability (207). The NS3 protein of BVDV has been purified and shown to possess RNA helicase (743) and RNA-stimulated NTPase (680) activities. Site-directed mutagenesis of the conserved helicase and NTPase motifs inhibited or destroyed these activities and viral replication coordinately (207,217).

The hydrophobic NS4A and NS4B proteins are similar in size, composition, and hydropathic properties to the NS4A and NS4B proteins of other family members. The only known function of these proteins is the serine protease cofactor activity of NS4A (771,779). As for HCV, cofactor activity involves interaction of a central domain of NS4A with the N-terminal region of NS3 (695).

The remaining two proteins, NS5A (about 58 kd) and NS5B (about 75 kd), are present as mature cleavage products as well as an uncleaved form, NS5AB (about 133 kd) (125,127). Neither protein contains the motifs postulated to be involved in methyltransferase or RNA triphosphatase activities, consistent with the lack of a 5' RNA cap structure on pestivirus genomes. Little is known about the function of NS5A. This protein was found to be phosphorylated by a cellular serine/threonine kinase with properties similar to enzymes that phosphorylate flavivirus NS5 and hepacivirus NS5A (561). NS5B contains motifs characteristic of RdRPs (124,126). The RNA polymerase activity of recombinant NS5B has been characterized *in vitro* and found to extend template-primed RNAs into double-stranded “copy-back” products (355,398,657,812) or to catalyze *de novo* initiation from short synthetic RNA or DNA templates (308,355). Polymerase activity is particularly responsive to GTP concentration, most likely because of a strong preference for initiation with guanylate (308,398).

RNA Replication

Analysis of pestivirus RNA replication is still at an early stage, although this process appears to be similar to that of flaviviruses. Minus- and plus-strand RNAs have been detected from 4 to 6 hours after infection, followed by the asymmetric accumulation of additional minus- and excess plus-strand RNAs (196). Accumulation of genome-length intracellular pestivirus RNAs, which co-migrate with virion RNA, generally follows the time course of infectious virus release, maximal virus titers being achieved about 12 to 24 hours after infection (196,458,542). Double-stranded RF RNAs and partial duplex RI RNAs, containing about six nascent strands, have been tentatively identified (195,196,542). The role of these RNA isoforms in replication remains to be elucidated.

As described later, there is good evidence that nonhomologous recombination can occur within pestivirus RNAs, and between pestivirus RNAs and host cellular RNAs (reviewed in ref. 441). Although the details of these recombination events are unknown, a likely mechanism is via copy-choice template recruitment during minus-strand synthesis, which is consistent with the coding orientation of cellular inserts. Given the frequency of pestivirus–cellular RNA recombinants compared to other positive-strand viruses, this is an interesting and potentially unusual characteristic of the pestivirus replicase.

Assembly and Release of Virus Particles

Other than the features of the virion structural proteins described before, little information is available on the assembly

and release of pestiviruses from infected cells. Pestivirus structural proteins are not found at the plasma membrane (209,748). Electron microscopic examination of virus-infected cells (46,208) suggests that pestiviruses mature in intracellular vesicles and are released by exocytosis. Formation of infectious BVDV-1 particles can be blocked by ER a-glucosidase inhibitors, presumably via misfolding of the viral envelope glycoproteins (816). A substantial fraction of infectious virus remains cell associated and some can be released from infected cells by successive freeze-thaw cycles (361,458). Interestingly, E^{rns} and E2 have been immunolocalized on isolated virus particles by electron microscopy, but E2 was not seen in particles undergoing secretion (or perhaps reattachment) at the cell surface (748). This suggests that E2 may be conformationally inaccessible to antibodies prior to a delayed maturation process.

Pathogenesis of MD and the Generation of Cytopathogenic Pestiviruses via RNA Recombination

Mucosal disease is the most severe outcome of BVDV infection and is usually fatal (reviewed in refs. 23,74,75,441, and 702). This disease can occur when a fetus is infected *in utero* with an ncpBVDV strain. If infection with ncpBVDV occurs between 80 and 100 days of gestation, animals may become tolerized to BVDV antigens and remain persistently infected for life. In the case of a persistently infected animal exhibiting MD, both cp and ncp biotypes of BVDV can be isolated (427). The close serologic relatedness of ncp–cp pairs isolated from an MD-affected animal led to the suggestion that cpBVDV might arise from ncpBVDV by a rare mutational event. Molecular characterization of a number of these ncp–cp pairs has verified this hypothesis and led to the remarkable discovery that some cpBVDV biotypes are generated via RNA recombination (211,434,438,439,698). In every case studied thus far, these rare events led to the production of NS3 (in addition to NS2–3), which is thought to be responsible for cpBVDV cytopathogenicity in cell culture and the pathogenesis of MD in the immunotolerant animal. Although less well characterized, a similar mucosal syndrome correlating with viral cytopathogenicity exists for BDV. Cytopathic CSFV strains have been described but do not seem to correlate with a particular disease state.

Sequence analysis of several independent cpBVDV isolates has revealed some common genome rearrangements resulting from RNA recombination (reviewed in ref. 441), and a few representative genome structures are illustrated in Figure 13. In the case of the Osloss and NADL strains, an in-frame insertion of cellular sequences is found in the NS2 gene (434,438). The Osloss insertion encodes a host ubiquitin monomer, which presumably directs cleavage at the ubiquitin/NS3 junction by the cellular enzyme ubiquitin C-terminal hydrolase. This role for the ubiquitin insertion has been confirmed for another cpBVDV with a similar genome rearrangement (697). The host sequence found in the NADL strain corresponds to a 270-nt portion of a bovine mRNA whose gene product has not been characterized (434). Deletion of this insertion from a cloned BVDV-NADL genome gave rise to a virus that did not produce NS3, was noncytopathic in culture, and exhibited reduced levels of RNA replication (430). Similar insertions of this same host gene have also been found to correlate with NS3 production and cytopathogenicity in two cpBDV isolates (37). Interestingly, a much smaller insertion is found in the NS2 gene of cpBVDV strain CP7, which contains a 27-nt duplication from an upstream region of the NS2 gene in an alternate reading frame (696). This insertion also leads to the production of NS3 (696) and a virus that is cytopathic in culture (437).

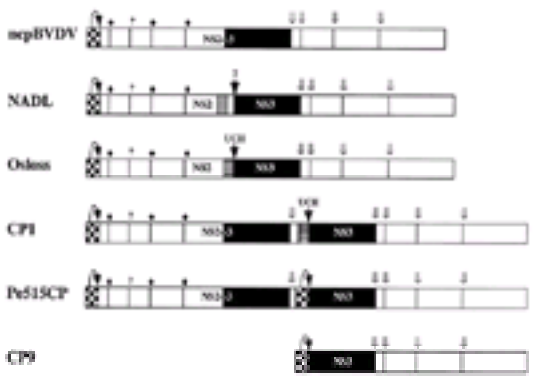

FIG. 13. Genome rearrangements associated with the generation of cytopathogenic bovine viral diarrhea virus (cpBVDV). The *top* diagram indicates the polyprotein of a typical ncpBVDV isolate. *Below*, the polyproteins encoded by five different cpBVDV isolates generated by RNA recombination are shown: NADL (432,433), Osloss (433), CP1 (438), Pe515CP (439), and CP9 (698). For all of the cpBVDV isolates, these polyprotein structures allow the production of both NS2-3 and NS3. In-frame insertions of host sequences are present in NADL (*shaded region*), Osloss (*striped region*), and CP1 (*striped region*). The enzyme responsible for NS3 production in the NADL strain is unknown, but the inserted ubiquitin sequences in Osloss and CP1 provide sites for processing by host ubiquitin C-terminal hydrolase (UCH) (697). For Pe515CP and the CP9 defective-interfering (DI) RNA, the N^{pro} autoprotease (*checkered box*) mediates the cleavage producing the NS3 N-terminus. The nomenclature and organization of the cleavage products and the symbols for the normal processing enzymes are defined in Figure 3 and Figure 10. The location of NS3 *(filled region*) is emphasized.

The CP1 and Pe515 cpBVDV strains contain large duplications encompassing the NS3 coding region and insertions of either ubiquitin sequences (438,439) or a duplicated copy of the N^{pro} autoprotease (439), respectively. Genome rearrangements with duplicated viral sequences flanking ubiquitin insertion sites have also been noted (545), which probably result from multiple independent recombination events (441). Additional cellular gene insertions have also been found in the context of genomic duplications, and they correlate with NS3 production and cytopathogenicity. These include the SMT3B ubiquitin homolog (544), a ribosomal S27a–ubiquitin gene fusion (41), and light chain 3 of microtubule-associated proteins 1A and 1B (436).

Several cpBVDV-1, cpBVDV-2, and cpCSFV strains consist of a paired DI particle and an ncp helper virus (38,350,440,698,710). An example of a cpBVDV-1 DI is represented by CP9, which contains a precise deletion of the C-E^{rns}-E1-E2-p7-NS2 genes resulting in an in-frame fusion of N^{pro} and NS3 (698). Such subgenomes are capable of

autonomous replication, express NS3, and induce cytopathic effects within cells (43,467,694). Pestivirus DIs interfere with the replication of helper viruses, which presumably provide packaging functions in trans.

Despite myriad ways that cp pestiviruses can be generated via RNA recombination, a few cpBVDV strains have been described that lack known genome alterations (136,210,512,545). For one such cp strain, BVDV-Oregon, NS2–3 cleavage was shown to be dependent on sequence information contained within NS2 and the first 66 amino acids of NS3 (348). Introduction of this NS2 gene into a heterologous ncpBVDV-1 genome led to NS2–3 processing and cytopathogenicity (347). Surprisingly, a relatively few amino acid changes in NS2 could account for this difference in cleavage efficiency. Nevertheless, due to the lack of an ncp counterpart, it can only be surmised that this strain had been derived by accumulated point mutations in the NS2 region.

Clearly, the unifying feature of cp pestiviruses is the production of NS3. As stated before, except for rearrangements that juxtapose ubiquitin or N^{pro} with NS3, the identity of the protease(s) responsible for NS2–3 cleavage in other cpBVDV strains, as well as in ncpCSFV and ncpBDV, is unknown. Involvement of the NS3 serine protease activity in NS2–3 cleavage has been excluded via mutagenesis or deletion in several cpBVDV polyproteins (348,436,696,779). One possibility is that foreign gene insertions in NS2 might signal a cellular protease to cleave at the NS2/3 junction, perhaps by inducing a conformation within NS2 similar to the NS2 proteins from ncpBDV, ncpCSFV, or BVDV-Oregon, which all cleave NS2–3 despite the absence of genome alterations. Alternatively, such changes might activate a latent or cryptic protease activity encoded by NS2. This could explain how the BVDV-Oregon NS2 gene is sufficient to direct NS2–3 cleavage, as well as explain NS2–3 cleavage in ncpCSFV and ncpBDV despite the absence of genome rearrangements. Further, in this regard, HCV and GBV-C (see the next section, The GB Viruses) NS2 proteins contain the catalytic residues of NS2–3 autoproteases, although there is scant homology to pestiviruses in this region. Thus if pestivirus NS2 does perform a catalytic role in NS2–3 cleavage, the mechanism is likely to be different from that of these other viruses. Regardless of the mechanistic details, it is interesting to note that pestiviruses differ among themselves and from other family members in NS2–3 cleavage efficiency. Perhaps inefficient cleavage may reflect a recent evolutionary adaptation, producing viruses that are less prone to cause disease but nevertheless successfully spread via congenital routes in domesticated animal herds.

Whereas there is a strong correlation between NS3 production and cytopathogenicity of cpBVDV, ncp strains of BDV and CSFV produce both NS3 and uncleaved NS2–3. This raises the issue of how NS2–3 cleavage leads to a cytopathic effect. One possibility is that NS3 may be directly cytopathic, perhaps involving the serine protease activity. Pestivirus cytopathology proceeds via apoptosis (264,621,809), a cell death pathway involving cellular protease effectors (reviewed in ref. 324). According to this model, the relative abundance of NS3 would determine its effect within a host cell. In this regard, cpBDV and cpCSFV seem to express increased amounts of NS3 (37,42,440). Alternatively, it has been noted that cpBVDV exhibits an increased efficiency of RNA replication compared with paired ncp viruses (347,430). Thus, NS2–3 cleavage might directly result in enhanced RNA replication, and a byproduct of this process could be responsible for cytopathogenicity. In this regard, enhanced levels of RI and RF RNA forms could activate the double-stranded RNA-activated kinase PKR, a known inducer of apoptosis (24,141,652). A larger question is how cytopathic effects on the cellular level lead to MD in infected animals. Increased cell death could directly contribute to tissue injury and induce inflammation. There is also evidence that animals with MD show increased numbers of infected cells (375), perhaps because of increased replication of cpBVDV. In addition, cpBVDV might exhibit differences in tropism that could contribute to disease (375).

THE GB VIRUSES

Discovery, Distribution, and Origin

In the early 1990s, a residual number of hepatitis cases were still not attributable to HAV, HBV, HCV, or the recently described hepatitis E virus (Chapter 89). Efforts aimed at identifying additional agents of hepatitis revealed three novel viruses that have been tentatively assigned to the *Flaviviridae*. Two of these viruses, GBV-A and GBV-B, were cloned via subtractive representational methods from the serum of a tamarin experimentally infected with a hepatitis agent originally derived from a human patient, GB (641). Although some human cases of non-A–E hepatitis showed serologic reactivity to GBV-A and GBV-B, RT-PCR analysis from these patients failed to detect either virus. Rather, a third related virus, GBV-C, was subsequently identified from one such patient (365,640). Cloning of novel viruses associated with non-A, non-B hepatitis by immunoscreening of a cDNA library identified an agent, initially termed hepatitis G virus (HGV), which turned out to be an independent isolate of GBV-C (386).

Based on sequence relatedness and overall genome structure, GBVs have been categorized within the *Flaviviridae*, although they remain unclassified at the genus level. GB viruses are most similar to HCV but phylogenetically distant enough to resist classification as hepaciviruses (see Fig. 1) (78,582). This is especially true for GBV-A and GBV-C, which share a number of unique features that distinguish them from other members of the family.

The inability to detect GBV-A or GBV-B in human samples led to investigations into their origins. Interestingly, GBV-A has been detected in several species of New World monkeys in the absence of experimental infection or overt disease (78,100,364,615). Viral sequences isolated from within a single primate species were highly related, whereas sequences isolated from separate species showed greater divergence, indicating that GBV-As are indigenous monkey viruses that have adapted to their hosts over extended periods of time (78,100,364,615). These results, and observations made during early cross-challenge experiments, provide support for the view that the original GBV-A isolate may have been acquired during passage of the GB agent in tamarins (78,502). The distribution of GBV-B in nature is unknown, as the only source of this virus is the original tamarin-passaged GB serum used to identify GBV-A and GBV-B.

Since its initial discovery, GBV-C has been found to be surprisingly common, with viral RNA detected in about 1% to 4% of healthy human volunteer blood donors (168,421,654,775). Phylogenetic analysis of GBV-C sequences has been complicated by an apparent bias against synonymous substitution in some parts of the genome, leading to differences in inferred relationships depending on the subgenomic regions under comparison (309,521,728). The molecular basis for

this bias is unclear; it may involve evolutionary constraints imposed by RNA secondary structures (637) or cryptic ORFs (507,643). Nevertheless, standardization of results has been achieved through the use of an appropriate molecular timepiece, such as the 5' NCR, the E2 gene, or complete genome sequences. These results indicate that variation among GBV-C isolates occurs in distinct genotypes that reflect their geographical distribution (183,387,469,643,644). GBV-C RNA has also been detected in the serum of wild chimpanzees (1) and chimpanzees infected with a putative hepatitis agent (48). Chimpanzee-infecting GBV-Cs were found to be more closely related to each other than to human-infecting GBV-Cs, suggesting coevolution of these viruses with their hosts (1,48,100).

Genome Structure and Expression

Complete or nearly complete genome sequences have been determined for a number of GBVs, and functional full-length infectious clones have been assembled for GBV-B (80) and GBV-C (777). Like the other family members, the GBV genomes encode a single long ORF containing structural protein genes [(C)-E1-E2] in the 5' one third, followed by nonstructural protein genes (NS2-NS3-NS4A-NS4B-NS5A-NS45B) in the remainder (365,468). The 5' NCR of GBV-B contains 445 nucleotides, which is about 30% larger than the HCV 5' NCR, but it has significant similarity in primary and presumed secondary structure to the HCV 5' NCR (see Fig. 11) (268,468). In contrast, GBV-A and GBV-C have 5' NCRs of 508 to 593 nucleotides that share about 50% identity to each other and can be folded into similar structures (see Fig. 11), but that do not resemble the 5' NCRs of HCV or GBV-B (639). The long 5' NCRs of all three GBVs contain multiple initiator AUG codons, and initiation of polyprotein translation is thought to be dependent on IRES activities, which have been demonstrated for all three GBV 5' NCRs (201,577,639). The GBV-B 3' NCR is 361 nucleotides in length, containing a short poly(U) stretch 30 nucleotides downstream of the stop codon, followed by 309 nucleotides of unique sequence (80,609). Although this region of the GBV-B genome does not contain sequence homology with HCV, the 3' 47 nucleotides of GBV-B is predicted to fold into a structure very similar to SL-I at the 3' end of the HCV genome (80,609). The 3' NCRs of GBV-A and GBV-C are highly conserved only within these virus groups, although a short 17-nucleotide stretch is also well conserved among all GBVs (609).

The relationships among GBVs and between HCV are also reflected in the organization of structural proteins. Like HCV, GBV-B encodes a basic capsid protein followed by two envelope glycoproteins. The genomes for GBV-A and GBV-C also contain E1 and E2 glycoproteins, but they lack any obvious capsid-like protein (386,468). *In vitro* translation of RNAs containing the GBV-A or GBV-C 5' NCR localized the translational start site to a conserved AUG immediately upstream of the E1 coding region (639). However, it has been observed that GBV-C-infected humans generate antibody responses against a small basic peptide that can be translated from an in-frame upstream AUG, suggesting that such a protein is expressed *in vivo* (777). Alternative explanations for the lack of a capsid-like protein include the possibilities that GBV-A and GBV-C might usurp a capsid-like protein of the host cell or a co-infecting virus, or that additional GBV proteins may be involved. In this regard, a region of the GBV-C NS5A gene exhibiting a bias against synonymous substitution has been noted to potentially encode a small basic protein (10 kd, pI 11.5) in an alternate reading frame (507). Furthermore, it remains possible that GBV-A and GBV-C virions might lack a distinct nucleocapsid. However, biophysical characterization of GBV-C particles indicate that GBV-C RNA can be found in low-density (1.07 g/mL) and intermediate-density (about 1.18 g/mL) fractions on a variety of density gradients (428,605,777). Pretreatment with detergents or organic solvents to remove membranes shifts the peak of viral RNA to a higher-density form that may represent nucleocapsids that have recently been visualized by electron microscopy (776).

The nonstructural proteins of GBVs show the greatest similarity to HCV, and the boundaries of cognate NS2, NS3, NS4A, NS4B, NS5A, and NS5B proteins have been proposed (365,468). Catalytic residues of the HCV NS2-3 autoprotease are conserved among GBV NS2 proteins (365,468), and this enzymatic activity has been described for GBV-C (45). Similarly, active sites of the HCV NS3 serine protease are retained in NS3 of GBVs. Biochemical characterization of the GBV-B serine protease activity indicates that it shares substrate specificity with the HCV enzyme and requires the virus-specific cofactor NS4A (83,610). The C-terminal region of GBV NS3 proteins retain the similarity to supergroup 2 RNA helicases that is common to the *Flaviviridae* (365,468), and NTP-dependent RNA unwinding activity has been demonstrated for NS3 proteins of GBV-B and GBV-C (229,813). NS5B contains similarity to the supergroup 2 RNA polymerases (365,468,641).

Association with Disease?

GBV-A has not been shown to cause disease in nonhuman primates, whereas GBV-B causes hepatitis in experimentally infected tamarins (80,614). As described before, although both of these viruses were thought to be derived from a source of human hepatitis, it appears that GBV-A had been acquired during primate passage and GBV-B has been isolated only from tamarin-passaged GB material. Attempts to identify a GBV in the original GB clinical sample have failed, most likely because of degradation during storage (614).

Human infection with GBV-C is well documented, although direct association of this virus with human disease has proven to be elusive (reviewed in ref. 638). Epidemiologic evidence suggests that it is primarily blood-borne, although other modes of transmission appear possible (105,169,240,624,654). These risk factors overlap with those of HBV and HCV, and the frequency of co-infections with GBV-C and other viruses have complicated etiologic determinations (13,386). It appears that a majority of GBV-C infections are subclinical, with only mild symptoms that typically resolve with the appearance of anti-E2 antibody (143,226,368,369,672,673). Viral persistence seems to occur in about 5% to 10% of GBV-C infections (226,421). A few studies have implicated GBV-C in acute and chronic non-A–E hepatitis (168), fulminant hepatitis (679,801,802), or other liver disease (129). However, recent work suggests that GBV-C is a lymphotropic virus (718). Clearly, more work needs to be done to establish the clinical significance of GBV-C and related viruses.

SUMMARY AND QUESTIONS

Although much has been learned about the general life cycle of the *Flaviviridae*, it is quite complex, and large gaps in our knowledge exist for every step. The ongoing development of improved genetic and biochemical tools to study these viruses will certainly enable a more complete picture of their biology. Recent progress has been made in identifying host

cell surface molecules that could be involved in binding and entry of viruses, although the details of such interactions, and how these control virus tropism and infection *in vivo*, is largely unknown. The general strategies of genome translation and polyprotein processing have been elucidated. Yet details such as the proteolytic processing mechanism of the flavivirus NS1-2A or pestivirus NS2-3 polypeptides need to be resolved. More effort is needed to understand the functional significance of polyprotein processing events for RNA replication, virus–host interaction, and virion formation. Furthermore, it is not understood how the competing processes of genome translation and genome replication are regulated for this virus family. New insights have emerged regarding the enzymology of some viral nonstructural proteins, as well as the identity of a few host proteins that most likely contribute to genome replication. However, the role of several nonstructural proteins in this process are unknown. More description is needed for how all these components, together with viral RNA, combine to produce a functional replicase. As for all positive-strand RNA viruses, the role of membranes in the process of RNA replication remains a mystery. It is interesting that phosphorylation of polypeptides upstream of the polymerase seems to be a conserved feature within the family. Identification of the relevant kinase(s) should allow the significance of this posttranslational modification to be addressed. Nearly all of what we know about virus–cell interaction for HCV has come from the study of heterologous systems, which have suggested important roles for HCV-specific gene products in interferon sensitivity and the development of HCC. The emergence of improved systems for studying HCV in culture will permit all of this work to be reevaluated, and moreover, it will undoubtedly reveal unexpected and novel aspects of HCV biology. Our understanding of virion formation is still at an early stage, and it is not yet clear how structural proteins combine to form nascent virions, where this process occurs, and whether packaging is temporally regulated within the replication cycle. The lack of obvious capsid proteins for GBV-A and GBV-C raises the questions of whether these virions actually contain nucleocapsids and, if so, what the identity of the capsid proteins is. It is fascinating that pestiviruses encode a ribonuclease within the extracellular domain of an envelope glycoprotein, although the biologic role of this activity remains to be clarified. Also, what is the role of the secreted flavivirus NS1 protein? Why does production of NS3 by cpBVDV correlate with cytopathogenicity and fatal MD? Are GBVs involved in human disease? What mechanisms are involved in the establishment and maintenance of chronic infections by pestiviruses and HCVs? Answers to these and other pressing questions should reveal the unique aspects to the replication of this evolutionarily distinct family of viruses. This will provide information useful for the development of effective immunization and therapeutic strategies to control diseases caused by these diverse and important pathogens. Clearly, the most exciting period of research into the *Flaviviridae* lies ahead.

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CHAPTER 33 Flaviviruses

Donald S. Burke and Thomas P. Monath

The genus *Flavivirus* is composed of about 73 viruses (Table 1). Of these viruses, 34 are mosquito borne, 17 are tick borne, and 22 are zoonotic agents transmitted with no known vector, often transferred between rodents or bats (Fig. 1).

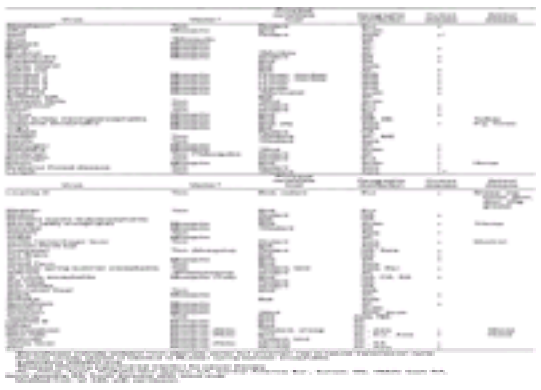

TABLE 1. *The Flaviviridae (arboviruses): vector–host relationships and disease associations*

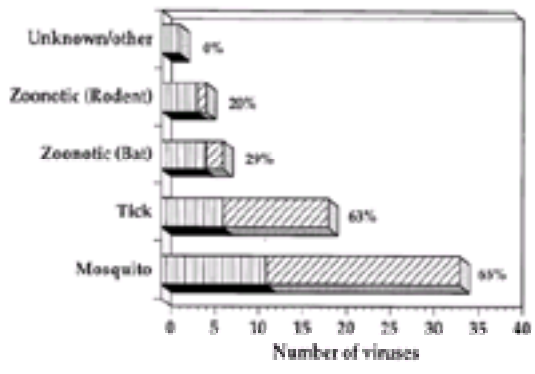

FIG. 1. Number of recognized flaviviruses, by mode of transmission. The number of viruses (*cross-hatched column*) and proportion (%) causing human disease in each transmission category are shown. Most viruses are mosquito or tick borne, and more than 60% of these agents cause human illness.

Forty species of the flavivirus family have been associated with human disease. Twenty-two of the 34 (65%) mosquito-borne viruses and 13 of 17 (107) of the tick-borne viruses can cause disease in humans. Only 5 of the 22 (23%) viruses with no known vector cause human disease. Dengue, yellow fever, the tick-borne encephalitis (TBE) complex, and Japanese encephalitis are the most important viral arboviruses in the world and cause extensive morbidity and mortality across every continent.

HISTORY

Yellow fever virus is the type virus of the family (hence the name, derived from *flavus,* Latin for “yellow”). It was the first filterable agent shown to cause a human disease and the first virus proved to be transmissible by an arthropod vector. Yellow fever virus was the first flavivirus to be isolated (in 1927) and cultivated *in vitro* (in 1932). During the first decade of this century, dengue virus was also shown to be a filterable virus transmitted by arthropods, but it was not isolated until 1943.

A number of diseases characterized by meningoencephalitis were recognized as nosologic entities during the 19th and 20th centuries and later proved to be caused by flaviviruses. Among these are louping ill (a disease of sheep, recognized in Scotland since 1807), JE (Japan, 1873), and Australian X disease (now known as Murray Valley encephalitis [MVE], Australia, 1917). Between 1931 and 1937, the viruses responsible for louping ill, St. Louis encephalitis (SLE), JE, and TBE were isolated. Common features of these viruses included neurotropism and arthropod transmission, but initially they were believed to be unrelated agents. In the late 1930s and early 1940s, relationships were demonstrated by neutralization and complement fixation tests between JE, SLE, and West Nile (WN) viruses. With the advent of the hemagglutination-inhibition (HI) test, which defined the broadest spectrum of antigenic relatedness, Casals and Brown (101) were able to separate the flaviviruses (group B arboviruses) from the alphaviruses (group A viruses) and to define the cross-reactions among a set of 10 flaviviruses. As the flavivirus family grew with continued isolations of new agents from wild vertebrates and arthropods, so did the complexity of their serologic classification (100). Thirteen new flaviviruses were isolated for the first time during the 1970s, and one new virus has been recovered since 1980.

Notwithstanding the lack of a serologic relationship, the group A and B arboviruses were originally linked on the basis of their mode of transmission and physicochemical characteristics into a single family, the family *Togaviridae,* encompassing small RNA viruses with lipid envelopes and cubic nucleocapsid symmetry. As knowledge of the morphogenesis, biochemistry, and replication strategy of the flaviviruses expanded, it was clear by 1984 that their differences when compared with other togaviruses were sufficiently great to place them in a separate family (764). Only members of the genus Flavivirus are discussed in the chapter; the Pestiviruses, Hepaciviruses, and other newly recognized *Flaviviridae*-like viruses (91) are not.

INFECTIOUS AGENTS

Physical and Chemical Properties

Flavivirus morphology, morphogenesis, protein composition, and genome structure are described in detail in Chapter 32 (see also 601). Here we focus on the characteristics of viruses that relate either to interactions with the environment or to laboratory manipulations in antigen and vaccine preparation. In the next section, the chemical and physical properties of gene products interacting with antibody and immunologically specified cells are described.

Flavivirus virions consist of a spherical ribonucleoprotein core surrounded by a lipoprotein envelope with small surface projections. The projections seen in electron micrographs are clarified by x-ray crystallography and represent molecules of envelope glycoprotein, which form rodlike structures anchored to the viral membrane at their basal ends. Envelope lipids constitute about 17% of the virion dry weight (718) and are derived from the host cell lipids.

Mature flavivirus virions contain three structural proteins: a nucleocapsid or core protein (C; 12 kd), a nonglycosylated membrane protein (M; 8 kd), and an envelope protein (E; 53 kd), which is usually glycosylated (718). The M and E proteins are both associated with the lipid envelope by means of hydrophobic membrane anchors. The E protein is the major component of the virion surface; it contains the important antigenic determinants subserving HI and neutralization and thus induces immunologic responses in the infected host. Structural elements of the E protein determinants are assumed to be involved in the binding of virions to cell receptors and in intraendosomal fusion at low pH.

Detergents and proteases have been used to characterize the structure of flaviviruses and to isolate immunologically reactive subunits (695,718). Nonionic detergents, such as Triton X, solubilize the entire envelope, releasing M and E proteins; whereas sodium deoxycholate appears to remove only E, leaving M associated with the nucleocapsid. Protease treatment showed that a portion of the E glycoprotein is located within the lipid bilayer (285). Analysis of the primary structure of the flavivirus glycoprotein has confirmed the presence of a hydrophobic membrane anchor region at the carboxyl terminus of the E protein molecule (535).

The flaviviral envelope protects the genome from cellular nucleases, and naked nucleocapsids released by detergent treatment are degraded by ribonuclease. Flavivirus infectivity and hemagglutinin are optimally stable at pH 8.4 to 8.8 (344). Sensitivity to acid pH (also to bile and proteolytic and lipolytic enzymes) generally precludes infection by the oral route. TBE may, however, be acquired by ingestion of infected milk (560). Flaviviruses are rapidly inactivated at high temperature. At 50°C, 50% of infectivity is lost in 10 minutes. As a practical measure, total inactivation of virus suspended in blood or other protein solutions occurs within 30 minutes at 56°C. Low temperatures preserve infectivity, with stability being greatest at –60°C or below. Aerosols present a hazard of laboratory infection (646). SLE virus is stable for 6 hours in aerosol suspension at room temperature and 23% to 80% humidity (344). For reasons that are not understood, aerosol laboratory infections with tick-borne flaviviruses are more frequent than with mosquito-borne viruses.

Flaviviruses are inactivated by ultraviolet light, gamma-irradiation, and disinfectants, including 3% to 8% formaldehyde,

2% glutaraldehyde, 2% to 3% hydrogen peroxide, 500- to 5,000-ppm available chlorine, alcohol, 1% iodine, and phenol iodophors. The tick-borne viruses appear to be relatively more resistant to these measures than mosquito-borne agents.

Antigenic Composition and Determinants

Antigenic Classification

All members of the genus *Flavivirus* share common antigenic sites, as revealed by HI tests with polyclonal immune sera, and this originally formed the basis for their classification (101). Neutralization tests (NT), on the other hand, are more discriminating and can be used to distinguish individual viruses in the genus and to define subgroups of closely related viruses.

On the basis of cross-neutralization using polyclonal hyperimmune antisera, the flaviviruses have been divided into a number of antigenic complexes. The original studies by de Madrid and Porterfield (150) classified 36 flaviviruses. A recent study classified 68 viruses into 14 clades within 8 antigenic complexes (Table 2). Eleven other viruses were not closely related enough to be included in any of the antigenic complexes (380). Because the envelope protein E is the viral hemagglutinin and the primary target for neutralizing antibodies (reviewed in 283), the cross-neutralization data reflect differences in the antigenic structure of this envelope protein and, together with the results of cross-HI tests, provide evidence for the presence of group-, serocomplex-, and type-specific determinants. This antigenic classification conforms to major biologic and epidemiologic characteristics of the flaviviruses (see Table 1).

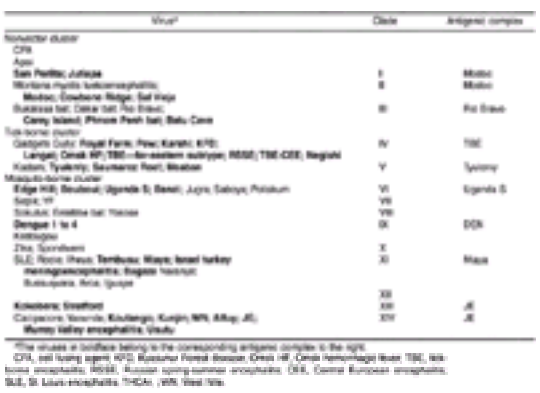

TABLE 2. *Assignment of flaviviruses to clusters and clades*

The complexity of the antigenic relationships among flaviviruses has further been emphasized by the application of protein E–specific monoclonal antibodies (reviewed in 284,291, and 614). These analyses have demonstrated the presence of flavivirus group-, serocomplex-, and type-specific antigenic determinants, generally confirming the relationships shown with polyclonal antisera. Monoclonal antibodies have also been found with subcomplex, subtype, strain, and even substrain specificity, allowing the further distinction of viruses at a level that has been difficult or impossible to demonstrate with polyclonal antisera (38,651). Thirteen different binding sites for monoclonal antibodies have been identified on the E protein of a dengue 2 strain (612).

Monoclonal antibodies have uncovered antigenic relationships at the epitope level that link different flavivirus antigenic complexes or that reveal previously unsuspected relationships between members of the same antigenic complex, thus complicating the simple concept of group-, complex-, and type-specific determinants. For example, among members of the dengue complex, subcomplex specificities were demonstrated for dengue 1 and 4 and for dengue 2 and 3 (294). Relationships were also revealed between dengue and members of other antigenic complexes (e.g., Tembusu virus). Moreover, monoclonal antibodies may show functional reactivity with heterologous, but not homologous, virus strains. For example, monoclonal antibodies prepared against yellow fever 17D virus neutralized the parental Asibi strain but not 17D (651). To complicate matters even further, binding and functional assays with the same monoclonal antibody may show completely different cross-reactivity patterns (283,291). These data are an apparent reflection of the complexity of antigenic determinants recognized by antibodies on the surface of the virion.

The complete or partial sequences of a number of flaviviruses have recently provided a classification based on sequence homologies and the construction of evolutionary trees (Fig. 2). The amino acid sequence comparisons of protein E, like those of other viral sequences (59,77,419), yield a picture that perfectly matches that of the flavivirus serocomplexes defined by cross-neutralization using polyclonal immune sera (see Table 2).

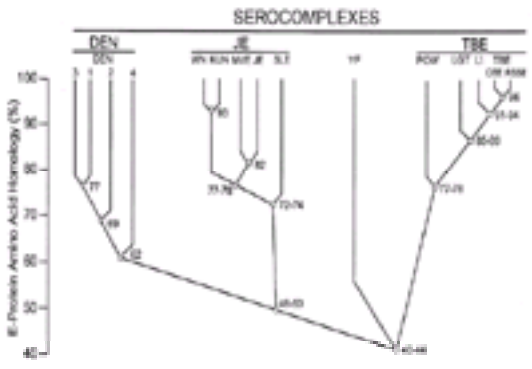

FIG. 2. Phylogenetic tree of the genus *Flavivirus,* using nucleotide sequence. The tree was constructed by the

neighbor-joining method. Each number at the nodes is the percentage of 500 bootstrap replicates supporting the relationships shown; *asterisk* indicates confidence probability higher than 90%. Vertical length is arbitrary. Scale is percentage of genetic distance. (From ref. 380, with permission.)

The TBE complex and yellow fever virus are most distantly related to each other and to the other flaviviruses, with a sequence homology of 40% to 44% (depending on the pair of viruses compared). There is a somewhat closer relationship between members of the JE serocomplex and the dengue viruses (46% to 53%). The different dengue types share 62% to 77% of their amino acids, with dengue 1 and dengue 3 being most closely related (77% homology), followed by dengue 2 (69%) and dengue 4 (62%). An extensive comparative sequence analysis of dengue 2 virus isolates has revealed a significant degree of diversity (up to 10% amino acid differences in protein E) and has allowed the identification of at least four genetic subtypes (419,604). Within the JE and TBE serocomplexes, the degree of nucleotide homology is at least 72% and 77%, respectively. As pointed out by Blok and colleagues (59), the viral proteins are very similar in topology.

The E protein is one of the most slowly evolving viral proteins (together with NS1, NS3, and NS5), indicating that immunologic pressure is not an important driving force in the evolution of flaviviruses.

Role of Structural and Nonstructural Proteins in Immune Response

The envelope protein E plays a dominant role in the generation of neutralizing antibodies and the induction of a protective immune response. This has been conclusively demonstrated by active immunization of animals with defined subviral components (291) and recombinant proteins (72,287,332,365,367,461,484,555,657) and by passive protection experiments with protein E–specific monoclonal antibodies (reviewed in 283 and 614). Protein E–specific nonneutralizing antibodies or neutralizing antibodies at subneutralizing concentrations, however, may also mediate the phenomenon of antibody-dependent enhancement (ADE) of infection, which has been implicated in the pathogenesis of dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS) (257).

Low-titer neutralizing activity and a significant degree of passive mouse protection has also been observed with monoclonal antibodies against the prM protein of dengue 3 and dengue 4 viruses (346). Mice immunized actively with prM/M also exhibit a degree of protection (70). The prM protein is part of immature virions, and its proteolytic cleavage, presumably by a cellular protease in the trans-Golgi network, generates mature virions. In certain instances, this precursor cleavage can apparently be incomplete, allowing the prM protein to function as an additional target for neutralizing and protective antibodies.

The nonstructural glycoprotein NS1 is expressed on the surface of infected cells (96,182,654,693) but is also secreted and, in this form, was previously designated as soluble complement fixing antigen (183,695). NS1 elicits an immune response in the course of flavivirus infections in humans and in experimental animals (227,385,783a). Its role as a protective antigen is discussed later. In the infected host, secreted forms of NS1 are found in the circulation.

Although passive protection in mice by a monoclonal antibody against the nonstructural protein NS3 of dengue 1 virus has been reported (704), the primary immunologic role of the nonstructural proteins, with the exception of NS1, appears to be their function as targets for cytotoxic T cells (Tc) (301,630).

The Antigenic Structure of Protein E

Protein E is the primary target for neutralization, and its antigenic structure has been extensively studied using both polyclonal and monoclonal antibodies (reviewed in 283,291, and 614). Because the location of disulfide bridges is also known (535), these data together have been used to generate structural models of protein E (94,289,361,448,613,615).

The x-ray structure of a soluble form of the TBE virus protein E has been determined (596). This soluble form was obtained by tryptic cleavage from purified virions (290) and lacked the carboxyl-terminal 80 to 90 amino acids including the membrane anchor. Because the cysteines forming all six disulfide bridges are absolutely conserved among flavivirus E proteins and a common 3D structure can be anticipated, the available data on other flavivirus epitopes will be discussed with reference to the TBE virus protein E.

Unlike the influenza hemagglutinin, this complex is a head-to-tail rather than a head-to-head oligomer. Thus, it apparently does not represent a spike protruding from the surface of the virion, but rather a 170-Å long rod that is probably anchored to the membrane at its distal ends. The side view shows that the dimer has a curved shape consistent with its attachment on the surface of a 500-Å particle. In each monomer, three structural entities are discernible, two of which are related to previously defined antigenic domains (compare Fig. 3**A** and 448). The carboxyl-terminal 100 amino acids (domain III in Fig. 3**A**) correspond to antigenic domain B and form a b-barrel composed of seven antiparallel b-strands resembling an immunoglobulin constant domain. This structure is connected by a flexible region to the central domain I, which is folded as an eight-stranded b-barrel with up-and-down topology. It includes the amino-terminal 50 amino acids as well as a sequence element that carries the single carbohydrate side chain and was previously referred to as antigenic domain C. Two long loops extending from this central part of the protein are primarily involved in dimer contacts (domain II in Fig. 3**A**) and correspond to antigenic domain A.

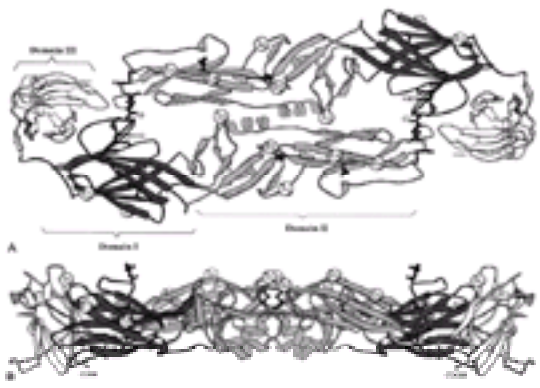

FIG. 3. Ribbon diagram representing the structure of the membrane anchor-free envelope (E) protein dimer isolated from tick-borne encephalitis virus. **A:** Top view. **B:** Side view. The positions of amino acid substitutions in monoclonal antibody escape mutants are indicated by *white circles* and designated according to Mandl and colleagues (448) and Holzmann and associates (311). Disulfide bridges and the single carbohydrate side chain (G) attached to domain I are shown as ball-and-stick representations.

Linear epitopes have been mapped using synthetic peptides and are found in areas of the glycoprotein predicted to be hydrophilic (325); however, the induction of neutralizing antibodies appears to be strongly dependent on the native conformation of protein E (761). Mutations leading to escape from neutralization have been mapped to each of the structural domains shown in Figure 3**A** (102,206,272,311,336,422,448), and the position of such amino acid substitutions in TBE virus mutants are indicated in Figure 3. Consistent with the suggested topology of the dimer on the virion surface, most of these mutations are located on the accessible upper side of the subunit, which also suggests that they indeed form part of the corresponding epitopes. In contrast, two of the mutations in TBE virus (B1 and B4) are not located on top but rather on the side of domain III, and may therefore alter the conformation rather than being part of the epitope itself. Interestingly, one of the mutations (designated O3) does not affect binding of the selecting monoclonal antibody to the virus but nevertheless abolishes its neutralizing activity. The scattered distribution of these neutralization-escape mutations over the entire subunit indicates that antibody binding to any of the structural domains can lead to virus neutralization. Taking into account the known sizes of Fab footprints on protein antigens, which are in the range of 500 to 900 $Å^2$ and involve 15 to 20 contact residues (147,676,752), most of the epitopes are expected to be discontinuous. This is consistent with the denaturation sensitivity of neutralization epitopes (761) and the finding that monoclonal antibodies usually do not react with synthetic peptides unless they recognize an epitope that is present on denatured protein E only (249,311,615).

Antigenic domain B (domain III in Fig. 3**A**) represents an independently folding domain that can be isolated as a trypsin-resistant fragment from purified virions (771) or generated as a recombinant protein by bacterial expression (458). In both cases, however, its reactivity with neutralizing monoclonal antibodies is dependent on the integrity of the single disulfide bridge located within this domain.

Because the dimeric subunit forms part of an as yet undefined lattice on the virion surface, it is likely that certain epitopes are composed of elements from different subunits. Although formal proof is lacking, some neutralization epitopes present on whole virions are lacking on the soluble dimer (290) and on the Triton X-100 solubilized protein. How the two-step process of rearrangement and trimerization of the E protein at low pH for the purpose of fusion alters the antigenic sites available for immune response is not clear (691). The first step is a reversible dissociation of the E dimers, followed by an irreversible formation of trimers involving the a-helical and conserved segments of the stem anchor region of the E protein.

The mechanism of antibody-mediated neutralization remains a matter of speculation. The functional sites of protein E have not yet been mapped, although it has been speculated that domain III may be involved in receptor binding (434) and that the highly conserved sequence from residues 98 to 111 at the tip of one of the domain II loops (see Fig. 3**A**) may be involved in fusion activity (616). Fusion activity requires conformational changes that affect several neutralization epitopes, primarily within the central domain I and domain II (292,616). These changes are apparently associated with a reorganization of the subunit interactions on the virion surface, with trimer contacts being favored in the low pH form, as opposed to dimer contacts in the native form (11). It can be assumed that the interference with these structural rearrangements by antibody binding represents one mechanism leading to virus neutralization.

HI and NT activity are not necessarily linked, and all possible combinations (HI+, NT+; HI–, NT+; HI+, NT) have been observed with monoclonal antibodies (reviewed in 283). Binding of certain monoclonal antibodies is enhanced in the presence of other antibodies (288,296). Such cooperative effects have been demonstrated in binding assays and also in neutralization and passive protection experiments, even with antibodies that are nonfunctional when tested alone.

Monoclonal antibodies have also been identified that do not neutralize the virus but are protective in passive immunization experiments (66,222). Nonneutralizing antibodies may even confer cross-protection between different flaviviruses. For example, a group-reactive nonneutralizing dengue antibody (4G2) provides efficient protection against yellow fever challenge (64). The ability of nonneutralizing antibodies to protect is not associated with complement-dependent lysis of infected cells, but a possible mechanism of protection is suggested by the finding that the protective antibodies inhibited virus replication in a neural cell line, although they showed no neutralization in Vero cells.

The Antigenic Structure of NS1

By the use of polyclonal and monoclonal antibodies, type-specific, subcomplex-specific, complex-specific, and flavivirus–cross-reactive epitopes have been identified (181,381), and a detailed epitope map of the secreted form of

dengue 2 NS1 has been established (295,782). Similar to the situation with protein E, most of the epitopes are conformation-dependent (181). Oligomerization is a general property of flavivirus NS1 proteins. The formation of dimers is favored in certain instances (772) and appears to be necessary for secretion (572).

Although antibodies to NS1 do not react with the virion and exhibit no neutralizing activity, passive transfer can confer protection against dengue and yellow fever virus infection in experimental animals (222,297,652). Enhanced protective efficacy was observed with combinations of monoclonal antibodies (297), suggesting that protection might be due to immunologic recognition and destruction of infected cells expressing NS1 (657). This phenomenon appears to be dependent on the Fc portion of antibodies (656) and could be due to complement-mediated cytotoxicity. However, other mechanisms may contribute as well because protection has also been observed in the absence of complement fixation (297).

Because the phenomenon of ADE of infection by antivirion antibodies has been implicated in the development of DHF/DSS, NS1 has been evaluated as a candidate vaccine for dengue and other flaviviruses (66,783,788). Active immunization experiments using NS1 purified from infected cells or recombinant NS1 demonstrated the induction of a protective immune response in experimental animals against homologous challenge with dengue, yellow fever, or TBE virus (331,652,653). The specific immunogenicity apparently depends on the physical structure of the NS1 preparation. Dimers were superior to monomers (181), and higher protection rates were observed with the soluble NS1 compared with its membrane-associated form.

Specificity and Targets for Cellular Immune Responses

The specificity of T-cell responses to flaviviruses in both the human and mouse systems has been most extensively studied with members of the dengue serocomplex and the JE serocomplex (WN, Kunjin, MVE) (301,398,734). In the course of dengue virus infection of humans, both CD4+, CD8– and CD8+, CD4– T-lymphocyte responses have been detected and characterized. In bulk cultures of CD4+ lymphocytes, as well as with CD4+ T-cell clones obtained from a dengue virus–infected individual, different specific cross-reactivity patterns with other dengue viruses, WN, and yellow fever virus were observed (392,792). Interestingly, these CD4+, CD8– T-cell clones were cytotoxic and exhibited class II major histocompatibility complex (MHC)-restricted cytotoxic activity against infected lymphoblastoid cell lines.

Similar results were also obtained with CD8+ T lymphocytes from infected humans and mice. Bulk responses and those of individual T-cell clones were cross-reactive with other dengue serotypes (82,227,630), supporting the concept put forward by Kurane and Ennis (398) that the activation of memory T cells during secondary infections with a heterologous serotype may contribute to the development of DHF/DSS (see later). In this context, it is of interest to note that the induction of cross-reactive T cells appears to differ in individuals with different genetic backgrounds. A predominantly type-specific CD4+ T-lymphocyte response was observed in an individual vaccinated with an experimental live-attenuated dengue virus type 1 vaccine (228), and the extent to which WN-immune CD8+ cells and CD4+ T cells from mice recognized Kunjin virus–encoded determinants varied considerably between mice of different MHC haplotypes (302,376).

Protein targets for T-cell responses have been identified (6,363). In the dengue virus system, NS3 appears to contain multiple (at least six) dominant epitopes for CD4+ and CD8+ T lymphocytes of both species (reviewed in 398), but T-cell epitopes were also recognized on E and NS1. Epitopes on NS3 have serotype, subgroup, and flavivirus group specificities (391,683). One epitope recognized by a dengue-specific human CD4+ T-cell clone was mapped to a peptide including amino acids 255 to 264 of NS3, which is conserved among all four dengue types (393).

NS3, NS4A, and NS4B are predominant targets for class I MHC-restricted anti-Kunjin, -WN, and -MVE responses in mice (302,431); whereas class II MHC-restricted responses to WN and Kunjin virus were predominantly directed against determinants on structural proteins or NS1 (376). In any case, a limited number of T-cell determinants appears to be recognized by a particular individual, and variation exists between different haplotypes (390,430).

Potential T-cell epitopes in protein E of MVE, JE, WN, and dengue virus were identified by the use of computer predictions and by analyzing *in vitro* T-cell proliferation responses against synthetic peptides (399,408,615). Functional identification of T-helper cell epitopes in protein E was achieved by measuring the B-cell response after immunization with synthetic peptides derived from MVE virus (465,617) and dengue 2 virus (618). T-cell help and enhanced antibody response was dependent on the linkage between the corresponding peptides, and the most efficient T-cell–B-cell epitope interaction occurred when the peptides were colinearly synthesized.

Antibody-Dependent Enhancement

ADE of flavivirus replication in Fc-receptor–bearing peripheral blood monocytes and macrophage-like cell lines has been demonstrated *in vitro* with a number of flaviviruses, including dengue, yellow fever, Wesselsbron, WN, and TBE viruses (260). Increased adsorption of virus particles (751) to host cell plasma membrane (mediated by subneutralizing antibodies), as well as increased efficiency of virion internalization, has been shown to increase viral replication fivefold to sixfold in monocyte- or macrophage-like cells (216,217).

ADE occurs through FcgRI and FcgRII (368,427,648), but a second type of enhancement dependent on the C3 complement receptor has also been described (98). Attempts with monoclonal antibodies to elucidate whether specific epitopes are responsible for ADE have revealed that (a) there is no correlation between serologic specificity and ADE, that is, it can be mediated by broadly cross-reactive as well as type- and even subtype-specific antibodies; and (b) antibodies with different functional activities (i.e., neutralizing, HI, or nonfunctional antibodies) can mediate ADE. Studies with a number of dengue 2 and dengue 4 strains and a panel of monoclonal antibodies have revealed significant heterogeneity, and strain-specific patterns of ADE reactivities have been discerned (262,511). The possible role of ADE in the immunopathogenesis of DHF is discussed later.

Molecular Basis of Virulence

Strain-specific differences in neurovirulence or neuroinvasiveness have been observed with several flaviviruses (38,39,158,314,315,500). Attenuation by serial passaging formed the basis for the development of the yellow fever 17D vaccine (708) and candidate vaccine strains for JE (534,784) and dengue virus (780). Attenuation during serial passage may occur quite rapidly. For example, mosquito-borne flaviviruses become rapidly attenuated after only six passages in HeLa cells (40,173,281). These observations are explained in part by the high rate of mutation of RNA viruses (40,308) and fixation of specific mutations during passage in a specific and homogeneous cell type.

Nucleotide sequencing of pairs of parental and attenuated vaccine derivatives has provided some clues as to the molecular basis of virulence, but the large number of mutations and their localization in many parts of the genome complicate interpretation of these comparative data (59,254,410,534,600). The number of nucleotide changes separating parental and vaccine strains is 67 for yellow fever virus (see later); 53 for dengue type 2 virus (59); and 45 for JE virus (534). In the case of yellow fever, although 67 nucleotide and 31 amino acid differences distributed across the genome were originally noted between 17D-204 vaccine and the parental Asibi virus (254), subsequent analyses of other vaccine strains derived from the 17D lineage and additional wild-type yellow fever viruses have significantly reduced the mutations that may explain attenuation (168a,333). Sequencing of the vaccine substrains 17D-204, 17DD, and 17D-213 and comparison with Asibi showed that only 50 nucleotide changes and 13 nonconservative amino acid substitutions were specific to the vaccine strains, 5 of which occur in the E gene. Because the 5 changes in the E gene occur at sites (amino acids 52, 173, 200, 305, and 380) that are conserved in virulent yellow fever viruses from both Africa (333) and South America (34) isolated many years apart, these mutations are likely to be implicated in attenuation.

The location of these mutations with respect to functional domains of the E gene lend further support to this conclusion (compare Fig. 3**A**). Two of the mutated amino acids (at positions 52 and 200) are present in the fusion sequence in domain II (448). Two changes (at amino acids 305 and 380) are present in domain III, and one (at amino acid 173) in domain I of the E protein. The mutations at positions 173 and 380 are very close to neutralization or virulence determinants mapped by monoclonal antibody escape mutants of TBE virus (309,448). Studies of envelope proteins of yellow fever viruses with monoclonal antibodies supports the notion that antigenic changes in the E protein are implicated in attenuation or virulence (40). In one study, the loss of a substrain-specific epitope, recognized by a monoclonal antibody, resulted in dramatic changes in neurovirulence ranging from avirulence to increased virulence (634,635). The role of protein E specifically in neurovirulence was further supported by studies with mouse and monkey brain tissue. In these tests, two to three mutations in the E protein altered viral ability to bind to monkey brain tissue or markedly enhanced neurovirulence (530,647). In the case of the NS1 gene, a 17D-specific mutation at the N-terminus of NS1 is similar to a change observed between parental and vaccine strains of dengue 2 virus (59). The 17D-specific alteration in NS3 yields a nonconservative amino acid change (D®E) in the C-terminal portion of the protein involved in linking the presumed helicase and RNA triphosphatase functions (762). In summary, a strong rationale exists implicating one or more of seven nonconservative changes in E, NS1, or NS3 in attenuation of yellow fever vaccine, a hypothesis that can now be empirically tested using site-specific mutations of an infectious cyclic DNA (cDNA) clone (599).

Yellow fever virus exhibits two distinct virulence factors reflecting its capability to induce encephalitis (neurotropism) and hepatitis (viscerotropism). The attenuated 17D vaccine virus has lost its capacity to cause hepatitis while retaining a reduced degree of neurotropism, particularly for the immature brain. A single fatal case of human encephalitis due to 17D virus has been reported (21), and the virus recovered from the brain was subjected to careful scrutiny (334). A monoclonal antibody specific for wild-type yellow fever virus recognized the case strain but not the parental vaccine (17D-204 substrain) and was considered a mutant at this epitope. The case strain also differed phenotypically in being neuroinvasive and neurovirulent for mice inoculated intranasally and appeared to be neurovirulent for monkeys inoculated intracerebrally. Sequencing of the E glycoprotein and comparison with other substrains of 17D vaccines revealed a single amino acid change (K®Q) at position 303 that could be correlated with the increased neurovirulence of the virus recovered from brain tissue. Presumably, this mutation arose during replication of the vaccine in the human host. Interestingly, this mutation is spatially close to two other mutations distinguishing wild-type from vaccine virus (including the nonconservative change at amino acid 305), suggesting that this region of domain III may represent an important locus defining neurovirulence.

Other data on the localization of virulence determinants have come from characterization of monoclonal antibody escape mutants (102,309,336,432), host range mutants (434), and virulence revertants (333) and by the engineering of infectious cDNA clones derived from dengue 4 virus and JE virus (702). Single amino acid substitutions in the E protein can have dramatic effects on virulence. A chimeric dengue 4 virus containing the envelope proteins from TBE virus exhibited the neurovirulence phenotype of TBE virus (558). In contrast to TBE virus, however, this chimera was not neuroinvasive, indicating that other regions in the genome contribute to the virulence properties of this virus.

Specific virulence mutations in protein E map to distinct structural elements (compare Fig. 3**A**). Domain III (C-terminal in the crystallized membrane anchor-free fragment), is the element that links the central domain I with the loops of domain II, the tip of domain II, and the carbohydrate side chain. The importance of domain III was directly demonstrated by the analysis of single amino acid mutants of MVE, TBE, louping ill, and JE viruses (102,309,336,434). Amino acid substitutions in the E protein of the yellow fever 17D vaccine strains also cluster within this relatively small domain (168a,254). Because this domain contains an RGD sequence in several mosquito-borne flaviviruses, it has been proposed to form part of the receptor-binding site, and this could explain its role in virulence (434). A mutation in the RGD sequence of MVE virus led to loss of virulence for mice (435).

Specific mutations within the element linking the central domain I with the domain II loops influence virulence with TBE (310), JE (102,272), and MVE viruses. The latter case is especially interesting since the attenuating mutation also lowered the pH threshold and the extent of fusion, suggesting a relationship between fusion activity and virulence (479a).

Using the TBE–dengue 4 virus chimera, Pletnev and associates (559) engineered specific mutations in the conserved

sequence element at the tip of domain II, which led to an increase in neurovirulence. This sequence interacts with domain III of the other monomer and thus forms an important contact point within the dimeric subunit. These interactions may also involve the juxtaposed carbohydrate side chain, the removal of which can reduce (in the case of the TBE–dengue 4 chimera) or enhance (in the case of a dengue 4–dengue 4 chimera) neurovirulence (347). As demonstrated with the TBE–dengue 4 chimera (559), such effects of carbohydrate side chains on virulence are not restricted to protein E but rather apply to the prM protein and NS1 as well. Also, a change in the prM cleavage site resulted in decreased neurovirulence of this chimera, consistent with the importance of prM cleavage to yield fully infectious virus (292,760). The role of the E protein in neurovirulence and neuroinvasiveness has been compared for several of the flaviviruses. The existing data indicate that determinants for both map to identical regions of the E protein, which include the lateral surface of domain III and the base of domain II (481). The attenuation of some of these variants has been associated with changes in binding to cell membrane proteins or changes in the pH-dependent fusion activity.

The development of stable genomic-length cDNA clones of various flaviviruses has accelerated the understanding of virulence factors. A good example of this is a dengue 2 virus that was stably cloned into *Escherichia coli* (236). Once the genome of the virus is stably cloned, infectious RNA can be generated. Although generating infectious clones is like a useful tool to elucidate the impact of mutations, it is not an easy task, as shown by the extensive work needed to generate an infectious Langat TBE clone (95). With a stable cDNA clone, it will possibly engineer mutations, so that the effect of specific alterations can clearly be determined (446).

Other regions of the flavivirus genome have not yet been precisely mapped with respect to virulence determinants, but much more information is expected to become available soon by the further extensive application of the infectious clone technology.

Virus–Cell Interactions

Host cell macromolecular synthesis is not markedly decreased until late in flavivirus infection, when cytopathic effects (CPEs) appear. As discussed further later, many arthropod and vertebrate cultured cells do not exhibit CPEs. In other cell–virus pairings, infection progresses to CPEs within an interval as short as 18 hours or as long as 5 to 7 days. A major change in infected cells is proliferation and hypertrophy of rough endoplasmic reticular membranes within which virus particles accumulate. At the ultramicroscopic level, cellular pathologic changes include mitochondrial damage, fragmentation of reticular membranes, formation of distended vacuoles and inclusion bodies, increase in lysosomal bodies, and rarefaction of cytoplasm (517). Activities of lysosomal enzymes increase in infected tissues. At the microscopic level, susceptible vertebrate cells, such a HeLa, BHK-21, porcine kidney (PS), and primary chick or duck embryo, display cell rounding, shrinkage, pyknosis of nuclei, and dislodgment from the growth surface (Fig. 4).

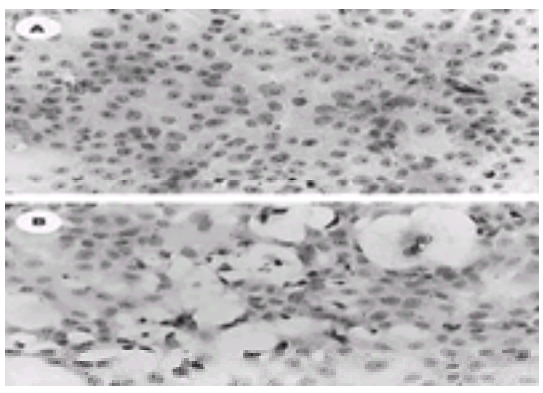

FIG. 4. Cytopathic effects of St. Louis encephalitis virus in Vero cell culture. **A:** Normal culture (Mayer's hematoxylin stain, x220). **B:** Culture after 5 days' incubation, showing focal cytopathic effects, shrinkage, and rounding of cells, pyknotic nuclei, and appearance of hole in the cell monolayer.

Cell fusion and syncytium formation (polykaryocytosis) have been observed in mammalian and arthropod cells. Fusion and infectivity are inhibitable with ammonium chloride, typical of viruses that enter cells by receptor-mediated endocytosis and low-pH–mediated membrane fusion in lysosomes. Fusion from without is demonstrable in mosquito cells after exposure of cell-bound virus to low pH (250,586).

In general, mosquito-borne viruses replicate in mosquito cell culture, and a few (such as SLE and WN, which have been isolated from ticks in nature) will also grow in tick cell cultures. Tick-borne flaviviruses replicate in tick cell cultures, but not consistently in mosquito cells. The antigenic reactivity of some flaviviruses is altered by replication in mosquito cells. For example, dengue and Kunjin viruses lose hemagglutinating activity on passage in *Aedes albopictus* cells (527).

Infected cells stained by the fluorescent antibody technique show viral antigen expression in cytoplasm, typically with a perinuclear concentration of reactivity.

Persistent noncytopathic infections by flaviviruses have been induced in a variety of arthropod and vertebrate cell lines. Depending on the virus–cell pairing, such infections can involve few cells in the culture (658) or nearly 100% of the cells (76). Progeny virus from infected cultures often exhibits altered antigenic reactivity, reduced virulence, temperature sensitivity, or other signs of mutation. The generation of defective interfering particles appears to be an important mechanism underlying persistence (566,658), and interferon production does not play an important role. Expression fo the premembrane protein in mosquito cells renders them resistant to dengue type 2 (203).

Propagation and Assay in Cell Culture

Flaviviruses produce CPEs and plaque formation in a variety of primary and continuous cell cultures derived from human, monkey, rodent, swine, and avian tissues (reviewed in 344). Cell cultures of reptilian, amphibian, and arthropod origin also support replication with or without CPE or plaques. Virus yields and titers, grade of CPE, plaque size and quality, and rate of growth vary with the specific virus and host cell (see discussion of individual viruses). In general, the BHK-21 (baby hamster kidney), SW-13 (human adrenal carcinoma), PS, *Aedes* species mosquito cells, and primary chick and duck embryo cells produce the highest virus yields, in the range of 10^{9}/ml to 10^{10}/ml. In comparison with alphaviruses, the latent period of flavivirus growth is relatively long, and growth is slow. At high multiplicity of infection in BHK-21 cells, SLE virus has a latent period of 11 hours and reaches peak titer at 28 hours. Plaques appear after 4 to 6 days; double overlay procedures are often required for optimal plaque assays in mammalian cell cultures. Addition of neutral red to agar overlay may enhance plaque size. Assay of dengue virus infectivity requires special techniques (see later).

Infection in Experimental Animals: Host Range

The susceptibility of experimental animals and host range vary widely and are considered in the discussion of individual viruses. In general, intrathoracic or intracerebral inoculation of live mosquito adults or larvae provides the most sensitive system for assay of mosquito-borne flaviviruses.

EVOLUTION

The evolution of the flaviviruses appears to have been driven in large part by their arthropod vector. Early in the classification of these viruses, it was recognized that the viruses that shared antigenicity also shared class of vector: mosquito, tick, or no vector. When more complete sequence-based phylogenies were generated, viruses with the similar vectors were more closely related (55,380).

One theory of flavivirus evolution is that the two distinct groups of tick- and mosquito-borne viruses evolved from a common ancestor. This would imply that nonvector progenitor viruses individually entered the tick and mosquito transmission cycle. However, some viruses that are mainly transmitted by one vector can still be competently transmitted by another (SLE, WN, and YF have been isolated from ticks). In addition, none of the nonvector flaviviruses can replicate in mosquito cell culture. This casual association of some mosquito-borne viruses may be a leftover trait from a past association the viruses had with ticks before adaptation to the mosquito vectors (380). This information supports another evolutionary path. Flaviviruses could have evolved from nonvector to tick-borne and finally into the mosquito-borne group. Mosquitoes feeding on viremic tick-borne virus hosts would provide the crossover opportunity. This theory is compromised by the phylogenetic placement of the nonvector Aroa, Entebbe bat, Saboya, and Sokuluk viruses (698,699) in the mosquito cluster. This could be partially the result of incomplete field information on these particular viruses.

There appear to be distinct differences between the evolution of the tick- and mosquito-borne viruses. The tick-borne viruses appear to have dispersed west over most of Asia and Europe and perhaps North America, with the ancestral lineage in the eastern portion of their range and the most recent strain populating the western end of the viral range (786). The mosquito-borne viruses show a much more varied and irregular dispersal and evolution. These differences in range are mirrored at the protein sequence level. The amino acid substitution in TBE viruses would usually be between physicochemically similar or related residues. This is contrasted with the mosquito-borne viruses in which the substitutions are much less conservative (786). Furthermore, the rate of amino acid substitution between the tick and mosquito viruses supports the observation of a lower evolutionary rate in the tick-borne viruses. The differences in vector life cycle, years for ticks and weeks for mosquitoes, and the fact that many of the tick-borne viruses can be transmitted vertically and in the absence of a viremic vertebrate host, supports the general idea of tick-borne viruses evolving at a slower rate. Additionally, it has been observed that the rate of mutation for RNA arboviruses overall is lower than other RNA viruses. This constraint has been explained by the virus having to maintain effective replication in the different environments of the vertebrate host and the arthropod vector (307,308).

The potential rapid rate of evolution is a direct concern to contemporary epidemiology of the flaviviruses. As the mosquito-borne viruses have expanded in range, their diversity has steadily increased (603,786). An expanding and evolving viral population is largely a mosquito-borne virus concern. For many of the mosquito-borne viruses, humans are a relevant host, part of the transmission cycle; therefore, that as the human population expands, the viral population will have a larger range to infect and evolve within. In contrast, for the tick-borne viruses, humans are generally regarded as a dead-end host; hence, the expansion of human populations are predicted to have a less significant effect. An expansion of the tick-borne viruses vertebrate hosts would probably have a more significant impact on their evolution than expansion of the human population. Perhaps it should be anticipated that, year after year, in hundreds of millions of mosquito-borne human flavivirus infections, there may appear a virus with altered tropisms that will develop into a significant public health concern.

PATHOGENESIS AND PATHOLOGY

The greatest body of information about flavivirus pathogenesis is derived from experiments on mice and other laboratory rodents. These animals provide a reasonably good model of flavivirus encephalitis but not of other syndromes associated with human flavivirus infection (i.e., fever, arthralgia, rash, and hemorrhagic fever). Viruses that produce these syndromes in humans, including dengue and yellow fever, cause encephalitic infections in laboratory rodents. The pathogenesis of dengue and yellow fever is discussed in their respective sections. The universal neurotropism of flaviviruses in rodents and even in arthropod vectors (in which brain and ganglia are major sites of replication) may reflect evolutionary conservation of viral polypeptide structures involved in receptor interactions and of cell membrane molecules that subserve virus–receptor interactions.

Three patterns of pathogenesis have been described in flaviviral encephalitis (339,497,524): (a) fatal encephalitis,

usually preceded by early viremia and extensive extraneural replication; (b) subclinical encephalitis, usually preceded by low viremia, late establishment of brain infection, and clearance with minimal destructive pathology; and (c) inapparent infection, characterized by trace viremia, limited extraneural replication, and no neuroinvasion.

Virus- and Host-Specified Factors Influencing Pathogenesis

The course and outcome of infection is influenced by both virus- and host-specified factors. High-dose and intracerebral or intranasal routes of virus infection predispose to fatal encephalitis. Virus strains may differ in neuroinvasiveness, neurovirulence, or both.

Among host factors influencing pathogenesis, the most important are age, sex, genetic susceptibility, and preexisting infection or immunity to heterologous agents. Neonatal animals are more susceptible to lethal encephalitis than older animals. Animals inoculated by the peripheral route are susceptible until 3 to 4 weeks of age, when resistance develops, but they may remain susceptible to lethal encephalitis when inoculated intracerebrally. Immature neurons are more susceptible to infection than mature neurons (538). Parallels are seen in some human flavivirus infections. For example, young infants are more susceptible to encephalitis after yellow fever vaccination. In the case of some flaviviruses (e.g., SLE), however, susceptibility to encephalitis increases with advancing age, with the elderly being most severely affected. The mechanisms underlying the increasing susceptibility with age are not known. Physiologic factors that lead to transient immunosuppression may also be responsible for increased susceptibility to flavivirus encephalitis. For example, mice exposed to cold or isolation stress and challenged with WN virus demonstrated higher virus replication in peripheral tissues and brain and higher mortality rates (44).

Sexually mature female mice demonstrate increased resistance to some flavivirus infections (15). Sex differences in susceptibility of humans (as opposed to exposure to infected vectors) have not been demonstrated, except possibly in the case of DHF, which preferentially affects girls.

Genetic determinants play a central role in the pathogenesis of flavivirus infections (642). Studies by a number of workers (487,636,758) demonstrated genetic resistance of nonimmune mice to flavivirus infection in a number of inbred mouse strains and showed that resistance was determined by a single autosomal dominant allele, currently designated as Flvr. The resistance allele was incorporated into susceptible C3H/He inbred mice to yield the resistant strain C3H/RV (C3H.PRI-Flvr). WN virus yields in brains of resistant C3H.PRI-Flvr mice were significantly lower than in susceptible mice, but there were no differences in interferon or humoral antibody responsiveness between the two mouse strains (219). *In vitro* studies with cells derived from the two mouse strains indicated that (a) macrophages from resistant mice do not support flavivirus replication as well as macrophages from the susceptible strain, and (b) the lower virus yields observed in cells of resistant mice were due to greater production of defective interfering virus particles (143). In the case of Banzi virus infection of C3H.PRI-Flvr mice, genetic resistance did not appear to depend on lack of permissiveness of tissues to virus replication (329), but instead had an immunologic basis (54,330). Virologic mechanisms may also operate in determining resistance in this model, however. Increased interfering virus found in lymphoid tissues of resistant mice may contribute to survival (673).

Studies in inbred mice derived from single subspecies of wild *Mus musculus* suggested that flavivirus resistance genes are carried by many wild mouse strains (735,736). In addition to Flvr, a second autosomal dominant allele at the Flv locus (designated Flvmr) was found to determine resistance. Although not fully mapped, the Flv locus is believed to reside on chromosome 5 (643). Haplotype restriction may also influence the immune response of the host to flaviviruses. Inbred mouse strains of the H-2d, H-2k, and H-2b haplotypes differed with respect to neutralizing antibody response to JE vaccine virus strains (770).

Concurrent infections with unrelated agents may enhance flavivirus neuroinvasion (reviewed in (497), presumably by disturbing the blood–brain barrier. This mechanism has been reported in mice doubly infected with JE, herpesvirus, *Trichinella* species, and visceral larva migrans; a similar phenomenon has been reported to occur in humans with neurocysticercosis (145,663). Impairment of the blood–brain barrier by needle puncture, hypercarbia, microwave radiation, or administration of bacterial lipopolysaccharide or sodium dodecylsulfate renders mice susceptible to lethal encephalitis by virus strains that are inherently neurovirulent but not neuroinvasive (358,406,441).

Extraneural Infection and Routes of Neuroinvasion

A general scheme for the dissemination of flaviviruses in the host is shown in Figure 5. Recent studies with dengue virus infection of humans show that skin Langerhans cells are primary targets (779a). After inoculation into the skin, the virus replicates in local tissues and regional lymph nodes. Virus is then carried through lymphatics to the thoracic duct and into the bloodstream (445). This primary viremia seeds extraneural tissues, which, in turn, support further viral replication and serve as a source for release of virus into the circulation.

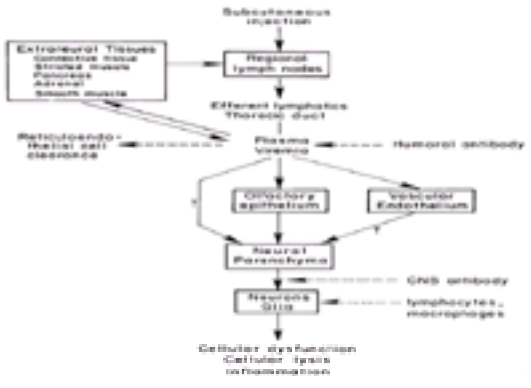

FIG. 5. General scheme of the sequential development of flavivirus infection. *Boxes* indicate sites of virus replication and

dashed arrows show immune defense mechanisms. (Modified from ref. 524, with permission.)

The viremia level is modulated by the rate of clearance by macrophages, and it is terminated by the appearance of humoral antibodies, usually by about 1 week after infection. Major extraneural sites of flavivirus replication include connective tissue, skeletal muscle and myocardium, smooth muscle, lymphoreticular tissues, and endocrine and exocrine glands. In baby hamsters infected with SLE and Rocio viruses, pancreas and heart were the most severely affected organs (269). Virus particles within secretory granules of exocrine and endocrine areas of the pancreas were released by exocytosis. Myocardial necrosis with productive viral infection of myocytes was a prominent finding. Correlations are possible between this experimental model and pathogenesis in clinical hosts. Infection of goat mammary glands, followed by secretion of virus in goat's milk, is an important mode of spread of TBE viruses. Interstitial myocarditis has been reported in WN and JE virus infections of humans and horses, and pancreatitis has been associated with human WN virus infection.

Investigation of experimental flavivirus encephalitis in mice has demonstrated a relationship between level of viremia, development of brain infection, and multisite appearance of viral antigen in nervous tissue (9), supporting the concept of hematogenous spread to the central nervous system (CNS) (339). As pointed out earlier, artificial disruption of the blood–brain barrier potentiates neuroinvasion and encephalitis. The mechanism by which flavivirus particles cross the blood–brain barrier during natural infection remains uncertain. The ability of these viruses to replicate in vascular endothelial cells suggests that they may grow across capillaries in the brain. However, viral antigen has been found only rarely in endothelial cells of brain capillaries *in vivo,* and flaviviruses did not replicate to high titer in brain endothelium cells *in vitro* (170).

The olfactory tract has long been recognized as (a) an alternative pathway to the CNS, and (b) an important mode of spread after aerosol exposure. Intranasal inoculation of flaviviruses may result in lethal encephalitis, presumably by direct infection of olfactory neurons and spread through the olfactory tract to the brain, whereas peripheral inoculation of the same virus strains does not result in neuroinvasion. However, olfactory neurons may also provide a route for CNS infection after peripheral virus infection. In an experimental model, mice and hamsters inoculated by the peripheral route with SLE virus developed low-level or undetectable viremias similar to those occurring in clinical hosts. This resulted in early infection of olfactory neurons (which are unprotected by the blood–brain barrier) and subsequent axonal transport of virions to the olfactory lobe of the brain (501). It is not known whether this pathway operates in humans, but a postmortem study of JE patients indicated a hematogenous, rather than an olfactory, route of neuroinvasion (340). Once in the CNS, virus spreads rapidly.

Neuronal centers vary in susceptibility. In the mouse, the hippocampal formation is particularly sensitive, whereas in monkeys and humans, the thalamus, substantia nigra, and cerebellum are most vulnerable (523,524).

Pathologic Changes

Pathologic changes observed in humans and experimental animals with flaviviral encephalitis include (a) neuronal and glial damage caused directly by viral injury and characterized by central chromatolysis, cytoplasmic eosinophilia and cell shrinkage, and neuronophagia; (b) inflammation, including perivascular infiltration of small lymphocytes, plasma cells, and macrophages (Fig. 6); (c) cellular nodule formation composed of activated microglia and mononuclear cells (Fig. 7); and (d) cerebral interstitial edema. In monkeys and hamsters infected with tick-borne viruses, astrocyte proliferation and hypertrophy appear as a late phenomenon (794). At the ultrastructural level, infection of neurons is characterized by marked proliferation and hypertrophy of rough endoplasmic reticulum (RER), accumulation of vesicular structures derived from the RER and containing virus particles, and progressive degeneration of the RER and Golgi apparatus (270). Neuronal destruction is infrequent, however, suggesting that dysfunction rather than cell death is responsible for lethal outcome of the host (271).

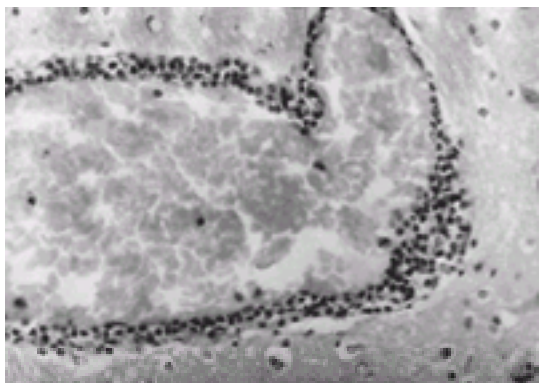

FIG. 6. Perivascular cuff of small lymphocytes around a vein in the hippocampus of a fatal human case of St. Louis encephalitis (hematoxylin and eosin, x400). (Courtesy of M. G. Reyes.)

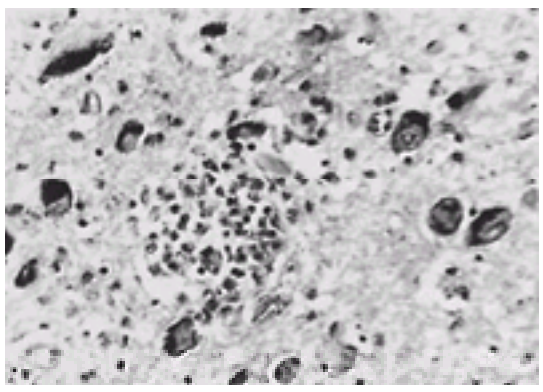

FIG. 7. Glial nodule in the substantia nigra from a fatal human case of St. Louis encephalitis (hematoxylin and eosin, x400). (Courtesy of M. G. Reyes.)

Residual neurologic deficits, electroencephalogram (EEG) changes, and psychiatric disturbances frequently persist after recovery from acute encephalitis. Pathologic lesions in cases with neurologic residua 12 to 67 years after recovery from acute JE were characterized by small areas of rarification (neuronal loss) surrounded by dense microglial scarring and were distributed in areas typically affected during the acute phase. In experimental animals, changes in behavior and learning ability have been documented (see next section).

Persistent and Congenital Infection

Neural tissue is a preferred site for *in vivo* persistence of latent infections with many viruses, and flaviviruses may be no exception. Studies with alphaviruses indicate that antibody may mediate viral clearance from neurons that lack surface class I MHC determinants for recognition by cytototoxic T lymphocytes (417,418). The mechanism of antibody clearance appears to involve intracellular uptake of antibody and restriction of gene expression. Passive administration of antibody to mice with established flavivirus (WN virus) brain infection prevented lethal infection (524). However, the role of antibodies in viral clearance as opposed to a mechanism involving cell killing by Tc cells suggests a mechanism for persistence of viral genomes in neural cells. Because abortive CNS infections with flaviviruses occur in humans and animals in nature, persistent infections may be established in neuronal tissue. In children infected with JE virus, recurrent neurologic disease has been observed (664), suggesting reactivation of latent neural infection. Chronic progressive human encephalitis and seizure disorders (Kozhevnikov's epilepsy) occurring years after infection with TBE virus has been reported (539). Yellow fever 17D virus has been recovered from the brains of monkeys more than 5 months after intracerebral inoculation. Hamsters and monkeys infected with tick-borne flaviviruses have developed progressive neurologic degeneration, with astrocytic proliferation, perivascular granulomas, and neuronal vacuolation (793,794). Chronic infection of neural or lymphoreticular tissues has also been reported with TBE and WN viruses in monkeys and with Kyasanur forest disease and SLE viruses in mice (reviewed in 497). In these models, some persistently infected animals either failed to develop neutralizing antibodies or reverted to seronegative, suggesting that productive viral replication occurred when immune responses were impaired or suppressed. Similarly, persistent infections with SLE, TBE, and JE viruses have been documented in hibernating bats and rodents, with reactivation following arousal, suggesting that immune suppression during hibernation may be responsible for persistence.

Virus isolates from persistently infected animals have exhibited phenotypic changes such as loss of hemagglutinin, reduced neurovirulence, and temperature sensitivity similar to that associated with persistent infection of cell cultures, indicating that persistent infections may be associated with attenuation of the virus phenotype, generation of defective interfering particles, or alteration of viral antigens to escape immune surveillance.

In Asia, JE virus is an important cause of epizootic abortion and stillbirth in swine and has been isolated from brain, liver, and placental tissues of these animals. Mice infected with JE virus show a high incidence of stillbirth and congenital malformations when inoculated 9 to 16 days before parturition. Mice can transmit JE virus to offspring of consecutive pregnancies (115), and immune suppression during pregnancy has been postulated to play a role in establishment of persistent maternal infection (467,468). Splenic T lymphocytes have been shown to harbor the virus in latently infected mice (469). Evidence for latent JE virus infection in humans is reviewed later in the section, Japanese Encephalitis Virus.

Immune Response in Protection, Recovery, and Pathogenesis of Encephalitis

Both humoral antibody and cellular immune responses have been implicated in protection and recovery from infection. Passive transfer of monoclonal antibodies against M and E proteins (284,346,458) as well as antibodies against the NS1 nonstructural protein (652,653,655) protects mice against flavivirus encephalitis. The mechanism of protection of antibodies to NS1, which is expressed on the surface of infected cells, appears to involve Fc-dependent effector functions, but the roles of complement-mediated cytolysis and ADCC *in vivo* are uncertain (656). As mentioned previously, passively transferred antibodies can abort experimental flavivirus encephalitis even when given after neuroinvasion, whereas transfer of immune spleen cells may not be effective at this stage. In severe or lethal infections characterized by high-titer viral growth and rapid accumulation of antigen in the critical target tissues, inflammatory responses may enhance lesions and accelerate death. Characterization of the inflammatory cell populations in brain tissue has shown that both natural killer (NK) cells and Tc cells are responsible for lysis of infected neurons and Tc cells for lysis of infected astrocytes (428,429). Infection of these target cells in the brain appears to result in an interferon-induced increase in class I and II MHC antigen expression, leading to increased recognition by Tc cells. In addition to cell-mediated clearance, antibodies have been implicated in early death in mice infected with yellow fever virus, an effect attributed to complement-mediated cytolysis of infected cells (220,221).

ARTHROPOD INFECTION

The dependency of certain arthropods upon blood feeding for energy requirements and egg development provides a mechanism for salivary transmission of flaviviruses and many other infectious agents. Although mechanical transmission from host to host by simple transfer of virus on the mouthparts of mosquitoes during the process of interrupted feeding may occur occasionally, biologic transmission is the rule. Biologic transmission of flaviviruses by arthropods depends on the following:

1. Ingestion of a blood meal containing virus and infection of epithelial cells lining the mesenteron (midgut)
2. Escape of virus from the midgut epithelium into the hemocele
3. Infection of the salivary gland
4. Secretion of virus in saliva during refeeding on a susceptible vertebrate host (Fig. 8)

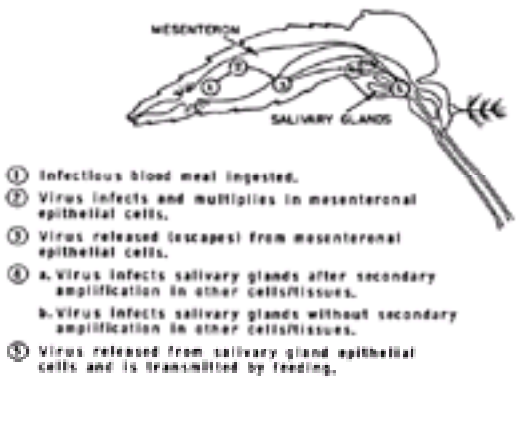

FIG. 8. Steps required for flaviviruses infection and transmission by an arthropod. (From ref. 268, with permission.)

Many flaviviruses exhibit a high degree of specificity in their ability to infect and be transmitted by individual insect or tick species (or even strains of individual species). Vector competence is under genetic control, with the susceptibility of the midgut epithelium being the primary determinant (268,727). In a susceptible arthropod, a sufficient concentration of virus must be ingested to exceed the mesenteronal infection threshold. The time interval between the ingestion of an infectious blood meal and the salivary secretion of virus (extrinsic incubation period) must not exceed the life span of the arthropod. Increased temperature shortens the extrinsic incubation period and may significantly increase the rate of virus transmission in nature. Other extrinsic factors affecting flavivirus transmission by arthropod vectors include (a) mosquito larval crowding and nutritional deprivation, which appear to increase transmission by adult vectors; (b) rearing temperature (349); and (c) co-infection with other infectious agents such as microfilaria, which may induce lesions in the midgut epithelial barrier and thereby facilitate viral dissemination to the hemocoel (728); or natural disasters (521).

Transfer of virus in mosquito saliva occurs during the process of probing host tissues with the piercing mouthparts in an attempt to cannulate a small vessel. The probing process often results in microhematoma formation, which facilitates location and cannulation of the vessel (177). Salivary enzymes play an important role in feeding, particularly apyrase, which prevents ADP-dependent platelet aggregation and coagulation (455). Salivary virus is deposited principally in the extravascular tissues of the host during probing, and saliva that is injected intravascularly is rapidly reingested by the mosquito during blood feeding (729,730). This results in virus replication at the site of inoculation and relatively slow spread of initial infection through lymphatic channels to regional lymph nodes, rather than a rapid viremic dissemination of the inoculum to the bloodstream.

For virus transmission to occur, the vector must find and successfully feed on a vertebrate host. Many factors influence the search for vertebrate hosts, including vector and host behavior and density, chemical signals emitted by the host, and environmental variables (reviewed in 177). By altering vertebrate host behavior, virus infection may itself influence rates of transmission because infected animals may be lethargic and exhibit reduced defense against mosquito and tick bite. This phenomenon has been demonstrated experimentally in the case of SLE virus–infected mice (149), but its relevance under field conditions remains to be demonstrated. Interestingly, the reduced defense against mosquito attack by immature nestling birds appears to be offset by their intrinsically lower attractiveness to blood-seeking mosquitoes (661).

Maintenance of the infection requires that the basic reproductive rate (number of new infections that arise from a single infection) be greater than one; that is, the host infected by one arthropod must serve as the source of infection for more than one new arthropod. Because mosquitoes feed on vertebrate blood for a very brief time, infection requires that the blood meal volume contain a concentration of virus sufficient to establish midgut infection in the vector. Thus, infected vertebrate hosts involved in transmission cycles generally develop viremia titers in excess of 10^5/ml. In contrast to mosquitoes, ticks imbibe blood over a much longer period of time. The requirement for host infection and viremia for virus amplification of tick-borne arboviruses has recently been questioned (402,536) because TBE virus is transmitted efficiently from infected ticks to uninfected ticks cofeeding on a vertebrate host in the absence of detectable viremia. Although the mechanism whereby infection of cofeeding ticks occurs has not been elucidated, it is logical to assume that the virus is blood-borne and below levels detectable by conventional techniques. Because vertebrate hosts are infected by multiple ticks in nature, virus amplification may occur without the requirement for active infection of vertebrate tissues and viremia. A component of tick saliva appears to enhance virus transmission between infected and uninfected ticks, possibly by suppressing early, nonspecific antiviral responses (NK cell activity) in the host (375,401). However, humoral immunity of previously infected vertebrate hosts would limit virus spread by the cofeeding mechanism in nature.

Vertical transmission from female arthropod to her progeny is an important mechanism for overwinter survival of certain mosquito- and tick-borne flaviviruses. Flaviviruses infect the genital tract of female mosquitoes so that the virus may

enter the fully developed egg through the micropyle at the time of fertilization and oviposition (625). As opposed to true transovarial transmission (infection of the egg at the time of development in the ovary), this mechanism allows infection of mature ova during the first ovarian cycle after feeding on a viremic host. Venereal transmission of flaviviruses from male to female mosquitoes has also been demonstrated (626). The mechanism of transovarial transmission of tick-borne flaviviruses remains to be elucidated.

Flaviviruses generally do not damage the vector, and the pathologic changes in the midgut seen in mosquitoes infected with some alphaviruses have not been observed in the case of *Flaviviridae.* In one study, vertical flavivirus infection in *Aedes* species mosquitoes appeared to induce a prolongation of development time from ova to pupa, suggesting a deleterious effect (706). However, no such effect was observed in the case of SLE virus in experimentally inoculated *Aedes* species larvae (731). These results contrast with those observed in the case of alphaviruses, which appear to induce more consistent and severe pathologic effects on mosquito larvae and pupae, possibly explaining the lower efficiency of vertical transmission of alphaviruses than of flaviviruses in their mosquito vectors. There have been conflicting results of studies on the effect that flavivirus infections, specifically dengue, have on the feeding patterns of the common vector *Aedes aegypti.* A study with dengue 2 virus indicated that the only alterations was actually a shorter feeding time, which would not be beneficial to the virus (577). In another study, dengue 3 virus–infected mosquitoes took a significantly longer time to finish their blood meals than did the uninfected mosquitoes, a behavior that could significantly increase the chance of infecting the vertebrate host (556a). The difference in the two studies could result from differences in mosquitoes: the earlier study used mosquitoes that had been colonized for many years and had adapted to feeding on the test animals, whereas the later study used mosquitoes that were between F1 and F3 generations from field-collected mosquitoes.

FLAVIVIRUSES ASSOCIATED PRIMARILY WITH THE ENCEPHALITIS SYNDROME

St. Louis Encephalitis Virus

The history of SLE has been reviewed in detail by Chamberlain (103). The disease was first recognized in 1932, when an outbreak occurred in Paris, Illinois. The following year, there was a large epidemic in St. Louis and Kansas City, Missouri, characterized by CNS infections of variable severity. The causative virus was isolated from human brain tissues inoculated into mice and rhesus monkeys. *Culex pipiens* mosquitoes were suspected to transmit the infection in this outbreak on epidemiologic grounds, but more than 20 years would pass before this species was confirmed as the principal vector in the east-central United States. During the early 1940s, the disease was recognized in the Pacific Coast states, where the virus was isolated from *Culex tarsalis.* A third transmission cycle involving *Culex nigripalpus* mosquitoes was suspected during SLE outbreaks in south Florida between 1959 and 1961, and later confirmed by entomologic studies. Since 1933, there have been numerous outbreaks involving the western United States, Texas, the Ohio-Mississippi Valley, and Florida. The largest epidemic occurred in 1975, with nearly 2,000 recognized cases. Epidemic activity in the subsequent decade is reviewed in reference 504. In the 1980s and 1990s, SLE epidemics occurred in Colorado, California, Florida, Texas, and Arkansas (453,588,723,724).

Infectious Agent

SLE virus is a member of the JE virus antigenic subgroup. Antigenic and structural properties of the virus have been described (344,718), and its nucleotide sequence has been partially elucidated (716). The organization of the genome is similar to all other flaviviruses. As expected, a higher order of sequence homology (about 65%) was found with members of the JE virus antigenic complex than with yellow fever or dengue viruses (about 40%). Cross-protection studies in experimental animals have confirmed the close relationship between SLE and other members of the JE complex, including WN and MVE viruses. However, immunization with dengue virus also confers cross-protection against SLE virus challenge in mice. During an epidemic of SLE in Florida, the incidence of encephalitis was significantly lower in people with prior dengue infection, as determined by the presence of antibodies, than in those lacking dengue immunity (60).

SLE virus produces CPE and plaques in a wide variety of vertebrate cell cultures (reviewed in 344). Mosquito cell cultures (derived from *Aedes, Culex,* or *Toxorhynchites* species) support growth of SLE virus, often with persistent infection and lack of CPE or with syncytium formation (585,586). Interestingly, SLE virus replicates to high titer and causes CPEs in lepidopteran cells (Sf9, *Spodoptera frugiperda*), whereas yellow fever 17D and dengue viruses, alphaviruses, and bunyaviruses do not (787).

Virus strains vary considerably in virulence for mice and may be classified as highly virulent, avirulent, or of intermediate virulence on the basis of the intraperitoneal-to-intracerebral LD_{50} ratio (500). Mouse virulence correlates with pathogenicity for rhesus monkeys inoculated intracerebrally and with the capacity of virus strains to induce viremia in the house sparrow (63). Chickens develop viremia without disease; newly hatched chicks have the highest titers. Young rats inoculated intracerebrally are partially susceptible, and survivors may develop cataracts (266). Guinea pigs, older rats, and rabbits develop antibody but not disease or consistent viremia. The SLE virus infects the chorioallantoic membranes of chick embryos. Horses are frequently infected in nature without known clinical consequences but are susceptible to lethal encephalitis after intracerebral inoculation of the virus.

The SLE virus has been isolated from a variety of wild vertebrate species during field studies, including many species of birds as well as raccoons, opossums, and bats in North America and birds, rodents, nonhuman primates, and the three-toed sloth in tropical America.

Pathogenesis and Pathology

The pathogenesis in experimental animals was described previously. The neuropathology associated with human disease is reviewed by Gardner and Reyes (208). Principal lesions include neuronophagia, cellular nodules, and

perivascular cuffing, most severely affecting substantia nigra, thalamus, and hypothalamus.

Clinical Features

Three clinical syndromes are described: encephalitis, aseptic meningitis, and febrile headache (Table 3). The severity of illness increases with advancing age, and people older than 60 years of age have the highest frequency of encephalitis. The incubation period varies between 4 and 21 days. Onset is characterized by generalized malaise, fever, chilliness, headache, drowsiness, anorexia, nausea, myalgia, and sore throat or cough, followed in 1 to 4 days by the acute or subacute appearance of meningeal and neurologic signs. Early urinary tract symptoms (frequency, urgency, dysuria) occur in nearly one fourth of patients (578). The case fatality rate increases with age, from 2% in young adults to more than 22% in elderly patients. Underlying hypertensive and arteriosclerotic disease, diabetes, and chronic alcoholism predispose to severe infection and fatal outcome. Four of the 41 recognized cases of SLE during an outbreak in Houston, Texas in 1991 were in patients with human immunodeficiency virus (HIV) infection, suggesting that immunosuppression caused by HIV may be a risk factor for expression of symptomatic encephalitis (542).

Encephalitis[a] (including meningoencephalitis and encephalomyelitis)
Acute febrile illness[b]
One or more signs in either of the following categories:
Altered level of consciousness (confusion, disorientation, delirium, lethargy, stupor, coma)
Objective signs of neurologic dysfunction (convulsion, cranial nerve palsy, dysarthria, rigidity, paresis, paralysis, abnormal reflexes, tremor, etc.)
Aseptic meningitis[a]
Acute febrile illness[b]
Signs[c] of meningeal irritation (stiff neck with or without positive Kernig's or Brudzinski's sign)
No objective signs of neurologic dysfunction
Febrile headache[a]
Acute febrile illness
Headache (may also have other systemic symptoms, e.g., nausea or vomiting)
No signs of meningeal irritation or neurologic dysfunction

[a] CSF pleocytosis present in patients with encephalitis and aseptic meningitis; it may also be found in patients with the syndrome of febrile headache.
[b] Oral temperature 100°F, 37.8°C.
From ref. 75, with permission.

TABLE 3. *Clinical syndromes caused by infection with flaviviruses associated with CNS disease*

The cerebrospinal fluid (CSF) shows moderate pleocytosis (£500 cells/mm ^{3}), mainly lymphocytes, although polymorphonuclear cells may predominate early in the infection. The CSF protein may be elevated (usually 45 to 100 mg%, rarely as high as 500 mg%). There are a few reports of hypoglycorrhacia. Hyponatremia and hypo-osmolarity occur in up to one third of the patients as a result of inappropriate secretion of antidiuretic hormone (767). Elevated plasma 17-hydroxycorticosteroids and loss of the normal diurnal pattern of glucocorticoid secretion indicate reaction to stress (169). A disproportionately high cerebral perfusion in relation to metabolic demands has been found, indicating a disturbance in cerebral autoregulation of blood flow, which was unrelated to changes in sensorium of acutely ill patients. Radionuclide brain scans and computed tomography have been normal. The EEG shows diffuse generalized slowing and amorphous generalized delta wave activity (75).

A period of prolonged convalescence occurs in 30% to 50% of cases, characterized by asthenia, irritability, tremulousness, sleeplessness, depression, memory loss, and headaches, lasting up to 3 years. About 20% of these patients have symptoms persisting for longer periods, including gait and speech disturbances, sensorimotor impairment, psychoneurotic complaints, and tremors (187). Old age and severity of acute illness appear to predispose to these sequelae.

Jaundice was reported in two cases of SLE occurring in Brazil (741). Hepatitis has also been a feature of WN virus infections in the tropics, raising the possibility that the clinical spectrum of infection with members of the JE virus complex may be broader than currently appreciated.

Diagnosis

The patient's age, season of the year, place of residence and exposure, and information about the occurrence and serodiagnosis of similar cases in the community are of paramount importance in the differential diagnosis. In the individual case, it is essential to rule out treatable bacterial, mycobacterial, spirochetal, and fungal infections as well as herpes encephalitis. Because SLE often strikes the elderly, it has occasionally been misdiagnosed as stroke.

Virus isolations from serum or CSF are very unusual, and testing is not profitable. In more than half of fatal cases, virus may be recovered by intracerebral inoculation of suckling mice with suspensions of brain tissues; occasional isolates have been made from liver, spleen, lung, and kidney (93). SLE viral antigen has been demonstrated by immunofluorescence (IF) examination of brain frozen sections (597). Flavivirus-like particles have been found by electron microscopy, and SLE antigen has been found by IF in urine sediment (440). Polymerase chain reaction (PCR) assays applied to blood, CSF, or tissue may provide a sensitive approach to early diagnosis in the future.

Specific diagnosis usually relies on serologic tests on appropriately timed acute and convalescent samples. The HI test detects mainly group-reactive antigens and is a useful screening procedure. Antibody titers increase rapidly during the first week after onset. In primary infection, titers to SLE antigen are usually higher than to heterologous antigens. In some areas of the southern United States, cross-reactions due to immunity to dengue virus confuse the diagnosis in older patients. The presence of CF antibody in a single serum sample is presumptive evidence of a recent infection. However, 20% of patients with confirmed SLE virus infections fail to develop detectable CF antibodies (93). Cross-reactions in the CF test are less than by HI. The NT is most specific. Antibodies appear during the first week and persist for many years, usually for a lifetime.

Local production of IgM antibodies in the CNS provides a potential means of rapid and early diagnosis. Demonstration of immunoglobulin M (IgM) antibodies in CSF by enzyme-linked immunosorbent assay (ELISA) is applicable to the diagnosis of SLE as early as 3 to 5 days after onset. The IgM-capture ELISA has replaced classic serologic methods in many laboratories. IgM antibodies in serum appear within the first 4 days after onset, peak at 7 to 14 days, and decline thereafter, generally reaching extinction by 60 days (503). Presence of IgM antibody in a single serum is presumptive evidence of recent infection. However, because IgM antibody may persist for up to a year in up to one fourth of patients, demonstration of a decrease in antibody titer between paired sera is preferable. IgA antibodies parallel those in the IgM class (724).

Treatment

Neither antiviral chemotherapeutic agents nor interferon has been evaluated for therapy of SLE. Treatment is supportive and consists of good medical management and nursing care, especially in the semicomatose and comatose patient.

There have been a number of laboratory accidents in which SLE was contracted through the aerosol route. Recombinant human interferon (IFN) hybrids (IFN-aA/D and IFN-aB/D) was tested to determine whether it could reduce mortality (78). Postexposure prophylaxis was not successful.

Epidemiology

Morbidity and Mortality

The epidemiology of SLE has been reviewed (491,504,588,721). Between 1964 and 1998, there were 4,478 confirmed cases of SLE (Fig. 9). Major outbreaks, which occur every 5 to 15 years, have involved up to 1,815 officially notified cases. Endemic transmission occurs during interepidemic intervals, with small numbers of cases (less than 50 per year). Attack rates in localities affected by epidemics have ranged from 1 to 800 per 100,000 population. The overall case fatality rate is about 7%, but it is significantly influenced by age. The ratio of inapparent to apparent SLE infection varies with age, from 806:1 in children to 85:1 in elderly patients.

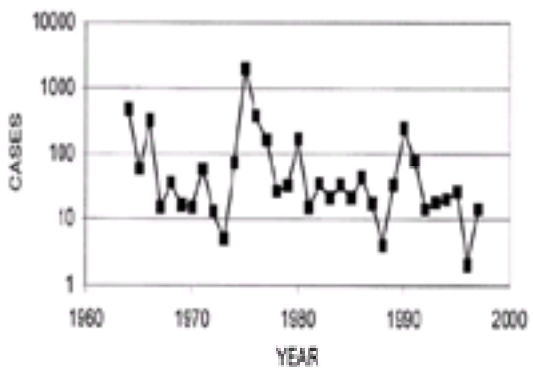

FIG. 9. Annual incidence of St. Louis encephalitis in the United States, 1964 to 1997. (Data from the Centers for Disease Control and Prevention.)

The disease appears in July, with the peak incidence in August and September, but outbreaks may occur later in the year at southern latitudes. SLE predominantly affects the Ohio-Mississippi Valley, eastern Texas, Florida, Kansas, Colorado, and California (Fig. 10). The primary transmission cycle involves wild passeriform and columbiform birds and the *Culex* spp. mosquitoes (Fig. 11). Mammals do not participate in the primary transmission cycle, but occasional isolates have been made from carnivores, and bats have been suggested to play a role in overwinter maintenance. The vectors responsible for virus amplification between birds and transmission from birds to humans vary regionally. In the Ohio-Mississippi basin and eastern Texas, the distribution of cases is urban-suburban, corresponding to high densities of the principal vectors, *Culex pipiens,* and *Culex quinquefasciatus,* which breed in polluted water (especially where poor sanitation exists). In Florida, the tropical mosquito *Culex nigripalpus* is the epidemic vector. In the western states, the principal vector, *Culex tarsalis,* breeds in irrigated or flooded dryland areas; its wide distribution leads to frequent human exposures in rural areas. *C. tarsalis* is also the vector of western equine encephalitis virus, and transmission of this virus and of SLE are often concurrent (588). SLE generally occurs as a rare, sporadic disease in many areas of tropical America (684).

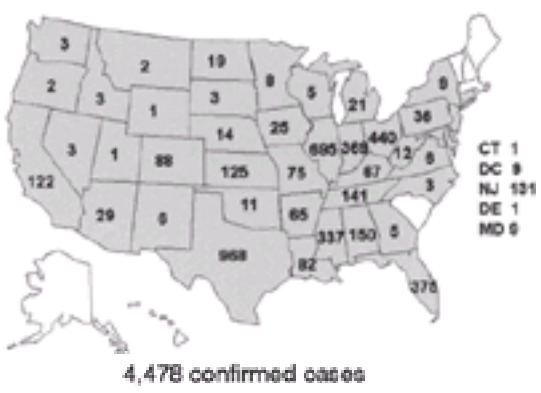

FIG. 10. State-by-state distribution of St. Louis encephalitis cases in the United States, showing cumulative number of

cases for the period 1964 to 1998. (Data from the Centers for Disease Control and Prevention.)

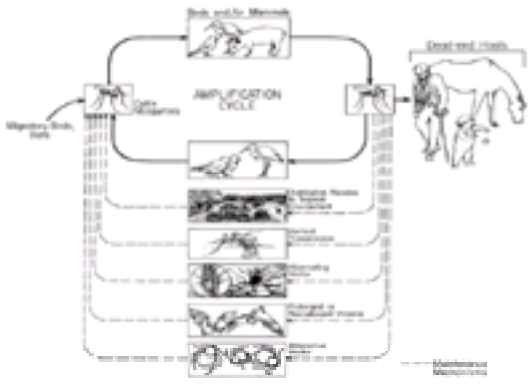

FIG. 11. Generalized transmission cycle of mosquito-borne flaviviruses causing encephalitis, showing summertime amplification and presumed overwintering mechanisms. Humans are dead-end hosts and do not participate in perpetuation of virus transmission. Vector species vary, but culicine mosquitoes are responsible for amplification cycles. Wild birds are the most important viremic hosts for most viruses, but in the case of Japanese encephalitis, pigs play an important role. The pattern shown applies to St. Louis, Japanese, Murray Valley, and Rocio encephalitis viruses as well as West Nile, Kunjin, space Usutu, and possibly other flaviviruses (see Table 1).

In the eastern and central United States, the incidence of disease and the case fatality rate are significantly higher in individuals older than 55 years of age than in younger people. Altered behavioral patterns of the human population (use of air-conditioning and television) may be responsible for decreased exposure to mosquitoes, as well as a decline in infection and immunity, in these endemic areas (200).

In rural areas of the western states, where *C. tarsalis* is the principal vector, cases in males outnumber those in females nearly 2:1 because of the greater exposure of males working outdoors. A predominance of cases in females has been found in the central and eastern states, where exposure to the household mosquito vectors (*C. pipiens, C. quinquefasciatus)* is responsible for infection. *Culex restuans* may be involved in early transmission during the cool spring months, preceding amplification by *C. pipiens* or *C. quinquefasciatus.* Risk factors for infection associated with low socioeconomic status include living in homes near open storm sewers and without screens and air conditioning, and sitting outside in the evening (453,491). In Florida, however, the black population was relatively spared during epidemics between 1959 and 1962; the higher prevalence of antibodies to dengue virus in the elderly black population may have provided a degree of protection (60).

Although historically a disease of rural agricultural communities in the western United States, SLE appeared in epidemic form in Los Angeles in 1984 (588). Subsequent investigations showed that virus transmission occurred principally in residential habitats, parkland, and vegetated undeveloped areas, with *C. tarsalis* as the principal vector (593).

Immunity

Surveys in several urban areas in the eastern United States have shown a 6% incidence of SLE virus infection during epidemics (299). A survey of urban and rural Indiana residents showed an overall seroprevalence of 3.6% and an estimated annual infection rate of 0.32% (233). A serologic survey in Los Angeles County during the outbreak in 1984 indicated evidence of infection in 1.6% of the human population.

Origin and Spread of Epidemics

Evidence indicates that SLE virus is maintained in local winter reservoirs in North America, but the possibility of annual reintroduction from warm regions of year-round transmission by migratory birds or bats cannot be excluded. The virus has been isolated from hibernating adult female *C. pipiens* (33), and there is annual recrudescence of virus activity throughout much of North America. Vertical transmission of virus has been experimentally shown in *C. pipiens, C. quinquefasciatus, C. restuans*, and other species. The virus can also be transmitted venereally in *Culex* species, providing a potential mechanism for virus spread from vertically infected males to female mosquitoes (669). SLE virus has been isolated from the gizzard of a cowbird 38 days after experimental infection (107) and from the blood of bats for prolonged periods (698). Transplacental passage of virus in bats has also been demonstrated. Despite these observations, the mechanism whereby SLE virus is maintained locally over the winter will require further elucidation.

Wild passerine birds serve as the main viremic hosts in these cycles of transmission. A comprehensive review of the avian species involved is given by McLean and Bowen (479). If the rate of virus transmission between birds and mosquitoes is sufficiently high, humans and other mammals (e.g., horses, dogs) may be tangentially infected but do not serve as viremic hosts. Many factors influence the rate of spread of virus (Table 4) and determine the course and extent of epidemics.

Virus	
	Strain differences in infectiousness for vectors and hosts
Vectors	
	Susceptibility to infection and efficiency of transmission ("vector competence")
	Vector population density, population dynamics, and age structure
	Longevity
	Preference for biting host species
	Distribution and dispersal (flight range)
Reservoir—hosts	
	Susceptibility to develop effective viremia
	Host population density, population dynamics, and age structure
	Active and passive immunity
	Attractiveness to vectors
	Distribution and dispersal
Clinical hosts (e.g., humans)	
	Population density
	Acquired immunity
	Exposure to vector bites

Modified from ref. 588, with permission

TABLE 4. *Some factors that affect rates of flavivirus transmission in nature*

In some areas in which there is an established enzootic cycle of SLE attempts have been made to determine the extent of transmission to humans and factors that affect the level of transmission (148,371,593). The use of sentinel birds has provided warning of an increase in the amount of SLE in circulation, which increases the potential for viral transmission to humans.

Molecular Epidemiology

SLE virus strains exhibit extensive genomic variability (717). Strains can be classified by their geographic origin; moreover, within a given area, both genetic drift and introduction of other geographic types have been demonstrated over time. Genome analysis thus provides a possible means of determining the origin and source of outbreaks. The presence of three unique genotypes was defined by RNAase T1 oligonucleotide fingerprinting: (I) Florida (associated with the *C. nigripalpus* cycle); (II) the Ohio-Mississippi River basin and eastern Texas (*C. pipiens* or *C. quinquefasciatus*); and (III) the western United States (*C. tarsalis*) suggests that the virus is maintained in each area in local reservoir hosts or vectors. Although relatively few strains from tropical America have been studied, they appear to represent distinct genotypes, again suggesting that the annual recrudescence of SLE virus transmission in North America is not dependent on reintroduction by migratory birds or bats. The segregation of North American virus genotypes by mosquito vector has evolutionary implications, and the selective forces have been partially elucidated (63,500,717). Virus genotype III is transmitted principally by *C. tarsalis,* despite the sympatric distribution of ample populations of *C. quinquefasciatus* in the western United States. This virus genotype is, however, less virulent than genotype II for a variety of hosts, including avian species, which develop relatively low viremias. Since *C. tarsalis* is highly susceptible to oral infection, whereas *C. quinquefasciatus* exhibits lower susceptibility, the former species is capable of amplified transmission of genotype III, whereas the latter is not. In the central United States, virus genotype II elicits high viremias in birds, sufficient to infect readily the more refractory *C. pipiens* and *C. quinquefasciatus.* Because *C. tarsalis* is not prevalent in this region, selective pressure for virulence is maintained on the virus genotype by the restricted vector competence of *C. pipiens* and *C. quinquefasciatus.*

Prevention and Control

Reduction of vector populations remains the most widely used method for prevention and control of SLE epidemics. Surveillance programs focus on early detection of increased levels of virus activity by testing avian sera for antibodies or mosquitoes for virus or viral genome by PCR. Sentinel flocks of chickens followed at frequent intervals for serologic conversions provide a sensitive indicator of SLE virus activity and have accurately predicted impending human epidemics.

Presently, there is no licensed SLE vaccine. However, there have been efforts to develop subunit, recombinant vaccinia, and DNA vaccines against this virus (554,743). One study tested the use of recombinant vaccinia virus expressing PreM/E protein and baculovirus-produced PreM/E proteins. In mice, the recombinant vaccinia virus followed by a boost with the purified baculovirus-produced proteins as 100% effective against viral challenge (743). An SLE DNA vaccine coding for the PreM/E protein has been tested. Although effective in mice, the level of protection varied (60% to 75% survival rate), with DNA dose and did not have the efficacy of the vaccinia-baculo immunization (554).

Japanese Encephalitis Virus

A disease resembling JE was recognized in horses and humans as early as 1871. A severe epidemic occurred in Japan in 1924, and a filterable agent was extracted from human brain and passed to rabbits, but the agent was not identified or characterized. In 1934, Hayashi reproduced the disease in intracerebrally inoculated monkeys. In 1935, the agent was recovered from the brain of a human in Tokyo and was virologically and serologically established as the prototype (Nakayama) strain. The virus was first recovered from brain tissue of a sick horse in 1937. Mosquito transmission was suspected during the early 1930s; in 1938, Mitamura and colleagues reported isolation from *Culex tritaeniorhynchus.* Classic studies in Japan by Scherer and associates (81,645) established that pigs and birds were the principal viremic hosts and that *C. tritaeniorhynchus* was responsible for transmission between these vertebrates and from them to humans.

Infectious Agent

Japanese encephalitis virus is the prototype of the JE antigenic complex (565). Cross-reactions in NT and cross-protection in animals have been demonstrated between JE and other flaviviruses, particularly members of the JE complex. The complete nucleotide sequence of the JE viral genome has been determined (472,701), and JE viral antigens have been expressed in recombinant systems (198,364,459,687). A full-length infectious clone was constructed

by *in vitro* ligation of cDNA fragments (702). Antigenic variation among JE strains has been shown by antibody-absorption HI, agar gel diffusion, CF, kinetic neutralization, antibody-absorption neutralization (537), and monoclonal antibody analysis (357). At least two immunotypes have been repeatedly distinguished: Nakayama (representing the prototype strain isolated from human brain in Japan in 1935) and JaGAr 01 (from *Culex* species mosquitoes, Japan, 1959). Virus strains isolated in 1969 to 1970 were immunologically placed between these types. Geographic variation between JE virus strains has been demonstrated by examination of RNA fingerprints and nucleotide sequencing (see later section, Molecular Approaches).

Considerable variation exists in neurovirulence and peripheral pathogenicity for mice among JE virus strains (314,315). Virus strains recovered from brain tissues of fatal human cases in Thailand differed by RNA fingerprint analysis from contemporary strains from pigs and mosquitoes (89), suggesting that the human isolates represent distinct neuroinvasive phenotypes. Virus strains from Malaysia and Indonesia, where JE occurs as a endemic infection with sporadic cases, are distinguishable by nucleotide analysis (122) from strains elsewhere in Asia, where the disease causes epidemic disease, suggesting that the Indonesian strains may have reduced virulence for humans. A comparison of highly neurovirulent and neuroinvasive JE strains and two strains that were neurovirulent and not neuroinvasive suggested that only a few differences distinguished the phenotypes. The different strains had 99% amino acid identity (529). The amino acid changes were centered on the E protein, which is suggestive that the envelope protein is responsible for the neuroinvasive qualities.

The virus replicates in a wide variety of primary and continuous cell cultures of hamster, porcine, chicken, monkey, and mosquito origin. Vero and LLC-MK2 cells are useful for plaque assays (692). Infant mice are highly susceptible to lethal infection by all routes of inoculation. Weanling mice succumb to intracerebral virus inoculation, but there is virus strain variation in pathogenicity by the intraperitoneal route. Hamsters and monkeys die after intracerebral or intranasal inoculation but develop asymptomatic viremia after peripheral infection. Rabbits and guinea pigs have asymptomatic infections by all routes of inoculation. The virus is pathogenic for embryonated chicken eggs. It produces disease in horses and swine (see later), whereas cattle are not susceptible.

Pathogenesis and Pathology

Sites of replication and dissemination of virus in the mouse have been described (315). During the acute stage, congestion, edema, and small hemorrhages are found in the brain. Microscopic lesions include neuronal degeneration and necrosis, neuronophagia, microglial proliferation forming glial nodules, and perivascular inflammation. These changes occur in gray matter and predominantly affect diencephalic, mesencephalic, and brainstem structures. Destruction of cerebellar Purkinje cells may be prominent.

In one study of fatal human cases, JE viral antigen was localized to neurons, with no evidence for glial cell infection (340). The highest concentration of infected neurons was in thalamus and brainstem. Among inflammatory cells recruited into perivascular infiltrates, CD4 T cells predominated, but a minority were CD8 T cells. Macrophages predominated among cells recruited into the brain parenchyma.

JE virus can gain access to the CNS at sites of blood–brain barrier disruption caused by herpesvirus (278). A similar role has been implicated for *Toxocara canis* and *Trichinella* species in experimental dual infections (141,550) and for *Taenia solium* (neurocysticercosis) in humans (145,663).

The pathways JE uses to cross over from circulation into the CNS are not well defined. A series of electron micrographs illustrate one possible pathway (426). The JE virion is transported across the cerebral blood vessels by attaching to the endothelial cell and being transported through an endocytotic vesicle. Then the virion is taken up a perivascular cell and transported into the CNS. The use of endocytotic vesicles means that the virus does not lose its own surface proteins and lipid bilayer by fusing with a cell.

Transplacental infection in swine results in fetal encephalitis, abortion, and stillbirth. The virus also produces hypospermia and aspermia in boars (253). Histopathologic changes include epididymitis, spermatogenic arrest, and inflammation of the tunica testis. Pregnant mice inoculated intraperitoneally also transmit JE virus to the fetus, with subsequent abortion (467,468). A curious feature of this model is that infected mothers, when mated again after 6 months, transmitted virus to the second litter. Latent infections of pregnant mice could be reactivated by cyclophosphamide or subsequent pregnancy. In other studies, latent infections were detected by cocultivation of mouse spleen cells and virus located principally to Lyt-1 (CD5+) cells (469).

There is also evidence for congenital and persistent infections in humans. In a series of nine pregnant women infected during an epidemic, four women infected during the first and second trimesters aborted, and virus was isolated from fetuses, whereas none of the five women infected in the third trimester miscarried or had abnormal babies (722). No other reports of human congenital infection are extant, and the frequency of this complication is unknown. Most women of child-bearing age in endemic areas of Asia are naturally or artificially immunized during childhood and are therefore not at risk. Evidence for latent infections of humans has been reported. By use of cocultivation techniques, JE virus was recovered from peripheral blood mononuclear cells of several children who developed recurrent disease as well as symptom-free children 9 months after an acute JE infection (664). Virus has been recovered from CSF samples as late as 117 days after onset of clinical symptoms (587). The frequency with which latent or persistent extraneural or neurologic infection occurs, whether such infections are associated with cytopathology or clinically relevant syndromes, and how the immune response modulates such infections remain to be elucidated.

Clinical Features

The incubation period is 6 to 16 days. As in SLE, illness may manifest as a febrile headache syndrome, aseptic meningitis, or encephalitis (163,210). In the full-blown encephalitic form, onset is rapid, beginning with a 2- to 4-day prodromal phase of headache, fever, chills, anorexia, nausea and vomiting, dizziness, and drowsiness. In children,

abdominal pain and diarrhea may be prominent. These symptoms are followed by the appearance of nuchal rigidity, photophobia, altered states of consciousness, hyperexcitability, and varying objective neurologic signs, including dull, masklike facies, muscular rigidity, cranial nerve palsies, tremulous eye movements, coarse tremors of the extremities, involuntary movements, generalized and localized paresis, incoordination, and pathologic reflexes. Sensory deficits are rare. Paralysis of the upper extremities is more common than paralysis of the legs. Spinal cord involvement (lower motor neuron deficits) may occur, and a bulboparetic syndrome has been described. Convulsions are frequent in children but occur in less than 10% of adult patients. Death occurs on the fifth to ninth day or during a more protracted course with cardiopulmonary complications. A poor prognosis is associated with respiratory dysfunction, positive Babinski's sign, frequent or prolonged seizures, prolonged fever, albuminuria, and evidence for high levels of virus replication in the brain, including infectious virus in CSF, high levels of IFN-a in CSF, and low levels of IgM antibodies in serum and CSF (84).

The peripheral white blood cell (WBC) count is mildly elevated. Urinary tract symptoms are common during the acute phase of illness and may be accompanied by sterile pyuria and microscopic hematuria and albuminuria. The CSF pressure may be elevated; microscopic examination shows 10 to 500 (rarely, up to 1,000) WBCs, with neutrophils predominating early, followed by lymphocytes. Cell counts fall gradually over 8 to 9 weeks. The CSF protein concentration is mildly elevated (50 to 100 mg%). The EEG is abnormal, with decreased electrical activity, slowing, and dysrhythmia. Brain echo and cranial tomography performed during the acute phase have shown characteristic thalamic and basal ganglia abnormalities correlating with poor prognosis.

The case fatality rate is 5% to 40%. Higher rates during epidemics (up to 70%) are reported but reflect poor medical care and recognition of only the most severe cases. Case fatality rates in affected American servicemen varied between 2% and 11%. Children and elderly patients are at highest risk for fatal infection. Neuropsychiatric sequelae occur in 45% to 70% of survivors and are particularly severe in children (378). Sequelae include parkinsonism, convulsive disorders, paralysis, mental retardation, and psychiatric disorders. The social prognosis of survivors is generally poor. Sequelae are more frequent in patients whose acute disease is severe, prolonged, and associated with coma and localizing neurologic signs. Magnetic resonance imaging in patients with sequelae has revealed abnormal-intensity areas in thalamus, globus pallidus, hippocampus, and substantia nigra, providing anatomic correlates of the emotional lability, memory impairment, and parkinsonism.

Diagnosis

Definitive diagnosis is similar to that described for SLE. In fatal cases, virus isolation and demonstration of viral antigen in brain tissue are feasible. Rapid diagnosis may be achieved by staining for antigen in mononuclear cells recovered from CSF (470). Virus isolation from blood is infrequent. However, virus may be isolated from CSF in up to one third of patients during the acute phase, and isolation is correlated with a poor prognosis. Serologic diagnosis depends on the demonstration of a fourfold or greater rise (or fall) in appropriately timed serum specimens. The HI, CF, and NT are applicable. Cross-reactions with heterologous viruses, particularly WN, may complicate the serodiagnosis in tropical areas of Asia. Serum IgM antibodies appear early, usually disappear by 3 to 6 months after onset, and are usually serologically specific (85). The IgM-capture ELISA is especially well suited for diagnosis by detection of locally synthesized antibody in the CSF (85,88,140), separating patients with virus infection of the CNS from those with other etiologies of encephalitis but serologic evidence for systemic (extraneural) JE infection. Intrathecal antibody synthesis can also be estimated by use of an IgG index or CSF IgG–to–CSF albumin ratio. The kinetics of the systemic and local CNS antibody response have prognostic importance; patients who survive have earlier and more vigorous responses. IgM antibody synthesis may persist in CSF for weeks after recovery, indicating persistent antigenic stimulation (587). Monoclonal epitope-blocking immunoassays may be used to identify JE-immunity among patients with cross-reactive flavivirus antibodies (86).

Treatment

There is no specific treatment. Good supportive care (as discussed for SLE) is essential. Vigilance should be maintained for severe hyperthermia and convulsions, and specific countermeasures should be applied. Cerebral edema has been postulated to be an important factor in pathogenesis. However, a controlled trial of high-dose corticosteroid therapy failed to show improvement in clinical outcome, mortality, or sequelae (306). There have been a few modest tests to determine whether there are any effective drug therapies for JE. In a Vero cell culture system, a furanonaphthoquinone derivative, FNQ3, showed some antiviral activity (703). Suramin, an antitrypanosoma drug, showed good antiviral activity against JE replication. Although these drugs are promising, their safety and *in vivo* effectiveness are still unknown. A technical guide to the clinical diagnosis and management of patients has been prepared by the World Health Organization (WHO) (777).

Epidemiology

Morbidity and Mortality

Japanese encephalitis virus is widely distributed in Asia, including Japan, China, Taiwan, Korea, Philippines, far-eastern former Soviet Union, all of Southeast Asia, and India (Fig. 12). About 50,000 cases and 10,000 deaths are recognized annually throughout Asia, but the disease is greatly underreported (732). The incidence in Japan, South Korea, and Taiwan has declined dramatically since the 1980s. Large numbers of cases (more than 10,000 per year) occur in China, but the annual incidence is also declining. In contrast, epidemic activity in northern and central India, Nepal, and the northern part of Southeast Asia has increased since the early 1970s (204). In one study of 740 consecutive hospital admissions with encephalopathy in India, 23% were due to JE virus, and 37% of these patients died (377). In northern Thailand and in Vietnam, outbreaks occur with attack rates of up to 10 to 20 per 100,000, an incidence similar to that of poliomyelitis in the United States before the advent of vaccines (305). The increase in JE virus transmission in these areas is linked to deforestation, agricultural development, and irrigation schemes for rice cultivation, leading to high-density breeding of *C. tritaeniorhynchus* (552). A recent episode in the island of Saipan illustrated the dynamics of JE infection after introduction of the virus into a susceptible area outside of the endemic zone (549). Virus introduction

was followed by intense amplification, universal infection of the pig population, infection of 4.2% of the human population, and a disease attack rate of 25 per 100,000.

FIG. 12. Distribution of Japanese encephalitis virus in Asia.

Children are primarily affected by the disease; attack rates in the 3- to 15-year age group are 5 to 10 times higher than in older individuals because of high background immunity in the older age groups. Epidemics in nonendemic regions have affected all age groups, but a bimodal age distribution (young children and the elderly) has appeared, indicating an increased risk in elderly people, as seen in SLE.

In endemic areas, nearly all people have sustained infection by young adulthood. The ratio of inapparent to apparent infections is 200:1 to 300:1 (45). Among factors that influence this ratio are age, differing virus strain virulence, and cross-protective immunity to other flaviviruses, especially dengue.

In tropical areas, there is an endemic pattern of infection, with occurrence of sporadic cases throughout the year. In temperate zones and in the northern part of the tropical zone, outbreaks have a marked seasonal incidence during the rainy (monsoon) season (July to September). Precipitation and temperature are important determinants of vector density and rate of virus transmission. The incidence is highest in rural agricultural areas. An excess of cases has been noted in males in many outbreaks, presumably because of increased exposure in areas of rice cultivation.

The reasons for the absence of epidemics in tropical countries are poorly understood. In southern Thailand, intense JE virus transmission to pigs has been demonstrated in the absence of epidemic human disease (89). The RNA genome of virus isolates from this area differed significantly from northern Thai epidemic strains, suggesting that a difference in virulence may be responsible. Other factors to be considered include vector–host relationships, vector abundance, vector competence, and cross-protecting heterologous immunity to dengue.

Transmission Cycle

Birds and pigs are effective viremic amplifying hosts, serving as the source for infection of mosquito vectors (see Fig. 11) (645). Cattle are frequently infected but have low viremias and do not perpetuate virus transmission (323). In temperate regions, virus transmission is detectable in July in mosquitoes, pigs, and birds, principally ardeid species (egrets, black-crowned night herons) and possibly ducks. Human infections occur several weeks later. The main epidemic vectors are *Culex* species, of which *C. tritaeniorhynchus* is the most important. It breeds in irrigated rice fields, shallow ditches, and pools, with peak population densities during the monsoon season. Infection rates in *C. tritaeniorhynchus* may exceed 1% during periods of peak transmission. Other species implicated in transmission include *Culex vishnui* (India), *Culex gelidus* and *Culex fuscocephala* (India, Malaysia, Thailand), *Culex annulus* (Taiwan), and *Culex annulirostris* (Guam). Isolations have been made from many other species of *Culex, Anopheles,* and *Aedes.*

The overwinter maintenance of JE in temperate areas has not been fully elucidated. Vertical transmission of JE virus by *Culex* and *Aedes* species has been demonstrated in the laboratory and by field studies (545,622,628). The virus is also sexually transmitted from male to female mosquitoes. Japanese encephalitis virus and antibodies have been found in bats in Japan. Experimentally infected bats held in simulated hibernation develop persistent infections and, upon warming, have viremias sufficient to infect mosquitoes. Japanese encephalitis virus and antibodies have been found in reptiles (668), but their role in the ecology of JE remains unknown. Certainly, the demonstration that latent and recrudescent infections occur in experimentally infected mice as well as in humans lends credence to the view that wild or domestic vertebrate hosts could play a reservoir role in overwinter maintenance. In tropical regions, year-round transmission of the virus among mosquitoes, birds, and pigs probably occurs (235).

Japanese Encephalitis in Travelers

Cases of JE have occurred among travelers to Asia. Between 1978 and 1992, 24 such cases were reported (24). Although the risk for overt disease among travelers is low (estimated to be 1 in 5,000 to 1 in 20,000 per week of travel, varying with location of travel, season, and rural exposure), the severity of the disease has encouraged use of vaccine (see Control, later). Among JE cases with known outcome among travelers, 40% died, and 36% had residual disabilities.

Molecular Epidemiology

Japanese encephalitis virus diversity has been determined by primer-extension sequencing of a region in the *C/prM* gene (122,321). These analyses demonstrated four geographically distinct genotypes of JE virus in Asia differing by more than 12% at the nucleotide level. Genotype I consists of virus strains from a broad region encompassing northern Asia (Japan,

China, Taiwan), Vietnam, Nepal, India, and Sri Lanka. Genotype II consists of strains from northern Thailand and Cambodia. A third genotype contains strains from Indonesia (Sarawak, Java), Malaysia, and southern Thailand. A fourth comprises isolates from eastern Indonesia (Java, Bali, and Flores) (213). It is of considerable interest that an epidemic pattern of disease expression prevails in areas where genotypes I and II circulate, but an endemic pattern is seen in areas represented by genotypes III and IV. Whether this reflects a difference in virus virulence or in epidemiologic and ecological factors remains uncertain. The very large and continuous geographic distribution of genotype I (from Japan to Sri Lanka) suggests the possibility of gene flow, but the virus distribution does not appear to fit patterns of bird migration.

Control

Vaccination

Vaccines have been used to prevent (a) encephalitis in horses and humans and (b) abortion and stillbirth in swine. Vaccination of horses with formalin-inactivated vaccines was the first successful application and afforded significant protection during an epizootic in Japan during 1948 to 1949. Since 1972, live attenuated vaccines have been licensed in Japan for use in pigs (722,781). Although immunization of pigs is a theoretical means of interrupting transmission and amplification of JE virus and thereby of preventing human infections, difficulties arise in practice. In many parts of Asia, pigs are only semidomesticated, and wide-scale immunization is difficult. In areas such as Japan, where swine husbandry is highly developed, pigs are born after the epidemic period, have interfering maternal antibody for 4 months, and are killed the following summer, leaving a narrow interval for vaccination.

Formalin-inactivated vaccines for use in humans are prepared from infected adult mouse brains or infected primary hamster kidney cell cultures in Japan and China, respectively (reviewed in 17 and 722). The mouse brain vaccine produced by the Research Foundation for Microbial Diseases (Biken), Osaka, Japan, is purified by protamine sulfate precipitation and ultracentrifugation and has been in wide use since the 1960s. A controlled trial of Biken vaccine in Thailand showed an efficacy of 91% (305). Mass vaccination campaigns have been carried out in Japan, Taiwan, and China, with children as the target population. Primary immunization requires at least two doses of 1.0 mL (0.5 mL in children younger than 3 years of age) at a 7- to 14-day interval. Although booster doses are recommended after 12 months, antibodies from the three-dose regimen have been shown to persist at protective levels for up to 3 years (202).

Because of the antigenic diversity of JE virus strains, efforts were made by Biken to improve on the original mouse brain vaccine produced from the 1935 Nakayama virus by incorporating a more recent isolate, Beijing-1 virus, representing a distinct (JaGAr-01) antigenic variant. Immunization of mice with Beijing-1 vaccine raised antibodies that were cross-reactive at higher titer with JE strains of different geographic origin. The Beijing-1 strain also replicates to higher titer in mouse brain. It is more potent and may be administered at a dose half that of Nakayama or bivalent vaccine. A monovalent Beijing-1 vaccine is currently produced for use in Japan, and it may ultimately replace the bivalent product for export. A comparison of Nakayama and Beijing vaccines in nonimmune Thai schoolchildren showed about 94% seroconversion against the homologous virus strain (620). At this time, there is no evidence to suggest that antigenic variation of vaccine or wild virus has a significant effect on vaccine efficacy.

Since 1981 when JE was proved to be a threat to travelers, there has been an international interest in vaccines. The Biken vaccine is licensed for use in the United States (marketed by Connaught Laboratories) and in Europe (Pasteur-Merieux). Studies in adults have shown that three (rather than two) inoculations are required to achieve protective immunity (neutralizing antibody titers of more than 10) in more than 90% of the vaccinees. Booster doses are recommended after 12 months. Side effects are generally inconsequential, consisting of local tenderness or mild systemic symptoms in 10% to 30%. Although produced from brain tissue of mice, the purified vaccine contains less than 2 ng/mL of myelin basic protein, an amount far below that capable of eliciting allergic encephalomyelitis in the sensitive guinea pig model. Only rare cases of postvaccinal encephalitis have been reported (540), and in such cases, a clear etiologic role for JE vaccine has been difficult to establish. Surveillance for postvaccinal neurologic events in Japan has not demonstrated an association between JE and neurologic complications.

Use of JE vaccine in adult travelers recently has resulted in a number of cases of allergic reactions, including urticaria, angioedema, bronchospasm, and erythema nodosum and multiforme (24). These reactions occurred acutely or after an interval of up to 7 days. The delay in onset appeared to lengthen when reactions followed the second or third vaccine inoculation. The incidence of these allergic phenomena has varied in different reports from 2 per 1,000 to as high as 1%. Allergic reactions in Western adult vaccinees appear to have increased in recent years, a phenomenon that was not explained by changes in vaccine manufacture or by use of specific vaccine lots.

Two other JE vaccines are currently in use in China: formalin-inactivated vaccine produced from primary hamster kidney (PHK) cells, and a live attenuated vaccine designated SA14-14-2, also prepared in PHK cell culture. Both vaccines are extensively used in China (719). Immunogenicity of the live vaccine in children exceeds 95%, but antibody titers appear to wane rapidly, and a booster dose is given at 12 months.

Efforts are underway to produce genetically engineered JE vaccines. Recombinant vaccinia (including the NYVAC vector, a highly attenuated strain of vaccinia suitable as a vaccine) encoding prM and E (or preM, E, and NS1) of JE virus elicited neutralizing antibodies and conferred a high level of protection against challenge of rhesus monkeys, mice, and pigs (366,367,581). Recombinant vaccinia vaccines expressing only the NS1 protein showed low levels of protection (364). The *PreM* and *E* genes were also inserted into a highly attenuated MVA strain of vaccinia virus (520), and immunized animals were completely protected against a lethal challenge of JE virus. Infection of mammalian cell cultures with recombinant vaccinia virus expressing the *prM* and *E* genes of JE virus results in the production of extracellular 20-nm subviral particles. These particles, composed of the prM/M and E viral proteins in a lipid bilayer, are free from nucleic acid, are easily purified, and are highly immunogenic, providing a practical approach to vaccine development (461). This type of extracellular particle has been effective in generating immunity to infection in Balb/c mice (367a). A full-length infectious clone of JE virus has been developed, allowing the introduction of attenuating mutations (702) and a potential means of constructing new live vaccines. Novel carrier and delivery systems for subunit or purified virion JE

vaccines have been investigated in preliminary studies, including polylactide-coglycolide–encapsulated vaccine and JE peptides linked to meningococcal proteosomes. In an attempt to simplify vaccination, a protocol calling for oral administration of an attenuated JE strain has been tested. Depending on the preparation, mice immunized in this manner showed 70% to 76% seroconversion. Although this shows that oral administration has potential, the level of protection needs to be improved for it to be a practical alternative.

DNA vaccinatons, one of the newest techniques in vaccinology, has been pursued in an attempt to generate new JE vaccines that will increase coverage. To date, three general strategies have been used in making DNA JE vaccines. One, the genes *PreM* and *E* are expressed by the DNA vaccine. This leads the cells transfected to secret virion-like particles, which are excellent immunogens. Tests with these vaccines have induced excellent and, in some cases, long-lived protection from challenge (109,360,362). In the second approach, only the E protein is encoded by the DNA vaccine. The E protein is either present in the cytoplasm of transfected cells or can be secreted as a dimer. These DNA vaccines have also demonstrated good protection against JE viral challenge (27,118). Third, some studies have involved DNA vaccines coding for the nonstructural protein NS1 (423). In previous studies, purified NS1 protein injected into test animals elicited varying levels of immunity. A slightly truncated version of NS1 (designated NS1') was able to induce good protection against challenge.

A chimera yellow fever 17D JE virus has also been designed (105). The virus has the yellow fever PreM/E proteins replaced by the JE preM/E protein. Therefore, this virus theoretically has the immunogenicity of JE and attenuated phenotype (determined by the nonstructural proteins) of the yellow fever 17D strain. Preliminary tests in animal models indicated that the chimera is immunogenic and safe (247,489). Potentially, any flavivirus preM/E protein, as long as a viable virus results, could be exchanged for the native 17D strain proteins, creating a number of immunogenic and safe vaccines.

Mice have been effectively immunized with live JE virus administered orally (583).

Vector Control

Field trials of organophosphate larvicides and adulticides have proved effective against vectors of JE. Use of agricultural pesticides in rice-growing areas has also reduced populations of *C. tritaeniorhynchus.* Integrated programs, which include use of chemical larvicides, larvicidal fish, and biologic larvicides (*Bacillus thuringiensis*), elimination of aquatic vegetation in irrigation canals, and spraying of residual insecticides in livestock pens have reduced the case incidence in China (314). Emergency epidemic control requires aerial ultralow-volume (ULV) spraying of organophosphate adulticides.

Murray Valley Encephalitis Virus

Epidemics of encephalitis (Australian X disease) in southern Australia in 1917, 1918, 1922, and 1925 are now believed to have been due to MVE virus. In 1917 and 1918, an infectious agent was recovered by inoculation of monkeys (73), but the virus was not established or characterized. During an outbreak in 1951, the virus was isolated from human brain and was shown to be a flavivirus related to, but distinct from, JE virus (195). *C. annulirostris* was suspected to be the vector in 1951, and the virus was subsequently isolated from this mosquito in 1960 (168). Epidemics occurred in southeastern Australia in 1956, 1971, and 1974. Small outbreaks also occurred in the Kimberley and Pilbara regions of western Australia in 1971, 1978, 1981, and 1984. The disease was recognized in New Guinea in 1956 (197).

Infectious Agent

MVE virus is a member of the JE antigenic complex. Antigenic comparison of five MVE virus strains isolated in Australia and New Guinea by HI, kinetic HI, and plaque-reduction NT showed four strains to be similar and showed the fifth to have minor but reproducible antigenic differences. Strains from the 1974 epidemic were indistinguishable by standard serologic tests from isolates recovered during previous outbreaks (164).

Mapping of the E glycoprotein of MVE virus with monoclonal antibodies and synthetic peptides has elucidated T- and B-cell epitopes (274,464). The nucleotide sequence of MVE has shown it to be most closely related to JE virus (142,411).

Many vertebrate and mosquito cell culture systems propagate the virus. Plaque assays can be performed in primary chick embryo and continuous lines of pig kidney, monkey kidney, and hamster kidney cells (763). Immune enhancement of virus growth has been demonstrated in primary chick embryo fibroblast cultures mediated by a subpopulation of macrophages having Fc receptors (353). The host range of MVE virus has been reviewed by French (196). Infant mice are highly susceptible by all routes of inoculation. Mice develop clinical resistance to peripheral (but not intracerebral) inoculation between 17 and 28 days of age. Hamsters 6 to 10 weeks old are susceptible to lethal infection by all routes. Monkeys, horses, sheep, and some birds develop encephalitis after intracerebral inoculation. Pigeons and chickens infected subcutaneously develop viremic infections without clinical illness. Rabbits and guinea pigs have subclinical infections after intracerebral and peripheral inoculations. Chicken embryos are highly susceptible and have been used for primary isolation from human cases (195).

MVE virus is suspected to cause neurologic disease in horses, but field and experimental studies have failed to incriminate the virus conclusively (457). During epidemics of MVE, there are reports of dogs dying; dogs are susceptible to infection, as shown by a high antibody prevalence, but no evidence has been obtained for a pathogenic role of MVE virus.

Pathogenesis and Pathology

There is little available information on the experimental pathology of MVE in animals. One study compared the spread of MVE strains of high and low neuroinvasiveness in mice (583). The high invasive strain infection quickly spread to the

lymph nodes and produced viremia and then a CNS infection with infection through the olfactory bulb. In contrast, the low invasive strain did not infect the lymph nodes, had a 20-fold lower viremia, and caused a much less intense infection of the CNS, also invading through the olfactory bulb. The main difference in the two strains is the speed and magnitude of infection. Thus, the neuroinvasion by MVE could be seen as a race between the magnitude and breadth of viral replication and invasion in comparison to the host immune response. Pathologic findings in human cases of fatal encephalitis are similar to those in JE (607).

Clinical Features

The disease begins with a 2- to 5-day prodrome characterized by fever, headache, myalgia, generalized malaise, anorexia, and nausea, followed by the appearance of nuchal rigidity and neurologic signs. In infants, the disease progresses rapidly, and patients are frequently comatose when first brought to medical attention. Bennett (46) divided patients into three groups on the basis of severity of illness: (a) mild cases with altered level of consciousness and variable neurologic abnormalities but without coma or respiratory depression, accompanied by stabilization of neurologic changes within 5 to 10 days; (b) severe cases with coma, paresis, and paralysis, including respiratory and pharyngeal impairment requiring respiratory assistance; and (c) fatal cases with spastic quadriplegia and progressive CNS damage or severe disease with superimposed bacterial infection. Neurologic sequelae occur in up to 40% of the mild cases and in all of the severe cases that recover.

Attempts to associate MVE virus with mild febrile illnesses without neurologic signs have failed (164).

Diagnosis

Specific diagnosis depends on isolation of virus from brain tissue or serologic tests. Virus isolation from blood or CSF has not been successful. Isolations from brain have been made in chick embryos and suckling mice. For serodiagnosis, the ELISA, HI, CF, and NT are useful. Cross-reactions with dengue and with Kunjin virus may confuse interpretation. An epitope-blocking ELISA with MVE monoclonal antibodies proved useful in distinguishing MVE infections from those with other flaviviruses (276). Some patients with encephalitis (presumably MVE) have shown rising antibody titers to Kunjin (165), possibly on the basis of a previous Kunjin infection and the original antigenic sin phenomenon. IgM antibodies appear to be quite specific and useful for early diagnosis (769). There has been some recent development of reverse transcription–polymerase chain reaction (RT-PCR) assays for MVE (482). These could provide earlier and more specific diagnosis than the conventional tests.

Treatment

Treatment is as described for SLE and JE.

Epidemiology

Epidemics have occurred principally in the Murray Valley region of New South Wales and Victoria, involving up to about 50 human cases (457). The most notable recent outbreak, in 1974, was unusual in its geographic extent, with patients also infected in east central Queensland, Northern Territory, northern and southeastern South Australia, and the Ord River Basin of Western Australia. Sporadic cases were reported in New Guinea. Outbreaks in Australia occur during the summer (January to May) and appeared to follow periods of abnormally high rainfall for two consecutive years. Water impoundment and irrigation schemes were responsible for the emergence of MVE in Western Australia in the 1970s. A mathematic model of MVE has provided hypotheses regarding amplification and dissemination of the virus in southern Australia (350).

Population-based incidence data on MVE are not available. Before 1974, children were predominantly affected. In the 1974 epidemic, however, 35% of the patients were younger than 10 years old, and a similar proportion were older than 50 years of age (165). A serologic survey conducted after the 1951 epidemic in various localities in Victoria showed 4.5% to 36% positive CF tests; the rate in children was about half that in adults (16). A statistically valid HI antibody survey conducted 8 months after the 1974 epidemic showed 4.5% positive overall. Follow-up surveys indicated no MVE virus activity after 1974, despite a high incidence of human infection with other flaviviruses (275). MVE is endemic in the tropical Kimberley area of Western Australia (675). Between 1978 and 1991, there were 26 reported cases of Australian encephalitis in Western Australia, of which 16 occurred in the Kimberley region (443). Of 20 cases serologically diagnosed, 18 had MVE, and 2 had Kunjin virus infections. Sixty-five percent of the encephalitis cases were in Aboriginal children, and 85% were male.

The principal vector is *C. annulirostris,* a transient pool breeder. The virus has also been isolated from *Aedes normanensis, C. tritaeniorhynchus,* and *C. pipiens. C. pipiens,* once considered to be a potential vector on epidemiologic grounds, has proved to be poorly susceptible or refractory to oral infection in experimental studies, whereas *C. annulirostris* is a highly efficient vector (348).

Large water birds (herons, egrets, and pelicans) appear to be the most important viremic hosts. However, mammals (including rabbits and kangaroos) may contribute significantly to the transmission cycle.

The overwintering mechanism and origin of intermittent epidemics are unknown (reviewed in 457).

Molecular Epidemiology

MVE viruses analyzed by mapping restriction enzyme digests and by nucleotide sequencing showed a remarkable homogeneity of strains from widely separated areas of Australia over a 23-year period (433). This finding suggests continuing gene flow, possibly by widely ranging water bird hosts. Strains from New Guinea differ, suggesting that this

area represents a separate focus.

Prevention and Control

In parts of Australia, MVE is a significant health risk to infants. Animal tests indicate that passive immunization with gammaglobulin of patients who were sublethally infected with MVE or JE can be an effective prophylactic measure (79,113). However, sera from patients who were immunized with the JE vaccine are not protective and instead appear to enhance the infection.

Recently, two types of vaccines have been investigated in a murine model: a DNA vaccine and a Semliki Forest virus–vectored vaccine (133,171,480). Both vaccines use the PreM/E proteins as immunogens and induce. In areas prone to recurrent epidemics (e.g., the Murray River basin), reduction of *C. annulirostris* breeding by use of larvicides is practiced. Use of rainfall data and surveillance of virus activity in mosquitoes and sentinel fowl to predict risk for epidemic allows targeted mosquito control efforts.

Central European and Russian Spring-Summer (Tick-borne Encephalitis) Viruses

TBE was clinically described in the far-eastern Soviet Union in 1934. In 1937, the causative virus was isolated from the human brain and demonstrated to be transmitted by ticks (679). The disease was first recognized in eastern Europe (Czechoslovakia) during an epidemic in 1948, and a virus isolated from a patient was shown to be similar to the Far Eastern virus. Transmission by ingestion of unpasteurized goat's milk occurs in both Russia and Europe. The disease has been recognized in all Central and Eastern European countries and Scandinavia as well as in France, Italy, Greece, and Albania.

Infectious Agents

The TBE complex consists of 17 antigenically closely related viruses, 13 of which cause human disease (see Table 1). Russian spring-summer encephalitis (RSSE) and Central European encephalitis (CEE) viruses are closely related antigenically and have been considered subtypes of the same virus. They share about 96% of their amino acids in the E protein and can be distinguished by means of protein E–specific monoclonal antibodies (312). No significant difference, however, has been observed in cross-protection experiments when mice were immunized with the European subtype and then challenged with different European and Far Eastern strains (312). As discussed below, the two subtypes also differ in their tick vectors and clinical expression. Remarkable stability of the CEE genome and antigenic determinants has been demonstrated among strains from different areas of Europe (248,312,766). The morphology, chemistry, and antigenic composition of TBE complex viruses are similar to those of other flaviviruses (285,293). Although the E protein of TBE virus undergoes specific conformational changes at acidic pH, which are associated with a reduction in infectivity (292), a significant degree of resistance to acid pH in sour milk (232) and gastric juice (560) has been reported. This feature is apparently responsible for oral infections through milk.

TBE complex viruses grow in a variety of cell cultures, including pig, bovine, and chick embryo, HeLa, Detroit 6, human amnion, Hep 2, Vero, and primary reptilian and amphibian cells (573), but CPE and plaquing are variable. Chicken embryos and a variety of animals are susceptible to infection. Infant and weanling mice develop fatal encephalitis by all routes of inoculation. Rats, guinea pigs, sheep, monkeys, and swine succumb to encephalitis after intracerebral inoculation. Hamsters are less susceptible to intracerebral and peripheral challenge than mice. Experimental inoculation of wild vertebrate species (including rodents, insectivores, foxes, birds, hares, and bats) results in viremia and antibody formation (230). Cows, goats, and sheep experimentally infected by inoculation or tick bite develop viremia and secrete virus in their milk. Mice can also be infected by the oral route, with subsequent shedding of virus in feces and milk (561).

The Far Eastern virus type (RSSE) is more virulent for sheep and monkeys inoculated intracerebrally than the Western (CEE) virus (789). However, no distinct difference has been noted in pathogenicity for mice or cell cultures (37). Variation has been found between wild virus strains in their ability to produce viremia in a natural host species, *Clethrionomys glareolus* voles (127).

Pathogenesis and Pathology

The pathogenesis in mice was reviewed by Albrecht (9) and does not differ significantly from the general scheme presented earlier. In monkeys, the anterior horn cells of the spinal cord and cerebellar cortex appear to be selectively more vulnerable to tick-borne viruses than other neuronal centers (525). An apparently unique feature of members of the TBE virus complex may be their propensity to cause persistent infections in experimental animals (and possibly also in humans). Mice infected with Kyasanur Forest virus may survive for months with paralysis, low titers of virus in the brain, and absence of detectable neutralizing antibodies (570). Hamsters infected with CEE virus develop late CNS lesions, including proliferation and hypertrophy of astrocytes (794), and have detectable viral antigen in tissues for up to 4 months. Louping ill virus persists in immunosuppressed guinea pigs for 50 days (793). Monkeys infected intranasally or intracerebrally with CEE virus develop a chronic encephalitis with degenerative spongiform lesions and astrocytic proliferation (794). The virus has been isolated from monkey tissues by cocultivation and explant procedures as long as 383 days after inoculation (563). Chronic progressive human encephalitis and seizure disorders (Kozhevnikov's epilepsy) have been associated with RSSE virus on serologic grounds (539), and virus has been isolated from the CSF of a patient with a disease clinically resembling amyotrophic lateral sclerosis (515). These observations support the notion that flaviviruses produce persistent infection in sequestered neuronal tissues of human hosts (see Pathogenesis and Pathology, earlier).

The neuropathology of experimental infection in mice has been reviewed by Vince and Grcevic (746), and the pathology of infection in humans has been reviewed by Zilber and Soloviev (790). Findings are generally similar to those of other flaviviral encephalitides. Gross changes include swelling, congestion, and petechial hemorrhages. Histopathologic alterations include meningeal and perivascular inflammation, neuronal degeneration and necrosis, neuronophagia, and

glial nodule formation involving cerebral and cerebellar cortex, brainstem, basal ganglia, and spinal cord. The anterior horn cells of the cervical cord are especially vulnerable, explaining the predominance of lower motor neuron paralyses of the upper extremities seen in many cases.

Clinical Features

The Central European form (CEE) is milder than RSSE and typically takes a biphasic course. The first phase is an uncharacteristic influenza-like illness with fever (temperature usually not exceeding 38°C), headache, malaise, and myalgia that arises after an incubation period of 3 to 7 days and lasts for about a week. After an asymptomatic period of up to a week, 20% to 30% of those affected develop the second phase of the disease, which involves the CNS. It may take the form of aseptic meningitis, meningoencephalitis, meningoencephalomyelitis, or meningoencephaloradiculitis (579,580). The case fatality rate is 1% to 2%, and the disease in children is less severe than in adults. Meningitis usually resolves completely. However, 10% to 20% of patients with the more severe clinical forms have long-lasting or permanent neuropsychiatric sequelae. These tend to be minor in degree, and motor defects are rare; CSF changes are similar to those in the other flavivirus encephalitides.

The disease in the Far East (RSSE) differs clinically from CEE. Onset of illness is more often gradual than acute, with a prodromal phase including fever, headache, anorexia, nausea, vomiting, and photophobia. These symptoms are followed by stiff neck, sensorial changes, visual disturbances, and variable neurologic dysfunction, including paresis, paralysis, sensory loss, and convulsions. In fatal cases, death occurs during the first week after onset. The case fatality rate is about 20% (229) but may be biased by the lack of hospitalization of mild cases. In contrast to CEE, the disease in children is more severe than in adults. Neurologic sequelae occur in 30% to 60% of survivors, especially residual flaccid paralyses of the shoulder girdle and arms.

Diagnosis

Fewer than half of patients give a history of tick bite. At the time of admission to hospital (i.e., after onset of CNS symptoms), specific antibodies are almost always detectable. Diagnosis is most often made by serologic tests, especially the detection of IgM antibodies by ELISA (304,619). Using sensitive tests, IgM antibodies may be detectable for up to 10 months after disease onset. The virus may be isolated from the blood during the first phase of illness and from brain tissue of patients dying during the early phase of the disease.

Epidemiology

TBE occurs in an endemic pattern over a wide area of Europe and the former Soviet Union, corresponding to the distribution of ixodid tick vectors (Fig. 13). The annual incidence is several thousands of cases, with considerable variation from year to year. In Europe, the highest morbidity has been recorded in Austria, Slovenia, Hungary, and the Czech and Slovak Republics, with several hundred cases occurring annually in these countries (57,230). In the former Soviet Union, 5,500 cases were reported in 1990. The disease occurs in foci characterized by ecological habitats favorable for ticks, and very little variation in geographic distribution is observed. The intensity of transmission varies from year to year; increases in small mammal populations (the principal hosts for immature ticks), with subsequent migration, are followed within 1 to 2 years by increased tick populations and a higher risk for human infections.

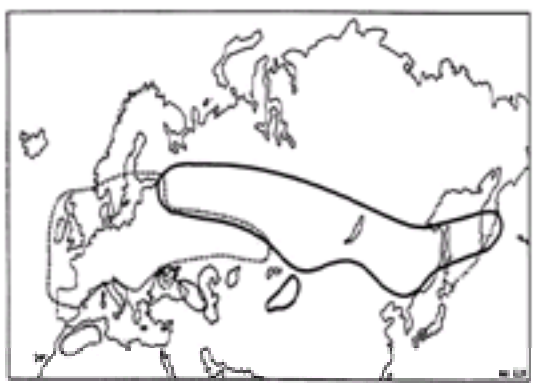

FIG. 13. Distribution of *Ixodes ricinus* (vector of Central European encephalitis and louping ill viruses; *dotted line*) and *Ixodes persulcatus* (vector of Russian spring-summer and encephalitis; *solid line*). (From ref. 57, with permission.)

The virus is maintained in nature in a cycle involving ticks and wild vertebrate hosts (Fig. 14). At least 10 species of rodents have been implicated as amplifying hosts (229,230). Insectivores (shrews, moles, and hedgehogs, which have relatively stable populations, in contrast to rodents), are believed to be important reservoirs. Large mammals, such as goats, sheep, and cattle, are important blood-feeding hosts for adult ixodid ticks but have low viremias and are not considered to be important sources of tick infection. The virus is excreted in the milk, however, and human infection may result from consumption of unpasteurized goat or sheep milk or cheese. Small outbreaks, involving all age groups and often in family groups, result from consumption of raw sheep or goat milk or cheese (231). Vertebrate hosts may also be involved in overwintering. Prolonged viremic infections have been demonstrated in hibernating dormice and hedgehogs, bats, and ducks.

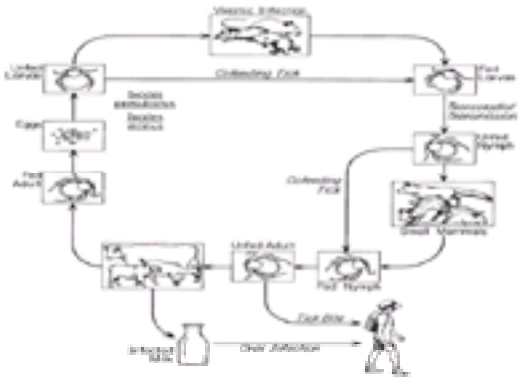

FIG. 14. Generalized transmission cycle of tick-borne flaviviruses, showing hosts for larval, nymphal, and adult ticks. Virus is passed to succeeding tick stages during molting as well as transovarially to progeny of adult ticks. Both male and female ticks are involved in transmission. Tick-borne encephalitis virus (and possibly other tick-borne flaviviruses) may be transmitted to uninfected ticks cofeeding on a vertebrate host without the requirement for active viremic infection of the host.

Ixodes ricinus and *Ixodes persulcatus* are responsible for transmission in Europe and the Soviet Union, respectively (see Fig. 13). Other tick species, of the genera *Dermacentor* and *Haemaphysalis,* have also been implicated in transmission, especially in areas that do not support *Ixodes* species ticks. Pre-imago ticks acquire infection by feeding on small mammals and pass the virus transtadially to the adult stage. Transtadial and transovarial virus transmission has been demonstrated in *Ixodes, Dermacentor,* and *Haemaphysalis* species. Considerable losses of virus occur with tick molting from stage to stage. About 1% of the progeny of an infected female *Ixodes* species tick acquire the virus. Although ticks may serve as a natural reservoir and overwintering mechanism, horizontal transmission between vectors and vertebrates is required for endemnicity. A mathematic model has been described that predicts age-specific infection rates and infection risk in hyperendemic foci (215).

The propagation of the TBE viruses does not appear to follow the classic model of the vertebrate host acting as an amplification and reservoir from which vectors are infected. Compared with other tick-borne diseases, such as Lyme disease, TBE has a far more focal distribution. This pattern suggests that the conditions of its maintenance are much more narrowly defined (584). When this pattern and the low and transitory nature of TBE viremia are considered, the persistence of TBE would seem to be very tenuous. Three factors help to define an alternative cycle in which TBE is propagated. First, the well-known, overall, synchronous seasonal activity of the vector. Second, TBE virus can be transmitted efficiently between infected and uninfected ticks cofeeding on the same host without the host supporting an infectious viremia. Finally, this transmission can occur at a high level, 89% (400), even on immune hosts.

In Central Europe, the activity of adult ticks occurs in two peaks (May to June and September to October), that are followed by two peaks of human disease at a 3- to 4-week interval, whereas in Scandinavia, only one peak is seen in late summer. Forestry workers, farmers, and others working in forest or scrub areas are at highest risk. In recent years, however, the infection has increasingly affected people in their leisure and travel activities. An imported case of TBE occurred in a child in Ohio (138).

Before the availability of an effective vaccine and biosafety precautions, laboratory infections with TBE virus were not uncommon (646).

Molecular Epidemiology

A phylogenetic analysis of the E protein of 25 TBE viruses revealed three genetic lineages. These correspond to European, Far Eastern, and Siberian subtypes (175). The variation between subtypes within strains was low, with a maximum of 2.2% at the amino acid level; between strains, the variation was 5.6%. This range is similar to that reported for other flaviviruses. The new Siberian subtype is more related to the Far Eastern than to the European subtype, which geographically makes intuitive sense. Although the differences between the subtypes are minor, they do have a significant effect on TBE virulence (277). Disease caused by TBE is more severe in Asia than in Europe, with case fatality rates of up to 30% and 1% to 2%, respectively. As with other flaviviruses, relatively few amino acid changes can have a significant effect on virulence (750).

Prevention and Control

Using Far Eastern strains of TBE virus, formalin-inactivated mouse brain vaccines were in used before World War II in the former Soviet Union. A live, attenuated heterologous (Langat) vaccine developed in Czechoslovakia was investigated (471) and abandoned. Formalized cell culture–derived vaccines were developed in Russia, and at the present time, an unpurified and a partially purified inactivated chick embryo cell vaccine are in use (180). An experimental vaccine has also been prepared using virus grown in a green monkey kidney cell line (126).

In Europe, a partially purified formalin-inactivated vaccine based on an Austrian virus isolate grown in chick embryo cells became commercially available in 1976 (372,388). This vaccine gave satisfactory seroconversion rates but caused a significant rate of side reactions, such as headache, malaise, and fever. This reactogenicity was almost completely eliminated when the vaccine was prepared in a highly purified form (286,386), which is licensed in several European countries. It contains Al(OH) as an adjuvant and has an efficacy of about 97% to 98% in the field. In Austria, mass vaccination was initiated in 1981 and resulted in a dramatic decline in the annual incidence of disease (389). A second European TBE vaccine has more recently been registered in Germany (267,355), and demonstration of efficacy will have to await results from longer-term usage. Vaccination may be warranted in people living in endemic areas, those working

under high-risk conditions (foresters, woodcutters, farmers, military personnel, laboratory workers), or travelers engaged in high-risk activities (e.g., field work, camping).

For passive immunization, a specific TBE-immunoglobulin is also available in several European countries that can be used for preexposure and postexposure prophylaxis (124). When given within 4 days after tick bite, protective efficacy was estimated to be 60% to 70% (387).

A difficulty in achieving complete TBE vaccination coverage has been the higher level of adverse reactions in children. In a controlled trial, a reduced amount of the Ecepur (TBE) vaccine was shown to induce an effective immune response and to have a much lower level of side effects (213). Another study showed that rapid vaccination protocols using inactivated vaccines could be successful in protecting recipients. This sort of rapid vaccination would be useful for individuals, such as those involved in military or relief operations, who have received short notice of a trip into an endemic area (689).

The high degree of relatedness of the TBE complex would seem to allow a vaccine against one subtype to be effective against another. This was confirmed in a murine study in which immunization with a vaccine against a European strain induced antibodies against a Far Eastern strain. Inactivated TBE vaccines have adjuvant/carrier activities for unrelated antigens and have been proposed as components of multivalent vaccines containing weak antigens (412).

Several new types of TBE vaccines are under development (282). A vaccinia virus vector expressing the PreM/E proteins induces protective immunity against challenge with virulent TBE virus (313). A chimera Langat/dengue type 4 attenuated vaccine induces neutralizing antibodies and resistance to fatal Langat virus–induced encephalitis (557). Naked DNA vaccines expressing PreM/E proteins of RSSE and CEE induced protective immunity against RSSE and CEE challenge (659). Another DNA vaccine study looked at how different forms of the E protein would effect its immunogeneity. In this study, the E protein was either intracellular, secreted as an E dimer, or secreted as a recombinant subviral particle (3). The best immunogen was the RSP. The E-dimer DNA vaccine elicited suboptimal protection, whereas the RSP DNA vaccine induced 100% protection. Synthetic infectious RNA has been used to immunize mice in a model system (447), and recombinant adenovirus carrying the gene for NS1 has also been tested in mice (712).

The use of repellents or protective clothing may reduce the risk for tick bite, although this is not a practical prophylactic measure, especially for people at permanent risk for exposure. In the past, widespread use of pesticides, including aerial applications of pesticides, was undertaken in some areas in attempts to interrupt transmission.

Other Viruses Causing Encephalitis

Rocio Virus

Rocio virus, a classic emerging virus, was first isolated from fatal human cases in 1975 during an explosive outbreak of encephalitis on the south coast of Sao Paulo State, Brazil (437,438). The virus is not placed in any of the flavivirus antigenic complexes, but it cross-reacts most closely with members of the JE subgroup, especially Ilheus virus. A plausible explanation for its sudden appearance is a mutation in a strain of Ilheus virus, which has a wide geographic range, including Brazil and Argentina. However, at this writing, neither Rocio nor Ilheus virus has been sequenced in an attempt to elucidate the origin of the epidemic.

The pathologic changes in humans are similar to those of JE and SLE infections: the most severely affected structures in seven patients studied were thalamus, dentate nucleus of the cerebellum, hypothalamic nuclei, and substantia nigra (629). In experimentally infected suckling hamsters, Rocio virus produced severe necrosis of myocardium and pancreas (269).

The clinical features in human cases are similar to those of SLE (713). The case fatality rate in hospitalized patients was 4%. Sequelae, including persistent cerebellar, motor, and neuropsychiatric signs, were noted in 20% of survivors. Diagnosis is by virus isolation from brain tissues of fatal cases or by serology.

Epidemics in coastal Sao Paulo State in 1975 and 1976 resulted in 971 cases, with attack rates of up to 38 per 1,000 inhabitants (326,327,438). The disease appears to have virtually disappeared, suggesting that the emergent virus may not have been well adapted for sustained transmission. Since 1976, only one serologically diagnosed case has been reported (a fatal case in an infant in 1980) (328), although other, clinically suspect cases continue to occur. The highest disease incidence during the outbreak of 1975 to 1976 was in young adult males engaged in outdoor work in impoverished agricultural areas. Wild birds are presumed to be viremic hosts in the transmission cycle. A single virus isolation was made from *Psorophora ferox* mosquitoes. *Aedes serratus* and *Aedes scapularis* were implicated as epidemic vectors of Rocio virus on epidemiologic grounds (194). Both *P. ferox* and *A. scapularis* transmit Rocio virus experimentally, whereas *A. serratus* does not (486). A mouse brain vaccine lacked potency (439).

Louping Ill Virus

Louping ill was recognized as a neurologic disease of sheep in Scotland during the late 19th century. Isolated in 1929 (567), the virus is a member of the TBE virus complex. By monoclonal antibody analysis, louping ill and CEE viruses belong to a distinct western subtype of the TBE virus complex (207,690). Phylogenetic analysis using the envelope gene sequence from various isolates revealed the presence of three major geographic populations the Irish, Welsh, and British variants (473). Louping ill is different from the other TBE viruses in that it is associated with a forest environment. This analysis also suggests that an ancestral virus was originally introduced to Ireland from Continental Europe and emerged as the distinct louping ill virus 800 years ago. From Ireland the virus dispersed, mostly in the last 130 to 300 years. There is suggestive evidence that the dispersal of louping ill followed the export of cattle from Ireland to England and spread further along routes of cattle transit. Although impossible to substantiate, this would be a classic dispersal of a viremic host into a virgin area, where the local arthropods feed off of the imported host and then form a new transmission cycle.

Louping ill virus kills suckling mice by all routes of inoculation; weanling mice, hamsters, and guinea pigs develop fatal encephalitis after intracerebral inoculation. Many cell cultures propagate the virus, and plaque assays may be performed in PS, Vero, and LLC-MK2 cells.

Experimentally infected sheep develop prolonged viremia followed by ataxia, paralysis, and tremors (591). Pathologic changes include diffuse meningoencephalitis with severe chromatolysis and destruction of Purkinje cells, reactive gliosis, and astrocytosis. Although clinical disease has been described in cows, horses, pigs, goats, deer, and dogs, louping ill is predominantly a disease of sheep, which develop a biphasic illness; the first phase is characterized by fever and weakness, followed by a neurologic illness with prominent cerebellar ataxia, hyperexcitability, and progressive paralysis. Epornitics of fatal disease in wild red grouse have been described (592). Experimentally infected red grouse also develop fatal infection and viremias sufficient to infect tick vectors (590).

The human disease was first described in a person with a laboratory infection (605). Thirty-nine human cases have been reported, of which 26 resulted from laboratory exposure (146,674). Of the natural infections, some followed tick bites, whereas others followed direct exposure of abattoir workers, butchers, and veterinarians to sick sheep. Shepherds and crofters are also occupational risk groups. Infection by the oral route has been documented in animals ingesting infected milk or lamb carcasses.

The human disease is biphasic and resembles CEE. The incubation period is 4 to 7 days. The first phase is influenza-like, lasting 2 to 11 days, followed by a remission of 5 to 6 days and then the reappearance of fever and a meningoencephalitic syndrome that last 4 to 10 days. There is leukopenia during the first phase and leukocytosis during the encephalitic phase. No deaths have been reported. The clinical features are reviewed by Smith and Varma (674) and Webb and colleagues (756). Asymptomatic infections are not infrequent, and abortive, influenza-like illness also occurs. In one laboratory-acquired case, a hemorrhagic diathesis developed, and the disease closely resembled Kyasanur Forest disease. Treatment is symptomatic. Diagnosis is by virus isolation from blood during the first phase of illness, from CSF during the early encephalitic phase, or by serologic tests (HI, CF, neutralization). Antibodies are produced locally in the CNS. The serum-to-CSF antibody ratio has been employed in human diagnosis (756).

Louping ill virus is distributed in Scotland, northern England, Wales, and Ireland. A virus serologically identical to louping ill virus was recovered from sick sheep in Norway (589). A closely related but distinct agent in Turkey has been associated with louping ill–like disease in sheep (Turkish sheep encephalitis) (205). The transmission cycle of louping ill involves *I. ricinus* ticks and both sheep and grouse (319). As with other TBE viruses, transmission can occur between infected and uninfected ticks that are cofeeding on the same host (341). Spread in this manner can occur on immune or nonimmune host animals, although it seems to be much more efficient on nonimmune animals. Control of the disease in sheep is by vaccination, dipping with residual acaricides, and environmental control of ticks. The vaccine now in general use is grown in sheep kidney cell cultures, is formalin inactivated, and is concentrated by methanol precipitation (80). A killed vaccine for use in laboratory and abattoir workers was once used on an experimental basis (178). It is likely that the TBE vaccine (prepared against the western subtype) would cross-protect against louping ill infection. A louping ill virus vaccine using Semliki Forest virus particles for delivery appears to provide excellent protection in several of the combinations (189).

Modoc Virus

Modoc virus was first isolated in 1958 from white-footed deer mice (*Peromyscus maniculatus*) collected in Modoc County, California. Isolations have subsequently been made from the same species in Oregon, Montana, and Alberta, Canada. The virus is antigenically related to other flaviviruses recovered from rodents (Cowbone Ridge, Jutiapa, Sal Vieja, and San Perlita viruses), and is probably transmitted by contact. Virus isolations have been obtained from throat swab specimens of deer mice trapped in the wild. It has not been possible to infect mosquitoes experimentally. The compartmentalization of virus transmission in a rodent–rodent cycle probably accounts for the scarcity of human infections. A single human case of illness attributed to Modoc virus was reported in California in 1966 (588). The patient was a 10-year-old boy who presented with a clinical picture of aseptic meningitis. The infection may have been acquired in Tulare County, where the patient played with deer mice near a rural cabin.

Powassan Virus

Powassan virus was first isolated in 1958 from a fatal case of encephalitis in Ontario (478). It is a member of the TBE virus complex but is more distantly related than other viruses in the complex both antigenically (92) and genetically (449). The virus is pathogenic for infant and weanling mice by the intracerebral and intraperitoneal routes; hamsters and rabbits develop subclinical infections. Experimental encephalitis has been demonstrated in rhesus macaques. Continuous cell lines of monkey kidney origin are useful for virus assay by CPE and plaque formation. Pathologic changes in the brains of mice, monkeys, and humans are typical of other flavivirus infections. Powassan infection is characterized by a variable period of fever and nonspecific symptoms, followed by neurologic signs of meningeal irritation and encephalitis, which are often severe. Serious neurologic sequelae have occurred in 35% of the reported cases, and in one, these resembled RSSE with shoulder-girdle involvement (134). In another case, the acute disease closely mimicked herpes encephalitis with temporal lobe involvement. In cases reported from the former Soviet Union, a characteristic syndrome with prominent cerebellar signs differentiated the disease from RSSE (414).

The disease occurs in Russia, Canada, and the United States. In North America, Powassan encephalitis has been reported in Ontario, New York, and Pennsylvania, with a total of 20 cases, 18 in people younger than 20 years of age, and one death (25,721). Most cases have been in males, probably reflecting increased outdoor activity and exposure to ticks. Human infections are rare; antibody surveys in the United States and Canada have generally shown prevalence rates of 0.5% to 4%. The virus has a much wider geographic distribution than indicated by case reports, and Powassan infection should therefore be suspected in cases of encephalitis throughout the United States and Canada. Treatment is

symptomatic; diagnosis is by serology or virus isolation from brain tissues of fatal cases.

In North America, the virus is transmitted in a cycle involving small mammals principally squirrels and ground hogs) and *Ixodes* species ticks, including (a) *Ixodes marxi* and *Ixodes cookei* in the East and (b) *Ixodes spinipalpus* in the West (25,26). The virus has also been isolated from *Dermacentor andersoni* in South Dakota. *I. scapularis* (the vector of Lyme disease) is a competent experimental vector. The widening geographic distribution of *I. scapularis* and its role in the spread of Lyme disease may herald an increased risk for human Powassan infection. Serologic surveys and virus isolations have shown infections in wild mammals, including rodents, hares, dogs, skunks, and foxes. In an experimentally infected lactating goat, virus secreted in the milk resulted in infection of the offspring; antibody has been demonstrated in naturally infected goats in New York State, indicating the possibility of milk-borne transmission to humans (775). The virus has been isolated from ixodid ticks (e.g., *Haemaphysalis neumanni*) and from mosquitoes in Russia (413). There is no evidence for mosquito transmission in North America.

Negishi Virus

Negishi virus was isolated from the CSF of a fatal human case in Tokyo in 1948 during an outbreak of JE. The virus is a member of the TBE virus complex (129). Monoclonal antibody analysis and nucleotide sequencing showed that the virus is most closely related to louping ill virus (744). Infant and weaned mice are highly susceptible to peripheral and intracerebral infection. A second fatal case occurred in 1948, and in 1950, a laboratory infection was reported with fever but no neurologic signs (541). Human cases of Negishi encephalitis have been recognized in China.

FLAVIVIRUSES ASSOCIATED PRIMARILY WITH FEVER, ARTHRALGIA, AND RASH

Dengue Fever Viruses

The first epidemic of a disease that is believed to have been dengue was described in Philadelphia in 1780 by Benjamin Rush. Epidemics were common during the 18th and 19th centuries in North America, the Caribbean, Asia, and Australia (99,670). The earliest recorded illness found thus far whose symptoms resemble dengue were found in a Chinese encyclopedia first published in the Chin Dynasty (265 to 420 A.D.). Outbreaks of an illness in the French West Indies in 1635 and a similar illness in Panama in 1699 could quite possibly have been dengue (237). These outbreaks and descriptions show that long before it became a significant health concern, dengue had a global distribution.

Dengue transmission by *A. aegypti,* first described by Bancroft in 1906, was later proved by Siler and colleagues (670) and Simmons and associates (671). Ashburn and Craig found a filterable, infectious agent in human blood in 1906; in 1926 and 1931, respectively, Siler and colleagues (670) and Simmons and associates (671) transmitted the virus to human volunteers and established the incubation period in mosquitoes. The virus was isolated in mice by Sabin and Schlesinger in 1944 (638), and the existence of more than one serotype was established by cross-protection studies in human volunteers (637).

In the years following World War II, the slow spread of dengue began. The number of nations and people affected have steadily increased, and today dengue is considered a critical global health concern; epidemics involving thousands of people and multiple strains recur in areas of tropical Asia, Africa, Australia, and the Americas where *A. aegypti* is present. Since the 1980s, dengue fever and a severe form of the disease described for the first time in 1954, DHF (see later, Dengue Viruses), have emerged as the most important arthropod-borne viral diseases of humans (238,241,506). More than 2.5 billion people inhabit tropical areas where they are at risk for dengue infection, and an estimated 100 million cases of dengue fever and 250,000 cases of life-threatening DHF occur annually on a worldwide basis.

Infectious Agents

Four serologic types (1 through 4) are recognized on the basis of plaque reduction NT (633) and constitute a distinct antigenic complex (92). Dengue 1 and 3 form a subcomplex defined by monoclonal and polyclonal antibodies (294). Some antigenic heterogeneity is apparent between strains of each type by conventional NT (632). However, monoclonal antibodies have also shown unexpected relationships at the subcomplex level (e.g., between dengue 1 and 3, 2 and 4, and between dengue and viruses of the JE and TBE complexes) (reviewed in 283 and 614). At a more detailed level, antigen signature analysis of dengue 2 strains by monoclonal radioimmunoassay (RIA) has revealed antigenic variation that correlates with genomic variation demonstrated by RNA oligonucleotide fingerprinting (505). The latter technique has now defined multiple geographic variants of dengue 1 and 2 virus (see Epidemiology, later).

Nucleotide sequences of all four dengue types have been reported (157,255,460). Within the dengue virus group, amino acid sequence positional homology of 63% to 68% was found, compared with 44% to 51% between dengue, yellow fever, and WN viruses. Genetic variants of dengue 2 show more than 90% similarity. A genetic relationship was found between dengue 2 virus and Edge Hill virus (58).

Dengue viruses grow in a variety of primary and continuous cell cultures; high yields and demonstration of CPE are difficult to obtain in many systems without adaptation and passage. Cells of human (BSC-1 and HL-CZ, a promonocyte cell line), monkey (LLC-MK2, Vero, primary monkey kidney), hamster (BHK-21), and mosquito origin are most susceptible. Yields of up to 10^7/ml to 10^8/ml, CPE, and plaque formation are obtained under appropriate conditions.

Dengue viruses replicate in the brains of suckling mice and hamsters inoculated intracerebrally (65). Neurovirulence for mice and monkeys increases with sequential passage in mouse brain. Guinea pigs, rabbits, cotton rats, adult hamsters, chickens, and lizards are not susceptible to infection (345,649). Embryonated eggs replicate some strains only after repeated passage. Adult mice inoculated with highly adapted dengue types 1 and 2 viruses become infected with or without overt encephalitis (61). Old World and New World monkeys and apes develop subclinical infection and viremia (621,671,768). Dengue viruses replicate to high titer in *Aedes* and *Toxorhynchites* species mosquitoes inoculated

intrathoracically or intracerebrally (627).

Protein E binds to Vero, CHO, endothelial, and glia cells in culture in a dose- and time-dependent manner and can be saturated (119). The cellular receptor used by dengue in this experiment is a highly sulfated type of heparan sulfate (121). The dengue envelope protein sequence has two putative glycosaminoglycan-binding motifs at the carboxyl terminus. Other members of the flavivirus family have similar motifs in their envelope proteins. Differences in the E protein and the differences among heparin sulfate structure and sulfation among cells may be the factors responsible for tissue tropism (320,409).

The CD14 receptor of monocytes and macrophages appears to be involved in dengue virus attachment to these cells because lipopolysaccharide, which binds to the CD14 receptor, is able to block dengue virus entry into monocytes and macrophages (120). The understanding of what surface receptors the flaviviruses use for cellular entry is expanding (575), but the diverse nature of the pathology and tropism within and between strains makes it possible that there are many different receptors (421).

Recently, it has been demonstrated that the initial target cells for dengue virus infection in humans are skin dendritic cells. Studies of virus growth in human cadaveric skin explants and in skin biopsy specimens from an infected human have demonstrated that a high proportion (60% to 80%) of local dendritic cells expressed dengue antigens (779a).

Pathogenesis and Pathology

Neuroadapted dengue virus produces typical encephalitic lesions, predominantly in the rhinencephalus of infant, weanling, and adult mice. Viral antigen is detectable by IF in reticuloendothelial cells of liver, lymph nodes, and spleen of intraperitoneally infected mice (61). Dengue infects liver cell lines *in vitro* and appears to induce cellular death or apoptosis, which could be involved in the disease pathology (425,454). Dengue viral antigens are detectable by Western blotting in suckling mouse brain or liver examined 7 days after intracerebral inoculation (114). Athymic nude mice peripherally infected with adapted dengue virus develop fatal encephalitis and viral antigen in neurons, skeletal muscle, myocardium, and Kupffer's cells. In mice, dengue replicates within and induces apoptosis in neurons of the cortical and hippocampal regions of the CNS (152).

In experimentally infected nonhuman primates, the role of mononuclear phagocytes as principal sites of dengue viral replication has been established by tissue titration and immunofluorescent staining of cells in skin, spleen, lymph nodes, liver, lung, and thymus; *in vitro* infection of monocytes supports these findings. Mild inflammatory lesions are found in the brain of monkeys inoculated by the intracranial route.

Classic dengue fever produces self-limited infection in humans. Biopsy specimens of skin lesions show swelling of endothelial cells of small vessels, perivascular edema, and infiltration of mononuclear cells.

Dengue viruses multiply in the midgut epithelium, brain, fat body, and salivary glands of mosquitoes (627). No detectable pathologic changes result from infection, and mosquitoes remain infectious for life. Dengue virus replicates in the female mosquito genital tract and may enter the ovum at the time of fertilization, thereby infecting a portion of her progeny (625). Sexual transmission also occurs from male *Aedes* species with inherited infections to susceptible females, which may subsequently pass the virus to their progeny (626).

Clinical Features

The clinical manifestations of dengue fever are described by Siler and colleagues (670), Simmons and associates (671), and Sabin (637). In the typical case, the disease begins abruptly, after a 2- to 7-day incubation period, with high fever, headache, retrobulbar pain, lumbosacral aching pain, conjunctival congestion, and facial flushing. Fever may be sustained for up to 6 to 7 days or may have a biphasic (saddle-back) course. Initial symptoms are followed by generalized myalgia or bone pain that increases in severity, anorexia, nausea, vomiting, weakness, and prostration. The pulse rate may be slow in relation to the fever. Respiratory symptoms (cough, sore throat, and rhinitis) are not uncommon, especially in children. A transient, generalized macular or mottled rash may appear on the first or second day. Coincident with defervescence (day 3 to 5) or shortly thereafter, a secondary rash, maculopapular or morbilliform in nature and nonirritating, appears first on the trunk and then spreads centripetally to the face and limbs but spares the soles and palms. The rash may desquamate. Fever may rise again, creating the second phase of the saddle-back course. Generalized lymphadenopathy, cutaneous hyperesthesia, and altered (metallic) taste sensation may accompany this step of the disease. The peripheral WBC count is depressed with an absolute granulocytopenia, and the platelet count may fall to less than 100,000/mm^3. Hemorrhagic phenomena are noted in a few cases and include petechiae, epistaxis, intestinal bleeding, menorrhagia, and a positive tourniquet test. Myocarditis and various neurologic disorders, including encephalopathy and peripheral mononeuropathy, polyneuritis, and Bell's palsy, have been associated with dengue fever (243,337,548). Central neurologic disorders appear to be more common in DHF than in classic dengue. Reye's syndrome has also been reported to follow dengue infection.

Convalescence may be prolonged, with generalized weakness, depression, bradycardia, and ventricular extrasystoles. Persistent arthritic symptoms are not a feature of dengue and suggest other viral etiologies, including alphavirus (Ross River or chikungunya) infection.

Diagnosis

Exposure by residence or travel in dengue-endemic areas and knowledge about the occurrence of other cases in the community are important clues to the diagnosis. Other infections that clinically may be confused with dengue include influenza, rubella, rubeola, malaria, scrub typhus, leptospirosis, and a variety of other arboviral infections. Rash is a helpful differential sign, but it may be difficult to discern in dark-skinned patients. Epidemic arboviral infections that resemble dengue fever and may be accompanied by rash include chikungunya, O'nyong nyong, WN, Sindbis, Mayaro,

and Ross River virus diseases.

Specific diagnosis depends on virus isolation or serologic tests. Virus may be recovered from the blood during the early febrile phase of the illness. Viremia titers in dengue 1, 2, and 3 infections range from barely detectable to 10^8 for 3 to 5 days; titers in patients infected with dengue 4 are about 100-fold lower (244,245). *Toxorhynchites* species mosquitoes are sensitive hosts for dengue virus isolation by intrathoracic inoculation; virus can be identified by IF staining or CF tests on mosquito tissues within 10 to 14 days after inoculation (374). Larval *Toxorhynchites* species inoculated intracerebrally are also useful for primary virus isolation. *Toxorhynchites amboinensis* (TRA-284), *A. albopictus* (C6/36), and *Aedes pseudoscutellaris* (AP-61) cell lines are now widely used for primary dengue virus isolation (384). The TRA-284 line adapted to serum-free medium provides the most sensitive assay. Syncytial CPE may be present, but it is an unreliable marker of infection; moreover, cultures must be examined by IF to detect virus. Monoclonal antibodies are used for type-specific identification by IF. These techniques allow isolation and identification in as short a period as 2 days, depending on titer of virus in the test sample.

Direct detection of dengue viral antigen in human serum has been reported by use of countercurrent immunoelectrophoresis and by monoclonal RIA. Rapid diagnosis has been achieved by immunocytochemical staining of peripheral blood mononuclear cells obtained during the acute phase of illness (518), and this method is more sensitive than virus isolation. RT-PCR has been applied to the rapid diagnosis of dengue infections, using serotype-specific primers and analysis of amplified sequences by agarose gel electrophoresis or hybridization with serotype-specific digoxigenin-labeled probes (159,404). This technique has permitted detection of viremia levels of less than 2 dex/ml.

Serologic diagnosis depends on the demonstration of a fourfold or greater rise (or fall) in antibodies by HI, CF, or NT. It is frequently difficult to establish the specific infecting serotype because of cross-reactions, especially in patients with preexisting heterologous immunity. In the case of sequential dengue infections, the antibody response to the initial infecting virus type may exceed that to the current infecting type (original antigenic sin) (261), but this is not a uniformly reliable method for retrospectively determining the identity of an earlier infection (383). The plaque-reduction NT is more specific than other tests. During the convalescent period after a sequential infection, cross-reactions make specific diagnosis difficult, but after about 6 months, the presence of neutralizing antibodies to multiple dengue serotypes is a reliable marker of prior infection with those serotypes. Epitope-blocking immunoassays employing monoclonal antibodies provide diagnostic specificity similar to the NT (86). The IgM antibody–capture ELISA is the favored assay for serologic diagnosis. IgM antibodies appear shortly after defervescence and wane after 1 to 2 months (324).

Because of the association between sequential infection and DHF, it is important to distinguish primary from secondary dengue infections. The HI test has been used to classify dengue infections. Secondary infections are characterized by the presence of HI antibodies in the acute phase sample and by high titers (more than 1:1,280) in convalescent sera. The ratio of IgM to IgG antibodies determined by ELISA is useful for distinguishing primary from secondary infections (324,382); in primary infections, the IgM-to-IgG ratio (expressed in optical density [OD] units) in acute sera or convalescent sera obtained during the first month after onset generally exceeds 1.5, whereas secondary infections are characterized by an excess of IgG. Western blotting of sera from patients with primary dengue infections reveals antibodies against the E glycoprotein and the NS3 and NS5 nonstructural proteins, whereas sera with high titers owing to secondary infections reveal a broader array of viral protein bands (113,114).

In recent years, several commercial kits have been designed to make dengue diagnosis simpler and more efficient. A good assay will allow dengue diagnosis without the need for a large, well-developed laboratory and highly skilled personnel, resources at a premium in dengue endemic areas. The clinical usefulness of one of these tests, a capture ELISA specific for IgM and IgG antibodies in distinguishing between primary and secondary infection, was demonstrated (140a). This distinction is important for the epidemiology of DHF.

Treatment

Treatment is supportive and includes bed rest, antipyretics, and analgesics. In case of dehydration, fluid and electrolyte replacement are used in addition. Perspectives on possible future antiviral treatments are reviewed by Leyssen, Declercq, and Neyts (420). Ribavirin has marginal value (444).

Animal Models

One of the problems in developing a better understanding of dengue infection and the various complications thereof has been the lack of a good animal model. Only humans, mosquitoes, and some primates are naturally infected.

Human cell lines have been engrafted into SCID mice, and some of the characteristics of systemic dengue infection are produced (13,424).

Epidemiology

Dengue virus is transmitted in a cycle involving humans and mosquitoes, *A. aegypti* being the most important vector (Fig. 15). Dengue occurs principally in tropical areas of Asia, Oceania, Africa, Australia, and the Americas. Temperate areas within the range of *A. aegypti* are susceptible to summertime introduction and spread of the virus. In areas having year-round vector activity and large human populations, one or more dengue virus types may be maintained endemically. Elsewhere, especially in small, insular populations, epidemics result from the introduction of a new type. Because most dengue cases are subclinical, many cases go unnoticed and can be silently transmitted, as was seen recently in Taiwan (117). Environmental changes can increase human infection and lead to outbreaks (110). Therefore, a long-term active surveillance system is needed to determine accurately the risk for a dengue outbreak.

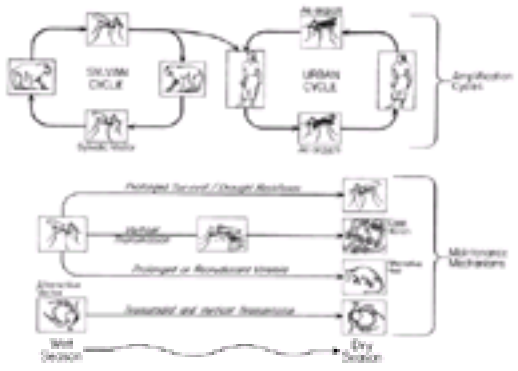

FIG. 15. Transmission cycles of yellow fever and dengue viruses. These viruses have enzootic maintenance cycles involving tree-hole breeding *Aedes* species vectors and nonhuman primates. Dengue viruses are transmitted principally between humans and *Aedes aegypti* that breed in domestic and peridomestic water containers. A sylvatic cycle has been documented in Southeast Asia and West Africa, but it is presently uncertain to what extent this cycle contributes to human infections. In the case of yellow fever, sylvatic transmission is widespread throughout the distribution of the virus. In tropical America, human yellow fever cases derived from contact with forest mosquito vectors, and no urban (*Aedes aegypti* mosquito-borne) yellow fever has occurred since the 1950s. In Africa, sylvatic vectors are responsible for intermonkey and interhuman virus transmission, and there is frequent involvement of *Aedes aegypti* in urban and dry savannah regions.

Protection against homotypic reinfection is complete and probably lifelong, but cross-protection between dengue types lasts less than 12 weeks (637). Consequently, it is possible to sustain multiple, sequential infections. In general, however, most clinically overt illness probably occurs during primary or secondary infections. Clinically apparent tertiary infections do occur, but infection with a fourth serotype is nearly always subclinical. Experimental infection in nonhuman primates has also shown incomplete cross-protection between pairs of dengue viruses, and sequential infection with three or four dengue types is required to achieve complete protection. The background of homotypic immunity in a human population group determines the incidence and age distribution of infections. In virgin-soil outbreaks, dengue attack rates are generally similar among age groups. In some outbreaks, a higher incidence of dengue fever has been found in females (22,343), probably indicating risk for infection around the home by the domestic, daytime biting vector, *A. aegypti.*

Vector density and factors determining exposure to infected female mosquito vectors are important determinants of the rate of dengue virus transmission. The domestic habits of the principal vector, *A. aegypti,* ensure that infection occurs in and around human habitations. Inhabitants of screened houses are at significantly lower risk for epidemic dengue (356). Where dengue is transmitted, it is not uncommon to find 10 to 20 female *A. aegypti* per room, of which 5% to 10% may be infected (512). *A. aegypti* commonly exhibit interrupted feeding behavior, and most females feed multiple times between egg laying; these factors contribute to the rapid transmission of dengue virus and the explosive nature of dengue epidemics.

The worldwide incidence of dengue has increased dramatically in the period following World War II, owing to expanding urban human populations and a coincident increase in *A. aegypti* density as well as the advent of air travel and rapid movement of viremic people. These changes are underscored in the Americas (241,506), where the frequency of epidemics has increased dramatically and multiple dengue serotypes have been introduced. Since the mid-1990s, the continent of South America was reinvaded by *A. aegypti* (Fig. 16), with the result that large dengue outbreaks, some involving more than 1 million people, occurred in immunologically naive populations. Attack rates in outbreaks in the Americas have ranged from 20% to 90% (179,186,242,369). In many areas, dengue has become established in endemic patterns of infection. In Puerto Rico, for example, dengue serotypes 1, 2, and 4 have circulated continuously since 1985 (512,611).

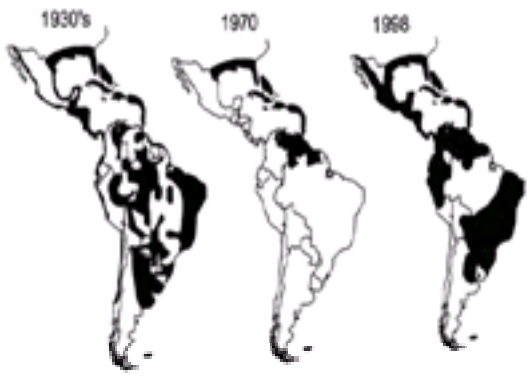

FIG. 16. Distribution of *Aedes aegypti* in the Americas in the 1930s, the 1970s, and in 1998. Reinvasion of the South American continent by *Aedes aegypti* occurred in the late 1970s and 1980s due to collapse of vector control programs, the increase inbreeding sites due to urbanization, and other factors. The result has been the emergence of epidemic dengue and dengue hemorrhagic fever in the continent, and an increased risk of urban outbreaks of yellow fever. From Gubler (237).

In hyperendemic areas of Southeast Asia, more than half of children experience infection with one or more dengue

serotypes by 7 years of age. In tropical areas, epidemics tend to occur during the monsoon or rainy season (18,322). Although increased rainfall results in expansion of vector mosquito breeding, human disease incidence does not correlate closely with vector population density. Other factors (in particular, increased temperature, which shortens the extrinsic incubation time of dengue virus in the vector) appear to be more important (191,594,754).

In the United States, most cases of dengue fever have been acquired abroad. Between 1980 and 1989, 1,457 cases of clinical dengue were reported in the United States, and 276 were confirmed by laboratory tests. There were several small outbreaks of dengue along the Texas–Mexico border in the mid-1990s, involving about 50 cases (587a).

In addition to *A. aegypti,* other species, including *A. albopictus, Aedes polynesiensis,* and *Aedes scutellaris,* play a role in rural and insular areas of Asia and the Pacific. *A. albopictus* was introduced from Asia into the United States and Brazil in 1985; an isolation f dengue virus has been made from this species in Brazil, but it has not yet been clearly implicated in epidemic dengue transmission (241,506). *A. aegypti* breeds in artificial containers with clean water in and around human habitations and bites principally during the daytime. In addition to biologic transmission, mechanical spread by *A. aegypti* and other mosquitoes may occur. Geographic variation exists between strains of *A. aegypti* and *A. albopictus* in terms of their vector efficiency (susceptibility to oral infection), but the epidemiologic importance of this variation has not been clearly established (240). In general, *A. aegypti* strains display low susceptibility to oral infection. This requires that virus titers in the blood of human hosts be high, exceeding 10^5/ml, for infection and transmission to be sustained. Thus, the vector serves as an important selection mechanism or biologic filter for maintaining virus virulence at a high level because only virus strains that replicate efficiently in humans and produce high viremias are transmissible by this mosquito.

Zoonotic cycles of dengue virus transmission involving monkeys and forest *Aedes* species have been documented in Malaysia (631), Sri Lanka (154), and West Africa (136,609) (see Fig. 15). The vector in Malaysia is *Aedes niveus;* the species implicated in West Africa are *Aedes furcifer, Aedes taylori, Aedes luteocephalus, Aedes opok,* and *Aedes africanus.* The possibility of a forest cycle in South America deserves study.

The maintenance of dengue viruses between epidemics has not been clearly defined, especially in rural areas with relatively sparse human populations (11). An alternative mechanism to year-round horizontal human–mosquito–human transmission, such as vertical transmission in *Aedes* species vectors, must be considered. Similar considerations apply to the maintenance of the sylvatic cycle over the dry season. Experimental studies have documented vertical transmission of dengue virus in *Aedes* species, and the virus has been isolated from field-collected larvae of *A. aegypti* and males of *A. furcifer-taylori* in West Africa (reviewed in 239 and 240). There is also evidence of a sylvatic cycle whereby jungle primates and mosquito vectors perpetuate the virus. This type of cycle is well known in jungle yellow fever. Dengue types 1 and 2 have been isolated from monkeys and sylvatic *Aedes* species in West Africa, and dengue 1, 2, and 4 have been isolated from monkeys in Malaysia (631). However, limited genetic studies of sylvatic dengue viruses in Africa appear to indicate that they are not involved in outbreaks of human dengue (604). The persistence of dengue in a jungle cycle would make it nearly impossible to eliminate the virus. However, once a good multistrain vaccine is available, the effect of the virus on the human population will be limited. As with yellow fever, any significant level of dengue introduction from a jungle cycle may require future vaccine program to continue in perpetuity.

Molecular Epidemiology

Nucleotide sequencing of the entire *E* gene has replaced the traditional techniques of RNA fingerprinting, RNA-DNA hybridization, antigenic analysis, short RNA oligonucleotide fingerprinting, and limited nucleotide sequencing of short regions of the genome as tools for determining the origin and spread of dengue epidemics (111,160,241,405,505,604,715). Phylogenetic and nucleotide sequence signature patterns of the *E* gene permit classification of each dengue serotype into a number of E genotypes, which generally correlate with geographic origin (Table 5). However, the current classification will expand as more strains of each dengue serotype are examined. Within a single geographic area, genetic changes in the virus population may be found over time (749), with the appearance of new variants by mutation and selection as well as by introduction from afar. Inconsistencies in the geographic classification, such as the finding of sequence homology between a virus genotype from one region and a strain from a widely separated region, imply that a new strain has been introduced by viremic humans or possibly by virus-infected mosquitoes or mosquito ova. Thus, genotypic classification has been useful as a tool to determine the origin and spread of epidemics. Two distinct genotypes of dengue 1 virus (see E genotypes I and II in Table 5) coexist in Thailand. Genotype II has been recovered in Africa. In 1977, it was introduced into the American region, where it caused a pandemic. The genotype has persisted in the Americas, and its distribution has expanded to include most Latin American countries except Argentina and Chile. Another example of a stably transmitted virus is the E genotype I of dengue 2 virus. This genotype, represented by the New Guinea C strain, the prototype dengue 2 virus, isolated in 1944, has persisted to the present day in Asia and was introduced into the Americas in 1981, causing the first epidemic of DHF in Cuba (252). This genotype now coexists in multiple areas with E genotype III, which had been present in the Caribbean since at least 1969. Similarity between the Caribbean E genotype III strains and dengue 2 strains isolated in the South Pacific between 1971 and 1976 indicated a potential route of spread.

Virus	Genotype[a]	Geographic distribution
Dengue 1	I	Japan (single isolate, 1943), Hawaii (single isolate, 1944), Thailand, Indonesia, Philippines, Taiwan, South Pacific
	II	Thailand, Malaysia, Burma, West Africa, Americas (introduced in 1977)
Dengue 2	I	New Guinea (prototype), Sri Lanka (before 1968), Philippines, Taiwan, Thailand, Malaysia, SE China, Americas ("Jamaica 'genotype'" introduced in 1981)
	II	Seychelles, Sri Lanka, Africa (introduced to East Africa in 1980–1981), Indonesia, Saudi Arabia
	III	India, South Pacific, Americas ("Puerto Rican genotype" introduced in 1969)
	IV	West Africa (sylvan cycle)
Dengue 3	I	Philippines, Indonesia, Malaysia, South Pacific (1980s)
	II	Thailand
	III	Sri Lanka, India, Africa
	IV	South Pacific (1960s), Americas (1960s, 1970s)
Dengue 4	I	Indonesia, South Pacific, Americas
	II	Thailand, Philippines

[a]Genotypes are defined on the basis of phylogenetic relationships of the E gene nucleotide sequence. The classification is a synthesis of several studies (see 111,405,604), and will change as sequences of other strains are reported and evolution of dengue virus genomes continues. The geographic distribution reflects virus importations and movements, and patterns of epidemic spread (see examples in text).

TABLE 5. *Classification of dengue viruses into E genotypes*

The dengue 4 virus, circulating in an endemic and epidemic pattern in the Americas since 1981, is closely related to strains from Niue Island and the Gilbert Islands, again indicating virus transfer between the Americas and Oceania. Comparison of dengue 2 strains isolated in the 1980s in Africa demonstrated that enzootic strains associated with sylvatic vectors and nonhuman primates in West Africa were genetically distinct from strains causing human epidemics. The West African epidemic strains were genetically related to isolates from Indonesia, Sri Lanka, the Seychelle Islands, and Somalia, suggesting a route of spread across the Indian Ocean to East Africa and thence to West Africa; the epidemics thus arose by introduction by viremic travelers rather than from a local jungle cycle (241,604).

Genetic analysis of strains has also suggested that dengue viruses vary in virulence (see later section, Dengue Viruses).

Evolution

Dengue viruses need to be able to survive in two different hosts, the vector and the reservoir. This dual requirement is thought to limit the degree of evolution because the virus must conserve amino acids that are critical to survival in the vector or reservoir.

A recent study indicates, however, that recombination may be an important aspect of dengue viral evolution (779). In a comparison of some 70 full-length dengue sequences, 7 were determined to have been the result of recombination. In the analysis, there were representatives of all four dengue types, but the recombination only appeared to take place within the same types. Recombination is difficult to detect because the complete sequences of many different isolates need to be compared. Questions about dengue recombination that need to be answered include the role it plays in dengue evolution and the degree of sequence homology needed for recombination.

ADE may play a role in the persistence of multiple strains of dengue. Computational-model analysis of dengue transmission dynamics determined that the ADE frequency plays a role in generating a persistent cycle of epidemic behavior (185,251). When modeled, the coexistence of all strains furthers the existence of the four distinct strains, whereas in its absence, there would be only one strain. Although this model is not definitive, it is interesting to note that among the other flavivirus (in which there is little or no ADE), even though there are many different isotypes, the same type of distinct strains as with dengue have not evolved. Nucleotide sequencing studies have been used to track the molecular epidemiology of specific dengue strains (602,672).

Prevention and Control

The development of a safe, effective dengue vaccine is a high priority of WHO, health ministries in some affected countries, the U.S. military, and at least one major pharmaceutical company, but has been an elusive goal (97,106). Classic live vaccines prepared by serial passage of dengue 1 and 2 in mouse brain were tested in humans 50 years ago (637,773) and found to be attenuated and immunogenic; however, the unacceptable substrate for these vaccines and the observation that neurovirulence of dengue viruses increased with continued mouse brain passage led efforts in a different direction (69,641). Propagation of mouse-adapted virus in chicken embryos (a technique similar to that used to develop yellow fever 17D vaccine) was successful and the passaged virus-induced abortive illness in human subjects (650). Although promising at the time, this approach was not pursued into development. In the 1970s, efforts to produce a live, attenuated vaccine by serial passage and cloning in cell culture (fetal rhesus lung or primary dog kidney) were initiated by the U.S. Army. By this time, it was clear that a combination vaccine that simultaneously induced protective immunity against all four serotypes would be required to avoid sensitizing the vaccinee to more severe disease (DHF). Candidate vaccines developed against the four serotypes had various markers of attenuation (small plaque size, temperature sensitivity, reduced viremia in monkeys) and were eventually tested in humans. All were found either to be overattenuated and poorly immunogenic or to induce dengue-like illness of varying severity. The dengue 2 vaccine (PR-159/S-1) was satisfactorily attenuated (660), but it elicited durable immunity only in subjects who had been immunized previously with yellow fever 17D, presumably on the basis of ADE (35). The experience gained in these studies suggested that *in vitro* (and even *in vivo* nonhuman primate) markers of attenuation were not reproducibly predictive of the response in humans, and current efforts are thus focused on stepwise testing of selected passage levels in small numbers of human volunteers.

Live, attenuated, small-plaque, temperature-sensitive dengue vaccines produced by serial passage in primary dog kidney cells without biologic cloning were developed in Thailand and have shown promise in human trials (50,52,53,737). The candidate vaccines have been administered to nonflavivirus-immune human subjects first as univalent and ultimately as tetravalent vaccines. Studies are currently underway in adults with prior dengue and JE virus immunity and in seronegative children. The results indicate that the quadrivalent vaccine is safe and immunogenic and that prior flavivirus exposure enhances the immune response to dengue. The precise dose requirements for each of the four serotype components of the vaccine mixture are still being refined, and it appears that children require significantly lower doses than adults to achieve adequate immunogenicity. Commercial production of the live dengue vaccine is expected in the near future.

Various strategies are also being explored toward the development of genetically engineered vaccines. Underlying assumptions of this work are that the immunity to protein E (and possibly also NS1) is required for durable protection; both humoral antibodies (especially neutralizing anti-E antibodies) and cytotoxic T-lymphocyte responses mediate protection; immunization with dengue virus is serotype specific (228), and, therefore, simultaneous immunization against all four serotypes is required. The major approaches are listed subsequently, with selected examples.

1. Recombinant subunit vaccines: expression of dengue structural proteins in eucaryotic systems has yielded immunogenic and protective antigens. Recombinant baculovirus expressing a fusion protein of E (lacking the C-terminal transmembrane domain) with MalE has been constructed. The fusion protein is secreted as a soluble product by *Spodoptera frugiperda* cells and is readily purified by maltodextrin affinity chromatography. The recombinant truncated E protein elicited neutralizing antibodies and protection in mice (151). In other studies, dengue *E* genes expressed in baculovirus also induced neutralizing antibodies and were protective (576,742). However, similar recombinant proteins have not always resulted in retention of conformational dengue epitopes required for neutralization (184). Immunization of monkeys with recombinant baculovirus-expressed structural and nonstructural proteins elicited binding antibodies but poor neutralizing antibody responses and poor protection against challenge (174). Other vaccines based on the highly immunogenic E protein have been tested. One vaccine was a fusion protein that combined 204 amino acids of E protein and 65 amino acids of NS1 protein from the Den-2 strain. This chimera protein was expressed as a fusion protein with staphylococcal protein A in *E. coli* and then administered to mice, where it was found to be very effective in protecting against lethal challenge by Den-2 virus (686). Another preparation involved the E protein with a six-histadine tag in place of the C-terminal amino acids expressed by recombinant baculovirus in *S. frugiperda* insect cells. The protein was purified under nondenaturing conditions and then administered to mice. These mice were able to survive a lethal Den-2 challenge (688). Subunit vaccines are of particular interest in generating successful dengue vaccine because of the difficulty in developing live-attenuated vaccines.
2. Production of recombinant noninfectious subviral particles: cells infected with recombinant vaccinia virus expressing the prM-E or prM-E-NS1 of JE virus were found to secrete the encoded proteins, which self-assemble into noninfectious, virus-like particles (461). The subviral particles appear to be highly immunogenic and protective (365). A similar approach to the production of dengue particles is now under investigation. A serious concern for the use of subunit vaccines (methods 1 and 2) is their ability to elicit sustained humoral immunity because waning antibody levels may precipitate aggravated disease as a result of ADE. The ability of subunit vaccines to induce protective responses based on cellular immunity is uncertain. Given classic concepts of antigen processing and presentation, such vaccines should induce predominantly CD4+ T-cell responses and would not be expected to elicit CD8+ class I MHC-restricted cytotoxic T cells that may play a role in clearance of infection. Nevertheless, protection has been demonstrated in mice immunized with recombinant structural proteins in the absence of detectable neutralizing antibodies (184), suggesting that linear T-cell epitopes inducing CD4+, CD8– class II MHC-restricted T cells (397,398) may be responsible.
3. Live attenuated, genetically engineered vaccines: full-length genomic cDNA of a Caribbean strain of dengue type 4 virus has been used to transcribe infectious RNA (403) and to produce stable progeny virus. The cDNA can be genetically manipulated to introduce deletions or substitutions. Mutations introduced in the 3'-noncoding region and at the NS1/NS2a cleavage site resulted in reduced replication of dengue 4 virus, suggesting that the virus had become attenuated. Other potential target sites of the genome for the introduction of attenuating mutations and deletions are under investigation. The infectious clone has been used to construct intertypic chimeric viruses in which the *C-prM-E* genes of dengue 1 or 2 or the *prM-E* genes of dengue 2 were inserted into the dengue 4 genomic framework (68,71). In animals, the chimeric viruses elicited protective homotypic immunity to the insert. Similar chimeras have been constructed using the *prM-E* structural genes of TBE virus in the dengue 4 genomic framework (559). Work is underway in several laboratories to use a similar approach, but with dengue virus structural genes inserted into the backbone of yellow fever 17D virus. In another approach, the structural gene of a dengue 1 vaccine strain, passive level PDK-13, were combined with the nonstructural proteins of the dengue 2 vaccine strain, passive level PDK-53. The initial mouse studies indicate that the resultant chimera dengue 1/dengue 2 vaccine induced higher levels of neutralizing antibodies to dengue 1 than the dengue 1 PDK-13 strain vaccine (316). Although this general approach shows considerable promise as a means of preparing rationally designed live vaccines, the genetic changes required to achieve the appropriate level of attenuation and immunogenicity in such constructs remain to be defined.
4. Live vectored vaccines: various prokaryotic and eukaryotic vectors expressing dengue genes are under investigation, including salmonella, vaccinia, rubella virus, and adenoviruses. Immunization of mice with recombinant vaccinia expressing dengue 1 *prM-E* genes elicits protective responses (70,192), which may depend on the expression of subviral particles (see previous paragraph number 2) during *in vivo* replication of the recombinant vaccinia.
5. DNA vaccine: the dengue 2 *PreM* and 92% of the *E* genes have been cloned into various expression vectors and administered to ALB/c mice (359). All of the mice developed antidengue neutralizing antibodies specific for dengue 2. However, when challenged with dengue 2, the immunized mice demonstrated no protection.

Until the successful development and implementation of dengue vaccines, prevention of epidemics will continue to rely on reduction or eradication of *A. aegypti* by breeding site elimination, use of larvicides, and perifocal spraying of insecticides. Eradication achieved 20 years ago in many countries of Latin America has now been reversed by repeated reinfestations, economic development with attendant expansion of *A. aegypti* breeding, reduction in program support, and insecticide resistance (see Fig. 16).

There are several different paths that mosquito eradication programs can follow. The most effective is to eliminate the mosquitoes at the larval stage. To accomplish this, there must be a complete understanding of the water containers in the community used by the mosquitoes. A small number of containers can support the majority of mosquito larva. In a study in Queensland, Australia, in one village, 13% to 29% of the water containers supported 60% to 63% of the immature forms (726). If access to these containers could be restricted, the mosquito population could be significantly decreased. It is also possible to treat these containers with larva-eating copepods that consume the larva while leaving the water in the tanks potable. Copepods of the genus *Mesocyclops* have been successfully used in mosquito control programs in areas as disparate as New Orleans, Louisiana and Phanboi, Vietnam. In both areas, the population of *A. aegypti* were significantly reduced or eliminated (519).

For the emergency control of epidemics, it is necessary to interrupt transmission by killing infected adult female *A. aegypti.* ULV aerial or ground applications of organophosphate insecticides have been used with variable success. Recent field studies in the Caribbean and South America suggest that ULV applications are relatively ineffective and do

not suppress vector populations long enough to interrupt transmission (526).

A reason for the inconsistent results of spraying are the flawed rationales in their application. The spraying is designed to kill the adult mosquitoes and halt the life cycle. Typically, the spraying is done 50 to 100 meters around the residence of the presumed or confirmed cases. This approach assumes that the adult mosquito's flight range is 50 to 100 meters. However, a recent study that marked eggs showed that the ovipositioning females can cover an area 840 meters in diameter (595). Therefore, limiting or stopping an epidemic in progress requires spraying over a larger area than was previously indicated.

West Nile Fever Virus

Infectious Agent

WN virus is a member of the antigenic complex of flaviviruses, which includes MVE, SLE, and JE viruses. The structures of the WN virus genome and glycoprotein spike have been partially analyzed (535). Kunjin virus (another agent in the virus complex) is more closely related to WN than to other members of the complex (132). A high degree of cross-protection was found in hamsters immunized with JE or SLE viruses and challenged peripherally with WN (265). Monkeys immunized with WN were partially protected from lethal intranasal JE virus challenge, whereas JE-immune animals were fully protected against WN challenge (223).

There is a considerable body of research on antigenic variation and restriction-length polymorphism analysis of WN virus. Strains from Africa, Europe, the former Soviet Union, and the Middle East as far east as Pakistan form one group distinct from strains isolated in India and the Far East (201,571). Other studies have shown considerable heterogeneity among strains isolated within a single region (49). In Madagascar, for example, monoclonal antibody analysis identified five antigenic variants, of which four represented the African-European antigenic group and one the India–Far East group (513). A Madagascan strain was found to be distinct from other WN viruses at the nucleotide sequence level (569). Additionally, similar subtypes have been identified in France, Senegal, and Central Africa (47). These antigenic and genomic analyses agree well with the model of migrating birds as important vertebrate hosts of WN that spread the virus to local mosquito populations at the site of infection and along their migratory routes. Even with long-distance travel, which gives rise to distant, but related virus populations, viruses with this method of dispersal still have local segregation of distinct genotypes in some areas. In the Central African Republic, for example, where human disease has been characterized by hepatitis, virus strains recovered from humans, mosquitoes, and ticks differed from the Egyptian prototype and from local avian isolates by monoclonal antibody analysis and restriction digest profiles (466). A mosquito isolate from the Central African Republic was placed (together with an East African strain) in a distinct group based on nucleotide sequence (569). The results suggest that the distinctive human disease pattern in this part of Africa may be caused by a local WN variant with a nonavian transmission cycle.

WN virus grows and produces CPE or plaques in a wide variety of cell cultures (including primary chick and duck embryo) as well as in continuous lines of human, primate, swine, rodent, and amphibian origin. It multiplies in *A. aegypti* and *Drosophila* species cells and produces CPE in *A. albopictus* cells.

Mice and hamsters of all ages are susceptible to lethal infection by the intracerebral route. Resistance to peripheral routes of inoculation develops with age; some virus strains are pathogenic for adult animals. Lethal oral infection of adult mice has been described. Hamsters transmit virus to their young through the milk. Rabbits, guinea pigs, and cotton rats develop antibodies without overt illness by all inoculation routes; rats succumb to intracerebral infection only. Rhesus and bonnet macaques develop fatal encephalitis after intracerebral or intranasal inoculation (223,523). Almost all birds tested develop viremia, including wild species, chickens, and pigeons (474,705). Generally, encephalitis and death appear to be rare, but in the recent American outbreak, there were numerous deaths among the American crow population, which have been used to track the geographic expansion of the virus (15a). Whether crows or other birds are instrumental in the virus spread through the eastern American seaboard is not known.

The developing chick embryo is highly susceptible to the virus. With one exception (namely, *Arvicanthus abyssinicus*), wild African rodents do not develop viremia.

Sporadic cases of naturally acquired WN encephalitis have been reported in horses in Egypt, France, and the United States (246); however, low-level viremia, antibody production, and absence of clinical illness are the rule. Bovine species do not develop viremia after experimental inoculation, but antibodies in cattle are prevalent. Dogs are susceptible to infection, and some develop mild illness, but low viremia levels appear to preclude a major role in transmission cycles (56).

Strain variation exists in the pathogenicity of WN virus for cell cultures, mice, pigeons, and lemurs (733). Experimental infections in various arthropods have been reported (reviewed in 280). *Culex univittatus,* the principal vector in Africa, is a highly efficient vector. The virus has been found to infect soft and hard ticks under natural and experimental conditions.

Pathogenesis and Pathology

The pathogenesis of WN virus is similar to that of other flaviviruses (379,450,524). WN virus is reported to produce persistent infection and a subacute inflammatory-degenerative process in the CNS of monkeys (562). Several studies have examined the role of intracellular adhesion molecule-1 up-regulation in virally infected cells as a mechanism associated with enhanced viral spread (665,666 and 667). Up-regulation of surface adhesion molecules leads to the attraction of important immune cells (667). Paradoxically, cell–cell adhesion may promote further viral infection of these cells and continued replication of the virus. Identification of sites where amino acid substitutions have significant effects on viral virulence and specificity has shown that the determinants for neuroinvasiveness are on the E protein, similar to other flaviviruses (104).

Pathologic observations in humans are limited to a small number of patients with fatal encephalitis and showed lesions of diffuse inflammation and neuronal degeneration. Autopsies performed on patients who died within 4 weeks after inoculation of WN virus (used as an experimental treatment for cancer) resulted in WN virus isolation from spleen, lymph nodes, liver, and lungs (681,682), a distribution similar to that described in laboratory animals.

Clinical Features

The incubation period is 1 to 6 days. The typical case is mild, characterized by fever, headache, backache, generalized myalgia, and anorexia. The course of fever may be biphasic. Rash occurs in about half of cases; onset of rash is either during the febrile phase or at the end of it. The rash is roseolar or maculopapular, is nonirritating, and principally involves the chest, back, and upper extremities. Rash may persist for up to a week and resolves without desquamation. Generalized lymphadenopathy is a common finding. Pharyngitis and gastrointestinal symptoms (nausea, vomiting, diarrhea, abdominal pain) may occur. The disease runs its course in 3 to 6 days, followed by rapid recovery (451). Children generally experience milder illness than adults. Infection may also result in aseptic meningitis or meningoencephalitis in a small proportion of patients, especially in elderly patients (28). Other neurologic presentations include anterior myelitis resembling poliomyelitis and encephalopolyradiculitis (83,224). In an outbreak of encephalitis in 12 of 49 aged patients who acquired the infection in Israel, four patients died. Three cases of encephalitis in young people were described by Flatau and associates (188); one patient had papillitis. Eleven percent of cancer patients inoculated with the prototype (Egypt 101) strain showed clinical signs of encephalitis (682), an incidence much higher than expected from experience in naturally acquired infection, probably owing to the altered susceptibility and poor immune responsiveness of the patient population. Nonneurologic, rare complications include myocarditis (8) and pancreatitis (553). In the Central African Republic, WN virus has been responsible for cases of hepatitis, including fatal disease resembling yellow fever (211).

Clinical laboratory findings include leukopenia and, in cases with CNS signs, CSF pleocytosis and elevated protein.

Inapparent and very mild infections are common. In the series of cancer patients intentionally inoculated by Southam and Moore (682), 89% of 78 infected patients had no clinical signs or symptoms other than fever; in 27%, temperature was never elevated more than 1°F.

Diagnosis

On clinical grounds, WN fever resembles dengue and other dengue-like fevers. Other manifestations include meningoencephalitis and (in Central Africa) hepatitis. Unlike SLE, JE, and MVE viruses, WN virus can be isolated from the blood of as many as 38% of patients (77% when the specimen is taken on the first day of illness) (214). In patients naturally infected, viremia was detectable up to 5 days after onset; titers were low (maximum titer, 3.3 dex/mL). In cancer patients inoculated with the Egypt 101 strain, viremias were more prolonged and of higher titer (682). Viremia has been demonstrated by chance during the incubation period. The reverse passive hemagglutination test has been used to detect viremia in the blood of nestling birds. Neither this technique nor immunoassays have been evaluated for detection of virus in human clinical specimens.

Serologic diagnosis is possible using any of the usual tests; cross-reactions with heterologous flaviviruses complicate the interpretation. In addition to standard serologic tests, the indirect IF and ELISA tests are applicable to the diagnosis of WN infection (704a). Radial hemolysis-in-gel has been used for the detection of IgG antibodies, has a similar sensitivity to the HI test, and has the advantage that there is no need to remove nonspecific inhibitors. RT-PCR has been effectively used to detect WN RNA from human clinical specimens, field-collected mosquitoes, and avian samples (74a,405a)

Treatment

Treatment is supportive.

Epidemiology

WN virus is widely distributed throughout Africa, the Middle East, parts of Europe and the former Soviet Union, India, and Indonesia, and during the summer of 1999, was introduced to the American Eastern seaboard. During the 1950s, classic studies on the epidemiology of WN virus in Egypt and the Sudan revealed that human infections were extremely common in the Nile Delta (705), where 22% of children and 61% of young adults were immune. A survey conducted 10 to 15 years later showed that the infection prevalence in adults had decreased somewhat (to 50%) (144). In a study conducted in 1968, 14.6% of febrile children attending the fever hospital in Alexandria were diagnosed as having WN infection (488). A survey showed that 6% of schoolchildren and 40% of young adults had been infected (137). The hyperendemic virus circulation has precluded sharp epidemics but places a high burden of infection on childhood populations, which experience largely unrecognized and clinically undifferentiated febrile disease. The risk for epidemic may increase (especially in areas such as Cairo) as endemic transmission declines and a larger segment of the adult population becomes susceptible.

Summertime epidemics of WN fever were recognized as early as 1950 in Israel and recurred there at frequent intervals during the 1950s (451). These epidemics involved hundreds of recognized cases, but the true incidence was undoubtedly much higher, and attack rates of more than 60% were reported in some localities. The epidemics were the result of amplified virus transmission and spillover to a human population with a low background of immunity.

In an area of South Africa with relatively low immunity to WN virus (13% to 20%), an outbreak in 1974 resulted in infection of 55% of the population. The epidemic involved an area of 2,500 km ^{2} of central and northern Cape Province. Hundreds to thousands of clinical cases occurred, but they were mild and without any recognized cases of encephalitis (476). Few clinical cases have been reported from West and Central Africa (211,714). In the central highlands of

Madagascar, a high annual incidence of WN virus infection (15%) has been documented, and the virus is suspected to be responsible for grippe-like illness in the population (513,514). A small outbreak of WN virus infection occurred between 1962 and 1964 in the Camargue region of France, in which 13 cases were documented, some with encephalitic complications (546). Infections in tropical Asia are frequent, and the virus appears to be hyperendemic in many areas.

The incidence of CNS infection has not been clearly defined, but this complication appears to be rare. Cases have been described in Israel, India, France, and Egypt. In Egypt, four of 133 patients with aseptic meningitis or encephalitis admitted to one hospital between 1966 and 1968 were shown to have WN infection (2).

In 1996, there was a major epidemic in Romania around Bucharest and the Danube valley. Between 1996 and 1997, 500 clinical cases were identified, and the case fatality rate approached 10% (317,318,720). WN virus was detected in the Czech Republic in 1997, and although several people were infected, the outbreak was not widespread (318).

In 1999, WN virus was first detected in the Western Hemisphere when it appeared in the New York City area. The initial outbreak affected about 60 people, 100 birds, and about a dozen horses, with 7 human fatalities (335). Although the overall numbers are small, the introduction and establishment of a new pathogen is a troubling occurrence. The strain responsible for the American outbreak is closely related to a Middle Eastern isolate (335). This could indicate that the introduction was a single event, whether it was an infected person, unlikely because of low viremia, or an imported animal is unknown.

Despite efforts to eliminate WN virus, it reappeared in the summer of 2000 (20). The geographic distribution of the virus is steadily increasing. The virus has been detected in sick birds as far north as Massachusetts and as far south as North Carolina. This spread is consistent with the infection of migratory birds and would appear to indicate that this virus has established itself in the northeastern part of the United States and will continue to spread along the migration routes of the birds it infects.

WN virus has been isolated from various mosquito species; *C. univittatus* and *C. pipiens molestus* appear to be the most important vectors in Africa and the Middle East (280). The virus has been isolated from an extensive list of *Culex, Aedes, Anopheles, Mimomyia,* and *Mansonia* species mosquitoes in Africa. *C. tritaeniorhynchus* is an important vector in tropical Asia. In the American outbreak, the virus was detected in *C. pipiens,* other *Culex* species, and *Aedes exans* mosquitoes (15a). A subspecies of *C. pipiens* is considered to be the primary vector. Isolations of the virus have been made from ticks, especially in bird rookeries in the former Soviet Union. There have been numerous isolations from wild birds in many areas, and high antibody rates in birds have been reported in Israel, Egypt, and South Africa. Birds sustain high viremia after experimental infection and are the only known amplifying host (474). WN virus has been isolated from a camel and a grass mouse in Nigeria and from a frugivorous bat in India (280,345). Bats have also been implicated as possible hosts in Madagascar. Humans and horses susceptible to clinical infections are incidental hosts and are not thought to play a role in the mosquito transmission cycle. The mechanism of virus survival over the winter or dry season has not been elucidated. Vertical transmission of WN virus in *Culex* and *Aedes* species has been demonstrated under laboratory conditions (42). Transtadial transmission by ticks may play a significant role in virus maintenance. It will be of interest to determine whether the phenomenon of nonviremic, saliva-enhanced transmission of WN virus occurs between cofeeding ticks (536).

Prevention and Control

There is no vaccine for WN virus, but experimental work is ongoing. In a chimera virus approach, the *Pre-M* and *E* genes of WN virus have been combined with the nonstructural gene portion of the attenuated yellow fever vaccine.

Until a successful vaccine is developed, space sprays and aerial sprays of insecticides are the only real option for attempts at control. As with any control program, a thorough understanding of the vector and its lifecycle is critical for efficient control.

Other Viruses

Several other flaviviruses cause human diseases but are not at present of major public health concern. These agents must be considered in the differential diagnosis of febrile illness in people inhabiting or traveling in endemic areas. Furthermore, with the spread of viruses formerly considered to be minor problems, such as dengue was before World War II, these viruses must be studied so that any increase in virulence or geographic distribution can quickly be recognized and appropriate response mounted.

Banzi Virus

Banzi virus was first isolated from the blood of a febrile child in South Africa in 1956 (678). Analysis of *NS5* gene sequences suggests that the virus is most closely related to the mosquito-borne flaviviruses, particularly yellow fever (199). It has been associated with human febrile disease in Tanzania and has been isolated from *Culex rubinotus* and other species of mosquitoes, rodents, and sentinel hamsters in Kenya, South Africa, Zimbabwe, and Mozambique. The virus is pathogenic for infant and weaned mice by all routes of inoculation; it causes CPE in HeLa and primary hamster kidney cells and causes plaques in Vero and LLC-MK2 cells. It has been used as a model to study the pathogenesis of flaviviruses in mice (329).

Bussuquara Virus

Bussuquara virus was originally isolated from a sentinel monkey in Brazil in 1956 (218). The virus is distinct from other flaviviruses by NT (92,139,150). Infant mice are susceptible to intracerebral and intraperitoneal infection, but older mice are susceptible only to intracerebral infection. Adult hamsters inoculated by all routes develop viremia and antibodies but no illness (345). Bussuquara virus replicates and produces plaques in a wide variety of cell cultures, including primary

duck and chick embryo, BHK-21, Vero, LLC-MK2, MA-104, and MA-111. Histopathologic lesions resembling yellow fever were found in the sentinel monkey from which the original isolation was made, but subsequent experimental infection failed to reproduce these findings. The virus is known in Brazil and Colombia, where it has been isolated from various mosquito species, and in Panama, where it was recovered from the blood of a patient with fever, anorexia, and joint pain lasting 4 days (the only known human case) (685). Antibody prevalence rates of 12% have been found in humans in Panama. The virus has been repeatedly isolated from *Proechimys guyannensis* rodents in Brazil; experimentally inoculated *Proechimys* rodents develop viremia. Mosquitoes of the genus *Culex* are the principal vectors. No control measures are applicable.

Edge Hill Virus

Edge Hill virus is a flavivirus that was isolated in 1961 from *Aedes vigilax* mosquitoes collected at Edge Hill, a suburb of Cairns, Queensland, Australia (166). On the basis of subsequent isolations and serosurveys, Edge Hill virus has a wide distribution in eastern Australia (167,273). By antigenic analysis, the virus was placed in the Uganda S virus complex (92). However, by nucleic acid hybridization, the virus is related to dengue 2 virus (58). Edge Hill virus causes CPE and plaque formation in PS, BHK-21, and Vero cell cultures. The virus is pathogenic for newborn mice inoculated intraperitoneally and intracranially and for weanling mice inoculated intracranially.

Human infections have been recognized by serologic surveys in eastern Australia. A single case of human disease occurring in 1990 was attributed to Edge Hill virus on the basis of IgM serologic responses (1). The patient, a 64-year-old farmer from southeast Queensland, had a syndrome resembling epidemic polyarthritis (Ross River virus disease), with fever, myalgia, arthralgia, and fatigue. Symptoms recurred when medication (prednisone) was withdrawn but finally resolved after 12 months. Sporadic cases must be distinguished from dengue fever (which cross-reacts serologically) and Ross River virus infection.

Edge Hill virus is transmitted between *A. vigilax* mosquitoes and mammals, principally wallabies and bandicoots. Virus isolates have been made also from *Cx annulirostris* and *Anopheles meraukensis.*

Ilheus Virus

Ilheus virus was first isolated from a pool of *Aedes* and *Psorophora* species mosquitoes collected in Brazil in 1944. It was originally placed in the WN virus antigenic complex but was shown to be distinct from other members of the complex by NT (92,150). The virus is pathogenic for infant and weanling mice by the intracerebral and intraperitoneal routes but not for other laboratory animals. It produces plaques in primary rhesus kidney cells and various continuous cell lines (BHK-21, Vero, LLC-MK2, and PS) but not in avian cells. Pathologic features are described only in mice, which develop typical encephalitis. A total of eight human infections have been documented by virus isolation, five with mild febrile illness, headache, and myalgias; one with encephalitis; and two without symptoms. These cases occurred in Brazil, Trinidad, Columbia, and Panama (345). The virus was inoculated into 20 cancer patients by Southam and Moore (681); three individuals developed CNS signs. Diagnosis is by virus isolation from blood or serologic tests, which are complicated by cross-reactions with other flaviviruses. The transmission cycle in nature involves wild birds and mosquitoes; at least eight genera of mosquitoes have yielded virus, but most isolations have been made from *Psorophora* species. The potential link to the emergence of Rocio virus was discussed previously. No control measures are applicable.

Kokobera Virus

Kokobera virus was first isolated in 1960 from *C. annulirostris* mosquitoes collected in northern Queensland. The virus is a member of the JE virus complex and is thus related to the other Australian pathogens MVE and Kunjin viruses. Kokobera virus causes CPE and plaques in BHK-21, Vero, and PS cells and is pathogenic for infant and weanling mice by the intracranial route and for infant mice inoculated intraperitoneally. Serologic surveys in New South Wales have demonstrated presumed human infections (273,275). Evidence for human disease was provided by serologic analyses of three patients from this region in 1984 and 1985 (62). The disease was characterized by fever; fatigue; headache; neck pains; painful, stiff, and (in one case) swollen joints; and (in two of three cases) maculopapular, slightly pruritic, desquamating rash. In all three patients, there was a prolonged convalescence, with joint pains persisting for months.

The virus is probably transmitted between *A. vigilax* and wallabies and kangaroos. However, virus isolations have also been made from *C. annulirostris.* Kokobera is distributed in widely separate areas of Australia, including New South Wales, Queensland, Western Australia, the Northern Territory, and Papua New Guinea. The use of molecular epidemiology has found that isolates of Kokobera cluster according to geographic location and time (564). There are three general topotypes covering Queensland and New South Wales, Papua New Guinea, and the Northern Territory and Western Australia. This distribution is different from the other Australian flaviviruses, MVE and Kunjin, which exist as single genetic types across the entire continent. MVE and Kunjin infect birds, which have large ranges and distribute the virus broadly, whereas Kokobera has macropods as its main host, so that the range over which a single isolate can spread is limited.

Koutango Virus

Koutango virus was isolated from a gerbil (*Tatera kempi*) in Senegal in 1968. Subsequent strains have been recovered from other rodent species (*Mastomys, Lemnyscomys*) and from several genera of ticks (*Rhipicephalus, Hyalomma, Alectorobius*) in Senegal and the Central African Republic. No naturally acquired human disease is reported, but a laboratory infection characterized by fever, headache, joint pains, and rash occurred in Senegal.

Kunjin Virus

Kunjin virus was first isolated from *C. annulirostris* mosquitoes in Australia in 1960. It is a member of the JE virus

antigenic complex and is thus antigenically closely related to MVE virus. The virus is pathogenic for infant mice by intracerebral and intraperitoneal routes and for weaned mice by intracerebral inoculation. It forms plaques in primary chick embryo, Vero, and PS cell cultures. Kunjin virus strains isolated in widely separated regions of Australia are genetically homogenous (4,190,436). The nucleotide sequence has a very high level of homology with respect to WN virus, implying an evolutionary relationship (132). Human disease has been reported from Australia. Two accidental laboratory infections were characterized by mild febrile illness, one with rash and one with lymphadenopathy (10). The first severe case with encephalitis mimicking MVE was described in 1986 (516). Of 45 cases diagnosed as MVE during the 1974 epidemic, five cases may in fact have been due to Kunjin virus infection. Cases of Kunjin encephalitis have been reported from Western Australia. Of 20 encephalitis cases diagnosed between 1978 and 1991, two (10%) were due to Kunjin and 18 were caused by MVE virus (443). The clinical disease resembles MVE but is probably less severe. Serologic diagnosis is complicated by cross-reactions with MVE. Kunjin virus was isolated from the spinal cord of a horse with severe encephalomyelitis in New South Wales (32). *C. annulirostris* is believed to be the principal vector, and wild birds and mammals are viremic hosts. The virus has also been isolated in Sarawak and Thailand.

Rio Bravo Virus

Rio Bravo virus was first isolated from a Mexican free-tailed bat (*Tadarida brasiliensis mexicana*) in California in 1954. Subsequently, many other isolations have been made from bats in California, Texas, New Mexico, and Sonora, Mexico (345). The virus is pathogenic for infant mice. Experimentally inoculated monkeys and hamsters develop infection but no illness. A single naturally acquired human case with febrile illness was reported. Laboratory infection (699) resulted in moderately severe illness in three of five patients who experienced aseptic meningitis, orchitis, or oophoritis. The virus is not arthropod borne and does not multiply in inoculated mosquitoes. It is probably transmitted bat to bat by direct contact or aerosol.

Sepik Virus

Sepik virus was isolated from *Mansonia septempunctata* mosquitoes in New Guinea in 1966. Several other isolates have been made from *Ficalbia* and *Armigeres* species mosquitoes in New Guinea. Antigenically, Sepik virus is a close relative of Wesselsbron virus. A single human case of febrile illness with headache, requiring hospitalization, has been recorded (345).

Spondweni Virus

Spondweni virus was originally isolated from *Mansonia uniformis* mosquitoes collected in Northern Zululand, South Africa, in 1955 (345). It is ungrouped but is most closely related antigenically to Zika virus. The virus is pathogenic for infant and weanling mice inoculated intracerebrally, produces CPE in primary hamster and monkey kidney cells, and produces plaques in Vero and LLC-MK2 cells. A virus isolated from the blood of an anicteric child with fever and headache and first thought to be Zika virus was later identified as Spondweni virus; however, recent analysis of this virus strain by monoclonal antibodies suggests that it is a mixture of yellow fever and another flavivirus most closely resembling Spondweni. A volunteer inoculated with this virus developed a mild febrile illness (43). During investigations in South Africa, two people acquired laboratory infections characterized by fever, chills, aches and pains, nausea, and epistaxis (477). Wolfe and colleagues (774) reported a case in an expatriate living in Burkino Faso; symptoms were fever, dizziness, nausea, myalgia, headache, photophobia, and a maculopapular pruritic rash. Treatment is symptomatic. Diagnosis is by virus isolation from blood or serology. Spondweni virus has been isolated from at least seven mosquito species, but most isolates have come from *Aedes circumluteolus* in South Africa. Antibody has been found in cattle, sheep, and goats in South Africa, but the vertebrate hosts involved in transmission are unknown. No control measures are applicable.

Usutu Virus

Usutu virus was first isolated from *Culex naevei* mosquitoes in South Africa in 1959. Newborn and weaned mice are susceptible to fatal encephalitis by the intracerebral route. A case of human infection with fever and rash was reported from the Central African Republic in 1982. The virus has a wide distribution in sub-Saharan Africa. *Culex* mosquitoes and birds are responsible for transmission in nature. *Culex perfuscus* appears to be an important vector. Isolations of the virus have also been made from *Mansonia africana* and *Praomys* species mice in the Central African Republic and from *Coquilletidia aurites* mosquitoes in Uganda.

Wesselsbron Virus

Wesselsbron virus was first isolated from a dead lamb during an epizootic in South Africa in 1955. It is antigenically closely related to Sepik virus and shows some cross-reactivity with yellow fever virus. Wesselsbron virus–immune monkeys develop reduced viremias when challenged with yellow fever virus (298). The virus kills infant and weanling mice by all routes of inoculation. It produces CPE in BHK-21 cells and plaques in Vero and LLC-MK2 cells. Wesselsbron infection in sheep is of veterinary public health importance in southern Africa, where it causes abortion and death of newborn lambs and pregnant ewes (759). Some goat strains are highly susceptible to lethal infection. A mild, febrile disease occurs in cows. A syndrome of hydrops amnii in pregnant ewes, with prolonged gestation, maternal deaths, and fetal malformation (arthrogyphosis, hydranencephaly, hypoplasia, or segmental aplasia of the spinal cord, neurogenic muscular atrophy, and inferior brachygnathia), has been associated with both wild virus infection and use of a live attenuated vaccine (130). Pathologic changes in lambs include jaundice, focal necrosis of liver cells, Councilman-like bodies, and mild periportal inflammation (416). The clinical features, epidemiology, and pathologic changes in livestock resemble Rift Valley fever, from which it must be distinguished. Naturally acquired human cases of febrile illness occurred in South and West Africa, and there have been several reports of laboratory infections. Human illness is characterized by a short incubation period (2 to 4 days) and sudden onset of fever, chills, myalgia, hyperesthesia of the skin, hepatosplenomegaly, and maculopapular rash. In severely ill patients, signs of CNS involvement have been noted (475). There have been no fatalities, nor have signs of severe liver dysfunction similar to those seen in livestock been

observed in humans. Clinical laboratory studies show leukopenia and elevated serum transaminase levels. Treatment is supportive. Diagnosis is by virus isolation from blood or by serology (30,31). The virus has been recovered from pharyngeal swabs. Serologic cross-reactions present difficulties in patients with prior flavivirus infection. The transmission cycle involves mosquitoes. Species incriminated most frequently are *Aedes caballus-juppi, Aedes lineatopennis,* and *A. circumluteolus* in South Africa and numerous other *Aedes* species elsewhere in Africa. The virus is known to occur in Zimbabwe, Cameroon, Nigeria, Senegal, Ivory Coast, Central African Republic, Uganda, Kenya, Madagascar, and Thailand (345). The vertebrate hosts involved in transmission are uncertain; domestic livestock and ostriches (12) develop high viremias, as do experimentally infected gerbils.

No specific control measures are recommended for prevention of human infection. Special care is required when manipulating this virus in the laboratory. Because of the veterinary hazard, work with the virus in the United States is restricted by the Department of Agriculture.

Zika Virus

Zika virus was first isolated from a sentinel monkey in Uganda in 1947. The virus is not assigned to a subgroup but is most closely related to Spondweni, yellow fever, and Uganda S viruses. Zika immunity suppressed viremia in monkeys challenged with yellow fever (298). The virus kills infant and weanling mice by all routes of inoculation. It produces viremia but no illness in monkeys. Rabbits and guinea pigs inoculated by peripheral routes develop antibodies. Plaque assays may be performed in primary chick or duck embryo cell cultures. Pathologic changes in infected mice include encephalitis, myocarditis, and myositis. Serologic surveys reveal a prevalence of human infection of up to 50% in many areas of Africa and in parts of Asia, but human disease has been rarely reported. Of about 14 diagnosed human cases, one was in a mosquito collector working in Senegal, 12 were naturally acquired cases that occurred in Senegal, Central African Republic (508), Nigeria (509), and Indonesia (543), and two were laboratory acquired. Illness is characterized by fever, malaise, headache, and a maculopapular rash. Treatment is supportive. Diagnosis is by virus isolation from blood or serology; cross-reactions confuse interpretation, especially in people with prior flavivirus exposures. The virus has been isolated from primatophilic *Aedes* species that also play a role in yellow fever transmission (e.g., *A. africanus, A. luteocephalus, A. vittatus, A. furcifer-taylori, A. metallicus,* and *A. opok*). Although the vertebrate hosts involved in transmission are not fully defined, it is likely that both nonhuman and human primates play a role and that the transmission cycle is similar to that of yellow fever. Multiple isolations have been made from *Cercopithecus* and *Erythrocebus* species monkeys in West and East Africa. Explosive epizootics (without recognized disease) occur in monkey populations in East and West Africa. A single isolate has been recovered from a rodent (*Taterillus* species) in Senegal, and isolations have been made from a large number of mosquitoes other than those listed previously, raising the likelihood that the ecology of Zika virus is considerably more complex. No control measures are applicable.

FLAVIVIRUSES ASSOCIATED PRIMARILY WITH HEMORRHAGIC FEVER

Yellow Fever Virus

Historical aspects of yellow fever have been reviewed by Strode (697) and Monath (493). The disease was recognized as a clinical entity in 1648 in Yucatan. Tropical areas of the Americas were subject to large outbreaks in the 17th, 18th, 19th, and early 20th centuries, and the disease occurred in epidemic foci as far north as Boston and Halifax; it also appeared during the 18th century in Italy, France, Spain, and England. As late as 1905, there was a significant outbreak in New Orleans, with 5,000 cases and 1,000 deaths.

Mosquitoes were suggested as the vector of yellow fever by Nott in 1848, but this theory was not seriously proposed until 1881 by Carlos Finlay. In 1900, Walter Reed demonstrated a filterable agent in the blood of patients and showed transmission by *A. aegypti* mosquitoes. Despite suggestions to the contrary, yellow fever was thought to be transmitted exclusively between humans by *A. aegypti.* Investigations by Soper (680) resulted in the concept of jungle yellow fever, later shown in both tropical America and East Africa to involve wild monkeys and sylvatic mosquito species.

In 1927, Mahaffy and Bauer first isolated the virus by inoculation of a rhesus monkey with the blood of a patient in Ghana; this was the source of the Asibi strain, parent of the 17D vaccine (694). In 1937, Theiler and Smith (708) reported attenuation of the Asibi strain by passage in chick embryo tissue and demonstrated use of the modified virus (17D) for human immunization.

Yellow fever continues to be a major public health problem in the Americas. Most cases are a result of humans intruding on the jungle or sylvatic cycle (41,740). Although there have not been any outbreaks in urban areas where the vector *A. aegypti* would be involved, there are some disturbing trends. The collapse of the *A. aegypti* control program has allowed this vector to reinfest Central and South America, and yellow fever has appeared in areas where it has been long absent. In 1998, the first case of yellow fever since 1902 occurred in French Guiana (300), and there is evidence of the urbanization of yellow fever. In 1996 and 1997, there were six cases reported in Santa Cruz, Bolivia (739). These cases were almost all localized to one neighborhood, and several of the subjects had not recently left the city, suggesting that transmission had occurred within the city limits and that *A. aeypti* was responsible. Although this particular incident was transient, all the factors to facilitate a significant outbreak are in place: a competent widespread urban vector, a natural sylvatic reserve that urbanites travel to and from, and a low level of immunization in many places in the Americas (about 34% to 41% in Santa Cruz).

In Africa, there continue to be epidemics involving large numbers of cases, such as the 1996 Kaffrine, Senegal outbreak (710). The disease incidence has risen dramatically in recent years (23) with the urban vector, *A. aegypti,* and sylvatic vectors have been responsible for epidemic transmission in Africa. At one time, yellow fever was an example of a successful public health policy with a reduced incidence in many countries. Today, 33 sub-Saharan countries with 450 million people are at risk, and yellow fever continues to be endemic in nine South American countries. WHO estimates, with adjustments for underreporting, that there are 200,000 cases of yellow fever each year, most in sub-Saharan Africa (606). Yellow fever can be prevented by vaccine, and its urban vector was almost eradicated. Today, the vector has

reinfested many areas, and many at-risk populations go unimmunized (490).

Infectious Agent

Yellow fever virus is the prototype of the *Flavivirus* genus. It has been used as a model for elucidation of the flavivirus genome structure and replication strategy (see Chapter 30) and for studies on the molecular basis of antigenic structure, function, and virulence.

Yellow fever is not placed within an antigenic subgroup or complex by plaque-reduction NT (92,150); however, it is antigenically more closely related to Banzi, Wesselsbron, Bouboui, Zika, and Uganda S viruses than to other flaviviruses. By virtue of its relationship to Zika virus, Spondweni virus may be considered indirectly linked to yellow fever. Cross-protection between yellow fever and many flaviviruses can be demonstrated using the sensitive intraperitoneal test in infant mice. In more epidemiologically meaningful tests of cross-protection, prior immunization with Wesselsbron, Zika, and dengue viruses causes a significant reduction in viremias of monkeys challenged with virulent yellow fever virus (298,707).

Antigenic differences have been shown between strains of yellow fever virus. By polyclonal antibody absorption techniques, strains from tropical America and Africa are distinct, as are 17D vaccine and parent Asibi viruses (128). Monoclonal antibodies have been described that are specific for yellow fever virus, for the 17D vaccine strain, or for vaccine substrains 17D-204 and 17DD (38,651). Monoclonal antibody analyses suggest that yellow fever virus is closer to mosquito-borne viruses than to tick-borne viruses. Some of these relationships (e.g., to Banzi, Uganda S, and Zika) fit previous concepts from polyclonal antibody analyses, but many others do not.

Monoclonal antibodies have not provided an antigenic classification of wild yellow fever strains by geographic origin, and further studies in this direction are required. In one limited analysis, Central and West African strains could be distinguished by immunoprecipitation of NS1 protein by anti-NS1 monoclonal antibodies (158). RNA fingerprinting and limited sequencing have delineated four yellow fever topotypes (see later section, Molecular Epidemiology). Strains from South America and Africa are also distinguishable by the electrophoretic migration and carbohydrate content of their E proteins.

Differences have been found between wild yellow fever virus strains in terms of their virulence for mice and monkeys and infectivity for mosquitoes (39,156), but some results are conflicting and reflect multiple variables, including mouse strain, virus passage level, and heterogeneity of virion subpopulations in virus stocks.

The molecular basis for yellow fever virulence has been partially elucidated by comparison between wild-type and vaccine viruses at the sequence level (254) and between vaccine strains with differing virulence patterns (168a,333,334). For a review, see previous section, Molecular Basis of Virulence.

Yellow fever virus can be propagated in a wide variety of primary and continuous cell cultures. Vaccine strains (17D and French neurotropic viruses) grow to higher titer and produce more evident CPE and plaques than do wild strains in various continuous monkey kidney (MA-104, Vero, LLC-MK2), rabbit kidney (MA-111), baby hamster kidney (BHK), and PS cell lines, as well as in primary chick and duck embryo fibroblast monolayers. Wild yellow fever virus strains can also be propagated in these cell cultures, but plaque formation is inconsistent and variable from strain to strain. Viral growth can be detected by IF in advance of the appearance of plaques. Both 17D vaccine and the parent Asibi virus grow in cell cultures of human origin (e.g., Chang liver cells, Henle embryonic intestine, HeLa, KB). Persistent infection of these cells has been described. The virulence properties of virus from carrier cultures can be altered; passage in HeLa cells, in particular, has been associated with a loss of viscerotropism for monkeys (40,172,173,281).

Mosquito cell cultures are useful for primary isolation and are more sensitive than Vero cells or infant mice. *A. pseudoscutellaris* (AP-61), cloned *A. aegypti,* and *A. albopictus* cells are susceptible; infection is generally assessed by IF or subpassage to mice or Vero cells. AP-61 cells consistently show CPE. Intrathoracic inoculation of mosquitoes (*Toxorhynchites* species or *A. aegypti*) is also useful for isolation or assay of yellow fever virus. After a 10- to 16-day incubation period, mosquitoes can be examined directly for virus by IF or can be subpassaged to a susceptible host (e.g., suckling mice). Passage of some wild-type and vaccine strains in C6/36 cells resulted in the appearance of temperature-sensitive virus.

In vertebrate species, yellow fever virus produces both neurotropic and viscerotropic patterns of infection. Viscerotropism reflects the pathogenicity of yellow fever virus for human or nonhuman primates infected by the peripheral route; disease is principally characterized by hepatic pathology. Rhesus and cynomolgus macaques, as well as certain neotropical monkeys, are highly susceptible. Monkeys intracerebrally inoculated with wild-type virus develop encephalitis but die of viscerotropic yellow fever.

The European hedgehog (*Erinaceus europaeus*) is the only nonprimate experimental animal that develops severe visceral lesions in response to yellow fever infection. Hamsters inoculated intraperitoneally with some virus strains (e.g., Asibi) are partially susceptible (scattered deaths), but they resist lethal infection with other virus strains. Rabbits resist intracerebral and peripheral challenge but form antibodies. Newly hatched chickens develop viremic infection without disease.

The neurotropic properties of yellow fever virus are most apparent in mice; infant mice are highly susceptible to encephalitis after intraperitoneal or intracerebral inoculation. Considerable variation in neuropathogenicity for mice exists between virus strains (39). Older mice are susceptible by the intracerebral route, as are guinea pigs.

Pathogenesis and Pathology

Neurotropic yellow fever infection of mice has been used as a model system for studies on the pathogenesis of flavivirus

encephalitis (see General Pathogenesis and Pathology, earlier). The discussion to follow deals solely with the viscerotropic disease in humans and nonhuman primate models.

The typical yellow fever lesion is marked by cloudy swelling, then by coagulative necrosis of hepatocytes in the midzone of the liver lobule, sparing cells bordering the central vein (Fig. 17). Eosinophilic degeneration of hepatocytes results in the formation of Councilman's bodies and intranuclear eosinophilic granular inclusions (Torres' bodies). On an ultrastructural level, Councilman's and Torres' bodies consist of amorphous material without any viral structures. Multivacuolar and microvacuolar fatty change is nearly always present, especially after the eighth day of illness. An inflammatory response is absent or mild. The reticulin framework is preserved, and healing is complete in recovered cases. Typical changes have been seen in biopsy specimens taken as early as the third day of illness; interpretation of biopsy or necropsy material obtained after the 10th day is often difficult. In cases in which death is delayed, partially disintegrated Councilman's bodies consisting of small, irregular, granular, ochre-colored bodies (Villela bodies) may be found, especially in the midzone.

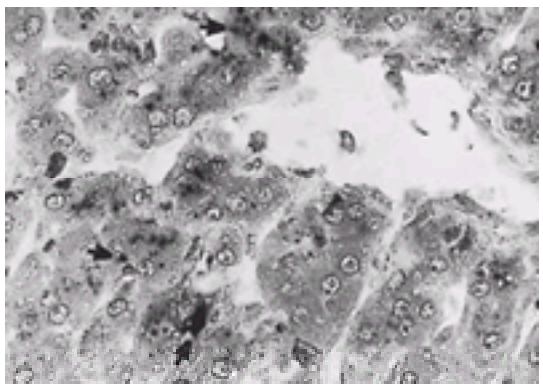

FIG. 17. Postmortem liver sample from a patient with yellow fever, showing midzonal necrosis and hemorrhage, as well as sparing of cells bordering the central vein. Yellow fever viral antigen (*arrows*) is demonstrated by immunohistochemistry (alkaline phosphatase–antialkaline phosphatase technique) and is present both in cells undergoing necrotic change and in the cells preserved around the central vein (×500).

Renal glomerular changes are relatively insignificant compared with acute tubular necrosis and fatty metamorphosis, which may be marked. Schiff-positive transformation of the glomerular basement membrane has been described and has been linked to altered permeability to proteins and albuminuria (496). The myocardial fibers show cloudy swelling, degeneration, and fatty infiltration. The brain may show edema and petechial hemorrhages. Lymphocytic elements in the spleen and lymph nodes are depleted, and large mononuclear or histiocytic cells accumulate in the splenic follicles.

Our present understanding of yellow fever pathogenesis and pathophysiology relies extensively on experimental observations in rhesus monkeys (498,711). After inoculation, the virus replicates in regional lymph nodes and then spreads to other tissues, including liver, spleen, bone marrow, and cardiac and skeletal muscle. The hepatic parenchyma is the principal target organ, and hepatocellular damage is undoubtedly mediated directly by viral infection. Serial examination of liver tissue from infected rhesus monkeys reveals the earliest cytopathologic changes and appearance of immunofluorescent viral antigen in Kupffer's cells (711). Lymphocytic necrosis in the germinal centers of the splenic periarteriolar lymphocytic sheath and necrosis of germinal centers in lymph node follicles are prominent findings in the rhesus monkey (498). Yellow fever (17D) virus replicates to high titer in human peripheral monocyte cultures and in peripheral blood lymphocytes stimulated by phytohemagglutinin (765).

The pathogenesis of the renal lesion is uncertain. In the experimentally infected rhesus monkey, changes in renal function are most revealing during the interval between 36 and 12 hr before death (498). Oliguria was interpreted to reflect intrarenal changes in blood flow secondary to decreased effective blood volume, whereas the development of acute tubular necrosis very late in the infection was the result of the generalized circulatory collapse. Acid-base disturbances, as well as changes in the distribution of water and electrolytes, in cardiac muscle and brain have been described and may be important pathophysiologic events.

The pathogenesis of the bleeding diathesis in yellow fever is also complex. Decreased synthesis of vitamin K–dependent coagulation factors by the diseased liver is an important part of the hemorrhagic disorder, but disseminated intravascular coagulation and altered platelet function may play a role in severe and fatal cases.

Genetic factors may be important in determining individual host responses to yellow fever infection but have not been well defined. Older reports emphasize the severity of the disease in whites compared with blacks, but it is not possible to separate genetic from acquired resistance factors (e.g., immunity to yellow fever or heterologous flaviviruses). Descendants of Dutch colonists in Surinam who survived typhoid and yellow fever epidemics had gene frequencies that were significantly different from those of a large Dutch control group, possibly indicating selection through genetic control of survival (161).

Clinical Features

The incubation period is usually 3 to 6 days. The clinical spectrum varies from very mild, nonspecific, febrile illness to a fulminating, sometimes fatal disease with pathognomonic features (496,697).

Severe yellow fever begins abruptly with fever, chills, severe headache, lumbosacral pain, generalized myalgia, anorexia, nausea and vomiting, and minor gingival hemorrhages or epistaxes. Despite a persistent or rising temperature, the pulse

may decrease (Faget's sign). This syndrome, lasting about 3 days, corresponds to the period of infection, during which yellow fever virus is present in the blood. It may be followed by a period of remission, with defervescence and mitigation of symptoms, usually lasting up to 24 hours. Fever and symptoms reappear with more frequent vomiting, epigastric pain, prostration, and the appearance of jaundice (period of intoxication). Viremia is generally absent, and antibodies appear during this phase. The bleeding diathesis is manifested by “coffee-ground” hematemesis (vomito negro), melena, metrorrhagia, petechiae, ecchymoses, and diffuse oozing from the mucous membranes. Dehydration results from vomiting and increased insensible losses. Renal dysfunction is marked by a sudden increase in albuminuria and diminishing urine output. Death (in 20% to 50% of severe yellow fever cases) occurs usually on the 7th to 10th day of illness and is preceded by deepening jaundice, hemorrhages, rising pulse, hypotension, oliguria, and azotemia. Hypothermia, agitated delirium, intractable hiccups, hypoglycemia, stupor, and coma are terminal signs. Leukopenia occurs during the acute phase of illness. Other laboratory abnormalities include elevation of bilirubin and serum transaminase levels, thrombocytopenia, prolonged clotting and prothrombin times, and ST-T wave changes in the electrocardiogram.

Convalescence is sometimes prolonged, with profound asthenia lasting 1 to 2 weeks. Late death, occurring at the end of convalescence or even weeks after complete recovery from the acute illness, is a rare phenomenon attributed to cardiac complications or renal failure. The duration of icterus in surviving cases is unknown. Elevations of serum transaminase levels have been documented to persist for at least 2 months after onset of yellow fever.

Diagnosis

Mild yellow fever cannot be distinguished clinically from a wide array of other infections, including viral hepatitis, falciparum malaria, leptospiral infections, Rift Valley fever, typhoid, Q fever, typhus, and surgical, drug-induced, and toxic causes. The other viral hemorrhagic fevers, which usually present without jaundice, include dengue, Lassa, Marburg, and Ebola virus diseases; Bolivian and Argentine hemorrhagic fevers; and Congo and Crimean hemorrhagic fever.

Specific diagnosis depends on histopathologic study, isolation of the virus, or demonstration of viral antigen or a specific antibody response. The virus is most readily isolated from serum obtained during the first 4 days of illness, but it may be recovered from serum up to the 14th day and, occasionally, from liver tissue at death. Isolation attempts from clinical specimens can be made by intracerebral inoculation of mice, intrathoracic inoculation of *Toxorhynchites* species mosquitoes, or inoculation of mosquito cell cultures. The *A. pseudoscutellaris* cell line has the advantage of high sensitivity and a relatively short incubation time (3 to 6 days) to detection of virus (by IF using polyclonal or monoclonal reagents). PCR or nucleic acid hybridization may find application for early detection of virus in such samples (574). Viral antigen or IgM–antigen complexes in serum may be detected by immunoassay (502), affording a rapid, early diagnosis. A comparison of various techniques showed that the antigen-capture ELISA has a sensitivity of about 70%, and it may detect noninfectious antigen in poorly handled specimens. RT-PCR has shown promise for rapid detection of viral genome in serum. A definitive postmortem diagnosis may be made by detection of yellow fever antigen in liver tissue sections by immunocytochemical staining (507). It should be emphasized that in living patients, liver biopsy is contraindicated as a diagnostic procedure due to the high risk for hemorrhage.

Serologic methods useful in the diagnosis of yellow fever include the usual HI, CF, and NT, single radial hemolysis, indirect IF, ELISA, and RIA. The HI, IFA, and neutralization antibodies appear within a week of onset; CF antibodies appear later. Paired acute- and convalescent-phase specimens are required to establish the diagnosis by the rise in antibody titer. Cross-reactions complicate serodiagnosis in cases with prior exposure to heterologous flaviviruses.

Antigens suitable for serologic assays are prepared from infected mouse brain tissue, cell culture fluids, or extracted mosquito cell culture membranes. Determination of IgM antibodies by the indirect fluorescent antibody technique or ELISA may indicate recent infection. The duration of IgM antibodies is uncertain, however, and appears to be quite variable. In people vaccinated with 17D virus, detectable IgM neutralizing antibodies are present as long as 18 months after immunization. IgM antibodies show relative specificity, but cross-reactions in ELISA are sometimes found in patients with prior flavivirus exposure.

The use of yellow fever 17D vaccine may confound serodiagnosis. In people without prior flavivirus exposure, the vaccine induces an NT seroconversion with a low titer (1:10 to 1:40) of HI antibodies and no detectable IF or CF antibodies. However, in people with preexisting flavivirus antibodies, vaccination may result in marked rises in yellow fever and heterologous HI and CF antibodies. The patterns produced may be broad or heterotypic; in some cases, however, homotypic responses are seen (a specific rise in yellow fever HI or CF antibodies), similar to those seen in recent natural infection.

Treatment

Treatment is supportive. Most patients with yellow fever have not benefited from the availability of modern intensive care, and it is unknown to what extent fluid management and correction of hypotension and electrolyte and acid-base disturbances would reverse the apparently inexorable course of severe yellow fever. A number of compounds with antiviral activity *in vitro* have been described, including ribavirin and derivative compounds (reviewed in 496). Ribavirin suppresses yellow fever replication *in vitro* but at concentrations higher than those achievable *in vivo.* Trials of ribavirin in experimentally infected monkeys showed no therapeutic effect. IFN-g treatment of monkeys resulted in delayed onset of viremia and illness but had no effect on survival.

Epidemiology

Yellow fever is a zoonotic disease. The primary transmission cycle involves wild nonhuman primates and various sylvatic (tree-hole–breeding) aedine mosquitoes. Humans may be tangentially exposed when they encroach on this cycle (so-called jungle yellow fever), and epidemic spread from human to human can subsequently be continued by sylvatic vectors (see Fig. 15). Alternatively, the domestic mosquito, *A. aegypti,* which lives in close relationship with humans, may

transmit the virus, with humans being the sole viremic hosts in the cycle (*A. aegypti*-borne yellow fever or urban yellow fever). Detailed reviews of the ecology of yellow fever are available (135,212,492).

Yellow fever occurs throughout much of tropical South America and sub-Saharan Africa. Within this region, viral activity may be intermittent and quite localized (19). The distribution of reported cases gives only a partial picture of the natural circulation of yellow fever virus and gives a misleading estimate of risk to travelers. The virus is potentially present throughout the tropical rainforest and bordering grassland areas of South America and Africa.

As previously mentioned, the annual global incidence of yellow fever is estimated to be about 200,000. These numbers include adjustment to estimate the true impact of the disease and are far above the reported confirmed cases, which are 50 to 300 per year in tropical America and up to 5,000 per year in Africa (Fig. 18). These data represent a significant underestimate of the true morbidity, as shown by investigations of various epidemics. The incidence in Africa has increased dramatically in recent years, principally owing to a series of annual outbreaks in Nigeria between 1986 and 1991 that probably involved hundreds of thousands of cases (Fig. 19) (493). The virus spread from Nigeria to Cameroon in 1990, causing an epidemic that may have involved up to 20,000 cases and 1,000 deaths (745). The most recent outbreak in West Africa occurred in Ghana (1993). In East Africa, the first yellow fever epidemic in more than 25 years occurred in 1992 to 1993 in western Kenya.

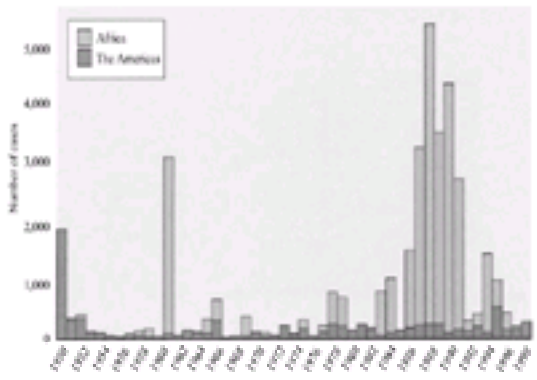

FIG. 18. Officially reported cases of yellow fever in South America and Africa, 1950 to 1998, showing the increase in incidence in Africa in recent years, owing to a series of epidemics in West Africa and (in part) to improved reporting.

FIG. 19. Countries in South America and Africa with officially reported yellow fever outbreaks. Areas considered to be at risk are also shown.

In tropical America, the incidence of jungle yellow fever is highest during months with peak rainfall, humidity, and temperature (January to March). In Africa, transmission by *A. aegypti* and tree-hole–breeding mosquitoes peaks during the late rainy season and early dry season. In tropical America, jungle yellow fever principally affects young adult males. The age and sex distribution reflects the higher incidence of exposure to *Haemagogus* species vectors during woodcutting and forest-clearing activities in the forest. In Africa, background immunity (natural and vaccine-induced) is the principal factor determining the age distribution of cases. In outbreaks affecting immunologically virgin populations (e.g., in Ethiopia, 1960 to 1962), all ages are equally affected. In West Africa, a high level of acquired immunity in adults has resulted in high attack rates in children. A slight excess of cases in males is typical of African epidemics and is probably due to increased exposure to sylvatic vectors during the period around sunset. The ratio of infections to clinical cases ranges between 2:1 to 20:1. Prior infection with one or more heterologous flaviviruses appears to mitigate severity and increase the infection-to-case ratio (499). The case fatality rate has varied widely in different epidemics, possibly reflecting virus strain variation in virulence.

Yellow fever has never occurred in Asia. Possible explanations include (a) cross-protection afforded by dengue immunity, and (b) low vector competence of Asian strains of *A. aegypti;* experimental evidence in support of both hypotheses has been presented (reviewed in 492).

Ecology of Yellow Fever in Tropical America

Howler monkeys (*Alouatta* species), spider monkeys (*Ateles* species), squirrel monkeys (*Saimiri* species), and owl monkeys (*Aotus* species) are effective viremic hosts and commonly develop fatal infections, whereas capuchin monkeys (*Cebus* species) and wooly monkeys (*Lagothrix* species) are susceptible to viremic infection but usually do not develop

clinical signs. This unstable host–parasite relationship may reflect the relatively recent introduction of the virus, possibly at the time navigation was established between Africa and America during the 15th century. Other South American vertebrates, including edentates, marsupials, and rodents, are now believed to play a negligible role in the yellow fever transmission cycle, although further study appears warranted.

Mosquitoes of the genus *Haemagogus* are the principal vectors of jungle yellow fever in tropical America. *Haemagogus* species mosquitoes breed in tree holes and feed in the forest canopy during the midday hours, but they also have been found biting humans in forest clearings and even inside houses in villages near the forest. Transovarial transmission of yellow fever virus in *Haemagogus* species has been experimentally demonstrated. This phenomenon may explain, in part, maintenance of the virus during prolonged dry seasons, when adult vector populations are diminished. The relatively drought-resistant mosquito *Sabethes chloropterus,* a relatively inefficient vector, may also play a role in virus survival.

The development of anti–*A. aegypti* campaigns in Latin America during the 20th century culminated in eradication of the vector from most countries surrounding the Amazon Basin and the disappearance of urban yellow fever after 1942. However, within the last 15 years, *A. aegypti* has reinvaded many areas (see Dengue Fever Viruses) (241,494). The urban vector again exists in or near areas of enzootic yellow fever, raising the specter of future urban outbreaks.

Ecology of Yellow Fever in Africa

All species of cercopithecid and colobid monkeys, and some prosimians tested have proved to be effective viremic hosts, circulating virus for several days or more at sufficient titers to infect mosquitoes (492,610). Infection infrequently results in illness or death, indicating a balanced host–parasite relationship.

A. africanus is responsible for year-round virus transmission in the humid equatorial African forests (see Fig. 15). The ecologic zones bordering equatorial forest have assumed great importance in yellow fever ecology. Appropriately named the *zone of emergence* by Germain and associates (212), the savannah vegetational zones support large and concentrated populations of monkeys and vector mosquitoes. Viral activity intensifies during the rainy season and wanes during the dry season, when vector populations virtually disappear. The principal species involved in sylvatic transmission and transmission to humans are *A. furcifer, A. africanus,* and *A. luteocephalus.* These species are responsible for interhuman spread during epidemics. Depending on location, other vectors that play a role in yellow fever transmission cycles include *A. vittatus, A. metallicus, A. opok, A. neoafricanus,* and *A. keniensis.* Surveillance of virus activity in sylvatic vectors collected in eastern Senegal over a 17-year period (1976 to 1993) has shown an impressive correlation with epidemics occurring throughout the West African region (23). The ecological events underlying the periodicity of virus amplification are unknown but may reflect regional fluctuations in rainfall patterns (494).

In areas subject to extreme drying (e.g., in the dry northern Sudan and Sahel savannah zones of West Africa), yellow fever occurs in intermittent epidemic form, and human immunity patterns indicate little or no infection during interepidemic periods. In these areas, domestic water storage is intensively practiced, domestic *A. aegypti* populations are high, and introduction of yellow fever virus may result in explosive outbreaks. Urban areas along the West African coast are also susceptible. A large urban epidemic occurred in western Nigeria in 1987.

Vertical transmission of yellow fever virus has been documented experimentally in *A. aegypti* (7). Evidence for vertical transmission in nature has been obtained by virus isolation from male *A. furcifer* and *A. aegypti* in West Africa (193). This mechanism ensures virus survival over the long dry season. Yellow fever virus has been isolated from *Amblyomma variegatum* ticks in the Central African Republic (640), raising the possibility that alternate vectors may play a role in dispersal or dry-season maintenance of the virus.

Yellow fever virus has been rarely isolated from other arthropods, including *Aedes dentatus, Coquilletidia fuscopennata,* and phlebotomine flies. Virus has been isolated from a bat in Ethiopia. These observations are of interest but probably bear little relationship to the ecology of yellow fever.

Molecular Epidemiology

The complete sequence of the E protein from 20 different isolates of yellow fever has been used to examine its evolution (108). Two major evolutionary lineages, E genotypes I and II, were characterized. Genotype I contains viruses isolated from East and Central Africa. Genotype II contains two sublineages: IIA from West Africa and IIB from America. One isolate from Trinidad was not characteristic of the IIB group, but it was related to African genotypes suggesting a recent introduction. The relationship between viruses supports the idea that yellow fever was introduced to the Americas from West Africa possibly through the slave trade. The recent reintroduction of an African subtype to the Caribbean shows that intercontinental transmission still occurs (112).

When yellow fever was transmitted to the Americas, it adapted to the local vectors and hosts. There are more unique amino acid substitutions relative to the nucleotide substitutions in the E protein in the American viruses than in the African subtypes (556). Five uniquely American amino acid signatures may possibly be the result of pressure by the new virus–host interaction. This evolution has altered the mouse virulence, antigenic character, and monoclonal antibody reactivity (9,21,155).

All of the African yellow fever virus genotypes are capable of rapid amplification, epidemic spread, and induction of highly lethal human infections, and all have demonstrated the capacity to cross from sylvan to urban (*A. aegypti*–borne) transmission cycles.

Prevention and Control

Vaccination

Yellow fever 17D is a safe, effective live viral vaccine prepared from infected chicken embryos under standards developed by WHO (608,677). Demonstrable immunity occurs in more than 95% of vaccinees within 10 days. For the purposes of international certification, immunization is valid for 10 years, but various studies have shown persistence of antibodies for as long as 30 to 35 years; immunity is probably lifelong (568). Many countries in South America conduct immunization campaigns, and a high vaccine coverage in enzootic areas has limited the incidence of human disease. Vaccination coverage in African countries is highly variable (608). In 1988, a strong recommendation was made to include 17D vaccine in the Expanded Program on Immunization. In 1992, coverage in 11 African countries ranged between 20% and 80%. Cost-effectiveness of routine preventive immunization of infants compares favorably to emergency mass immunization for epidemic control (495).

Serious adverse reactions to 17D vaccine are extremely uncommon. No abnormalities in liver function tests are associated with 17D vaccination. Fewer than 10% of vaccinees experience headache and malaise. Allergic reactions occur at a very low rate (about 1 in 1 million), predominantly in people with an allergy to eggs. The vaccine should not be given to patients with immunodeficiency states (including clinically overt HIV infection) or in those taking immunosuppressive drugs.

Neurologic accidents are extremely uncommon, and 14 of 20 such cases involved infants younger than 6 months of age (485,495); vaccine is now contraindicated in this age group. Encephalitis following yellow fever vaccine is due to virus neuroinvasion and direct viral injury; in all but one fatal case (21), the encephalitis resolved without sequelae.

The safety of yellow fever vaccination during pregnancy is a subject for debate. Taking the results of two studies into consideration, the risk for congenital infection (assessed by IgM antibody present in cord blood) approximates 1% to 2% and is not associated with fetal abnormality or illness (522,725). The stage of pregnancy during which vaccine was administered was uncertain in many cases, and further studies are warranted. Given the susceptibility of the immature nervous system to 17D virus, as noted by the occurrence of postvaccinal encephalitis in infants, and the fact that transplacental infection occurs in a small number of cases, immunization during pregnancy should be avoided. This recommendation must, however, take into account the relative risk for natural infection, which may be as high as 20% to 30% during epidemics.

Vaccination results in a low-level viremia lasting 1 to 2 days and beginning 3 to 4 days after inoculation. The low magnitude of viremia and the fact that *A. aegypti* is refractory to oral infection with 17D virus preclude the possibility of natural transmission (and possible reversion) of vaccine virus.

Factors that may affect seroconversion to the vaccine include (a) nutritional state, (b) simultaneous administration of other vaccines, (c) preexisting heterologous flavivirus immunity, and (d) pregnancy. Children with kwashiorkor showed marked impairment in antibody production after 17D vaccination. People administered 17D yellow fever and cholera vaccines simultaneously or 1 to 3 weeks apart showed reduced antibody responses to both vaccines (209). Other vaccine combinations and immune serum globulin can be used without interference. Studies with 17D vaccine produced in mouse brain and administered by scarification have shown a reduction in vaccine seroconversion in African population groups with prevaccination heterologous flavivirus antibodies. In patients given 17D chick embryo vaccine by the subcutaneous route, however, preexisting heterologous immunity did not interfere with the immune response. In one study (522), only 38% of pregnant women seroconverted, compared with 82% to 90% of nonpregnant controls. There are no data on the safety or efficacy of yellow fever vaccination of HIV-infected patients. Present recommendations are for vaccination of infected individuals without signs or laboratory parameters indicating immunosuppression.

At the present time, vaccines produced by some of the world's 12 manufacturing institutes are contaminated with avian leukosis virus. Although undesirable, this contaminant has not been associated with the development of leukemia, lymphoma, or other cancers (753). Fully effective 17D vaccine virus can be genetically cloned and recovered (452). Intranasal administration of the 17D vaccine (531) induces an immune response in rhesus monkeys. Although there has been no pathogen challenge, if this intranasal route were as successful as the injection, immunization would become much easier, and vaccine coverage would be simplified.

The French neurotropic vaccine, produced from infected suckling mouse brains, is no longer manufactured. The vaccine had the advantage of high stability and ease of administration (by scarification or multiple puncture). However, about 20% of vaccinees developed systemic symptoms, 3% to 4% developed meningeal signs, and 0.5% to 1.3% developed postvaccinal encephalitis. Neurologic accidents were more frequent in children than in adults; fatalities and permanent neurologic sequelae have been reported.

Other Preventive Measures

Areas infested with the domestic form of *A. aegypti* are at risk for the introduction and urbanization of yellow fever. Elimination of breeding sites (e.g., tires, artificial containers), treatment of potable water with temephos (Abate), perifocal spraying with organophosphorus insecticides, and use of Gambusia minnows are effective if applied in a well-administered and continually supported program.

In the case of an outbreak of *A. aegypti*-borne yellow fever, ground or aerial ULV application of adulticides may be used. The control of yellow fever epidemics involving wild vector species is more difficult, and little experience with vector control has been accumulated.

Aerial ULV applications of malathion are effective for the control of the yellow fever vector *Aedes bromeliae* breeding in false banana (*Musa ensetta*) plantations in Ethiopia. Ground and aerial applications of malathion rapidly suppressed populations of *A. africanus* in forest habitats in West Africa for a period of time believed sufficient to interrupt virus

transmission. Aerial ULV was also used for the control of *Haemagogus* species vectors in forested areas in eastern Panama in 1974.

Dengue Viruses (Dengue Hemorrhagic Fever)

Cases of hemorrhage and death had been described during outbreaks of classic dengue fever in Australia (in 1897), Greece (1928), and Formosa (1931) (338). In 1954, a febrile disease with hemorrhagic signs (Philippine hemorrhagic fever) occurred in epidemic form in Manila and was shown to be caused by dengue 3 and 4. DHF was subsequently described in many other areas of Southeast Asia (264). The disease was first reported in China in 1985. The incidence of DHF in Asia has risen steeply since the 1980s, and more than 450,000 cases are now reported annually, making it a leading cause of morbidity. Case fatality rates currently range from 1% to 10%, depending on the availability and sophistication of hospital care. Sporadic cases of DHF were recognized in the Americas during the 1970s, and epidemic DHF appeared in the 1980s. The pathogenesis of DHF remains incompletely understood. Halstead proposed that the severe form of the disease had an immunopathologic basis and occurred in individuals previously sensitized by infection with a heterologous dengue serotype (257,259).

Infectious Agents

All four dengue virus serotypes cause DHF and DSS. In Thailand, dengue type 2 was the predominant virus associated with these syndromes until the 1980s, when dengue 3 and 4 emerged as important serotypes. In Indonesia, dengue 3 was most frequently isolated and associated with fatal cases between 1976 and 1978. In Cuba, Venezuela, and Brazil, DHF epidemics occurred when a specific strain of dengue 2 was introduced 3 to 4 years after an outbreak of dengue 1. The sequence of infecting serotypes, the interval between infections, and strain differences in virulence may be important determinants of the clinical and epidemiologic patterns of DHF (87,153,240,257,258,639). Because an animal model of DHF has not been found, this question has eluded study. Two studies have indicated that viral replication in human mononuclear cells may provide a correlate of virulence (354,510). Comparison of strains associated with different disease outcomes at the genome level have also provided clues that virus-specified factors may contribute to the pathogenesis of DHF (506,604).

Pathogenesis and Pathology

The development of a satisfactory animal model of DHF has been an elusive goal. Unsuccessful attempts to develop models have included the sequential infection of nonhuman primates and of SCID mice reconstituted with human cells. The human disease is characterized principally by diffuse capillary leakage of plasma and a hemorrhagic diathesis. Increased vascular permeability results in hemoconcentration, decreased effective blood volume, tissue hypoxia, lactic acidosis, and shock. These pathophysiologic events are probably mediated by cytokines, and pathologic findings in fatal human cases consequently reveal little evidence of direct viral injury (51,90,598). Gross pathological findings include petechiae, ecchymoses, and focal visceral hemorrhages; serous and bloody effusions; retroperitoneal edema; and, in some cases, hepatic enlargement. On histopathologic examination, there are diffuse small-vessel changes in viscera and soft tissues with leakage of plasma (perivascular edema) and erythrocytes. The spleen and lymph nodes show proliferation of immature and mature lymphoid cells and plasmacytoid elements. Necrosis of thymus-dependent areas of the spleen may be prominent. Hepatic lesions similar to other hemorrhagic fevers, although generally less severe, consist of central or paracentral focal necrosis, sinusoidal acidophilic (Councilman's) bodies, hypertrophy of Kupffer's cells, variable and mild fatty change, and patchy portal mononuclear cell infiltration. Bone marrow changes include maturational arrest of megakaryocytes. Focal dengue viral antigen has been demonstrated in skin, liver, and mononuclear leukocytes.

Prospective studies in Thailand have shown that more than 90% of cases of DHF occur in patients who have previously been infected with at least one heterologous dengue serotype (87,259,639). Children with secondary immune responses were at significantly higher risk for DHF than those experiencing their first infection. About 3% of people who ustain secondary dengue infections develop DHF, whereas less than 0.2% of those with primary infections develop the syndrome. In infants who develop DHF as a consequence of primary dengue infection, the disease occurs mainly between 6 and 12 months of age in the presence of waning, passively acquired maternal antibody. The concept of antibody-dependent immune enhancement of dengue viral replication in monocytes has been proposed (257,258,259 and 260) to explain these observations (see previous section, Antibody-Dependent Enhancement). Dengue viruses were shown to replicate to higher titer in human mononuclear cells in the presence of cross-reactive nonneutralizing dengue antibodies than in the absence of antibodies. Infectious complexes of virions and IgG antibody gained access to monocytic cells through their Fcg receptors. These findings led to the concepts that the presence of enhancing antibodies increased the number of dengue-infected cells and that lysis or immune clearance of these cells led to the release of vasoactive mediators and procoagulants (738). Attempts to model this phenomenon *in vivo* by studying viremia levels in immunologically sensitized nonhuman primates provided equivocal results (256,623). However, recent evidence for the occurrence of ADE *in vivo* has been obtained from studies on the presence of NS1 antigen in the sera of patients with primary and secondary dengue infections (783a). Acute-phase sera from patients with secondary, but not primary, dengue infections were found to contain high levels of NS1 antigen, indicating enhanced numbers of infected cells in secondary infections.

Recent studies have focused on the role of T-cell activation and cell-mediated immunologic clearance of dengue-infected monocytes in the pathogenesis of DHF. Both CD4+, CD8– and CD4–, CD8+ T cells have been detected in people naturally infected with dengue viruses or immunized with live attenuated vaccines (396,397). Cytotoxic CD4+, CD8– T-cell clones established from immune donors have demonstrated dengue serotype-specific, dengue serotype–cross-reactive, and flavivirus cross-reactive specificities (791). The CD4+ clones lyse autologous dengue virus-infected cells in an HLA class II–restricted fashion; proliferate; and produce IFN-g, interleukin-2 (IL-2), and granulocyte-macrophage colony-stimulating factor when stimulated with dengue virus antigens (396,544). Memory CD4–, CD8+ cell clones with HLA class I–restricted cytotoxicity also show dengue serotype cross-reactivity (82). The NS3, NS1, and E proteins appear to contain important epitopes for recognition by both CD4+ and CD8+ T cells (392). Further complicating the model, it has been shown that there can be a partial agonist effect on the cytotoxic T-lymphocyte

response to the homologous protein of different dengue serotypes (791). Under this assumption, the effector cells are partially activated, thus producing some of the soluble mediators, which could be involved in the DHF pathogenesis. But the limited nature of activation induces an ineffective viral clearance. Although seemingly counterintuitive to the present model of DHF development, there has been some evidence of impaired T-cell proliferation during an acute dengue infection (462).

A unifying hypothesis linking ADE of virus replication and T-cell activation has been proposed by Halstead (258) and Kurane and Ennis (398) (Fig. 20). In this model, the host has been previously infected with a dengue serotype and has cross-reactive serum antibodies and memory T cells. Upon infection with a heterologous dengue serotype, the formation of virus–antibody complexes leads to complement activation and enhanced infection of Fcg receptor–bearing monocytic cells. Memory CD4+ T lymphocytes activated by dengue antigens proliferate and produce IFN-g, which in turn up-regulates both the expression of Fc receptors on monocytic cells (augmenting the infection of these cells by immune complexes) and the expression of HLA class I and II molecules involved in recognition of these cells by Tc cells. The dengue-infected monocytic cells are targets for CD4+ and CD8+ Tc cells, resulting in the release of cytokines or cell lysis and release of intracellular enzymes and activators. Evidence for enhanced CD4+ and CD8+ T-cell activation in DHF patients was provided by the finding of higher levels of soluble IL-2 receptor, soluble CD4, and soluble CD8 in sera of DHF than in dengue fever patients (394). However, the precise role of cytokines and tissue factor mediators of capillary leakage, shock, and hemorrhage in DHF remains a subject of research. Although several lines of evidence suggest that T-cell activation and release of mediators are key to the pathophysiology of DHF, it has proved difficult to demonstrate clear correlations with disease severity (226). Elevated levels of IL-2 have been found in children with both dengue fever and DHF (394). IL-2 induces capillary leakage and complement activation when administered to humans. Elevated levels of tumor necrosis factor (TNF), IL-6, C3a and C5a, and histamine have been described in DHF (303,747). In children with DHF, levels of TNF-a and IL-6 were highest on the day of appearance of shock. TNF-a is released from activated monocytes and macrophages, is implicated in septic shock induced by bacterial endotoxins, and is thus a potentially important mediator in DHF and DSS. Bradykinin has not been implicated (176) as a mediator in DHF. High IFN-a levels are sustained for a week or more after onset (395), but the role of this molecule in immunoregulation and pathogenesis, clinical symptomatology, and control of dengue infection in the host remains to be defined.

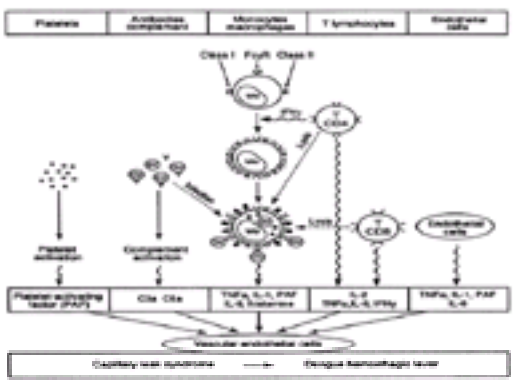

FIG. 20. Diagram of the immunopathogenesis of dengue hemorrhagic fever induced by cytokines and other chemical mediators of increased vascular permeability. (From ref. 398, with permission.)

The role of cytokine mediators in DHF has been investigated in mice by Chaturvedi and colleagues. These investigators described a unique cytokine called *cytotoxic factor* (CF) produced by splenic T cells. When administered to uninfected mice, cytotoxic factor induced vascular and blood–brain barrier permeability and lymphoid cell depletion, effects mimicking elements of DHF pathophysiology (116,162). Immunization of mice with cytotoxic factor prevented these effects. A recent study has demonstrated that the CF was found only in the CD4+ T cells (5,662) and that the amount of CF correlated with the degree of illness. Although implicated in the pathogenesis of DHF, the exact nature and properties of CF need to be determined so that its role can be better defined.

Another uncertainty in the DHF puzzle is the role of infected endothelial cells (14,29). Human umbilical vein endothelial cells become activated when exposed to culture fluids from infected monocytes and up-regulated expression of adhesion molecules VCAM-1 and ICAM-1. Infected human endothelial cells also up-regulate the production and secretion of chemokines RANTES and IL-8 (342), which can increase vascular permeability through transient recruitment and local activation of neutrophils. Additionally, cross-reactive antibodies activate complement on infected endothelial cells. In addition, it was shown that in the absence of outside influence, dengue-infected endothelial cells die, possibly through programmed cell death. All of these factors could contribute to the development of DHF. It is possible that these particular effects play a role along with the various cross-reactive T cells.

The etiology of the hemorrhagic disturbance in DHF appears to be complex. Microvascular injury, thrombocytopenia, platelet dysfunction, and disseminated intravascular coagulopathy have been variously implicated (reviewed in 258). Liver injury of significant degree may impair synthesis of vitamin K–dependent coagulation factors. Increased platelet turnover has been attributed to the direct attachment of dengue virus to platelets and to the presence of antiplatelet antibodies, with subsequent immune elimination. At a cellular level, it is probable that procoagulants are released from infected monocytic cells or endothelial cells activated by IL-2 or other cytokines. In one study, dengue-infected monocytes were found to contain significantly higher levels of plasminogen activator-inhibitor, suggesting a mechanism for interference with the fibrinolytic system (373). A possible link between the immune response to dengue infection and the hemorrhagic diathesis has been suggested (456) on the basis of homology between the amino acid sequence of plasminogen and a 20-residue region of the dengue E glycoprotein. Cross-reactive antibodies between the dengue E peptide and the corresponding site of plasminogen were demonstrated in sera of dengue patients.

Why only a small subset (about 3%) of people with secondary infections develop DHF remains uncertain. Both virus-specified and host-related factors may operate. Virus strains may vary with respect to the presence of enhancing epitopes or other intrinsic virulence factors. In the Americas, dengue 2 has been responsible for repeated outbreaks of classic dengue fever as well as by the explosive appearance of DHF in Cuba, Venezuela, and Brazil. This apparent paradox could be explained solely on the basis of the epidemiologic events leading to the establishment of hyperendemic transmission of multiple serotypes and an increased incidence of sequential infections. However, circumstantial evidence from molecular analysis suggests that the dengue 2 strain responsible for the occurrence of severe disease may represent a variant with increased virulence for humans.

A comparison of the gene sequences of dengue 2 viruses from the Caribbean region indicate that two distinct variants were cocirculating over a period of many years (604). One genetic variant represented the Puerto Rican strain, that had been introduced into the region in 1969 and has persisted in an endemic and epidemic pattern, associated with classic dengue fever. A second variant represented a strain first isolated in Jamaica in 1981. The Jamaican genotype was responsible for the DHF epidemics in Venezuela (1989) and Brazil (1990). Sequence homology between the Jamaican variant and virus strains from Southeast Asia suggested the original source of introduction. In contrast, the Puerto Rican genotype resembled contemporary virus strains from Polynesia, where for the most part disease was mild. These observations infer that the Jamaican genotype of dengue 2 is more likely to cause DHF than the Puerto Rican genotype. The virus responsible for the large DHF epidemic in Cuba in 1981 was distinct from the Jamaican genotype, and more closely resembled older strains from Southeast Asia (252).

Several workers have noted an apparent increase in the severity of disease expression during the chronologic progression of dengue epidemics (623,624). For example, during the 1981 Cuban epidemic, the incidence of severe grades of DHF, and the case fatality rate increased with time and was highest in the later stages of the outbreak (370). During an epidemic of dengue fever in Queensland, the incidence of hemorrhagic phenomena rose as the epidemic progressed (696). These observations suggested that dengue viral virulence may have been enhanced by rapid passage through the human population. No comparisons of early and late virus strains by sequencing or virus growth in human cells or mosquitoes have yet been reported to support this hypothesis.

A preliminary analysis demonstrated variation in the ability of dengue virus strains to replicate in human peripheral blood mononuclear cells and higher replication of isolates from DHF patients than from dengue fever patients (354). A second study of dengue 2 isolates also demonstrated higher replication in human peripheral blood mononuclear cells of strains from DHF patients than those from dengue fever patients (510). These studies were performed in human peripheral blood mononuclear cells and in the presence of enhancing antibodies from donors unrelated to the subject from whom the virus isolate was obtained.

Host genetic factors may influence severity of dengue infection (463). An association between HLA haplotype and disease expression was found in Cuban patients with DHF. HLA-B “blank,” HLA-A1, and HLA-Cw1 were more frequent and HLA-A29, less frequent than in control subjects (547). In Thailand, HLA-B blank and HLA-A2 were associated with DHF (125). In the Cuban epidemic, whites had a higher incidence of DHF than blacks, a finding that could not be explained on the basis of the background of immunity (67). Among acquired factors that influence disease expression, nutrition and underlying diseases may play a role. The prevalence of malnutrition is lower in children with DHF patients than in the general population (709), possibly reflecting the requirement for robust immunologic responsiveness in the genesis of DHF. Chronic diseases such as sickle cell disease, diabetes mellitus, and bronchial asthma appear to increase the risk for severe disease (67).

Clinical Features

The clinical manifestations of DHF and DSS are described by Cohen and Halstead (131) and by Nimmannitya and associates (532). DHF is distinguished from dengue fever by the presence of fever, thrombocytopenia (platelet count less than 100,000 per mm^3), and hemoconcentration (hematocrit increased by more than 20%). DSS is a more severe form of the disease, characterized by hypotension (pulse pressure, 20 mm Hg; cold and clammy extremities), or profound shock (Table 6). The disease initially presents in a manner similar to that of classic dengue fever (see previous section, Dengue Viruses) but progresses after 2 to 5 days to a rapidly progressive, severe form with prostration, restlessness, irritability, shock with cold clammy extremities, diaphoresis, circumoral and peripheral cyanosis, rapid respiration, rapid pulse, and hypotension. Spontaneous hemorrhages occur, including petechiae, ecchymoses, oozing from venipuncture sites, epistaxis, and so forth. Clinically relevant hemorrhage occurs in 10% to 15% of cases. Gastrointestinal hemorrhages and intracerebral bleeding may be life-threatening. Physical findings include skin hemorrhages, pleural effusions, changes in vital signs, and hepatomegaly. Jaundice is a rare manifestation (528). Neurologic abnormalities have been reported in some cases, the most frequent manifestation being encephalopathy (stupor, coma, convulsions, paresis), generally with normal CSF. Cases meeting the criteria for Reye's syndrome have been described. Laboratory abnormalities in DHF include elevated hematocrit and hypoproteinemia (reflecting the capillary leakage), thrombocytopenia, elevated serum transaminases, depression of complement (especially C3) and fibrinogen levels, and the presence of fibrin split products in plasma. The progression of shock is rapid; without physiologic treatment, up to 50% of patients with profound shock die. However, early recognition and appropriate treatment have resulted in overall case fatality rates of less than 1%.

Grade	Hemo-concentration[b]	Spontaneous hemorrhage	Hypotension[c]	Shock
I	+	0	0	0
II	+	+	0	0
III	+	+/−	+	0
IV	+	+/−	+	+

[a]Defined by the World Health Organization.
[b]Hematocrit increased by ≥20%.
[c]Hypotension and pulse pressure >20 mm Hg, physical signs of impaired circulation.
From ref. 778, with permission.

TABLE 6. *Clinical grades of dengue hemorrhagic fever*[a]

Diagnosis

Geographic location and epidemiologic setting are important clues to the diagnosis. DHF has generally not occurred in areas affected by other viral hemorrhagic fevers. An exception is the recent appearance of DHF in South America, where individual cases of the disease resemble and have been confused clinically with yellow fever. Chikungunya occasionally produces an illness with minor hemorrhagic manifestations similar to those of classic dengue fever, but cases with severe hemorrhage, hemoconcentration, thrombocytopenia, and shock are rare or do not occur. Nonviral causes must be considered in the individual case, including bacterial sepsis, scrub and epidemic typhus, leptospirosis, severe malaria, and typhoid fever.

Virus isolation from tissues of fatal cases is less often successful than in classic dengue (533). Techniques for virus isolation from blood of acutely ill patients were discussed in a previous section, Dengue Viruses. Serodiagnosis is possible, but identification of the infecting subtype is difficult because most cases of DHF occur in people with prior dengue exposure. Secondary antibody responses characterized by high HI, CF, and ELISA antibody titers, which are broadly cross-reactive, are typical in cases of DHF. Various attempts to examine the patterns of antibody responses to individual dengue proteins have not revealed consistent differences between dengue and DHF patients. In general, antibodies to the nonstructural proteins, including NS1, NS3, and NS5 are more prominent in sera of patients with secondary infections (113,385).

DHF has a sudden onset and thus can be difficult to predict and treat quickly and efficaciously. It is believed that cytokines play a key role in the pathology; if this is true, it should be possible to predict the course of the disease by the production of different cytokines. There are several detectable factors that appear to predict DHF. CF, IL-8, soluble tumor necrosis factor receptor, and transforming growth factor are all elevated during DHF (48,407,582).

Treatment

WHO has formulated specific guidelines for the management of cases (778). Principles of treatment are dictated by the need to monitor closely the patient's vital signs and hematocrit and to replace plasma volume by judicious fluid replacement. Oxygen should be administered; moreover, if disseminated intravascular clotting is documented, consideration may be given to heparin therapy. Blood transfusion is indicated only in the case of severe hemorrhage. Salicylates and hepatotoxic drugs should be avoided. Corticosteroids are widely used; evidence for their usefulness is conflicting, but some studies indicate that they are of no value (700).

Specific, antiviral therapy has not been extensively evaluated. An uncontrolled trial of IFN was conducted during the 1981 epidemic in Cuba, with some indication that deaths may have been averted. Although ribavirin shows *in vitro* activity against dengue virus, concentrations required for *in vivo* efficacy are not achievable without toxicity. A study of ribavirin in dengue-infected monkeys failed to show an antiviral effect (444).

Epidemiology

Nearly 3 million cases of DHF have been officially reported worldwide through 1990, most cases occurring in Asia. In Thailand alone, 874,207 cases of DHF were reported between 1958 and 1990, with a case fatality rate of 1.57%; in Thailand, the disease ranks fifth as a cause of morbidity, third as a cause of death, and fifth as a cause of years of productive life lost (225). Factors responsible for the emergence of DHF as an epidemic disease in Asia in the 1950s and 1960s and for its recent dramatic rise in incidence (Fig. 21) include the following:

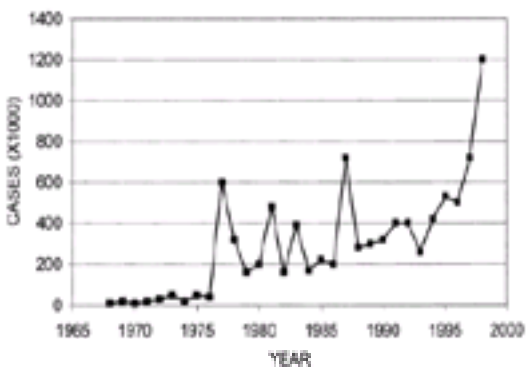

FIG. 21. Incidence of reported cases of dengue hemorrhagic fever, worldwide, 1968 to 1998, and showing a dramatic

increase in incidence in the recent past. The factors responsible are described in the text and include changes in human and vector ecology leading to increased intensity and geographic expansion of virus transmission and cocirculation of multiple dengue serotypes.

1. Demographic changes (human population growth and urbanization) favoring contact with the domestic mosquito vector, *A. aegypti*
2. Ecologic changes linked to urbanization (poor sanitation, inadequate piped water, necessitating domestic water storage) favoring *A. aegypti* breeding
3. The rapid rise in air travel, providing the means for movement of viremic humans and dissemination of multiple dengue serotypes
4. Establishment of hyperendemic dengue infection and increasing frequency of sequential infections of children (241,506)

The factors responsible for the emergence of DHF in Asia have recently come to bear on the Americans. The distribution of *Aedes aegypti* changed dramatically, due to the collapse of mosquito control efforts (see Fig. 16). The pattern of dengue infections changed from outbreaks at intervals of multiple years caused by a single serotype to annual outbreaks and cocirculation of three of the four dengue serotypes. Sporadic cases of DHF were recognized in the 1970s, and major epidemics occurred in Cuba, Venezuela, and Brazil in the 1980s. Eleven countries in the region have now reported the occurrence of DHF (Fig. 22).

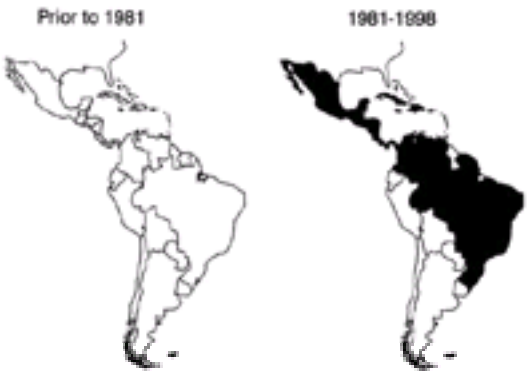

FIG. 22. Distribution of dengue hemorrhagic fever in the Americas before 1981 and during the period from 1981 to 1998, showing spread of the disease as a result of reinvasion of the region by the *Aedes aegypti* vector and the introduction and transmission of multiple dengue serotypes. (From ref. 237, with permission.)

DHF in Asia is a disease of childhood. Two peaks have been noted in age-specific incidence rates: children younger than 1 year of age and children 3 to 5 years of age. The disease in infants is associated with primary infection in the presence of maternal antibody, whereas most cases in older children are the result of secondary infections. Studies in Thailand have estimated the frequency of DHF and DSS in children 1 to 14 years of age to be 31 and 11 cases per 1,000 secondary dengue infections, respectively, whereas the incidence of DHF and DSS in primary infections is 1.9 and 0.07 per 1,000, respectively (87). An age-dependent excess in cases of severe DHF with shock syndrome in girls, compared with boys, has been noted and appears to be related to host factors because serosurveys have shown no difference in sex-specific antibody prevalence.

Other Flaviviruses Associated with Hemorrhagic Fever

Kyasanur Forest Disease Virus

Kyasanur Forest virus was isolated from a sick monkey (*Presbytis entellus*) in the Kyasanur Forest, Shimonga District, Karnataka (then Mysore) State, India, in 1957 (36,776). The virus belongs to the TBE virus antigenic complex. No antigenic differences between strains have been found. The virus is lethal to infant and weanling mice by both the intracerebral and intraperitoneal routes; produces CPE or plaques in chick embryo, hamster, and monkey kidney cell cultures; and replicates without CPE in a continuous cell line of *Haemaphysalis spinigera* tick cells. Pathologic findings in human patients include parenchymal degeneration of the liver and kidneys, hemorrhagic pneumonitis, and an increase in reticuloendothelial tissue in liver and spleen, with marked erythrophagocytosis (442). Similar changes have been seen in experimentally infected monkeys. Bonnet macaques are susceptible to severe or lethal infections and demonstrate necrosis of lymphoid tissues (351). Some primate species also develop encephalitic lesions (chromatolysis of neurons and focal demyelination) (755). Lactating monkeys shed small amounts of virus in their milk. The clinical illness in humans is characterized by fever, headache, myalgia, cough, bradycardia, dehydration, hypotension, gastrointestinal symptoms, and hemorrhages (551). In some patients, a biphasic course is seen; the first phase, as described previously, lasts 6 to 11 days, followed by an afebrile period of 9 to 21 days and then the reappearance of fever and signs of meningoencephalitis. In such patients, the disease closely resembles Central European TBE (757). Leukopenia is a frequent finding during the acute phase of illness, and serum transaminase levels are raised. The cause of hemorrhage is unknown, but disseminated intravascular coagulation has been suspected. Diagnosis is by virus isolation from blood or serology. Standard serologic tests and enzyme-linked immunoassays are applicable. Viremia is detected between the 2nd and 12th days of illness and is maximum between the 3rd and 6th days, with a mean titer of 3.0 dex/mL. The disease is limited to Mysore State, India, but is gradually spreading. Epizootics occur in wild monkeys. Human infections occur

principally during the dry season and in people with close contact with forested areas.

Thousands of cases have been reported since the recognition of the disease in 1957; the annual incidence of virologically diagnosed cases varies between 400 and 500 cases. The case fatality rate is 3% to 5%; no sequelae are reported. The basic transmission cycle involves ixodid ticks and wild vertebrates, principally rodents and insectivores (36). Bats and ground-dwelling birds may play a role in transmission. Large animals (goats, cows, sheep) become infected, but viremias are low, and their importance is principally as hosts sustaining tick populations. At least 10 species of ixodid ticks have been implicated in transmission; *Haemaphysalis spinigera* is a major vector. Transtadial and transovarial transmissions occur in ticks. The density of tick vectors in a given year correlates with the incidence of human disease. A formalin-inactivated vaccine produced in chick embryo fibro-blasts has been licensed and is currently in use in the endemic area.

Omsk Hemorrhagic Fever Virus

Omsk hemorrhagic fever (OHF) virus was first isolated in 1947 from the blood of a patient with hemorrhagic fever during an epidemic in Omsk and Novosibirsk Oblasts, former Soviet Union. As demonstrated by serologic relationships and nucleotide sequence homology (234), OHF virus is a member of the TBE virus complex. Strain heterogeneity has been noted in virulence and antigenic characteristics. The virus is pathogenic for infant and weanling mice by all routes of inoculation. Guinea pigs inoculated by the subcutaneous route develop fever and scattered deaths; rabbits develop antibodies only. Unlike Kyasanur Forest disease, OHF virus does not induce disease or significant pathology in experimentally inoculated bonnet macaques (351). The virus causes hemorrhagic disease and death in experimentally inoculated muskrats and narrow-skulled voles (*Microtus gregalis*). The virus has been propagated in pig kidney, Hela, and Detroit G cells. The disease in humans closely resembles Kyasanur Forest disease except that sequelae (hearing loss, hair loss, neuropsychiatric complaints) are relatively frequent. The case fatality rate is 0.5% to 3%. Between 1945 and 1958, a total of nearly 1,500 cases were recorded in the Omsk region (352). Small numbers of cases acquired by tick bite continue to occur among rural residents engaged in field work during the spring and summer. Muskrat hunters are at risk for the disease by direct contact with blood and tissues, and such cases may occur during the winter months.

The basic transmission cycle remains uncertain. The ixodid tick vector *Dermacentor reticulatus* has been incriminated by field evidence and experimental infection (352). Other species, especially *Ixodes apronophorus,* are suspected to play a role in virus maintenance. Rodents, particularly water voles (*Arvicola terrestris*), are the principal viremic hosts. Direct rodent-to-rodent transmission may occur. Muskrats are epizootic hosts, and human infections occur by direct contact with urine, feces, or blood. No specific Omsk hemorrhagic fever vaccine has been developed, but TBE vaccines apparently provide cross-protective immunity and have been used in high-risk population groups.

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CHAPTER 34 Hepatitis C Viruses

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HISTORY

Recognition of the Disease

Viral hepatitis had been separated into two distinct diseases termed *infectious hepatitis* and *homologous serum hepatitis* during World War II (211). Later, the terms *hepatitis A* (HA) and *hepatitis B* (HB), respectively, were applied, and the infectious agents associated with these diseases were named *hepatitis A virus* (HAV) and *hepatitis B virus* (HBV). Until sensitive and accurate tests became available to distinguish patients who were infected by these viruses, however, the diagnosis was based on the clinical and epidemiologic features of the individual patient. With the discovery of Australia antigen, later termed *hepatitis B surface antigen* (HBsAg), in 1965 (37) and its association with hepatitis B in 1968 (244,266), a clearer picture of viral hepatitis began to emerge. In 1971, a radioimmunoprecipitation test for antibody to HBsAg was developed that reached a sensitivity sufficient to identify virtually all patients who had previously been infected with HBV (186). Soon after, highly sensitive radioimmunoassays (RIAs) for HBsAg became available as well as a test for antibody to the hepatitis B core antigen (HBcAg). With these three tests, virtually all patients infected by HBV in the present or past could be identified and their current clinical status determined.

Surprisingly, using these assays, it was found that most patients with transfusion-associated hepatitis did not have hepatitis B (12). Because the only other known hepatitis agent was HAV, which did not fit well with the epidemiologic or clinical picture of posttransfusion hepatitis, there began to emerge the belief that there may be other, yet to be identified, hepatitis agents. In 1973, the hepatitis A virus was identified by immune electron microscopy (IEM) (96). Although this assay was cumbersome, not only was it capable of detecting in HAV some clinical samples but also it was reasonably sensitive for detecting antibody to HAV. To determine the role of HAV in posttransfusion hepatitis, a study was undertaken in which very well characterized patients from the National Institutes of Health (NIH) Clinical Center prospective trials of posttransfusion hepatitis were examined for the development of antibody to HAV after their acute hepatitis. Of 22 patients whose pretransfusion and posthepatitis sera were studied, none developed new antibody to HAV or rising levels of preexisting antibody as measure by IEM (97). A similar group of patients was characterized by Prince and colleagues, who also concluded that these cases were not hepatitis A based on clinical characteristics (261). Finally, in a study of 13 patients with a total of 30 distinct multiple bouts of community-acquired hepatitis, only 2 cases could be diagnosed as hepatitis A, 12 were hepatitis B, and the remaining 16 were termed *non-A, non-B hepatitis* (NANBH) (230). From these studies, the concept of NANBH became accepted, and the search began for the causative agent.

Identification of the Virus

During the ensuing 15 years, a large number of reports appeared claiming to have discovered the virus, a viral antigen, or a specific antibody associated with NANBH. None of these reports proved accurate. Nevertheless, many things were learned about the etiologic agent, the disease, and the epidemiology of NANBH. The agent was passed to chimpanzees (13,137). It was clearly shown that NANBH caused chronic infections, as would be predicted by the fact that it was frequently transmitted by blood transfusion from presumably chronic carriers. It was shown that NANBH could be a very serious disease that could result in chronic liver disease and cirrhosis, whereas the acute disease was often relatively mild compared with that of transfusion-associated hepatitis due to HBV (11). The pathology was described at both the

light and electron microscopic levels (83,255). Several viral characteristics were also described using the chimpanzee model for the infectivity readout. It was shown that the virus could be inactivated by lipid solvents, suggesting that the virus was enveloped (98). The virus was also shown to be able to pass through a 50-nm filter (126). Thus, a picture of a small, enveloped virus emerged.

With the development during the 1980s of increasingly powerful and sensitive molecular biologic techniques, the detection of previously unrecognized infectious agents became possible. Using a large volume of plasma that had been collected over time from a chronically infected chimpanzee and that had a relatively high titer of infectious virus (40), a group of scientists at the Chiron Corporation, led by Michael Houghton, made a lambda phage complementary DNA (cDNA) expression library from the nucleic acid extracted from the plasma (64). This library was screened with serum from a patient with chronic NANBH as a likely source of antibody to the virus. They identified a clone termed *5-1-1* that expressed an antigen that was shown to be specific for NANBH. They were able to show that the origin of the clone was a single-stranded RNA molecule of about 10,000 nucleotides in length and that the RNA had one continuous long open reading frame (ORF). The expression product of clone 5-1-1 became the basis for the first serologic assay for antibody to the NANBH agent, which was now termed *hepatitis C virus* (HCV) (181). The specificity of the clone and the antibody assay was determined by testing sera from patients who had previously been used to transmit NANBH to chimpanzees, implicated blood donors, and transfusion recipients who developed NANBH. They found that at least 80% of patients with posttransfusion NANBH developed antibody to 5-1-1, and 58% of patients with chronic NANBH without a known exposure to blood had antibody. The 5-1-1 antigen remains a part of the commercial multiantigen test for HCV antibody in use today.

Later, sequence analysis of clones representing nearly the complete HCV genome showed that the virus most closely resembled the Flaviviridae. Since the discovery of HCV by the technology of modern molecular biology, many of the fine details of this virus have been determined, even though it still has not been grown to useful levels *in vitro.*

INFECTIOUS AGENT

Classification

HCV has a similar genomic organization and polyprotein hydrophobicity profile as the pestiviruses and flaviviruses (65) and has been classified as a separate genus in the family Flaviviridae (102). The HCV viral particle is about 50 nm in diameter (126,292) and consists of an envelope derived from host membranes into which are inserted the virally encoded glycoproteins (E1 and E2) surrounding a nucleocapsid and a positive-sense, single-stranded RNA genome of about 9,500 nucleotides (65). The genome contains highly conserved untranslated regions (UTRs) at both the 5' and 3' termini (127,173,315), which flank a single ORF encoding a polyprotein of 3,000 amino acids (67,313). This is processed cotranslationally and posttranslationally by cellular and viral proteases to produce the specific viral gene products outlined in Figure 1. The structural proteins, core, E1 and E2, are located in the N-terminal quarter, with the nonstructural (NS) proteins (NS2, NS3, NS4A, NS4B, NS5A and NS5B) in the remaining portion of the polyprotein (117,131).

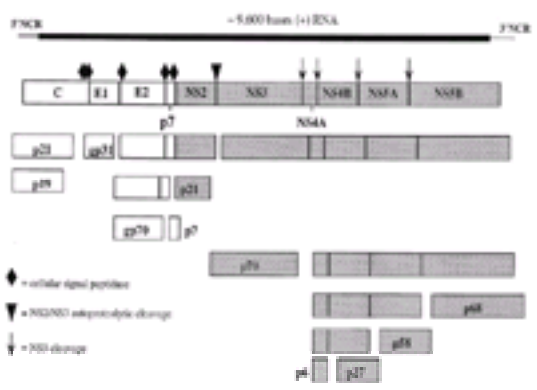

FIG. 1. Structure and processing of the hepatitis C virus (HCV) polyprotein by cellular signal peptidases and virally encoded proteases (NS2/3 and NS3). The viral coding region is represented across the *top. Boxes* below indicate precursors and mature proteins generated by proteolytic processing events. Approximate sizes by sodium dodecyl sulfate-polyacrylamide electrophoresis of the mature proteins (*p*) and glycoproteins (*gp*) are indicated. See Table 1 for nucleotide and amino acid locations of the mature products and Table 2 for the amino acid sequences of the cleavage sites.

Genome region, protein	Nucleotide location[a]	AA location of the mature product in the HCV polyprotein[a]	Approximate size by SDS-PAGE	Function
5' UTR	1–341		None[b]	Initiation of translation, replication[b]
C	342–914	1–191/ 29/182	p21/19	Structural, encapsidation of viral RNA[c]
E1	915–1490	192–383	gp31[c]	Structural, receptor binding, cell entry[c]
E2	1491–2579	384–746	gp70[c]	Structural, receptor binding, cell entry[c]
E2-p7	1491–2755	384–809	gp70[c]	NK, possible precursor or structural function
p7	2580–2755	747–809	p7	NK
NS2	2756–3419	810–1029	p21	Part of NS2-3 protease
NS3	3420–5312	1027–1657	p70	Part of NS2-3 protease, serine protease, helicase, NTPase
NS4A	5313–5476	1658–1711	p6	Cofactor for NS3 serine protease activity
NS4B	5477–6257	1712–1972	p27	Replicase component[c]
NS5A	6258–7600	1973–2420	p56	Replicase component[c]
NS5B	7601–9374	2421–3011	p68	RNA-dependent RNA polymerase
3' UTR	9375–9621		None	Replication,[b] packaging of viral genome[b]

[a]Based on HCV-H strain nucleotide and amino acid sequence (145).
[b]5' UTR contains several short open reading frames (ORFs), whether there is production of polypeptides or their possible functions is unknown.
[c]Indicates proteins are N-glycosylated.
[d]Designates putative function based on comparisons with other viruses.
AA, amino acid; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide electrophoresis; NTPase, nucleoside triphosphatase; NK, not known.

TABLE 1. *Features of the HCV genome and polyprotein products*

Cleavage site	Amino acid sequence[a]	Protease	cis/trans	Cotranslational (Co) or posttranslational (Post)	Cofactor requirement
C/E1	LTVPASA–YQVRN	Signal peptidase	NA	Co	No[b]
E1/E2	LPAGVGA–ETHVT	Signal peptidase	NA	Co	No[b]
E2/p7	LISCAEA–ALENL	Signal peptidase	NA	Post	No[b]
p7/NS2	LPQRAYA–LDTEV	Signal peptidase	NA	Post	No[b]
NS2/NS3	SKGWRLL–APITA	NS2-NS3 protease	cis	Co	No[b]
NS3/NS4A	ADLEVVT–STWVL	NS3 protease	cis	Co	NS4A
NS4A/NS4B	PEDWEEC–SCHLP	NS3 protease	cis/trans	Post	NS4A[c]
NS4B/NS5A	SECTTPC–SGSWL	NS3 protease	cis/trans	Post	NS4A
NS5A/NS5B	TEDVVCC–SMSYS	NS3 protease	cis/trans	Post	NS4A[c]

[a]Based on the HCV-H published sequence (145).
[b]No cofactor requirement has been demonstrated.
[c]Cleavage at these sites still occurs in the absence of NS4A but at a lower efficiency.

TABLE 2. *Clevage in the HCV polyprotein*

Nomenclature and Genotypes

HCV strains show extensive genome heterogeneity, although the variation is not uniform throughout. Relatively well-conserved regions of the HCV genome (core, E1, and NS5) have been extensively studied and used as the basis for classification of isolates into six distinct genotypes (1 to 6) and more than 50 subtypes (1a, 1b, 1c, and so forth) (296; reviewed in 46). It has been shown that antibodies to the core or NS4 regions can be used to distinguish between some HCV genotypes due to variation in the amino acid sequence at the sites of reactive B-cell epitopes (299). These serologic differences, however, do not equate with distinct serotypes based on antibody neutralization as generally defined for most viruses. As yet, there is no classification of HCV on the basis of serotyping, the antibody data for HCV merely represents type-specific antibody that can represent a simpler and quicker way to determine the genotype of a virus. Other genotyping methods include (a) restriction fragment length polymorphism of the 5' UTR (298); (b) reverse dot blot hybridization analysis of 5' UTR, or LIPA (Line Probe Assay, Innogenetics, Zwijnaarde, Belgium) (303); and (c) nested polymerase chain reaction (PCR) amplification of the core gene using type specific primers (242). Overall, however, PCR amplification and sequence analysis is the most reliable method for determining genotype differences of HCV isolates.

Through sequence analysis, as many as 11 genotypes had been defined, although these were not always accepted as distinct from previously existing genotypes. A broader system was recently proposed to unify the nomenclature for HCV, define the requirements for classification, and accommodate isolates that did not fit into the Simmonds scheme, which is based on phylogenetic analysis of the NS5 region (274,297). The concept of six major groups for HCV based on sequence homology would remain, but these would be distinguished phylogenetically and described as *clades.* Genotypes 1, 2, 4, and 5 would be identified as clades 1, 2, 4, and 5, respectively; genotypes 3 and 10 would become members of clade 3, and genotypes 6 to 9 and 11 would be members of clade 6. Despite the discussions surrounding the classification of HCV sequences, clinical and virologic data have not uncovered significant phenotypic differences between genotypes. It appears that patients infected with types 1a and 1b respond somewhat less well to interferon (IFN) treatment and are associated with more rapid progression to chronic liver disease than those infected with other genotypes (reviewed in 46). It must be remembered that all types so far isolated have the potential to cause serious liver disease.

Virologic and Molecular Characteristics

Most of the reports regarding the processing of HCV proteins and genome function are based on *in vitro* studies using mammalian, insect cell, or bacterial expression systems. Kolykhalov and colleagues demonstrated an essential requirement for the enzymatic activities of the NS2/3 protease, the NS3-4A serine protease, the NS3 nucleoside triphosphatase (NTPase) and helicase, and the NS5B polymerase for chimpanzee infection (172). In addition, a requirement for elements contained within the 3'-UTR of the HCV genome for successful replication of the virus has also been shown using the chimpanzee (172,340). These observations suggest that the *in vitro* data provide a good representation of events occurring during productive viral infection.

The 5'-Untranslated Region

The complete 5'-UTR consists of 341 nucleotides (125), the proposed secondary structure (138) of which is shown in Figure 2**A**. This is one of the most conserved portions of the HCV genome (47,300), and while nucleotide variations characteristic of different HCV types exist and have been used in PCR-based genotyping assays (300), the overall secondary structure is still conserved. Several groups have confirmed an internal ribosome entry site (IRES) that can direct translation in a cap-independent manner (104,273,327). This occupies most of the HCV 5'-UTR, as depicted in Figure 2**A**, with a requirement for sequence downstream of the initiator AUG for efficient IRES function. In the genome, coding sequence from the core region of 12 to 30 nucleotides is sufficient for this IRES function (140,273), but some reporter gene sequences can be substituted (104,139). It has been suggested that specific sequences in this region downstream of the initiating AUG contribute to IRES function through RNA–RNA interaction (138,139). The 5'-UTRs from different genotypes have been shown to direct translation with different efficiencies (72,141), which may be dependent on subtle sequence variations within this region affecting RNA–RNA interactions or RNA–protein interactions. Several cellular proteins have been shown to bind the 5'-UTR of HCV and play functional roles in HCV internal initiation (8,123,301), including polypyrimidine tract–binding protein and the eukaryotic translation initiation factor eIF3 (7,49). However, the precise mechanism of translational control in HCV infection is still to be determined.

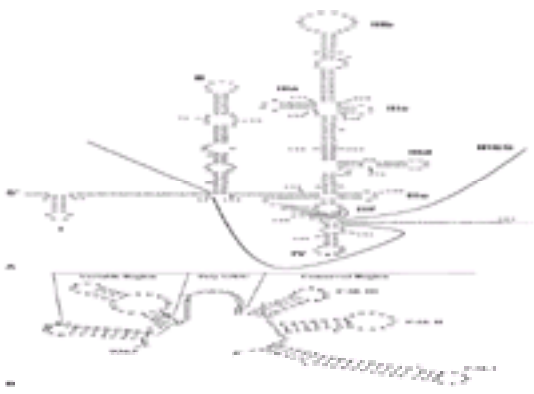

FIG. 2. A: Model showing the predicted RNA secondary and tertiary structure of the hepatitis C virus (HCV) 5'untranslated region (H strain) and the adjacent core coding sequence showing the individual stem-loop stuctures important for internal ribosome entry site (IRES) function. The minimal region required for IRES function (nucleotides 43 to 354) is indicated. *Solid lines* indicate base pair interactions within the pseudoknot (loop IIIf); *highlighted* AUG indicates the site of translation initiation. (From ref. 138, with permission from American Society for Microbiology). **B:** Computer-predicted secondary structure of the HCV 38UTR (H strain) and limited upstream sequence. Stem-loop (SL) structures and individual regions are labeled. The open reading frame UGA termination codon is located at nucleotide 9375. (From ref. 171, with permission of the American Society for Microbiology).

The 3'-Untranslated Region

After the ORF stop codon is the 3'-UTR. This contains, in the 5' to 3' direction, a region of about 30 nucleotides, which shows nucleotide variability between genotypes, a poly(U) tract of variable length, a polypyrimidine C(U)n stretch, and a highly conserved 98-base sequence, thought to represent the 3'-terminus of the genome (171,313). Computer predictions of the secondary structure and cleavage analyses show that the region can form stable stem-loops (36,148,171) (see Fig. 2**B**), although the upstream two stem-loops have not been confirmed experimentally. Specific interactions between the 3'-UTR, in particular the conserved 98 base region at the 3'-terminus, and cellular proteins, including polypyrimidine tract–binding protein (148,323) suggest that this region is involved in viral replication and possibly translation (149). Studies in chimpanzees using infectious clones of HCV deletion mutants have shown that the poly(U/UC) region and the conserved 98 base region are critical for infectivity but that the variable region, or its secondary structure, is not (172,340). This further confirms the involvement of the proximal 3' region of the HCV genome in replication.

The Viral Proteins

The functional HCV proteins are generated from the polyprotein following cotranslational and posttranslational cleavage by cellular and viral proteases (see Fig. 1). The amino acid sequences and positions of the cleavage sites at each junction are listed in Table 2). The structural proteins are characterized by hydrophobic domains at the C termini that are important for membrane association and subsequent cleavage by the signal peptidases, localized in the lumen of the endoplasmic reticulum (ER) (117,131). Cleavage at the junction between NS2 and NS3 occurs autoproteolytically by a protease encoded by NS2 and the N-terminal portion of NS3 (115,132), whereas the cleavages of the other downstream NS proteins are mediated by a distinct virus-encoded serine protease located in the N-terminal third of NS3 (27,88,116,132).

The Core Region

The first 191 amino acids of the HCV polyprotein represent the putative nucleocapsid or core protein with a molecular weight of 21 to 23 kDa (131). This region is highly conserved (48) and includes several T-cell and B-cell epitopes (114,162). Different molecular weight forms representing C-terminally truncated products have been identified in sodium dodecyl sulfate polyacrylamide electrophoresis (SDS-PAGE) analysis (131,279,341). The truncated form of 19 to 21kDa is thought to represent the mature protein (202,341), terminating at amino acid 173 to 174 (202,279). Some of these truncated forms have been identified in the nucleus (202,203,307,341). The basic nature of the core N-terminus, which contains several putative nuclear localization signals and a DNA-binding motif (59), may result in translocation to the nucleus, but there are no clinical data indicating the presence of the core in the nuclei of infected liver cells (24). The RNA-binding activity has been localized to the N-terminus, amino acids 1 to 75 (279), and is characterized by a cluster of basic residues showing a high degree of conservation, suggesting an important biologic function. *In vitro,* it has been shown to interact with cellular proteins and other viruses (264,290) performing multiple functions in cells, such as transactivation and suppression (217,342). These numerous reports have implications for possible adverse effects of core on cellular functions, which may contribute to pathogenesis (see Pathogenesis).

The Envelope Region

The glycoproteins (gp) E1 (gp31) and E2 (gp70), representing the putative viral envelope proteins, are targeted to the ER by aminoterminal signal peptides and are N-terminally glycosylated at 5 or 6 and 11 sites, respectively (87,117,263). It has been shown that the proteins are retained in the ER rather than retrieved from the Golgi complex during processing through the C-terminal hydrophobic transmembrane domains (73). The sequence of cleavage events from the viral polyprotein appears to be such that two possible precursors for E2 are produced (see Fig. 1 and Table 1), E2-NS2 and E2-p7 (87,117,197,226). Signalase cleavage at the p7 site is inefficient, leading to the presence of two forms of the second envelope protein, E2 and E2-p7 (197,226), differing only in their C-termini (see Fig. 1). Neither the function of p7 nor the role of the E2-p7 protein in viral particle formation is known.

Kinetic studies show that E1 and E2 interact slowly to form a stable complex (80,87,117). Properly folded complexes

require N-linked glycosylation (67,221) and are noncovalently bonded into heterodimers (80,87,263), a proportion of disulfide-bonded complexes have been identified that are thought to represent misfolded structures (80,87). The interaction seems to be totally membrane associated as the proteins remain localized to the ER and are not transported to the cell surface (87), although deletion of the E2 C-terminal hydrophobic domain results in secretion of this protein from cells (222).

An unusually high degree of amino acid variation has been observed in the N-terminus of the E2 protein (329). This hypervariable region, referred to as *HVR1,* is located between amino acids 384 to 410 of the polyprotein (amino acids 1 to 27 of E2). The HVR1 varies within any one isolate (231,289), generating a form of quasi-species, and between different genotypes. It should be stressed that no one HVR1 sequence pattern has been associated with any particular genotype, and therefore, this region cannot be used to make distinctions at this level.

Observations that specific antibodies to HVR1 epitopes change during the course of a chronic infection suggest that this region is subject to immune pressure, with the potential for escape mutants (reviewed in 101). Puntoriero and associates, however, developed a consensus profile of HVR1 sequences and indicated that there may be more cross-reactivity between variants than previously thought (262). These data provide important considerations for prophylactic vaccine development.

The NS2 Region

The NS2 protein is cleaved from the polyprotein at its N-terminus by a cellular signal peptidase and at its C-terminus by an HCV protease encoded by most of the NS2 region and part of the NS3 domain. This NS2-3 protease is believed to mediate autoproteolytic cleavage at the 2/3 site (115,132), appears to be zinc dependent (132), and can function in *trans,* although inefficiently (115,267). The region that is required for processing at the 2/3 site in mammalian cells lies between residues 849 and 1207 of the HCV polyprotein (115,280). This cleavage event, however, is not required for NS3-serine protease–dependent cleavages downstream, and mutations inactivating the NS3-serine protease have no effect on 2/3 cleavage (115,132). Residues His-952 and Cys-993 of the NS2 region are essential for the NS2/3 cleavage and are thought to be involved in zinc binding (115,132), whereas mutations of Cys-1123, Cys-1125, and Cys-1171 in the NS3 region significantly decrease autoproteolytic activity (132). *In vitro* studies on NS2-3 have shown that the efficiency of processing is at least partially dependent on the presence of microsomal membranes (115,280), suggesting the involvement of a cellular cofactor in this event. The exact mechanism of autoproteolysis at the 2/3 site is still to be determined, alternate zinc protease or cysteine protease mechanisms have been suggested (334); however, further structural and biochemical studies are still required to characterize this viral protease fully.

Whether NS2 itself plays a separate role in viral replication is still to be determined. It has a hydrophobic profile and is a transmembrane protein with the C-terminus located in the ER lumen and the N-terminus in the cytoplasm (280). It has been suggested that NS2 may play a role in virus assembly because it has been coprecipitated with the envelope proteins and the cleavage between E2-p7 and NS2 occurs after the complete folding of E2 (87). Whether this is the case remains to be determined and requires the analysis of NS2 in the context of productive viral replication, as opposed to *in vitro* expression systems.

The NS3 Region

The NS3 encodes serine protease, NTPase, and RNA helicase activities. The serine protease activity has been particularly well characterized and probably plays an essential role in HCV processing because a flavivirus homolog has been previously shown to be indispensable for viral growth (56), making it a desirable target for antiviral drugs. The activity resides in the N-terminal third of the NS3 protein and functions independently in two sets of processing events: first, the cleavage of the NS2/3 site, in conjunction with NS2 (115,132), and second, the release of the remaining downstream NS proteins, by mediating cleavage at the 3/4A, 4A/4B, 4B/5A, and 5A/5B sites (Table 2) (27,115,132,139). The first 181 amino acids of NS3 (amino acids 1027 to 1207 of the polyprotein) have been shown to contain the domain necessary for all of these cleavages (198), with the minimal protease region narrowed down to amino acids 1059 to 1204 (336). Amino acid sequence comparisons of several HCV isolates showed that the NS3 protein contains three highly conserved residues, His-1083, Asp-1107, and Ser-1165, which are known to represent the catalytic triad of the serine proteinase family (56). Substitutions at any one of these residues abolish processing at the downstream sites (27,116,319). The cleavage between NS3/4A occurs in *cis* (28), and although those at the other sites can occur in *trans* (27,198,319), it is yet to be established which type of cleavage dominates during viral replication.

Each of the NS3-dependent cleavage sites contains certain residues, which are conserved both between sites and genotypes, suggesting an importance for substrate specificity (see Table 2) (132,173). These residues include an Asp or Glu at P6, a Cys or Thr at P1, and a Ser or Ala at P1', although *in vitro* studies show that certain sites are more tolerant of substitutions than others (29,170,345). This is distinct from the flaviviruses, in which the NS3-dependent cleavages usually occur after dibasic amino acids (56).

The NS4A protein functions as a cofactor in NS3 activity. It is essential for the NS3/4A and NS4B/5A cleavage events and enhances cleavage at the NS4A/4B and NS5A/5B sites (91,198,134). The N-terminal 22 amino acids of NS3 have been shown to interact with NS4A (30,91,314), creating a stable complex between the two proteins.

The crystal structure of the NS3 protease has been elucidated (160,219,337), and the solution structure of the N-terminal domain has been solved by NMR (25) (reviewed in 27). The protein has a structural zinc-binding site coordinated by residues Cys-1123, Cys-1125, Cys-1171, and His-1175 (160,209,302), mutation of any of which reduces protease activity (132,302). These analyses of the NS3 structure indicated that the substrate-binding pocket (SBP) is primarily determined by residues Leu-1161, Phe-1180, and Ala-1183, with Phe-1180 forming the bottom of the SBP. Substitutions at several sites around the SBP showed that only mutations of Phe-1180 substantially affected specificity (166), confirming the importance of this residue in substrate recognition.

The RNA helicase (157,309) and NTPase (306) activities of NS3 reside in the C-terminal 465 amino acids of the NS3 protein. These functions are less well characterized than the serine protease, although NS3 appears to possess properties similar to the helicases of other positive-stranded RNA viruses and to contain characteristic sequence motifs (306). The helicase unwinds double-stranded RNA (dsRNA) and RNA–DNA heteroduplexes in the 3' to 5' direction using any NTP or deoxyribonucleoside triphosphate (dNTP) as an energy source (121,309). Biochemical characterizations (256,259) and the x-ray crystallographic structure (159) of the helicase have recently been reported, confirming structural features common to other helicases. Mutations of certain residues within the conserved sequence motifs severely affect the functions of the enzymatic activities (128,158,196). These data, combined with biochemical and structural analyses, provide an understanding of the mechanism of action for the helicase and the basis for therapeutic drug design.

The NS4 Region

The NS4 region encodes two viral proteins designated *NS4A* and *NS4B.* The function of NS4B, a very hydrophobic protein, is unknown, although it is required for the phosphorylation of NS5A together with NS4A and NS3 (232,315). NS4A has also been shown to act as a cofactor in NS3 protease activity (see earlier).

NS4A is 54 amino acids in length and has a hydrophobic N-terminal region followed by a hydrophilic C-terminus (90). NS4A enhances the NS3 serine protease cleavages in *cis* and in *trans* (28,90,198,314). The precise region of NS4A essential for the activation of NS3 has been mapped to the central, hydrophobic portion of the protein encompassed by amino acids 21 to 34; a peptide spanning this region is sufficient to enhance cleavage by NS3 (28,91,199,291). A stable, physical interaction occurs between the two proteins that can be destabilized by mutations in NS4A (91,199) and inhibited by a peptide carrying a single residue mutation at position 28 in the 21– to 34–amino acid stretch (291). Although NS4A is not essential for all the processing functions of the serine protease, it appears to form an integral part of the protease complex (160), possibly stabilizing the protein in an active conformation and directing it to membranes where proteolytic processing and RNA replication take place (91,160,199). NS4A has also been shown to form a complex with the NS4B/NS5A polyprotein product (200), in which the NS4A central hydrophobic region is again essential. This interaction may explain the requirement of NS4A as a cofactor for cleavage by NS3 at this site.

The NS5 Region

The NS5 region is processed into the proteins NS5A and NS5B. The primary function of NS5A is unknown, although from comparative studies with other viruses, it is predicted to play a role in RNA replication. The mature NS5A exists as a phosphoprotein (p56); the phosphorylation occurs primarily on serine residues through the action of unknown cellular kinases (268,269). Two phosphoproteins (p56 and p58), termed *phosphorylated* and *hyperphosphorylated* forms, respectively, have been identified for 1b genotypes (20,167,232), for which the production of p58 requires NS3, NS4A, and NS4B expression in *cis* (167,232). A requirement of two domains for phosphorylation of NS5A has been shown (19,315), located around the center (amino acids 2200 to 2250) and C-terminus (amino acids 2350 to 2419). The major phosphorylation site for HCV H strain (genotype 1a) has been mapped to Ser-2321 (268), whereas mutation analysis has identified residues Ser-2197, Ser-2201, and Ser-2204 as putative hyperphosphorylation sites for p58 production by HCV J strain (type 1b) (315).

In addition to its potential involvement in RNA replication, clinical studies suggest that NS5A may play a role in conferring sensitivity to IFN-a through interaction with the IFN-induced cellular protein kinase PKR (106) (see Mechanisms of Persistence).

NS5B forms the C-terminal protein of the HCV polyprotein. It contains a GDD motif, residues 2737 to 2739, common to other RNA-dependent RNA polymerases (RdRps) (254) and appears to be a membrane-associated phosphoprotein (144). RdRp activity has been demonstrated for recombinant NS5B expressed in insect cells (34,205) and *Escherichia coli* (99,240), and RNA-binding domains have been identified within this region (61,205). Four amino acid motifs have been shown to be essential for polymerase activity (205). These were designated A (amino acids 2640 to 2645), B (amino acids 2702 to 2711), C (amino acids 2737 to 2739), and D (amino acids 2762 to 2766) and appear to be important for nucleotide binding and catalysis (A), template/primer positioning (B), and NTP binding and catalysis (C and D).

Most of the recombinant NS5B proteins studied direct replication of RNA in a primer-dependent manner (35,205). The primer may be an exogenous RNA molecule or generated through folding of the input RNA at the 3' end. A full-length, *E. coli*–expressed form of NS5B, however, was able to synthesize genome-length HCV in a primer-independent manner (240). This protein also recognized specific sequences at the 3' end of positive- and negative-stranded HCV RNAs for the initiation of RNA synthesis, suggesting this may more closely represent the authentic HCV RdRp (240). It is probable that replication of the HCV genome involves negative-stranded RNA synthesis initiated by interaction between the NS5B polymerase or replicative complex and the 3'-terminus of the viral genome. Specificity of binding by recombinant NS5B is not limited to RNA templates containing the 3'-UTR of HCV (35,205,240), although conserved stem-loop structures within the 3'-coding region of the HCV RNA have been shown to interact specifically with NS5B (61). The mechanism of HCV replication is still to be determined, as are the other components of the replicative complex; these analyses of the RdRp provide important contributions toward understanding this process and the development of possible antiviral agents targeting this protein.

Virion Morphology

The genome of HCV was sequenced and characterized long before virus-like particles were described. It was shown early on that HCV is inactivated by chloroform (98), indicating that particles are enveloped, and it was shown through filtration that the diameter is between 30 and 60 nm (126). Low levels of virus in plasma samples and problems of *in vitro* cultivation have made visualization of this virus difficult; however, virus-like particles have been identified using electron microscopy (292). Figure 3 shows spherical particles about 50 nm in diameter that specifically reacted to HCV

antibodies, observed in thin-section electron microscopy of an *in vitro* infected cell line (292).

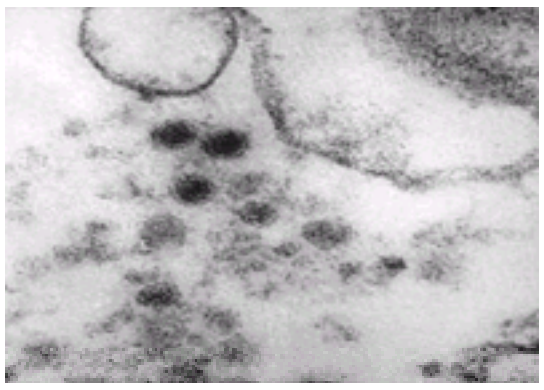

FIG. 3. Standard thin-section electron microscopy of *in vitro* infected HPBALL cells harvested on day 25 postinoculation showing hepatitis C virus–like particles. (From ref. 292, with permission.)

Sucrose gradient analyses of HCV-positive sera and plasma have isolated both low-density (1.06 to 1.13 g/mL) and high-density (1.17 to 1.25 g/mL) fractions containing HCV RNA (133,152,317). It has yet to be established whether these fractions represent intact virions or forms of subviral HCV RNA–containing material. It is probable that association with low-density lipoproteins (LDL) is responsible for the low-density fraction. A density of 1.06 g/mL is unusually low, even for an enveloped RNA virus such as HCV, and is close to that of plasma LDL (133), whereas the higher-density fractions (1.1 to 1.18 g/mL) possibly represent free virus or particles complexed with immunoglobulin (Ig) (66,133). Association with LDL could confer two advantages to the virus: (a) particles may be protected from antibody-mediated neutralization, and (b) virions may gain entry into cells through the cellular LDL receptor. These data argue in favor of a protective association between HCV virions and LDL, and recent reports suggest that the LDL receptor can mediate uptake of HCV, but it is unclear whether this leads to productive infection (3,227).

Replication

One of the major hurdles in testing both neutralizing antibodies and antiviral drugs to combat HCV infection is the lack of an effective cell culture system for the virus. The data regarding the functions of HCV genes and replication of the virus have been derived using established mammalian cell expression systems or the chimpanzee animal model. Infectious RNA transcripts used in chimpanzees have demonstrated requirements for 3'-UTR elements and functional enzymatic activities in HCV replication (172,340). This model, however, is too expensive for extensive analyses such as these. Unfortunately, a tissue culture system reliable enough to support HCV growth to the extent that experiments on replication can be carried out is yet to be developed.

Tissue Culture

There have been several reports of mammalian cells able to support the growth of HCV. The cell lines used include MT2, peripheral blood mononuclear cells, lymphocyte cell lines, and hepatocytes from humans and chimpanzees (reviewed in 213). An enhancement of HCV replication in MT2 cells by Epstein-Barr virus was recently reported; in particular, the *EBNA1* gene was thought to be involved (305). These systems rely on the use of reverse transcription–polymerase chain reaction (RT-PCR) for the detection of virus and, in particular, strand-specific RT-PCR as evidence of virus replication. Data indicating the presence of negative-stranded RNA need to be interpreted with care because specificity and sensitivity can vary depending on the choice of primers and reaction conditions (187,194). Data indicate, however, that there exist limited cell culture systems for HCV; with further development, these may provide an effective *in vitro* study system for the virus.

Alternative approaches to studying virus assembly or the role of viral antigens in replication have involved the production of virus-like particles (34) or pseudotype vesicular stomatitis virus (VSV) particles containing E1 or E2 (183). These systems provide a potential means of studying envelope glycoprotein interactions, essential elements required for particle assembly, and neutralizing antibody activity. A subgenomic replicon containing most of the nonstructural proteins of HCV was shown to replicate to high levels in a hepatoma cell line (206); radiolabeling of viral RNA and proteins was possible. Systems such as this may provide the basis for defining functional HCV replication units and testing of antiviral drugs.

Experimental Animal Models

Chimpanzee

The chimpanzee (*Pan troglodyte*) is genetically more than 98.5% identical to humans and is the only proven animal model for HCV infection and disease. Chimpanzee studies provided the first evidence of HCV transmission (137), a means of measuring infectivity, and material for cloning and characterizing the HCV genome (45,64). Evidence of a lack of protective immunity against HCV, understanding of the role of HVR1, and insights into possible vaccine strategies have also been gained from chimpanzee studies. Finally, this model has provided the only reliable means of testing the infectivity of transcribed RNAs from molecularly cloned, putative full-length HCV cDNA (169,338).

In most aspects, the virologic and clinical presentation of acute HCV infection is similar in humans and chimpanzees. This includes onset of disease, level of viremia, and timing of serologic response and liver damage, as indicated by alanine aminotransferase (ALT) elevation. There are, however, some differences. Only about 30% to 50% of

chimpanzees develop persistent HCV infections (31), compared with as many as 70% to 80% of humans.

The onset of hepatitis C in chimpanzees is typically mild. Although about 25% of patients with transfusion-associated hepatitis C become jaundiced, there are no reports of jaundice in a chimpanzee due to HCV infection. This milder disease in chimpanzees is also reflected in lesser degrees of hepatic injury measured by ALT levels or histopathology. Chimpanzees rarely develop significant chronic liver disease, although both chronic hepatitis and hepatocellular carcinoma have been reported (326). This low incidence may be biased, however, by the young age at which many animals are infected and the short observation period.

Humoral immune responses to HCV structural proteins are observed less frequently and are found to be significantly weaker in chimpanzees than in humans (4,54,63). Even in the absence of strong humoral immune responses, however, the cellular immune response has been described to be quite vigorous in chimpanzees with acute, self-limited hepatitis C (77). Termination of HCV infection correlates precisely with the onset of a multispecific intrahepatic cytotoxic T-lymphocyte (CTL) response that can persist for more than 1 year after resolution of infection (75).

The development of infectious RNA transcripts from a functional molecular clone allowed infection and pathogenesis studies with a single, well-defined viral polyprotein sequence. This greatly simplified the study of HCV evolution during acute and chronic infection and the analysis of HCV-specific immune responses (169,214,338,339).

After intrahepatic injection of transcribed full-length uncapped RNAs, circulating HCV RNA was generally detectable in the serum by 1 week after inoculation and increased during the first 10 to 12 weeks (169). ALT levels peaked at weeks 10 to 14 after infection, and in most animals, seroconversion was observed after 2 to 3 months (169). The severity of intrahepatic histologic changes, especially portal inflammation and focal necrosis, was typical for hepatitis C in chimpanzees and paralleled ALT elevations. These pathogenic profiles are strikingly reminiscent of those obtained in chimpanzees inoculated with the infectious HCV-containing samples. These experiments proved that circulating RNA is indeed due to authentic virus replication, rather than release of inoculated nucleic acid, and defined the elements of a functional HCV genome. In addition, the demonstration that RNA transcribed from cloned HCV cDNA could initiate infection and cause hepatitis in chimpanzees provided the formal proof that HCV alone is the causative agent of this disease.

Mouse

Although HCV does not infect mice, several mouse models have proved valuable in hepatitis C research. Mice that are transgenic for human major histocompatibility complex (MHC) molecules, for example, have been used efficiently to identify HCV epitopes and to test vaccination strategies. By immunization with peptides, DNA vaccines, or vaccinia viruses expressing HCV sequences, multiple human leukocyte antigen (HLA)-A2–restricted T-cell epitopes of the hepatitis C virus have been defined (248,294,330). These epitopes were endogenously processed and also recognized by HCV-infected humans. Thus, mice transgenic for human MHC molecules represent an excellent model system to develop immunotherapeutic vaccines that may be used in humans. For example, a multiepitope vaccine has been shown to induce simultaneous CTL responses to a pool of five different epitopes (248).

Transgenic mice that express individual or several HCV proteins have been used to analyze the biosynthesis of HCV proteins and their pathogenic role. Liver-specific expression of HCV core and E2 proteins resulted in the predominantly cytoplasmic presence of core protein with occasional nuclear staining and both cytoplasmic and membrane expression of the E2 protein. Importantly, the liver of two independent mouse lineages remained histologically normal for the entire observation period, that is, until 6 months and 18 months of age, respectively (156,250). In contrast, transgenic mice expressing HCV core under the control of a regulatory region from HBV developed progressive hepatic steatosis (229), and one fourth of the male transgenic mice developed hepatocellular carcinoma (238). This effect was specific for the expression of HCV core as a transgene because transgenic mice that carried HCV E1 and E2 under the same transcription control region did not have neoplastic lesions in the liver (168).

Finally, immunodeficient mice have been used to test the hypothesis that HCV persists in peripheral blood mononuclear cells (PBMC). Specifically, severe combined immunodeficiency (SCID) mice have been intraperitoneally inoculated with hematopoietic cells from HCV-infected subjects (43), and HCV-infected human liver fragments have been transplanted under the kidney capsule of immunodeficient mice (108). The studies suggest that HCV persists in mononuclear cells at a low rate of multiplication.

Tree Shrew

Tree shrews, tupaia belangeri chinensis, have been shown to be susceptible to HCV inoculation. So far, these animals have been experimentally infected with HCV genotypes 1a, 1b, and 3, but viremia was only transient or intermittent, with a low rate of anti-HCV seroconversion (335).

PATHOGENESIS

Immunobiology of Infection

Viral Entry into the Host

Although the primary source of HCV infection has not been determined in up to 40% of cases, HCV infection is generally thought to occur through parenteral routes. HCV virions spread through the bloodstream, and as early as 2 days after intravenous infection of chimpanzees, HCV RNA becomes detectable in the liver, the primary site of HCV replication.

A candidate protein for the cellular HCV receptor is human CD81, a transmembrane molecule that is expressed on most

human cells except red blood cells and platelets. Binding of CD81 coated on beads to the HCV E2 domain and to HCV RNA–containing particles in chimpanzee infectious plasma is species specific and can be inhibited by anti-E2 antibodies and by serum from chimpanzees vaccinated with E2 protein (253).

Another candidate receptor is the low-density lipoprotein receptor (LDLR) (3,227). Endocytosis of the HCV receptor complex, as well as its inhibition by anti-LDLR antibody, has recently been demonstrated *in vitro.* It has yet to be shown whether either of these receptors provides a route of entry that leads to productive infection in cells.

Cell and Tissue Tropism

As the primary site for HCV replication, the liver contains the highest levels of HCV RNA, ranging from 10^{8} to 10^{11} copies per gram of tissue (304). However, only a small percentage of hepatocytes (5% to 19%) are HCV RNA positive by *in situ* hybridization. Accordingly, expression of HCV antigens is rather low (1% to 10%). In addition, only 1% to 5% of mononuclear cells, and an even smaller proportion of biliary epithelial or sinusoidal lining cells, express HCV antigens (237). Most infected cells display little or no hepatocellular damage, which may suggest weak stimulation of the immune system due to low antigen expression (23) (see later).

RT-PCR techniques with a high specificity for the negative-stranded HCV RNA also commonly detected RNA in lymph nodes and pancreas and less frequently in adrenal glands, bone marrow, thyroid tissue, and spleen (189). Whether HCV also replicates in PBMC is controversial. Indirect evidence for extrahepatic replication of HCV in PBMC is derived from the observation that HCV HVR1 sequences isolated from PBMC of HCV-infected patients displayed considerable differences in the complexity of quasi-species as compared with HVR1 sequences isolated from serum and liver of the same individuals. The predominant sequences from each source were mutually different in 23% of patients (245).

Immune Responses

Humoral Immune Response

Although individuals vary in both specificity and timing of anti-HCV seroconversion, HCV-specific antibodies are generally detectable 7 to 31 weeks after infection (62). The humoral immune response is usually multispecific and targeted against epitopes within the HCV core, envelope, NS3, and NS4 proteins (4,146,299).

A major problem in assessing the relevance of these antibody responses is the lack of a convenient neutralization assay for HCV. As in other viral infections, antibody binding could interfere with HCV entry into host cells or viral replication, and it could opsonize virions for elimination by macrophages.

Evidence for a protective role of antibodies stems from limited studies in which chimpanzee or tissue culture infectious HCV was neutralized *in vitro* by incubation with antibody (95,293,344). Thus far, the HVR1 of the HCV E2 protein has been identified as the major target for neutralizing antibodies (95,293). This N-terminal sequence of 30 amino acids exhibits high sequence variability (130,155). Sequence variability in HVR1, however, does not appear to evolve by random drift, because virologic data from a single-source infection showed that amino acid replacements either are limited to particular sets of amino acids with shared biochemical properties or are completely conserved (218). Thus, amino acid substitutions appear to be influenced by both negative and positive selection, presumably by the immune response of the host.

Neutralizing antibodies appear to be targeted to sequences within the envelope glycoproteins in addition to the HVR1 sequence. Vaccination with recombinant envelope proteins expressed in mammalian cells elicited high titers of glycoprotein-specific antibodies that protected chimpanzees from low-dose homologous HCV challenge (63). In this experiment, the strongest correlate of protection was the level of specific antibodies that were capable of inhibiting binding of the HCV E2 to the candidate HCV receptor CD81 expressed by the cell line Molt-4 (253,275). This neutralization of binding (NOB) assay was also employed to detect high titers of antibodies that interfered with binding to the HCV E2 protein in the serum of patients who resolved chronic but not acute hepatitis C (147). The data suggest that the epitopes responsible for priming of NOB antibody responses are conserved in differing viral genotypes.

Despite these observations, however, the role of antibodies targeted to the envelope proteins, especially the HVR1, remains less clear. Several studies have suggested that neutralizing antibodies target the envelope proteins (63,94,95,275), that an early antibody response to the HVR1 is associated with viral clearance (9,347), and that more complex initial quasi-species distribution and viral complexity of the HVR1 correlate with HCV persistence (266). A recent study, however, reported that clearance of HCV in 28 chimpanzees was not associated with an antibody response to E1 and E2 (32). In addition, in 12 chimpanzees with persistent infections analyzed from two different studies, there was no evidence that the persistence was related to the development of immune escape of variants in the E1, E2, and specifically, HVR1 region (32,214). These results suggest that additional mechanisms are involved in maintaining viral persistence (see later).

Cellular Immune Response

The cellular immune response is thought to play a particularly important role in the host's defense against infections with noncytopathic viruses such as HCV because of its ability to recognize viral antigens in and to eliminate virus from infected cells. Although it is known that the liver is significantly enriched for cells of the innate immune response, such as natural killer cells, as well as for T-cell receptor-g– and d–positive cells and Va24-positive T cells, the role of the innate and antigen-nonspecific immune response to HCV has not yet been sufficiently studied. Most analyses have concentrated on the induction, effector function, and maintenance of HCV-specific CD4+ helper T cells and CD8+ cytotoxic T cells.

CD4+ Helper T Cells

Recent studies suggest that the HLA class II restricted T-cell response is particularly important to the outcome of acute HCV infection (84,111,185,224). Specifically, it has been demonstrated that patients with acute self-limited HCV mount an early as well as vigorous and multispecific helper T-cell response that is readily detectable in the peripheral blood (84,185). If, in contrast, this immune response is weak, less efficient, or not maintained for a sufficient length of time, patients proceed to persistent infection and chronic hepatitis (111). Thus, the intensity, epitope specificity, and cytokine profile of the T-cell response during the early stages of HCV infection appear to be predictive of viral clearance and recovery. Interestingly, these findings correlate well with the clinical observation that recovery from hepatitis C usually occurs within 3 months of clinical onset and that it is more common in patients with clinically evident than with asymptomatic disease (112).

A list of HCV sequences and epitopes targeted by CD4+ helper T cells is presented in Table 3. The most frequently recognized epitopes are contained within HCV core 21 to 40, NS4 1767 to 1786, NS4 1909 to 1929, and NS3 1251 to 1272, with the putative minimal optimal epitope within the NS3 sequence located at amino acids 1251 to 1259. Remarkably, these epitopes not only are highly immunogenic and immunodominant but also are conserved among HCV genotypes, indicating that mutations in these sequences may not be compatible with the HCV life cycle. Moreover, all of these T-cell epitopes bind promiscuously to a large variety of different HLA class II molecules with high affinity, suggesting that they can be efficiently presented and recognized by patients with many different HLA haplotypes. Thus, they are ideal candidates to be included in prophylactic or therapeutic T-cell–based vaccines against HCV.

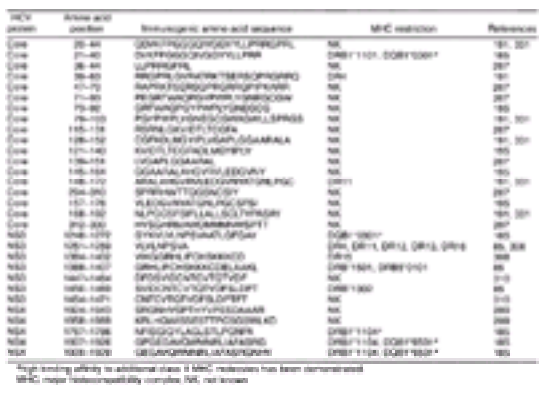

TABLE 3. *HCV peptides recognized by human class II MHC restricted CD4$^+$ T cells*

Helper T cells mediating this HCV-specific immune response have been shown to display a helper T-cell subtype 1 (T_H1) or T_H0 cytokine profile, whereas a T_H2 profile is associated with a chronic course of infection (322). In another study, the levels of circulating interleukin-2 (IL-2), IL-4, IL-10, and IFN-g were significantly higher in patients with chronic hepatitis C than in uninfected controls (50).

After recovery from acute hepatitis C, the HCV-specific helper T-cell response has been observed to persist. Whether this HCV-specific memory T-cell response is able to protect against reinfection with homologous or heterologous virus is not yet known and can only be analyzed in chimpanzees. Similarly, a frequently discussed, controversial question is whether maintenance of a virus-specific memory response requires persisting antigen or even low levels of replicating virus for periodic de novo induction of virus specific T cells. Although trace amounts of persisting, replicating virus have been detected in patients who have clinically and serologically recovered from hepatitis B virus (272), this question cannot yet be answered for HCV.

CD8+ Cytotoxic T Cells

Only three recent studies analyze the role of the CTL response in acute hepatitis C of humans and chimpanzees (56,75,192). In all three studies, an early, vigorous, multispecific CTL response was associated with a self-limited outcome of infection. In the chimpanzee model, the onset of this multispecific intrahepatic CTL response correlated precisely with termination of infection (75).

To identify potentially protective CTL responses, several other studies have analyzed the CTL response of HCV-seronegative, healthy people with frequent exposure to HCV. Interestingly, individuals who were repetitively exposed to HCV either occupationally (178) or by HCV-infected spouses (284) mounted peripheral blood HCV-specific helper and CTL responses in the absence of either a humoral immune response or viremia. This observation is compatible with the hypothesis that T-cell memory may be induced by periodic reintroduction of viral antigen by recurring, subclinical viral exposure.

Similar to helper T-cell epitopes, CTL epitopes have been identified in all viral proteins (33,55,89,175,176 and 177) (Table 4). As in other viral infections, with influenza and human immunodeficiency virus (HIV), HCV-specific CTLs have been shown to be broadly cross-reactive and able to recognize different viral subtypes. Several epitopes have also been demonstrated in the context of several HLA haplotypes, which have been grouped to HLA supertype families. To date, nine HLA class I supertypes are defined according to the A2-like, B7-like, B44-like, A1-like, A24-like, B27-like, B58-like, B62-like, or A3-like binding motif that a given viral peptide needs to contain in order to bind to a specific HLA allele. In total, more than 95% of the world population displays at least one of these HLA supertypes, an important consideration

for the potential applicability of a CD8+ T-cell–based vaccine.

TABLE 4. *HCV epitopes recognized by class I MHC restricted CD8+ T cells*

Because repetitive *in vitro* stimulation with HCV-derived peptides is required to expand CTL from the peripheral blood, the frequency of progenitor CTL in the circulation is assumed to be rather low (55,271). This has been confirmed by the recently developed technique of staining peptide-specific T cells with MHC peptide tetramers, a technique that does not require *in vitro* expansion of T cells. In the peripheral blood, 0.01% to 1.2% of circulating CD8+ T cells have been shown to be specific for two analyzed HCV NS3 epitopes, whereas at the site of inflammation, the infected liver, the frequency of epitope-specific CD8+ T cells was at least 30-fold higher (127). All of the intrahepatic NS3-specific CD8+ T cells were CD69+, suggesting that they were activated and presumably contribute to immune-mediated liver injury. Notably, however, this virus-specific intrahepatic response is not sufficient to induce viral clearance in most chronically infected patients presumably because the frequency of intrahepatic HCV-specific T cells in chronic hepatitis C is still significantly lower than that for other self-limiting infections (212).

The apparent weakness of the HCV-specific CTL response is not due to generalized immunosuppression because the CTL response against influenza virus (273) and Epstein-Barr virus (135) is normal in HCV-infected patients. The HCV-specific CTL response may therefore be too weak to achieve viral clearance but may exert some control over viral load during chronic infection. Indeed, a stronger polyclonal CTL response in the peripheral blood (270) and the liver (233) is associated with lower levels of HCV viremia. It appears, however, that this occurs at the expense of an inflammatory liver disease, which after many years to decades, may lead to irreversible liver cirrhosis and hepatocellular carcinoma.

Mechanisms of Immune-Mediated Liver Injury

Several histologic features allow a clear differentiation between hepatitis C and other types of inflammatory liver disease. Specifically, the triad of steatosis, bile duct damage, and lymphoid follicles within the portal tracts is characteristic for hepatitis C (21,282).

Steatosis manifests as large droplets of fat vacuoles in the cytoplasm of hepatocytes (193). Importantly, transgenic mice that expressed the HCV core protein intrahepatically also developed progressive hepatic steatosis (239). Bile duct damage is a frequent finding in hepatitis C but is uncommon in other forms of chronic viral hepatitis (21,193). Finally, eosinophilic material (Mallory-like bodies) in the cytoplasm of periportal hepatocytes is found in about 20% of chronic cases of HCV hepatitis (193).

The level of necroinflammatory activity, fibrosis, and cirrhosis can vary widely and is not necessarily associated with an increased serum alanine aminotransferase activity. Disease progression to liver cirrhosis is promoted by various cofactors, such as co-infection with other hepatotropic viruses (e.g., HBV [73,100]) and alcohol intake (220,249). HCV infection also appears to increase the susceptibility to liver injury after iron loading in chimpanzees (31).

Primary hepatocellular carcinoma (HCC) is a frequent complication of longstanding chronic active hepatitis. The mean intervals between infection and onset of cirrhosis and of HCC are relatively long and are estimated to be 21 and 29 years, respectively (164). As recently shown in hepatitis B virus infection, HCC is assumed to be induced by chronic immune-mediated hepatocellular injury and regeneration. Indeed, in regions of active inflammation, CD8+ T cells can be found surrounding hepatocytes expressing Fas antigen, and the generation of acidophil Councilman's bodies is interpreted as CTL-induced hepatocyte apoptosis (134).

Immune-mediated liver disease is initiated by HCV-specific liver-infiltrating T cells. These cells, which are estimated to represent about 1% of the total body lymphocytes, are continuously recruited from the peripheral blood (238) and do not undergo clonal expansion in the liver. With recruitment of T cells from the blood, a certain local and functional compartmentalization has been observed. Although CD4+ helper T cells are found in the portal areas, CD8+ lymphocytes infiltrate the hepatic lobules. B cells, on the other hand, are predominantly seen in germinal centers of lymphoid follicles located in the portal tracts (246). Studies of the effector function of intrahepatic T cells revealed that the intrahepatic helper T-cell response is focused on the HCV NS4 protein and mediated by T cells that express a selective T-cell receptor not found in the peripheral blood compartment (223). The predominant use of Vb5.1 by intrahepatic T cells (154) also indicates oligoclonal expansion and a restricted specificity for a common immunodominant HCV antigen.

Presentation of HCV antigens on infected hepatocytes, recognition by CTL, and induction of liver injury appear to be enhanced by HCV-induced expression of HLA-A, -B, and -C and by intracellular adhesion molecule 1 (23). In more aggressive forms of HCV infection, even CD68+ macrophages and monocytes are found. These activated cells are

thought to mediate and enhance antigen presentation and hence the inflammatory process as a whole (215). As a result, most cases of circulating HCV are thought to be produced through continuous rounds of *de novo* infection and rapid turnover of cells, not through release from chronically infected cells (103).

The mechanisms that regulate T-cell–induced viral clearance versus T-cell–induced liver injury are not completely understood. Liver-infiltrating HCV-specific T cells are T_H1 dominant and have been shown to produce IFN-g and tumor necrosis factor-a (TNF-a). These cytokines can suppress replication and gene expression of other viruses, such as HBV, without provoking liver disease. Thus, it is possible that HCV is less susceptible to these cytokines and that destruction of infected hepatocytes plays the predominant role and promotes chronic inflammatory liver disease.

Destruction of infected hepatocytes may occur in several ways: HCV-infected hepatocytes can be killed by HCV-specific CTL clones by means of Fas ligand, TNF-a, or perforin-based mechanisms. Fas ligand–induced apoptosis of hepatocytes is especially feasible because expression of Fas, a mediator of apoptosis, is upregulated in HCV-infected hepatocytes as well as in uninfected bystander cells in response to inflammatory cytokines (225) and because Fas (CD95) is expressed on activated, liver-infiltrating T cells (204). TNF-a is predominantly produced by macrophages (324) but is also released by (176) and expressed on the surface of (161) CTL. Finally, perforin-mediated mechanisms may contribute to lysis of antigen presenting, anti-Fas and TNF-a–resistant cells (18).

After exerting their effector functions, most of these liver-infiltrating T cells undergo programmed cell death themselves. T_H1 cells are preferentially attracted into HCV-infected livers and are especially sensitive to Fas- and FasL-induced apoptosis. Nuti and colleagues estimated a loss through apoptosis of 2×10^8 cells, with is 0.1% of the total body lymphocytes, per day (238).

Mechanisms of Persistence

The mechanisms whereby HCV circumvents the immune response, persists, and causes chronic inflammatory liver disease are currently undefined. One hypothesis is that the HCV-specific immune response is too weak to clear HCV from all infected hepatocytes once a persistent infection is established. Hypothetically, this may be due either to an insufficient induction of the primary immune response during acute infection (75) or to the inability to maintain the T-cell response at high levels during chronic hepatitis C (111). Generally, the intensity of the initial immune response depends on antigenic load, co-stimulatory signals, the type of the antigen-presenting cell, and differentiation and cytokine profile of helper T cells. Each of these factors may not be optimal in acute HCV infection because, for example, display of HCV antigens is relatively low and co-stimulatory molecules are predominantly expressed on professional antigen-presenting cells in the lymph nodes and not on hepatocytes, the preferred cell type for HCV replication. It has also been shown that dendritic cells (DC) from HCV-infected individuals exhibited an impaired stimulatory potential against allogeneic CD4 T cells (153). Exogenous IL-2 or IL-12 restored such defective DC capacity, which offers promise for the modulation of DC functions that are presumably due to the observed lower expression of IFN-g levels, lower CD86 expression, and IL-12 p35 and p40 transcripts (153).

Second, recent reports indicate that individual HCV proteins may actively suppress the immune response of the host. For example, expression of HCV core by recombinant vaccinia viruses suppresses the generation of vaccinia virus-specific cytotoxic T cells in mice (188). Although the mechanisms by which this inhibitory effect of HCV core is mediated have not been identified, numerous regulatory roles of HCV core that affect signal transduction, expression of viral and cellular genes, and cell growth and proliferation are known and can potentially mediate these effects (60,217,264,265,290,343).

In addition, two separate proteins of the hepatitis C virus have been shown to interfere with the antiviral actions of IFN. IFN induces antiviral genes, such as double-stranded RNA-activated protein kinase (PKR) at the transcriptional level. PKR inhibits protein synthesis by phosphorylation of the translation initiation factor eIF2a. Strikingly, an E2 sequence of the IFN-resistant HCV genotypes 1a and 1b, but not the IFN-sensitive HCV genotypes 2a, 2b, and 3a, exhibits homology with the phosphorylation sites of the IFN-inducible protein kinase PKR and the translation initiation factor eIF2a (316). These E2 sequences of the HCV genotype 1a and 1b are able to bind to and inhibit the kinase activity of PKR.

Particular sequences in NS5A between amino acids 2209 and 2248, termed the *interferon sensitivity-determining region* (ISDR), appear to correlate with the sensitivity of HCV to IFN treatment. This function is highly controversial, and many groups have found no association between NS5A sequences and IFN resistance or sensitivity in infected patients (70,251,252). This HCV nonstructural protein has been shown, however, to bind and inhibit the IFN-induced protein kinase PKR (105,106,312), an immediate-early effector of the cellular antiviral response induced by IFN. Interestingly, the interaction of NS5A with PKR is modulated by mutations within the ISDR, which has also been shown to be the PKR-binding domain (107). Therefore, the observations that HCV antigens interact with an IFN-induced protein may in part explain the mechanism of relative IFN resistance observed with some genotypes.

A third hypothetical explanation for HCV persistence could be the high genetic variability of the virus due to a lack of proofreading activity of the viral RNA polymerase. Recent studies suggest that circulating HCV has a half-life of only about 3 hours, indicating relatively efficient virus replication and release, at least in patients with high levels of viral load (234). This dynamic process, capable of continuously generating viral variants, allows genetic variation to be an important strategy for the establishment and maintenance of persistent infection in order to adapt quickly to humoral or cellular immune selection pressure. Weiner and associates reported the emergence of an HCV mutant that was able to escape the HCV-specific CTL response in a chimpanzee with chronic evolution of HCV infection (328). Sequence variations in T-cell epitopes have also been found in patients with acute and chronic hepatitis C (57,151,321), although at a low frequency. Evidence of selection pressure exerted by the humoral immune response has been obtained from several clinical studies (reviewed in 101). More complex initial quasi-species with sequence changes in E1 and HVR1 of E2 have been reported more often in patients who develop persistent infection than in patients who clear HCV (266). In immunocompromised patients, the viral nucleotide sequence variability in the HVR1 of HCV was markedly lower than in immunocompetent HCV-positive patients, suggesting that immune selection, primarily by HVR1-specific antibodies,

influences sequence variation in this region (239).

EPIDEMIOLOGY

Hepatitis C is a disease with a worldwide distribution, although the prevalence varies broadly among geographic areas. It is estimated that there are 170 million chronic carriers of HCV in the world, and reported prevalence rates range between about 0.1% in countries like Iceland and Norway to the 18.1% reported in Egypt (129). Because most infections with HCV become persistent, spread is primarily by parenteral exposure to the blood of chronically infected individuals. Therefore, factors such as social and cultural customs, medical practices, available medical technology, and individual behaviors that lead to exchange of blood between individuals all contribute to the infection rates in a specific area or country.

Prevalence and Incidence

The Centers for Disease Control and Prevention (CDC) has estimated that there were about 28,000 new infections with HCV in the United States in 1995. This compared with an incidence of about 180,000 new cases per year in the mid-1980s (14) (Fig. 4). This dramatic drop off in new infections is partially due to screening blood donors for antibody to HCV. More important, however, this decline likely reflects changes in drug use and other behavior patterns possibly associated with the AIDS epidemic. The Third National Health and Nutrition Survey, a large-scale serologic survey, was conducted between 1988 and 1994 by the CDC. Serum samples were tested for anti-HCV and for HCV RNA from 21,241 people 6 to 89 years of age (17). The prevalence of anti-HCV in this population was 1.8%, which extrapolates to about 3.9 million Americans infected by HCV. HCV RNA was detected in 73.9% of the HCV-positive subjects. Thus, it is estimated that at least 2.7 million Americans are chronically infected with HCV. The age-stratified prevalence rates are shown in Figure 5. It is clear that HCV infections in the United States are most common in the middle adult years, peaking in the fourth decade, with 65% of all infections occurring among people 30 to 49 years of age. Because drug use is the most important risk factor, the age distribution may represent a cohort effect of the rapid increase in use of illicit drugs among young adults around the time of the Vietnam War. In other parts of the world, the epidemiology may be different. In Italy, it was found that the prevalence of anti-HCV in the population increased with age. It is believed that there was a lot of HCV transmission before the Second World War due to the use of unsterilized injection equipment (120). Demographic factors associated with increased prevalence of HCV infections, including race, economic status, and marital status, are shown in Table 5. Behavioral factors that most strongly correlated with HCV infections were illegal drug use and high-risk sexual behavior. The adjusted odds ratio for any cocaine use was 5.16 compared with people who denied ever using cocaine and was 2.99 for those who admitted to using marijuana more than 100 times compared with those who denied ever using it. The use of injection drugs was not determined in this survey, and it is assumed that the use of marijuana was a surrogate marker for other high-risk behavior. The number of sexual partners and the age at first sexual intercourse also correlated with HCV. The adjusted odds ratio for greater than 50 sexual partners was 5.16 compared with people with no partners or one partner (17).

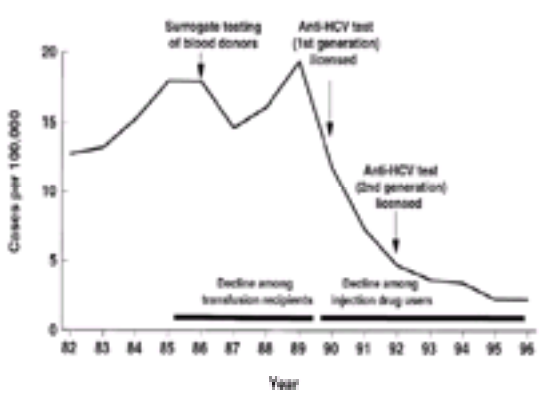

FIG. 4. Estimated incidence of acute hepatitis C virus infections in the United States between 1982 and 1996. (Data from the Centers for Disease Control and Prevention.)

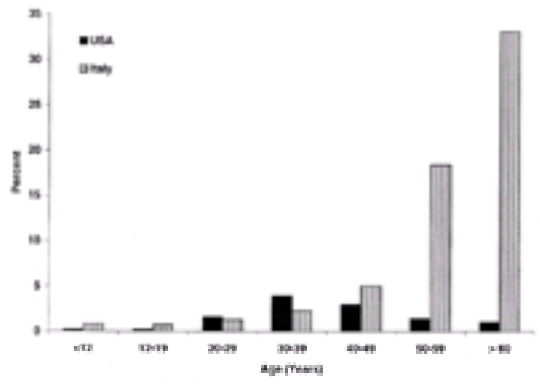

FIG. 5. Age stratified prevalence of antibody to hepatitis C virus in the United States and Italy. (Data from refs. 17 and 120, with permission.)

Characteristic	Prevalence of Anti-HCV (%)
Race or ethnic group	
Non-Hispanic white	1.5
Non-Hispanic black	3.2*
Mexican American	2.1
Other	2.9
Sex	
Male	2.5*
Female	1.2
Marital status	
Divorced or separated	5.1*
Never married, married, or widowed	1.8
Poverty index	
Below poverty level	3.2*
At or above poverty level	1.6
Education	
<12 y	2.8*
>12 y	1.3

*$p < 0.05$ for the comparison with non-Hispanic whites.
†$p < 0.05$
Data from ref. 17, with permission.

TABLE 5. *Prevalence of antibody to HCV (ANTI-HCV) according to demographic characteristics*

Modes of Transmission

Most hepatitis C infections become chronic, with a resulting persistent viremia. Transmission of the virus from person to person by parenteral exposure to blood from a persistently infected individual is believed to be the most common means of infection. However, nonparenteral or nonapparent parenteral exposure probably is also likely to occur (17). Although it appears that HCV can be transmitted by mucosal exposure, compared with HBV infection, this route is very inefficient. The low titers of HCV in the blood relative to hepatitis B likely accounts for the difference in mucosal transmission, although other virologic factors could also be important. Although HCV is a member of the Flaviviridae family, there is no epidemiologic or experimental evidence for transmission by an insect vector, although it remains theoretically possible.

The single most important mode of transmission of HCV in the United States and probably most Western countries is the illicit use of injectable drugs. At this time, HCV has been virtually eliminated from the blood supply, but studies from before the HCV-screening era indicated that even then, drug use was the most common identifiable exposure found in patients with chronic HCV infections (16). Sexual transmission of HCV is not as common as it is with hepatitis B virus, but it seems to occur. It is difficult to control for other behavior-related risk factors when studying spousal transmission, rates among sex workers, or rates observed in sexually transmitted disease clinics. However, very low rates (2.7% to 2.9%) of HCV positivity have been observed in sexual partners of HCV-positive hemophiliac patients, and the few positive partners had alternative risk factors (42,124). In a study of household contacts of 60 women who had been infected with HCV by a contaminated batch of anti-D Ig, there were no male partners positive and only one of 170 children positive. However, several studies correlate HCV positivity with number of sexual partners, employment in the sex industry, or sexually transmitted diseases (22,210,278). Male homosexuals also tend to have higher rates of HCV, but transmission by male homosexual practices occurs less frequently than that of HBV, and several studies have found that male homosexual sexual practices appear to play a minor role, if any, in transmission (39,247). An analysis of asymptomatic blood donors who were found to be anti-HCV positive found an association between admitted cocaine snorting and HCV infection (10). It is possible that this represents transmission by trauma to the mucous membranes with a shared cocaine straw. It is also possible that admitted cocaine use correlates with intravenous drug use that was not admitted (17).

Risk Factors

Any behavior, occupation, or medical condition that results in frequent percutaneous exposure to blood presents the most important risk for acquiring hepatitis C. Figure 6 shows the changing importance of several selected risk factors associated with acute HCV infection from 1983 through 1996. In studies performed before specific screening tests for HCV infections were available, there was a significant association of acute NANBH and a history of transfusion within the 6 months preceding the transfusion (15). With the understanding that HIV could be transmitted by blood transfusion, blood donors began to be screened by a rigorous interview that included lifestyle questions. Many of the behaviors that relate to increased risk for HIV are also related to HCV risk. In addition to the enhanced interview, donor blood was screened by two nonspecific tests that could identify some high-risk donors. A test for antibody to HBcAg identified individuals who had been exposed to HBV. It is presumed that people who had been exposed to HBV would be at increased risk for HCV infections as well. In one retrospective analysis, it was found that 11.9% of people receiving blood transfusions including at least one unit that was anti-HBc positive, compared with 4.2% of recipients of all anti-HBc–negative blood, developed NANBH (179). It was calculated from this study that if the blood had been screened for anti-HBc, 43% of the posttransfusion NANBH cases would have been prevented.

The second nonspecific screening assay to reduce posttransfusion hepatitis was the measurement of the ALT level in the donor blood. A French study found that the risk from HCV infection from blood transfusion dropped from 4.1 per 1000 transfused units to 3.4 per 1000 units in recipients of ALT-screened blood and to 1.4 per 1000 units in recipients of ALT plus anti-HBc–screened blood (150). Surrogate testing was instituted in 1986 in the United States, and with the addition of specific tests for anti-HCV in 1991, it has been estimated that the risk for acquiring HCV from a blood transfusion is now about 1 in 103,000 transfusions (283).

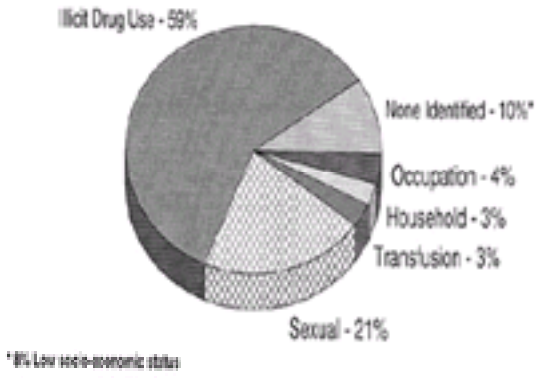

FIG. 6. Proportion of acute hepatitis C cases related to selected risk factors in the United States between 1983 and 1996. Before testing, transfusion accounted for about 20% of acute non-A, non-B hepatitis C cases. At present, transfusion associated hepatitis C has been virtually eliminated. (Data from the Centers for Disease Control and Prevention.)

In addition to blood transfusion, the use of many plasma products was associated with HCV infections. Before the use of screened and treated clotting factor concentrates, more than 90% of hemophiliac patients treated with these products were infected with HCV. At present, screening of source plasma, as well as specific viral inactivation steps added to the manufacturing process, has eliminated HCV from clotting factors as well as other plasma products.

Because source plasma is now screened for anti-HCV, it was thought that a potential paradoxical effect on HCV transmission might occur in the use of Ig for hepatitis A prophylaxis and to treat patients with hypogammaglobulinemia. This product had never been reported to transmit HCV or NANBH. However, because the screening assay did not eliminate all HCV-positive plasma units and the plasma pools often included more than 10,000 units, the previously safe Ig might now contain infectious HCV because it would no longer be neutralized by specific antibody previously present in the pool. Recently, that prediction was realized when transmissions of HCV by a preparation of intravenous Ig were observed (41). At this time, all Ig preparations for either intramuscular or intravenous use are screened for HCV RNA by a sensitive PCR assay or undergo viral inactivation steps during manufacture, and it is believed that no infectious HCV remains in any Ig preparations.

The majority of acute cases of HCV now occur in injection drug users (IDUs) (Fig. 7). Between 1991 and 1996, surveillance studies performed by the CDC found that 59% of adults with acute HCV infections had a history of drug use. A study of IDUs in Baltimore revealed that 76.9% were positive for antibody to HCV, compared with 65.7% for HBV, 20.5% for HIV, and 1.8% for human T-cell lymphotropic virus. In addition, it was found that 64.7% of these IDUs were already HCV seropositive within 1 year of initiating injection drug use (110). It was also found that seroprevalence was independently associated with reusing syringes, injecting the first time with someone more than 5 years older, and injecting cocaine (109). Among similar studies from around the world, HCV-seropositive rates among IDUs were reported to be 60% to 70% in Victoria, Australia (76), 60% to 70% in Iceland (208), 95.7% in Switzerland (82), and 74.6% in Saudi Arabia (236). The use of drugs and sharing of syringes, needles, or other drug paraphernalia is an extremely efficient means of transmission of HCV. The other major risk factors include sexual exposure (see Mode of Transmission, earlier), occupation, and household contact. At least 10% of acute HCV cases have no epidemiologic factors identified. In a study of the risk factors associated with chronic hepatitis among blood donors who tested positive for anti-HCV, a history of drug use, including cocaine snorting, was the most common risk factor, followed by sexual promiscuity, transfusion, imprisonment, tattooing, needle stick, and acupuncture. Only sexual promiscuity would not be considered a parenteral exposure (74).

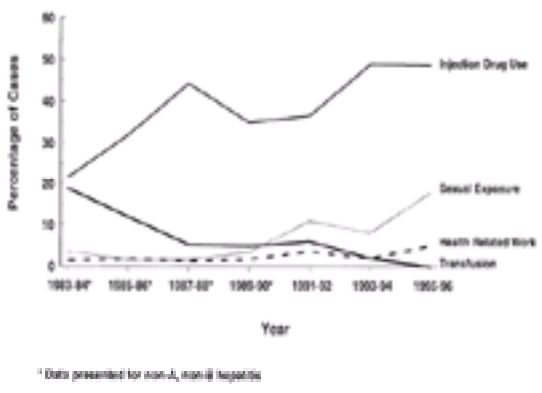

FIG. 7. Exposure risks associated with acquiring hepatitis C among adults in the United States between 1991 and 1996. (Data from the Centers for Disease Control and Prevention.)

CLINICAL FEATURES

Acute Hepatitis

The incubation period for hepatitis C averages about 7 weeks, with a range of 2 to 26 weeks, after exposure as measured by prospective studies of transfusion-associated hepatitis and needle sticks (285,288). The clinical picture of acute hepatitis C (illustrated in Fig. 8**A**) resembles other forms of acute viral hepatitis. However, symptoms of malaise, nausea, and right upper quadrant pain followed by dark urine and jaundice appear in only about one third of patients.

Biochemical evidence of hepatitis, such as elevated levels of serum ALT, are observed in more than 80% of cases, with levels typically 10 times normal or higher. ALT elevations usually coincide with symptoms, and both tend to resolve in 2 to 12 weeks. Hepatitis C RNA can be detected in the serum early after exposure, usually within the first 3 weeks (93). In acute resolved cases, the RNA disappears as the disease resolves (see Fig. 8**A**). HCV is rarely associated with fulminant hepatitis, but it has been reported (92,333). There are no recognized sequelae in patients who resolve hepatitis C infections. However, patients in whom HCV resolves do not in general appear to be protected from reinfection. Multiple bouts of hepatitis C have been reported in patients repeatedly treated with blood or plasma products (184). Liver biopsy is not recommended for the diagnosis of acute viral hepatitis, but in cases in which it has been performed, the histologic changes in addition to lymphocytic infiltrates include distinctive eosinophilic clumping of hepatocyte cytoplasm, acidophilic bodies, microvesicular steatosis, and activation of the sinusoidal cells (83).

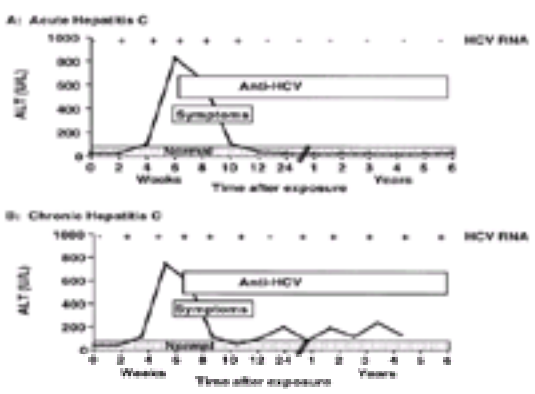

FIG. 8. Representation of clinical responses in patients after infection in acute (**A**) and chronic (**B**) hepatitis C. ALT, alanine aminotransferase.

Chronic Hepatitis

The clinical responses observed in chronic hepatitis C are illustrated in Figure 8**B**. The single most important feature of HCV infection is the propensity of the virus to cause persistent infection. About 85% of patients infected remain so for more than 6 months, most for the remainder of their lives. These chronically infected patients are the source of almost all new infections and themselves are at increased risk for the development of significant chronic liver disease, cirrhosis, and HCC. Many chronically infected patients do not have symptoms and would not be detected unless tested by their physicians or if they donate blood. Others may develop severe chronic liver disease over only a few years or, more commonly, over several decades. Figure 9 shows typical histologic features observed in chronic hepatitis C infection. Patients with chronic HCV infection may experience increased fatigue as their only symptom. Others may have overt symptomatic liver disease with anorexia, nausea, right upper quadrant pain, dark urine, and pruritus. ALT levels frequently fluctuate over time and may be normal or significantly elevated in the same patient measured at different times, whereas others have persistently normal or persistently elevated ALT levels. A summary was published of 10 reports in which 233 patients with chronic HCV infection but with normal ALT levels were studied by liver biopsy; it revealed that 12% had normal liver biopsies, 25% had only nonspecific changes, 41% had histologic findings of chronic persistent hepatitis, 22% had chronic active hepatitis, and only 0.5% had cirrhosis (143). Therefore, normal ALT levels do not always correlate perfectly with benign disease.

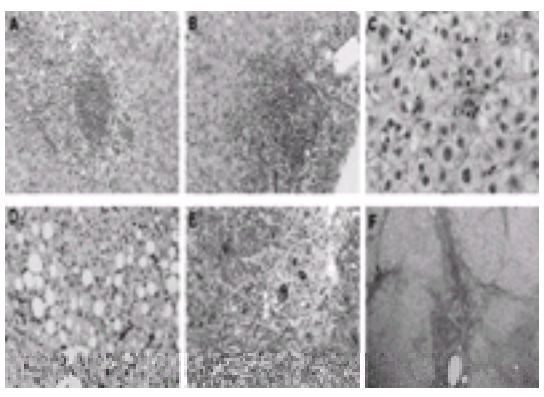

FIG. 9. Typical histologic features of chronic hepatitis C. **A:** Portal area with a lymphoid aggregate. Lymphoid aggregates in hepatitis C have an organization similar to primary lymphoid follicles in lymph nodes (H&E, ×200). **B:** Portal area showing piecemeal necrosis and Poulsen lesion. The edge of the portal area is disrupted by lymphocytic infiltration. Within the portal area is a lymphoid aggregate surrounding a duct that shows reactive epithelial changes. This “duct injury” is termed a *Poulsen lesion.* It is not associated with chronic cholestasis or duct loss (H&E, ×100). **C:** Lobular inflammation. Small foci of inflammation are seen within the hepatic parenchyma in chronic hepatitis C. Hepatocellular apoptosis can sometimes be observed. **D:** Steatosis. Accumulation of intracellular lipid within hepatocytes is a common feature in chronic hepatitis C and is seen more often in hepatitis C than in hepatitis B. Severe steatosis is uncommon. **E:** Periportal fibrosis. The earliest fibrotic change in hepatitis C is an expansion of the portal area by fibrosis. This is seen as thin collagen fibers extending out from the portal area to separate hepatocytes. This gives the portal area a stellate appearance. **F:** Advanced fibrosis. As the disease progresses, more scarring takes place. Fibrous bridges form between adjacent portal areas and between portal areas and central veins. The change shown here falls short of a diagnosis of cirrhosis. (Courtesy of Dr. David Kleiner, Laboratory of Pathology, National Cancer Institute, National Institutes of Health, Bethesda, MD.)

Outcomes of chronic hepatitis C infections in individual patients are difficult to predict. Hepatitis C patients presenting with significant chronic liver disease develop serious sequelae. For instance, of 100 Japanese patients presenting with chronic hepatitis C and liver disease and followed an average of 11 years earlier, 42 developed cirrhosis and 19 HCC (310). When patients are followed prospectively from infection, however, the picture is quite different (286). The National Heart Lung and Blood Institute conducted a series of five studies on posttransfusion hepatitis between 1968 and 1980 in which 8% to 18% of blood recipients developed hepatitis; about 90% of these cases were designated NANBH, almost all due to HCV (287). Follow-up studies of the 568 cases of NANBH an average of 18 years after transfusion as well as of 984 matched controls who were transfused but did not develop hepatitis revealed that death from all causes occurred in 51% in the hepatitis group and 52% in the controls. The mortality rate from liver disease was 3.2% among the hepatitis patients and 1.5% among controls, which was a small but significant difference. About one third of the patients who underwent liver biopsy after 18 to 20 years had cirrhosis, but presumably only the sicker patients had biopsies.

A group of children in Germany who had undergone cardiac surgery before 1991 when HCV screening of blood donors was begun were studied a mean of 19.8 years after their first surgery (325). Sixty-seven of 458 (14.6%) patients studied had anti-HCV, but only 37 of these (55%) had detectable HCV RNA. Only one HCV RNA–positive patient had elevated ALT levels, and this patient had right-sided heart failure. Seventeen HCV RNA–positive patients underwent liver biopsy. There was periportal fibrosis in two patients, both of whom had congestive heart failure, and micronodular cirrhosis in another. The remaining 14 patients had minimal lymphocytic periportal infiltrates. Therefore, in this group of patients infected as children and evaluated nearly 20 years later, the infection had cleared in 45% and was following a relatively benign course in the others. It is possible that in subsequent decades, these patients will begin to develop significant liver disease.

In contrast to the prospective studies reviewed previously, studies of patients with hepatitis C referred for evaluation of chronic liver disease show a rather different picture (320). Liver biopsies were performed in 101 patients in the United States with transfusion-associated chronic liver disease due to HCV. An additional 30 patients were studied but did not undergo biopsy because of abnormal coagulation tests. The mean interval from transfusion to diagnosis in this group was 19.5 years. This study showed the progressive nature of HCV-associated chronic liver disease. The mean interval from transfusion to diagnosis for patients with chronic hepatitis was 13.7 years, for chronic active hepatitis was 18.4 years, for cirrhosis was 20.6 years, and for HCC was 28.3 years. During the follow-up, which averaged about 4 years, 8 patients died from cirrhosis, and 11 died from HCC. These results were very similar to those reported earlier from Japan, in which the mean intervals to chronic hepatitis, cirrhosis, and HCC were 10.0, 21.2, and 29 years, respectively (16,165). Thus, chronic liver disease due to HCV infection generally follows an indolent but progressive course.

Hepatocellular Carcinoma

The most serious late outcome of chronic HCV infection is HCC. Soon after specific tests became available to detect patients with HCV infection, the association of chronic HCV infections with HCC was made. Worldwide, most HCC cases are related to either chronic HBV or HCV infection, and the prevalence of HCC and the proportion of cases related to either virus is directly reflected by the prevalence of each virus in that population (Fig. 10). Chronic infection with either virus results in chronic inflammation, necrosis, regeneration, and cirrhosis, which are all related to increased risk for oncogenesis. Cirrhosis of almost any cause carries an increased risk for HCC, and about 80% of patients with HCC have cirrhosis (281). HBV is a DNA virus that can integrate into the host genome, and it expresses the X protein, which is a transactivator, interacts with p53, and has other properties that may contribute to tumorigenesis. On the other hand, HCV is a typical RNA virus without the potential for its genome to be reverse-transcribed by a viral polymerase into DNA. The question of whether any of the HCV gene products have an effect on cellular metabolism that could lead to oncogenic transformation is under investigation. For instance, studies have shown that the HCV core protein can enter the nucleus and protect the cells from apoptosis mediated by anti-Fas or TNF-a, probably through activation of nuclear factor kappa B (216,346). In addition, NS3 has been reported to transform 3T3 cells in which it is expressed, and NS5A contains a potential transcriptional transactivator domain near its C terminus (69,277). In addition, transgenic mice expressing the core protein developed primary liver cancer resembling HCC after 16 months of age (228). Either of these phenomena could be related to carcinogenesis. Interaction of HCV proteins with cell regulatory factors may exert only small effects on the cell. However, because it typically takes 20 to 30 years for tumors to appear in persistently infected patients, it is possible that even such a small effect when exerted over a long time could be responsible for hepatocarcinogenesis.

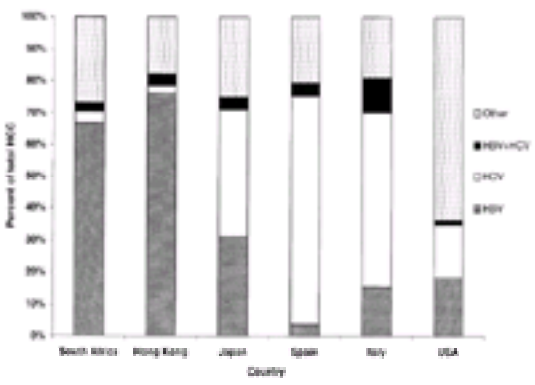

FIG. 10. The relative proportion of hepatocellular carcinoma cases associated with chronic hepatitis B virus (HBV) or hepatitis C virus (HCV) infections in different countries. Some parts of the world still have relatively high numbers of HBV carriers, resulting in a high proportion of hepatocellular carcinoma HCC cases due to HBV. In other countries such as Japan, HBV is coming under control resulting in a higher proportion of HCC cases related to HCV. (From ref. 81, with permission.)

Extrahepatic Manifestations of Infection

A variety of clinical syndromes that may be either autoimmune or mediated by immune complexes have been associated with chronic HCV infections. The diseases most closely associated with chronic HCV infections are essential mixed cryoglobulinemia, membranoproliferative glomerulonephritis, and porphyria cutanea tarda (122). Other diseases that have been related to HCV include keratoconjunctivitis sicca, lichen planus, autoimmune thyroiditis, Mooren corneal ulcers, idiopathic pulmonary fibrosis, and diabetes mellitus (122). Type II or essential mixed cryoglobulinemia (MC) is characterized by vasculitis with palpable purpura, arthralgias, and weakness and may involve the kidneys or central nervous system. This association has been reported from many areas of the world but appears to be particularly strong in Italy. A prospective study of MC in patients with chronic liver disease revealed that 46% of patients with chronic HCV infections and only 5% of patients with HBV infections had cryoglobulinemia, and about half of these patients had the clinical syndrome (1). The cryoglobulins have been shown to be composed of HCV, anti-HCV Ig, rheumatoid factor, and complement, and the cutaneous vascular lesions contain HCV, rheumatoid factor, and IgG (2). Successful treatment of HCV with IFN generally improves the MC (53). There is an additional association among HCV, MC, and lymphoproliferative disorders. Among 25 patients with MC, 7 had B-cell non-Hodgkins lymphoma. Serum anti-HCV was detected in 88% of the patients with MC and in all the patients with lymphoma (348). It is possible that the putative replication of HCV in lymphoid cells contributes to the pathogenesis of MC and the associated lymphoproliferative disorders. Membranous or membranoproliferative glomerulonephritis has also been linked to HCV infection and is most often seen together with MC (276). However, immune complex deposition, including HCV core protein, in the absence of cryoglobulins has also been reported (241).

Porphyria cutanea tarda also has a strong association with chronic HCV infection, although there appears to be a marked geographic variation in the rate of HCV positivity in patients with porphyria. A recent study in the United States found that 16 of 17 porphyria patients tested positive for anti-HCV, whereas only 17% in Northern Europe, 20% in Australia and New Zealand, and 65% in Southern Europe tested positive (68). Iron overload is often associated with this form of porphyria, and patients are often improved by frequent phlebotomies to deplete the excess iron stores; antiviral therapy also appears to have a beneficial effect (243,257).

DIAGNOSIS

The common tests used for diagnosis of HCV infection were designed primarily for screening of blood donors. These assays are based on detection of serum antibody to various HCV antigens because these antibodies are nearly universally present in patients who are chronically infected with HCV. Acute HCV infections are relatively rare among blood donors, but the antibody tests often fail to detect these patients in the window period between the time of infection and the time of appearance of antibody detectable by the assay. Therefore, the antibody assays also have limited utility for diagnosis of patients with acute HCV infections. Tests for HCV RNA genome detection based on the PCR or other highly sensitive RNA detection systems have been used for the diagnosis of acute hepatitis. Although these tests are available for clinical testing and are being used in blood banking, they have not yet achieved final approval for these uses by the U.S. Food and Drug Administration.

Serologic (Antibody) Assays

The components of the different diagnostic assays that have been developed are shown in Table 6. The locations of the antigens and peptides on the HCV polyprotein are shown in Figure 11.

Diagnostic test	Components
ORTHO HCV ELISA Test System	c100-3
ORTHO HCV 2.0 ELISA Test System	c100-3 c22-3 c200
ORTHO HCV Version 3.0 ELISA Test System	c22-3 c200 NS5
RIBA HCV 3.0 Strip Immunoblot Assay (SIA)	c22 Peptide 5-1-1 Peptide c100 Peptide c33c Antigen NS5 Antigen

[a]Locations of antigens and peptides on the HCV polyprotein are shown in Figure 11.
ELISA, enzyme-linked immunosorbent assay.

TABLE 6. *Components of previous and current commercial anti-HCV antibody test systems licensed in the United States*[a]

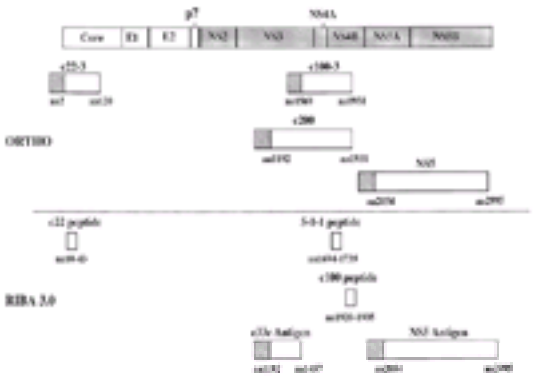

FIG. 11. Location on the HCV polyprotein of recombinant antigens and peptides used in the Ortho enzyme-linked immunosorbent assay (ELISA) (Ortho-Clinical Diagnostics, Rochester, NY) and the Chiron recombinant immunoblot assay (RIBA) antibody test systems (Chiron Corporation, Emeryville, CA). Recombinant antigens are expressed in yeast as fusion proteins with superoxide dismutase; this protein tag is represented by the *hatched area.* The antigens and peptides used in individual test systems are shown in Table 6.

The first diagnostic assay for HCV infection was developed as part of the studies that led to the initial detection of the agent. The first clone identified, 5-1-1, was used in hybridization assays to identify three overlapping clones that together constituted a 363–amino acid peptide termed *C100-3,* which was then expressed at high levels in yeast (181) and used in the first-generation enzyme-linked immunosorbent assay (ELISA). Other antigens were added later to make the second-generation commercial ELISA. The newest approved system, the Ortho HCV version 3.0 ELISA test system (Ortho-Chemical Diagnostics, Rochester, NY), includes three recombinant antigens, c22-3, c200, and NS5. Each of these polypeptides is expressed in yeast as a fusion protein with superoxide dismutase. At present, there are two licensed anti-HCV ELISA systems available in the United States, and several other systems are available in other countries. The latest test kit, version 3.0, may shorten the interval between infection and the first detection of serum antibody. Nevertheless, studies of patients with a defined exposure, such as transfusion, have shown that antibodies are not generally detectable for at least 6 weeks and may not appear for several months (118). HCV RNA may often be found in the patient's serum within the first week after exposure; therefore, the patient is probably infectious during this period before antibodies can be detected, but these patients with acute hepatitis C usually have not yet developed detectable antibody, making positive diagnosis difficult.

Supplemental tests have been developed to help exclude false-positive ELISA results. The most commonly used supplemental assay is the recombinant immunoblot assay RIBA (Chiron Corporation, Emeryville, CA). In this assay, several recombinant peptide antigens (locations are shown in lower half of Fig. 11 are applied on a strip, which is then probed with the patient's serum. In this way, the response to individual antigens can be recognized, and some false-positive ELISA results can be eliminated.

Virologic Assays

Sensitive tests for HCV RNA based on RT-PCR or other nucleic acid amplification techniques can be used during the window period. Although some of these tests are available commercially, the assays are not yet licensed as diagnostic tests in the United States, and the performance, sensitivity, specificity, and reproducibility of any given assay may not be well established. Until these tests are licensed, the user must rely on published results or reputation to judge the quality of the assay. Quantitative assays of HCV RNA can be performed by end-point dilutions of RT-PCR assays, competitive RT-PCR assays, or one of the commercial tests, such as the AMPLICOR Monitor (Roche Diagnostics, Nutly, NJ) or the QUANTIPLEX or bDNA assay (Chiron Corporation, Emeryville, CA). These quantitative tests may have utility in monitoring viral load in patients undergoing antiviral therapy (119).

Immunoassays using monoclonal antibodies for the detection of the core antigen of HCV (HCVcAg) have been developed with the claim of sensitivities comparable to nucleic acid amplification systems (19). In this assay, the viral envelope and the preexisting anti-core antibodies are stripped off the HCVcAg by detergents; the core antigen is then detected by a standard immunoassay. Although the HCVcAg was detected in 94.5% of 59 patients who had detectable HCV RNA, it was not shown that the assay actually had similar sensitivity to detect virus at a very low level. More studies are needed to determine whether such an assay can have the necessary sensitivity to close the serologic window between the time of the first appearance of viremia and the development of serum antibody.

Assays to determine the HCV genotype have recently become useful in clinical decision making. It has been know for some time that patients with genotype 1 infections are relatively resistant to IFN therapy. With combination therapy of IFN plus ribavirin, it has been found that patients with genotype 2 and 3 HCV infections may need only 6 months of therapy, whereas those with type 1 infections must be treated for a full year (see later). Many systems for determining genotypes have been published (see Nomenclature and Genotypes, earlier), and at least one system (LIPA assay) is available commercially, although not licensed (303).

TREATMENT AND PREVENTION

HCV presents a nearly perfect target for an antiviral drug approach to therapy. Hepatitis C is a chronic disease that progresses slowly over many years. Therefore, there is a long period of time in which antiviral therapy could be initiated as well as a long treatment window when therapy might be effective. The viral genome codes for several enzymatic activities that are vital to replication and quite distinct from human analogs. These include proteases, a helicase, and the RNA-dependent RNA polymerase, which all could be targeted by inhibitors.

One of the most promising approaches to anti-HCV drug discovery is the development of inhibitors of the virally encoded protease NS3. This chymotrypsin-like serine protease is essential for the maturation of the viral polyprotein, and efficient enzymatic activity requires complex formation between NS3 and its cofactor, NS4A. Targets for inhibition could include the NS3–NS4A interaction, zinc binding in the NS3 domain, and direct protease inhibitors. Of these, inhibitors of protease activity appear the most realistic (reviewed in 26 and 182).

Licensed Therapeutics

Two drugs have been approved for the treatment of chronic HCV infections. IFN-a has been approved for the treatment of compensated chronic NANBH (hepatitis C) since 1991, and recently ribavirin, a guanosine analog, has been approved

for use in combination with IFN-a. There are now several IFNs available, including IFN-a $_{2a}$; IFN-a$_{2b}$; IFN-a$_{n1}$, which is a combination of nine IFNs; and consensus sequence IFN, in which a consensus sequence of a variety of IFNs was synthesized. All these forms of IFN are produced by recombinant DNA technology and therefore are consistent and free of potential contamination by human pathogens. Clinical trials have not indicated major differences in the response rates among these products. Pegylated forms of IFN (bound to polyethylene glycol) are under development that may offer advantages over the presently available IFNs (235).

IFNa$_{2b}$ was the first approved form of IFN. It was originally used in a dose of 3 million units administered subcutaneously three times a week for 6 months. Later, it was found that improved virologic responses were obtained if therapy were extended to 1 or even 2 years, and it is now approved for treatment for that period. A meta-analysis of 32 clinical trials revealed that the biochemical (normalization of ALT) end of treatment response (ETR) rate was 47% of treated patients compared with 4% of controls. Virologic ETR rates (no detectable HCV RNA by RT-PCR) was 29% of treated patients, compared with 5% of controls (52). However, 5 months after the cessation of therapy, the sustained biochemical and virologic response rates had fallen to 23% and 8%, respectively. Although the ETR rates for patients treated for 6 months or 12 to 24 months were not different (45% versus 47%), the sustained response rate was 27% in those receiving longer-term therapy, compared with 14% for those receiving 6 months of treatment. Therefore, the present recommendation is for at least 12 months of therapy. Because the first IFN treatment trials occurred in the mid-1980s, few treated patients have been followed for long periods of time. The data that are emerging, however, are encouraging. Ten patients who were treated with IFN-a $_{2b}$ between 1984 and 1987 were followed 6 to 13 years after therapy. All 5 patients who had a sustained responses 6 months after the cessation of therapy remained HCV RNA negative, and only one had a minimally elevated ALT level (190). All 5 patients had improved scores for inflammation and fibrosis in their liver biopsies. The 5 patients who did not have responses at 6 months continued to have HCV RNA detectable in their blood, elevated ALT levels, and progressive disease on biopsy.

Patients who never respond or who relapse may be retreated with the same or a different form of IFN, at the same or a higher dose, and for a longer period of time. Only about 2% to 3% of nonresponders treated with higher doses and for longer periods had sustained responses after retreatment (5,51); however, sustained responses were achieved in about 15% of relapsers treated for an additional 6 months at the same dose, in 29% treated 6 more months at a higher dose, and in about 43% of patients retreated for 12 months.

Ribavirin is a nucleoside analog of guanosine that is absorbed orally and has been extensively studied as an antiviral agent for more than 20 years. Monotherapy of hepatitis C with ribavirin resulted in a nonsustained reduction of ALT levels without significant effects on HCV replication (38). However, combination therapy of ribavirin with IFNa $_{2b}$ resulted in a sustained response rate of 49%, compared with only 5% observed in matched patients treated with IFN alone (78). Figure 12 represents a sustained response to IFN-a and ribavirin treatment in chronic hepatitis C. In addition, as many as 50% of patients who relapse after an initial response to IFN alone have had sustained responses to the combination therapy (77). The combination of IFN-a$_{2b}$ and ribavirin has now been approved for both the initial treatment of patients with chronic HCV infections and for patients who have relapsed after initially responding to IFN alone. The utility of the combination therapy in treating individuals who never responded to IFN monotherapy is not yet clear.

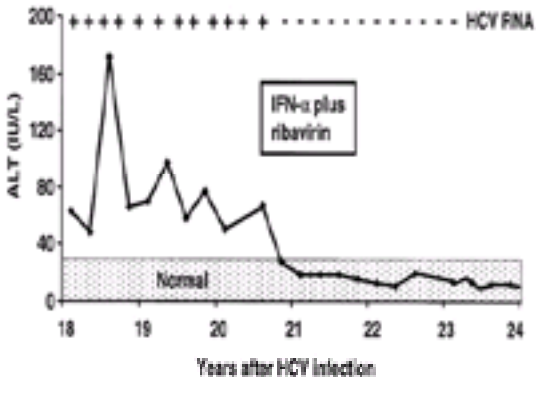

FIG. 12. Representation of spontaneous fluctuations in serum alanine aminotransferase (ALT) over time in chronic hepatitis C virus (HCV) infection and a sustained response to antiviral treatment. During treatment with interferon-ax, 3 million units weekly, and ribavirin, 1000 mg/d, ALT values decrease and remain within normal range, and serum HCV RNA remains undetectable by reverse transcription–polymerase chain reaction (RT-PCR).

Although all patients with chronic hepatitis C are candidates for therapy, at present, treatment is recommended for only a defined subset. These are patients with chronic hepatitis C who are at risk for progression to cirrhosis. These patients have persistently elevated ALT levels and liver biopsy findings of either portal or bridging fibrosis and at least moderate degrees of inflammation and necrosis (201). It is less clear whether patients with milder forms of liver disease are at significant risk for progression, and it is recommended that these patients be followed carefully, including repeat liver biopsies at 3- to 5-year intervals. Patients with more severe disease, such as established cirrhosis, probably would benefit from therapy, and patients with decompensated liver disease should be considered for transplantation. At present, treatment is not recommended for children younger than 18 years of age. Co-infection with HIV or HBV is not a contraindication to treatment, and HIV-infected patients with good CD4 levels have responded well to therapy (86). Treatment of drug or alcohol abusers should not be initiated without careful consideration. Contraindications to treatment include history of depressive illness, cytopenia, hyperthyroidism, and renal transplantation. Because markers of autoimmunity are common in chronic HCV infection, it is often difficult to determine the cause of the liver disease. A treatment decision between immunosuppression and antiviral therapy is often difficult and needs careful consideration because of the potentially negative effects of immunosuppressive therapy on hepatitis C.

Factors that influence treatment outcomes have been studied extensively. The NS5A region has been shown to interact with the protein kinase PKR, which is a known mediator of IFN-induced antiviral activity (see Mechanisms of Persistence, earlier). It is also known, however, that patients with genotype 1 viruses in general respond less well than patients infected with genotype 2 or 3 viruses (79). It has now been shown that patients with genotype 2 or 3 infections can be treated with IFN-a_{2b} plus ribavirin for only 6 months, whereas those with type 1 infections must be treated for at least 12 months (219,258). Thus, patients being evaluated for antiviral therapy should have their infecting virus genotyped. Poor treatment response rates have also been correlated with high pretreatment viral RNA levels. This may reflect the sensitivity of the virus to endogenous IFN.

Prevention

Prevention of hepatitis C at present is based on prevention of exposure to contaminated blood by screening of blood and plasma donors, identification of carriers by testing high-risk individuals, and public health measures designed to prevent spread. No effective vaccine has been developed to prevent HCV infection. In developed countries, screening of blood donors has virtually eliminated transmission of HCV by transfusion. Even before the specific screening tests were available, HCV transmission by blood had been significantly reduced by elimination of paid donors, ridged donor screening implemented to reduce HIV transmission, and possibly the use of surrogate tests for hepatitis C infection (see Modes of Transmission, earlier).

Source plasma for manufacture into various plasma products, such as clotting factors, is now all screened by the specific assays. Because these products are all made from pools containing plasma from thousands of donors, most of these pools continue to have low levels of HCV. Presently, these plasma-derived products all must undergo some specific process designed to eliminate or inactivate viruses, especially lipid-enveloped viruses that include HCV, HBV, and HIV. At the present time, plasma products are all considered to be free of infectious HCV.

Passive Immunization

Some recent studies indicate that the passive immunoprophylaxis of HCV infections may be possible. Pooled Ig with a high titer of anti-HCV was mixed with HCV *in vitro,* and this mixture was shown to be noninfectious when injected into a chimpanzee (344). Using the same Ig preparation, chimpanzees were inoculated with HCV and then given the Ig weekly for 13 weeks. Relative to the untreated control chimpanzees, the treated animals had a short, low-level viremia with minimal disease. When the treatment was stopped, however, the HCV viremia reappeared with acute disease (180). These two experiments show that antibody alone can have a profound effect on HCV infection and can neutralize the virus under limited conditions. The place for passive immunization with high titered Ig preparations in the prevention of HCV infection in humans has not been determined.

Active Immunization

HCV itself presents numerous problems that complicate the development of an effective vaccine. The lack of a suitable *in vitro* culture system makes production of vaccine quantities of whole virus impractical. The genetic diversity of the virus and the high level of mutability also complicate vaccine development. A vaccine based on the E1 and E2 glycoproteins was tested in chimpanzees (63). The vaccine produced an antibody response to E1 and E2, but it was short lived and required frequent boosting. Animals that were challenged intravenously with a very low dose of HCV were protected if the challenge virus was the same as the virus that was used to make the vaccine. Although protection against infection was not produced against other strains of HCV or higher levels of challenge virus, infections in the vaccinated chimps tended to be mild and short lasting. It is possible that a vaccine that only prevents chronic infections would be useful. Such a vaccine would prevent the most serious problems associated with chronic HCV and would greatly reduce the chance of transmitting the virus to others. Phase 1 clinical trials are in progress with a related vaccine based on the E2 glycoprotein only.

The challenges to the development of an effective HCV vaccine may require nonclassic approaches. A strong T-cell response, including both helper T cells and cytotoxic T lymphocytes, may be very important for both clearing and preventing infection. It has been suggested that specifically inducing CTL responses to well-conserved epitopes included in nonstructural proteins may be important for a prophylactic vaccine. Several laboratories are now studying this possibility in the chimpanzee model.

PERSPECTIVES

The etiologic agent of hepatitis C has been known for just over 10 years. Even without a cell culture system to study this virus carefully, progress in all aspects of hepatitis C has been remarkable. High-priority areas for future investigation include elucidating the mechanisms of persistence and carcinogenesis. Detailed understanding of viral replication and the development of efficient, high yield *in vitro* culture systems and small animal models remain important problems. The pharmaceutical industry currently has large efforts in antiviral therapy, which should begin to yield results soon. However, on a global basis, it is not likely that antiviral drugs that need to be taken over long periods of time will have a major impact owing to the expense, logistics, and compliance. Therefore, vaccines against hepatitis C designed to either prevent new infections or treat existing, chronic infections remain the highest priority for future research. HCV presents numerous problems that make the development of effective prophylactic or therapeutic vaccines immensely difficult. As some of the basic questions are answered, the path to a useful vaccine may become clearer.

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CHAPTER 35 *Coronaviridae:* The Viruses and Their Replication

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CLASSIFICATION

Coronaviruses, a genus in the family *Coronaviridae*, are large, enveloped, positive-stranded RNA viruses that cause highly prevalent diseases in humans and domestic animals (Table 1). They have the largest genomes of all RNA viruses and replicate by a unique mechanism, which results in a high frequency of recombination. Virions mature by budding at intracellular membranes, and infection with some coronaviruses induces cell fusion.

Antigenic group	Virus	Host	Respiratory infection	Enteric infection	Hepatitis	Neurologic infection	Other[a]
I	HCoV-229E	Human	X			?	
	TGEV, PRCoV	Pig	X	X			X
	CCoV	Dog		X			
	FECoV	Cat		X			
	FIPV	Cat	X	X	X	X	X
	RbCoV	Rabbit		X			X
II	HCoV-OC43	Human	X	?		?	
	MHV	Mouse	X	X	X	X	
	SDAV	Rat					X
	HEV	Pig	X	X		X	
	BCoV	Cow	X	X			
	TCoV	Turkey	X	X			
III	IBV	Chicken	X		X		X
	TCoV	Turkey	X	X			

[a]Other diseases caused by coronaviruses include infectious peritonitis, immunologic disorders, runting, nephritis, pancreatitis, paralysis, myocarditis, and sialodacryoadenitis.
HCoV-229E, human respiratory coronavirus; TGEV, porcine transmissible gastroenteritis virus; PRCoV, porcine respiratory coronavirus; CCoV, canine coronavirus; FECoV, feline enteric coronavirus; FIPV, feline infectious peritonitis virus; RbCoV, rabbit coronavirus; HCoV-OC43, human respiratory coronavirus; MHV, murine hepatitis virus; SDAV, sialodacryoadenitis virus; HEV, porcine hemagglutinating encephalomyelitis virus; BCoV, bovine coronavirus; IBV, avian infectious bronchitis virus; TCoV, turkey coronavirus.

TABLE 1. *Serotypes, natural hosts, and diseases of coronavirus*

Coronaviruses were first recognized as a distinct virus group by their characteristic virion morphology in negatively stained preparations (1,36) (Fig. 1). The viral envelopes are studded with long, petal-shaped spikes, giving coronaviruses the appearance of a crown (Latin, *corona*), and the nucleocapsids are long, flexible helices. Other characteristics that define Coronaviridae include the 3'-coterminal, nested-set structure of the mRNAs, unique RNA transcription strategy, genome organization, nucleotide sequence homology, and the properties of their structural proteins (36,138). The Coronaviridae family includes two genera, coronavirus and torovirus, which share many features of genome organization and replication strategy but have different virion morphology and genome lengths. The Coronaviridae, together with the Arteriviridae family, are classified in the order Nidovirales, whose member viruses share a unique feature: they all synthesize a nested set of multiple subgenomic mRNAs (34). Toroviruses and all members of the Arteriviridae family infect only animals. This chapter discusses coronaviruses exclusively.

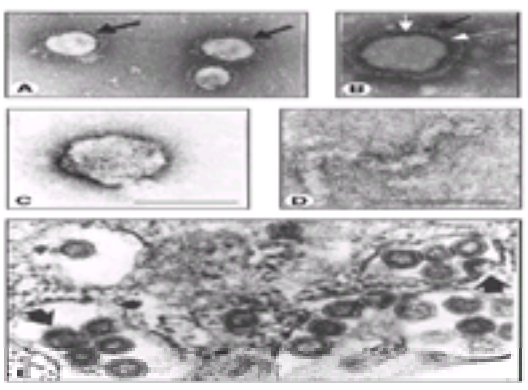

FIG. 1. Morphology of coronaviruses. Human respiratory coronavirus HCoV-OC43 (original magnification, ×90,000) **(A)** and turkey enteric coronavirus TCoV **(B)** in negatively stained preparations. The large, petal-shaped spikes (*black*

arrows), composed of the S glycoprotein, seen on the envelopes of both viruses distinguish coronaviruses from other enveloped viruses. Some coronaviruses also exhibit a fringe of shorter spikes composed of the hemagglutinin-esterase (HE) glycoprotein (*white arrows*). **C: I**nternal core of porcine transmissible gastroenteritis virus (TGEV). The core was obtained after removal of envelope by treatment with NP-40 and appears to have a spherical symmetry. **D:** Helical nucleocapsid released from the TGEV core structure by Triton X-100 treatment. *Bar*: 70 nm. **E:** Human cell infected with human respiratory coronavirus HCoV-229E. Spherical virions are seen budding at membranes of the ER and in smooth-walled vesicles (*arrows*). The electron-dense helical nucleocapsid is visible as slender, flexible tubules within the virions. The virus particles of some coronaviruses may mature further into electron-dense particles (215) (original magnification, ×60,000). (**B** courtesy of Peter Tijssen; **C and D** courtesy of Luis Enjuanes.)

Coronaviruses can be divided into three serologically distinct groups (see Table 1). Within each serogroup, the viruses are classified according to their natural hosts, nucleotide sequences, and serologic relationships. Most coronaviruses naturally infect only one animal species or, at most, a limited number of closely related species. Virus replication *in vivo* can be either disseminated, causing systemic infections, or restricted to a few cell types, often the epithelial cells of the respiratory or enteric tracts and macrophages, causing localized infections (see Table 1). Nucleotide sequences of the entire genomes of several coronaviruses, including avian infectious bronchitis virus (IBV), murine hepatitis virus (MHV), human coronavirus 229E (HCoV-229E), and porcine transmissible gastroenteritis virus (TGEV), confirm the similarity of the genome organization of these different coronaviruses (28,72,94,95,144).

VIRION MORPHOLOGY AND STRUCTURE

The structure of coronavirus virions is diagrammed in Figure 2. The virions are spherical enveloped particles about 100 to 120 nm in diameter. Inside the virion is a single-stranded, positive-sense genomic RNA 27 to 32 kb in size, the largest of all RNA virus genomes (28,72,94,138,144). The RNA genome associates with the N (nucleocapsid) phosphoprotein (50–60 kd) to form a long, flexible, helical nucleocapsid (175,254). When released from the virus particles, the nucleocapsids appear as extended tubular strands 14 to 16 nm in diameter (211,254) (see Fig. 1, panel **D**). Recent studies showed that in at least two coronaviruses (TGEV and MHV), the helical nucleocapsid is enclosed within an "internal core structure," 65 nm in diameter, that is spherical, and possibly icosahedral, in form. The core is composed of M (membrane) glycoprotein (and probably N protein as well). It can be released from virions by NP-40 treatment (211) (see Fig. 1, panel **C**). The virus core is enclosed by a lipoprotein envelope, which is formed during virus budding from intracellular membranes (91,201,264). Two types of prominent spikes line the outside of the virion. The long spikes (20 nm), which consist of the S (spike) glycoprotein, are present on all coronaviruses; the short spikes, which consist of the HE (hemagglutinin-esterase) glycoprotein, are present in only some coronaviruses (see Fig. 1, panels **A** and **B**). The envelope also contains the M glycoprotein, which spans the lipid bilayer three times (171,172 and 173); thus, the M protein is apparently a component of both the internal core structure and the envelope. The envelope also contains an E (envelope) protein, which is present in much smaller amounts than the other viral envelope proteins (87,271,295).

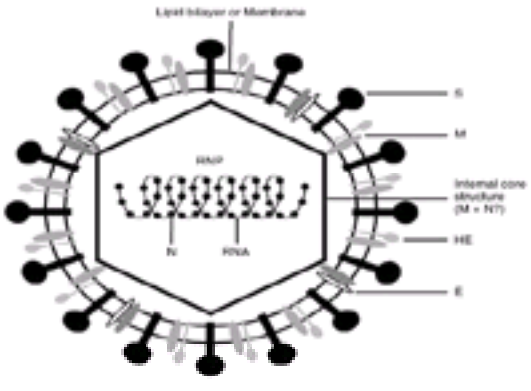

FIG. 2. Model of coronavirus structure: a schematic diagram of virion structure. S, spike glycoprotein; M, membrane glycoprotein; E, small envelope protein; HE, hemagglutinin-esterase glycoprotein; N, nucleocapsid phosphoprotein; ICS, internal core-shell composed of M glycoprotein; NC, nucleocapsid.

Structural Proteins

The S glycoprotein forms the large, petal-shaped spikes on the surface of the virion. This heavily glycosylated protein has a molecular mass of about 150 to 180 kd. S can be divided into three structural domains (from N- to C-terminus): a large external domain that is further divided into two subdomains, S1 and S2; a transmembrane domain; and a short carboxylterminal cytoplasmic domain. The S1 subdomain includes the N-terminal half of the molecule and forms the globular portion of the spikes. It contains sequences that are responsible for binding to specific receptors on the membranes of susceptible cells. S1 sequences are variable, containing various degrees of deletion and substitutions in different coronavirus strains or isolates. Mutations in S1 sequences have been associated with altered antigenicity and pathogenicity of the virus (74,99). In contrast, S2 sequences are more conserved. S2 is acylated and contains two heptad repeat motifs that suggest a coiled-coil structure (53,167). Indeed, evidence suggests that the mature S protein forms an oligomer, most likely a trimer (58). Thus, S2 subdomain probably constitutes the stalk of the viral spike. In most MHV strains and in bovine coronavirus (BCoV), the 180-kd S protein is cleaved during or after virus maturation by a cellular protease to yield the S1 and S2 proteins that remain noncovalently associated in the viral spikes. The extent of S cleavage varies among different coronaviruses and also depends on the host cell types (81,253). The cleavage of S into S1 and S2 may enhance the cell fusion activity or viral infectivity (89,255), but even uncleaved S protein can mediate

cell–cell fusion and fusion of the viral envelope with host cell membranes, though at a lower efficiency (26,245,259). The S proteins of coronaviruses in group I are not cleaved; even so, some of these viruses, such as feline infectious peritonitis virus (FIPV), can induce cell–cell fusion (54).

The S glycoprotein has several important biologic functions (Table 2). Monoclonal antibodies against S can neutralize virus infectivity (47,77), consistent with the observation that S protein binds to cellular receptors (86,128). The receptor-binding domain of the S protein of MHV is localized within the N-terminal 330 amino acids of the S1 domain (257). Consequently, the amino acid sequences of the S1 domain can determine the target cell specificity of coronaviruses in animals (99,219).

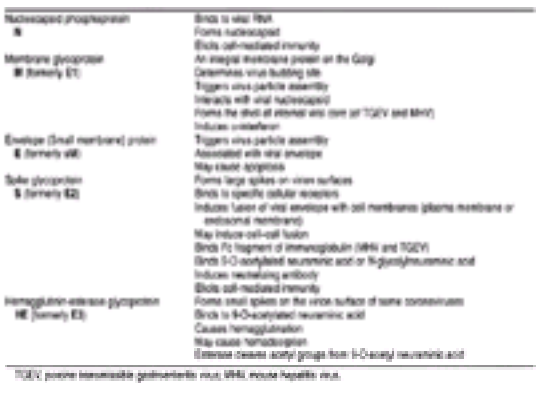

TABLE 2. *Properties and functions of coronavirus structural proteins*

S protein also induces fusion of the viral envelope with host cell membranes and, sometimes, cell–cell fusion. Expression of coronavirus S protein alone can induce fusion of receptor-bearing cells (54,259,294). The membrane fusion activity is most likely conferred by the internal hydrophobic sequences within the S2 (167). However, the fusion activity can be affected by sequences at multiple sites in the S2 as well as S1 (84,217).

Several other biologic activities have been associated with the S protein (see Table 2). The presence of MHV S protein on the plasma membrane of infected cells makes the cells susceptible to B-cell–mediated cytotoxicity (108,280), and the S glycoproteins of several coronaviruses share a similar sequence with the Fcg receptor for immunoglobulin (Ig), enabling the S protein to bind to the Fc fragment of IgG (198). This molecular mimicry may play a role in viral pathogenesis. The S proteins of BCoV and HCoV-OC43 can also bind 9- *O*-acetylated neuraminic acid-containing glycans and possess a hemagglutinating activity (227,228 and 229). This binding may be involved in the first step of virus infection for these viruses. Thus, S is a large, multifunctional protein that plays a central role in the biology and pathogenesis of coronavirus infections.

The HE glycoprotein is found on the virions of some group II coronaviruses and turkey coronavirus (TCoV) (group III) as a disulfide-linked dimer of a 65- to 70-kd protein that forms short spikes on the virions (see Fig. 1 and Fig. 2) (104,117,129,231). The presence or absence of this protein is highly variable even among group II coronaviruses, and its structure is frequently mutated or completely deleted during serial virus passaging in culture (288), suggesting that the HE protein is not essential for viral replication. Interestingly, the HE protein of coronavirus shares about 30% amino acid sequence similarity with the hemagglutinin protein of influenza C virus and may have been derived by a recombination between an HE mRNA of influenza C virus and the genomic RNA of an ancestral coronavirus (168). Like the S proteins of BCoV and HCoV-OC43, HE protein of various coronaviruses binds 9- *O*-acetylated neuraminic acid residues (229,231,274) and likely contributes to the hemagglutination and hemadsorption activities of coronaviruses. As the name implies, HE has an acetylesterase activity that cleaves acetyl groups from 9- *O*-acetylated neuraminic acid (274,290), thereby preventing or reversing the hemagglutination or hemadsorption induced by S or HE. These properties suggest that HE may be involved in either virus entry or virus release from infected cells. Indeed, some monoclonal antibodies against HE of BCoV can neutralize virus infectivity (64). In this virus, HE may mediate initial adsorption of the virus to cell membranes, but subsequent steps of virus entry most likely require the interaction of the S protein with a specific receptor, in a mechanism similar to that seen in other coronaviruses. This conclusion is supported by the finding that infection of murine cells by an HE-containing strain of MHV is inhibited by the monoclonal antibodies against the cellular receptor (83) or the viral S protein, despite the presence of HE protein in the virion (77). Thus, HE appears to be a “luxury” protein and is not absolutely necessary for virus infection in culture. Nevertheless, the presence of HE in a virus may alter its pathogenicity in animals (287).

The M glycoprotein differs from other coronavirus glycoproteins in that only a short aminoterminal domain of M is exposed on the exterior of the viral envelope. This domain is followed by a triple-membrane–spanning domain, an a-helical domain, and a large carboxylterminal domain inside the viral envelope (7,163,171,217). In some coronaviruses, such as TGEV, the carboxylterminus of the M protein is exposed on the virion surface (212). Glycosylation of the aminoterminal domain is O-linked for MHV and N-linked for IBV and TGEV (65,106,142,197). Monoclonal antibodies against the external domain of M neutralize viral infectivity, but only in the presence of complement (47). M proteins of some coronaviruses can induce interferon-a (43). The M proteins are targeted to the Golgi apparatus and not transported to the plasma membrane (172,173,258). In TGEV and MHV virions, the M glycoprotein is present not only in the viral envelope but also in the internal core structure (211).

An additional small envelope protein (E, formerly called sM), which is 9 to 12 kd, was recently shown to be associated with the viral envelope (87,159,295). Both E and M proteins are required for budding of virions (see Assembly and Release of Virions) (27,271). The topology and abundance of the E protein in the viral envelope have not been precisely

determined.

The nucleocapsid protein, N, is a phosphoprotein of 50 to 60 kd that interacts with viral genomic RNA to form the viral nucleocapsid. N has three relatively conserved structural domains, including an RNA-binding domain in the middle (187) that binds to the leader sequence of viral RNA (249). N protein in the viral nucleocapsid further interacts with M (254), leading to the formation of virus particles. N may also play a role in viral RNA synthesis, as suggested by a study in which an antibody directed against N inhibited an *in vitro* coronavirus RNA polymerase reaction (49). N protein also binds to cellular membranes and phospholipids (5), a property that may help to facilitate both virus assembly and formation of RNA replication complexes.

Coronaviruses, like other positive-stranded RNA viruses, do not incorporate RNA-dependent RNA polymerase into mature virions. Protein kinase activity is present in purified virions, but it is not yet clear whether it is a viral or cellular enzyme (240).

Viral Genome

The genomes of several coronaviruses are illustrated in Figure 3. Coronavirus genomic RNAs are capped, polyadenylated, positive-stranded RNAs of 27 to 32 kb in length. They can function as mRNAs, and the purified genomic RNA is infectious (164, 226). At the 5' end of the genome is a sequence of 65 to 98 nucleotides, termed the *leader RNA,* that is also present at the 5' ends of all subgenomic mRNAs (139, 238, 243). An untranslated region (UTR) of 200 to 400 nucleotides follows this leader sequence. At the 3' end of the RNA genome is another UTR of 200 to 500 nucleotides, followed by poly(A) of variable length. The sequences of both the 3'- and 5'-UTR are important for RNA replication and transcription. The remaining genomic sequence includes 7 to 10 open reading frames (ORFs). Gene 1, which comprises two thirds of the genome from the 5' end, is about 20 to 22 kb in length. It consists of two overlapping ORFs (1a and 1b). These ORFs are translated into a polyprotein, which is the precursor of viral polymerase. The order of the genes encoding the polymerase (*Pol*) and the four structural proteins that are present in all coronaviruses is 5'- *Pol*-S-E-M-N-3'. These genes are interspersed with several ORFs encoding various nonstructural proteins and the HE glycoprotein, which differ markedly among coronaviruses in number, nucleotide sequence, gene order, and method of expression (138) (see Fig. 3). The functions of most of the nonstructural protein gene products are unknown; some are absent in genomes of some coronaviruses (232, 291). The marked variability of coronavirus genome structure may be the result of RNA recombination.

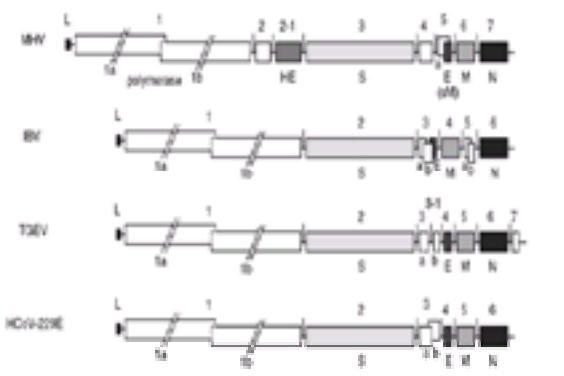

FIG. 3. Genomic organization of coronaviruses. The structures of four coronavirus genomic RNAs, for which complete sequences are available, are shown. The sizes of the genes are drawn about to scale, except for gene 1. The murine hepatitis virus (MHV) genome is 31.2 kb, and its gene 1 is 22 kb. The genomes for avian infectious bronchitis virus (IBV), porcine transmissible gastroenteritis virus (TGEV), and human respiratory coronavirus (HCoV)–229E are 27.6, 28.5, and 27.2 kb, respectively. The 5' end consists of a cap and a 65- to 98-base long leader sequence (L). *Shaded boxes* represent open reading frames (ORFs) encoding structural proteins; *unshaded boxes* encode nonstructural proteins. The *vertical lines* represent intergenic sequences (IG). The area between two IGs represents one gene; the separate ORFs within each gene are translated from a single mRNA species. Note the variations in the number, sequences, and locations of the ORFs encoding the nonstructural proteins. Coronavirus genomes are polyadenylated at the 3' end.

GROWTH OF CORONAVIRUSES

Most coronaviruses infect only the cells of their natural host species and a few closely related species. Some coronavirus strains may infect a wider range of cells under certain experimental conditions; for example, intracerebral inoculation of some MHV strains into owls and African green monkeys can result in central nervous system infection (33, 192). Primary isolation of human respiratory coronaviruses often requires human fetal tracheal organ cultures (see Chapter 36). Expansion of the viral host range can be achieved by serial passaging of the virus in a heterologous cell line or can occur spontaneously during persistent infection of cells of the natural host species. A variety of MHV variants capable of infecting hamster and human cells have been isolated by these processes (18, 19, 224). This expansion of host ranges often results from the ability of the virus to use an expanded repertoire of receptor molecules (18).

In their natural host species, coronaviruses exhibit marked tissue tropism; for example, TGEV causes mainly gastrointestinal diseases in pigs, whereas a closely related virus strain, porcine respiratory coronavirus (PRCoV), causes predominantly respiratory infections in pigs (51). The species and tissue specificity of the virus is at least partially determined by the nature and distribution of cellular receptor molecules and by sequence variations in the viral S glycoprotein (219).

Coronavirus replication takes place in the cytoplasm of infected cells and can occur in enucleated cells, although at a low

efficiency (29,281). However, actinomycin D has been reported to inhibit the replication of some coronaviruses in some cell lines (67,73,148). Thus, the possible role of the nucleus in coronavirus replication is still debatable. Infectious coronaviruses can be isolated from culture fluids and from infected cells disrupted by freezing and thawing. Infectivity of MHV virions is fairly stable at pH 6.0, but the virus is rapidly inactivated at a mildly alkaline pH (256). Coronaviruses can cause either cytocidal or persistent infections of cells in culture and in animal infections, depending on the virus strain and the host cell. In cytocidal coronavirus infections, cells may form multinucleated syncytia, lyse, or both. Plaques on infected cell cultures can be visualized by cell lysis, fusion, neutral red staining, or, in the case of BCoV, hemadsorption. Persistent HCoV-229E infection can be established in some human cell lines, producing virus for weeks without cytopathic effects or cell death (39). After an acute lytic infection, many coronaviruses establish persistently infected carrier cultures with minimal cytopathic effects; these cultures are resistant to superinfection by the wild-type virus (39,50,89,105).

Coronaviruses and their replication *in vitro* and *in vivo* can be detected by sensitive immunoassays with various monoclonal antibodies, nucleic acid hybridization, and reverse transcription–polymerase chain reaction (RT-PCR) as well as by plaque assay, immunofluorescence, and, in some cases, hemadsorption (34,48,193).

CORONAVIRUS REPLICATION

The major events in the coronavirus replicative cycle are summarized in Figure 4.

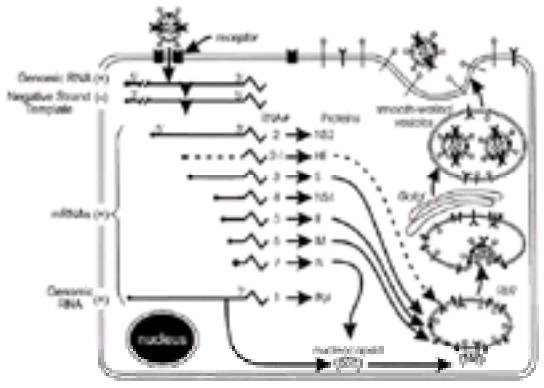

FIG. 4. Coronavirus replication cycle. Virions bind to the plasma membrane by interaction of the spikes with specific receptor glycoproteins or glycans. Penetration occurs by S protein–mediated fusion of the viral envelope with the plasma membrane or endosomal membranes. The gene 1 of viral genomic RNA is translated into a polyprotein, which is cotranslationally or posttranslationally processed to yield an RNA-dependent RNA polymerase (Pol) and other proteins involved in viral RNA synthesis. The Pol products use the genomic RNA as a template to synthesize negative-stranded RNAs, which are, in turn, used to synthesize genomic RNA and subgenomic mRNAs. The mRNAs in infected cells consist of an overlapping nested set of 3' coterminal RNAs containing a common leader RNA sequence at the 5' end. The mechanism of subgenomic mRNA synthesis has not been established (see Fig. 5). With a few exceptions, each mRNA is translated to yield only the protein encoded by the 5' most open reading frame of the mRNA. These proteins include structural proteins N, M, E, S, and HE and several nonstructural proteins. The N protein and newly synthesized genomic RNA assemble in the cytoplasm to form helical nucleocapsids. The membrane glycoprotein M is inserted in endoplasmic reticulum (ER) and anchored in the Golgi apparatus. Nucleocapsid (N plus genomic RNA) probably first binds to M protein at the budding compartment that lies between the RER and the Golgi. Similarly, E protein is also transported through the ER to the Golgi, where E and M proteins interact to trigger the budding of virions, enclosing the nucleocapsid. The S and HE glycoproteins are also translated on membrane-bound polysomes, inserted into the RER, and transported to the Golgi complex. These proteins are cotranslationally glycosylated, then trimerized and transported through the Golgi apparatus, where they undergo further modifications and, in some coronaviruses, are cleaved into two subunits, S1 and S2. During transport, some S and HE proteins associate with M protein and are incorporated into the maturing virus particles. Excess S and HE proteins that are not incorporated into virions are transported to the plasma membrane, where they may participate in cell–cell fusion or hemadsorption, respectively. Virions are apparently released by exocytosis-like fusion of smooth-walled, virion-containing vesicles with the plasma membrane. Virions may remain adsorbed to the plasma membranes of infected cells. The entire cycle of coronavirus replication takes place in the cytoplasm.

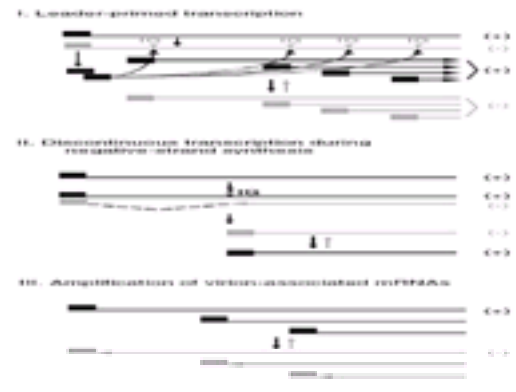

FIG. 5. Proposed models of coronavirus mRNA transcription (see text). *Solid lines* represent positive-stranded RNA; *dashed lines* represent negative-stranded RNA. *Boxes* represent the leader RNA or antileader. *Arrowheads* indicate the direction of transcription.

Attachment

The first step in the viral replication cycle is the binding of virions to the plasma membranes of target cells. Coronaviruses containing the HE glycoprotein (e.g., BCoV, HCoV-OC43) may bind to 9- *O*-acetylated neuraminic acid (sialic acid), a common moiety on membrane macromolecules (229,231,275). Enzymatic removal of the 9-*O*-acetylated neuraminic acid from cell membranes or treatment of virus with HE-specific monoclonal antibodies inhibits BCoV infection but does not completely block it (63,64,229), suggesting that the binding of HE to 9- *O*-acetylated neuraminic acid residues is necessary for, or at least can facilitate, virus infection. Inhibition by diisopropylfluorophosphate of the esterase activity of HE also markedly reduces BCoV infectivity (274); however, the esterase activity is likely involved in virus release rather than in virus entry because the newly synthesized virions may be trapped by the neuraminic acid residues on cell surface molecules and require esterase for virus release. In any case, virus binding to neuraminic acid residues may not be a crucial step for virus infection because many coronaviruses do not bind sialic acids. Furthermore, because sialic acid is a common cell surface carbohydrate and the tissue tropism of coronaviruses is restricted, this HE-sialic acid binding may not be sufficient to determine viral tropism. Recent studies have shown that the S protein of TGEV binds *N*-glycolyl neuraminic acid, whereas S protein of PRCoV, which is related to TGEV but causes primarily respiratory infections, does not, raising the possibility that this binding may contribute to the enterotropism of TGEV (230).

The main determinant of viral tropism is likely the binding of the S protein to a specific receptor glycoprotein on the cell surface. Receptors have been identified for several coronaviruses. The MHV receptor is a murine biliary glycoprotein belonging to the carcinoembryonic antigen (CEA) family in the Ig superfamily (69,283). It has an extracellular domain with four Ig-like loops, a transmembrane region, and a short intracytoplasmic domain. The viral S glycoprotein binds to the N-terminal Ig-like domain of MHV receptors (70). A monoclonal antibody against the receptor blocks MHV infection in murine cell culture and in infant mice (203,238). Subsequent studies have shown that other members of the murine CEA family and several human CEA-related glycoproteins can also serve as MHV receptors (44,45,68,194,292,293).

The MHV receptor is expressed predominantly in the liver, gastrointestinal tract, macrophages, and B cells, but not on thymic T cells (51,88), consistent with the target cell specificity of MHV. Surprisingly, the level of MHV receptor in the brain is very low, although many MHV strains can infect brain. Thus, MHV may use other receptors for central nervous system infections. A mouse strain SJL expresses a mutant MHV receptor that is functional when expressed at high levels in hamster or human cells, and yet SJL mice are highly resistant to MHV infection (68,293). Therefore, receptor expression is not the sole determinant of viral tropism. Several MHV variants can efficiently use human CEA as receptors for infection of human cells (18,19). The expression level of the MHV receptor may be a limiting factor for MHV infections under some conditions; increased levels of receptor expression can enhance the susceptibility of the cells to MHV infection (46,220). It is interesting to note that MHV infects polarized epithelial cells through only the apical, not the basolateral, surface (214), consistent with the MHV receptor expression pattern in respiratory and enteric epithelium in mice (88).

HCoV-229E, TGEV, FIPV, and probably canine coronaviruses (CCoV) use the cell membrane–bound metalloprotease, aminopeptidase N (APN), of their respective host species (23,57,285), rather than CEA-like molecules, as a receptor. APN is widely distributed on many cell types, including respiratory and enteric epithelial cells and neuronal and glial cells (131). Some monoclonal antibodies against porcine or human APN block the binding of TGEV or HCoV-229E virions to their target cells (57,285). However, the protease activity of APN is not necessary for the receptor function; inhibitors of APN and mutations of APN that abrogate the enzymatic activity do not block virus infection (57,283).

Penetration and Uncoating

After virus binds to a specific receptor, it enters the cell, a step that involves fusion of the viral envelope with either the plasma membrane or the endosomal membranes. MHV, BCoV, and IBV exhibit a neutral or slightly alkaline pH optimum for induction of cell–cell fusion (149,204,256,279), suggesting that these viruses may cause fusion of the viral envelope with the plasma membrane at the normal physiological pH of the extracellular environment. Therefore, these viruses probably enter cells by virus–cell fusion at the plasma membrane. In contrast, infectivity of some MHV strains is reduced, but not completely inhibited, by lysosomotropic drugs, suggesting that these viruses may enter cells through the endosomal pathway (84,127,190). Current experimental evidence indicates that different coronaviruses can enter cells either by acidic pH–dependent endocytosis or by pH-independent fusion at the plasma membrane (124).

The mechanism by which a virus uncoats and releases its genomic RNA into the cytoplasm is not yet clear. Cellular factors may be required for both virus penetration and uncoating. Some murine cell lines are resistant to MHV infection despite the presence of a functional MHV receptor; MHV infections in these cells are blocked at penetration, uncoating, or other steps of virus entry (8,79,286). These resistant mouse cells can be grouped into three complementation groups, suggesting that at least three cellular genes are involved in the processes of virus entry (8).

Transcription of Viral mRNA

Once viral RNA is released into the cytoplasm, it is used to synthesize a viral RNA-dependent RNA polymerase, which then initiates the transcription of viral mRNAs. Coronaviruses synthesize multiple subgenomic mRNAs, each of which translates one or, occasionally, more than one protein. There is a subgenomic mRNA for the expression of every viral gene except gene 1, which is expressed from the genomic RNA. Five to seven subgenomic mRNAs are synthesized in infected cells, depending on the coronavirus strain or isolate. These viral mRNAs are represented by numbers in order of decreasing size (138); mRNAs discovered after these numbers were assigned are denoted by a hyphen and a second number (e.g., mRNA 2-1) (37). The mRNAs form a nested set with a common 3' end and poly(A) (see Fig. 4). Although each mRNA, except the smallest, contains two or more ORFs, only the 5'-most ORF is translated, with minor exceptions, as noted later. Thus, these mRNAs are functionally monocistronic.

A characteristic feature of coronavirus subgenomic mRNAs is the presence at their 5' end of a leader sequence, 65 to 98 bases long, that is identical to the sequence at the 5' end of the genomic RNA (139,238,243). This leader sequence is not found elsewhere in the genome; however, the sequence between each gene, termed the *intergenic sequence* (16) (Fig. 5; see also Fig. 3), or transcription-associated sequence (TAS) (100) shares a sequence homology of 7 to 18 nucleotides with the 3' end of the leader (238). The core sequence of the intergenic sequence for MHV is UCUAAAC or a variant of it (112), whereas the 3' end of the leader consists of several tandem repeats of UCUAA. Sequence comparison of viral genomic and mRNAs suggests that subgenomic mRNAs are derived by juxtaposition of the leader sequence to the mRNAs through the UCUAA repeat sequence (238). The mechanism of joining these two RNA elements is discussed later in this section.

The viral genomic RNA is first transcribed into negative-stranded RNA, which is then used as the template for synthesis of mRNAs and genomic RNA. Studies have shown that both genomic- and subgenomic-sized negative-stranded RNAs are present in relative ratios comparable to those of the corresponding positive-stranded genomic and subgenomic mRNAs (222,235,236). All the negative-stranded RNAs were in double-stranded form; no free negative-stranded RNA was detected (210,221,222). The mechanism of synthesis and the functions of the subgenomic- and genomic-length negative-stranded RNAs are subjects of controversy. There are several models to explain how the subgenomic mRNAs are synthesized so that the leader RNA is fused to the mRNAs (see Fig. 5). Several pieces of experimental data are relevant to the formulation of these models: ultraviolet (UV) light transcriptional mapping studies (108,248,289) indicate that the UV target sizes of subgenomic mRNAs are roughly equivalent to the physical sizes of the individual mRNAs, suggesting that they are transcribed independently. Early in infection, the UV target sizes of the subgenomic mRNAs are equivalent to that of the genomic RNA (176,288), indicating that synthesis of a genomic-sized RNA is a prerequisite for subgenomic mRNA transcription. The difference in UV target size between the early and late stages of viral infection suggests that different mechanisms of RNA synthesis may operate at different stages of the viral replication cycle. Furthermore, in cells simultaneously infected with two different MHV strains, as much as half of the mRNAs may contain a leader sequence derived from one strain and coding sequences from the other strain (184), indicating that the leader sequence can be freely exchanged during transcription. This observation has been reproduced using a defective-interfering (DI) RNA vector system (see later), in which most of the DI-derived subgenomic mRNAs contain a leader sequence derived from the helper viral RNA molecule (109,297). Therefore, all models of coronavirus transcription include a discontinuous transcription process by which the leader sequence and mRNA sequences are derived from two different RNA molecules (109,297).

The first model for coronavirus mRNA synthesis involves *leader-primed transcription* (135) (see Fig. 5**I**). According to this model, discontinuous transcription occurs during positive-stranded RNA synthesis. The genomic RNA is first transcribed into a full-length negative-stranded RNA. Transcription of the leader RNA begins at the 3' end of the full-length, negative-stranded template RNA and terminates at the end of the leader sequence. The leader dissociates from the template, possibly with the polymerase, and subsequently binds to any intergenic sequence on the negative-stranded template and serves as the primer for mRNA transcription, resulting in an mRNA that contains a leader RNA fused to the mRNA. Several pieces of evidence are consistent with this model:

- MHV-infected cells contain free leader RNAs ranging in size from 50 to 90 nucleotides (16); however, free leader RNAs were not found in BCoV-infected cells (42).
- Leader RNAs transcribed from two different MHV strains in the same cell can be randomly joined to mRNAs from either strain (184).
- In an *in vitro* transcription system using lysates derived from MHV-infected cells, exogenously added leader RNA can be incorporated into subgenomic mRNAs (10).
- Studies using a DI RNA vector system showed that the appearance of full-length, genomic-sized negative-stranded RNA precedes the appearance of subgenomic negative-stranded RNA and subgenomic positive-strand mRNAs (4). Furthermore, the appearance of subgenomic mRNAs preceded the synthesis of subgenomic negative-stranded RNA, suggesting that the subgenomic mRNAs are most likely synthesized from the genomic-sized negative-stranded RNA template, at least early in infection. The subgenomic negative-stranded RNA could be generated from amplification of the subgenomic mRNA (4). However, the possible differences in the relative sensitivity for detection of the different RNA species in this study have not been rigorously excluded.

According to this transcription model, the IG or TAS sequences serve as promoters for mRNA transcription. In the case of MHV, these sequences include a minimal core promoter sequence of seven nucleotides (UCUAAAC) (111,178). When this seven-nucleotide sequence is inserted into the DI RNA and expressed in the MHV-infected cells, a subgenomic mRNA is transcribed from this intergenic sequence site (179). The transcribed MHV mRNA contains a leader sequence derived from the 5' end of the same RNA molecule (in *cis*) or a different RNA molecule (in *trans*) (297). Fusion of the leader RNA occurs within the UCUAAAC sequence. Additional sequences neighboring these seven nucleotides may also contribute to the joining of these two RNA regions (267).

The intergenic sequences preceding the various ORFs show some variability. Certain mRNAs, such as MHV mRNA 2-1, can be transcribed only when the leader RNA sequence (particularly its 3' end) and the intergenic sequence preceding the gene (i.e., HE gene) are compatible (133), suggesting that the interaction between the leader RNA and intergenic sequence regulates mRNA transcription. Deletion or mutation of the leader RNA abolishes or severely reduces mRNA transcription (152,297). According to this model, the subgenomic negative-stranded RNAs are synthesized from the subgenomic mRNA and may serve as the templates for transcription later in the viral replication cycle. This possibility would account for the UV target sizes observed later in infection.

An alternative model for coronavirus mRNA transcription is *discontinuous transcription during negative-stranded RNA synthesis* from the full-length genomic RNA template (222) (see Fig. 5**II**). According to this model, the polymerase pauses at one of the IG or TAS sequences and then jumps to the 3' end of the leader sequence in the genomic RNA template, generating a subgenomic negative-stranded RNA with an antisense leader sequence at its 3' end. This subgenomic negative-stranded RNA then serves as a template for synthesis of mRNAs. The positive-stranded intergenic sequence serves as the transcriptional termination sequence or the sequence that interacts with the leader RNA to facilitate

polymerase jumping during negative-stranded RNA synthesis. This model is compatible with the following experimental observations:

- Subgenomic negative-stranded RNAs have a poly(U) sequence at the 5' end and an antisense leader sequence at the 3' end (101,235); thus, they are exact complementary copies of the viral subgenomic mRNAs.
- The different subgenomic negative-stranded RNAs and their corresponding mRNAs are equally abundant in infected cells (236).
- Subgenomic replicative intermediate RNAs actively replicate in infected cells. When subgenomic RNAs were separated by size and then denatured, the smaller RIs generated smaller mRNAs, and the larger RIs generated only larger mRNAs. This finding suggests that each subgenomic mRNA may be transcribed from a corresponding subgenomic-sized, negative-stranded template (222). Furthermore, subgenomic negative-stranded RNAs were detected in the membrane-associated replication complexes (234).
- The UV target sizes for subgenomic mRNA synthesis at a later stage of viral replication correspond to the sizes of the individual subgenomic mRNAs (108,248,289). However, this model does not explain the kinetics of the appearance of various RNA species (4) or the UV target sizes of these mRNAs early in viral replication (176,289).

In this model, the IG or TAS sequences involved in the leader-mRNA fusion will operate during negative-strand RNA synthesis.

A third model (see Fig. 5**III**) is based on the finding that subgenomic mRNAs are incorporated into virions of some coronaviruses, including BCoV, TGEV, and IBV (103,236,298), along with the full-length genomic RNA. This model proposes that, after virus entry, the subgenomic mRNAs associated with the incoming virions are used directly as templates for the synthesis of subgenomic negative-stranded RNAs, which, in turn, serve as templates for the synthesis of additional subgenomic mRNAs (232,233). This model is not compatible with the discontinuous nature of coronavirus RNA synthesis. Furthermore, not all coronaviruses have been found to contain virion-associated subgenomic mRNAs. Coronavirus positive-stranded mRNAs cannot be amplified when transfected into infected cells (41,152). Nevertheless, it is possible that this model may operate at some stages of the viral replication cycle for some coronaviruses.

Data available at this time cannot unequivocally establish or exclude any of these models. The difficulty in differentiating these models is partially due to the fact that subgenomic negative-stranded RNAs can be copied from subgenomic mRNAs and vice versa because the subgenomic RNAs contain the complete cis-acting signals for negative-stranded RNA synthesis (155). So far, the synthesis of the subgenomic mRNAs and the synthesis of subgenomic negative-stranded RNAs have not been uncoupled in infected cells under any experimental conditions. Therefore, it is difficult to determine experimentally which subgenomic RNA species, positive- or negative-sense, comes first. Furthermore, most of the studies were performed late in infection when both positive- and negative-stranded subgenomic RNAs were already present and presumably could be further copied from each other. It is possible that elements from each of these models may operate at various stages of the viral life cycle. Comparative information about RNA transcription mechanisms in other related viruses, such as toroviruses and arteriviruses, may help to elucidate coronavirus RNA transcription because they have smaller RNA genomes and polymerase genes (59,242); furthermore, infectious viral complementary DNA (cDNA) or its transcripts are available for arteriviruses (269,270). However, there are no leader sequences on torovirus subgenomic mRNAs, and coronavirus replication has a high frequency of leader switching that is not seen in arterivirus replication.

Coronavirus RNA synthesis takes place on membranous structures associated with the endoplasmic reticulum (ER), Golgi complex, or late endosomes, depending on the cell types (61,237,265). Most of the gene 1 proteins are associated with the newly synthesized viral RNA. This observation is consistent with the finding that MHV temperature-sensitive mutants with defects in gene 1 can be classified into five complementation groups, all of which are defective in viral RNA synthesis (15). Anti-N antibody has been shown to inhibit MHV RNA synthesis *in vitro,* suggesting that N protein is involved in RNA transcription (49). Host components may also be involved in viral RNA synthesis (150,151).

In addition to the leader RNA and intergenic sequences, several other RNA elements, including the 3' end of the viral genome (155,156), are involved in the regulation of coronavirus mRNA transcription. About 55 nucleotides at the 3' end of the genomic RNA and the poly(A) sequence are required for the initiation of negative-stranded RNA synthesis (155); in contrast, the presence of the entire 3'-UTR region (305 nucleotides) is necessary for mRNA transcription (156). The difference in the lengths of the 3' end sequences required for negative-stranded RNA and mRNA synthesis suggests that most of the 3' end cis-acting sequence is involved in the synthesis of positive-stranded RNA. Thus, the 3' end of viral RNA (or 5' end of negative-stranded RNA) likely interacts with the leader or intergenic sequence to regulate mRNA transcription.

Replication of Genomic RNA

The viral genomic-sized RNA in the infected cell consists of two components: the mRNA encoding gene 1 products (5%) and the genomic RNA to be packaged into nucleocapsids and virions (95%) (210,244). Unlike the discontinuous synthesis of subgenomic mRNA, the production of genomic RNA presumably requires uninterrupted synthesis using a full-length, negative-stranded template. Therefore, the mechanism of genomic RNA replication conceivably differs from that of subgenomic mRNA transcription. It has been shown, however, that some genomic-sized RNAs of MHV may be synthesized by discontinuous transcription, that is, fusion of the leader sequence to a UUUAUAAAC sequence immediately downstream of the leader RNA, thereby creating a genomic-sized RNA with a small deletion (296). This mechanism has also been demonstrated in DI RNA replication. During MHV DI RNA replication, the leader sequence of the DI RNA can be rapidly replaced by that of the helper virus RNA (181), suggesting that MHV RNA replication involves a free leader RNA (of either positive or negative sense), similar to the discontinuous transcription of subgenomic mRNAs. In addition, the leader sequence of MHV RNA genome undergoes rapid evolution in the number of UCUAA repeats at the 3' end of the leader (180). These UCUAA sequences are also hot spots of RNA recombination, possibly as a result of discontinuous synthesis (116). Therefore, the replication of genomic RNA appears to occur by discontinuous synthesis

involving a leader RNA as well.

Studies of MHV DI RNA have also revealed information regarding the minimum cis-acting signals for coronavirus genomic RNA replication. Replication of the MHV DI RNA requires sequences of about 400 to 800 nucleotides at both the 3' and 5' ends of the genomic RNA and an internal replication signal of about 135 nucleotides (119,154). However, other DI RNAs of MHV and BCoV do not require this internal sequence for replication (41,169). This difference is likely due to differences in the secondary structure of the RNA genomes between different viral strains (120). The lengths of the cis-acting signals for RNA replication at both ends of the viral RNA differ slightly from those required for mRNA transcription (152,154,156). Additionally, the 3' end cis-acting signal for genomic RNA replication (154) is longer than that required for negative-stranded RNA synthesis (155), again suggesting long-range interactions between the two ends of the genomic RNA during RNA replication.

Translation of Viral Proteins

Although all but the smallest coronavirus mRNAs contain multiple ORFs, only the 5'-most ORF of each mRNA is generally translated by a cap-dependent ribosomal scanning mechanism (see Fig. 4). Most of the structural proteins, including S, M, N and HE, are translated from separate mRNAs by this mechanism. Because each of the coronavirus mRNAs contains a 5' leader sequence, which enhances translation in virus-infected cell lysates (260), virus-specific mRNAs may be preferentially translated in the face of translational shutoff in the infected cells. Other viral proteins are translated by other distinct mechanisms (see Fig. 3). First, the mRNA 1 of all coronaviruses contains two large overlapping ORFs that are translated into one polyprotein by a ribosomal frame-shifting mechanism (28,30,72,94,144). Translation of this gene, however, is initiated by the conventional cap-dependent translation mechanism. Second, in several coronavirus mRNAs, it is the second or third ORF that is most efficiently translated. For example, the E protein is translated from the second ORF of MHV mRNA 5 and the third ORF of IBV mRNA 3. These ORFs are preceded by an internal ribosomal entry site sequence that allows ribosomes to bypass the upstream ORFs and translate the downstream ORF by a cap-independent translation mechanism (161,262). Some of the upstream ORFs are also translated, but the translational efficiency and functional significance of these proteins are not clear. Third, several other mRNAs contain two overlapping ORFs, both of which are translated by unknown mechanisms. For example, mRNA 5 of IBV contains two ORFs that are both translated *in vitro* and *in vivo* (160), and an internal ORF (termed I) within the N genes of MHV and BCoV is translated *in vivo* (75,233).

Processing and Intracellular Transport of Viral Proteins

Polymerase Precursor Polyprotein 1a and 1b

Polyprotein 1a and 1b is presumably the first and only viral protein synthesized from the incoming viral genome because it is required for viral RNA synthesis. (Other viral proteins are translated from subgenomic mRNAs.) Once viral RNA synthesis begins, more gene 1 protein products are translated from the newly synthesized genomic RNA. The primary gene product (from ORF 1a and 1b), which is predicted to be nearly 700 to 800 kd, is cotranslationally or posttranslationally processed into multiple proteins by its own proteases. ORF 1a contains at least two protease domains: a papain-like cysteine protease (PLP) and a chymotrypsin-picornavirus 3C–like protease (3CLp) (90,144). In some viruses (e.g., MHV, HCoV-229E, and TGEV), there are two copies of the PLP domain, PLP1 and PLP2. PLP and 3CLp cleave the polyprotein into multiple proteins through a complex series of processing intermediates, some of which have not been unequivocally identified. In MHV, PLP1 is responsible for cleaving the aminoterminal proteins p28 and p65 from the polyprotein (11,12,24,60,62) (Fig. 6). The remaining portion of the polyprotein (including both ORF 1a and ORF 1b) is most likely processed by 3CLp (157,162,299,300). 3CLp itself is cleaved from the polyprotein by its own autoprotease activity into a protein of 27 kd, which has both trans- and cis-acting proteolytic activities (158,165,166,299). The 3CLp-mediated cleavage sites were predicted by computer analysis to be mostly at Q/S or Q/A sites (90,144); many of these have been confirmed by experimental analysis. A 100-kd protein from ORF 1b is thought to contain the polymerase domain (92,157), although the polymerase activity has not been directly demonstrated. A genetic complementation group thought to be responsible for mRNA transcription has been mapped to the coding region for this protein (223). A 67-kd protein has been shown to include the helicase domain (61). Many of these proteins and possibly their processing intermediates are colocalized with the newly synthesized viral RNA and, thus, are presumably present in the RNA replication complex, which is associated with the membranous structures in the infected cells (61,237,265). PLP2 has recently been shown to cleave at the upstream border of MP1 domain (112).

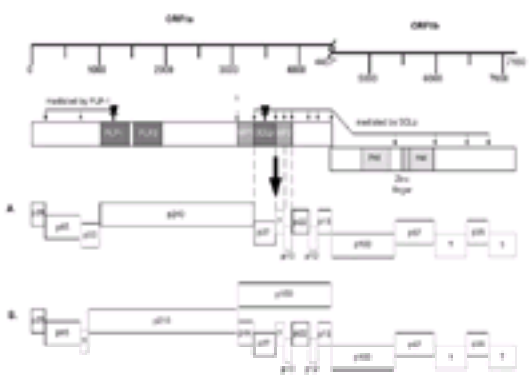

FIG. 6. Presumptive proteolytic products of the murine hepatitis virus (MHV) gene 1 (polymerase). Unlike the structural proteins of coronaviruses, which are each encoded by a separate mRNA, the proteins composing RNA-dependent RNA polymerases are formed by virus-encoded proteolytic processing of a precursor polyprotein. Open reading frames (ORFs) 1a and 1b are translated into a polyprotein by a ribosomal frame-shifting mechanism. The predicted and confirmed functional domains of ORF 1a and 1b are indicated. Two versions of the presumptive proteolytic processing pathways are shown. The *dashed lines* represent the unconfirmed products or cleavage sites. The full-length primary translation product and intermediates are not shown. The functions of most of these peptides are not yet known. PLP1,

PLP2, papain-like cysteine protease 1 and 2; 3CLp, poliovirus 3C-like protease; Pol, predicted polymerase; Hel, predicted helicase; MP, membrane-binding domain. (Courtesy of Susan C. Baker and Mark Denison.)

S Protein

The S protein is cotranslationally inserted into the rough endoplasmic reticulum (RER) and glycosylated with N-linked glycans. The protein is linked by multiple intramolecular disulfide bonds to form a complex structure and then oligomerized into homotrimers (58,199). Transportation of the S protein to the Golgi complex is accompanied by trimming of high mannose oligosaccharides and the addition of terminal sugars to form mature glycans. The S2 domain undergoes fatty acylation, probably on cysteine residues (225,255). Cleavage of S into S1 and S2 occurs after the conversion of glycans from simple to complex forms (272), either in the Golgi complex or extracellularly. Typically, the cleavage site is flanked by several basic amino acids (38,170); coronavirus S proteins that lack these residues, such as those of HCoV-229E or FIPV, are not cleaved. Most of the S protein accumulates in the Golgi of infected cells, where it participates in virus particle assembly; however, some S oligomers are transported to the plasma membrane, where it may mediate cell–cell fusion (91,272).

M Protein

The M protein is synthesized on membrane-bound polysomes and inserted into the ER membranes by an internal signal sequence (215). M protein is highly hydrophobic and spans the membrane three times (7,216). In IBV and TGEV, the aminoterminal domain of the M protein, which is located on the luminal side of the ER, is cotranslationally glycosylated with N-linked glycans (247); however, the corresponding domain in MHV is posttranslationally glycosylated by O-linked glycans in the Golgi apparatus (106,196,197). The mature M protein accumulates in the Golgi apparatus, but unlike S, it is not transported to the plasma membrane. The nature of the membrane-targeting sequence in the M proteins of different coronaviruses is variable (163,171).

HE Protein

The HE protein is cotranslationally glycosylated by N-linked glycans in the RER and forms disulfide-linked dimers of 165 to 170 kd (104,117,290). It is transported to the Golgi, where the N-linked glycans are converted to the complex form (290) and incorporated into the envelope of budding virus particles. HE protein that is not incorporated into budding virions is transported to the plasma membrane and expressed on the cell surface (117).

N Protein

The N protein is translated on free polysomes and rapidly phosphorylated on serine residues in the cytosol (247), but the extent of phosphorylation and its functional significance are not yet known. Some N protein may be associated with cellular membranes (4,247), where it participates in viral RNA synthesis and virus budding. Interestingly, some antigenic peptides of the N protein can be recognized on the surface of infected cells by T cells (25,251,277).

E Protein

The acylation of the MHV E protein is the only posttranslational modification known to occur in E protein (295). This protein is localized primarily in the perinuclear region in infected cells but is also expressed on the cell surface (87,295).

Assembly and Release of Virions

The first step in virus assembly is the binding of N protein to viral RNA to form helical nucleocapsids. Efficient packaging of genomic RNA into MHV virions requires a specific RNA signal that consists of a stretch of 61 nucleotides in the 3' end of gene 1b, about 20 kb from the 5' end of the genome (80,266,284). This sequence, found only in the genomic-length RNA, specifically binds the N protein (188). Thus, the interaction between the N protein and this packaging signal is likely the initial step of nucleocapsid formation. BCoV, TGEV, and IBV virions also package small amounts of all subgenomic mRNAs, which do not contain this packaging signal (103,235,236,298). These virion-associated mRNAs have been postulated to serve as templates for amplification of the subgenomic mRNAs for these viruses (237). Even DI RNAs without a typical packaging signal can be incorporated into virions, albeit inefficiently (40,182). The packaging of subgenomic mRNAs and these DI RNAs into virions may be mediated by the binding of N protein to the leader sequences of these RNAs (249).

Once the nucleocapsid is formed, it interacts with the M protein at the cellular membranes (254), most likely the ER or the Golgi complex. Studies have shown that the N protein by itself cannot be packaged into virions; it can be enveloped only together with the viral RNA (27,271), suggesting either that M protein interacts directly with the viral RNA or that the viral RNA–N protein interaction induces a conformational change in the N protein, enabling it to interact with the M protein. This interaction may lead to the formation of the spherical internal core shell surrounding the nucleocapsid (211) and, at the same time, enable the nucleocapsid to be packaged into budding virus particles, which are formed on the membranes of the ER and Golgi.

Although the interaction of the nucleocapsid with M protein likely triggers the packaging of the nucleocapsid into virions, the formation of virus particles cannot occur without an additional viral protein, the E protein. In fact, formation of virus-like particles, which do not have nucleocapsids, can occur in the presence of the M and E proteins alone (27,271). Studies showed that most mutations of the E protein resulted in altered virus morphology (76), and most alterations of the M protein abrogated virus-like particle formation (56). MHV virions can be released from tunicamycin-treated, virus-infected cells as spikeless, noninfectious particles that do not contain the S protein (106), indicating that S protein

is not essential for virus particle formation.

Virus budding is first detected at a specialized membrane structure called the *budding compartment,* located between the ER and the Golgi (66,121,263,264), where the M protein is anchored (121). Virus budding is most likely triggered by the interaction of E protein with the M protein located on the membrane. Because E protein in cells can be found at sites other than the ER or Golgi complex (86,292), the virus budding site is likely dictated by the M protein. It should be noted that coronavirus budding never occurs on the plasma membrane probably because the M protein is never found on the plasma membrane.

E protein is considered to be a minor component of the virion. The ratio of M to E protein in virions can be as high as 100:1 (271); thus, although the E protein is required for the initiation of virus assembly and budding, it may serve only as a scaffolding protein that is not an essential part of the mature virion. The expression of E protein induces curvature of the M-containing intracellular membrane (271); thus, E protein may be involved in the pinching off of the budding virions into the budding compartment.

The S and HE proteins are incorporated into virions through their interactions with M protein. The formation of S–M and HE–M complexes occurs in the pre-Golgi complex (195,200). These two complexes then form an S–M–HE ternary complex before the glycoproteins undergo trimming of their sugar chains (197). Thus, the processing of virion-associated glycoproteins most likely occurs as virions pass through the Golgi. After budding, virus particles may undergo further morphologic changes within the Golgi, resulting in the appearance of mature virus particles with a compact, electron-dense internal core, as they reach the secretory vesicles (213,218). Virions accumulate in large, smooth-walled vesicles, which eventually fuse with the plasma membrane to release virus into extracellular space (91). Virus release is restricted to certain areas of cells; for example, MHV-A59 virions are released from the basolateral surface of certain polarized murine cells (214). The mechanism of release site restriction is unknown.

Effects on Host Cells

Coronavirus-induced cytopathic effects vary with the virus strain and the host cell. Infection with certain coronaviruses, such as MHV, BCoV, IBV or FIPV, induces cell fusion in culture and in infected tissues. Even those coronaviruses that cause little or no cell fusion induce rounding and lysis of cells (84,89). The mechanisms of coronavirus-induced cytopathic effects are poorly understood. Some MHV strains are known to inhibit translation of cellular proteins (98,239) and cause selective inhibition of transcription of some cellular genes (132). Infected cells may undergo fragmentation and rearrangement of the Golgi apparatus late in infection (143). TGEV and some MHV strains may also induce apoptosis in some cell types, which may be mediated by the E protein (3,22,71). A combination of these virus-induced effects may contribute to the cytopathic effects observed in host cells late in infection.

Persistent coronavirus infection occurs readily in cell culture. Generally, after the initial cytocidal infection, carrier cultures arise, in which only a fraction of the cells produce infectious virus at any time, and virus production continues for months (89,105,140). Cells in persistently infected cultures are resistant to superinfection with wild-type virus, and various replication-defective mutants are frequently selected from these cultures (84,89,102,105,252). These mutant viruses may modulate virus dissemination in culture. Virus persistence in culture may also be due to the selection of coronavirus-resistant cells, whereby only a small number of cells remain susceptible to coronavirus infections, thus limiting the extent of viral dissemination (103,174,220). Both viral and cellular evolutions likely contribute to the maintenance of persistent infections. In fact, the virus and host cells often coevolve during persistent infection, resulting in the selection of both resistant cells and virus mutants with an expanded host range (46,224). HCoV-229E has been demonstrated to establish persistent infection in human oligodendrocyte and glial cell lines (6).

Although most coronavirus infections in animals are acute and self-limited, persistent coronavirus infection *in vivo* can occur in immunocompromised hosts, such as nude or newborn mice (20,21,209,261,278). Some temperature-sensitive or monoclonal antibody neutralization-escape mutants of MHV are particularly prone to cause persistent infection in animals (32,78,122). Persistency also has been noted in natural infections of feline enteric coronavirus (FECoV) in cats (96). In such cases, FECoV may undergo serial evolution, forming a distinct virus quasi-species in each cat. These chronic FECoV carriers would be resistant to superinfection (96).

CORONAVIRUS GENETICS

Virus Mutants

Like most RNA viruses, coronaviruses are thought to mutate at a high frequency because of the high error frequencies of RNA polymerases (246). However, in one case in which the entire MHV genome was sequenced after repeated passages of the virus in culture, surprisingly few mutations were detected (146). Nevertheless, multiple types of mutants have been isolated.

Temperature-sensitive (ts) MHV mutants have been classified into at least seven complementation groups (145), five of which are RNA negative, that is, they cannot synthesize RNA at the nonpermissive temperature (145,223). All of the RNA-negative mutations have been mapped to locations within gene 1 (15), suggesting that at least five separate functions of gene 1 products are required for viral RNA synthesis. Several complementation groups of RNA-positive mutants replicate viral RNA at the nonpermissive temperature but show altered cytopathic effects or fail to produce infectious virions (15,145). The RNA-positive mutants have defects in the genes encoding the S or N structural proteins (123,188,205). Some ts mutants differ in virulence from wild-type virus in animal infections. For example, although the wild-type JHM strain of MHV causes acute encephalitis in mice, some of its ts mutants cause a subacute demyelinating disease (93,125).

Deletion mutants also occur frequently. Many natural MHV strains have deletions of up to 200 amino acids in a hypervariable region of the S protein and show altered biologic properties *in vivo* (14,85,203). The most striking example

of the biologic importance of deletion mutations is the emergence of PRCoV from TGEV. TGEV causes epizootic enteric infections in pigs. In the early 1980s, PRCoV emerged in Europe as a new virus that caused widespread epizootic respiratory infections in pigs (141). Nucleotide sequence comparison of the two viruses showed that PRCoV was a derivative of TGEV with a large deletion within the S protein (31,202,281). Similar PRCoV strains have emerged independently in the United States as deletion mutants in the S gene of TGEV (9,141). FIPV most likely arose from deletion mutations of FECoV in a similar manner (273).

RNA Recombination

A unique feature of coronavirus genetics is a high frequency of RNA recombination, particularly in MHV (136). Although nonsegmented genomes of RNA viruses generally exhibit very low or undetectable recombination frequencies, the recombination frequencies for the entire coronavirus genome have been calculated to be as high as 25% (15). RNA recombinants were first isolated during co-infection with different ts mutants of MHV at the nonpermissive temperature (137). Additional recombinants were isolated using selectable genetic markers such as neutralization-escape mutants and strains that differ in cell fusion activity (113,115,116,181). To date, RNA recombination has been demonstrated for MHV, TGEV, and IBV, both in tissue culture and in experimental and natural animal infections (13,114,126,276).

The high frequency of RNA recombination in coronaviruses is probably the result of the unique mechanism of coronavirus RNA synthesis, which involves discontinuous transcription and polymerase jumping (136). It is possible that the viral polymerase associated with the incomplete nascent RNAs dissociates from its template at a random point and switches to a homologous site on a different RNA template to complete RNA synthesis by a copy-choice mechanism (14,136).

RNA recombination is an important mechanism in the natural evolution of coronaviruses. For example, new strains of IBV in poultry flocks are the result of natural recombination between different field strains (34,110,131,276). RNA recombination may also play a role in the evolution of different coronavirus species. For example, a reversal in the order of the M gene and gene 5 on the IBV and MHV genomes (see Fig. 3) may have occurred because of homologous recombination at consensus intergenic regions between the two genes in a progenitor of one of the viruses. Recombination may also explain the acquisition of the HE gene from an mRNA of influenza C virus by a progenitor of the group II coronaviruses (168). Some biotypes of FECoV appear to have arisen by recombination between FECoV and canine coronavirus (97).

RNA recombination has been developed into a potent genetic tool to introduce desired RNA sequences into coronavirus genomes. Cells are transfected with a DI RNA containing RNA sequences with desired mutations and then infected with a ts mutant that has a defect in the N gene. Recombination can take place between the DI RNA and the viral RNA at any homologous site; some of the resulting wild-type viruses may incorporate the part of a DI RNA sequence that contains the introduced RNA sequences and can be selected by using the appropriate selection markers (123,130,147,188,205). This approach, termed *targeted RNA recombination* (123), is an important tool for genetic manipulation of coronaviruses because full-length infectious cDNA clones of coronavirus genomic RNA are not yet available for most coronaviruses.

Defective-Interfering RNA

When a coronavirus such as MHV is passaged at a high multiplicity of infection, new RNA species smaller than the genomic RNA emerge (185). These are DI RNAs, which can replicate only in the presence of a helper virus and may interfere with the replication of the helper virus. DI RNAs have been detected for MHV, IBV, and TGEV during viral replication in cell culture (185,189,207). Three different types of DI RNAs have been demonstrated (177). The first type, typified by the DIssE RNA (177) of MHV, replicates efficiently but is not packaged efficiently into virions. However, small amounts of this DI RNA can be nonspecifically incorporated into virions; thus, it can be maintained and amplified during serial virus passages in culture (182). The second type, typified by the DIssF RNA (177) of MHV, contains a packaging signal that enables it to be incorporated into virions efficiently (186,266). The third type, typified by the DIssA RNA (177) of MHV, is not a true DI RNA because it has retained the ability to replicate. It contains small deletions at multiple sites, is nearly the size of the viral genomic RNA, encodes a functional polymerase, and can replicate even in the absence of a helper virus (118,182). All of the DI RNAs contain both the 5' and 3' ends of the viral genome, which include the cis-acting signals for RNA replication. Most of them also contain an ORF encoding a protein that is fused from different viral proteins (183). The presence of an ORF, which may or may not encode functional viral proteins, may enhance the fitness of the DI RNA during evolution (55,119,153), although the ORF or its protein product is not necessary for RNA replication (153,208).

During serial passages of viruses in cell culture, the original DI RNAs frequently disappear, and new ones of different sizes are generated (177). The continuous evolution of DI RNAs is often due to recombination between the original DI RNA and the RNA of helper virus (82). DI RNAs with a longer ORF have a selective advantage over those with a shorter ORF, contributing to the evolution of DI RNA (119,268). DI RNAs have not been detected during natural coronavirus infections; thus, their biologic significance in coronavirus infection is not clear.

CONCLUSIONS

Coronaviruses are unique among RNA viruses in many aspects of their viral structure and replication. They are characterized by exceptionally large RNA genomes, nested subgenomic mRNAs, discontinuous transcription mechanism, high-frequency RNA recombination, and the unusually large number of gene products associated with RNA synthesis. Coronavirus RNA incorporates two different gene expression strategies: polyprotein processing for gene 1 and one–gene–to–one–mRNA expression for the other genes, probably a reflection of their unusual evolutionary origin. Many questions regarding the coronavirus replication cycle remain unresolved, including the mechanism of mRNA transcription, the functions of gene 1 proteins and other nonstructural proteins, and the mechanisms of virus entry and assembly. Recent findings suggest that the structure of virions and the role of cellular factors in viral replication need to

be reevaluated.

Coronavirus research has been hampered by the lack of infectious cDNA or RNA transcripts, so that standard reverse genetics approaches are inapplicable. Construction of a functional full-length genomic cDNA has been difficult because of the large size of the coronavirus RNA genome. Only recently did the construction of an infectious coronavirus cDNA (for TGEV) succeed (2). Such infectious recombinant genomes will undoubtedly faciltate the coronavirus research. In addition, studies of the related but less complex toroviruses and arteriviruses should also further enhance our understanding of the exceptionally complex coronavirus replication cycle.

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CHAPTER 36 Coronaviruses

Kathryn V. Holmes

HISTORY OF CORONAVIRUSES

Coronaviruses cause common respiratory and enteric diseases of humans and domestic animals (209,246). The structure of the coronaviruses was first determined by negative-stained electron microscopy. Avian infectious bronchitis virus (IBV) was found to have an array of large spikes on the viral envelope that resembled a crown (14). Mouse hepatitis virus (MHV), human respiratory coronaviruses (HCoV), and coronaviruses of domestic animals were found to share the same structure and, later, the same genome organization and replication strategy (1,30,31,209). In virions of some coronaviruses, including HCoV-OC43 and bovine coronavirus (BCoV), an additional layer of shorter spikes composed of the hemagglutinin-esterase (HE) glycoprotein was also observed on the viral envelope (103,104,258). Thin sections of coronavirus-infected cells and tissues showed that virions formed by budding at intracellular membranes, in a site between the rough endoplasmic reticulum and the Golgi apparatus called the *budding compartment* (43,227). Numerous virions of HCoV-229E were observed adsorbed to the plasma membranes of infected cells, but coronaviruses do not bud from the plasma membrane (170). The structure of the coronavirus nucleocapsid was more difficult to visualize than that of paramyxoviruses. The long, monopartite positive-stranded RNA genome of coronaviruses is encapsidated by the viral nucleocapsid protein, N, to form a long, flexible, helical nucleocapsid (136).

Classification and Nomenclature

Coronaviruses, toroviruses, and arteriviruses are now classified as members of the order Nidovirales, a group of enveloped, positive-sense RNA viruses that all synthesize a 3' co-terminal set of subgenomic mRNAs in infected cells (30). The toroviruses and coronaviruses are genera within the *Coronaviridae* family that differ in genome length, structural proteins, and nucleocapsid structure. The arteriviruses are classified as a separate family within the Nidovirales order. Arteriviruses have icosahedral nucleocapsids and markedly smaller genomes than coronaviruses. Like many coronaviruses, toroviruses cause enteric disease in humans and animals (109,212). Toroviruses in negative-stained stool specimens have sometimes been confused with coronaviruses by electron microscopy. This chapter discusses the coronaviruses that infect humans and studies on related coronaviruses of animals that shed light on the replication, pathogenesis, epidemiology, and prevention and treatment of human coronaviruses.

There are three groups of coronaviruses, with little or no serologic cross-reactivity between antigens on virions of the three groups (see Chapter 35). There are two groups of mammalian coronaviruses, and avian coronaviruses IBV and turkey coronavirus (TCoV) form a third group (67,176). The prototype of the first group is HCoV-229E. Coronaviruses of pigs (transmissible gastroenteritis virus [TGEV] and porcine respiratory coronavirus [PRCoV]), cats (FCoV), and dogs (CCoV) are related viruses in this group (176). The second group of mammalian coronaviruses includes the prototype MHV, and coronaviruses of humans (HCoV-OC43), rats (RCoV), cattle (BCoV), pigs (HEV), and other species (176). Antigenic cross-reactivity of the nonstructural proteins of coronaviruses has not yet been studied.

A brief summary of coronavirus genome organization, RNA transcription, and replication is presented below. More detailed discussions of these topics are found in Chapter 35 and in several reviews (24,121,209,213).

Genome Organization

Coronavirus genomes are monopartite, single-stranded, positive-sense, polyadenylated, and capped RNAs ranging from 27 to 32 kb in length (121,213). The complete nucleotide sequences of many coronavirus genomes have been determined (18,52,76,127). The order of the genes encoding the viral RNA-dependent RNA polymerase and the four common structural proteins, the spike (S), envelope (E), membrane (M), and nucleocapsid (N) proteins is the same for all coronaviruses: Pol-S-E-M-N (213). Genomes of coronaviruses in the MHV group also have a gene encoding the hemagglutinin-esterase (HE) glycoprotein located between Pol and S (131,213,258). Coronavirus genomes also include a variety of additional open reading frames (ORFs) that encode two to four nonstructural proteins of unknown functions.

These genes are not highly conserved among different coronaviruses. They are inserted into the genome in various sites between the genes that encode the polymerase and structural proteins (213,257). In the genome, a common intergenic sequence (IS) of about 7 bases is found at the 5' end of each gene (121). This IS sequence is essential for the formation of subgenomic RNAs because mutations in the IS can prevent the expression of the subgenomic RNAs, and insertion of an IS into a replicon can cause transcription of a subgenomic mRNA (96,142,226).

Subgenomic RNAs

Coronavirus-infected cells contain multiple overlapping subgenomic, capped, and polyadenylated mRNAs with a common 3' end (121). Each subgenomic mRNA and the viral genomic RNA, which also serves as an mRNA, is translated to yield only the protein encoded by the 5' gene on the mRNA. After the cap at the 5' end of every mRNA is a leader sequence about 70 bases in length that is the same as the sequence at the 5' end of the genomic RNA.

Small quantities of subgenomic negative-stranded RNAs corresponding to each subgenomic mRNA are found in infected cells (23,199,200,202). These have a poly(U) sequence at the 5' end and a sequence complementary to the leader at the 3' end. Several alternative mechanisms have been proposed to explain the presence of the leader at the 5' end of each subgenomic mRNA and the generation of the subgenomic negative strands in infected cells (121,201). These models are described in Chapter 35.

In infected cells, there are considerable differences between the numbers of subgenomic RNA transcripts that encode each of the structural genes. Because it is near the 3' end of the genome and present in all subgenomic viral mRNAs, the *N* gene is found in infected cells in much larger amounts than the *S* gene, which is about 8 kb upstream of *N.* The *N* gene is more highly conserved among isolates of the same coronavirus than the *S* gene. Therefore, the *N* gene has been used for initial studies on reverse transcription–polymerase chain reaction (RT-PCR) detection of viral RNA in infected cells. A highly conserved region from the polymerase gene has also been used to develop a consensus RT-PCR assay that can detect 11 coronaviruses in all 3 groups (217).

Replicase

The replicase gene found at the 5' end of all coronavirus genomes is enormous, about 20-kb long, with two partially overlapping ORFs, ORF1a and ORF1b, joined by an elaborated pseudoknot that acts as a ribosomal slippery site (77,121). Expression of this mRNA is different from that of the coronavirus subgenomic mRNAs. Like the nonstructural proteins of picornaviruses and alpha-viruses, the coronavirus gene 1 is translated as a polyprotein that is cotranslationally cleaved by a chymotrypsin-like protease (3CLpro) and one or two papain-like cysteine proteases found in the nascent protein (75,78,79,225,259,260). The proteins generated by these cleavage events in coronavirus-infected cells are now being characterized (75,249). Domains of the polyprotein that have homology to RNA polymerase and helicase, and an ATPase activity associated with the 1b protein of HCoV-229E have been identified (84,121). Some of the ORF1 gene products are associated with membranes in the cytoplasm of infected cells, and RNA synthesis occurs at or near this site (15,47,239). Complementation between temperature-sensitive, RNA-negative mutants of MHV have revealed at least eight complementation groups associated with ORF1 (8,214). Analysis of the processing of the polymerase polyprotein and characterization of the functions of the cleavage fragments may lead to identification of new targets for drugs to treat coronavirus infections.

Genetic Heterogeneity

In general, RNA-dependent RNA polymerases have a much higher error frequency than DNA-dependent polymerases. Based on an estimation of one error per 10,000 bases, the coronavirus RNA polymerase may generate about three random mutations in each about 30-kb genome produced in an infected cell. This would generate a population of genetically different viruses or quasi-species even in virus preparations derived from a single plaque. In addition to this high frequency of mutation, coronaviruses use RNA recombination as another way to generate genetic diversity in virus populations (24). RNA recombination between genomes of different strains of a coronavirus has been shown to occur with a high frequency in MHV, and recombination also occurs in other coronaviruses (120,141,143). Recombinants between vaccine strains and virulent IBV strains have been isolated from flocks of chickens in the field (117,245). Recombination between canine and feline coronavirus genomes has apparently generated variant coronaviruses that can be horizontally transmitted in cats (83). Nucleotide sequence analysis indicates that recombinants have probably also occurred naturally between TGEV and CCoV, generating a virus that is virulent for dogs (251).

RNA recombination may also generate viruses with deletions in their genomes. Many coronaviruses have strains that differ from each other by large deletions in or near a site within the N-terminal domain of the spike glycoprotein, S. Strains of MHV that differ in virulence and tissue tropism have deletions of different sizes in a hypervariable region in the N-terminal domain of S, as well as point mutations in S (194). The position of the deletions is correlated with the presence of a predicted stable stem-loop structure in the corresponding region of the S gene (195). Virulent enterotropic strains of porcine TGEV spontaneously give rise to strains that have deletions of more than 200 amino acids in a hypervariable region in the amino-terminal domain of the S glycoprotein (6). These strains are called *porcine respiratory coronavirus* (PRCoV) because they fail to cause enteric disease, replicate only in the respiratory tract, and are much less virulent than TGEV. Very large deletions in the genomes of coronaviruses generate subgenomic defective interfering (DI) RNAs that can be replicated in cells co-infected with a virus with an intact genome (53,80,132,144,155,177,226). Some DI RNAs that can be encapsidated in nucleocapsids and released from cells in enveloped virions were used to identify an about 69-nucleotide packaging signal on the positive-stranded genomic RNA (17,42,155,254).

Genetic studies of coronaviruses have been done using targeted RNA recombination (145,146,197). Cells inoculated with an infectious virus are transfected with cDNA of a minireplicon RNA derived from the same virus that has been altered by site-directed mutation. Within the cell, RNA recombination generates viral genomes in which the mutated gene or genes are substituted for the wild type. Selectable markers such as host range, temperature sensitivity, or monoclonal antibody selection are used to select the desired recombinants for studies of virus replication and pathogenesis. Targeted RNA

recombination has been used to map determinants of coronavirus pathogenesis, such as host range, tissue tropism, and virulence (116,126,129,145,181,197).

The high frequency of RNA recombination in coronavirus-infected cells may also account for the observation that the genomes of different coronaviruses contain a variety of genes that encode nonstructural proteins, and perhaps for the presence of the *HE* gene of the group 2 coronaviruses (162,242). The coronavirus *HE* genes have about 30% amino acid sequence similarity to the *HE* gene of influenza C virus (131). Possibly in a cell that was simultaneously infected with influenza C and a coronavirus ancestral to the group 2 coronaviruses, a nonhomologous RNA recombination event occurred between the coronavirus genome and the mRNA that encodes the HE glycoprotein of influenza C. Expression of an HE glycoprotein that binds to a sialic acid moiety on cell membranes would potentially help the coronavirus to bind to cells, increasing viral infectivity (244). Genes that are inserted into a coronavirus genome downstream of ORF1 are only expressed if a short conserved IS is located immediately upstream of the ORF so that a subgenomic mRNA with this ORF at its 5' end will be made and translated (121). Several of the nonstructural genes of coronaviruses are not required for virus replication in tissue culture or in animals, and their functions are not yet known (160). Because they are in helical nucleocapsids, coronavirus genomes may not be constrained in length like the genomes of viruses with icosahedral capsids. Therefore, if convenient methods are devised to insert foreign genes into coronavirus replicons as expression vectors, quite large insertions might be feasible.

HUMAN CORONAVIRUSES

Virus Isolation

Although coronaviruses of birds and many animal species can readily be isolated from infected tissues and serially propagated in continuous cell lines, isolation of human coronaviruses from infected individuals has only rarely been achieved (22,98,99,135,148,189,235,258). Hamre and Procknow isolated HCoV-229E from a patient with an upper respiratory infection by serial blind passages in secondary cultures of human embryonic kidney cells (68,69). Several other human coronaviruses were similarly isolated from colds at the same time. Some human coronaviruses that could not be isolated in cell lines were isolated from patients with colds by inoculation of human fetal tracheal or intestinal organ cultures (1,22,235). Most of the human coronaviruses that were grown in this way were never successfully adapted to serial passage in continuous cell lines and are no longer available for study. HCoV-OC43 and the antigenically related human coronavirus OC38 were isolated in human fetal tracheal organ culture, and then serially passaged by intracerebral inoculation into suckling mouse brain (148). Several human coronaviruses were isolated from adults with colds by a combination of growth in human diploid fibroblasts and inoculation of human volunteers (124). Human enterotropic coronaviruses were isolated from infants or children with gastroenteritis or necrotizing enterocolitis by inoculation of human fetal intestine or human rectal or kidney cell lines, and several of these viruses are closely related to BCoV (36,158,189,235,258). Differences in clinical symptoms were observed in human volunteers inoculated with several HCoV-229E–related viruses (188). Because very few isolates of human coronaviruses are available for study, little is known at the molecular level about the extent of genetic variability of the coronaviruses that commonly circulate in the human population (73,115). Strains of most avian and mammalian coronaviruses show extensive variation in their spike genes that can sometimes be correlated with differences in virulence and tissue tropism (94,102,118,119,187,194). Sequence analysis of HCoV genes in clinical specimens and in additional virus isolates is needed to reveal the extent of genetic diversity among human coronaviruses. This information will show how much antigenic drift occurs in human coronavirus S proteins over time and whether specific mutations in human coronavirus S glycoproteins are correlated with differences in pathogenesis.

Detection of Human Coronaviruses

Human coronavirus replication in organ cultures was initially detected by visualization of virions by electron microscopy (1). The genomes of the prototype HCoV-229E and HCoV-OC43 strains have been sequenced, and monoclonal antibodies to viral structural proteins are available (37,76,211). Therefore, it is now feasible to detect human coronavirus replication in human cell cultures and in some clinical specimens using RT-PCR and immunoassays (64,165,184,185,211). HCoV-OC43–related virions that express an HE glycoprotein on the viral envelope can also be detected by hemagglutination and acetyl esterase assays, and growth of this virus in cell cultures can be detected by hemadsorption, binding of erythrocytes to infected cells (100,258). Assays to detect antibodies directed against coronaviruses use infected cells in culture, extracts of infected cells, recombinant coronavirus proteins, or, for HE-expressing viruses, hemagglutination inhibition assays (64,138,139). HCoV-OC43 forms plaques in the HRT-18 line of human rectal tumor cells and grows in human rhabdomyosarcoma cells, human macrophages, primary human fetal neural cells, and human neural cell lines (3,37,204). HCoV-229E forms plaques in several human respiratory epithelial cell lines, such as MRC-5, WI-38, and L132, and replicates in human rhabdomyosarcoma cells, human type II pneumocytes, primary human neural cells, and human neural cell lines (4,16,204,222,237). HCoV-229E also replicates and causes cell fusion in human macrophages and macrophage cell lines (174). HCoV-OC43 can be passaged in suckling mouse brain (148). HCoV-229E has been serially passaged by intranasal inoculation into human volunteers (10,13,20,26,188,233).

PATHOGENESIS AND PATHOLOGY OF HUMAN CORONAVIRUSES

Entry into the Body

Natural infection of humans with human respiratory coronaviruses occurs through exposure to respiratory secretions. Intranasal inoculation of human volunteers with these viruses results in common colds (10,13,20,26,188,233). Coronaviruses of animals and birds are transmitted by the respiratory or enteric routes, depending on the virus. Because coronaviruses are enveloped, they are less stable in the environment and in clinical specimens than most nonenveloped viruses, such as rhinoviruses. In general, coronavirus infectivity can best be maintained at neutral or mildly acidic pH (122,221).

Receptors

Human aminopeptidase N (hAPN), a 150-kd zinc-binding glycoprotein with endopeptidase activity, is the receptor for HCoV-229E (256). Human APN is expressed on the apical surfaces of respiratory and intestinal epithelium as well as on myelocytic cells, on kidney tubular epithelium, and at synaptic junctions (5,169,208). Expression of recombinant hAPN in nonhuman cells makes them susceptible to HCoV-229E infection (256). In human cell cultures, treatment with some monoclonal antibodies directed against hAPN blocks virus infection, as do some inhibitors of hAPN activity, such as phloridzine (256). However, some mutants of hAPN that lack protease activity can still serve as receptors for HCoV-229E (44). Porcine coronaviruses TGEV and PRCoV, which cause enteric and respiratory symptoms, respectively, both use porcine APN (pAPN) as receptors (45), and many feline coronaviruses (FCoV) use feline APN (fAPN) as receptors (45,46,230). There are two biotypes of FCoV that may differ in the use of fAPN as a receptor (89,159).

Interestingly, HCoV-229E cannot use pAPN as a receptor, and TGEV cannot use hAPN (108), although the human and pig APN genes are 80% identical in nucleotide sequence. Inoculation of cells expressing recombinant chimeras of human, feline, or porcine APNs showed that 8 amino acids in the N-terminal region of hAPN near amino acid 291 are required for HCoV-229E receptor activity, whereas a different region of pAPN, amino acids 717 through 813, is a determinant of TGEV receptor activity (12,44,106,107 and 108). Perhaps these regions lie close together on the receptor glycoprotein in its native configuration. Feline APN can serve as a receptor not only for FCoVs but also for HCoV-229E, TGEV, and CCoV, which cause disease in humans, pigs, and dogs, respectively (230). These observations suggest that the progenitor of the group 1 coronaviruses may have been a feline-like coronavirus that used fAPN as its receptor, and that the S glycoproteins of HCoV-229E and the porcine coronaviruses TGEV and PRCoV may have later become adapted to recognize APNs of their respective hosts. Because feline cells and murine cells expressing recombinant fAPN can be infected with the group 1 coronaviruses of humans, pigs, cats, and dogs, it is possible that cats co-infected with feline and nonfeline coronaviruses serve as “mixing vessels” in which recombination between the coronavirus genomes could occur (83,230).

HCoV-OC43 does not use hAPN as its receptor (256). This virus binds to cells by means of the HE glycoprotein or the viral S glycoprotein, which can recognize 9- *O*-acetylated sialic acid moieties on the cell surface (115,244). Treatment of susceptible cells with neuraminidase makes the cells more resistant to HCoV-OC43 and BCoV infection but does not abrogate infection (114,206). It is not yet clear whether interaction of the S glycoprotein of HCoV-OC43 with another as yet unidentified receptor is also necessary for virus infectivity. Interestingly, like the HE glycoprotein of group 2 coronaviruses, the S glycoprotein of TGEV, a group 1 coronavirus, also recognizes *N*-acetyl 9-*O*-acetylated sialic acid moieties as well as *N*-glycolylneuraminic acid on plasma membranes of porcine cells (205,207). Point mutations in two amino acids between amino acids 145 and 155 in the amino-terminal domain of S of TGEV block virus binding to 9-*O*-acetylated sialic acid and block hemagglutination, but do not prevent virus entry through the pAPN receptor glycoprotein (113). These mutations lie within the hypervariable region of S that is spontaneously deleted in PRCoV, and PRCoV does not bind to this sialic acid moiety. The nonhemagglutinating mutant TGEV viruses have markedly reduced enteropathogenicity. Possibly, binding of virions to sialylated residues somehow increases viral stability and facilitates virus infection in the gastroenteric tract (113). The HE glycoproteins of HCoV-OC43, BCoV, and some MHV strains have acetyl esterase activity that can release sialic acid residues from 9- *O*-acetylated sialic acid moieties (115,244). This receptor-destroying activity of coronavirus HE glycoprotein, like that of the neuraminidase glycoprotein of influenza A virus, may aid in releasing virions adsorbed to 9- *O*-acetylated neuraminic acid moieties on the plasma membranes of infected cells and facilitate spread of virus to uninfected cells.

Replication in Polarized Cells

In polarized epithelial cells *in vitro,* HCoV-229E adsorbs preferentially to the apical plasma membrane where the hAPN receptor glycoprotein is concentrated (Blau and Holmes, in preparation). Coronaviruses are apparently released from infected cells by an exocytosis-like process that follows budding of virions into the pre-Golgi budding compartment (228). Virus release can be at the apical or basolateral membranes, or both, depending both on the virus and the host cell type (192,193). Coronavirus-infected epithelial cells die after several days and are shed from the epithelium. Apical release might efficiently spread virus among cells in the respiratory or enteric tracts, whereas basolateral release might facilitate systemic spread of the virus (192). *In vitro,* HCoV-229E virus can be released both from apical and basolateral surfaces of polarized epithelial cells expressing hAPN (Blau and Holmes, in preparation).

Cell and Tissue Tropism

Most coronaviruses first replicate in epithelial cells of the respiratory or enteric tracts. Some, including PRCoV and HCoV-229E, are believed to replicate principally in respiratory epithelial cells such as type II pneumocytes, where they produce virus and cause local respiratory symptoms (237). Other coronaviruses, including TGEV, BCoV, HEV, CCoV, FCoV, and MHV, infect respiratory epithelial cells and epithelial cells in the enteric tract. These viruses cause diarrhea that is particularly severe, sometimes fatal, in young animals (11,179). Inapparent enteric infection of adult animals maintains the virus in the population (56,255). It is not clear how these enveloped viruses retain their infectivity in the presence of the bile and proteolytic enzymes in the enteric tract. Perhaps because coronavirus envelope glycoproteins are extensively glycosylated, the virions are highly resistant to degradation by proteases in host tissues (33). In fact, when BCoV in group 2 is grown in certain cell lines, treatment of virions with trypsin is required to activate cell fusion activity and virus infectivity *in vitro* (220). On virions, the protease cleaves the spike glycoprotein only at one specific site near the middle of the molecule. Cleavage of newly synthesized S glycoproteins may be done by furin or other trypsin-like proteases in infected cells or by extracellular enzymes after release of virions (57). Some mutants of group 2 viruses that lack this specific protease site in S are delayed in virus entry and cell fusion and form small plaques (85), but other mutants that are not cleaved in S can cause extensive cell fusion (215). In contrast, the coronaviruses in the HCoV-229E serogroup do not have a protease cleavage site in their spike glycoproteins, and protease treatment of virions is not required for activation of virus infectivity. The virulence and tissue tropism of TGEV are determined by the spike glycoprotein, as demonstrated by targeted RNA recombination (197). Although the general structure of S proteins in different coronaviruses is conserved, there is relatively low sequence homology between *S* genes of the three

coronavirus groups.

Like coronaviruses of many other species, human coronaviruses have occasionally been implicated in enteric disease, particularly in newborns (36,63). The evidence for human enterotropic coronaviruses depends on visualization by electron microscopy of coronavirus-like, virus-like particles (VLP) in stools of infants with diarrhea, serologic studies of outbreaks of infant diarrhea, and infrequent isolation of coronaviruses from stools of infants and children with diarrhea (28,62,135,175,189). However, the role of coronaviruses in human diarrhea is not yet firmly established. The electron microscopic evidence is not compelling because other enveloped viruses such as human toroviruses (50,93,109,110 and 111) and even fragments of intestinal brush-border membranes resemble coronaviruses in negative-stained fecal preparations. In studies of nursery outbreaks of enterocolitis, VLPs were also observed in stools from infants without diarrhea (61). Because the immunologic responses of neonates are not strong, serologic evidence of infection in these patients is difficult to obtain. Rises in antibody titers against HCoV-OC43– related coronaviruses detected in sera of adults in contact with the sick infants could be due to respiratory infection, rather than enteric infection. There is significant serologic cross-reactivity among coronaviruses in group 2, so that rises in antibody titer do not unequivocally implicate a particular virus within this serogroup as a cause of disease (87,134,152). Isolation of putative human enterotropic coronaviruses has only rarely been achieved. Some coronavirus-like VLPs observed by electron microscopy in stools of patients with diarrhea could be passaged only once in organ cultures of human fetal enterocytes *in vitro* or in human embryo kidney cells (36). A putative human enterotropic coronavirus was isolated from stools of an infant with diarrhea by growth in human fetal intestinal organ cultures. The virus was neutralized by sera from adults in contact with the infant (189). The virus was adapted to growth in the J774 line of murine macrophages and the C6/36 mosquito cell line (130). Nucleotide sequence information on this viral isolate is not yet available. Several coronaviruses in group 2 that were propagated from human diarrheal stool samples in human or murine cell lines were found to be more closely related to bovine coronavirus strains than to the prototype HCoV-OC43 strain by reaction with monoclonal antibodies and by nucleic acid sequence analysis (158,240,258). Either these patients were infected with bovine coronavirus or viruses very similar to BCoV can circulate among humans and cause enteritis.

In addition to local infection of the respiratory or enteric tracts, several coronaviruses spread to other epithelial cell types where they cause symptoms of disease. For example, rat coronavirus strains cause respiratory infection or sialodacryoadenitis due to infection of the salivary and lacrimal glands (178). RCoV can also interfere with reproduction by infecting the female urogenital tract (238). Human coronaviruses have been associated with middle ear infections (184).

Several coronaviruses cause systemic infection in their natural hosts. HEV of swine causes enteric infection and is neurotropic (2). Infection spreads to nerves that innervate the stomach of infected piglets, prevents emptying of the stomach, and results in vomiting and wasting disease. Feline coronaviruses cause enteric disease in cats, but in some infected animals, virus mutants arise that cause a fatal systemic disease called *feline infectious peritonitis,* in which virus is detected in the plasma and ascitic fluids and infects many tissues (81,82). Strains of IBV that are nephrotropic cause death of chickens from acute kidney disease (95). Some strains of MHV, such as MHV-RI and MHV-Y, are strictly enterotropic, whereas other strains are respiratory tropic, neurotropic, or hepatotropic (11). Some MHV strains can spread to the liver, spleen, thymus, and brain, where they cause hepatitis, prolonged immunomodulation, acute encephalitis, and subacute or chronic focal demyelinating encephalomyelitis (39,85,123). Infection of macrophages is important in the spread of virus *in vivo* (7). SJL/J mice, which have many mutations in the virus-binding domain of the CEACAM1 glycoprotein isoforms that serve as MHV receptors in susceptible mice, are highly resistant to MHV infection, and macrophages isolated from these animals are also resistant to infection (19,51,105,219).

Coronaviruses Associated with Systemic Human Diseases

Human coronaviruses have occasionally been associated with pneumonia, particularly in immunosuppressed patients, or with neurologic symptoms and myocarditis (55,191). Of particular interest because of the induction of a multiple-sclerosis (MS)-like disease in rodents by murine coronavirus, is the possible role of human coronaviruses in MS. Coronavirus-like particles were observed by electron microscopy in a perivascular lesion in the brain of a patient with MS (224), and coronaviruses related to HCoV-OC43 and very similar to MHV were isolated from brain of two MS patients by passage in mice or murine cell lines (60,248). RT-PCR studies have identified sequences from the nucleocapsid genes of HCoV-229E and HCoV-OC43 in human brain tissue and cerebrospinal fluid (40,48,218). HCoV-229E RNA was detected in about 44% (40 of 90) of human brains tested, with about equal frequency in brain from MS patients and patients who died from other neurologic diseases or normal control subjects (218). HCoV-OC43 sequences were detected in about 23% (21 of 90) of the brains tested, with 36% incidence in brain from MS patients and 14% in that of controls. Both HCoV-229E and HCoV-OC43 can cause persistent infection in primary cultures of human fetal brain cells *in vitro* (3,4). The importance of human coronaviruses as a cause of diseases outside the respiratory tract remains to be determined.

Immune Responses

Studies in human volunteers were done to analyze the pathogenesis and immune responses of colds caused by human coronaviruses (13,20,21 and 22,25,26 and 27,233,234,236). Virus was inoculated intranasally, and patients were studied for development of symptoms, shedding of virus in respiratory secretions and changes in titer of antiviral antibody in serum and respiratory secretions. Fifty percent of volunteers inoculated with HCoV-229E developed colds. The incubation period was 2 to 4 days, and the mean duration of disease was 7 days (20). In this study, colds due to coronaviruses were generally classified as mild, although they were associated with significantly more nasal discharge than rhinovirus colds in volunteers. Other clinical features of coronavirus colds included headache (85%), sore throat (54%), malaise (46%), cough (31%), chills (31%), and fever between 37.3° and 38°C (23%). Eighty percent of the inoculated volunteers, including some who had clinically inapparent infection, excreted virus in respiratory secretions by day 3, and virus shedding had ceased by day 6 (20).

More than 80% of the adult volunteers had neutralizing antibodies against HCoV-229E in their sera before virus inoculation due to previous infections. However, most of the seropositive volunteers could be reinfected by the same virus. After virus inoculation, significant rises (fourfold or greater) in the level of virus-neutralizing antibody titers were

observed in 60% of volunteers (21), and rising serum antibody titers during convalescence could also be observed using hemagglutination inhibition assays and enzyme-linked immunoassays (64,138,139).

Treatment of volunteers with intranasal interferon-a before and after human coronavirus inoculation reduced the incidence of colds and the severity of symptoms as well as the amount of virus shed. Children who have more than six upper respiratory infections per year were found to have lower interferon responses to infection with coronaviruses and other respiratory viruses than children who have one or no upper respiratory infections per year (183).

Further studies on responses of natural killer (NK), B, and T cells to human coronavirus infection and on cytokine and chemokine expression during coronavirus infection are needed to elucidate the role of the immune response in symptoms, clearing of viral infection, and resistance to reinfection.

Virus Shedding

Coronaviruses are shed from the infected respiratory or enteric tracts. Pigs infected with PRCoV readily transmit the virus to other animals, likely by means of aerosols (125). Enterotropic coronaviruses of animals, such as TGEV and bovine, canine, and feline coronaviruses, can be spread by feces from infected animals (81). Enterotropic BCoV spreads efficiently among cattle and is also transmitted to sheep, deer, and other ungulates, perhaps by exposure to contaminated pastures of BCoV-infected cattle (140,229,231). Humans inoculated with respiratory coronaviruses shed virus from the respiratory secretions on day 3, but virus shedding was undetectable by day 6 after inoculation (20).

Virulence

There is no convenient small animal model for the study of the pathogenesis of respiratory infections caused by human coronaviruses. In human volunteers inoculated intranasally with a human coronavirus, the severity of symptoms varied markedly among infected individuals (20). Clearly, host factors play an important role in human coronavirus disease. In general, coronaviruses of animals cause the most severe diseases in primary infections of neonatal animals (11,179). Adult animals may develop clinically inapparent infections when exposed to the virus, shedding virus that is capable of causing severe disease in neonates (255). Genetic factors of the host can affect susceptibility to coronavirus diseases. At least three murine genes have been identified in inbred strains of mice that make animals more resistant to MHV infection than highly susceptible strains (7,19,49,51). These genes affect the virus yield, the innate immune response, and the functions of virus receptors. Cheetahs are highly vulnerable to fatal disease caused by feline coronaviruses, possibly because cheetahs have little polymorphism in their histocompatibility genes (54).

Variations in virulence may also be attributed to coronavirus strain differences. Strains of coronaviruses that vary significantly in virulence have been isolated from infected animals, and differences in the virus genomes have been identified. Strain differences in susceptibility to coronavirus virulence have been attributed to susceptibility of the S protein to cleavage by protease; expression of HE glycoprotein on the viral envelope and in infected cells; point mutations or deletions in the gene encoding the S glycoprotein; ability to use alternative cellular receptors; tissue tropism of virus strains; and the ability of some strains to cause persistent infections *in vivo.* In human coronaviruses, the extent of virus strain differences and the role of such differences in the virulence of human coronavirus disease are not known. As described previously, although coronavirus infection of humans occurs frequently throughout life, few strains of human coronaviruses can be propagated *in vitro,* and only a few of these strains have been used for infection of human volunteers (21,188). The extent of sequence variability among the human coronaviruses in groups 1 and 2 that circulate in humans remains to be determined.

Persistence

Some coronaviruses can cause persistent infection *in vitro* and *in vivo,* but the molecular mechanisms of persistence of these RNA viruses are not fully understood. Persistent infection *in vitro* has been studied for several coronaviruses, including MHV in murine fibroblasts and neural cell lines, and HCoV-229E and HCoV-OC43 in human respiratory cell lines or human neural cells (3,4,9,32,58,65,90). Detailed studies on the mechanism of persistence of the human coronaviruses and on possible changes in virus during persistence have not yet been done. Persistent infection of MHV in mouse fibroblast cell lines generates carrier cultures in which 10% to 30% of cells express viral antigens, expression of the virus receptor CEACAM1a is markedly reduced, and small plaque viruses with mutations in the S glycoprotein replace the wild-type virus and continue to be produced for months or years (9,35,65,86,198,203). MHV viruses isolated from persistently infected cells *in vitro* have point mutations in the S glycoprotein, including some that eliminate the protease cleavage site between S1 and S2 in the S glycoprotein. These mutations are associated with reduced infectivity of the virus, entry of the virus at endosomes rather than at the plasma membrane, loss of cell-fusing activity, reduced virus yield, and altered virus tropism *in vivo* (58,168). Some MHV mutants from persistently infected murine cells exhibit extended host range (35,203). Mutations in other viral genes in addition to S may also be associated with virus persistence (34).

Persistent coronavirus infection *in vivo* may result from failure of the immune response to clear virus infection, insufficient neutralizing antibody in the respiratory or enteric fluids, virus mutation to escape the immune response, absence of cytotoxic T-lymphocyte (CTL) epitopes on viral proteins that could promote clearance of infected cells, sequestration of virus in an immunologically privileged site, or virus-induced immunomodulation, among other factors. TGEV was persistently shed for 18 months in the feces of a sow with inapparent enteric infection, and the virus could be transmitted to susceptible 3-day-old piglets that developed acute gastroenteritis (255). As is true for enteric infection with several other types of viruses, the presence of neutralizing antibodies in the serum of the sow did not necessarily correlate with the ability to prevent reinfection or to clear virus infection from the enteric tract. Feline coronavirus can be shed in the feces for up to 7 months (82). An epitope in the S protein that is recognized by CTLs in C57BL/6 mice is deleted in MHV mutants isolated from persistently infected animals (180). The neurotropic MHV-JHM strain can cause prolonged persistent infection in mouse brain and spinal cord. Virus from brain from persistently infected animals had point mutations and a variety of deletions in the hypervariable region of S (194). Persistence of MHV in mice depends on many

factors, including the strain, age, and immune status of the mouse, and the strain, inoculum dose, and route of inoculation of the virus (11,223). Acute transient inapparent infection of adult mice with MHV can alter the cytokine profile of the animals acutely and for months after clearance of infectious virus (39). The virus-induced alterations in cytokine expression may alter the responses of the animal to other infectious agents. Persistence of human coronavirus RNA in a substantial proportion of human brain tissue or spinal fluid was detected by RT-PCR, although it is not yet certain whether the entire genome is present in the brain (40,48,163,218). Infectious virus has not been isolated from these specimens. The route by which human coronaviruses reach the brain and the clinical significance, if any, of the persistence of coronavirus RNA in human brain have not been determined.

EPIDEMIOLOGY

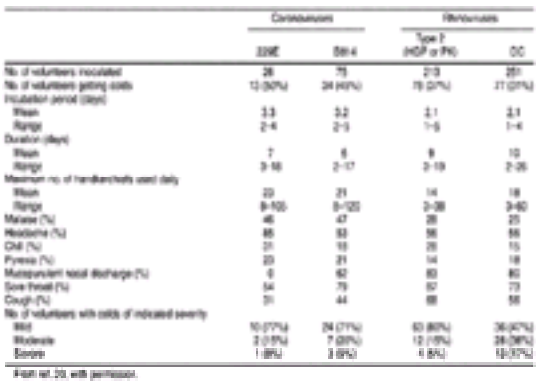
Neutralizing antibodies to coronaviruses related to HCoV-229E or HCoV-OC43 are detectable in the sera of pediatric and adult populations (20,21,101,153,185). Studies with human volunteers show that despite the presence of anticoronavirus antibody in the serum, reinfection of the respiratory tract with clinical symptoms can occur (234). Individuals can be reinfected with the same strain of virus within 1 year after the first inoculation, although there is considerable variation in the degree of illness associated with the two inoculations (20,74). The frequent susceptibility of adults to reinfection with a closely related coronavirus suggests that a variety of antigenically distinct human coronaviruses circulates in the population (188).

Natural outbreaks of colds caused by coronaviruses occur predominantly during the winter months, although in children, two peaks in late autumn to early winter and early summer were detected (91). In general, outbreaks of HCoV-229E in a community occur at 2- to 4-year intervals, and outbreaks of HCoV-OC43 occur in alternate years when the incidence of HCoV-229E infection is low (157). Prospective studies of respiratory infections in families, schools, and military populations were done in the 1970s and early 1980s using coronavirus isolation in tissue culture or organ culture, as well as electron microscopy, enzyme-linked immunosorbent assay (ELISA), and detection of rises in complement-fixing or neutralizing antibodies in the serum in association with clinical symptoms (71,91,99,124,150,153,156). These studies indicated that the frequency of colds due to coronaviruses varied in different populations from 5% to 30% depending on the time of year, the population studied, and the methods used in the study. The recent development of RT-PCR assays for human coronavirus infections and the availability of monospecific and monoclonal antibodies to detect viral antigens in clinical samples will allow more accurate estimates of the incidence and epidemiology of colds in humans (40,73,167,184,185,211,253).

It is generally believed that there are no animal reservoirs for human coronaviruses and that the viruses spread only among humans. However, S glycoproteins of human and animal coronaviruses within each serogroup are antigenically related, and it is not yet certain whether some coronaviruses circulate among humans and one or more mammalian species (88,152,243,258). The extent of genetic variation among human coronaviruses from different outbreaks is unknown. There are biologic differences among human coronaviruses isolated from different outbreaks of disease (147,188). Nucleotide sequences of the genes encoding the S glycoproteins of only a few HCoV-299E–related viruses and HCoV-OC43 virus have been determined to date (73,76,115,161). The S protein of the prototype HCoV-229E strain isolated by Hamre in the United States in 1966 differed from the LP strain isolated in the United Kingdom in the 1970s by only one amino acid, whereas the sequence of the *S* gene from respiratory washings of an adult with upper respiratory disease from Ghana in the 1990s differed from the prototype HCoV-229E *S* gene by only 47 of 1012 amino acids (73). There is considerably more amino acid sequence variation in *S* genes of group 2 coronaviruses of humans and animals, particularly in the S1 region. The deduced amino acid sequence of the S protein of the HCoV-OC43–related hemagglutinating coronavirus isolated from a child with diarrhea was 99% identical to a BCoV *S* gene sequence (258). In addition, other characteristics of the genome of the human virus resembled BCoV more than HCoV-OC43, including the presence of several ORFs downstream of *S* that are found in the BCoV genome, but absent in the genome of the prototype HCoV-OC43 (258). The spike glycoproteins of TGEV, CCoV, and FCoV are more closely related to each other than to that of HCV-229E (252). Cats and dogs must at least occasionally have been co-infected with feline and canine coronaviruses to permit the recombination in the spike gene that generated the FCoV biotype II viruses (83). To understand how human coronaviruses spread, new epidemiologic studies are needed that employ sensitive and strain-specific monoclonal antibodies, RT-PCR analysis of viral genes, and improved methods for isolation of infectious viruses from clinical specimens.

CLINICAL FEATURES OF HUMAN CORONAVIRUS INFECTION

HCoVs in both the 229E- and OC43-related serogroups cause upper respiratory signs and symptoms in adults and children that vary in frequency and severity in different outbreaks. The symptoms of common colds in volunteers inoculated with human coronaviruses and human rhinoviruses are similar (20,22,188,234,236) (Table 1). Volunteer studies showed that in adults, the mean incubation period of colds caused by coronaviruses is 3.3 days, with a range of 2 to 4 days (20). Many of the volunteers inoculated intranasally with HCoV-229E got febrile colds. Other symptoms included rhinorrhea, headache, malaise, chills, sore throat, and cough (20). Symptoms lasted for a mean of 7 days, with a range of 3 to 18 days. Symptoms varied considerably among different patients inoculated with the same coronavirus, perhaps because of differences in previous exposure to related viruses (20,188). About half of volunteers inoculated intranasally with HCoV-229E developed symptoms of the common cold, and 30% of volunteers had asymptomatic virus infection with shedding of virus from the upper respiratory tract (20). Shedding of infectious virus from apparently healthy individuals and from people with cold symptoms is an important factor in the epidemiology of coronavirus infection.



	Coronaviruses		Rhinoviruses	
	229E	OC43	Type 2 (HGP or PK)	OC
No. of volunteers inoculated	26	75	213	251
No. of volunteers getting colds	13 (50%)	34 (45%)	78 (37%)	77 (31%)
Incubation period (days)				
Mean	3.3	3.2	2.1	2.1
Range	2-4	2-5	1-5	1-4
Duration (days)				
Mean	7	6	8	10
Range	3-10	2-17	2-19	2-26
Maximum no. of handkerchiefs used daily				
Mean	23	21	14	18
Range	8-103	0-120	2-38	2-60
Malaise (%)	46	47	39	37
Headache (%)	85	93	90	88
Chill (%)	31	16	25	15
Pyrexia (%)	23	21	14	18
Mucopurulent nasal discharge (%)	0	62	83	80
Sore throat (%)	54	79	87	73
Cough (%)	31	44	68	58
No. of volunteers with colds of indicated severity				
Mild	10 (77%)	24 (71%)	63 (80%)	36 (47%)
Moderate	2 (15%)	7 (20%)	12 (15%)	28 (36%)
Severe	1 (8%)	3 (9%)	4 (5%)	13 (17%)

From ref. 20, with permission.

TABLE 1. *Clinical features of colds produced by inoculating four viruses*

When inoculated with the same virus a second time, an individual may have either a similar syndrome or a milder or more severe one (188). Patients who had symptomatic coronavirus infection showed rises in neutralizing and complement fixation antibody titers in the serum after inoculation that waned after months (26,112). The severity of colds caused by coronaviruses differs in different outbreaks, but most people with coronavirus colds develop only mild to moderate symptoms (20). There are no pathognomonic features of coronavirus-induced upper respiratory infections, so it is impossible to establish the etiology of a virus cold based on clinical findings alone (22).

Lower respiratory tract infections with coronaviruses were observed in some pediatric populations, but not in other studies (149,153,154). Asthma may be triggered by infection with coronaviruses as well as other respiratory viruses (128). In military recruits, coronaviruses accounted for about 30% of pneumonia cases (250).

DIAGNOSIS

Most human coronavirus infections are not diagnosed because the upper respiratory disease is relatively mild and self-limited, and no drugs are available to provide more than symptomatic relief. Only a small number of prospective studies on the etiology of human viral upper respiratory infections included sensitive assays to detect coronavirus infection (124,147,157,165,184). Although early studies relied on detection of coronavirus infection of animals and humans by immunoelectron microscopy, isolation of infectious virus in cell culture or organ culture, and rises in antibody titers detected by complement fixing or neutralizing antibodies, more recent studies have used much more sensitive and specific nucleic acid hybridization, RT-PCR, nested RT-PCR, quantitative PCR, and *in situ* hybridization assays to identify the presence of several viral genes and mRNAs in clinical specimens (59,66,164,165,166 and 167,172,210,217). Sensitive assays have been developed to detect coronavirus RNA in the feces of infected animals (70,81,82,92,172,186,232). Coronavirus infection can also be detected by immunofluorescence of cells shed into respiratory secretions, and serologic responses to coronavirus infection can be detected in human sera using recombinant viral proteins (154,185). Both group-specific antibodies and strain-specific monoclonal antibodies are being used for immunofluorescence and ELISA studies to detect coronavirus antigens (137,173,190). Although these sensitive and specific diagnostic tests for human coronavirus infection are not yet commercially available, assays are used for diagnosis of animal coronavirus infection.

Upper respiratory tract infections of humans caused by coronaviruses are associated with a variety of clinical signs that differ from one outbreak to another and from one individual to another (29,156,157,188). Clinical symptoms depend in part on previous infection with the same strain of human coronavirus (188). Therefore, colds caused by coronaviruses cannot be distinguished by clinical criteria alone from colds caused by rhinoviruses and other respiratory viruses (124). Previous studies on the etiology of colds have determined that about 15% to 30% of colds are caused by coronaviruses in adult and children (91,133,137,153). Recently, prospective studies to identify viral causes of upper respiratory infections in a pediatric population using RT-PCR have found that HCoV-229E and HCoV-OC43 may cause about 15% of colds (184). In addition, HCoV-OC43 was detected by RT-PCR in middle ear fluids of children with middle ear infections associated with colds (182,184).

Viral infections of the upper respiratory tract can precipitate asthma attacks (216). Coronaviruses are among multiple viruses that can precipitate wheezing or asthma attacks in susceptible individuals (128,151). In a prospective study of children younger than 6 years of age who had colds and HCoV antigens in the upper respiratory tract demonstrated by ELISA, 30% had wheezy bronchitis (91). It is likely that virus-induced inflammation and cytokine production play important roles in virus-induced asthma. Further studies are needed to understand the pathogenesis of increased reactivity of airways during human coronavirus infection.

PREVENTION AND CONTROL

The three principal reasons for developing sensitive assays for rapid diagnosis of human coronavirus infection are to elucidate the epidemiology and pathogenesis of coronavirus infections in humans, explore the possible significance of virus strain variation in virulence, and use only appropriate drug therapy for coronavirus infections when drugs to treat or prevent colds become available. Several strategies for treating human rhinovirus infection are being investigated, including specific inhibitors of the essential viral proteases that process the virus polyprotein; new inhibitors of the cascade of cytokines and inflammation that cause many of the symptoms of colds; molecules that block rhinovirus-receptor interactions, such as soluble receptors for rhinoviruses or antibodies to a rhinovirus receptor; and molecules that intercalate into the pocket beneath the floor of the canyon in the rhinovirus capsid, preventing receptor binding or blocking essential receptor-induced conformational changes in the virus capsid (41,72). Most of the potential drugs against rhinovirus colds would be unlikely to be effective for coronavirus colds because of differences in the

structures, receptors, and replication strategies of rhinoviruses and coronaviruses.

At present, there is no drug therapy for human coronavirus infections other than over-the-counter drugs that provide symptomatic relief. Potential drugs against coronaviruses include inhibitors of viral RNA-dependent RNA polymerase; the virus-encoded proteases essential for processing of the virus-encoded RNA-dependent RNA polymerase; coronavirus budding or release from infected cells, which may involve hemagglutinin-esterase; virus binding to specific cell surface receptors, such as hAPN for HCoV-229E; and the receptor-induced conformational changes in the virus spike glycoprotein that are required for virus entry.

No vaccines are available to prevent human coronavirus infection, but vaccines against common veterinary coronaviruses, such as IBV and CCoV, are routinely used to prevent serious disease in young animals, and various strategies to improve immunizations for veterinary coronaviruses are being tested (171,196). PRCoV acts as a naturally attenuated vaccine against TGEV. Infection of the respiratory tract of pigs with PRCoV causes inapparent infection or mild disease and may help to protect the animals from serious enteric disease on subsequent exposure to virulent TGEV (125).

There are several potential problems associated with development and use of live attenuated coronavirus vaccines. First, natural infection with virulent coronavirus strains does not prevent repeated episodes of disease caused by the same strain of virus. Therefore, new ways to stimulate protective immunity must be devised to create vaccines that will be more effective than natural infection. Second, for human coronaviruses, the number of different viral epitopes that must be included in a single vaccine is not known because the extent of the genetic heterogeneity of virus strains in circulation is not known. Third, because of the high frequency of RNA recombination in cells that are co-infected with two coronaviruses, recombination between live attenuated vaccine virus and virulent wild-type virus can occur in infected individuals. Variant strains of IBV that arose by recombination of vaccine and wild-type strains have caused outbreaks of disease in chicken flocks (117,245). Finally, an important problem that has prevented development of a vaccine to prevent feline infectious peritonitis (FIP) is the recognition of immune potentiation of feline coronavirus disease (179). FIP occurs most commonly in seropositive animals that have previously been infected with an enterotropic feline coronavirus (38,179,247). Recent studies suggest that FIP results from mutation of enterotropic FCoV strains that lead to systemic spread and virus persistence in some cats (241). The mechanism of immune enhancement of FCoV disease is not yet understood.

PERSPECTIVES

Coronaviruses in two distinct serogroups cause repeated episodes of upper respiratory infection in humans. Although the disease is generally mild to moderate, self limited, and patients recover within a week, common colds are an important economic problem because of the high incidence of disease with consequent loss of productivity. In addition to causing colds, human coronaviruses have been implicated in middle ear infections, exacerbations of asthma, pneumonia, diarrhea in infants, and myocarditis. Very few isolates of coronavirus can be propagated in cell culture. The extent of genetic variation among human coronaviruses that may be associated with differences in virulence and protective antigens has not yet been determined. Although considerable progress has been made in developing diagnostic tests to detect human coronaviruses, and in understanding the epidemiology of human coronaviruses, significant efforts are needed to develop small animals that provide models for diseases caused by human coronaviruses in which to test future drugs that may block coronavirus infection. Vaccines to prevent human coronavirus diseases do not appear feasible at this time. Potential targets for development of new drugs to treat coronavirus infections are being identified by studies on viral genes, gene products and replication strategy. In order to use such novel drugs wisely, sensitive and rapid techniques are needed to distinguish coronavirus infections from infections caused by the many other viruses that cause colds such as rhinoviruses. RT-PCR and immunoassays specific for coronavirus RNA and proteins provide powerful and rapid new diagnostic tools for study of the pathogenesis and epidemiology of human coronaviruses. Identification of the viral cause of an upper respiratory infection prior to selecting the appropriate antiviral therapy is likely to be expensive. Therefore, therapy for the common cold may depend upon use of combinations of several drugs, each of which inhibits one type of common cold virus.

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CHAPTER 37 Arteriviruses

Eric J. Snijder and Janneke J. M. Meulenberg

The family of *Arteriviridae,* which was established in 1996, comprises four enveloped, positive-stranded RNA viruses: equine arteritis virus (EAV), lactate dehydrogenase–elevating virus (LDV), porcine reproductive and respiratory syndrome virus (PRRSV), and simian hemorrhagic fever virus (SHFV). The unification of these previously unclassified viruses was the direct result of the sequence analysis of their genomes during the 1990s, which significantly advanced our understanding of the molecular properties and replication cycle of arteriviruses. In combination with a number of distinct biologic properties, including the unique properties of their structural proteins, their virion morphology, and the ability to establish persistent infections, this provided the basis for the classification of arteriviruses as an independent family. The same molecular studies also revealed an intriguing relationship with the family *Coronaviridae* (see Chapter 35) at the level of genome organization and expression. The replicase genes of the two virus groups are assumed to share common ancestry, despite striking differences in virion structure, genome size, and biology. The evolutionary link between arteriviruses and coronaviruses is most convincingly reflected in their common genome expression strategy, which includes the generation of a nested set of subgenomic (sg) mRNAs. It is the latter property that formed the basis for the name Nidovirales (*L. nidus,* meaning nest) that was given to the novel order comprising the arterivirus and coronavirus families in 1996.

HISTORY OF ARTERIVIRUSES AND ARTERIVIRUS DISEASE

Three of the four arteriviruses (EAV, LDV, and SHFV) were first isolated and characterized 30 to 40 years ago (30,84,101). The porcine arterivirus PRRSV emerged in the late 1980s to early 1990s (18,116), causing vast epidemics of a previously unknown reproductive and respiratory swine disease in both North America and Europe. Remarkably, the subsequent molecular characterization of PRRSV strains from both continents (1,66,74,76) revealed extremely large genetic differences, especially considering the short time period (about 4 years) between the appearance of the disease in North America and Europe. These observations strongly suggest that the two PRRSV genotypes evolved separately from non–disease-causing viruses and are only distantly related to a common ancestor.

The consequences of arterivirus infection can range from an asymptomatic persistent or acute infection to abortion or lethal hemorrhagic fever. Macrophages appear to be the primary target cell for all arteriviruses (82,83). The natural host range of arteriviruses is restricted to horses and donkeys (EAV), pigs (PRRSV), mice (LDV), and several genera of African and Asian monkeys (SHFV). Recently, chickens and Mallard ducks, which were exposed to PRRSV in drinking water, were reported to shed the virus in their feces, suggesting that they may be susceptible to PRRSV infection (120).

In nature, EAV and PRRSV are transmitted primarily by the respiratory route (64,103,115). The severity of disease can vary greatly and is influenced by the strain of the virus and the condition and age of the infected animal. EAV and PRRSV infections can be subclinical but can also lead to a variety of symptoms, including the necrosis of small muscular arteries from which the name of the family prototype EAV was derived. Both viruses can cause abortion in pregnant animals and may persist in the semen of infected males, which makes sexual transmission an important secondary route of infection (100,103).

The name of the mouse arterivirus LDV is derived from the increase in the level of lactate dehydrogenase (LDH) caused by the virus in the blood of infected mice (84). LDV has been used extensively as an *in vivo* research model (11,83). The virus infects a renewable subpopulation of macrophages and is able to escape immune surveillance. Usually, this persistent infection is largely asymptomatic, but in certain inbred mouse strains, neurovirulent LDV variants can cause a fatal age-dependent poliomyelitis (19).

SHFV was isolated from outbreaks of fatal hemorrhagic fever in macaque colonies (101). The virus appears to be

endemic among several species of African monkeys (*Erythrocebus patas, Ceropithecus aethiops, Papio anuibus,* and *Papio cyanocephalus*), in which it causes asymptomatic acute or persistent infections depending on the virus strain (44,59). The epizootics and disease in captive macaques, during which mortality approached 100%, are probably explained by inadvertent SHFV transmission by humans from African monkeys to macaques.

On the basis of morphologic criteria (size, an isometric core, and the presence of an envelope), arteriviruses were regarded as unclassified togaviruses or flaviviruses or as members of a “floating genus” for many years. An important milestone was the sequence analysis of the EAV genome in 1991 (22), which revealed the unexpected relationship with members of the coronavirus family. Since then, the availability of cloned arterivirus sequences has rapidly increased our understanding of the properties of the viral RNAs and proteins. The recently developed infectious cDNA clones of EAV (26,105) and PRRSV (65) will allow an even more sophisticated analysis of the replication cycle and biologic properties of arteriviruses.

VIRION STRUCTURE AND PROPERTIES

Arteriviruses are spherical, are 40 to 60 nm in diameter, and possess a relatively smooth surface that lacks large projections (Fig. 1; reviewed in 10,82, and 91). They consist of an isometric, possibly icosahedral, nucleocapsid of 25 to 35 nm, which is surrounded by an envelope carrying cuplike structures. The buoyant density of arteriviruses is 1.13 to 1.17 g/cm^3 in sucrose, and their sedimentation coefficient ranges from 214S to 230S. The virions are highly unstable in solutions containing low concentrations of nonionic detergents or at a pH other than 6 to 7.5. Furthermore, they lose their infectivity quickly when stored at temperatures of 4°C or higher.

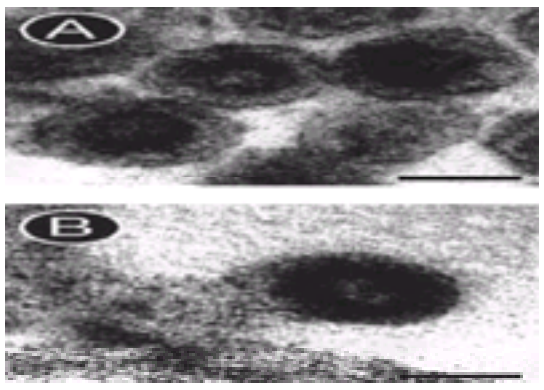

FIG. 1. Electron micrographs of arterivirus particles. **A:** Negative staining of extracellular porcine reproductive and respiratory syndrome virus (PRRSV) particles (*bar* is 27 nm). **B:** Equine arteritis virus (EAV) particle budding from smooth intracellular membranes (*bar* is 25 nm). (From ref. 91, with permission.)

The arterivirus nucleocapsid is composed of the 12.7- to 15.7-kb RNA genome and the nucleocapsid protein N (Fig. 2). The lipid bilayer that surrounds the nucleocapsid contains five or six envelope proteins, an unusually large number compared with other positive-stranded RNA viruses. Two of these proteins are major structural components: the nonglycosylated integral membrane protein M and the “major” glycoprotein (25,35,68). The nomenclature of arterivirus envelope glycoproteins has not yet been standardized. For example, the major glycoproteins have been designated G $_L$, VP-3P, GP$_5$, and p54 in the case of EAV, LDV, PRRSV, and SHFV, respectively (Table 1). For convenience and clarity, in this chapter we refer to the glycoproteins as GP $_x$, where x indicates the number of the open reading frame (ORF) in the genome from which the protein is expressed (Fig. 3; see Table 1 and later discussion).

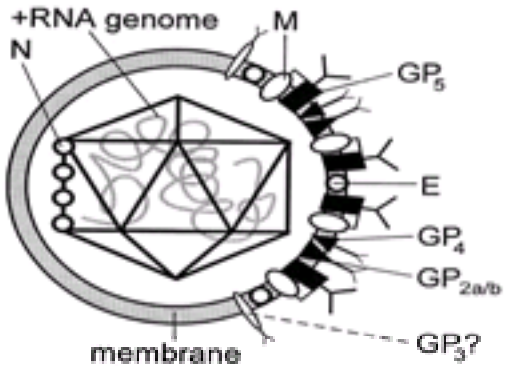

FIG. 2. Schematic representation of an arterivirus particle. The location of the structural proteins GP $_{2a/b}$ to GP$_5$, E, M, and N is shown (see also Table 1 and Fig. 3). Note that the glycoprotein nomenclature of simian hemorrhagic fever virus (SHFV) deviates from that of the others, that not all proteins depicted here have been identified in all four arterivirus particles, and that conflicting data on the presence of GP $_3$ in virions have been reported for porcine reproductive and respiratory syndrome virus (PRRSV).

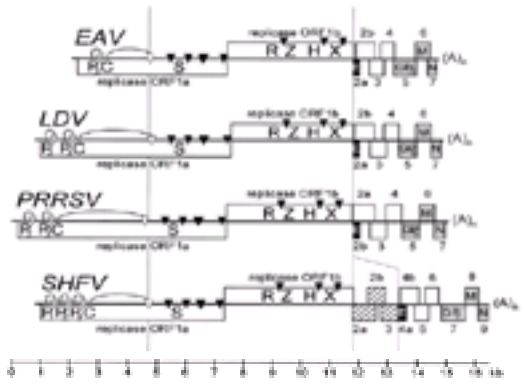

FIG. 3. Overview of arterivirus genome organizations. The family prototype equine arteritis virus (EAV) is shown at the *top.* The replicase open reading frames (ORFs) 1a and 1b are followed by the gene encoding the E protein (*black*), three genes (ORFs 2a and 2b to 4) encoding minor glycoproteins, and the genes for the major structural proteins GP_5 (GP_7 in simian hemorrhagic fever virus [SHFV]), M, and N (*gray*). The 3'-proximal region of the SHFV genome contains a large insertion (*hatched area*) containing three ORFs that may encode additional glycoproteins. In the replicase ORFs, the position of known (EAV) or predicted (lactate dehydrogenase–elevating virus [LDV], porcine reproductive and respiratory syndrome virus [PRRSV], and SHFV) polyprotein cleavage sites are depicted. *Black arrowheads* represent sites cleaved by the nsp4 serine protease (*S*). The papain-like protease domains (*P1* to *P3*) in the nsp1 region, the nsp2 cysteine protease (*C*), and their (predicted) cleavage sites are also shown. In the ORF 1b polyprotein the four most conserved domains of nidovirus replicases are depicted: the RNA-dependent RNA polymerase (*R*), putative zinc finger (*Z*), RNA helicase (*H*), and nidovirus-specific domain (*X*).

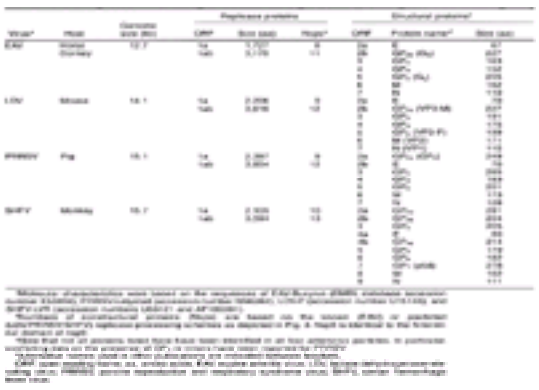

TABLE 1. *Molecular properties of arteriviruses*

The glycoproteins $GP_{2a/b}$ (PRRSV, EAV, and LDV) and GP_4 (EAV and PRRSV) were experimentally identified as minor virion components (29,35,67,92,111). A fourth glycoprotein, GP_3, was suggested to be a minor structural glycoprotein in a European PRRSV isolate (111) but a nonstructural protein in a Canadian isolate (63). A novel nonglycosylated structural protein was recently detected in EAV particles and was provisionally designated E (for envelope) protein (92).

GENOME STRUCTURE AND ORGANIZATION

The arterivirus genome is a positive-stranded, 3' polyadenylated RNA molecule. At the 5' end, the SHFV genome was reported to contain a type I cap structure (86); in addition, cap analog was found to be an essential component of *in vitro* transcription reactions to generate infectious PRRSV and EAV RNA from full-length cDNA templates (65); L.C. van Dinten and E.J. Snijder, unpublished data). Full-length genomic sequences (see Table 1) have been obtained for EAV (22), European and North American isolates of PRRSV (1,66,74,76), two LDV strains (40,81), and recently also for SHFV (85; T. R. Maines and colleagues submitted for publication).

The arterivirus replicase gene, which consists of the large ORFs 1a and 1b, roughly occupies the 5'-terminal three fourths of the polycistronic arterivirus genome (see Fig. 3). In contrast to the more conserved ORF 1b region, the size of ORF 1a is variable (encoding between 1,727 and 2,502 amino acids), a property that largely explains the genome size differences among arteriviruses (see Table 1). The significance of the small potential ORF found in the leader sequence upstream of the replicase gene in EAV, PRRSV, and SHFV is unclear (52,91). However, inactivation of the EAV leader ORF was recently shown not to affect virus replication in cell culture (73).

The region downstream of the replicase gene contains a set of 7 to 10 relatively small genes, most of which have both 5'- and 3'-terminal sequences that overlap with neighboring genes (see Fig. 3 and Table 1). These genes encode mostly (or exclusively) structural proteins, they are translated from sg mRNAs (see later), and their organization is generally well conserved in the arterivirus genome. An exception is a region downstream of the SHFV replicase gene, which contains three additional ORFs, comprising about 1.6 kb (90). Sequence comparisons suggested that these ORFs may have arisen from the duplication of ORFs 2 to 4 by an RNA recombination event (41).

THE ARTERIVIRUS REPLICATION CYCLE

Attachment and Entry

Upon transfection of genomic RNA, arteriviruses can replicate in several cell lines that are not susceptible to infection with virus particles (49,65). Furthermore, cell lines that are resistant to LDV can be infected by murine coronavirus and LDV pseudovirions (32). Certain cell lines have been reported to become LDV susceptible when they are first infected with murine leukemia virus (50), although these results have not been reproduced by others (82). These data suggest that cell tropism is determined by the presence of an as yet unidentified receptor on the cell surface. In the case of PRRSV, infection of MARC-145 cells could be prevented by prior incubation of virus or cells with heparin and also by treatment of the cells with heparinase (51). Therefore, it was proposed that a heparin-like molecule on the surface of MARC-145 cells may serve as an attachment molecule for PRRSV. More recently, a 210-kd membrane protein from porcine alveolar macrophages was identified as a putative receptor for PRRSV (31). Two monoclonal antibodies directed against this as yet uncharacterized protein were found to block attachment of the virus to these cells.

The entry pathway of PRRSV was investigated using drugs that affect the pH of intracellular membrane compartments (55). It was shown that a low pH in these compartments is required for virus entry, which most likely occurs through the standard endocytotic route. Entry was also blocked by cytochalasin D (75), a compound that inhibits receptor-mediated endocytosis. Electron microscopy revealed PRRSV particles contained in relatively small vesicles, which appeared to be clathrin coated (55). Similar observations were made during the initial stages of LDV infection in macrophages (54).

Genome Replication

The arterivirus replication cycle (Fig. 4)occurs entirely in the cytoplasm of the infected cell. A genome-length negative strand is generated that serves as the template for genome replication. In addition, nested sets of sg negative strands and sg mRNAs are generated, which are each other's complement. The transcripts in each set are both 5'- and 3'-coterminal with each other and with the corresponding genome-length RNA (Fig. 5 and see later discussion).

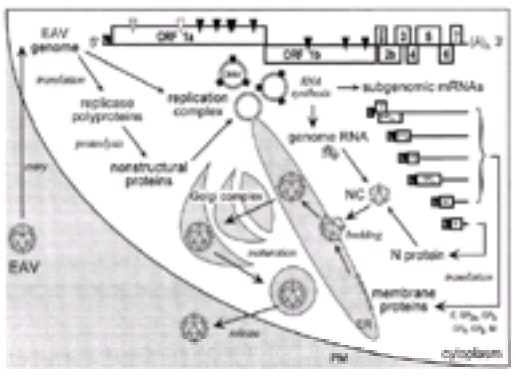

FIG. 4. Overview of the life cycle of the arterivirus prototype equine arteritis virus (EAV). The EAV genome organization, including replicase cleavage sites (*arrowheads;* see Fig. 3), is shown at the top of the figure. The nested set of subgenomic (sg) mRNAs with their common leader sequence (*L*) is depicted at the right. ER, endoplasmic reticulum; PM, plasma membrane; DMV, double membrane vesicle; NC, nucleocapsid.

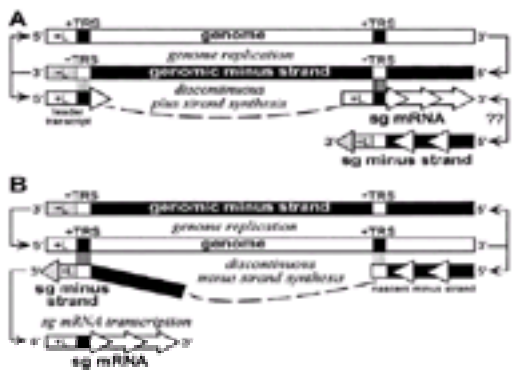

FIG. 5. Transcription models illustrating the discontinuous step during arterivirus mRNA synthesis, which involves TRS base pairing (109) and has been postulated to occur either during positive-stranded synthesis (model A) or during negative-stranded synthesis (model B). See also references 9,87,91, and 109 as well as Chapter 35 and references therein. Depicted are the genomic RNA and one of the subgenomic (sg) RNAs. Positive and negative strands are shown as *black bars* and *white bars,* respectively. The position of the positive transcription-regulating sequences (TRSes; *black boxes*), negative TRSes (*white boxes*), and leader (*+L*) and antileader (-L) sequences is indicated. **A:** Leader-primed transcription model. Discontinuous transcription was first proposed to occur at the level of positive-stranded RNA synthesis, through positive leader priming on complementary (negative) body TRS sequences in the full-length negative-stranded template. Transcription of sg negative strands from mRNA templates may occur, but in this model, such negative transcripts are assumed to be nonfunctional (“dead-end”) products. **B:** Discontinuous negative-stranded extension model. Negative-stranded synthesis is either continuous, yielding the full-length negative strand for genome replication, or discontinuous, yielding an sg negative strand that subsequently serves as the template for sg mRNA synthesis. The latter model is supported by data recently obtained from mutagenesis studies using an EAV full-length cDNA clone (109) and is also consistent with most of the observations made for coronaviruses using biochemical and genetic methods.

Little is known about the RNA signals involved in arterivirus genome replication. The coding regions of the genomes are flanked by 5'- and 3'-untranslated regions of 156 to 221 and 59 to 117 nucleotides, respectively. However, natural and synthetic defective interfering RNAs, which were recently generated for EAV, invariably required at least 300 nucleotides from both genome termini for efficient replication. This suggests that replication signals partially overlap with coding sequences (72). The interaction of these RNA signals with viral or host proteins is a largely unexplored field. However, four host proteins from MA-104 cells were reported to bind to *in vitro* generated transcripts representing the 3' end of the SHFV genomic negative strand (48). The same four proteins, which remain to be identified, apparently interact with transcripts representing the corresponding region of the LDV and EAV negative strands, suggesting the involvement of a common set of host factors in the initiation of arterivirus positive-stranded RNA synthesis.

Subgenomic mRNA Transcription

One of the hallmarks of the replication cycle of arteriviruses (and other Nidovirales) is the generation of a nested set of sg mRNAs (Fig. 5) from which the (mostly structural) genes in the 3' end of the genome are expressed. The sg mRNAs are 3' coterminal, but they also have a common 5' leader sequence (24), which is derived from the 5' end of the genome. After conventional cis-splicing was ruled out as the mechanism of EAV leader-to-body fusion (23), it was postulated that arterivirus sg mRNAs are synthesized by a discontinuous transcription mechanism similar to that previously proposed for coronaviruses. Clearly, this concept is supported by the presumed common ancestry of the arterivirus and coronavirus replicase genes (22,27,91). Furthermore, in both virus groups, short conserved transcription-regulating sequences (TRSes) are present at the 3' end of the leader sequence and at the 5' end of the transcription units for each of the sg mRNA bodies (reviewed in 91). The observation that arterivirus-infected cells contain a nested set of sg negative-stranded RNAs, complementary to the sg mRNAs, was another important parallel with coronaviruses (14,21).

A substantial number of models for nidovirus sg mRNA synthesis have been proposed, in particular for coronaviruses, and have been reviewed extensively (see Chapter 35; references 9,87,91; and references therein). Initially, the observation that the positive TRS at the 3' end of the leader could base pair with the negative TRS upstream of each transcription unit in the genomic negative strand led to the proposal of a leader-primed transcription model (see Fig. 5**A**) for coronaviruses. In this model, base pairing between the positive and negative TRSes is followed by extension of the leader to yield an sg mRNA. The detection of sg negative strands, however, indicated that the discontinuous step could also occur during the synthesis of negative-stranded rather than positive-stranded sg RNAs. This concept was subsequently supported by biochemical and genetic studies with coronaviruses and resulted in a model (see Fig. 5**B**) in which discontinuous extension of negative strands yields sg templates for sg mRNA synthesis (87).

Recently, direct proof of base pairing between the positive leader TRS and the negative body TRS was obtained from reverse genetics studies using an EAV infectious copy DNA (cDNA) clone (109). Site-specific mutations introduced in the positive leader TRS can be compensated by the introduction of the same mutation in the positive body TRS, thereby preserving the potential for base pairing between the positive leader TRS and the negative body TRS (Fig. 5). It was even possible to maintain partial transcription activity after substituting all five conserved nucleotides of the EAV TRS, which indicates that this sequence per se does not serve as a recognition signal for viral or cellular proteins involved in discontinuous transcription. Furthermore, the TRS sequences found in mRNAs generated by TRS mutants with partial transcription activity (109) provided additional support for the model of discontinuous negative-stranded synthesis (see Fig. 5**B**). The mechanism by which the transcription complex is translocated between body and leader TRSes, a step that may resemble copy choice RNA recombination (9,109), remains to be elucidated. Obviously, specific interactions between proteins (viral or cellular) and RNA sequences in both nascent strand and template are likely to be its driving force. Future studies may also reveal why several arterivirus mRNAs are generated from multiple or nonconsensus TRSes in the 3'-terminal region of the genome (reviewed in 91).

Translation and Posttranslational Processing

Translation of the arterivirus genome, the first step in virus replication (see Fig. 4), yields two large replicase polyproteins: the ORF 1a protein (1727 to 2502 amino acids) and the ORF 1ab protein (3175 to 3959 amino acids). ORF 1b translation requires a –1 ribosomal frame shift just before ORF 1a translation is terminated (22). The ORF 1a and 1b overlap region contains two signals that are assumed to promote this event: a so-called slippery sequence, which is the actual frame-shift site; and a downstream RNA pseudoknot structure. The functionality of the frame-shift signal has been demonstrated for EAV: using a reporter gene construct, a frame-shifting efficiency of 15% to 20% was observed (22).

The posttranslational fate of the arterivirus replicase polyproteins has only been studied extensively for EAV, and an apparently complete processing scheme has recently been obtained (94,106,108,112). The EAV ORF 1a and ORF 1ab polyproteins are cleaved 7 and 10 times, respectively, by three different ORF 1a–encoded proteases (see later). In combination with the ribosomal frame shift, this leads to the generation of 12 processing end products, or nonstructural proteins (nsp1 to nsp12) and a large number of processing intermediates. Of the processing end products, nsp1 to nsp8 are generated from the ORF 1a polyprotein, whereas nsp10 to nsp12 are entirely ORF 1b–encoded. The putative RNA polymerase subunit, nsp9, consists of a small, ORF 1a–encoded N-terminal domain, which is identical to nsp8, and a large, C-terminal part that is encoded by the 5'-proximal region of ORF 1b. In addition to the three proteases described for EAV, both LDV and PRRSV possess a fourth nonstructural proteolytic activity (20,40,66), which mediates the rapid release of an additional N-terminal cleavage product. The additional protease resides in the extreme N-terminus of the ORF 1a protein and may be a duplication of the papain-like cysteine protease (PCP) domain present in the C-terminal domain of EAV nsp1 (20). The recent sequence analysis of the SHFV replicase gene has revealed an even more complex situation: in this virus, an array of 3 PCP domains appears to be present in the 480-residue region upstream of the nsp1–nsp2 junction (see Fig. 3). This raises the total number of arterivirus nonstructural proteases to five (T. R. Maines and colleagues, submitted for publication).

With the exception of the smallest sg species, the arterivirus mRNAs are structurally polycistronic. However, they are generally assumed to be functionally monocistronic. A notable exception is mRNA2 of EAV (see Fig. 4), which was

recently proposed to be a functionally bicistronic mRNA from which both GP_{2b} and the newly discovered E protein are expressed (92). Likewise, mRNA2 of LDV and PRRSV and mRNA4 of SHFV are probably used to express the corresponding genes of these other arteriviruses. Another mRNA of SHFV, mRNA2, was proposed to be functionally bicistronic, but in this case, the expression of two ORFs (2a and 2b) from this mRNA remains to be demonstrated (41).

In contrast to most other major groups of animal positive-stranded RNA viruses, virus-encoded proteases are apparently not involved in the maturation of nidovirus structural proteins. In the case of arteriviruses, structural protein proteolysis appears to be limited to the (cotranslational) removal of N-terminal signal sequences from each of the glycoproteins.

Replicase Proteins and Replication Complex

The nidovirus replicase gene encodes two polyproteins of extraordinary size and complexity. Although a number of (putative) functions have been inferred from comparative sequence analysis, the detailed functional dissection of the complex array of nidovirus nonstructural proteins is yet to be started. In view of the two-fold size difference between the replicases of arteriviruses and coronaviruses, these future studies will undoubtedly reveal both novel similarities and differences between the two groups. At this stage, it appears that the functions encoded by replicase ORF 1a and ORF 1b fall into different categories. The role of ORF 1a–encoded functions in the regulation (by proteolytic processing) of the expression of replicase subunits has been firmly established. Furthermore, a number of hydrophobic ORF 1a–encoded subunits have been implicated in replication complex formation. The ORF 1b–encoded proteins, on the other hand, appear to be more directly involved in viral RNA synthesis.

For EAV, protease domains have been identified in nsp1, nsp2, and nsp4. These proteases and their corresponding cleavage sites are well conserved in other arteriviruses. EAV nsp1 and nsp2 both contain a cysteine autoprotease domain that mediates, probably exclusively in *cis,* their rapid release from the polyprotein (93,95). The presence of one (LDV or PRRSV) or possibly even two (SHFV) additional protease functions upstream (see Fig. 3) is a remarkable feature of the arterivirus replicase, suggesting considerable genetic flexibility and adaptation to host-specific circumstances.

The nsp4 serine protease (SP) is the arterivirus main protease and a member of a relatively rare group of proteolytic enzymes, the 3C-like serine proteases (96). The SP combines the catalytic triad of classic chymotrypsin-like proteases with the substrate specificity of the 3C-like cysteine proteases, a subgroup of chymotrypsin-like enzymes named after the picornavirus 3C proteases. Specific residues in the substrate-binding region of the SP are assumed to determine its specificity for cleavage sites containing a (Glu/Gln)-(Gly/Ser) dipeptide (96, 106,112). Eight SP cleavage sites have now been identified: five in the C-terminal half of the ORF 1a protein, a region in which alternative processing occurs (112); and three in the ORF 1b–encoded polypeptide (106).

With the exception of part of nsp1, all EAV replicase subunits localize to the perinuclear region of the infected cell (104,108), where they are associated with intracellular membranes, predominantly or exclusively those of the endoplasmic reticulum. This complex has also been shown to be the site of *de novo* viral RNA synthesis. Recent biochemical and electron microscopy studies have implicated hydrophobic ORF 1a–encoded subunits (in particular, nsp2, nsp3, and nsp5) in replication complex formation (36,104). Upon arterivirus infection, host cell membranes are modified into vesicular double membrane structures that carry the arterivirus replication complex (80,104). The formation of such closely paired membranes and double membrane vesicles (DMVs) is a typical feature of arterivirus-infected cells and had been described many years ago (8,97,117). Expression of EAV nsp2 to nsp7 in a heterologous expression system induced strikingly similar structures, indicating that DMV formation does not depend on other viral proteins or EAV-specific RNA synthesis (80).

Predictions relating to the functions of the arterivirus ORF 1b–encoded replicase subunits are largely derived from comparative sequence analysis. Putative RNA-dependent RNA polymerase and NTP-binding RNA/helicase motifs were identified in nsp9 and nsp10, respectively (22,40,43,66). Recently, both the NTPase and duplex-unwinding activities of EAV nsp10 were demonstrated using *in vitro* assays (89). These studies also revealed a close functional relationship to the helicase of coronaviruses (88,89). Furthermore, ORF 1b encodes two additional conserved domains that appear to be unique to nidoviruses: a conserved domain in nsp11 and a Cys/His-rich domain in the N-terminal region of nsp10, upstream of the helicase. The nsp10 Cys/His-rich domain was previously proposed to have metal-binding properties (43) and was recently implicated in a remarkable mRNA transcription defect. The serendipitous replacement of Ser-2429 by Pro, which is located at the C-terminal border of the nsp10 Cys/His-rich domain, rendered the EAV full-length cDNA clone noninfectious owing to an almost complete block of sg mRNA transcription (105,110). However, this mutant replicated its genomic RNA efficiently, showing that arterivirus replication and sg mRNA transcription are (at least in part) separate processes. Recently, a more extensive mutagenesis study of the EAV nsp10 Cys/His box has strengthened the notion that this domain is involved in both genome replication and sg RNA transcription and may have unique zinc-binding properties (107).

A second (putative) zinc finger protein with a specific role in sg RNA synthesis is EAV nsp1 (102). This most N-terminal replicase subunit was shown to be dispensable for genome replication, but absolutely required for sg RNA transcription.

Major Structural Proteins

Arteriviruses possess a set of six or seven structural proteins, a number that is unusually large compared with other positive-stranded RNA viruses. The three major structural proteins GP_5 (and its equivalent GP_7 in SHFV), M, and N are encoded in this order by the three most 3' ORFs of the arterivirus genome (see Fig. 3 and Table 1). The N proteins are small (12 to 15 kd) and highly basic, which may facilitate their interaction with the genomic RNA during assembly of the nucleocapsid. The regions of N that are important for this interaction have not yet been defined. The EAV N protein was shown to be phosphorylated and constitutes about 20% to 40% of the protein content of the virion (25).

The nonglycosylated M protein (16 to 20 kd) is the most conserved structural protein of arteriviruses. It resembles the

coronavirus M protein in that its N-terminal half presumably traverses the membrane three times (25,35,66). This results in a N_{exo}-C_{endo} configuration with a short stretch of only 10 to 18 residues exposed at the virion surface. One of the membrane-spanning fragments is thought to function as a signal sequence. Although little is known about the function of the arterivirus M protein, it probably plays an important role in virus assembly and budding, as has been shown for coronaviruses. The M protein accumulates in the endoplasmic reticulum, where it forms disulfide-linked heterodimers with the 24- to 54-kd major glycoprotein, GP_5 or GP_7 (28,34). A Cys residue in the short N-terminal ectodomain of the M protein is most likely involved in the formation of an intermolecular disulfide bridge with a Cys residue in the GP_5 ectodomain. The GP_5–M heterodimers, which are essential for infectivity, are transported to the Golgi complex (28) and are incorporated into virus particles at some point during this process.

Despite considerable differences in primary structure, the major glycoproteins of arteriviruses (GP_5 or GP_7) share common structural features. They contain an N-terminal signal sequence that is assumed to be cleaved from a short ectodomain. In the case of LDV and PRRSV, the latter is about 30 amino acids long and contains one to three N-linked glycans (35,66,74). The putative ectodomain of EAV GP5 is 95 residues long and usually possesses a single N-linked polylactosamine side chain (25). In some strains, however, an additional N-glycan chain is present (39). Also, in the case of LDV, GP_5 glycosylation occurs by the addition of variable numbers of lactosamine repeats (56). Nonneuropathogenic and neuropathogenic strains typically contain three and one polylactosaminoglycan chains, respectively (see later). The internal hydrophobic region of GP_5 is assumed to span the membrane three times and to be followed by a cytoplasmic domain of 50 to 72 amino acids. Although one might speculate that the arterivirus major envelope protein is involved in receptor recognition, no direct experimental evidence is available thus far.

Minor Structural Proteins

The minor envelope protein encoded by EAV and LDV ORF 2b, PRRSV ORF 2a, and SHFV ORF 4b (see Fig. 3) is a typical class I membrane protein. It contains an N-terminal signal sequence, a C-terminal transmembrane segment, and an ectodomain possessing complex-type N-glycans when the protein is incorporated into virions. It has been shown that intracellular EAV GP_{2b} exists as four monomeric species owing to the formation of intrachain disulfide bonds but that only disulfide-linked GP_{2b} homodimers are incorporated into virus particles (23,27). In contrast, the equivalent PRRSV GP_{2a} protein is present in virions as a monomer (67).

The PRRSV and EAV GP_4 proteins were also identified as minor glycoprotein constituents of the virus particle (92,111). They contain an N-terminal signal sequence and a potential C-terminal membrane anchor, which are typical for class I integral membrane proteins. Conflicting data on the presence of the GP_3 glycoprotein in PRRSV particles have been reported. Whereas GP_3 was detected in purified virions of a European PRRSV strain using specific monoclonal antibodies (111), the protein could not be immunoprecipitated from a purified virus preparation of a Canadian strain (63). However, small amounts of the latter protein were detected in a soluble form in the medium. Topologic studies on the corresponding LDV protein also suggested that it is secretory protein, but thus far, this protein has not been detected in cell lysates or the medium of infected cells (37).

The recently discovered E protein of EAV, which is encoded by a gene conserved in all arteriviruses, has further extended the collection of arterivirus structural proteins (92). The hydropathy profile of this small hydrophobic nonglycosylated protein suggests that it is an integral membrane protein with an uncleaved signal-anchor sequence in the central part of the molecule. Although the specific functions of the envelope proteins of arteriviruses remain to be elucidated, it has been shown by reverse genetics that the EAV E, GP_{2b}, GP_3, GP_4, GP_5, and M proteins are essential for the production of infectious progeny virus (73,92).

Assembly and Release

The EAV N protein was found to co-localize partially with the replication complex. Because it has been established that N is not required for genome replication or sg mRNA transcription (73,105), this observation suggests that genome encapsidation may occur (or begin) at the site of viral RNA synthesis. Arteriviruses acquire their envelope by budding of preformed nucleocapsids into the lumen of the smooth endoplasmic reticulum or the Golgi region (62,97,117). Most arterivirus envelope proteins are retained in intracellular membranes, but their specific roles in assembly have not yet been established. After budding, virions accumulate in intracellular vesicles, which are transported to the plasma membrane and release the progeny virus. The generation of aberrant nucleocapsid structures or unusual elongated virions was observed for various arteriviruses.

VIRUS PROPAGATION AND PATHOGENESIS

Growth Features

With the exception of EAV, arteriviruses have a very restricted host specificity. LDV grows in primary cultures of mouse macrophages but not in macrophage or other cell lines (83). In addition to primary macrophages from their respective hosts, SHFV and PRRSV replicate in cell lines of African green monkey kidney cells (MA-104 and derivatives thereof) (6,44,53). Most European PRRSV strains, however, have to be adapted to these monkey kidney cell lines before reasonable titers are obtained. EAV replicates efficiently in primary cultures of horse macrophages and kidney cells as well as in a variety of cell lines, such as baby hamster kidney, rabbit kidney, and African green monkey kidney cell lines (83,91).

One-step growth experiments have shown that the replication cycle of arteriviruses is short, with maximal progeny virus titers released by 10 to 15 hours after infection. The maximal titers obtained in cell culture are 10^6 to 10^7 tissue culture infectious dose ($TCID_{50}$) per milliliter for PRRSV but may exceed 10^8 plaque-forming unit (PFU) per milliliter for EAV and SHFV (91). The infection of macrophages and cell lines is highly cytocidal, resulting in rounding of the cells and

detachment from the culture plate surface. PRRSV, SHFV, and EAV are titrated by end-point dilution or plaque assays. However, LDV is titrated *in vivo* in mice because the percentage of susceptible cells in primary mouse macrophage cultures is too low to detect their destruction (83).

Epidemiology and Pathogenesis of Equine Arteritis Virus

Despite its worldwide distribution, EAV has rarely caused disease outbreaks. The first recognized and most severe epizootic occurred in 1953 in Bucyrus, Ohio (30). Subsequently, milder outbreaks have been reported from Austria, Canada, Italy, Poland, Switzerland, and the United States. The apparent discrepancy between the high incidence of the virus and the low number of recorded disease outbreaks is explained by the predominantly subclinical course of infection. Transmission of EAV occurs primarily through the respiratory and venereal routes (64,103). The latter route of infection involves transmission to brood mares by persistently infected “carrier” stallions, which shed the virus in their semen (103). So far, no natural hosts other than horses and donkeys have been reported for EAV. EAV initially replicates in lung macrophages. The virus then spreads to bronchial lymph nodes, from where it becomes disseminated throughout the body. By the third day of infection, a viremia has developed, and virus can be isolated from practically all tissues. In the few cases that EAV causes disease, clinical features are characteristic vascular lesions, necrosis of small muscular arteries, acute anorexia, and fever, usually accompanied by palpebral edema, conjunctivitis, nasal catarrh, and edema of the legs, genitals, and abdomen. Infection of pregnant mares frequently results in abortion (30).

Epidemiology and Pathogenesis of Porcine Reproductive and Respiratory Syndrome Virus

PRRS was detected first in 1987 in the United States and subsequently spread rapidly to Canada. In Europe, the first outbreaks were recognized in Germany in 1990 and were followed by outbreaks in the Netherlands, Belgium, Spain, the United Kingdom, France, and Denmark (114). Today, PRRSV infection is ubiquitous in all swine-producing areas of the world, including North and South America, Europe, and Asia. Severe abortion storms had a resurgence in 1996 to 1998 in the United States. During the past several years, however, the number of acute disease outbreaks has decreased, and mild subclinical infections are more common, especially in areas in which the virus is endemic. PRRSV is shed in saliva, respiratory tract secretions, oropharyngeal secretions, urine, and semen. Although it has been proposed that PRRSV is primarily transmitted through aerosols, airborne transmission was extremely difficult to achieve under experimental conditions (121). PRRSV can also cross the placenta and infect the fetus.

The clinical signs and pathogenesis of PRRSV infection are influenced by many factors, including host age, breed, sex, immune status, and virus strain. Typical signs include fever, anorexia, labored respiration, lymphadenopathy, gross and microscopic lesions in the lung, and reproductive failure characterized by delivery of weak or stillborn piglets or autolysed fetuses (114). The virus primarily infects alveolar lung macrophages and macrophages of other tissues. Twelve to 24 hours after exposure, young pigs, sows, and boars become viremic, a state that can last from 1 to 2 weeks in mature animals to 8 weeks in young pigs. The virus infection may persist in swine for prolonged time periods (up to 2 to 5 months). PRRSV was shown to replicate in testicular germ cells, such as spermatids and spermatocytes, a property that may explain the excretion of infectious virus in semen (99). PRRSV induces apoptosis *in vitro* in macrophages and in germ cells (98,99). The GP_5 protein is thought to play a role in the induction of this process (98), but the relevance for pathogenesis *in vivo* is unclear.

Epidemiology and Pathogenesis of Lactate Dehydrogenase–Elevating Virus

LDV was first discovered in laboratory mice (84). The virus was also isolated from wild mice in several countries, although the worldwide incidence is not known. Despite a lifelong viremia and virus secretion in urine, feces, and saliva, horizontal transmission of LDV between mice is inefficient, except in the case of fighting males (77). In contrast, transmission from mother to offspring through the placenta or breast milk is much more efficient as long as anti-LDV immune responses have not yet been elicited (83).

Infection of mice with LDV leads to a lifelong viremic but asymptomatic infection that is maintained by continuous rounds of cytocidal replication of LDV in a renewable subpopulation of macrophages (78). Twenty-four hours after infection, LDV titers of 10^{10} infectious dose (ID_{50}) per milliliter are present in the plasma, which then decrease to a level of 10^{4} to 10^{6} ID_{50}/mL. These titers remain present throughout the life of the mouse, together with elevated levels of LDH and other serum enzymes, owing to the destruction of macrophages that play a role in the clearance of this enzyme. LDV can be detected in the spleen, lymph nodes, thymus, and liver of persistently infected mice. Immune complexes of 150S to 300S are formed, most likely as a consequence of the polyclonal activation of B cells (12). Neurovirulent LDV variants can cause a fatal age-dependent poliomyelitis in certain inbred mouse strains that are of the Fv-1 $^{n/n}$ genotype and carry N-tropic, ecotropic MuLV proviruses (19). The replication of these ecotropic MuLVs in the glial cells of the spinal cord was proposed to render the anterior horn neurons susceptible to cytocidal LDV infection. Consequently, the development of age-dependent poliomyelitis might result from a combination of increased expression of the ecotropic MuLVs and a decreasing ability to mount a motor neuron–protective anti-LDV response.

Epidemiology and Pathogenesis of Simian Hemorrhagic Fever Virus

SHFV was first isolated in 1964 from diseased macaques in research centers in the Soviet Union and the United States (101). The virus was readily transmitted from the initially infected rhesus monkeys (*Macaca mulatta*) to other macaque species (*Macaca fascicularis* and *Macaca arctoides*), whereas members of other monkey genera did not show clinical symptoms. Subsequently, similar epizootics among macaques occurred in various primate centers in the United States and Europe. SHFV can spread rapidly within a colony, most likely by direct contact and aerosols. In contrast to the other arteriviruses, SHFV is not transmitted transplacentally from mother to offspring (45). Natural SHFV outbreaks among macaques have not been reported, but the incidence of persistent subclinical infections in wild patas monkeys appears to be high. It has been proposed that these persistently infected monkeys may be the source of virus causing the epizootics in macaque colonies (59).

Clinical signs in macaques consist of early fever and mild facial erythema and edema, followed by anorexia, dehydration, and various hemorrhagic manifestations. The macaques usually die within 2 weeks after infection, with mortality rates approaching 100%. Macrophages are the primary target cells for SHFV, and a causal relationship exists between the cytocidal infection of these cells by SHFV and the clinical symptoms of hemorrhagic fever (44).

IMMUNOLOGY AND VACCINES

Humoral Immune Response

Antigenic cross-reactivity between different arterivirus species has not been demonstrated. Although antibodies to arterivirus structural proteins can be detected within 1 week after infection, the four viruses can all establish persistent infections. The mechanisms by which arteriviruses can evade the host immune response remain to be elucidated.

Sera from EAV-infected horses recognize the N, M, GP_5, and GP_{2b} proteins (17,60). PRRSV-infected pigs contain antibodies directed against GP_{2a} to GP_5, M, and N, the antibodies directed to N being most abundant (58,68). Different epitopes of the PRRSV N protein were identified using monoclonal antibodies. Some of these were specific for European strains; others were specific for U.S. strains. An immunodominant antigenic domain was mapped to the central region of the N protein (70,85).

Neutralizing antibodies from arterivirus-infected animals are predominantly directed to the major glycoprotein GP_5 (12,16,42). Furthermore, various neutralizing MAbs directed against GP_5 of EAV, LDV, and PRRSV have been isolated. The neutralization site of EAV and LDV GP_5 was mapped to the ectodomain of the protein (2,16,56). Recent immunization studies using alphavirus-based expression vectors have shown that the coexpression of EAV GP_5 and M is required for the induction of neutralizing antibodies in mice (3). For PRRSV, a second neutralizing MAb-binding domain was identified in the ectodomain of the GP_4 protein (71). Because this region is highly heterogeneous among different strains of PRRSV, it is most likely subject to immunoselection. PRRSV-specific MAbs directed against GP_5 were more effective in virus neutralization than those directed against GP_4 (113). The immunogenicity of the SHFV structural proteins has not been studied in detail.

The production of neutralizing antibodies in EAV-infected horses coincides with virus clearance, suggesting that the humoral immune response plays an important role in recovery (38). In contrast, neutralizing antibodies against LDV and PRRSV are first detected 1 to 2 months after infection, are produced at low levels, and do not or hardly reduce viremia (12,58,118). It has been proposed that LDV- and PRRSV-specific antibodies actually enhance virus replication because it was observed that virus uptake in macrophages *in vitro* was increased in the presence of antibodies (13,118). Although anti-LDV antibodies do not prevent LDV replication in macrophages, several studies indicated that they specifically protect motor neurons from infection by neuropathogenic LDV strains and thereby prevent the onset of age-dependent poliomyelitis in mice (46,82). In contrast to nonneuropathogenic LDV strains, these neuropathogenic LDV strains are not able to establish persistent infections in mice. *In vitro* neutralization assays with plasma from infected mice and neutralizing MAbs showed that neuropathogenic LDV strains were more efficiently neutralized than nonneuropathogenic strains (15). This was not due to sequence differences in the GP_5 neutralization domain because synthetic peptides representing these sequences were bound with equal efficiency by neutralizing antibodies in enzyme-linked immunosorbent assays (56). In the case of nonneuropathogenic strains, however, the GP_5 ectodomain was found to contain three polylactosaminoglycan chains, as opposed to the presence of a single polylactosaminogly can in the ectodomain of neuropathogenic strains. Hence, it was postulated that the nonneuropathogenic strains can establish persistent infections because neutralizing antibodies bind less efficiently to the highly glycosylated ectodomain of their GP_5 surface protein (15,56).

The humoral immune response against SHFV varies with the species of monkey and the virus isolate that is tested (44,45). The rapid death of macaques after SHFV infection precludes an effective host immune response. Virulent SHFV strains, which cause acute disease in patas monkeys, induced neutralizing antibodies 7 days after infection. The production of neutralizing antibodies correlated with the complete clearance of the virus from the circulation by 21 days after infection. On the other hand, SHFV strains that cause a persistent infection in these monkeys induce very low titers of nonneutralizing antibodies.

Cell-Mediated Immune Response

Cellular immune responses to arterivirus infection have not yet been characterized in detail. Cytotoxic and helper T-cell responses were detected in LDV-infected mice but did not reduce LDV replication (33). T-cell proliferation responses in pigs infected with PRRSV were detected at low levels between 4 and 12 weeks after infection (4). These responses were mainly directed to the M, GP_{2a}, and GP_5 proteins (5). Additional studies are required to determine whether there is a correlation between T-cell responses *in vitro* and protection *in vivo.*

Vaccines

For the economically important arteriviruses EAV and PRRSV, live attenuated (Arvac, Ford Dodge Laboratories, Fort Dodge, IA; RespPRRS, Boehringer Ingelheim Animal Health, St. Joseph, MO; Prime Pac PRRS, Schering Plough, Kenilworth, NJ) and killed vaccines (Artervac, Ford Dodge Laboratories, Sligo, Ireland; Suipravac-PRRS, Laboratorios Hipra, Gerona, Spain) are commercially available. The live vaccines induce long-lasting protection but do not completely prevent reinfection with wild-type virus and virus transmission. Recently, adverse effects of vaccination of Danish pig herds with a modified live PRRS vaccine have been described (7), which were probably caused by reversion of the vaccine virus to virulence. Acute PRRS-like symptoms, including an increased number of abortions and stillborn piglets, were experienced in vaccinated herds. Furthermore, vaccine virus was transmitted from vaccinated to nonvaccinated

boars in several cases, resulting in viremia and shedding of vaccine virus into semen (61). In this respect, the killed vaccines are safer but are less efficacious in the induction of protection. Promising results have been obtained in DNA vaccination experiments with plasmids expressing the GP_5 protein of PRRSV (81). Neutralizing antibodies and lymphocyte proliferation responses were detected in DNA-vaccinated pigs, and the spread and clinical signs of challenge virus were reduced. These results suggest that GP_5 of PRRSV might be a good candidate for a DNA-based subunit vaccine.

EVOLUTION

The comparison of nidovirus genome organizations and the phylogenetic analysis of conserved replicase domains indicate that nidoviruses represent a distinct evolutionary lineage among positive-stranded RNA viruses (10,22,27,40,91). In arteriviruses and coronaviruses, related replicase genes and replication strategies have been combined with seemingly unrelated sets of structural genes. The different sets of structural genes that have been acquired by various nidovirus genera suggest the extensive involvement of RNA recombination in their evolution. The RNA recombination frequency during coronavirus replication is remarkably high (see Chapter 35), a characteristic that is probably determined by replicase properties and that may also be reflected in the mechanism of sg mRNA synthesis (109). It has been hypothesized that the acquisition of a novel N protein gene, giving rise to a virus core with different structural properties, may have created the possibility for an ancestor of the coronavirus branch to increase its genome size and diverge from the arterivirus branch (40).

The occurrence of (frequent) RNA recombination during arterivirus evolution has been postulated and is supported by recent experimental studies. Upon co-infection of mice with two phenotypically distinguishable LDV strains, a high recombination frequency in the 3'-terminal part of the genome was observed (57). Furthermore, two closely related North American PRRSV strains were reported to recombine during *in vitro* mixed infections in MA-104 cells (119). RNA recombination was also implicated in two apparent rearrangements of arterivirus genomes (see Fig. 3): the presumed duplication or triplication of the arterivirus nsp1 protease domain (20; T. R. Maines and colleagues, submitted for publication), and the insertion of three genes in the SHFV genome (41). As a result of the last mentioned event, SHFV contains three additional ORFs that are absent in the other arteriviruses (see Fig. 3). Because the proteins encoded by SHFV ORFs 4, 5, and 6 display similarity with the proteins encoded by ORFs 2a, 2b, and 3, it was proposed that these genes may have been either duplicated by an SHFV RNA recombination event or derived from a more distantly related arterivirus by heterologous RNA recombination (41).

The variation in arterivirus genome length is mainly due to major sequence differences in the N-terminal part of replicase ORF 1a. A phylogenetic analysis of the RNA-dependent RNA polymerase motifs of EAV, LDV, and PRRSV suggested that the EAV-branch diverged from a common ancestor before the LDV and PRRSV lineage (40). The relatively large evolutionary distance between EAV on one hand and LDV and PRRSV on the other is further illustrated by the amino acid sequence differences between structural proteins (69). Similar sequence comparisons indicate that SHFV occupies an intermediate position but is more closely related to LDV and PRRSV than it is to EAV (10,90). The close relationship between LDV and PRRSV structural proteins (32% to 53% amino acid sequence identity) has led to the hypothesis that the latter virus may have originated from interspecies transfer of LDV from mice to pigs or vice versa, an event that must have been followed by a substantial period of independent evolution of the virus in its new host (82).

Comparison of the amino acid sequences of the M and N proteins of two LDV strains revealed that these are more conserved than the polypeptides encoded by ORFs 1 to 5 (95% to 99% versus 75% to 87% identity, respectively) (82). Among EAV field isolates, the M and N protein sequences are also more conserved than those of other structural proteins. Similar conclusions were drawn from a recent analysis of the structural genes of sequential virus isolates from persistently infected carrier stallions (47). In the latter study, an overall sequence variation of about 1% per year was observed for the structural protein-coding region of the genome. However, the proteins encoded by ORFs 3 and 5 were found to evolve much more rapidly, suggesting that these polypeptides are subject to strong selective pressure in the host (47).

Striking sequence diversity was detected between North American and European isolates of PRRSV: the structural proteins of virus isolates from the two continents shared only 54% to 79% sequence identity (77). The GP_5 protein is the most variable structural protein, with only 51% to 55% sequence identity between North American and European isolates, whereas the M protein is the most conserved structural protein, with 78% to 81% of the residues being identical (69). Sequence comparisons between the replicases of the European Lelystad virus isolate and two U.S. isolates also revealed major differences (1,76). Replicase subunit nsp2 was found to be the most variable nonstructural protein: the sequence of the U.S. strains was 102 amino acids longer than that of Lelystad virus and shared only 32% of identical amino acids. On the basis of these large genetic differences, the PRRSV isolates from North America and Europe were concluded to represent two distinct genotypes, which must have evolved independently in swine populations before PRRS emerged on both continents.

PERSPECTIVES

The molecular characterization of four arterivirus genomes has revealed that these viruses generate about 20 protein species (excluding replicase-processing intermediates) and 14 to 18 RNA molecules (positive and negative strands). Most of these molecules have been defined in basic terms only, and understanding the molecular details of their role in the viral life cycle is one of the important challenges for the next decades of arterivirus research. For example, the characterization of the six or seven structural proteins and their role in the assembly of the arterivirus particle promises to be a complex issue. Likewise, the regulatory role of replicase proteolysis in the viral life cycle and the assembly and function of the arterivirus replication–transcription complex are largely unexplored fields. Both assembly and RNA synthesis depend on host cell membranes and probably also on the host proteins they contain. Thus, intracellular virus–host (protein–protein and protein–lipid) interactions are likely to be uncovered. The identification of the arterivirus receptor and possibly other host factors involved in entry and uncoating might explain the highly specific tropism of this group of viruses. At the RNA level, primary sequences and RNA structures involved in replication, discontinuous

transcription, and encapsidation are being defined. The recently developed systems for arterivirus reverse genetics are expected to be of great value for these molecular studies as well as for research into molecular pathogenesis, viral persistence, immunity against arterivirus infections, and the development of improved arterivirus vaccines.

An intriguing final question is whether arteriviruses will emerge or be identified in other host species. On the basis of the plasticity of arterivirus genomes and the fact that (at least) a number of arteriviruses can be transmitted by aerosols, these viruses can be assumed to have ample opportunity to cross species barriers. In fact, it is quite possible that they may already have done so. Looking at the inapparent and persistent infections caused by the known members of this virus group, it may be difficult to detect arterivirus-induced disease in other species. Also in this respect, the availability of complete genomic sequences for these viruses, which display no antigenic relationship, is essential and may form the basis for the development of rapid assays for detecting known and novel arteriviruses.

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CHAPTER 38 *Rhabdoviridae:* The Viruses and Their Replication

John K. Rose and Michael A. Whitt

The *Rhabdoviridae* are enveloped RNA viruses that are the simplest of all the nonsegmented negative-stranded RNA viruses classified in the large order of Mononegavirales. The genetic organization of rhabdoviruses is similar to that of the larger *Paramyxoviridae* and *Filoviridae* of the same order, and many aspects of rhabdovirus transcription and replication are similar as well. Because the genomes are in the negative (noncoding) sense, all of these viruses must carry an RNA-dependent RNA polymerase in the virion. The first such polymerase was identified in a rhabdovirus (8). Soon after infection when the viral genome is released into the cell, the RNA-dependent polymerase transcribes the genome to make subgenomic viral mRNAs that are translated to make viral proteins. Viral genome replication requires ongoing viral protein synthesis and results in synthesis of full-length copies of the antigenome followed by synthesis of full-length genomes that are subsequently packaged to form progeny virions.

CLASSIFICATION

Viruses of the family *Rhabdoviridae* are widely distributed in nature, where they infect vertebrates and invertebrates as well as many species of plants. The rhabdoviruses that cause rabies and economically important diseases of fish appear to have life cycles confined to vertebrate species. All other rhabdoviruses are thought to be transmitted to vertebrates and plants by infected arthropods, which may be the original hosts from which all rhabdoviruses evolved. Shope and Tesh (191) have provided an excellent review of the ecology and classification of rhabdoviruses that infect vertebrates. Characteristically, all rhabdoviruses have a wide host range, although many have adapted to grow in specific hosts and at their particular ambient temperatures.

In addition to numerous plant rhabdoviruses (85), more than 70 rhabdoviruses of vertebrates have been identified and classified. The viruses of the family *Rhabdoviridae* known to infect mammals, including humans, have been classified into two genera: the *Vesiculovirus* genus, whose name is derived from vesicular stomatitis virus (VSV); and the *Lyssavirus* genus, otherwise known as the rabies and rabies-like viruses. The well characterized viruses of these two genera are listed in Table 1, which was adapted from the review by Shope and Tesh (191), with modification based on Bilsel and colleagues (17). Unlike vertebrate rhabdoviruses that replicate in the cytoplasm, the related *Bornaviridae* replicate in the nucleus (as do plant rhabdoviruses) and are now classified as a separate virus family.

Virus	Source of virus in nature
Vesiculovirus (VSV) genus	
VSV—New Jersey	Mammals, mosquitoes, midges, blackflies, houseflies
VSV—Indiana	Mammals, mosquitoes, sandflies
VSV—Indiana 2 (Cocal)	Mammals, mosquitoes, mites
VSV—Indiana 3 (Alagoas)	Mammals, sandflies
Jurona	Mosquitoes
Carajas	Sandflies
Maraba	Sandflies
Piry	Mammals
Calchaqui	Mosquitoes
Yug Bogdanovac	Sandflies
Isfahan	Sandflies, ticks
Chandipura	Mammals, sandflies
Hirame	Mosquitoes, sandflies
Porton-5	Mosquitoes
Lyssavirus genus	
Rabies	Dogs, cats, wild carnivores, bats, cattle, humans
Lagos bat	Bats
Mokola	Shrews, humans, dogs, cats
Duvenhave	Humans, bats
Duvenhage	Humans, bats
Obodhiang	*Aaneyxus* mosquitoes
Kotonkan	*Culicinides* mosquitoes

Adapted from refs. 17 and 191, with permission.

TABLE 1. *Members of two major rhabdovirus genera*

Among the animal rhabdoviruses, many of those belonging to the genus *Vesiculovirus* infect biting insects. At least some of these insects can transmit the viruses to animals, and insect transmission may explain the seasonal nature of the disease. The list of insect vectors that may transmit vesiculoviruses to animals includes blackflies, sandflies, and mosquitoes. Evidence for insect-to-animal transmission has been reviewed in detail recently (112). The VSVs found commonly in the Western Hemisphere are divided into two distinct serotypes, called *New Jersey* and *Indiana,* and these share about 50% amino acid sequence identity in their glycoproteins (60,174). Detailed subclassification of the viruses within these serotypes has been described based on extensive nucleotide sequence analysis (17,18,143). In addition, these studies noted a remarkable genetic stability of the viruses over many years of natural infection in particular geographic locations, despite the well-known high rate of mutation of VSV and other RNA viruses passaged in tissue culture (197).

The New Jersey and Indiana serotypes of VSV infect insects and mammals (see Table 1) and cause the economically important disease called *vesicular stomatitis* in cattle, horses, and swine. The disease, which has been thoroughly and recently reviewed (112), is characterized by vesicular lesions of the tongue, gums, teats, and hooves, and its symptoms resemble those of foot-and-mouth disease. Infected animals recover from VSV infections within about 2 weeks. Vesicular stomatitis is enzootic in Mexico, Central America, and northern South America, and small-scale outbreaks occur relatively frequently in the southwestern United States. Other serotypes of VSV, such as Chandipura and Isfahan, are found outside the Western Hemisphere in India and Iran and have also been associated with vesicular disease in domestic animals (226). The Chandipura glycoprotein sequence shares about 40% amino acid sequence identity with either the Indiana or the New Jersey glycoproteins (129). In contrast, the glycoprotein of the distantly related rabies virus shares only about 20% amino acid sequence identity with the VSV G protein (173).

VSV infection has been observed in people exposed to infected animals or inadvertently exposed in the laboratory (49,88). Infection in humans can result in influenza-like symptoms lasting a few days or in vesicular lesions of the mouth, but infection may often be asymptomatic. A very high percentage of humans living in rural areas where VSV infection of animals is common (eg, rural Panama) have high titers of antibody to VSV (207), but VSV infection is not normally associated with human disease in these areas. The pathogenesis of the rabies viruses is quite distinct from that of the vesiculoviruses and is described thoroughly in Chapter 39.

VIRION STRUCTURE

Rhabdovirus virions are composed of an external membrane derived from the cell in which the virus grew and an internal ribonucleoprotein core (Fig. 1). The single viral glycoprotein spans the membrane once and forms an array of about 400 trimeric spikes that extend from the viral membrane. The glycoprotein spikes are clearly visible in the electron micrograph of bullet-shaped VSV particles, shown in Figure 2. About 1,800 molecules of the viral matrix (M) protein are inside the viral envelope between the membrane and the nucleocapsid core. This core contains the single negative strand of genomic RNA, which ranges from about 11 to 12 kb in length in different rhabdoviruses. The bullet-shaped particles, 180 nm long and 75 nm wide, are typical of all rhabdoviruses, except certain plant rhabdoviruses that are bacilliform in shape and almost twice the length (85).

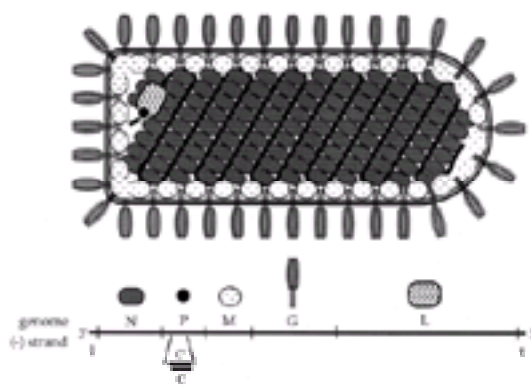

FIG. 1. Schematic representation of vesicular stomatitis virus (VSV) showing the two major structural components: the nucleocapsid (RNP) core, which contains single-stranded RNA tightly encased in the major N protein and the minor polymerase proteins L and P; and the membrane bilayer containing the transmembrane glycoprotein (*G*) and the peripheral matrix (*M*) protein, which adheres to the inner membrane surface and binds to the RNP core. Shown below is the gene order of the VSV genome with symbols representing the expressed proteins. The region of the P gene encoding the C proteins is indicated. (Modified from a drawing by Dr. Randall Owens.)

FIG. 2. Electron micrograph of vesicular stomatitis virus (VSV), negatively stained with phosphotungstic acid. Disrupted virions show the helical nucleocapsid core. The surface of intact virions shows protruding glycoprotein spikes.

The genomic RNA is in a tight complex with about 1,200 molecules of nucleocapsid protein that are arranged like beads on a string along the RNA, with each N protein molecule covering about nine nucleotides. This ribonucleoprotein complex is coiled into a tightly packed helix of about 35 turns within the virion. This helix is clearly visible in the two disrupted particles seen in Figure 2, and extended nucleocapsids from disrupted particles are visible as well. The viral RNA-dependent RNA polymerase, composed of the L and P proteins, is associated with the nucleocapsid core, and L and P proteins are present in about 50 and 500 molecules per virion, respectively. The gene order for VSV is diagrammed in Figure 1, with symbols for the structural proteins encoded by each of the genes. As discussed in detail later, separate mRNAs corresponding to each gene are transcribed sequentially beginning at the 3' end of the genomic RNA. Even during transcription and replication of full-length genomes, the genome and antigenome RNAs remain in a tight complex with the N protein.

Table 2 lists the molecular weights of VSV (Indiana serotype) proteins, predicted from the gene sequences, the number of molecules of each found per virion, and the physical characteristics of virus particles. The protein composition of the particles is constant except for the glycoprotein (G), which can vary considerably. VSV particles produced early in infection can have at least three-fold less G protein per virion than those produced late in infection (116). In addition, VSV incorporates cellular and other viral membrane proteins into its envelope quite promiscuously. For example, when foreign membrane proteins are expressed at high levels from VSV recombinants, the foreign proteins can represent as much as 25% of the total protein in the VSV envelope (182). These studies emphasize the imprecise nature of VSV membrane assembly.

Proteins	Calculated molecular weight[b]	Molecules per virion
N	47,355	1,258
P (NS)	29,878	466
M	26,064	1,826
G	63,416	1,205
L	240,707	50

[a]Length, 180 nm; width, 75 nm; S_{20w}, 660 Svedbergs; density, 1.16 g/mL (in sucrose); RNA length, 11,161 nucleotides.
[b]Calculated from the predicted amino acid sequences, including 6,000 daltons for the two N-linked glycans on G protein.
Adapted from refs. 169 and 209, with permission.

TABLE 2. *Composition and physical characteristics of vesicular stomatitis virus*[a]

GENOME STRUCTURE AND ENCODED PROTEINS

Rhabdovirus genomes contain a minimum of five genes in the order 3' N-P-M-G-L 5', as shown for the VSV Indiana genome in Figure 3. In VSV, two small proteins termed C and C' are encoded in a second reading frame within P, but these appear specific to vesiculoviruses and are not found in lyssaviruses. There are also significant variations in the genome structure of other rhabdoviruses. For example, fish rhabdoviruses generally have an extra small gene between G and L (100), plant rhabdoviruses can have an extra gene between P and M (72), and sigma virus of *Drosophila* has three genes between N and G as well as a 33-nucleotide overlap of the G gene with the preceding gene (206). The functions of these extra gene products are not known.

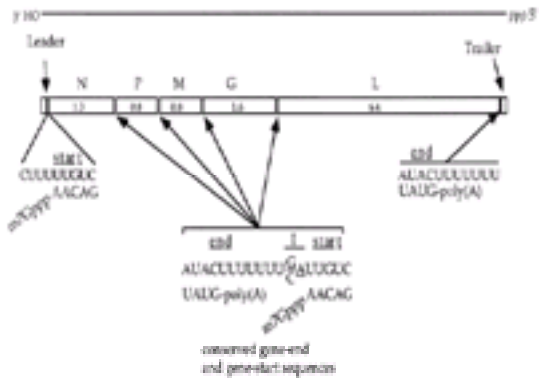

FIG. 3. Diagram of the vesicular stomatitis virus (VSV) genome showing the sequences at the gene junctions, including the polyadenylation signals, intergenic dinucleotides, and transcription initiation sites.

Rhabdovirus genomes have an extremely simple and compact arrangement with little wasted space. This is exemplified in the diagram of the VSV (Indiana serotype) genome (see Fig. 3). Each mRNA begins with the identical five nucleotide start sequence cap-AACAG, and terminates with the sequence UAUG-poly(A), and there are only two nucleotides between each of the genomic RNA regions encoding mRNAs (170). Only the very minimal conserved sequences, including the transcription termination and polyadenylation signals, intergenic dinucleotides, and transcription start signals, are required to signal mRNA termination, polyadenylation, and reinitiation of the subsequent transcript (183). The 5'- and 3'-noncoding regions on the mRNAs are typically short, although there are some virus-specific exceptions. The leader and trailer regions preceding and following the sequences complementary to the mRNAs are also short. Presumably, the genetic selection favoring rapid replication has prevented accumulation of extra sequences that would slow genome replication.

In light of this conservation of genome size in the rhabdoviruses, it is interesting to note that VSV can replicate its genome and assemble virus particles efficiently even when large additions are made to the genome. As discussed under Molecular Genetics of Rhabdoviruses, the reverse genetic system for VSV, first established by Lawson and associates (102), has allowed expression of foreign genes within the VSV genome when appropriate transcription start and stop sequences are included (183). Many VSV recombinants have been made that express extra genes. The addition of single genes of less than 2,000 nucleotides in length generally has little effect on VSV growth in tissue culture or on pathogenesis in mice (98,165,166,182,183), whereas the addition of two extra genes totaling 4,200 nucleotides, a 40% increase in genome size, decreased virus titers only about three-fold (68). Because the helical nature of the VSV nucleocapsid does not put major constraints on virus length, virus particles can grow longer without large effects on viral replication or packaging. The increase in particle length is as expected when particles are imaged by electron microscopy (182).

Nucleocapsid Protein (N)

The rhabdovirus nucleocapsid protein serves the critical function of tightly packaging the RNA genome into a RNase-resistant core that is the template for both transcription and replication. From the length of the VSV Indiana genome and the number of N protein molecules per virion (see Table 2), each N protein molecule would cover about 9 nucleotides of RNA. This N protein–RNA complex interacts with the P–L polymerase complex during transcription and replication, and with the M protein during nucleocapsid condensation, membrane binding, and budding. As discussed under Genome Replication, the VSV N protein recognizes specific packaging sequences in the 5' ends of both the genomic and antigenomic RNA, and the level of VSV N protein is thought to regulate the switch from transcription to replication.

Relatively little is known about the domain structure of the nucleocapsid protein, but deletion of the five C-terminal amino acids abolishes RNA-binding activity, and certain point mutations in this sequence can also prevent RNA binding (37). Other studies indicate that this domain of N protein is required for N–P protein interactions (204). The formation of N–P complexes is important for preventing N protein aggregation (40), which occurs when N protein is expressed at high levels in the absence of other viral proteins (76).

P Gene and Encoded Proteins (P and C)

The VSV P protein, or phosphoprotein, in combination with the L protein, forms the viral transcriptase–replicase complex (47). The P protein of VSV is an acidic protein of 265 amino acids (numbers from P of the Indiana serotype) (59) and is found in various phosphorylated forms in cells and virions. Sequence comparison studies have defined a domain structure for VSV P protein (18). It has an acidic N-terminal domain of 150 amino acids containing phosphorylation sites, followed by a highly variable region from residues 150 to 210 called the *hinge.* Domain II, also containing phosphorylation sites, spans from residues 210 to 244. Domain II is followed by a basic C-terminal domain III consisting of 21 amino acids. P protein is phosphorylated by casein kinase II on serine and threonine residues located in the N-terminal acidic domain (12), and, as discussed in the transcription section below, this phosphorylation is required to generate polymerase activity (11). The P protein forms trimers after phosphorylation, and only after trimerization is it able to bind the L protein and the N–RNA complex to form the active transcriptase (61). Binding of L protein also stabilizes the P trimer (61). The active polymerase complex is therefore an L–P3–N–RNA complex.

Recent studies have implicated phosphorylation of sites in domain II in regulation of P protein function during replication (81). It was suggested in that study and in an earlier study (33) that differential phosphorylation might regulate the switch from transcription to replication. As discussed under Genome Replication, differential phosphorylation may result in the formation of two distinct polymerase complexes: one that functions as a transcriptase and another that functions as a replicase (151). The P protein of Sendai virus and the P proteins of other *Paramyxoviridae* are also phosphorylated, and phosphorylation has been implicated in controlling P protein function. In a recent study, however, the major phosphorylation sites were eliminated from the Sendai P protein by mutagenesis of the gene. Replacement of the wild-type gene with the mutated gene in virus had no measurable effect on Sendai virus transcription or replication, and the mutant virus showed normal pathogenesis in animals (77). Although the paramyxovirus and rhabdovirus P proteins are highly divergent in sequence, these results suggest caution in interpretation of *in vitro* studies of rhabdovirus P protein mutants until the effects of the mutations are analyzed using reverse genetics of infectious virus.

In addition to encoding the P protein component of the viral polymerase, the VSV *P* gene encodes two small basic proteins of 55 and 65 amino acids from a second reading frame. This reading frame is presumably accessed by

ribosomal scanning and initiation at the second and third AUGs in the P mRNA (see Fig. 1). Expression of these proteins, called C and C', was first identified in cells infected with the New Jersey serotype of VSV (196), but similar proteins are also made by the VSV Indiana serotype (99).

Potential functions of the VSV C proteins were investigated by incorporating a mutated *P* gene eliminating C protein expression into infectious VSV. A stop codon was incorporated into the C and C' coding sequence in such a way that the P protein sequence was unchanged (99). Virus that no longer expressed C proteins showed growth kinetics identical to wild-type virus. The amounts of viral mRNAs and proteins synthesized and incorporated into virions were indistinguishable for mutant and wild-type viruses, and the kinetics of host protein synthesis shutoff were also identical for both (99). Although the C and C' proteins are dispensable for VSV growth in tissue culture, they are known to be conserved in all vesiculoviruses (196) and thus may play a role in viral pathogenesis or transmission by insect vectors. Studies on pathogenesis of C negative mutants in mice showed that they were no less pathogenic than the parental virus (A. Roberts and J. Rose, unpublished results). Pathogenesis in normal host animals, such as cattle, or replication in insects has not yet been examined.

Large Protein (L)

The first complete rhabdovirus *L* gene sequence was determined by Schubert for the VSV Indiana serotype (188), and since that time, numerous rhabdovirus *L* genes have been sequenced. All of these proteins consist of more than 2,100 amino acids in a single polypeptide chain. As discussed previously, the complex of L and P proteins constitutes the active RNA polymerase that copies the N–RNA template to produce mRNA, or complete antigenomes and genomes. The large size reflects the multifunctional nature of these proteins in transcription, replication, mRNA capping (2), methylation of 5' cap structures (74), polyadenylation (189), and replication of the viral RNA.

The domains of rhabdovirus L proteins involved in the multiple enzymatic activities have not been clearly mapped except for a potential methyltransferase domain (74). Rhabdoviruses and paramyxovirus L proteins all contain a highly conserved GDN motif (155), which is a variant of the GDD motif found in positive-stranded RNA virus polymerases. Mutations within this core motif in the VSV polymerase block transcription activity *in vitro,* and a mutant GDD retains partial activity (194). Mutations in the GDN sequence of rabies L protein abolish both transcription and replication activities in a mini-genome replication system (184).

The addition of poly(A) to rhabdovirus mRNAs is thought to result from repetitive copying by the polymerase of the U7 sequence found at the end of each gene (189). Mutations in L protein of VSV are known to cause aberrant polyadenylation of mRNAs and are consistent with a role for L in this process (79,80). In addition, a compound, *S*-adenosyl homocysteine, which blocks methylation of cap structures, also promotes synthesis of extremely long poly(A) on VSV mRNAs (175). This effect presumably reflects interference with normally coordinated functions of the polymerase.

There is also recent evidence that the VSV polymerase requires cellular translational elongation factors EF-1a, -1b, and -1g to be fully active (38). These proteins had not previously been identified as components of the VSV virion, perhaps because they are present only at very low levels. A precise stoichiometry of these factors relative to L protein needs to be determined in virions because the virion polymerase is active in generation of all viral mRNAs without addition of host factors (140).

Glycoprotein (G)

All rhabdoviruses encode a membrane glycoprotein consisting of about 500 amino acids in a single polypeptide chain. The VSV envelope contains about 1,200 molecules of this single transmembrane G protein that form 400 trimeric spikes (42,97) arranged in a coat on the virion membrane (see Fig. 1 and Fig. 2). The VSV G protein (Indiana) is synthesized as a precursor of 511 amino acids (174), from which an N-terminal signal sequence of 16 amino acids is cleaved after the protein is inserted into the endoplasmic reticulum (ER) (115). The VSV G protein is a typical type I membrane glycoprotein, with most of the amino acids exposed on the virion surface (Fig. 4). There are two sites of N-linked glycosylation, and both glycans are processed to the complex type (159). A hydrophobic domain of 20 amino acids spans the membrane, and a 29–amino acid cytoplasmic domain extends into the virion. A single molecule of palmitate is esterified to a cysteine residue in the cytoplasmic domain of some rhabdovirus G proteins (171,180), but the function of the palmitate is unclear because a mutant G protein lacking palmitate has normal membrane fusion activity, assembles normally into virions (224), and reconstitutes fully infectious virus (L. Buonocore and J. Rose, unpublished data).

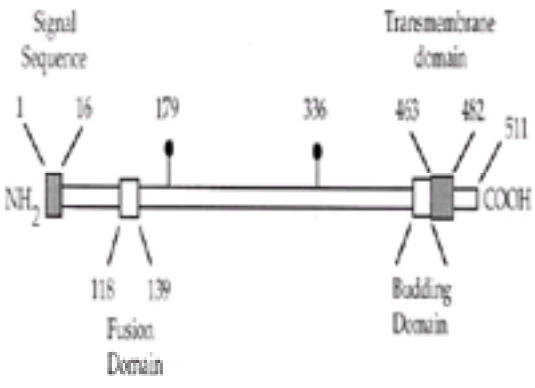

FIG. 4. Schematic of the vesicular stomatitis virus (VSV) glycoprotein illustrating the functional domains.

The VSV G protein has served as an important model for many studies on protein folding, assembly, and transport to the cell surface (43). Immediately after synthesis and before the correct disulfide bonds are formed, G protein associates with a molecular chaperone in the ER called *BiP* (GRP78), which assists in protein folding (125). Correctly folded monomers are released from BiP within about 3 minutes after synthesis, and these then assemble into trimers in about 7 minutes before leaving the ER. A second molecular chaperone, calnexin, recognizes monoglucosylated oligosaccharides on VSV G protein and is bound and released from VSV G with kinetics that are slightly delayed compared with those of BiP, suggesting sequential action of these two chaperones (69). Transport of G protein to the Golgi occurs within about 15 minutes, and protein appears on the cell surface after about 30 minutes (16).

Mutations in all three domains of the VSV G protein that affect transport from the ER have been analyzed in detail. Mutations in the extracellular domain typically prevent correct folding of G monomers and induce aggregation with BiP protein before trimerization (44). When VSV G protein monomers have severe folding defects, for example, because of an absence of glycosylation sites, they are found in large, disulfide-bonded aggregates that remain permanently bound to BiP protein in the ER (44,126,127,154). Mutations in the transmembrane domain can also affect transport by inducing aggregation and preventing transport, whereas mutations in the cytoplasmic domain that reduce the transport rate do not appear to affect the folding of the extracellular domain (44,172). These results indicate that the cytoplasmic tail of the glycoprotein contains a signal that accelerates protein exit from the ER, perhaps by signaling concentration of the protein at sites of vesicle budding from the ER. Detailed mapping of the cytoplasmic tail has implicated a diacidic signal Asp-X-Glu, where X represents any amino acid in accelerating export (144). Further studies have shown that this transport signal extends to a Y-X-X-aliphatic motif in the cytoplasmic tail (190). The VSV G cytoplasmic tail is required for sorting to the basolateral membrane of polarized MDCK cells (158), and the same signal (Y-X-X-aliphatic) that is involved in promoting G protein export from the ER is also involved in sorting to the basolateral membrane (210). Basolateral sorting of VSV G protein is presumably important for systemic spread of the virus, as occurs during natural infections of livestock.

VSV G protein trimers are not tightly associated, but instead are in a constant and rapid equilibrium with monomeric subunits in the ER (235) and at the cell surface (234), or when examined in solution after solubilization with detergent (124). Once assembled into virions, it is likely that a trimeric or higher-order G protein structure is stabilized by lateral interactions among trimers on the virion surface and by interactions with the internal virion components.

VSV G protein undergoes a conformational change at pH values below about 6.1, and this conformational change stabilizes the trimer (42). The low pH-induced conformational change presumably exposes a hydrophobic domain that can insert into a target membrane and mediate membrane fusion (52,163). A linear hydrophobic domain typical of fusion domains in other viral fusion proteins is not obvious in the VSV G protein sequence, although the N-terminus is somewhat hydrophobic. Initial studies with small hydrophobic peptides corresponding to the N-terminus of the mature VSV G protein showed that these could mediate hemolysis, and it was suggested that hemolysis might be equated with membrane fusion activity (178). However, a change in the peptide sequence that abolished hemolysis had no effect on fusion activity when introduced at the N-terminus of the VSV G protein (228). These results indicate that the hemolysis assay employing peptides is not always relevant to membrane fusion, but they do not rule out a direct role for the N-terminus in the membrane fusion process.

Studies with mutants of the VSV G protein have implicated the involvement of other domains in the fusion process. A site for N-linked glycosylation introduced at amino acid 117 completely abolished the membrane fusion activity of G protein without blocking folding, transport to the cell surface, or incorporation into virions (225). The proximity of this mutation to a sequence of 19 uncharged amino acids (amino acids 118 to 136) suggested that this domain might be involved in fusion. Analysis of linker insertion mutants that failed to induce cell fusion identified the same site as well as two other distant sites in G protein that affected membrane fusion (114). Additional studies pointed to the importance of amino acids 118 to 136 because mutations in this region either abolish fusion or affect the pH threshold for fusion (54,55,236). A hydrophobic photolabeling technique identified a peptide from residues 59 to 221 of VSV G protein interacting with the hydrophobic membrane core (45). Because this region encompasses residues 118 to 136, the result also provides support for the role of the 118 to 136 domain in membrane insertion during fusion. A region in a similar position in rabies virus G protein (residues 103 to 179) was identified using the same probe (45).

Matrix Protein (M)

The matrix (M) protein is the smallest and most abundant protein in the rhabdovirus virion. Despite its small size, M protein has many functions. VSV M protein plays critical roles in condensation of nucleocapsids during virus assembly, in disruption of the cytoskeleton, and in inhibition of host functions as described in the sections that follow. It also has an intrinsic ability to cause budding of vesicles from cells when expressed alone (89), a property that is probably important in natural budding and appears related to the presence of proline-rich motif that binds to WW domains of cellular proteins (71). A fraction of VSV M protein is associated with membranes in infected cells; most is cytoplasmic (30,31,131,231) whereas the rest of M protein is located in the nucleus of infected cells (123). Because of M protein association with the plasma membrane and with nucleocapsids, it has been proposed that M acts as a bridge between the viral envelope and the nucleocapsid (see Fig. 1). Recent studies of rabies virus support this location for its M protein (136), but other studies of VSV suggest that M protein may form a cigar-shaped internal structure, which has been proposed to form a scaffold around which the nucleocapsid is wound (9,10). It seems highly improbable that M protein could occupy different positions in rabies and VSV, but this issue awaits resolution with more definitive analytical techniques. A report that M protein can stabilize G protein trimers *in vitro* (119) and that membranes from cells expressing M protein bind nucleocapsids (30) also supports the idea that M bridges the gap between the nucleocapsid and the G protein. The VSV M protein is highly basic and does not contain hydrophobic domains that are sufficient to act as membrane spans. However, the amino terminus of VSV M may be membrane associated because it can be labeled with a hydrophobic photoaffinity probe (108).

The VSV M protein is phosphorylated on serine and threonine residues between amino acids 20 and 35, but the assembly functions of M protein are not disrupted when the major sites of phosphorylation are disrupted (92), and the

function of phosphorylation, if any, remains to be determined.

STAGES OF REPLICATION

The replication and assembly of members of the *Rhabdoviridae* family can be divided into distinct stages as depicted in Figure 5. Although many of these events occur simultaneously in an infected cell, it is convenient to consider the process of infection as a linear series of events that proceeds in the following order: adsorption, entry and uncoating, transcription, replication, assembly, and budding.

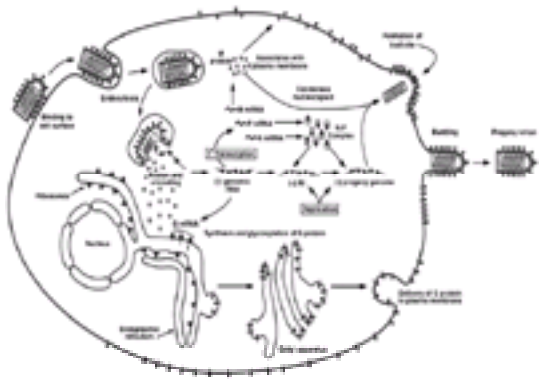

FIG. 5. Diagram of the life cycle of a rhabdovirus.

Adsorption

Rhabdoviral infection is initiated by attachment of virus to a receptor on the host cell surface. The receptors for the attachment of rhabdoviruses have been difficult to identify because of the generally broad host range and the binding properties of rhabdovirus particles. Virus adsorption is inefficient and difficult to quantitate for viruses in this family. For example, VSV binding is pH dependent, and maximal binding occurs between pH 6.5 and 6.0 (56,130); yet even at the optimal pH, binding fails to reach equilibrium (130). Furthermore, when virus is prebound at 4°C, only a limited amount of virus is internalized when cells are warmed to 37°C (130). However, virus infectivity can be increased more than 20-fold when virus is adsorbed at pH 6.3 versus when virus is bound at neutral pH (56). The pH dependence of binding correlates well with pH-induced conformational changes in the G protein (56), suggesting that the receptor-binding site in the envelope protein becomes exposed during the transition from the neutral to the acid conformation.

Kinetic analysis of VSV binding to Vero cells suggested that two separate sites (saturable and unsaturable) exist for attachment of VSV (179). To identify the saturable binding component, Vero cell membranes were disrupted with the dialyzable, nonionic detergent octyl- D-glucopyranoside. When analyzed in a competitive binding assay, the extract specifically inhibited the saturable, high-affinity binding of 35S-methionine–labeled VSV to Vero cells (177). The binding inhibitor was resistant to protease and neuraminidase but was inactivated by phospholipase C, suggesting it was a phospholipid. Of all phospholipids tested, only phosphatidylserine totally inhibited the high-affinity binding of VSV to Vero cells and also inhibited VSV plaque formation by 80% to 90% but did not block herpesvirus plaque formation (177). Based on these results, it is thought that phosphatidylserine may be one of the VSV receptors, at least on Vero cells. The identification of phospholipid binding domains in other rhabdovirus glycoproteins supports this idea and suggests that phosphatidylserine binding is a common feature of rhabdovirus envelope proteins (48).

The receptor for rabies virus has been controversial, and recent evidence indicates that several different receptors can be used. Sequence similarities between a region in the rabies glycoprotein and snake venom neurotoxins initially led to the hypothesis that the nicotinic acetylcholine receptor (AChR) is a receptor for rabies virus (109), and evidence supporting this has been obtained (62,70). Infection of cell lines that lack AChR, however, indicated that other molecules must also be used by rabies virus for entry (160). Recently, two additional receptors for rabies virus entry were identified. Using a random-primed cDNA expression library constructed from neuroblastoma cell (NG108) mRNA, the murine low-affinity nerve-growth factor receptor was identified as a functional receptor for street rabies virus (212). In a separate study, it was shown that the neural cell adhesion molecule can also serve as a receptor for the CVS (challenge virus standard) strain of rabies virus both in cultured cells and in mice (211).

Entry and Uncoating

After binding, the virions are endocytosed through a clathrin-dependent pathway typical of receptor-mediated endocytosis (130). Subsequent reductions in the pH of the endocytic compartment eventually trigger a membrane fusion reaction between the envelope of the endocytosed virion and the endosomal membrane. This fusion event is catalyzed by the G protein and results in the release of the ribonucleoprotein (RNP) core into the host cell cytoplasm (130). Either concomitant with membrane fusion or immediately after, M protein dissociates from the RNP core (164). The combined processes of membrane fusion and M protein dissociation constitute the uncoating event for rhabdoviruses. The trigger for M protein dissociation from RNPs is not known.

Transcription

The first synthetic event that occurs after uncoating of RNPs is transcription of viral-specific mRNAs by the L–P3 polymerase complex brought into the cell by the virion. Primary transcription occurs in the absence of protein synthesis, unlike genome replication, which requires newly synthesized N and P proteins and ongoing translation (41). The template for VSV transcription is the genome RNA complexed with the N protein in a ribonuclease-resistant form. As discussed

previously, both the L and P proteins are required for polymerase activity (47). The P protein does not possess any known enzymatic activity, but instead acts as an accessory factor that may modify the activity of L protein through the phosphorylation status of P. The importance of P protein phosphorylation for transcription has been studied most extensively for VSV. Phosphorylation of serine and threonine residues in the N-terminal domain I of P protein by casein kinase II converts P to a transcription-competent state using an *in vitro* transcription assay (12). Although the absolute requirement for P protein phosphorylation in transcription has been questioned (195), studies using a transcriptionally active VSV mini-genome expressed from plasmid DNA support the conclusion that phosphorylation is required for transcription *in vivo* (151). The additional negative charge of the phosphates presumably induces conformational changes and results in the formation of P protein trimers that bind to L protein to constitute the active polymerase (61). There is also a recent report that the L protein of VSV requires cellular translational elongation factors EF-1a, -1b, and -1g to be fully active in an *in vitro* transcription assay (38), indicating that host factors may also contribute to the activity of the polymerase.

Transcription begins at the exact 3' end of the genome, where the polymerase first synthesizes a small 47-nucleotide RNA called the *leader.* Each of the five mRNAs encoding the viral proteins is then synthesized in the order it appears from the 3' end of the genome. The leader is neither capped nor polyadenylated, but it is encapsidated once sufficient quantities of the N protein, in the form of N–P complexes, are synthesized.

Sequential transcription of VSV genes was first shown indirectly through the effect of increasing doses of ultraviolet (UV) irradiation on synthesis of the individual transcripts detected in a coupled transcription-translation system (7) and also by direct analysis of the transcripts separated by electrophoresis (1). Sequential transcription was also demonstrated directly by nucleic acid hybridization of sequences synthesized *in vitro* or by detection of specific oligonucleotides from the 5' ends of the mRNAs (83,84). The latter studies were inconsistent with independent initiation of mRNA synthesis on each gene because the synthesis of 5' mRNA sequences was dependent on transcription of the preceding mRNA. Studies involving reconstitution of purified nucleocapsid template with solubilized transcriptase showed that only leader RNA sequences are made initially and that mRNA oligonucleotides appear only after full-length leader has been synthesized (46). Thus, the VSV polymerase is not free to initiate transcription independently on each gene, and sequential transcription appears as a result of an obligatory entrance of all polymerases at the leader gene.

In vitro transcription studies also demonstrated that the VSV polymerase pauses for 1 to 2 minutes at each gene junction and then reinitiates synthesis on the downstream gene with only 70% to 80% frequency (83). The pause time may represent, at least in part, time required to copy repetitively the U7 sequence to generate the poly(A) on each mRNA.

Transcriptional attenuation is a common feature of nonsegmented negative-stranded RNA viruses, and this phenomenon results in a gradient of mRNA synthesis such that the N message is the most abundant in infected cells and the L message is the least abundant (83,214). Therefore, VSV transcription is both sequential and polar. The polarity of transcription is an inherent property of the polymerase, and attenuation results from events that occur when the polymerase interacts with cis-acting signals at each gene junction. Extra foreign genes inserted into VSV are subject to and exert the same 20% to 30% attenuation effect (98,167,183), and when VSV's own genes are rearranged, there is still a 20% to 30% attenuation at each gene junction regardless of gene order (6,217).

The mechanism leading to the generation of individual transcripts during a rhabdoviral infection is not completely understood, but there is strong evidence supporting a start and stop model for rhabdoviral transcription. In this model, the polymerase initiates transcription at the 3' end of the genome and then terminates transcription at the leader– *N* gene junction. The polymerase then reinitiates at the conserved start site 3' UUGUC . . . . 5' of the *N* gene, and sequential transcription ensues. There is evidence from a VSV mutant called *polR* that the polymerase can initiate internally at the *N* gene start site (32), but this seems to be a unique property of the *polR* mutant that results from a mutation in the N protein, and once the leader–*N* gene junction is traversed, subsequent transcription occurs by the start and stop mechanism.

The use of transcriptionally active VSV mini-genomes (described later under Molecular Genetics of Rhabdoviruses) has allowed the dissection of cis-acting signals that are important for transcript initiation, capping and methylation, and polyadenylation and termination. Using a dicistronic mini-genome system, it was found that the first three nucleotides of the conserved 3' UUGUC . . . . 5' start sequence (complementary to the cap-AACAG at the beginning of each mRNA) were the most important for VSV gene expression. The U at position 1 and G at position 3 were absolutely required for transcript accumulation in infected cells, whereas the second position could be a pyrimidine, although U was preferred (201). Remarkably, mutations at the second position and substituting a C for a U at the first position did not prevent initiation per se, but instead affected modifications at the 5' end of the mRNA as well as polymerase processivity (203). Because the polymerase is responsible for addition of the 5'-7mGpppG cap, the conserved sequences at the beginning of each gene appear to contain separate but overlapping signals for capping and for transcript initiation. Thus, transcript initiation and 5' end modification (presumably capping) are linked, yet separable, events.

The mechanism by which VSV transcripts are capped has not yet been defined, but because the entire VSV life cycle is carried out in the cytoplasm of the cell, it is thought that the host nuclear enzymes are not involved. Analysis of the cap structure found on VSV transcripts revealed that both the a and b phosphates of the 5'–5' triphosphate linkage of the guanosine cap are contributed by a guanosine diphosphate (GDP) donor (2). Therefore, the addition of the 5'–5' guanosine cap cannot be mediated by a covalent nucleotidyl transfer reaction with guanosine monophosphate (GMP) as occurs during typical eukaryotic capping. Available evidence indicates that the polymerase complex mediates the capping reaction because mRNAs generated during *in vitro* transcription reactions with detergent solubilized purified virions are efficiently capped (2). Although the L protein does share some sequence homology to nucleotide binding proteins, it shows no homology to covalent nucleotidyl transferases (192), and there have been no mutations identified in L protein that affect capping. However, viruses that are deficient in methyltransferase activity have been isolated, and the defect has been mapped to L protein (74). Recently, it was suggested that the GTP- and GDP-binding properties of the a subunit of EF-1 associated with L protein may play a role in the VSV capping reaction (38). It is possible that other host proteins may associate with L to mediate transcript capping, but as mentioned previously (L protein section), these must

be found on only a fraction of the polymerase complexes found in virions.

After transcript elongation, the polymerase encounters the conserved stop-polyadenylation signal consisting of 3'-AUAC(U7)-5'. Two events can occur once the polymerase reaches this sequence. For most transcripts, the polymerase pauses and reiteratively copies, or “stutters,” over the seven uridinylate residues to produce a poly(A) tail about 150 nucleotides in length before terminating. Occasionally, the stop-polyadenylation signal is ignored, and transcription continues past the intergenic region and through the next gene, generating a read-through transcript that becomes polyadenylated at the downstream stop-polyadenylation site (75). Studies to define the stop-polyadenylation signal revealed that reducing the length of the U7 tract, even by one nucleotide, or changing the C of the preceding 3'-AUAC(U7)-5' to any other nucleotide prevents termination and causes exclusive read-through transcription at the mutated gene junction (13).

After polyadenylation, the polymerase scans through the intergenic dinucleotide in a nontranscriptive mode until the next transcription start sequence is encountered (14,201,202). The attenuation of expression observed for downstream genes presumably results from some of the polymerases dissociating from the template after polyadenylation or during the transition from polyadenylation and termination to reinitiation at adjacent downstream gene.

Genome Replication

During transcription, the VSV polymerase responds to signals that result in the synthesis of the leader RNA and individual mRNAs. In contrast, during replication, the polymerase must ignore these signals and synthesize genome-length positive-stranded RNA. This RNA is then replicated to form the genomic negative-stranded RNA. Unlike transcription, VSV replication requires ongoing translation, particularly of viral N and P proteins. This was found initially because inhibition of protein synthesis by cyclohexamide would prevent RNA replication but not transcription (41). The original concept that the N protein alone is sufficient for replication of VSV RNPs has been superseded by evidence that ongoing synthesis of both N and P proteins, complexed in a 1:1 molar ratio, is far more efficient for supporting viral replication *in vitro* (152). The basis for this requirement was initially explained by a widely accepted model for negative-stranded virus replication, which also accounted for the switch from transcription to replication. In this model, encapsidation of leader RNA by newly synthesized N protein (22) causes an antitermination event that signals the polymerase to begin processive RNA synthesis such that the signals for mRNA initiation and termination are ignored (3,21). Therefore, once sufficient quantities of N protein (or more appropriately, N–P complexes) have accumulated, there is a switch from primarily discontinuous mRNA transcription to the synthesis of genome-length, positive-sense replicative intermediates (RI). The RI, like the genome, must also be fully encapsidated to serve as a template for the synthesis of progeny negative-sense genomes. This model is attractive because immediately after virus entry, transcription would be favored over replication. At later times, when the concentration of N protein increases, replication would be favored.

More recently, a modification of this model has been developed that suggests that transcriptase and replicase are two different complexes, each with a unique composition. The two-polymerase model arose from studies using P protein mutants that were defective in transcription but that could support efficient replication of VSV defective interfering (DI) RNAs (39,151). The P protein mutants either had substitutions in the conserved C-terminal basic residues and could bind N protein but did not interact efficiently with L protein (39), or were defective in domain I phosphorylation (151). Because phosphorylation is required for P protein oligomerization, transcriptional activation, and interaction with L protein, the transcriptase would be composed of an L–P3 complex, whereas the replicase would consist of an L–(N–P) complex in which P protein is not phosphorylated. Regulation of P protein phosphorylation and ongoing viral protein synthesis would therefore affect the formation of the two different complexes at different times after infection.

Encapsidation of the genome and antigenome by N protein is intimately tied to and essential for virus replication. Encapsidation occurs as the genomic RNA is synthesized rather than after synthesis is complete, which fits well with the antitermination model described previously for the switch from transcription to replication. It was recognized early that the RNA encapsidation signal must reside in the 5'-terminal sequences of the genome (the trailer region) and the antigenome (the leader) because the leader RNA, the RI, and progeny genomes are the only virus-specific products that are encapsidated (3,21,110). Sequence comparisons between different serotypes of VSV revealed that 14 of the 18 terminal nucleotides of the genome are highly conserved. From this observation, it was postulated that five adenine residues repeated at every third position from the 5' end of either the genomic or antigenomic RNA are important for the selective encapsidation of these RNAs (21,23). *In vitro* encapsidation reactions using synthetic RNAs containing the 19 terminal nucleotides of VSV and either purified N protein or N protein synthesized *in vitro* (36,141) supports this hypothesis. More recent studies using subgenomic replicons have confirmed that the 5'-terminal residues of the genome are critical for efficient encapsidation (151); however, it appears that sequences beyond the first 19 nucleotides, yet within the 5' terminal trailer region, are also important and greatly affect the efficiency of the process (221).

For rabies virus, leader encapsidation may also be regulated by the phosphorylation status of N protein. Unlike the VSV N protein, which is not phosphorylated, rabies N is phosphorylated on serine residues, and the unphosphorylated form of N protein binds more tightly to leader RNA *in vitro* (230).

One of the challenges in understanding rhabdovirus replication is that two distinct events must occur sequentially: first, production of a complete positive RNA strand complementary to the entire parental template, followed by production of complete negative-stranded (progeny) RNAs. There is also a significant asymmetry in the amount of positive-sense RI to negative-sense genome that is made in infected cells. For both rabies and VSV, the genome is about 20- to 50-fold more abundant than the RI. The basis for this asymmetry appears to result solely from the activity of the promoter sequences found at the 3' end of the genome or RI.

It had long been assumed that sequences at the extreme 3' end of the genomic and antigenomic RNA were important for polymerase binding and that the complementary sequences found at the 5' ends were required for encapsidation by N protein. Recently, specific sequences have been identified that play a role in regulating the balance between transcription of mRNAs, which are not encapsidated by N protein, and replication of the genome and antigenome, which

are encapsidated. Using deletion analysis and cDNA-derived model subgenomic RNAs based on a naturally occurring defective-interfering virus of VSV, Pattnaik and colleagues have shown that the signals necessary for both replication and encapsidation are present within the 5'-terminal 36 nucleotides and the 3'-terminal 51 nucleotides (149). This result confirms the long-standing hypothesis that the promoter for polymerase binding is contained within the terminal sequences of the genome. A subsequent study further delineated the minimal replication signal as being the 3'-terminal 24 nucleotides of the trailer complement; however, the sequence of the next 20 nucleotides, specifically those found at the 3' end of the positive-sense RI, serves as a specific enhancer of replication (113). Therefore, sequences at the 3' end of the antigenome of wild-type VSV provide the basis for differential (enhanced) replication of the genome over the antigenome in infected cells. Similarly, distinct elements in leader region have been identified that are important for transcription but that are not required for replication (221).

In an elegant demonstration of the power of reverse genetics, Finke and Conzelmann constructed an ambisense rabies virus genome that contained identical, transcriptionally active (genomic) promoters at the genomic and antigenomic termini and that transcribed a foreign gene from the antigenomic RNA. Analysis of replication products revealed that the ratio of genome to antigenome was 1:1 in infected cells, indicating that promoter strength was primarily responsible for maintaining the balance of genome to RI. This study also showed that packaging of rabies genomic or antigenomic RNPs into virions is random and depends only on the ratio of the RNPs in infected cells. For wild-type rabies virus, genomic and RI RNPs were present in cells at a ratio of 49:1 and were incorporated into virions at the same ratio. In contrast, the ambisense mutant genomic and antigenomic RNAs were packaged equally into virions.

Although the ratio of genome to antigenome is primarily affected by sequence-specific elements in the antigenomic promoter, there is evidence that other factors may also contribute to the replication efficiency of rhabdovirus genomes. For example, it has been suggested that the extent of terminal complementarity can also affect whether the template (in this case, a defective interfering RNA) predominantly directs transcription or replication (218). Thus, replication may also be regulated by interactions between the ends of the genome. These authors suggested that there is communication between the ends of rhabdoviral genomes through base pairing and that this somehow affects replication efficiency (218).

Assembly and Budding

The process of rhabdovirus assembly can be divided into three distinct phases: (a) encapsidation of newly replicated genomic RNA by N protein, (b) simultaneous condensation of the ribonucleocapsid core by M protein and association with the plasma membrane, and (c) particle envelopment and release. The first step, encapsidation, occurs in the cytoplasm and results when nascent genomic RNA associates with newly synthesized N, P, and L proteins to form RNP complexes. These are often referred to as *nucleocapsids* or *RNP cores.* Because RNA encapsidation is required for RNA replication and transcription, this step was described in the preceding section.

After encapsidation, the RNP complex associates with the plasma membrane and condenses into a tightly coiled structure called a *skeleton* before release from the cell. Numerous studies have demonstrated that the matrix protein is responsible for both of these events (10,64,93,121,122,136). Thus, M protein plays a critical role in virus assembly and budding. Most of our understanding of RNP condensation is derived from *in vitro* studies in which RNP cores are isolated from detergent solubilized virions in high salt buffers, which results in the release of M protein in a process somewhat analogous to virus uncoating after membrane fusion. RNPs isolated under these conditions appear as loosely coiled, extended filaments and are transcriptionally active. The process can be reversed by the addition of purified M protein to the extended RNP cores in low ionic strength buffers, which results in RNP condensation and the formation of skeleton-like structures similar to those seen within virions (142). The addition of M protein to RNPs also inhibits VSV transcription *in vitro* (29); therefore, the reversible nature of RNP condensation by M protein is thought to be critical for virus infectivity (121).

Condensation of RNP cores provides an explanation for the inhibitory activity of M protein, but it also poses a dilemma. How can viral transcription levels remain high when the cytoplasm of infected cells contain high concentrations of RNP cores and M protein? One explanation is that RNP condensation does not occur until the RNP cores associate with the inner leaflet of the plasma membrane just before budding (131,147); therefore, RNP condensation is a highly regulated process. The demonstration that 10% to 20% of M protein associates with the inner leaflet of the plasma membrane (30) and that the membrane-associated fraction can bind nucleocapsids partially stripped of M protein (31) provided an explanation for the regulated assembly of RNPs at the cell surface. However, recent evidence suggests that an additional priming event is needed because neither cytoplasmic nor membrane-associated M protein can bind to RNPs that are completely free of residual M protein (51). The priming event may involve the formation of specific M protein complexes (63) or may require modification of the RNP core by host factors (51). The topology of M protein with respect to the RNP core during RNP condensation has been a topic of debate and was discussed previously.

After RNP condensation, rhabdovirus particles are released from infected cells by budding from the cell surface. During budding, the condensed core becomes enclosed within a membrane envelope that, for VSV, has about 1,200 molecules of virally encoded G protein and smaller amounts of normal cellular membrane proteins. Early models of rhabdovirus assembly postulated that the incorporation of G protein into virions results from specific interactions between the cytoplasmic tail of G protein and components of the condensed RNP core (139,193). Evidence supporting this model came from studies showing that G proteins with truncated cytoplasmic tails were not efficiently incorporated into virions (223) and that the addition of the G tail was necessary for the incorporation of heterologous viral glycoproteins into virus particles (133,148). G protein, however, is not needed for VSV or rabies budding, although it greatly enhances budding efficiency (96,134,185,205). Cells infected with recombinant viruses recovered from plasmids in which the G gene was deleted produced noninfectious particles with the characteristic bullet-shaped morphology of rhabdoviruses. Therefore, interactions between G protein and the condensed RNP core are not required to initiate the budding process, although they enhance its efficiency 10- to 30-fold.

Studies to define a role of the G protein cytoplasmic tail in virus assembly have revealed that rabies virus and VSV have distinct requirements. Except in one case in which the cytoplasmic tail of a heterologous protein contained a sequence

that greatly reduced incorporation of the glycoprotein into VSV particles (87,148), appending the cytoplasmic tail of VSV G protein to heterologous proteins does not increase virus budding or glycoprotein incorporation (90,168,182). Similarly, the recovery of infectious VSV containing G proteins with truncated cytoplasmic tails or chimeric G proteins with foreign cytoplasmic sequences indicated that a short cytoplasmic domain is important for efficient virus budding but that the specific amino acid sequence in that domain was not important (181). In contrast, the assembly of foreign glycoproteins into rabies virus appears highly dependent on the rabies G cytoplasmic tail (132,133), although infectious rabies virus can be recovered when the entire cytoplasmic domain is deleted (134). The basis for this difference between rabies virus and VSV is not known, but it may reflect a fundamental difference in the way that the M–RNP core interacts or organizes the G protein spikes in the viral envelope.

In comparison with the role of G protein in virus budding, mutation or deletion of the M gene has a much more drastic effect on virus assembly. Temperature-sensitive M protein mutants of VSV produce very low levels of spherical or pleomorphic-shaped particles at the restrictive temperature (122). Similarly, a recombinant rabies virus lacking the M gene produces about 10^5 fewer particles than the wild-type virus, and these particles have a filamentous morphology (136). Complementation with the wild-type M protein expressed from plasmids increases virus budding (136) and converts both the VSV and rabies M mutants to the typical bullet-shaped morphology of rhabdoviruses (122,136). The observation that transient expression of M protein, in the absence of other viral proteins, causes membrane evagination and the release of small membrane-enclosed vesicles (89) supports the idea that M protein has an intrinsic budding activity. Such an activity is reminiscent of the ability of retrovirus Gag proteins, when expressed alone, to produce virus-like particles, and it has been proposed that rhabdovirus M proteins and retrovirus MA proteins may be structurally and functionally similar (107). The recent demonstration that a highly conserved motif (PPxY) in the N-terminus of rhabdovirus M proteins can functionally substitute for the late-domain (L domain) of an avian retrovirus gag protein provides support for this hypothesis (35). Point mutations in the PPxY motif of VSV M protein do not affect membrane localization but significantly reduce both M-dependent vesicle budding (71) as well as the amount of virus released from infected cells (86). Retrovirus L domains function in the last step of virus budding, namely membrane fission and virus release (227). Therefore, the PPxY motif in rhabdovirus M proteins may also be involved in virus release but not RNP condensation or the initiation of virus budding. Thus, high-level virus production results from the concerted action of both the M and G proteins, and only when M protein is absent do rhabdoviruses fail to assemble.

Although far from complete, the available data support a model of assembly in which nucleocapsids bind to regions of the plasma membrane enriched in both M and G proteins. Once nucleocapsids bind to these regions, RNP condensation occurs, and formation of the bud site is initiated. In VSV, specific interactions between the cytoplasmic tail of G protein and the condensed RNP core appear not to be required, although a short cytoplasmic tail of G may insert into a pocket on the condensed core and promote a specific arrangement of G trimers in the viral membrane (181).

VSV buds preferentially from the basolateral surface of polarized epithelial cells (25), a property that is likely important in systemic spread of the virus in infected animals. The sorting of VSV G protein to the basolateral domain of polarized cells independent of other VSV proteins (65,199) and the presence of a dominant basolateral sorting signal in VSV G (210) could explain the preferential polarized release of VSV from the basolateral surface. There is also evidence for independent polarized sorting of M protein to the basolateral membrane (15), but signals in M protein involved in the sorting have not yet been identified.

Because specific sequences in the transmembrane and cytoplasmic domains of VSV G are not required for efficient budding, it seemed likely that G protein sequences important for driving efficient budding would reside in the ectodomain. The recent identification of a “budding domain” in the membrane-proximal region of the G ectodomain provides support for this hypothesis (168). The mechanism by which this domain acts to enhance budding is not understood. It could act to promote association of large arrays of G protein, which would induce membrane curvature at the bud site, or it could have more indirect effects on modifying the local lipid environment to promote interactions with areas of the membrane containing M–RNP complexes. The final step in virus budding requires a membrane fission event that results in release of the virus from the host cell. Little is known about how this occurs, but it has been suggested that the PPxY motif in M protein facilitates virus release through the recruitment of host factors that contain WW domains (35,71).

The use of reverse genetic systems has greatly advanced our understanding of protein domains involved in rhabdovirus assembly, but there are still many questions that remain unanswered. For example, little is known about the contribution that host factors play in virus assembly and release. A complete understanding of the roles of individual viral proteins and of potential host factors involved in virus budding will likely require detailed structural information on the individual components as well as sophisticated biochemical assays to measure directly protein–protein and protein–lipid interactions.

Production of Defective Interfering Particles

As with many other viruses, passage of rhabdoviruses in tissue culture leads to accumulation of defective interfering viruses called DI particles. These particles have a normal VSV protein composition but have truncated RNA genomes lacking some or all of the viral structural protein genes and therefore they can only propagate in cells co-infected with parental virus. The DI particles out-replicate parental virus and rapidly accumulate to higher levels than parental virus during passage at high multiplicity. Elimination of DI particles from virus cultures can be accomplished by low-multiplicity infection and repetitive isolation of virus from single plaques. DI particles can also be separated from the parental virus based on velocity of sedimentation on sucrose gradients. Perrault (153) has provided an excellent review of the DI particle structure and formation.

Typical types of VSV DI particles are illustrated in Figure 6. The most common type of DI particle contains RNAs with complementary terminal sequences that rapidly self-anneal to form short stems or longer snap-back structures after the template-associated N protein is removed. These DIs lack the 3' leader sequence (Le) used to initiate transcription of the viral genes and replication of the antigenomic RNA. These DIs have a strong replicative advantage over standard genomes. Because of the self-complementary nature of the DI genome ends, replication of either strand initiates on the

complement of the 5' trailer sequence (Tr'). This is the same sequence that serves as the promoter for synthesis of VSV genomic RNA and is a stronger promoter than the 3' leader sequence (Le), which initiates synthesis of antigenomic RNA. This difference in relative promoter strengths discussed in the preceding section on genome replication would explain the excess synthesis of genomic RNA relative to antigenomic RNA in infected cells. DI RNAs that retain both ends of standard genome at their termini are less common and do not compete as well against the standard genome, probably because they, like the standard genome, are involved in transcription as well as replication and do not have as strong a promoter for their positive-stranded RNA replication.

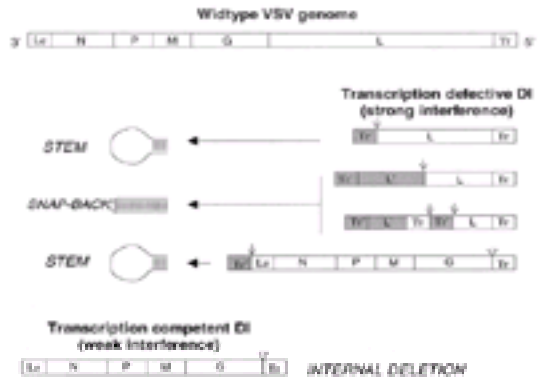

FIG. 6. Schematic representation of different classes of rhabdovirus DI particles. (Courtesy of Dr. J. Perrault, San Diego State University.)

Generation of DI RNA molecules from a standard virus is believed to occur after a viral polymerase with its attached nascent RNA chain dissociates from its template and resumes synthesis at a new site either on its own nascent chain or on a different template molecule (153). Generation of the first two types of DIs illustrated in Figure 6 could occur by a single switch (*arrow* indicates switch site) of the polymerase to copy its own nascent chain. Other, more complex types of DIs can result from multiple polymerase switches, including switches to downstream sites on a template to generate internal deletions (*triangles* in Fig. 6). Multiple switch events are possible, and DIs with more extensive genome rearrangements have been reported (146,176).

MOLECULAR GENETICS OF RHABDOVIRUSES

The ability to perform precise molecular genetic analysis of rhabdoviruses required the development of systems for recovering infectious viruses from DNA copies. Recovery of rhabdoviruses and other nonsegmented negative-stranded RNA viruses (NNS viruses) from DNA presented a unique problem because neither their genomic nor their antigenomic RNAs serve as mRNAs, and neither can be used directly to recover infectious virus. This contrasts with the positive-stranded RNA viruses in which the genomic RNA transcribed from DNA can be used to initiate infection. The minimal infectious unit for the NNS RNA viruses is the genomic RNA complexed with nucleocapsid and RNA polymerase proteins in a RNP complex. Because the NNS RNA viruses have genomes that are quite large (11 to 18 kb), it has not been possible to assemble their RNAs into infectious RNPs *in vitro,* an approach that did work earlier to introduce individual RNA segments into influenza virus (118).

Recovery of Infectious Rabies and Vesicular Stomatitis Virus from Plasmid DNA

Before the recovery of complete, infectious negative-stranded RNA viruses from DNA, several laboratories developed systems that allowed replication of small, defective negative-stranded RNA genomes or RNA mini-genomes that were derived from DNA copies. The RNA mini-genome constructs contained one or more genes flanked by short 3' and 5' sequences derived from the natural virus genome and containing the signals required for replication (reviewed in reference 167). The first such system for VSV employed a defective interfering particle RNA that was expressed from plasmid DNA (150). The VSV proteins required for replication of this RNA (N, P, and L) and for assembly of infectious particles (M and G) were provided by a vaccinia virus-based expression system (57). A related system was developed for a rabies virus mini-genome (34). The rabies construct contained short 3' and 5' rabies genomic sequences flanking an expressed bacterial gene (*CAT*). A similar mini-genome system was also developed for VSV and contained VSV *G* or *G* plus *M* genes flanked by VSV terminal genomic sequences (200). The replicating mini-genome and DI RNA systems were important for the eventual development of systems for recovery of complete infectious rhabdoviruses from DNA. As described later, however, they did not work to generate infectious virus starting with expression of the the negative-stranded genomic RNA.

The basic system for recovery of all nonsegmented negative-stranded RNA viruses was established first in the rhabdoviruses. In 1994, Schnell and colleagues (186) reported successful recovery of rabies virus from cDNAs. This was accomplished by transfecting cells with plasmids encoding the viral transcription complex components under T7 promoter control (N, L, and P) into cells that had first been infected with recombinant vaccinia expressing T7 polymerase (57). In addition, a plasmid encoding the full-length viral antigenome under T7 promoter control was transfected into the cells (Fig. 7). Supernatants from these cells contained infectious rabies viruses. Use of an antigenome instead of the genome is important because it avoids the problem of hybridization of genomic RNA to the mRNAs transcribed from the plasmids encoding N, P, and L proteins. When one starts with the positive-stranded antigenome, this RNA can form an RNP without any interference from the mRNAs. Once in RNP form, the positive strand can then be replicated to form full-length negative-stranded RNPs that are wrapped into RNPs as nascent RNA chains and thus are immune to interference from mRNAs.

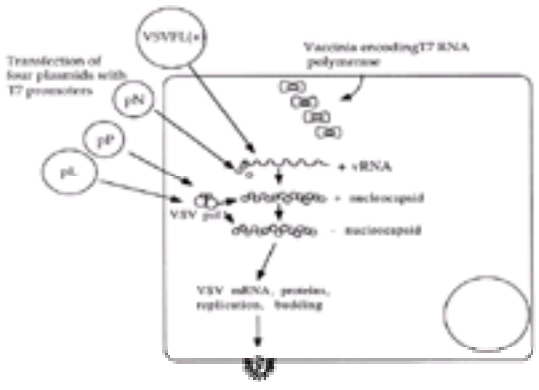

FIG. 7. Diagram of the system for recovery of rhabdoviruses from plasmid DNA.

Within a few months of the rabies report, infectious VSV was recovered from a plasmid DNA copy using a protocol very similar to that reported for rabies (102), and this was subsequently repeated (220). An infectious recombinant in which the Indiana serotype glycoprotein gene was replaced with the New Jersey serotype glycoprotein gene was also recovered, demonstrating the potential of the system for generating chimeric viruses (102). Subsequent application of the same or similar methodologies has led to recovery of numerous other NNS viruses from DNA copies (reviewed in 167).

Rhabdoviruses as Gene Expression, Vaccine, and Targeting Vectors

As described previously, the reverse genetic systems for rhabdoviruses have been crucial to dissecting numerous aspects of virus replication and assembly. Additional foreign genes can be introduced into recombinant rhabdoviruses, and foreign gene expression is generally stable (135,183). In VSV vectors, the expressed proteins can represent as much as 5% of the total cell protein 7 hours after infection (167,183).

The potential of recombinant VSVs as vaccine vectors was shown in a study in which a VSV recombinant expressing an influenza hemagglutinin gene was used in mice. A single intranasal inoculation with as little as 10 plaque-forming units induces a potent and protective immune response to influenza (166). VSV vectors have also been attenuated through mutations in the cytoplasmic tail of VSV G protein such that there is little or no pathogenesis even in 6-week-old mice (165), yet strong and protective immune responses are still generated. Ongoing studies of VSV vectors in rhesus macaques indicate a lack of pathogenesis and induction of strong cellular and humoral immune responses to encoded HIV envelope protein (J. Rose and colleagues, unpublished observations). These studies indicate that VSV may have potential as a vaccine vector in humans.

Other studies have illustrated the potential of recombinant VSVs as targeting vectors. A recombinant virus lacking its glycoprotein gene but encoding instead the human immunodeficiency virus (HIV) receptor and coreceptor was shown to incorporate both foreign glycoproteins into its coat. Furthermore, this virus could specifically target, infect, and kill HIV-infected cells *in vitro* (185). Related studies performed with rabies virus pseudotypes also suggested the potential of rabies as a targeting vector (133).

The potential of rhabdovirus recombinants as surrogate viruses for viruses that are dangerous or impossible to grow was illustrated by a recent study in which the VSV glycoprotein gene was replaced with an HIV envelope gene. In addition, a gene encoding green fluorescent protein was included (24). This virus infects cells using the HIV receptor and coreceptor and can be used as a fast and convenient assay for HIV neutralization. In another strategy, investigators have used a virus lacking G protein but encoding green fluorescent protein (GFP) to generate pseudotyped virus containing the envelope glycoprotein of Ebola virus. The pseudotypes allowed the identification of cell surface determinants important for Ebola virus infection (205) and resulted in the identification of a putative fusion domain in the Ebola glycoprotein (82) without the need to work under BSL-4 containment conditions. The promiscuous nature of VSV assembly that allows for extensive incorporation of foreign proteins into the envelope suggests that numerous similar applications are possible.

Given the extensive progress with reverse genetics of rhabdoviruses made recently, we can expect to see many new applications of this exciting technology in the years to come.

MOLECULAR BASIS OF PATHOGENESIS

General Aspects

Despite the similarities in structure between the lyssaviruses and vesiculoviruses, the former generally cause a relatively slow and progressive disease, and the latter cause acute disease that is eliminated rapidly by host immune responses. The pathogenesis of rabies virus is addressed in a subsequent chapter, and the veterinary aspects of vesiculovirus pathogenesis have been thoroughly and recently reviewed (112).

The basis for VSV pathogenesis appears intimately tied to the replication potential of the virus. For example, a mutation truncating the VSV G cytoplasmic domain from 29 amino acids to 9 amino acids reduces the virus yield only two-fold in tissue culture (181) but nearly eliminates pathogenesis in a mouse model (165). Complete elimination of the cytoplasmic domain reduces the virus yields about 10-fold (181) and completely eliminates pathogenesis in the mouse model while still allowing replication sufficient to induce strong immune responses (165). Similar results have been obtained using rearranged VSV genomes in which reduction in virus yield in culture correlates with reduced pathogenesis in animals (217). Rapid production of new virions is probably crucial to virus spread before immune responses result in virus clearance. In an infected animal, in which virus replication is likely an exponential process initially, even two-fold differences in final virus yield per cell in tissue culture are rapidly amplified to much greater differences in virus load in an

animal.

Almost all vertebrate cells tested and many invertebrate cells are susceptible to VSV infection. The replication of VSV can vary widely in different cell types, and infectious virus yields also vary, but little is known of the molecular basis for this variation. For example, VSV yields in baby hamster kidney cells (BHK-21) are about 100,000 particles per cell, of which about 10% can be infectious. Optimal VSV titers obtained in BHK cells range from 10^9 to 10^{10} per milliliter of medium. In contrast, VSV yields in HeLa cells are typically less than 5% of those obtained in BHK cells. The human lymphoblastoid cell line Raji is infected by VSV, but the viral RNA synthesis is low, and the VSV particles produced have an abnormal protein composition with only low levels of matrix protein (145,219). Rabbit corneal cells are infected by VSV but produce less than one infectious particle per cell. Co-infection with vaccinia virus reverses the block to VSV replication through an unknown mechanism (208). In *Drosophila* melanogaster cells, VSV establishes a persistent, noncytopathic infection, and virus titers are low (229). This may be due in part to a deficiency of VSV G protein in the viral particles that are produced (229).

At high multiplicities of infection in BHK cells, VSV cytopathic effects (cell rounding) can be evident 1 to 2 hours after infection. At lower multiplicity, the cytopathic effects may not be evident for 4 to 5 hours in BHK cells. In other cell types in which VSV replication is less robust, cytopathic effects often require longer to become evident.

The cell rounding effects of VSV appear to be due largely to expression of the VSV M protein. M protein expression alone in cells causes rounding and detachment by rapidly depolymerizing actin as well as tubulin and vimentin. This causes the breakdown of stress fibers, microtubules, and intermediate filaments (20,120,137,231). The VSV glycoprotein is also toxic to cells when expressed in large quantities, although stable cell lines can be made constitutively expressing low levels of G protein (53,158). The mechanism of toxicity of VSV G protein is not known, but it may involve a low level of membrane fusion activity even at neutral pH.

Inhibition of Cellular Protein Synthesis

VSV infection results in substantial inhibition of cellular protein synthesis within 3 to 4 hours after infection when the VSV proteins are the major proteins synthesized by the infected cells. When total poly(A)-positive mRNAs are purified from infected cells and translated *in vitro* in reticulocyte lysates, the mRNAs encode predominantly the VSV proteins, suggesting a major change in the ratio of viral mRNAs to host mRNAs in the infected cells (95). Lodish and Porter (117) carried out a study that suggested a competition mechanism for the inhibition of cellular mRNA translation. They found that the total amount of translatable mRNA in cells increased about three-fold after infection owing to substantial viral mRNA synthesis, and they suggested that inhibition of cellular protein synthesis could be explained at least in part by competition for ribosomes by the large excess of viral mRNAs. Based on analysis of polysome size, the efficiency of initiation of translation on cellular and viral mRNAs was found to be about the same in infected cells, but cellular ribosomes were distributed among more mRNAs after infection (117). A subsequent study (187) challenged this purely passive shutoff mechanism because severe limitation of VSV transcription did not substantially reduce the inhibition of host mRNA translation. However, a specific mechanism that discriminates between cellular mRNAs and viral mRNAs has not been identified. The fact that host mRNAs expressed from VSV recombinants are translated with efficiencies similar to viral mRNAs (98,182,183) suggests a lack of discrimination based on mRNA sequence alone, unless the selective specificity is present solely within the minimal 5'- or 3'-terminal nucleotides required on each mRNA as part of the VSV transcription initiation or termination signals.

Inhibition of Cellular Transcription, mRNA Transport, and DNA Synthesis

VSV infection results in inhibition of host cell mRNA synthesis and expression, and even a low level of the VSV matrix protein is sufficient for this inhibition (19). The mechanism of inhibition appears to result at least in part from inactivation of the transcription factor TFIID (233). In addition to having direct effects on host transcription, the VSV matrix protein blocks transport of mRNAs from the nucleus (73). This study showed that M protein interferes with transport that is dependent on the nuclear guanosine triphosphatase Ran-TC4 and its associated factors.

Other studies suggested a role for the VSV leader RNA in inhibition of RNA synthesis. VSV transcription is known to be required for inhibition of cellular RNA synthesis (216). Because the VSV leader RNA is transcribed first from the VSV genome, its transcription is most resistant to inactivation by UV light. Virions exposed to high doses of UV are still able to synthesize leader RNA and inhibit cellular mRNA synthesis (66), suggesting a role of the leader in this inhibition. *In vitro* transcription experiments using oligodeoxynucleotides having sequences homologous to leader RNA also supported a role for the leader in inhibition of transcription (67). However, other experiments showing a requirement for protein synthesis in inhibition of host RNA synthesis are not consistent with leader RNA alone being responsible for the inhibition of host RNA synthesis (156). The interpretation of the UV inactivation experiments was questioned by Whitaker-Dowling and Youngner (222), who showed that the doses of UV used to implicate leader RNA were sufficient to cause direct damage (cleavage) of VSV virion proteins.

The same types of UV inactivation experiments that implicated leader in inhibition of RNA synthesis also implicated leader in inhibition of cellular DNA synthesis. The mechanism of the inhibition is not known, but studies performed in an *in vitro* system for adenovirus replication showed that oligodeoxynucleotides corresponding in sequence to the VSV leader RNA could inhibit adenovirus DNA replication (162). The VSV leader is know to be rapidly transported into the cell nucleus after infection (101), a property consistent with a role in inhibition of both DNA replication and DNA-dependent transcription.

IMMUNE RESPONSES

There has been relatively little research on immunity to VSV in natural host animals, such as cattle, in which the virus infects epithelial cells and causes vesicular lesions. The virus is cleared effectively by the immune system, but the types of immune responses required for clearance have not been studied in the natural hosts. Interestingly, the immunity to

VSV after natural infection in cattle is not long-lived, and the same strain of VSV appears to be able to infect cattle in subsequent years, despite the presence of extremely high serum neutralizing antibody titers to the infecting strain (213). The mechanism of reinfection does not involve genetic drift in the virus glycoprotein as the sequence is unchanged in the reinfecting virus. There is also evidence that some VSV genomic RNA but not mRNA sequences can persist in experimentally infected cows after infectious virus has been cleared, but the mechanism of this persistence is not known (111).

Most studies of immunity to VSV have been performed in more tractable mouse systems, and the pathogenesis in laboratory mice is quite different from that in the natural host animals. When inoculated intranasally in some mouse strains, such as Balb/c or C57BL/6, VSV can infect the brain and cause a lethal encephalitis (78,161). VSV has also been found to replicate in the lungs of Balb/c mice after intranasal inoculation (165,166). Other routes of infection in mice, such as the intraperitioneal route, can induce immunity to VSV without significant pathogenesis, even when high titers of virus are inoculated. High and protective levels of antibodies to VSV persist in mice and diminish only two- to four-fold even 2 years after infection (165).

Role of Interferons

In VSV infection, as in infection with many other pathogens, interferons (IFNs) provide a critical front line of defense. Although VSV isolates differ in their ability to induce IFN (128), VSV replication is extremely sensitive to IFN action. The IFN response in mice slows the spread of VSV infection until an antibody response can completely control the infection. The importance of the IFN response was shown in IFN-a- and IFN-b-receptor–deficient mice (198). Infection of these mice results in rapid, disseminated VSV replication in multiple organs, and the mice die within a few days. Other studies with mice deficient in the JAK-STAT IFN signaling pathway have also revealed extreme sensitivity to VSV infection (138).

Roles of Antibody and Cytotoxic T Cells

The relative importance of antibody and cytotoxic T cells in immunity to viral infection varies depending on the virus and host in question. Antibody is a crucial component of immunity to VSV in mice. All neutralizing antibodies to VSV recognize the single exposed transmembrane glycoprotein G (94). Studies in IFN-receptor–deficient mice showed that passively transferred antibody to VSV G protected these mice efficiently against systemic infection (198). In contrast, passively transferred VSV-specific T cells were unable to protect these mice. The relative unimportance of T cells in immunity to VSV has also been shown in experiments involving mice lacking CD8+ T cells. These mice are as resistant to VSV as control mice with normal levels of CD8+ cells T cells (58). However, T-cell responses, although insufficient on their own, can protect from VSV-induced disease in an immunocompetent mouse. This was shown in experiments in which a T-cell response to purified VSV nucleocapsid protein protected mice from a normally lethal VSV infection (4).

Neutralization

Infection of mice by VSV induces a rapid production of neutralizing immunoglobulin M (IgM) antibody, which occurs independently of T-cell help, followed by appearance of neutralizing IgG antibodies that are dependent on T-cell help for their production (106). The ability of VSV to induce IgM independent of T-cell help appears to be related to the presence of G protein in a dense array on the viral surface (5).

The molecular requirements for antibody-mediated neutralization of VSV *in vitro* and protection against lethal disease *in vivo* have been studied in detail using a monovalent single-chain antibody and antibody fragments generated from VSV-neutralizing antibodies (91,103,104). These experiments demonstrate that neutralization of VSV and protection against disease do not require Fc-mediated mechanisms and that antibody cross-linking is not crucial for protection. Although the mechanism by which antibodies neutralize VSV has not been studied, it probably requires antibody physically shielding most VSV G spikes on the virion and thereby blocking attachment and fusion. This is likely because in rabies virus neutralization, the number of antibody molecules required for complete neutralization of a virion is at least half the number of viral glycoprotein spikes (50). Multiple epitopes binding neutralizing monoclonal antibodies have been described for the G proteins from the Indiana and New Jersey serotypes of VSV (26,105,215)

T-Cell Epitopes

Early studies in mice revealed that epitopes in both the VSV N and G proteins are recognized by cytotoxic T cells (157,232). The VSV Indiana nucleocapsid protein contains an immunodominant H-2 Kb epitope binding class I major histocompatibility complex in C57BL/6 (B6) mice. This peptide, N52-59 (RGYVYQGL), was among the first viral cytotoxic T-lymphocyte epitopes to be identified and sequenced (191). Subsequent studies have shown class II major histocompatibility complex restricted epitopes recognized by cytolytic T cells in Balb/c mice (27). The helper T-cell epitopes in the G protein have been analyzed with a complete panel of overlapping synthetic peptides. Three different helper T-cell epitopes in C57BL/6 mice and two in BALB/c mice were defined in VSV G (Indiana) (28).

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CHAPTER 39 Rhabdoviruses

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The family *Rhabdoviridae* consists of more than 175 different viruses isolated from both plants and animals. These enveloped viruses share a common elongated rodlike shape, encompassing single-stranded, negative-sense RNA as a primary unifying feature of the group. This distinctive morphology, of uncertain functional significance, separates rhabdoviruses from other taxa in the order *Mononegavirales*, the *Filoviridae* and the *Paramyxoviridae.* Rhabdoviruses can replicate in plants, in invertebrates, or in vertebrates. The *Rhabdoviridae* contain many members that are significant medical, veterinary, and botanic pathogens. This chapter concentrates on biologic features of the animal rhabdoviruses; it does not discuss either of the plant genera, *Cytorhabdovirus* or *Nucleorhabdovirus.* Currently, animal rhabdoviruses include three genera: *Lyssavirus*, *Ephemerovirus*, and *Vesiculovirus.* Many other rhabdoviruses have not received adequate study and are assigned to the family solely on the basis of morphology.

LYSSAVIRUS

History

Rabies is an archaic entity, one of the oldest recognized infectious diseases. Despite the historical preeminence of smallpox, influenza, and, more recently, AIDS, the continuing biomedical preoccupation with rabies is understandable because its “alarming manifestations in man and dog alike . . . and its almost inevitable progression to a fatal outcome have ensured unparalleled notoriety” (488). These concerns extend beyond the material to the spiritual plane, as revealed by the following prayer: “San Roque, San Roque, que este perro no me toque!” This supplication for protection to the patron saint against pestilence, taught to children in both the Old and the New World for invocation whenever they encountered a dog on the street, literally translates to “St. Roque, St. Roque, do not allow this dog to touch me!”—classically linking dogs, bites, and resulting misfortune. There is an even earlier passage that mentions the dangers of dog bites, in the pre-Mosaic Eshnunna Code of Mesopotamia, circa the 23rd century B.C.: “If a dog is mad and the authorities have brought the fact to the knowledge of its owner; if he does not keep it in, and it bites a man and causes his death, then the owner shall pay two-thirds of a mina [40 shekels] of silver. If it bites a slave and causes his death he shall pay 15 shekels of silver” (24).

Thus, ancient civilizations were quite familiar with rabies. In *The Iliad* (700 B.C.), Hector is compared to a rabid dog. Chinese scholars warned of the dangers of rabid dogs in 500 B.C., and Aristotle (4th century B.C.) correctly associated the disease with animals, but erroneously exempted humans from contracting it from a mad dog's bite. In Rome, Cordamus guessed that a poison (i.e., a “virus”) was present in saliva. Similarly, in the 1st century A.D., another Roman, Celsus, described clinical aspects of human infection: “The patient is tortured at the same time by thirst and by invincible repulsion toward water.” For prevention, he recommended immediate excision of the bitten tissue, cauterization of the wound by a hot iron, and, unfortunately, ducking the victim into a pool. The Hebrew Talmud, also dating from the 1st century, makes several references to the disease. Throughout the ages, ingestion of a wide variety of substances (e.g., the liver from a mad dog, crayfish eyes, a cock's brain or comb, or the cast slough of snakes pounded in wine with a male crab) and carrying sacred talismans were believed to be cures for rabies (24). Some have even suggested the basic

vampire and werewolf legends harken from human rabies cases (182).

A gradual transition from the medieval era of religious beliefs and superstitions to the Renaissance period of pragmatism and experimentation resulted in a remarkable treatise in 1546, entitled “The Incurable Wound” by Fracastoro. This Italian physician clearly stated that human beings are susceptible to rabies, and he vividly described a clinical case: “Its incubation [following a bite by a rabid animal] is so stealthy, slow and gradual that the infection is very rarely manifest before the 20th day, in most cases after the 30th, and in many cases not until four or six months have elapsed. There are cases recorded in which it became manifest a year after the bite. [Once the disease takes hold,] the patient can neither stand nor lie down; like a madman he flings himself hither and thither, tears his flesh with his hands, and feels intolerable thirst. This is the most distressing symptom, for he so shrinks from water and all liquids that he would rather die than drink or be brought near to water; it is then that they bite other persons, foam at the mouth, their eyes look twister, and finally they are exhausted and painfully breathe their last” (236). His portrayal of human rabies is accurate in that the incubation periods may extend from months to years after initial exposure (402), but a biting attack upon others by a rabid patient with resultant disease is an uncommon event (146).

Although rabies is known to have been widespread in the Old World for thousands of years, its occurrence in the New World is less understood because of a dearth of records prior to European arrival. Rabies in the Americas was reported by the Reverend Marmolejo in Mexico as early as 1709, but there is a suspicion that it was present before Columbus's arrival in the 15th century. For example, not long after the discovery of the Americas, the bishop Petrus Martyr-Anglerius wrote in his *De Rebus Oceanicis et de Orbi Novi Decades Octo*, “In several places bats not much smaller than turtle doves used to fly at them [the Spanish sailors and soldiers] in the early evening with brutal fury and with their venomous bites brought those injured to madness . . . [and] bats . . . come in from the marshes on the river and attack our men with deadly bite” (251). This may have been one of the first descriptions of rabies transmission by vampire bats. Elsewhere, in North America, some 200 years after initial Spanish invasion, canine rabies was described in 1753 from the Virginia colony, and later in foxes.

The bite of a rabid animal was considered a likely source of rabies infection by many, but it was only in 1804 that Zinke used dog saliva for transmission. Later in 1879, Galtier is credited with experimental rabies transmission to rabbits, and from rabbit to rabbit (253). Clinical descriptions formed the basis for diagnosis until the advent of light microscopy. A clear description of viral and neuronal interactions was made by Negri in 1903, with the detection of cytoplasmic inclusions (Negri bodies) in neurons of rabid animals (256). Reports of intraneuronal bodies specific for rabies stimulated debate concerning both their diagnostic utility and their nature. Although the diagnostic value of Negri bodies was established by 1913, their viral composition had to wait until the later development of electron microscopy.

Pasteur's research on rabies is perhaps the most well known historical achievement in the field. First, through adaptation of “street” (wild-type) virus to laboratory animals, he was able to change its properties. Today, one could apply the term *mutant* to his “fixed” virus strains. Second, Pasteur and his team developed concepts and experimental approaches to the first protective vaccination against rabies (253). Desiccated spinal cords from rabies-infected rabbits became the first rabies vaccine, and they were supposedly safe, although now it is known that the fixed viruses from which the vaccines were derived were not apathogenic but could actually cause the disease. July 6, 1885, is a milestone in the history of rabies. On that day, 9-year-old Joseph Meister was bitten at multiple sites by a rabid dog and received the first postexposure prophylaxis with Pasteur's vaccine. Remarkably, Joseph survived (253).

Pasteur's vaccine, with all its modifications, became the accepted rabies prophylactic throughout the world in the early 20th century. However, problems remained, because improperly inactivated virus caused rabies, and animal brain tissue induced allergic reactions leading to neuroparalytic accidents. Moreover, perhaps most importantly, the vaccine was not very effective in cases of severe bites, such as those inflicted on the face and neck by rabid wolves.

Postexposure prophylaxis against rabies through simultaneous administration of antirabies serum and vaccine was introduced in 1889 by Babes (15). This approach found few adherents and was forgotten until 1940, when interest in the use of serum containing rabies antibodies was revived. In a trial organized by the World Health Organization in 1954, the combined use of serum and vaccine was found to be more protective than vaccine alone (192), an observation later corroborated by Chinese findings (143). Today, the combination of serum and vaccine is the recommended standard for prophylaxis in human rabies exposure.

In the 1960s, a rabies virus grown in human diploid cells was used for production of a safe and efficacious vaccine (252,253), eliminating many of the problems connected with vaccines produced in brain tissue. This vaccine is being used widely throughout the world, although for economic reasons several developing countries still use nervous tissue vaccines. New generations of rabies vaccines will have both maximal efficacy and safety while still being affordable in regions with enzootic dog rabies.

Infectious Agent

Taxonomy

Classically, only one major serogroup was established within the *Lyssavirus* genus, although various serotypes were defined (367). Placement within the genus was determined by serologic cross-reactivity of viral antigens (e.g., complement fixation, immunofluorescence tests), primarily based on antigenic sites on the nucleoprotein (the N protein). Historically, placement of a species as a rabies or rabies-related virus was determined by recognition of antigenic sites of the glycoprotein (the G protein) via virus neutralization tests. More recently, sequence data became available for a number of lyssaviruses (48,245,280,403,433,505), and a trend toward genetic classification should continue. Virus species currently or putatively assigned to the *Lyssavirus* genus, all isolated from mammals, include rabies virus (RABV); Australian bat virus (ABV); Duvenhage virus (DUV); European bat virus, type 1 (EBV-1); EBV-2; Lagos bat virus (LBV); and Mokola virus (MOK) (Table 1). Two other viruses, Obodhiang and Kotonkan, found only in Africa and originally isolated from mosquitoes and midges, respectively (390), and Rochambeau virus (237), isolated from a pool of

mosquitoes, *Coquillettidia albicosta*, in French Guiana during 1973, have been suggested for consideration in the genus based on serologic data, but this awaits ultimate taxonomic assignment.

Lyssavirus	Host(s)	Geographic location
Rabies virus	Domestic and wild canids, mustelids, viverrids, and insectivorous and hematophagous bats	Worldwide, except for the Australian and Antarctic continents and some islands
Lagos bat virus	Fruit bats (Megachiroptera)	Africa
Mokola virus	Probably insectivore or rodent species	Africa
Duvenhage virus	Probably insectivorous bats	Africa
European bat lyssavirus 1	Insectivorous bats	Europe
European bat lyssavirus 2	Insectivorous bats	Europe
Australian bat lyssavirus	Fruit bats (Megachiroptera) and insectivorous bats	Australia

TABLE 1. *Members of the genus Lyssavirus of the Rhabdoviridae family*

Morphology and Physical Properties

Rhabdoviruses are enveloped, rod-shaped particles (Fig. 1), approximately 100 to 430 nm long and 45 to 100 nm in diameter, when mature (186). Animal rhabdoviruses are usually less than 180 nm, but those isolated from plants may be longer. Typically, mature virions appear either as bullet-shaped particles with one rounded and one flattened end, or as bacilliform particles that appear hemispherical at both ends (501). Defective-interfering (DI) particles have the same basic configuration but are shorter (20% to 50% the length of standard infectious viruses). DI particles resemble standard viruses in their protein and lipid composition, but they are not infectious, and 50% to 80% of the genome may be deleted (460). The linear RNA is encapsidated along its length (507). Typically, five polypeptides are encoded. The nucleocapsid or ribonucleoprotein (RNP) core exhibits helical symmetry with a diameter of approximately 30 to 70 nm, and it is surrounded by a lipid envelope. The virion outer surface is covered with projections (peplomers) approximately 5 to 10 nm long and 3 nm in diameter, except for the quasi-planar end (501). Peplomers are composed of trimers of the viral G protein (127,174).

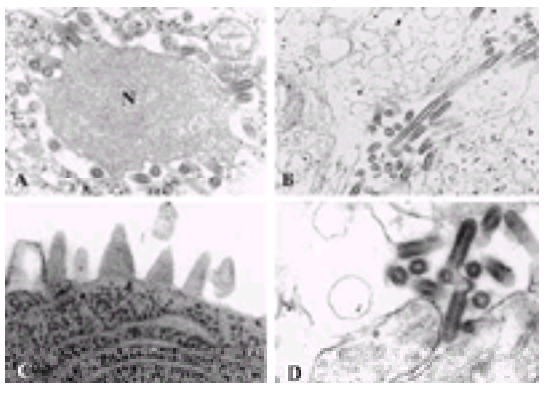

FIG. 1. Electron Micrographs of selected animal rhabdoviruses. **A:** *Lyssavirus*, Lagos bat virus. A typical Negri body (*N*) in the brain, with budding virions (×42,000). **B:** *Vesiculovirus*, vesicular stomatitis virus. Occasional anomalous elongated form of the virus (×35,000). **C:** *Ephemerovirus*, bovine ephemeral fever virus. Isolated from a cow in Australia, 1969. Late infection of VERO cell (×110,000). **D:** Unassigned, sawgrass virus. Isolated from a tick in Florida (×137,500). (Photographs courtesy of Sylvia Whitfield, CDC, NCID, DVRD, VRZB.)

The molecular weight is 300 to 1,000 × 10^6, and the buoyant density is 1.19 to 1.20 g/cm^3 in cesium chloride (CsCl), and 1.17 to 1.19 g/cm^3 in sucrose. Virus infectivity is stable in the pH range of 5 to 10, but it is rapidly inactivated at 58°C, by ultraviolet or x-ray irradiation, or by exposure to lipid solvents and oxidizing agents. Viral RNA accounts for 1% to 4% of the particle weight and has an estimated molecular mass of 4.2 to 4.6 × 10^6 daltons (11 to 15 kb in size). Virion RNA has a sedimentation coefficient of 42 to 45 Svedberg units and a buoyant density of 1.66 g/cm^3 (406). The RNA has a 5' terminal triphosphate and is not polyadenylated. The terminal sequences are complementary. Defective RNAs are significantly shorter than full-length RNA (by less than half), and replicate only in the presence of usually homologous helper rhabdoviruses (217).

Biologic Properties

Host Range and Infection of Experimental Animals

Rabies is an acute encephalitis caused by a lyssavirus. It is enzootic in all major regions of the world except Antarctica. The prototypic species of the *Lyssavirus* genus is RABV, which can infect warm-blooded animals under experimental conditions, but only mammals are significant natural hosts. Reservoirs include dogs, foxes, jackals, coyotes, raccoons, skunks, mongooses and their allies, and bats. Several countries, such as the United Kingdom and Japan, have become rabies free, once infected terrestrial animals were eliminated, but bats can reach even distant islands (370,477). Dogs are the most frequent vector for transmission to humans. The lyssaviruses DUV, LBV, and MOK are restricted to Africa (242). Although bats and small mammals such as shrews have been suggested as potential reservoirs (243), relatively

little is known about these African agents compared to RABV. In Europe, phylogenetic analysis of EBV genes after bootstrap resampling using maximum parsimony and neighbor-joining methods indicated low intrinsic heterogeneity, and it suggested at least two genetically distinguishable lineages, possibly related to spatiotemporal introduction from a different geographic location such as North Africa (12). Bats, such as *Eptesicus serotinus* and *Myotis dasycneme* and *M. daubentonii*, appear to be principal reservoirs for EBV1 and EBV2, respectively. Australia was believed to be free of the disease until 1996 (186), when indigenous lyssaviruses were discovered. These viruses appear to utilize either frugivorous *Megachiroptera* or insectivorous *Microchiroptera* as primary reservoirs, and they have caused at least two human deaths.

With the exception of RABV, the importance of the lyssaviruses as disease entities remains a matter of speculation (479). All mammals are probably susceptible to some degree, but experimental results suggest a hierarchy of species susceptibility (498). Very susceptible species include foxes, coyotes, jackals, and wolves (391,492). Cats (known as vectors but not as reservoirs), and some wildlife species, such as raccoons, are moderately susceptible to infection. The opossum (*Didelphis virginiana*) appears especially resistant (25). However, most experimental animal studies were performed before antigenic or genetic differences between viruses were appreciated as to their role in pathogenesis (193,327).

Since Pasteur's experimental inoculation of dogs and rabbits (335), many other species have been experimentally infected with RABV (452), including birds. No lyssaviruses have been isolated from cold-blooded vertebrates. Laboratory rodents, such as mice, have been extensively used for rabies diagnosis, vaccine potency testing, and pathogenic studies, although these taxa are epidemiologically insignificant as lyssavirus vectors or reservoirs (84,259), compared to the Carnivora and Chiroptera.

Propagation and Assay in Cell Culture

Early 20th century attempts to propagate RABV focused on central nervous system (CNS) tissue culture. In 1958, serial passage of fixed (laboratory strains) as well as street (primary field isolates) viruses was accomplished using primary hamster tissue cultures (246). Primary kidney cell cultures from hamsters, dogs, pigs, and other species are used for both primary isolation and laboratory propagation of fixed and street RABV (89,480). Continuous cell lines, particularly BHK-21, are commonly used for isolation and propagation of RABV because of their susceptibility to infection and high virus yields (376). The human diploid cell lines WI-38, MRC-5, and Vero can be used for commercial human rabies vaccine production (405,481,484), as well as avian and primate cell cultures (68). Although BHK-21 cells have been extensively used for propagation of fixed viruses, street RABV isolates may not be consistently recovered using these cells. Primary cell cultures or mouse neuroblastoma cell lines are frequently used for isolation of street RABV, although a wide variety of other cell types can be used for propagation (104). Two methods of virus titration for assessment of infective foci are the fluorescent antibody test (114,482) and the plaque assay (379). In general, lyssaviruses do not cause a specific cytopathic effect in cell culture. The plaque assay is useful for fixed strains of RABV that are well adapted to BHK-21 cells, but it provides inconsistent results with street viruses.

Chemical and Antigenic Properties of Viral Proteins

Rhabdoviruses generally encode five proteins, designated G (glycoprotein), N (nucleoprotein), P or NS (phosphoprotein), M (matrix protein), and L (RNA-dependent RNA transcriptase).

Glycoprotein

The G protein (102) is the primary surface antigen capable of inducing and reacting with virus-neutralizing antibodies (VNAs), and it is associated with receptor activities. Almost all major human and veterinary vaccines are based on the functional aspects of the G protein of RABV. The G protein gene has been cloned and sequenced from a number of lyssaviruses (9,95,436,505,509). Sequences contain a single open reading frame encoding a 522- to 524-amino-acid protein in lyssaviruses, with approximately 90% homology at the amino acid level. Mature G protein has 505 amino acids, resulting from cleavage of the N-terminal 19-amino-acid hydrophobic signal peptide, and it has three structural domains: the C-terminal cytoplasmic domain of 44 amino acid residues, the hydrophobic 22-amino-acid transmembrane domain, and the external antigenic domain extending from the transmembrane to the N-terminus (501). Within the antigenic domain are three to four putative glycosylation sites for addition of asparagine (N)-linked oligosaccharides, but not all sites are actually used (119,384,501,503). Fatty acid (palmitate) has also been found covalently linked to the cytoplasmic domain (175). A soluble form of G protein (Gs) is secreted from virus-infected cells; it lacks 58 amino acids from the C-terminus (129), which may result from proteolytic cleavage of newly synthesized protein. The Gs protein is antigenically identical to full-length protein but does not confer protection against lethal challenge (129). This difference may be related to the ability of G protein to aggregate or to form a multimeric structure more immunogenic than Gs monomers (501). With intracellular transport, G protein produced in the rough endoplasmic reticulum may exist in an inactive conformational state during transit through the Golgi apparatus, perhaps to avoid nonspecific membrane fusion (176).

Antigenic determinants of G protein to either B or T cells have been identified by using cyanogen bromide (CNBr)-cleaved peptide fragments or escape mutants resistant to neutralization. The CNBr fragments can be mapped and tested for antigenicity and immunogenicity (127). When administered to laboratory rodents, CNBr fragments induced antibody that bound to virus and G protein, but with low neutralizing activity, suggesting that antigenic structure of neutralizing epitopes was conformational. Based on groupings of escape mutant viruses of RABV from neutralization by one or more anti-G protein monoclonal antibodies (MAbs), three major antigenic sites (I, II, and III) were operationally delineated from both Evelyn-Rokitnicki-Abelseth (ERA) and challenge virus standard (CVS)-11 strains, and two additional sites (IV and V) were described on the G protein of the ERA strain (159,262,264). A minor antigenic site separable from site III by three amino acids was also delineated (34). Operationally defined antigenic sites correlated with the physical topography of the G protein by nucleotide sequence analysis of mutant variants representing different antigenic sites (502,504). Most of the antigenic structure of site III comprised a continuous distribution of epitopes closely arranged in

the primary structure. In contrast, antigenic site II consisted of discontinuous epitopes, and site I was revealed as an independent site containing a single epitope, not shared with epitopes of other defined sites. In general, most MAbs do not recognize G protein under conditions of reduction and alkylation, further suggesting that conformation derived from higher-order structure (secondary and tertiary) of the G protein is needed (127,502), but a few MAbs can bind to denatured G protein, indicating that these epitopes are probably continuous (57). Escape mutants to MAbs can possess a single amino acid change, with a histidine at position 264 (instead of arginine in the parent virus), and a methionine residue at position 44 replaced by isoleucine (122), and immunization with a synthetic peptide comprising amino acid residues 253 to 275 conjugated to keyhole limpet hemocyanin can result in VNA production and protection upon challenge with a lethal dose of virus.

Both CNBr cleavage fragments of G protein, and synthetic peptides corresponding to G protein amino acids, have been used to identify likely T-cell determinants. The CNBr peptides stimulated *in vitro* proliferation of T cells obtained from spleens of immune animals (293). Stimulation was observed using a 27-residue peptide corresponding to amino acids 18 to 44, and a 13-residue peptide corresponding to amino acids 32 to 44, believed to be T-helper-cell epitopes. The G protein has also been reported to be a cytotoxic-T-lymphocyte (CTL) stimulator (293). Analysis of secondary CTL responses *in vitro* of lymphocytes obtained from A/J mice by tryptic peptides and a CNBr fragment allowed identification of a single nominal determinant located between amino acids 130 and 178 of the G protein (292).

Compared to other lyssaviruses, MOK appears to be the most disparate member. The G protein of MOK is at least two amino acids shorter than RABV G protein (434), and it carries two potential glycosylation sites at positions 221 and 338. The MOK G protein shares only 54% homology with RABV G protein at the amino acid level. The most divergent amino acid changes occurred in the antigenic sites mapped to rabies virus G protein, partially explaining the absence of cross-protection (125).

Nucleoprotein

The N protein is the major component of the viral RNP. Amino acid sequences of RABV N protein have been deduced from the primary nucleotide sequence of several strains, including production virus (PV), CVS, ERA, and street Alabama Dufferin (SAD) (95,139,435,504). The amino acid sequence is 450 residues long, with a high degree (98% to 99.6%) of homology between different N proteins, correlating with antigenic similarity analyzed with MAbs (116,504), with the partial exception of the Flury high egg passage (HEP) strain. Sequencing of the N protein genes of LBV, DUV, and EBV showed an overall amino acid similarity of N-protein sequences with RABV of 77% (48). The phosphorylation site on RABV N protein is the serine residue at position 389 (124). Association of N protein in the RNP complex can diminish the opportunity for degradation by ribonucleases, may partially restrict RNA configuration, and could have regulatory functions during transcription (501).

Antigenic sites of RABV were suggested using competitive enzyme-linked immunosorbent assay (ELISA) (263). Most MAbs recognized conformational determinants, but some delineated sequential determinants from denatured N protein. Further mapping of B-cell epitopes was achieved by generating peptides, and by synthesizing peptides corresponding to amino acid sequences of immunoactive fragments, resulting in the identification of two linear epitopes on the N protein: an overlapping epitope located between residues 374 and 383, and another between amino acids 313 and 337 (124). Some linear epitopes may be expressed on the immature form of the N protein, whereas others may be created or exposed only after N-protein maturation (185).

The N protein is a major target antigen for T-helper cells, which cross-react among lyssaviruses (63,64,139). A human T-cell epitope on the N protein was been identified by using cloned T-cell hybridomas and synthetic peptides (65). Thus, viral RNP can prime T cells, elicit N-protein-specific antibodies, and serve to enhance immune responses (219). The N protein is a superantigen in Balb/c mice but not in other mouse strains (261).

Nominal Phosphoprotein Protein

The NS protein constitutes a part of the viral RNP and contains 297 to 303 amino acids (95,268). In contrast to divergence in the vesiculoviruses, sequence comparisons of RABV NS proteins show a high degree of homology, from 92% to 98% (268). The NS protein is phosphorylated (406), but sites of phosphorylation have not been precisely determined. Only threonine and serine residues are phosphorylated, and 16 of 40 serine plus threonine residues may provide phosphorylation sites within the N-terminal half of the NS protein (501). A unique cellular protein kinase has been discovered that phosphorylates NS protein, suggesting an important role in protein structure or function (191).

A B-cell antigenic determinant has been mapped between residues 75 and 90 of the NS protein (124). The NS protein expressed in vaccinia virus stimulates strong CTLs and weak T-helper cells in H-2 ^{k} mice (270), and the CTL epitope has been mapped to residues 191 to 206.

Matrix Protein

The M protein is believed to link the viral envelope with the RNP, and it contains 202 amino acids (95,351,436). Comparison of the amino acid sequences among different RABV strains showed 91% to 94% homology (214). The M protein also contains covalently linked palmitic acid (175). One primary function of M protein appears to involve the interaction with the cytoplasmic domain of the G protein and the RNP during virus assembly and budding (501). Its antigenic structure has not been widely studied.

RNA-Dependent RNA Transcriptase Protein

The L protein is the largest lyssavirus protein, containing 2,127 to 2,142 amino acids (95,437). Its functions include RNA synthesis, capping, methylation, and polyadenylation of viral RNAs (31,460,501), but because it is present in minute amounts, it is one of the least well studied lyssavirus antigens. Sequence homology among rhabdoviruses and other

members of the *Mononegavirales* (342,432) suggests evolution from a common ancestor. These regions may be functional domains responsible for the multiple catalytic activities of the L protein. A 400- to 450-nucleotide area, located between the beginning of the L gene and the end of the G gene, is bounded by transcription termination and polyadenylation-like signals, but it is a noncoding region, without apparent pseudogene functions (349,432).

Clinical Features

Rabies cases are almost always attributable to the bite of a rabid animal. For example, animal bites were the cause of 99.8% of 3,920 human rabies cases examined at various Pasteur Institutes between 1927 and 1946 (300). Nonbite exposures, which rarely cause rabies, include inhalation of aerosols (220,223,495), licks (273), transdermal scratches, or other unusual events that lead to contamination of an open wound or mucous membrane (154), such as corneal transplantation from an infected donor (228). Virus does not enter through intact skin. Recent bat RABV–associated human deaths in the United States may not have a reported exposure source (70,330), but these cases are most likely caused by bat bites in which either the risk was not appreciated or the bites were not immediately recognized by the patient. Disease development after exposure may depend in part on the location and severity of a bite, the species of animal responsible for the exposure, and the virus variant (15,23,328,391,453). Highest mortality tends to occur in persons bitten on the head and face (40% to 80%), with intermediate mortality in those bitten on hands or arms (15% to 40%), and least in those bitten on the trunk or legs (5% to 10%), or through clothing (<5%), when no specific vaccination was initiated (15,383,393,453).

The incubation period (the length of time between exposure to virus and development of clinical signs) is usually 1 to 2 months (154,466), but because it can vary from less than a week (210,337,340) to several years (3,16,402,485,491), rabies is one of the most variable of infectious diseases. The length of the incubation period may depend on the bite site and relative proximity to the CNS (3,230,393,419), severity of the bite, type and quantity of virus introduced, host age, and immune status (3,8,132,138,204,209,300,329,391).

Development of clinical rabies can be divided into three general phases: a prodromal period, the acute neurologic phase, and coma preceding death (154,210). During the prodromal period, lasting 2 to 10 days, symptoms are usually mild and almost entirely nonspecific and include general malaise, chills, fever, headache, photophobia, anorexia, nausea, vomiting, diarrhea, sore throat, cough, and musculoskeletal pain. One specific early symptom is abnormal sensation around the bite site, such as itching, burning, numbness, or paresthesia (132).

During the acute neurologic phase, patients exhibit signs of nervous system dysfunction such as anxiety, agitation, dysphagia, hypersalivation, paralysis, and episodes of delirium. Occasionally, priapism or increased libido may be observed (133). Cases in which hyperactivity is predominant are classified as “furious” rabies. When paralysis dominates, it is classified as paralytic or “dumb” rabies (86,248,454). From 17% to 80% of patients exhibit a pathognomonic sign of rabies, hydrophobia, believed to be caused by an exaggerated respiratory tract protective reflex (3,8,470,491). Hydrophobic episodes, initially triggered by attempts to drink and a variety of tactile, auditory, visual, and olfactory stimuli (210,491), may last from 1 to 5 minutes. Paralytic rabies can occur in about 20% of patients and may appear more frequently in persons exposed to certain variants, such as vampire bat rabies virus strains (221). In marked contrast to furious rabies, the sensorium is largely spared (212,221). Patients initially develop paresthesia and weakness, and finally flaccid paralysis, usually in the bitten extremity (86). Paralysis progresses to paraplegia and quadriplegia.

In furious rabies, the neurologic period ends after 2 to 7 days with coma or sudden death from respiratory or cardiac arrest (41). In paralytic rabies, the course is usually less rapidly progressive, with some patients living up to 30 days without intensive care (290). The final stage of the disease is coma, which lasts 3 to 7 days and results in death (41). In patients receiving respiratory assistance, survival may be prolonged for weeks (41,330), with death caused by other complications (36,137,204). At least four human cases have been documented in which recovery occurred. Three had exposure to animal bite (4,205,343), and one case occurred after suspected inhalation of rabies virus in the laboratory (66). None were spontaneous recoveries but rather were nonfatal vaccination failures, because the patients had either pre- or postexposure prophylaxis.

Clinical disease in animals is not unlike that of humans, except for the absence of hydrophobia. Signs are quite variable but may include altered phonation, pica, cranial nerve deficits, altered activity patterns, and loss of fear of humans (18).

Pathology and Pathogenesis

After inoculation of infectious saliva by bite, virus may either persist and replicate at the inoculation site for hours to weeks (74,77) or follow a relatively rapid centripetal course directly to the CNS (17), with replication and dissemination prior to the development of a significant immune response (75). Virus may replicate in muscle tissue (74,148,319) before progressing to the peripheral nervous tissue via neuromuscular connections. Mortality may be markedly reduced when infected limbs are cauterized or amputated (22) arguing for an initial productive infection in muscle cells. Several studies collectively suggest that RABV can infect muscle cells and replicate before invasion of the peripheral and central nervous systems (78), while others demonstrate that virus can enter the nervous system without prior local replication (17,385). Thus, during the incubation period, virus may reside in the periphery, it may remain sequestered within neurons, or perhaps it can persist in macrophages (350).

Neurotropism is a main feature associated with natural infection, with viral replication restricted almost exclusively to neurons. Virus may be taken up by unmyelinated sensory nerve endings of neuromuscular and neurotendinal spindles and motor end plates (203,282,472). Based on ultrastructural and immunofluorescence studies demonstrating viral clusters at neuromuscular junctions, and on the activity of substances such as a-bungarotoxin or D-tubocurarine, which specifically compete with the nicotinic acetylcholine receptor (AChR) and can reduce viral infectivity of chick myotubules *in vitro*, it has been suggested that RABV recognizes cholinergic binding sites (59,281,282). A similarity between amino acid residues 174 to 202 of the G protein and snake venom neurotoxins that bind at the site of the AChR have provided further evidence for virus attachment sites. However, other investigations have reached different conclusions based on

the relative susceptibility of cells in culture that express high-density AChR versus cells lacking AChR (352,441). Growth factors, neural cell adhesion molecules, and various phospholipids and glycolipids are also probably operative in determining viral neurotropism (352,431,442).

After peripheral nerve entry, virus moves centripetally within axons to the CNS at an estimated rate of 3 mm/hr (319,440). Evidence of transport by axonal flow in peripheral nerves comes from experiments in which transport of RABV was inhibited with colchicine and vinblastine (206,440). The form of the virus during axoplasmic transport, either as intact virus or as an RNP complex, remains unclear (90,183). Synaptic transfer of virus may ensue in the form of bare nucleocapsids (184).

Virus spread may be facilitated by movement across cell-to-cell junctions, supported by observations of budding on axonal membranes and viral inclusions at nodes of Ranvier (203). Viral distribution via cerebrospinal fluid (CSF) may also contribute to virus spread within the CNS (375).

Once virus reaches the brain, it spreads centrifugally to a variety of organs. The spread into the salivary gland, which represents the final phase of the infection, is important for transmission from animal to animal and from animal to human. Virus is found in salivary glands in the majority of rabid foxes, raccoons, skunks, and dogs (27,76,492,497). Much of the virus is produced in mucogenic acinar cells and is delivered into the saliva by normal secretory flow (318).

Other sites in which virus can be found include sensory nerve end-organs in oral and nasal cavities, taste buds, adrenal glands, pancreas, kidney, heart muscle, brown fat, hair follicles, retina, and cornea (321). Replication with large amounts of matrix and anomalous products may occur more frequently in tissues that contain low amounts of virus, such as the adrenal medulla, compared to tissues that produce large amounts of virus, such as the salivary glands (30).

Molecular mechanisms involved in virus pathogenesis remain largely unresolved. Several studies indicate that the G protein plays an essential role. Certain RABV variants belonging to a group that defined antigenic site III were found to be nonpathogenic (99,100,129). Use of antigenic variants representing operationally defined antigenic sites on the G protein showed that pathogenicity correlated with the presence of a determinant located within antigenic site III (99,100,130). The change in pathogenicity corresponded to a single amino acid substitution at position 333 of the G protein, which is arginine in the parent CVS and ERA viruses, and glutamine or isoleucine in the corresponding variant viruses (130). Study of MAb-resistant mutants isolated from a 5-fluorouracil-mutagenized stock confirmed that arginine-333 was replaced in nonpathogenic mutants (380).

Biologic relevance of arginine at position 333 in the rabies G protein was shown by *in vivo* and *in vitro* studies in which the pathogenic parent and nonpathogenic variant viruses were compared for their ability to infect neuroblastoma cells, to spread from cell to cell *in vitro*, and to spread through the brain (128). Entry halftime of pathogenic parental viruses was seven to eight times shorter than that of nonpathogenic variant viruses. Furthermore, apathogenic variant viruses were unable to spread from cell to cell in neuroblastoma cell cultures and displayed a lower rate of spread throughout the CNS (128). Other investigators showed that pathogenic viruses can apparently use different receptors or routes of entry from those used by their nonpathogenic derivatives (101,257), as well as differentially affect the expression of major histocompatibility complex (MHC) class II mRNA in the CNS (224).

Fusion of virus-infected cells may also be an important pathogenetic mechanism (313). irulent-type virus G protein but not the avirulent variant G protein induced fusion of neuroblastoma cells at neutral pH, suggesting that the syncytium-forming ability of the virulent type G protein is responsible for the more efficient spread of virulent virus in the brain.

Gross lesions in the CNS do not usually occur. Microscopically, inflammatory lesions are commonly noted in the spinal cord and brain stem (132,234,317,338,387). Demyelination and axonal degeneration of cranial nerves have been reported (309). Intensity of inflammatory lesions may depend in part on the host species, the virus strain, and the course of the disease (338). Relative immune suppression can result in less inflammation and abundant virus replication (1). Severe inflammation may be associated with virus isolates from bats, with Flury low egg passage (LEP), and with MOK and LBV (90). Prolonged virus infection can also produce intense encephalitic lesions (320). Characteristic changes in the CNS include formation of inclusion bodies within the cytoplasm of neurons (324). These Negri bodies are eosinophilic masses measuring 1 to 30 nm in diameter, and they consist predominantly of viral nucleocapsids. Negri bodies may not be readily evident, they can be confused with other inclusions, and their importance has been diminished with the advent of specific fluorescent antibody or immunohistochemical techniques.

The pathogenic pattern of street virus infections in the CNS can differ from that related to fixed virus infection. Often, street virus leaves neurons intact (225,317). Cytopathic differences between street and fixed virus infection include the appearance of large Negri bodies within neurons of street virus–infected mice (225). Many differential findings with experimental street virus infections in animals, such as the relative size or distribution of Negri bodies, are also observed in human rabies pathology.

Histopathologic changes in the CNS are often limited, not matching marked neurologic symptoms. Impairment of neurologic function rather than overt anatomic change may be responsible for clinical sign development. RABV infection has a selective effect on the electrophysiologic activity of neurons: It induces sleep alterations (187,188) and dramatic changes in the expression of immediate-early and late response genes (170). Expression of immediate-early genes such as egr-1, c-fos, and junB is strongly increased, whereas expression of late response genes, such as the gene that encodes the neurotransmitter enkephalin, is markedly decreased (170).

Programmed cell death, or apoptosis, may be an effective host response after viral infection of certain regenerating tissues, but it can clearly impact disease status when affecting neurons (226,427). The down-regulation of G protein expression by some viruses may contribute to viral pathogenesis by preventing apoptosis (312).

Immune Response

Antibodies induced by vaccination, particularly those with neutralizing activity, play a prominent role in immune defense against infection (218). Immunity may also be naturally acquired after multiple exposures to virus, on rare occasions (164). The G protein represents the only antigen that induces VNA and is able to confer immunity against a lethal challenge infection (102). The ability to induce VNA depends on the intact secondary and tertiary structure of the G protein (127,503). Protective activity induced by Gs is reduced compared with that of the virion-associated G protein (129). VNAs exert their protective effect by neutralization of extracellular virus, by complement-mediated lysis of virus-infected cells, and by antibody-dependent cytotoxicity (113). VNA can mediate viral clearance from the CNS without other immune effectors (123). The RNP is a major antigenic complex that induces a virus-specific antibody response, and antibodies directed against RNP can contribute to protection against infection (121,126,286). Animals treated with anti-N sera can be protected against a subsequent challenge with RABV, and anti-N sera can exhibit an antiviral activity *in vitro* (286). However, the mechanism by which anti-RNP antibodies inhibit viral replication remains unclear.

Infection with RABV results in the generation of virus-specific CD8+ and CD4+ T cells. The G protein is one of the antigens that induce CTL responses (293). Some mouse strains infected with virus also develop strong CTL responses to the NS protein (269). However, the role of CD8+ T cells in immune defense is unclear. Some investigators report clearance of rabies virus after transfer of RABV-specific T cells and protection against rabies by a CTL clone, whereas other investigators showed that CTLs are insufficient to protect against challenge, and *in vivo* depletion of CD8+ T cells had no effect on host resistance to street virus infection (238,269,339,344). In contrast, CTLs may actually be involved in the immunopathology and have been implicated in neuritic paralysis (418,476). By comparison, the induction of CD4+ T cells is an integral part of the protective immune response against rabies (120). Elimination of CD4+ cells abrogates the production of IgG neutralizing antibody in response to virus infection (339). The RNP contains major epitopes that induce CD4+ cell responses, and the majority of these T cells cross-react with other lyssaviruses (139). The RNP-specific T cells, which can augment the production of VNA through a mechanism known as intrastructural antigen recognition, are believed to be the major factor that mediates the protective immune response induced by internal viral antigens (124,168).

Diagnosis

Clinical diagnosis is not difficult if there is a documented, relevant history of exposure and subsequent compatible clinical signs or symptoms. However, because an exposure history may be lacking, rabies should be considered in any acute, unexplained neurologic disease of suspected viral origin that rapidly progresses to coma and death (21). Routine diagnosis is established by standard laboratory tests for specific virus isolates, antigens, nucleic acids, or VNA (198,400,469). Postmortem diagnosis should be performed on CNS specimens, especially the brainstem, hippocampus, and cerebellum (421). The fluorescent antibody test (400) and the avidin-biotin immunohistochemical technique (194) are sensitive and specific methods for detecting virus antigen (Fig. 2). Antibodies, particularly those that recognize continuous epitopes of the N protein, are useful because they provide consistently reliable results, even if the tissues have been fixed with formalin or embedded in paraffin (147,468). Examination of skin biopsies from the face (55) or hair-covered occipital portions of the neck for virus antigen (42,330) is a rapid method to diagnose human rabies before death. Rabies virus can be isolated from saliva by direct intracerebral inoculation into mice (254) or by infection of neuroblastoma cells (395). Fluorescent antibody examinations of corneal impressions may also occasionally lead to the diagnosis of human rabies (249). The reverse transcriptase–polymerase chain reaction assay (RT-PCR) has been used to amplify and sequence parts of the lyssavirus genome directly from brain, saliva, and other affected tissues (207,330,400). This not only allows detection of rabies virus–specific RNA but also permits insights into the identity of the virus variant by genetic sequencing. Detection of VNA in serum (330,398) late in the clinical course can be diagnostic for rabies, if the patient has not been previously vaccinated. Except for certain cases of postvaccinal encephalomyelitis, CSF antibodies are produced only in rabies-infected, not in vaccinated, individuals (471).

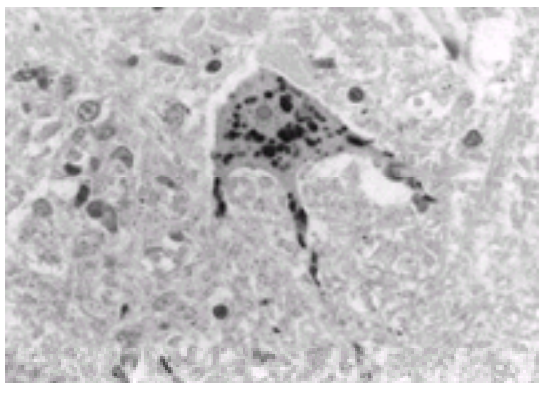

FIG. 2. Light microscopic photograph of multiple intracytoplasmic rabies virus inclusions in a neuron from the brain of a naturally infected bison, revealed by immunohistochemistry (×400). (Photograph courtesy of Michael Niezgoda, CDC, NCID, DVRD, VRZB.)

Treatment

No successful treatment of clinical rabies is available (134,210). The disease is fatal, with few exceptions, and no spontaneous recoveries are reported. Sedation and life support measures have been used to prolong life but not to prevent death. Thus, rabies treatment is a misnomer and usually refers to medical aid related to animal bite and disease prevention by postexposure prophylaxis (478). Future *in vivo* experimental approaches by a combination of virus

neutralization and inhibition of viral replication in the CNS should be encouraged, provided that an early diagnosis of rabies would be entertained (123,171,210).

Epidemiology

The domestic dog is the principal host and major vector of rabies throughout the world (145,465), particularly in the equatorial regions of Asia, Latin America, and Africa. International reporting of both human and animal rabies cases grossly underestimates the magnitude of the problem (307,455). Predominant wild reservoirs belong to the family Carnivora (21,244) and include foxes (40,103) in the Arctic (*Alopex lagopus*), Canada, central and western Europe (*Vulpes vulpes*), and scattered foci elsewhere throughout North America (e.g., *Urocyon cinereoargenteus*); the raccoon dog (*Nyctereutes procyonoides*) in eastern Europe, Scandinavia, and portions of Asia (83,258,332); coyotes, jackals (*Canis* species), and other wild canids in North America, Asia, and Africa (173); skunks (*Mephitis mephitis*, *Spilogale putorius*) in North America (79,80,190); procyonids, such as the raccoon (*Procyon lotor*), in the eastern United States (496); and viverrids (e.g., the yellow mongoose, *Cynictis penicillata*) in Africa and Asia. The Indian mongoose (*Herpestes auropunctatus*) was introduced into several Caribbean islands for snake and rat control during the 19th century and persists as a major agricultural and public health threat (142,285). Rabies detection in rodents is uniformly rare (84,259,315,493). Bat rabies predominates as a New World phenomenon, described primarily among the insectivorous species of the United States and Canada (some 40 species), and the three hematophagous vampire species (principally *Desmodus rotundus*) ranging from northern Mexico to Argentina (20,85). Renewed investigations suggest that other bat species may also be important throughout Latin America (116,117). Other lyssaviruses utilize bats in Africa, Europe, and Australia (12,26,183,381).

Lyssavirus epidemiology is influenced in part by host species distribution, abundance, demographics, behavioral ecology, dispersal, and interactions with humans (370). Because of its consequences when ignored, rabies is a reportable disease in many countries, although surveillance is inadequate, particularly in sylvatic hosts. Biased epidemiologic information usually derives from clinical reports or the examination of suitable brain material submitted to public health or veterinary diagnostic laboratories only after infectious contact with animals is suspected. Exposure is generally defined as transdermal contact, typically by a bite, or mucosal contamination with potentially infectious material, such as saliva or CNS tissue (499). The relative risk associated with other scenarios is difficult to define (71).

The spatiotemporal distribution and relative intensity of rabies infection in various mammalian species is often described based on the passive monitoring of suspicious contact cases. Active surveillance by structured examination of a field sample, irrespective of human–animal contact, is expensive, time consuming, and rarely performed. Unless justified, it may waste precious health sector resources and may lead to unnecessary exposure to the public or to improperly trained staff. Thus, compromise between active and passive monitoring remains the format for primary lyssavirus detection, disease prevention, programmed control, and, in some instances, attempted elimination assessment.

Recent surveillance efforts in the United States (255) illustrate the benefits of a systematic surveillance approach that serves to define rabies among single or multispecies assemblages. For example, during 1998, 7,961 rabies cases (a decrease of 7% since 1997) were tallied. In contrast to indigenous canine rabies of the 1940s, more than 90% of existing cases were from wildlife. Most (44%) resulted from the continued spread of raccoon rabies, which resulted from the progression of an outbreak initiated in the late 1970s when animals from an infected nidus in the southeastern United States were translocated to the Virginias (438). Cases increase as infection spreads within local populations. Other major wildlife contributors are bats (13%), skunks (29%), and foxes (6%). Cases of coyote rabies from an outbreak in southern Texas (91) continued a substantial decline. Domestic species included cats (4%), cattle (2%), and dogs (1%). Historically, Hawaii remained the only rabies-free state, never having reported a case of indigenously acquired rabies, partly because of its isolation (155,374).

The application of MAb technology (367) to the study of lyssaviruses has provided evidence for considerable antigenic variation, based on the N and G protein reactivities among both fixed and street RABV and between related viruses (215,247,302). Such MAbs have been particularly useful in determining the extent of natural antigenic variation among lyssaviruses isolated from wildlife within a restricted geographic area or from separate continents. In particular, distinctions between viruses isolated from bats and terrestrial carnivores became obvious (26,367,396,397,401,403). Notwithstanding genetic variation and the general tendency or relatively rapid evolution of RNA viruses toward the persistence of quasi-species (311), striking global patterns have emerged that are highly suggestive of a conservative strategy and stabilizing selection represented by any particular variant (46,323,397,401).

Extensive lyssavirus lineage analyses have been conducted to delineate genetic clusters or viral genotypes, to evaluate the phylogenetic diversity within each genotype, and to more precisely appreciate the divergence of wild isolates in each genotype. RT-PCR followed by sequencing or restriction endonuclease digestion using material extracted directly from infected brain tissues distinguishes at least seven genotypes (Fig. 3) (47,48 and 49). The African DUV and EBV lyssaviruses appear related, whereas the LBV and MOK viruses are phylogenetically distant from RABV. The ABV is most closely related to classical RABV. Intragenotypic viral clusters are also distinguishable, suggestive of historical, geographic, or host species relationships. Results are generally consistent with previous classifications using antigenic criteria (322,371,372,404).

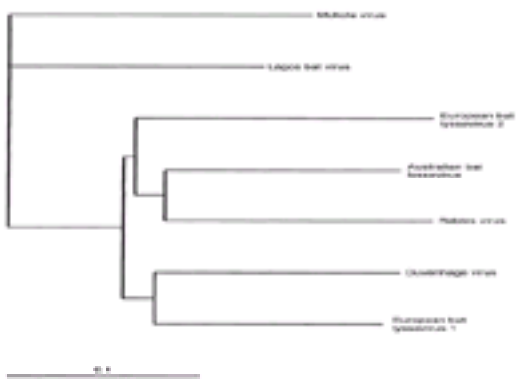

FIG. 3. Phylogenetic relationships among members of the *Lyssavirus* genus based on the nucleotide sequence homology of the nucleoprotein gene. GenBank accession numbers: AF006497, M31046, S59448, U22842, U22844, U22847, U22488.

Combined with historical, temporal, and spatial disease surveillance data, antigenic characterization with MAbs and nucleotide sequence analysis can assist in the assignment of isolates to different animal reservoirs (401,403,404). Recent North American surveillance data (399) suggest that two fox rabies foci in Canada, Alaska, and the northeast United States are related (369). Skunk rabies isolates appear to be distinct variants defining separate outbreaks in the north-central and south-central parts of North America, and California. Additionally, smaller independent foci involve foxes, dogs, and coyotes in Texas, as well as foxes in Arizona and portions of the southwest (91,255,397,401,403).

Antigenic or molecular characterization is useful for investigation of unusual or unexpected mortality, especially in domestic animals or humans with no obvious exposure history, because data obtained can be compared with variants from known animal reservoirs. For example, analysis of recent human rabies cases from the United States implicates viruses associated with insectivorous bats in the etiology of infection (330), and some bat isolates appear to possess unique pathogenic properties compared with isolates from the Carnivora (314).

In contrast to parts of Europe and North America where wildlife rabies predominates, in southeast Asia, Africa, and much of Latin America, dogs continue to be the principal causative vectors of rabies to humans. Realizing the critical importance of dogs as the primary reservoirs, the Pan American Health Organization began a program in 1983 to eliminate urban rabies from the principal cities of Latin America by the year 2000 (141). Results have been very encouraging (10). From the 1980s to the 1990s, the annual number of human rabies cases decreased from 350 to less than 114; rabies-specific mortality declined from 1.3 to less than 0.2 per million, and the proportion of 414 cities free of rabies increased from less than 75% to more than 80%. These results suggest that widespread vaccination of canine populations in Latin America and elsewhere (35,45,156,157) can reach the levels of herd immunity needed to prevent rabies epizootics, and that elimination of canine rabies, at least in urban areas, may be an achievable goal. Rabies transmitted by hematophagous bats, unique to the Americas, is an emerging public health problem (289,469), in addition to being a historically important disease of livestock with widespread economic implications (189).

Prevention of Rabies in Humans

Rabies is a significant global disease that results in death, with only a few exceptions (4). More than 4 million humans annually undergo antirabies prophylaxis, but an estimated 50,000 others die, primarily from the bite of an infected dog (307,455). Public health expenditures are thought to be substantial but have been poorly quantified (305). Regional epidemiologic surveillance and knowledge of viral pathogenesis can significantly reduce human morbidity from inappropriate prophylaxis and direct mortality ascribed to lyssaviruses by identifying reservoirs and unlikely exposures (386,493), developing treatment algorithms, and communication of risk to different occupational groups. Largely because of surveillance and veterinary efforts in developed countries with controlled canine rabies, actual human rabies deaths are now infrequent in these regions. Nevertheless, vigilance to minimize human mortality requires the annual treatment of tens of thousands of potential exposure cases, even in Europe and North America (197).

Postexposure prophylaxis in humans includes proper wound care and the administration of multiple doses of rabies vaccine, together with antirabies immune globulin (68,499). Although the inclusion of serum or antirabies immune globulin in the treatment protocol is not new (60), it is infrequent. Most cases of human rabies prophylaxis in Africa, Asia, and Latin America are with vaccine only (307), often with nervous system tissue vaccine (28,229,267,331,341). Human diploid cell rabies vaccines (HDCV) have been used in much of the developed world and form the standard for comparison with the Pasteurian neural vaccines from the 19th century (456,478), including its later phenolized derivatives (Fermi, Semple, and others). Modern inactivated cell culture vaccines (416) and antirabies immune globulin are major improvements over crude biologicals derived directly from animals, and they decrease the adverse events related to anaphylaxis or serum sickness (265,486). HDCV is one of only three vaccines now licensed in the United States (68) for either pre- or postexposure prophylaxis (Table 2 and Table 3). Other rabies vaccines are produced in avian embryo fibroblasts or in rhesus monkey renal cell culture, and aluminum phosphate may be used as an adjuvanted (67). Production of HDCV is relatively difficult, with limited viral yields, resulting in high production costs. Primary hamster kidney cell vaccines are used in China and parts of Asia (284). Biotechnologic advances, such as automated purification techniques, have resulted in vaccines that are more readily available, and thus at lower cost, such as a purified Vero cell rabies vaccine. Efficacy trials of these alternative vaccines using reduced doses, various immunization schedules, and alternative routes have been conducted worldwide in populations of highest risk (88). Serologic evaluation and long-term clinical observations of exposed individuals have demonstrated both high efficacy and safety of these new vaccines and schedules (87). At present, there is no evidence to suggest that prophylaxis failure is caused by antigenic variation of RABV (499). Rather, factors such as inadequate wound care, omission of potent serum, failure to infiltrate the wound with immune globulin, and delay or failure to follow recommended procedures have been implicated in human rabies cases

after vaccination (211,334,354,487). Future tactics for global rabies control will continue to focus on the need for potent, inexpensive pre- and postexposure biologicals (167,265,266) and schedules for humans, and on safe, effective vaccines for animals that are reservoirs for the disease (21,56,149,151,158,494,499).

Type of vaccination	Route	Regimen
Primary	Intramuscular	HDCV, PCEC, or PVRV, 1.0 mL, [deltoid area], one each on days 0,* 7, and 21 or 28
	Intradermal	HDCV, 0.1 mL, one each on days 0, 7, and 21 or 28
Booster	Intramuscular	HDCV, PCEC, or PVRV, 1.0 mL, [deltoid area], day 0, only
	Intradermal	HDCV, 0.1 mL, day 0, only

*Day 0 is the day the first dose of vaccine is administered.
HDCV, human diploid cell vaccine; PCEC, purified chick embryo cell vaccine; PVRV, rabies vaccine adsorbed.

TABLE 2. *Human rabies vaccine preexposure schedule—United States, 1999*

Vaccination status	Treatment	Regimen*
Naive	Wound cleansing	Treatment should begin with thorough cleansing of all wounds with soap and water. If available, an agent such as a povidone-iodine solution should be used to irrigate the wounds.
	RIG	Administer 20 IU/kg body weight. If anatomically feasible, the full dose should be infiltrated around the wound(s) and any remaining volume should be administered IM at an anatomic site distant from vaccine administration. The RIG should not be administered in the same syringe as vaccine, because RIG might partially suppress active production of antibody, and no more than the recommended dose should be given.
	Vaccine	HDCV, PVRV, or PCEC 1.0 mL, IM [deltoid area†], one each on days 0,* 3, 7, 14, and 28.
Previously vaccinated§	Wound cleansing	All postexposure treatment should begin with immediate thorough cleansing of all wounds with soap and water. If available, an agent such as a povidone-iodine solution should be used to irrigate the wounds.
	RIG	RIG should not be administered.
	Vaccine	HDCV, PVRV, or PCEC 1.0 mL, IM [deltoid area†], one each on days 0 and 3.

HDCV, human diploid cell vaccine; PCEC, purified chick embryo cell vaccine; RIG, rabies immune globulin; PVRV, rabies vaccine adsorbed; IM, intramuscular.
*These regimens are applicable for all age groups, including children.
†The deltoid area is the only acceptable site of vaccination for adults and older children. For younger children, the outer aspect of the thigh may be used. Vaccine should never be administered in the gluteal area.
§Day 0 is the day the first dose of vaccine is administered.
¶Any persons with a history of preexposure vaccination with HDCV, PVRV, or PCEC, prior postexposure prophylaxis with HDCV, PVRV, or PCEC, or previous vaccination with any other type of rabies vaccine and a documented history of antibody response to the prior vaccination.

TABLE 3. *Human rabies vaccine postexposure prophylaxis schedule—United States, 1999*

Rabies Control in Animals

Rabies is not considered a serious candidate for disease eradication at this time because of numerous and diverse wild reservoirs (72). However, the correlation between canine rabies and human fatalities has led to the successful application of herd health initiatives using vaccines of modified live agents and inactivated products (56), particularly in developed countries. Development of a suitable lyssavirus vaccine is not synonymous with either disease prevention or control, because a comprehensive domestic animal program also requires responsible pet ownership and the complexities inherent in the social, cultural, and religious aspects of the human–animal bond. Such a program entails stray animal management; leash law amendments; humane population curtailment (e.g., early spay/neuter programs); animal importation, translocation, and quarantine regulations; schedules for early preexposure vaccination of companion animals (in light of potential maternal immune inhibition); and rational postexposure management (68,69). Unlike postexposure prophylaxis of humans, euthanasia is usually recommended for the naive animal exposed to rabies, but this may eventually change with the development of safe and effective biologicals and protocols.

Current veterinary vaccines are more potent than the initial attenuated and inactivated vaccines used in the early part of the 20th century (13,345). However, because no vaccine is 100% effective, given poor cross-reactivity with some genotypes (457), and because correct identification of the properly immunized animal may be confusing, the vaccinated dog or cat is not exempt from confinement and close observation, particularly if human exposure occurs, whether provoked or not. This strict period of quarantine applies only to dogs, cats, and in some countries domestic ferrets (69). Human prophylaxis may be delayed during this time in nonenzootic canine rabies areas (394). In addition, pet vaccination status does not necessarily alter the need for euthanasia of an offending animal, regardless of vaccine potency or efficacy, if rabies is suspected.

In the case of free-ranging, nondomestic mammals, population reduction of major rabies reservoirs has been practiced for centuries. Except on rare occasions, it has not been generally regarded as a humane, long-term, cost-effective, or ecologically sound tool to control lyssavirus infection (115,195). A notable exception relates to hematophagous bats in Latin America. One of the first specific control methods was the use of anticoagulants applied topically to bite wounds on cattle, followed by systemic treatment of exposed cattle at risk, and finally topical treatment with anticoagulants of vampire bats themselves, exploiting their behavior of mutual grooming at the roost (161). Properly applied, these control efforts can spare the needless destruction of many beneficial nontarget bat species from nonspecific environmental alteration (316), perhaps some day to be augmented with novel vaccination strategies (382).

For more than four decades, efforts have been made to protect free-ranging wildlife against virulent street virus challenge by oral consumption of a potent vaccine contained within an attractive bait (494). In the past several years, millions of rabies-virus-vaccine-laden baits have been distributed over huge areas in western Europe (14,19,158,464), eastern Canada, and the United States for wildlife rabies control, with effective strategies designed in both rural and urban areas (271,359,360). Concerns about residual virulence and ineffectiveness (for other reservoir species) of the first generation of modified live oral viruses has led to the development of other attenuated and recombinant rabies vaccines

(19,39,158,260,274,336,365,368,483). After initial laboratory results with red foxes, efficacy, safety, and field studies with a number of oral vaccine candidates have been extended to other relevant carnivore species, including Arctic and gray foxes, coyotes, raccoon dogs, raccoons, skunks, and, domestic dogs (52,150,163,332,368,464).

A vaccinia–rabies glycoprotein (V-RG) vaccine was the first recombinant rabies vaccine to be constructed, field tested, and considered for regulation in Europe and North America for wildlife rabies control. This vaccine has been extensively reviewed to ensure safety (tested in over 40 species of mammals and birds) and efficacy (proven against severe rabies challenge in target species). Thermostability of the vaccine has been demonstrated under laboratory and field conditions. Following the success of the V-RG vaccine against fox rabies in Belgium (52) and France, preliminary field trials suggest its potential utility for rabies control in raccoons, foxes, and coyotes in the United States (144,196,356,361). Other orthopoxviruses (150,288) have been considered as vectors of lyssavirus antigens, but these have not been field tested to date.

The rabies G protein gene also has been inserted into the human adenovirus genome (346). Preliminary experiments have been encouraging, with adequate G protein expression in cell culture and efficacy demonstrated in several immunization trials in captivity (73,291,508). Newer constructs (e.g., rabies glycoprotein inserts utilizing the adenovirus E1 region, incapable of replication in host tissue) are also under consideration in a variety of species. In addition, an oral baculovirus-vectored vaccine induces protective immunity in raccoons (169). The potential for manipulation of the RABV genome has been simplified by the generation of infectious virus entirely from cloned cDNA (377). If future recombinant, replication-incompetent, inactivated, or DNA-based (140,227,272,287,310,333,366,506) vaccines prove both efficacious and economical (304,306), they may render a majority of previous biosafety concerns obsolete (308), paving the way for more widespread free-ranging wildlife and dog rabies control, particularly in developing countries (298).

VESICULOVIRUS

History

Vesicular stomatitis virus (VSV) is the best-studied member of the genus *Vesiculovirus* of the family *Rhabdoviridae.* It produces an acute disease in cattle, horses, and pigs characterized by fever, and vesicles in the mucosa of the oral cavity and in the skin of the coronary band and teat. Clinically, it is very similar to foot-and-mouth disease (FMD). VSV can also cause a disease with flu-like symptoms in humans.

Although VS was first reported in the United States in 1916 during an event that reached epidemic proportions in cattle and horses (420), a clinically similar disease was previously described in army horses in 1862, during the U. S. Civil War (299). In 1915, during World War I, French veterinarians described a disease clinically similar to VS in horses imported to Europe from the United Sates and Canada. At that time, the etiology of this disease could not be determined with certainty, but it could be transmitted from horse to horse by rubbing the saliva of a sick animal on the tongue of a healthy one, establishing the infectious nature of the disease (200).

In 1925, cattle transported from Kansas City, Missouri, to Richmond, Indiana, developed vesicular lesions in the mouth and initiated an outbreak of VS in the area. The disease was experimentally transmitted to horses and the infectious agent was maintained by serial passages in animals. This strain became the VS Indiana (VS I) strain (98). In 1926 an outbreak of VS in cattle occurred in New Jersey. The causative agent was found to be a filterable agent that could infect cattle, horses, and guinea pigs. This virus, serologically different from the VS I strain, is currently known as the VS New Jersey (VS NJ) strain (97,98). The VS I and VS NJ viruses represent the two serotypes most commonly isolated in the Americas. In the United States, the last reported outbreak of the VS I serotype occurred in 1965 (474). The VS NJ serotype was responsible for outbreaks in the United States in 1944, 1949, 1957, 1959, 1963, 1982–1983, 1985, and 1995 (51). In 1997 isolated cases were diagnosed in several horses in New Mexico, but this did not initiate an outbreak (11).

Between 1946 and 1954, during an important outbreak of FMD in Mexico (180), the joint Mexico-American commission for the control of this disease developed techniques for the differential diagnosis of FMD and VS based on the isolation of the agent and complement fixation methods (200). The VS I and VS NJ serotypes were isolated in Mexico in 1947 and 1948, respectively. The availability of a more efficient diagnostic methodology demonstrated that VS was prevalent throughout the year in the tropical areas of Mexico (200).

In South America, the disease was reported in 1939 in La Plata, Argentina (200). Later, VSV was isolated in Barinas, Venezuela, in 1941 and in Colombia in 1943 (200). Currently, VS is endemic in many Latin American countries and is responsible for important economic losses in the livestock industry. Disease caused by the VS NJ and VS I serotypes was reported in 11 countries of Latin America in 1996 (500).

Although the presence of VS was previously suggested in Africa in 1884 to 1887 and in Asia in 1944 (200), presently the disease is considered enzootic only in the Americas (500).

Other members of the VS serogroup are endemic in Asia and the Americas. Pyri virus was isolated from an opossum (*Philander opossum*) in Brazil in 1960 (428). Cocal virus was isolated from mites of the genus *Gigantolaelaps* from 11 rice rats (*Oryzomys laticeps velutinus*) trapped during September, 1961, on Bush Bush Island in the Nariva swamp in Eastern Trinidad Island (232,233). The VSV Alagoas serotype was isolated from domestic animals in the state of Alagoas, Brazil, during a VS outbreak (422). Chandipura virus was obtained from the sera of two patients with a febrile illness in Nagpur City, Maharashtra State, India, in 1965 during an epidemic of chinkungunya and dengue (37). This virus was also isolated from phlebotomine sand flies in West Africa in 1991 (165).

Isfahan virus was isolated from sand flies (*Phlebotomus papatasi*) collected in Dormian, Isfahan Province, Iran, during 1975 (426). Maraba virus was also isolated from sand flies (*Lutzomyia* sp.) collected in the state of Pará, Brazil. Although humans are infrequently infected based on serology, the actual public health significance of Maraba virus has not been

assessed (439).

Infectious Agent

Taxonomy

Based on the serologic cross-reactivity patterns and sequence analyses of the members of the *Vesiculovirus* genus, a unique serogroup, VS, has been established. The VS serogroup includes VSI (the type species of the genus), VS NJ, VS Alagoas, Chandipura, Cocal, Isfahan, Maraba, and Piry viruses (451). Other species tentatively assigned to this genus are BeAn 157575 virus, Boteke virus, Calchaqui virus, Carajas virus, eel virus American, Gray Lodge virus, Jurona virus, Klamath virus, Kwatta virus, La Joya virus, Malpais Spring virus, Mount Elgon bat virus, Perinet virus, Pike fry rhabdovirus (grass carp rhabdovirus), Porton virus, Radi virus, spring viremia of carp virus, Tupaia virus, ulcerative disease rhabdovirus, and Yug Bogdanovac virus. VSV will be the only member of the *Vesiculovirus* genus discussed extensively in this section.

Morphology and Physical Properties

The members of the *Vesiculovirus* genus share the general morphologic and physical properties described for the other members of the *Rhabdoviridae*, such as *Lyssavirus*. The viral genome is 11.2 kb in length. It presents a leader sequence of 50 nucleotides anteceding the N gene, an untranslated region of about 60 nucleotides located after the L gene, and intergenic regions formed by two nucleotides (451).

Biologic Properties: Host Range and Infection of Experimental Animals

The virus has been isolated from a variety of animals, including mammals and invertebrates. Naturally, the disease affects horses, cattle, and swine; sheep and goats are only rarely infected (180,200). In humans, VSV infections can induce symptoms that vary from influenza-like to encephalitic (348). Human infection is most common in rural areas where the disease is enzootic (153,200). In Panama, serologic evidence suggested a prevalence of 29% (388). Serologic surveys have shown that some species of wild ruminants, carnivores, marsupials, and rodents can be infected with the virus (428,474). Wild ungulates may play a role in the maintenance of enzootic cycles (160,475). Virus has been isolated from numerous insect species such as mosquitoes, midgets, and sand flies. In Panama and in Georgia, United States, VSV was obtained from phlebotomine sand flies (*Lutzomyia* spp.) (96,423). The virus has also been isolated from blackflies (*Simuliidae*) in Colombia (428), *Musca domestica* (166) and *Culicoides* spp. in Colorado (463), and mosquitoes (*Aedes* spp.) in New Mexico (417).

Other members of the VS serogroup have also been isolated from a wide variety of vertebrates and invertebrates. During the Rockefeller Foundation Virus Program (1951 to 1970), five human infections with Piry virus occurred in the New York and Belen laboratories (428). Serologic surveys revealed the presence of antibodies against Piry virus in specimens from *Proechimys* sp., *Caluromys* sp., *D. marsupialis*, and *Oryzomys goeldii* (428). Cocal virus was isolated from a *Heteromys* rat in Trinidad (429). Antibodies against Cocal virus were found in 68% of the *Oryzomys* rat species in Belen, Brazil, during an outbreak of the disease (429). The VS Alagoas virus was isolated from sand flies (*Lutzomyia* sp.) collected in Durania, Colombia, and affects humans, cattle, horses, and mules (422). Alagoas VNAs were found in sera collected from humans, opossums (*Didelphis marsupialis*), porcupines (*Coendou* sp.), and dogs in Arboledas and Durania, Colombia (422). After the first isolation of Chandipura virus, a retrospective serologic investigation in populations from different regions of India demonstrated that the prevalence ranged from 10% to 89%. VNAs were also found in camels, horses, cows, buffaloes, sheep, goats, rhesus and other monkeys (37), and hedgehogs (*Atelerix spiculus*) in Nigeria (241). This virus was isolated from *Phlebotomus* spp. in Aurangabad, Maharashtra State, India (118). Serologic studies on Isfahan virus found a high prevalence of VNAs in humans from several regions of Iran. The highest prevalence of VNAs was found in gerbils (*Rohmbomys opinus*), but no antibodies were found in domestic animals in Isfahan (426).

A wide variety of vertebrates and invertebrates have been experimentally infected with VSV by various routes of inoculation. The discovery that guinea pigs are highly susceptible to VSV provided one of the first and most important tools for virus detection (97). The intracerebral inoculation of suckling and adult mice, hamsters, rats, and chinchillas produces a fatal infection (200,474). Mice die of an acute encephalitis within 2 to 3 days after intracerebral inoculation, and the most important histopathologic lesions consist of occasional perivascular mononuclear cell infiltration and rare foci of necrosis (107). Horses, cattle, swine, and sheep are easily infected by inoculation with the virus into the epithelium of the dorsum of the tongue (180). Agouti (*Dasyprocta* sp.), anteater, aracari, armadillo, bats, birds, coati, and other animals have been experimentally inoculated by several routes to determine their role in VSV ecology (425). The experimental infection of cattle, sheep, goats, pigs, and ponies with Piry virus failed to produce lesions in the first four species and produced only limited ulcerations in ponies (489). In experimental infections of domestic animals with Isfahan and Chandipura viruses, only lesions at the intradermal lingual sites of inoculation were observed. Ponies, steers, and goats were affected by Chandipura virus, but only ponies showed lesions with Isfahan virus (490).

VSV has been experimentally inoculated in *A. aegypti*, with the infection successfully transmitted to mice (428,474). Sand flies (*Lutzomyia* spp.) (94) and blackflies (*Simulium* spp.) (105) are capable of transovarial transmission and infection of susceptible hosts. All members of the VS group are readily propagated by experimental inoculation of mosquitoes (429). Alagoas virus was replicated in *Lutzomyia longipalpis* by intrathoracic inoculation. Experimentally infected sand flies were able to transmit the virus transovarially to their progeny and to infect mice by bite (422).

VSV replicates efficiently in a broad spectrum of vertebrate and invertebrate cell lines. Most mammalian cells sustain virus replication, but they vary significantly in their susceptibility (459). Commonly, BHK-21 cells are used to obtain high virus titers (459) and Vero cells are used for virus isolation (474). Avian-derived cells such as primary chick embryo cultures support VSV replication, yielding high-titer preparations (89). VSV can also infect and replicate to high titers in cell culture systems derived from reptiles and fish, and replication can occur in several insect cell lines (89). The virus replicates in *Aedes albopictus* and *Ae. aegypti* cell lines without producing cytopathic effect (CPE), and it is easily

isolated in *Ae. albopictus* (C6/36) cell lines (89,459,474). A persistent infection with VSV can be established in *Drosophila melanogaster* cell cultures (458).

In vitro replication of VSV proceeds over a wide range of incubation temperatures, depending on the cell line. Highest titers have been obtained in mammalian and avian cells with incubation temperatures ranging from 31°C to 37°C (89,458,459). Temperatures of 11°C have been used with snake-derived cells (89). Several other factors can influence the virus yield in different cell systems, such as the presence of DI and interferon (89,459). Cell differentiation influences the cell susceptibility to VSV infection (458). Cultured human lymphocytes are resistant to VSV infection but support VSV replication after mitogen stimulation (44).

In mammalian and avian cell lines and in some clones of insect cells, VSV rapidly induces CPE (296). Fibroblasts and epithelial cells show characteristic pathologic changes during the early stages of VSV infection. In fibroblasts, the initial microscopic changes consist mainly of an increase of membrane pseudopods, while vacuolization is the principal change observed in epithelial cells. At the final stages of infection, cell rounding is present in both cell types (110). The observed CPE of VSV is the consequence of viral transcription (295) and not the inhibition of cellular RNA synthesis (162). In enucleated vertebrate cells, the virus propagates to nearly normal titers (162). Although VSV does not generally induce CPE, a clone of an *Ae. albopictus* cell line showed CPE at 28°C or 34°C. The ability of other clones from the same cell line to show CPE are temperature dependent (373). Plaque assay is the method of choice for the determination of virus titers. Any cell type in which VSV induces CPE can be used to titrate the virus, but BHK-21 cells are commonly used because the cell line is especially sensitive (89,458).

The VS NJ and VS I strains can be replicated by inoculation of embryonated chicken eggs using the chorioallantoic membrane route. Embryos inoculated at 7 days of incubation and maintained at 35° to 36°C die in less than 24 hours and yield the highest titers. However, embryos at 39° to 40°C may survive infection and present only proliferative lesions in the chorioallantoic membrane (392).

The other six members of this group can also be replicated in numerous mammalian cell lines such as Vero, BHK-21, HeLa, L, and MKTC (54,232,422,426,430,439). Piry and Chandipura viruses have been grown in chick embryo cell cultures (37,54), AP61 insect cells (165), and BSC-1 mammalian cells (37). Piry virus has been replicated in the allantoic cavity of 5-day-old embryonated chicken eggs (489).

Chemical and Antigenic Properties of Viral Proteins

Glycoprotein

The G protein is the serotype-specific antigen (62). There is only 50% homology at the amino acid level between the G proteins of the two major serotypes, I and NJ. The VS I G protein has 511 amino acids with an N-terminal signal peptide of 16 residues (363). The mature protein can be divided into three structural domains: the first 29 amino acids from the C-terminus comprise the cytoplasmic domain, the next 20 hydrophobic residues form the transmembrane domain, and the major segment of the G protein, extending from the transmembrane to the N-terminus, is the external antigenic domain. The VS I G protein is N-glycosylated at positions 178 and 335 (363) and also contains covalently linked fatty acid (palmitate and myristate) in the cytoplasmic domain of the molecule (82,362).

The VS NJ G protein contains 517 amino acids (172). Signal peptide sequence, glycosylation sites, and position and size of the transmembrane domain of VS NJ G protein are identical to those of VS I G protein. The VS NJ G protein is not acylated.

Using MAbs to either VS I or VS NJ G protein, several antigenic determinants have been operationally delineated. Four major antigenic epitopes that reacted with these neutralizing MAbs were found on each G protein (276,277 and 278). Those MAbs that recognized these epitopes did not cross-react between the G proteins of the two serotypes. A fifth neutralizing epitope shared by both G proteins was identified with a MAb derived from VS I that cross-reacted with VS NJ G protein. Many nonneutralizing epitopes also have been defined for the G protein of both serotypes (50,277). Most of the epitopes to MAbs recognize the G protein of VS NJ map to the center (residues 193 to 289) of the molecule, whereas the epitopes to MAbs reactive to the G protein of VS I map either to the cysteine-rich N-terminal domain (residues 80 to 183) or to the cysteine-poor C-terminal region (residues 286 to 428) (239). The VSV G protein also induces a CTL response. The CTLs generated by one serotype can lyse cells infected with the other serotypes (364). This heterotypic cell killing may be the result of cross-reacting epitopes of the VS I and VS NJ G proteins that are recognized by heterotypic nonneutralizing MAbs (277). A panel of overlapping synthetic peptides and CD4+ I-A-restricted T-cell hybridomas was used to analyze T-helper-cell epitopes of the VS I G protein (58). Of nine hybridomas, five were specific for amino acids 415 to 433, three recognized amino acids 52 to 71, and one reacted with amino acids 316 to 335. All three epitopes activated specific T-helper cells *in vivo* that were able to provide functional help in the production of anti-VSV-specific neutralizing immunoglobulin (Ig)G antibody. The comparison of the nucleotide and amino acid sequences of the G protein of the VS I and VS NJ serotypes with those of Cocal, Chandipura, and Piry virus identified several structural similarities between them (38). These proteins have N-linked glycosylation sites at identical positions. The cytoplasmic domains of the five proteins showed very little conservation, sharing conserved tryptophan/proline and cysteine residues at identical and multiple positions, indicating their importance in protein folding and function. The five proteins shared several regions of amino acid identity (38). Regions between positions 124 to 137 and 395 to 424 are involved in the membrane fusiogenic activity of the G protein. The functions of the region between positions 83 and 99 and other conserved regions of four to six amino acids are unknown (38).

Nucleoprotein

The VSV N protein has 422 amino acids and is the group-common antigen of the *Vesiculovirus* genus (33). The N proteins of the two major serotypes share 68.7% identity. The N protein of the VSV serotypes and that of Chandipura virus share 50% to 51% identity (297). Anti-N-protein MAbs have been generated and used extensively to study the

interaction of the N protein with viral RNA and other proteins during the process of virus transcription and replication (31). The VSV N protein may be the predominant cross-reactive antigen recognized by primary anti-VSV CTLs and also the major target antigen for secondary anti-VSV CTL responses (347). An octamer peptide (residues 52 to 59) of the VSV N protein has been defined that is recognized by VSV-specific CTLs in the context of the Kb histocompatibility antigen (449). The comparison of the deduced amino acid sequence of the N protein of Piry virus with VS NJ, VS I, and Chandipura showed an homology that varied from 51.1% to 55.5%. The hydropathic profiles of these four vesiculoviruses showed a high degree of similarity, and three potential functional domains were delineated.

Phosphoprotein

The VSV NS or P protein contains 274 amino acids for both VS I and NJ (179). The VSV P proteins among VSV strains are extremely divergent, and only 33% homology at the amino acid level was reported between the P proteins of the major serotypes, although the overall structure of the protein was retained (179). The P protein is phosphorylated at Ser-59 and Ser-61 in the NJ serotype, and at Ser-60, Thr-62, and Ser-64 in the I serotype. The P protein is sequentially phosphorylated first by cellular casein kinase, CKII, and second by a protein kinase associated with a cellular kinase that is tightly attached to the L protein and the N protein–viral RNA complex (81). Although constitutive phosphorylation of the P protein is not indispensable for VSV RNA synthesis, it could play a role in the multimerization of the P protein and possibly P–L complex formation (407). Three functional domains have been identified on the P protein in relation to its binding to the N protein–RNA template and the L protein. Domain I, an L-binding domain, is located at the N-terminal region of the P protein from amino acids 1 to 137. Domain II is located from residues 214 to 247 and may bind the L protein for transcription activation. Domain III is an RNA-binding domain located in the C-terminal region from residues 250 to 274. Domains I and II of the P protein from VS I are important for the association of the P protein with the viral L protein. The C-terminal region of the P protein is essential for its efficient association with the viral N protein (32).

Matrix Protein

The M protein of VSV contains 229 amino acids (363). The VSV M protein is known to be phosphorylated in VSV-infected cells, primarily on serine (93). The known functions of the protein are its participation in the virus budding process, mediating the binding of the viral nucleocapsid to the cell plasma membrane, and the inhibition of the host-directed transcription by unknown mechanisms (2). The M protein possibly contributes to the production of VSV CPE through its interaction with cellular tubulin (303). The antigenic structure of VSV M proteins has not been studied.

RNA-Dependent RNA Transcriptase

The VSV L protein, the largest protein, contains 2,109 amino acids and has a molecular weight of 241 kd (32). The L protein appears to be closely associated with the P protein in the virion. This association is essential for the L protein to achieve its complete functional capacity (32). Recent studies have shown that the L protein also needs to be associated to the cellular elongation factor-1abg for its activity (109). The L protein functions include RNA synthesis, capping, methylation, and polyadenylation of viral RNAs (32). The amino acid comparison of the L proteins of VS, RABV, Sendai, Newcastle, and measles viruses showed that there are at least four stretches of amino acids that are conserved among rhabdoviruses and paramyxoviruses (342). These regions may be the functional domains responsible for the multiple catalytic activities of the L protein.

Pathogenesis and Pathology

The mechanisms of VSV transmission are not completely understood. Ecologic factors and special conditions regarding the host and the etiologic agent have been implicated in the clinical presentation of the disease (199,200 and 201). Experimental transmission from animal to animal by direct contact has produced irregular results. The virus is unable to penetrate intact skin or mucosa, and for a successful transmission it needs to be introduced beneath the skin and mucous membranes via wounds and abrasions (200,235). The virus can also be transmitted by the bite of insect vectors such as mosquitoes (*Aedes* spp.) (428,474), sand flies (*Lutzomyia* spp.) (94), blackflies (*Simulium* spp.) (105), and other diptera (152).

In natural infection, the incubation period varies from 2 to 9 days, but usually the lesions develop between 2 and 5 days after exposure (208,235). The lesions of VS are indistinguishable from those of FMD. In cattle, initially the lesions are characterized by pink to white papules in the mouth that progress in 1 to 2 days to vesicles. The vesicles may coalesce and rupture, leaving a denuded area that heals in 1 or 2 weeks if there are no secondary infections (235). These lesions may also occur in the dental pad, lips, gums, muzzle, nose, teats, and feet. In the experimental inoculation of horses, it was observed that in the mouth, vesicles appeared 42 hours after inoculation, and 2 days after that, part of the dorsal epithelial covering the tongue sloughed off. In this experiment, vesicles also appeared in the feet. At necropsy, the spleen was enlarged, but no other lesions were observed in the internal organs (97). In the natural infection of horses, lesions can be found in lips, the corners of the mouth, muzzle, nostrils, ears, belly, prepuce, and udder (208). The lesions in pigs are similar to those described for cattle (201). The main microscopic changes consist of intercellular edema in the stratum spinosus that leads to cell dissociation and necrosis. Concomitantly, there is an inflammatory reaction of the mucosa and the lamina propria, with the presence of macrophages, neutrophils, and lymphocytes (235).

Although the development of secondary lesions in places other than the point of inoculation is suggestive of viremia, the virus could not be isolated from blood even at 6 hours after the experimental inoculation of pigs (92,353,408). The virus is present at its highest titer in the vesicular fluid, which represents a transient but very efficient source of virus for contact transmission (92). The virus can be isolated from specimens taken from saliva, tonsils, vesicular fluids of feet, and, in some cases, feces (92,353,408).

Persistent infection of livestock with VSV was postulated as a possible mechanism for viral maintenance in nature (202). Experimental studies were unable to demonstrate the presence of virus in tissues of inoculated pigs (202) and failed to corroborate this hypothesis. Viral RNA was detected in the tongue and draining lymph nodes in cattle 5 months after

experimental inoculation, but no viral RNA was detected in naturally infected cattle (283). No infectious virus could be isolated from these animals (283).

Immune Response

Antibodies, particularly those with virus-neutralizing activity, play a prominent role in the immune defense against infection. VSV has been extensively used as a model to study the immune response in mice (513). However, very little information is available about the immune response elicited in different natural hosts. All neutralizing antibodies are directed toward the G protein (240). In experimentally inoculated mice, VSV induces a very early neutralizing IgM response. This early response is T-helper-cell independent, and it is induced by the ability of VSV G protein to activate B cells directly (513). This ability is the result of the expression of the VSV G protein as repetitive epitopes that are in a rigid, densely packed “procrystalline” form in the virus envelope (513). A switch to an IgG neutralizing response occurs on day 6 to 8 after inoculation. Neutralizing titers of 10^{-4} and 10^{-5} are reached by days 9 to 12 and maintained for more than 6 months (279). The VNA response is sufficient to control the infection in affected animals (275). Adoptively transferred hyperimmune serum protected B-cell-depleted agammaglobulinemic mice from paralytic disease after VSV inoculation (181). Mice are susceptible to intravenous or intraperitoneal VSV inoculation until they are 3 weeks old.

Interferon seems to play an important role in the natural resistance of older mice to VSV infection (178). Eight- to 12-week-old mice devoid of interferon-mediated natural antiviral immunity were highly susceptible to VSV infection despite the ability to mount a normal humoral and cellular immune response against the virus (412). Also, an enhanced secondary antibody response was observed in mice and cattle when interferon gamma was included in the primary immunization (7).

Experimental infection of mice with VSV induces a strong CTL response that is not serotype specific (364). Although anti-G protein CTLs have been demonstrated, the N protein is the predominant cross-reactive serotype antigen recognized by anti-VSV CTLs (347). Humoral immunity develops after natural infection in horses, cattle, and swine, and antibodies can be demonstrated for more than 7 years after infection (201). Experimentally inoculated swine showed a VNA response that peaked at the third week after inoculation. VNAs were still detected at 18 weeks after inoculation at relatively high titers. When challenged for a second time, these animals did not develop lesions and showed a high secondary antibody response. The study of the antiviral T-cell response by stimulation of peripheral blood mononuclear cells with specific antigen indicated that T-cell immunity remained elevated for prolonged periods (353).

Epidemiology

The disease is present only in the Western Hemisphere, and it is enzootic in southern Mexico, Central America, and some regions of South America (473). In the United States, Ossabaw Island, off the coast of Georgia, is the only confirmed area where VS is enzootic (408). In the rest of the United States, the disease is sporadic. Historically, the disease appeared in approximately 10-year cycles, but in recent years it has occurred more frequently. Transmission of VSV during an outbreak remains unclear. Animal-to-animal contact plays a role in transmission between domestic species (51). Mechanical vector transmission has also been documented during epizootics. Virus isolations from nonbiting insects, such as *Musca domestica*, have been reported (166). Many potential biologic vectors of VSV have been suggested, but the phlebotomine sand fly, *Lutzomia shannoni*, is the only confirmed suspect in the United States (474).

In temperate zones, VSV outbreaks begin in late summer and end with the arrival of frost (180). In the United States, the outbreak during 1982–1983 was unusual because it continued throughout the winter months until the following spring (51). In tropical areas, the disease appears at the end of the rainy season and disappears with the advent of the dry season (474).

During outbreaks, morbidity rates in a herd usually range from 10% to 15% (473). Cattle generally recover in few days, but horses and pigs may develop lameness (200,473). A broad spectrum of wild mammals can also be affected (473). Typically, the disease affects only horses, cattle, and swine. Factors that influence the disease spread in dairy cattle have been studied. Coarse roughage, hard pelleted concentrates, poor general and milking hygiene, and insufficient teat sanitation play an important role (199). In the southeastern United States, feral swine had 10% to 100% antibody prevalence from 1979 to 1985 (410). Feral swine on Ossabaw Island showed 12% and 60% seroconversion between June and September in 1982 and 1983, respectively (411). In some enzootic areas of Central America, over 80% of the cattle have antibodies against VSV, but only 9% of the animals may present clinical signs in a particular year. In the same regions, wildlife also have a high seroprevalence to VSV (424). In tropical areas where the disease is enzootic, VSV seroprevalence in the human population may be as high as 48% (231). Serologic studies during outbreaks in Panama demonstrated a seroprevalence of 71% and 34% in personnel working with infected and noninfected cattle, respectively (53). A similar situation has been observed during VSV outbreaks in Colorado, where personnel (veterinarians, researchers, and regulatory staff) handling sick livestock showed an antibody prevalence of 13% whereas unexposed humans had a 6% seroprevalence (355).

The mechanism by which VSV is maintained in enzootic regions is not fully understood. Sand flies may transmit the virus from a putative reservoir (e.g., plants, wildlife, cattle) to livestock. Alternatively, VSV may be maintained in the sand fly population by transovarial transmission, and the insects infect susceptible animals during feeding (94). In some enzootic areas, feral swine have been suggested as a potential amplifying host (409). Molecular epidemiologic studies indicated that enzootic areas may be the origin of the virus responsible for outbreaks in epizootic zones (325). VSV may be introduced in a particular area by the movement of infected animals, wildlife, or insects, but the actual mechanism is unknown. Viruses circulating in enzootic areas present a high genetic diversity, with several lineages coexisting in the same region (326). Within enzootic areas, the viruses seem to adapt under selective pressures exerted by ecologic factors. Viruses from different ecologic areas within enzootic regions belong to different genotypes. Viral adaptation to different insect vectors or mammalian reservoirs might be determinant factors for this divergent evolution (357). Viruses

obtained from a particular outbreak showed a marked genetic homogeneity (325).

Clinical Signs

In cattle, after an incubation period of 3 to 9 days, the body temperature may reach 105° to 107°F. Temperature rise usually begins before vesicle development. Body temperature falls when the vesicles are fully formed, and the depression remains until rupture of the vesicles (98). Affected animals have increased salivation and a sharp reduction in milk production. Eating is difficult because of the sore mouth, with a consequent loss in condition. Lameness develops with foot lesions. If there are no complications, recovery occurs in 4 to 5 days (98). The disease follows an almost identical course in horses and swine (201).

Diagnosis

Because VS is clinically similar to FMD, differential diagnosis between the two diseases is of utmost importance, especially in countries free of the latter disease. Originally, inoculation of natural hosts with material from vesicular lesions was used for differential diagnosis. With the development of a complement fixation technique, a more rapid, sensitive, and accurate method was available for the differentiation of VSV and FMDV (200). Isolation of VSV from vesicular fluid or epithelium of the lesions in mice, embryonated eggs, or cell culture is not difficult (89). The virus can be identified by virus neutralization, complement fixation, or immunofluorescence (201). A rise in antibody titer in serum samples taken during the clinical and convalescent phases of the disease is evidence that the infection was caused by VSV. Virus neutralization and ELISA techniques are commonly used for this purpose (5). Recently, detection of VSV in clinical samples by RT-PCR has been described. This method is highly sensitive and specific, providing a rapid diagnosis and material for genetic characterization of the virus (216,358).

Treatment, Prevention, and Control

Supportive veterinary care of affected animals helps to prevent complications that may delay recovery. Vaccination against VSV has been practiced to only a limited extent. A modified live vaccine was used in parts of the United States, Central America, and Peru. VSV was attenuated in cell culture or chicken embryos. This vaccine, administered intramuscularly in cattle, induced protection from the disease for at least 1 year (201). During the 1985 epizootic in Colorado, an inactivated VSV NJ vaccine with virus propagated in cell culture was used in the field. Later, this vaccine was tested in an experimental trial and it induced antibodies that lasted for 175 days. Viral challenge of the experimental animals was not performed to assess the protection conferred by this vaccine (177). Recently, a recombinant vaccinia virus expressing the VSV I glycoprotein was developed and used to experimentally immunize cattle. Inoculated animals developed antibodies and resisted intradermal lingual challenge (294).

EPHEMEROVIRUS

History

The first reference to bovine ephemeral fever (BEF) can be found in the book *In the Heart of Africa* (378). Not until the mid-20th century was the disease reported in much of its natural range (415). Until 1966, when BEFV was grown in mice (450), research on the agent was restricted to transmission studies in cattle (89). The ability to cultivate BEFV in another system besides its natural host was a fundamental step in the study of the disease and its control (415).

Infectious Agent

Taxonomy

The members of the *Ephemerovirus* genus show a very low cross-neutralization reaction, but they are highly cross-reactive in complement fixation or indirect immunofluorescence tests. No serogroup has been defined within this genus. Phylogenetic studies suggested that the *Ephemerovirus* is more closely related to the *Vesiculovirus* genus than to the other members of the *Rhabdoviridae.* The viruses assigned to this genus include BEFV, Adelaide River virus, and Berrimah virus. Other viruses tentatively placed in this genus are Kimberley, Malakal, and Puchong virus (451). BEFV is the only member of the *Ephemerovirus* genus discussed extensively in this section.

Morphologic and Physical Properties

Although members of the *Ephemerovirus* genus share general morphologic characteristics with the *Rhabdoviridae*, they also present unique features. The viral genome of BEFV is 14.8 kb in length and contains 10 genes (3'-N-M1-M2-G-G_{NS}-a_1-a_2-b-g-L-5') separated by intergenic regions of 26 and 53 nucleotides (451). In contrast to BEFV, the viral genome of the Adelaide River virus is 14.6 kb in length and contains nine genes (3'-N-M1-M2-G-G_{NS}-a_1-a_2-b-L-5') separated by intergenic regions of one to four nucleotides (451).

Biologic Properties: Host Range and Infection of Experimental Animals

Only cattle and water buffalo are clinically affected by BEF (413), but on the basis of serologic evidence a wider range of wild ruminants can be infected (6,111). Suckling mice are susceptible to BEFV when inoculated by the intracerebral route (450), as are suckling hamsters and rats with mouse-adapted BEFV (222). In Kenya, virus isolation was reported from a pool of five *Culicoides* spp. (112), and in Australia BEFV was obtained from *C. brevitarsis*, *Anopheles brancoftii*, and a pool of several species of mosquitoes (415). BEFV can be isolated from infected blood by intravenous inoculation of chicken embryos (135). Cells of bovine origin are poorly susceptible to BEFV (136). The virus grows well in BHK-21 cells inoculated with infected mouse brain or bovine leucocytes. BEFV has also been adapted to hamster lung, Vero, and C6/36 cell lines. Primary inoculation of *Ae. albopictus* C6/36 cells with the leukocyte fraction of the blood with subsequent

passage through BHK-21 cells has been used for virus isolation, but this method is too slow for practical diagnosis (414).

Chemical and Antigenic Properties of Viral Proteins

Glycoprotein G

The glycoprotein G is the viral protein that induces protective neutralizing antibodies and is a structural component of the viral envelope (213). The BEFV G protein consists of 623 amino acids with a calculated molecular weight of 72.7 kd, and it has five potential glycosylation sites. The BEFV G protein has a 30.8% identity and a 53.4% similarity with the homologous protein of Adelaide River virus (467). Distinct neutralizing antigenic sites (G1, G2, $G3_a$, and $G3_b$) have been identified in BEFV G protein using MAbs in competitive assays and cross-neutralization tests (106). Selection of escape mutants allowed identification of several epitopes in the extracellular domain. Two B-cell epitopes were mapped in the G1 antigenic site, at positions 488 to 491 and 499 to 502. The mutation in site G2 mapped at amino acid 169 or 187. Antigenic site G3 appears to combine complex discontinuous epitopes, because mutations were located at positions 49, 57, 218, 229, and 265 (250). Alignment of BEFV G protein with the homologous protein of representatives among the *Vesiculovirus* and *Lyssavirus* showed similarities in the location of neutralizing epitopes. The conservation of cysteine residues suggests a similar folding pattern for these proteins (250).

Glycoprotein G_{NS}

The genome of BEFV and its close relative Adelaide River virus encodes a second glycoprotein, G_{NS}, that is located downstream of the G gene. G_{NS} is synthesized in approximately the same amount as G during cell infection (461), but it is not found in the mature virion. Intracellularly, G_{NS} is localized in the endoplasmic reticulum/Golgi complex, and it is associated with amorphous structures in the cell surface but not with viruses in the budding process. It is highly glycosylated, with a molecular weight of 90 kd. G_{NS} protein shares significant amino acid sequence homology with the G protein, but it does not induce protective neutralizing antibodies (213). The G_{NS} function is unknown. It was proposed that the gene coding for this protein originated by gene duplication by a copy-choice mechanism involving relocation of the polymerase in an upstream position during viral replication (467).

Nucleoprotein

The N protein of BEFV is 431 amino acids long with an estimated molecular weight of 49.519 kd (462). The N protein is phosphorylated (461). In amino acid positions 349, 362, and 371 there are serine residues that are potential phosphorylation sites and optimal substrates for CKII (462). Amino acid sequence comparison between N proteins of BEFV and members of the vesiculoviruses and the lyssaviruses showed regions of high homology (462). A more detailed analysis identified five potential domains with conservation of identical or structurally similar amino acids in the N protein of BEFV, VSV, and RABV (462). Serologic cross-reaction between BEFV and RABV N protein has been described (462). Polyclonal antibodies against BEFV, RABV, MOK, LBV, and DUV reacted with the N protein of BEFV and RABV viruses (462). Extensive cross-reactivity between rhabdoviruses has been reported (61). There has not been a detailed study of the other viral proteins. According to their molecular weight, relative abundance, and other characteristics, other viral proteins have been tentatively assigned putative functions, taking as a model the RABV proteins. The RNA polymerase activity L protein has been assigned to a 180-kd polypeptide that is core associated. The M protein function was tentatively assigned to a core-associated protein of 43 kd. The M2 function has been assigned to a phosphorylated core-associated protein of 29 kd (461). The functions of a1, a2, b, and g gene products are unknown (451).

Pathogenesis and Pathology

BEFV enters the body through the bite of an infected hematophagous arthropod (389) and is not transmitted by animal-to-animal contact (414). The site of primary replication is unknown (389). After an incubation period of 4 to 7 days, there is a viremia that lasts 3 to 4 days, with the virus in the neutrophil fraction (510). There is a marked neutrophilia with neutrophil infiltration of the serous membranes. Viral antigen can be detected in neutrophils and in the mesothelial cells (511). Neutrophils play an important role in the development of clinical symptoms and pathologic lesions of BEF. Neutrophil-depleted animals experimentally infected with BEFV did not develop clinical signs or detectable neutralizing antibodies, although they were viremic (510). While the clinical signs are being manifested, there is a marked increase of vasoactive amine activity that may be responsible for areas of increased permeability in the serous membranes. The increase in amine activity coincides with the peak of neutrophilia (445). Also, there is an elevation of plasma fibrinogen, regarded as a nonspecific response of the inflammatory process, and hypocalcemia (445). The hypocalcemia is responsible for some of the clinical signs such as anorexia, ruminal and intestinal stasis, and muscle fasciculation and paralysis. The basic lesion in BEF is a fibrinous polyserositis (511). This lesion involves synovial, pericardial, pleural, and peritoneal cavities. There is a characteristic accumulation of synovial fluid in the joints, as well as fluid accumulation in the abdominal, thoracic, and pericardial cavities. This fluid contains a large amount of neutrophils. Viral antigen can be demonstrated in these cells. This accumulation of fluid is the result of an arteritis of the small vessels (389).

Immune Response

Observation of sentinel cattle during a BEF outbreak showed that the animals had neutralizing antibodies by the third day after the clinical signs appeared. Sick animals were protected during later outbreaks (414). Transient protection against BEFV was observed in animals infected with arboviruses. Protection of these animals was thought to be the result of the production of interferon (414). Calves vaccinated with two doses of vaccine showed high and persistent neutralizing antibodies and resisted experimental challenge and natural infection (443).

Epidemiology

BEF is distributed throughout Africa, the Middle East, Southeast Asia, and northern Australia (415). It has never been

reported in the Americas. The disease occurs during summer and autumn and disappears with the arrival of the first frosts in subtropical areas. In the tropics, yearly occurrence is associated with the rainy season (415). Although transmission of the virus occurs via insect vectors, the difficulty of isolating the virus from insects hampers the recognition of the vector species involved in its transmission. BEFV was isolated from *Culicoides* spp. and from mosquitoes. In Australia, the geographic range of the disease is larger than that of the *Culicoides* species from which the virus was isolated (415). Two species of mosquitoes, *Culex annulirostris* and *Anopheles annulipes*, may be implicated in transmission in these areas (415). In general, morbidity is low, but in some outbreaks, all of the animals in a herd may be affected. In other instances, only 2% or 3% of the animals show clinical signs (415).

Natural disease occurs only in cattle and water buffalo. Although seroprevalences of 13% to 38% were reported in cattle in enzootic areas, a higher prevalence of 64% has been observed during outbreaks (108). The role of wild ruminants in the maintenance of BEFV in nature is not understood. Seroprevalences ranging between 28% and 54% were found in wild ruminants in Kenya, Zimbabwe, and Tanzania (6,111).

Clinical Signs

After an incubation period of 2 to 4 days, the first clinical sign is fever (40° to 42°C), accompanied by malaise and a severe drop in milk production. In 12 to 24 hours, fever remits, followed by a second febrile phase. During this second phase, the animals are depressed and anorexic and show muscle stiffness and lameness. Ruminal stasis, nasal and ocular discharges, and swelling of one or more joints are present. Subcutaneous emphysema may develop in some animals (413). The clinical signs persist for 1 or 2 days, and thereafter animals recover rapidly and totally. Clinical signs are much more severe in adults than in calves. Calves under 6 months of age show no clinical signs (43).

Diagnosis

Clinical diagnosis of BEF is based on its rapidity of spread and transient nature (43). For confirmation, virus isolation or the demonstration of an increase in antibody titer in paired serum samples is needed. Although impractical for routine diagnosis, cattle inoculation with blood from BEFV-affected animals is the most sensitive method for viral isolation. Isolation of BEFV in *Ae. albopictus* cells from the blood of infected animals, followed by direct immunofluorescence to detect the presence of viral antigens, has been used in experimental studies (446). Virus neutralization is commonly used to detect a rise in antibody titer between clinical and convalescent-phase serum (131). A complement fixation test was developed to detect antibodies using antigen prepared in BHK-21 cells, but it has not been widely applied (29). A blocking ELISA test compared favorably with the virus neutralization technique and did not detect cross-reactions with antibodies to Kimberly and Berrimah viruses (512).

Treatment, Control, and Prevention

Animals affected with BEFV should be rested and protected from inclement weather. The administration of phenylbutazone blocks the host inflammatory response, responsible for the clinical signs. The drug suppresses the synthesis of cyclooxygenase-dependent prostaglandins, hence modifying the inflammatory response *in vivo* (447). Parenteral administration of calcium solutions helps in recovery (444).

Vector control is very difficult, and vaccination is the only practical method for prevention of BEF. Several attenuated vaccines were produced by serial passage in mouse or cell culture (413). They provided protection against experimental challenge when mixed with adjuvants and given in several doses (413). Inactivated vaccines were developed in Japan and Australia (413), but they induced poor and unreliable immunity. More recently, vaccinia virus expressing the G protein elicited neutralizing antibodies in cattle, which were resistant to a subsequent viral challenge (213). Additionally, an experimental subunit vaccine consisting of purified G protein mixed with Quil adjuvant conferred protection against viral challenge when administered in two doses, 21 days apart (448).

PERSPECTIVES

Although there is a wealth of information about the chemical and biologic characteristics of some animal rhabdoviruses, other important aspects such as pathogenesis, mechanisms of virus maintenance, sylvatic transmission in endemic regions, and factors that contribute to the reemergence of disease in epizootic areas after long periods of epidemiologic silence, remain largely unknown. For example, no definitive reservoirs of either VSV or MOK have been identified with certainty. Moreover, rabies was believed absent from the entire continent of Australia until a novel bat lyssavirus was discovered, largely by accident, in 1996. From the standpoint of vaccine cross-reactivity, will the next discovery be as benign? Clearly, insights provided by innovative avenues of investigation may help to unravel remaining secrets of rhabdovirus transmission and perpetuation. As an example, co-feeding studies of infected and noninfected blackflies, *Simulium vittatum*, on nonviremic mice, recently demonstrated the possibility of infection of an insect vector on a nonviremic host (301), and findings such as these may contribute to an overall better understanding of the mechanisms for virus maintenance in nature, even in the absence of viremia in certain mammalian taxa. Given the dramatic global changes that have occurred during the last few decades, the perceived impact of environmental alterations on the relatively rapid evolution of the known *Lyssavirus*, *Vesiculovirus*, and *Ephemerovirus* groups, and the plethora of poorly studied and still unclassified rhabdoviruses, only the development of new interdisciplinary studies via international collaborations can evaluate the potential risk these agents may present as emerging public health and veterinary pathogens of tomorrow.

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CHAPTER 40 *Filoviridae:* Marburg and Ebola Viruses

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HISTORY

In 1967, multiple cases of severe hemorrhagic fever with nosocomial transmission among European laboratory workers led to the spectacular initial identification of Marburg virus and this new virus family (126,142). The virus came with African green monkeys imported from Uganda and the epidemic was controlled, with limited interhuman spread, when the monkeys and contaminated materials were disposed of. The next recognized filovirus activity was in 1976, when Ebola virus appeared simultaneously in the Democratic Republic of the Congo (DRC; then called Zaire) and Sudan (20,231,232). These epidemics were actually caused by two distinct forms of the virus, a fact not recognized until years later (26,47,144,197). In the ensuing years, only sporadic filovirus activity came to biomedical attention (6,76,93,112,162) and progress was made in the basic laboratory study of filoviruses.

In 1989, Ebola returned to center stage and borrowed a theme from the Marburg 1967 episode: It first came to the United States with imported macaques from the Philippines (105,166,169). This Reston species appears to be less pathogenic for humans, but outbreaks in macaques have shown it to cause a high degree of mortality in these monkeys. Furthermore, the quarantine rules put in place to prevent a recurrence of the 1967 Marburg epidemic limited the infection to the actual monkey quarantine facilities in this and subsequent importations (50). The actual origin of the Reston species of Ebola virus was never determined, although multiple importations occurred from the same monkey export facility (Table 1) (145).

Marburg virus	1967	Marburg and Frankfurt, Germany Belgrade, Yugoslavia	Uganda	32 (23)
	1975	Johannesburg, South Africa	Zimbabwe	3 (33)
	1980	Nzoia and Mabole, Kenya	Western Kenya	2 (50)
	1987	Kisumu, Kenya	Western Kenya	1 (100)
	1999–2000	Watsa, DRC	DRC	*
Ebola virus				
(Zaire)	1976	Yambuku, DRC	DRC	318 (88)
	1977	Tandala, DRC	DRC	1 (100)
	1994	Mikoulou, Gabon	Gabon	49 (59)
	1995	Kikwit, DRC	DRC	315 (77)
	1996	Mayibout, Gabon	Gabon	31 (64)
	1996	Booué, Gabon and Johannesburg, South Africa	Gabon	60 (75)
(Sudan)	1976	Maridi, Sudan	Southern Sudan	284 (53)
	1979	Nzara and Yambio, Sudan	Southern Sudan	34 (65)
	2000	Gulu District, Uganda	Uganda	>400 (~50)
(Reston)	1989	Reston, Virginia (also Pennsylvania and Texas)	Philippines†	1 (0)†
	1992	Siena, Italy	Philippines	0
	1996	Alice, Texas	Philippines	0
(Côte d'Ivoire)	1994	Taï Forest, Ivory Coast and Basel, Switzerland	Taï Forest, Ivory Coast	1 (0)

*Reston virus has been traced only to a single monkey-breeding facility in the city of Calamba, Philippines.
†In one study, mortality in monkeys was estimated at 60% (89).
DRC, Democratic Republic of the Congo.

TABLE 1. *Outbreaks of Filovirus Disease*

Then from 1994 to 1996, Ebola virus apparently became active in broad areas in, or adjacent to, the central African rain forest. Small epidemics of the Zaire species occurred in Gabon (2,82) and a large outbreak caused severe disruption in Kikwit, DRC (33,119,194). A new species was noted to be active in Côte d'Ivoire (135). Subsequently, there has been no sign of the Zaire species of Ebola, but in September of 2000, serial human infections by the Sudan species appeared in northern Uganda. Active analysis of a string of new infections with Marburg viruses derived from different phylogenetic lineages in the eastern DRC in 1999 and 2000 is also underway (230).

With the exception of the recent Marburg outbreak, all the filovirus epidemics have followed a similar pattern. One

presumed index case leads to a chain of infection that is amplified by needle use or close human contact. The application of appropriate parenteral practices and barrier nursing as well as simple quarantine of sick patients has stopped all these outbreaks. All recognized filovirus outbreaks are summarized in Table 1. The high case fatality (25% to 80%) among infected humans, and the public imagination have amplified the apparent impact far beyond the actual disease significance. Nevertheless, we remain profoundly ignorant of the natural history of the viruses, there is no treatment, and relatively little is understood of the pathogenesis and immunology of the infections. They will continue to be a problem in Africa until proper hospital hygiene becomes practical and routine.

THE INFECTIOUS AGENTS

Classification

Filoviruses are taxonomically classified in the order *Mononegavirales* (160), which contains a large variety of enveloped viruses with nonsegmented, negative-stranded (NNS) RNA genomes. Initial morphologic observations of Marburg virus suggested that it might best be placed in the family *Rhabdoviridae*, but subsequent morphologic, genetic, physiochemical, and virologic studies of Marburg and Ebola viruses led to the creation of a separate family, the *Filoviridae* (120). The family is divided into two genera, currently designated as “Ebola-like viruses” and “Marburg-like viruses.” There are four species within the “Ebola-like viruses,” Zaire (type species), Sudan, Reston, and Côte d'Ivoire (Ivory Coast). A single species, Marburg virus, makes up the “Marburg-like viruses.” Division into two genera, *Ebolavirus* and *Marburgvirus*, is under consideration, with species names being assigned to the different Ebola virus lineages. There is no antigenic cross-reactivity between Marburg and Ebola viruses, but the Ebola viruses have common and unique epitopes.

Because of their aerosol infectivity, high mortality rate, and potential for person-to-person transmission, Marburg and Ebola viruses are classified as biosafety level 4 (BSL4) (WHO: risk group 4) pathogens, for which maximum containment facilities are required for all laboratory work (36,122). The biosafety level of the Reston subtype of Ebola may eventually be reconsidered because several documented human infections have not been associated with disease, but there is insufficient experience to recommend a change at this time.

Virion Morphology and Structure

Electron microscopy of Marburg and Ebola virions produced in tissue culture have shown them to be pleomorphic, appearing as either long filamentous (and sometimes branched) forms or in shorter U-shaped, 6-shaped, or circular configurations (Fig. 1, Fig. 2, and Fig. 3) (57, 78,157,173). The filamentous forms vary greatly in length (up to 14,000 nm), and an early study showed that the unit length associated with peak infectivity is 790 nm for Marburg virus and 970 nm for Ebola virus (120). A more recent comparison of filovirus isolates grown in various cell lines revealed the mean unit length of virions to be about 860 nm for Marburg virus and about 1,200 nm for Ebola viruses (78). Virions have a uniform diameter of 80 nm and are composed of a helical ribonucleoprotein (RNP) complex (nucleocapsid) with a closely apposed unit-membrane envelope (derived from host cell plasma membrane), whose surface is studded with 10-nm-long peplomers (Fig. 4). Nucleocapsids are 50 nm in diameter, with an axial space 20 nm in diameter and a helical periodicity of about 5 nm. Virion density is 1.14 g/mL as determined by centrifugation in a potassium tartrate gradient (121). Marburg and Ebola virions contain one molecule of noninfectious, linear, negative-sense, single-stranded RNA with a Mr of 4.2×10^6, constituting about 1 percent of the virion mass (177).

FIG. 1. Ebola virus. Unfixed diagnostic specimen, from the first Vero cell passage of the first human blood specimen examined in the 1976 epidemic. Filamentous virions unpenetrated (*top*, ×35,000) and penetrated (*bottom*, ×63,000) by negative-contrast medium (sodium phosphotungstate).

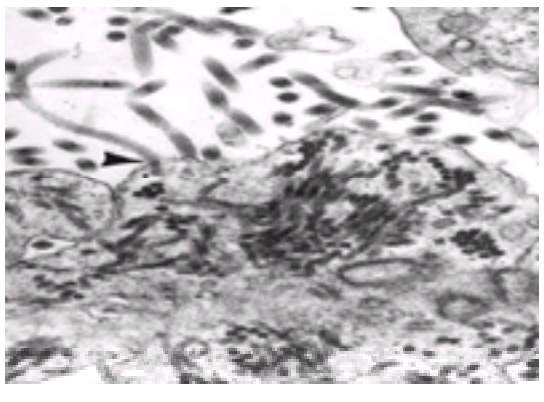


FIG. 2. Ebola virus. Vero cell culture, day 2; virions budding from plasma membrane (*arrow*) and preformed

nucleocapsids in cytoplasm (×28,000).

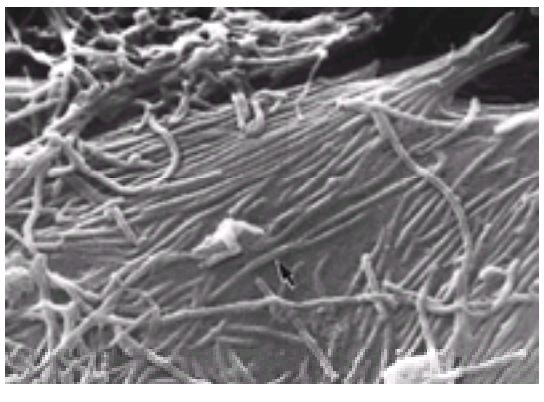


FIG. 3. Scanning electron micrograph of Ebola-infected cultured cell showing numerous virus particles (*arrow*) along the cell surface (×13,500). (Courtesy of C. S. Goldsmith and J. Carr.)

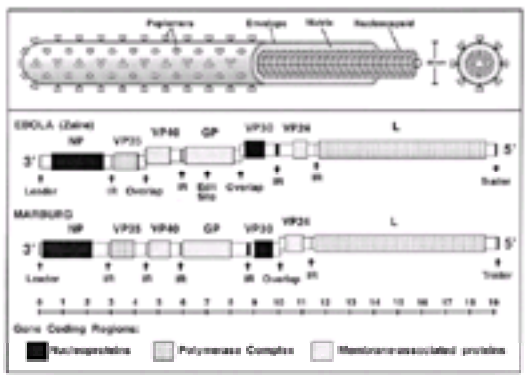


FIG. 4. Schematic representation of the structure of a filovirus virion and the genome organization of Ebola virus (Zaire species) and Marburg virus (Musoke strain). The virion is depicted as a full-length particle with the internal nucleocapsid exposed, and also as a cross-section. Virion proteins as noted in Table 2. IR, intergenic region.

Gene order	Gene	Protein function	MW [kDa][a]
1	Nucleoprotein (NP)	RNA encapsidation	98–104
2	VP35	Cofactor in polymerase complex	35
3	VP40	Matrix protein; virion assembly and budding	26–40
4	Glycoprotein (GP)	GP[b] = Virus entry (surface peplomer)	150–170
		SGP[c] = Unknown	50–60
5	VP30	RNA encapsidation	27–30
6	VP24	Unknown	24–25
7	Polymerase (L)	Transcription and replication	270

[a]Molecular weight based on sodium dodecyl sulfate–polyacrylamide gel electrophoresis migration.
[b]Virion GP (peplomer) is in the form of a trimer of the GP_1–GP_2 heterodimer; MW is for the heterodimer.
[c]Expressed only by Ebola viruses (primary gene product of GP gene); MW is for the monomeric form.

TABLE 2. *Filovirus genes (3' to 58) and the functions and relative molecular weights of gene products*

Marburg and Ebola virus infectivity is quite stable at room temperature (20°C) and can resist desiccation (18), but it is largely inactivated in 30 min at 60°C. Infectivity is also greatly reduced or destroyed by high doses of ultraviolet light and gamma irradiation (40,56,146,223), lipid solvents, b-propiolactone, and commercial hypochlorite and phenolic disinfectants. [*Note:* Decontamination requires specific, controlled use of virus inactivating agents (34,36,122).]

GENETICS, VIRUS PROTEINS, AND REPLICATION

Genetics

As do all members of the order *Mononegavirales*, filoviruses contain a single-stranded, negative-sense RNA molecule as their genome. The primary structure of the genomic RNA has been determined for two Marburg virus isolates (from 1967 and 1980), as well as a 1976 isolate of the Zaire species of Ebola virus (27,28,60,61,124,191,192,218). The genomes of filoviruses are approximately 19,000 bases in length and contain seven sequentially arranged genes (see Fig. 4). These genomes are the largest of the order *Mononegavirales*, and their organization generally conforms to that seen for paramyxoviruses and rhabdoviruses. However, their complexity is more akin to viruses of the family *Paramyxoviridae.* The extragenic sequences at the extreme 3' (leader) and 5' (trailer) ends of the genomes of filoviruses are conserved, show a high degree of complementarity, and can form stem-loop structures (148,218). For all NNS RNA viruses, these sequences are important in the initiation of transcription and the replication of full-length genome RNA, and they serve as promoters for the virus polymerase; certain adjoining sequences may also be involved in promoter activity. Analyses of

defective interfering particles have shown the Ebola virus promoters to be contained within 156 and 177 nucleotide regions of the genomic and antigenomic RNA 3' termini, respectively (30). Genes are delineated by conserved transcriptional signals, beginning with a start site at the 3' genome end and terminating with a transcriptional stop (polyadenylation) site. Termination of transcription occurs at a series of five or six Us, where stuttering (repeated copying) by the polymerase results in the addition of long poly(A) tails to the transcripts. Polyadenylation/stop signals generally conform to the following consensus sequence: 3'-UAAUUC(U) $_{5–6}$. Transcriptional signals of filoviruses contain a common sequence, 3'-UAAUU, at the 5' end of start sites and at the 3' end of stop sites, a property that is so far unique to these viruses. In addition, there are other characteristics that distinguish filovirus genomes from those of other NNS RNA viruses. One unique characteristic is that filovirus genes tend to possess long noncoding regions at their 3' and/or 5' ends, which contribute to the increased length of the genome and may function in the stability of transcripts. Analysis of the 5' ends of filovirus transcripts shows a potential for the formation of conserved, stable, stem-loop structures, which might affect their stability and capacity to bind ribosomes (150,191).

Another unique feature of filoviruses is the positioning of certain neighboring genes. The genes of rhabdoviruses and paramyxoviruses are usually separated by short intergenic regions of one or more nucleotides, but some genes of filoviruses overlap one another (61,191,197,218). These overlaps are short, are limited to the conserved transcriptional signals, and are centered on the common 3' UAAUU pentanucleotide sequence described earlier. There is one overlap in the Marburg genome (VP30 to VP24 genes), but in the Zaire species of Ebola virus there are two overlaps (VP35 to VP40, and GP to VP30). The positioning of the Marburg virus overlap and a long intergenic region (that precedes the VP30 gene) appears to be shifted one gene with respect to the genome of the Zaire species of Ebola virus. Preliminary sequence analyses of other Ebola virus species (Sudan and Reston) have indicated that overlaps are also present in their genomes, indicating that this is a general feature of the family. Overlaps have been identified in the genomes of human and bovine respiratory syncytial virus (68 bases between M2/22K and L genes) (44) and two rhabdoviruses, Sigma virus (207) and Adelaide River virus (222), but these overlaps are not common features of their respective families. The rhabdovirus overlaps are shorter and are most like those of Ebola and Marburg viruses, but nonconserved sequences separate the transcriptional signals and do not overlap. The mechanism by which initiation of transcription of the 5' overlapping gene occurs is unknown, but for the short overlaps it is possible that the proximity of the polymerase allows recognition of the start site. The function of all these overlaps is at present unclear, but transcription attenuation of the downstream gene has been proposed for human respiratory syncytial virus (44). For Ebola virus (Zaire species), transcription of the VP40 and VP30 genes do not appear to be attenuated, and in the case of the VP40 gene, expression is very high.

The organization of the glycoprotein (GP) genes of Ebola viruses is quite unusual and provides an important distinction between Marburg virus and Ebola viruses. The Marburg virus GP gene encodes a single product, the GP, in a conventional open reading frame (ORF), whereas all Ebola viruses encode the GP in two ORFs (0 and -1 frames) that are expressed through transcriptional editing (197,216). The primary gene product of the Ebola GP gene is not the GP, however, but rather a smaller, nonstructural, secreted glycoprotein (SGP) that is released from infected cells in large quantities. The transcriptional editing event that leads to GP production occurs at a series of seven Us on the genomic RNA template and results in the insertion of an additional single A, which connects the GP open coding frames; approximately 20% to 25% of the transcripts are edited. The mechanism of insertion most likely evolved out of the polymerase's ability to polyadenylate by stuttering on a poly(U) template. However, insertion of a single nucleotide at the editing site appears to occur with a high degree of fidelity. Transcriptional editing is not unprecedented in the order *Mononegavirales*, as a similar type of editing has been demonstrated for the P gene of certain paramyxoviruses. However, the editing of Ebola virus GP gene transcripts is the only example of a virus glycoprotein that is expressed through this type of mechanism. Sequence analysis of the GP genes of Marburg virus isolates indicates that a nucleotide sequence that corresponds to the editing region of Ebola virus GP genes is absent (198), and efforts to detect a similar secreted form of the Marburg virus GP have been unsuccessful. The difference in filovirus GP gene organization provides important evidence pointing to a divergent evolution for Ebola and Marburg viruses.

Phylogenetic analyses of the family *Filoviridae* have been performed using the coding region of the glycoprotein gene (82,197,198,204,212). The evolutionary profile of the family (Fig. 5) indicates that (a) Ebola and Marburg viruses represent distinct lineages of filoviruses, (b) the four species of Ebola viruses also represent distinct lineages, and (c) there is an extraordinary level of genetic stasis within the lineages of Ebola virus. Nucleotide and amino acid differences between Marburg and Ebola viruses are both approximately 55%, whereas Ebola virus species show 37% to 41% differences in nucleotide and amino acid sequences (197). These same levels of sequence variation are also seen when other genes are compared. Within species of Ebola viruses, however, there is a remarkable degree of genetic stability (<2% change over up to 19 years), indicating that these viruses have most likely reached a high degree of fitness as they have adapted to their respective niches.

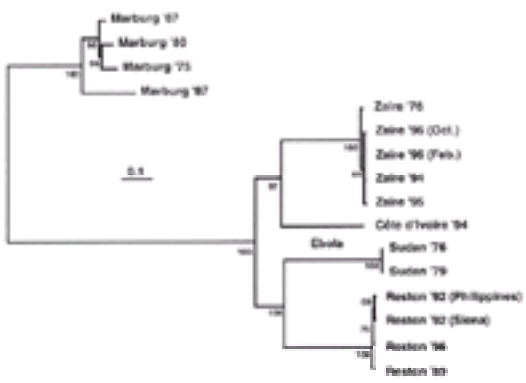


FIG. 5. Phylogeny of the family *Filoviridae.* This phylogenetic tree is derived from comparisons of nucleotide sequences for the GP gene coding regions. Analysis was performed using the Puzzle computer program (version 4.0) developed by Korbinian Strimmer and Arndt von Haeseler and the Treeview program of Roderic D. M. Page. The tree is a quartet puzzling tree derived from 10,000 quartet puzzling steps and is based on maximum likelihood values estimate using the

HKY model (88) and a uniform rate of heterogeneity. *Bar length* equals 0.1 nucleotide substitutions per site.

Marburg virus isolates have not shown the degree of variation seen among Ebola virus species, but two lineages of Marburg virus have been described (112,198). The 1987 isolate from Kenya appears to represent a separate lineage from the three previous isolates, but the difference (21% to 23% amino acid change) is not enough to constitute a separate species (198). Preliminary analysis of the 1999–2000 outbreak in Durba, DRC, suggests that both the recognized lineages are present (230). Phylogenetic analyses of deduced amino acid sequences of the entire L-gene coding region indicate that filovirus L proteins are more like those of paramyxoviruses than those of rhabdoviruses (A. Sanchez, unpublished data, 1995).

Virus Proteins

Filovirus proteins can be subdivided into two categories, those that form the RNP complex and those that are associated with the envelope (see Fig. 4 and Table 2). Those associated with the nucleocapsid are involved in the transcription and replication of the genome, whereas the envelope-associated proteins primarily have a role either in assembly of the virion or in receptor binding and virus entry.

Proteins Forming the RNP Complex

Nucleoprotein

The nucleoprotein (NP) is the primary structural protein associated with filovirus nucleocapsids (15,55,60, 121,123). The NPs of filoviruses are phosphorylated and range in size (Mr) from 96 to 104 kd, as determined from sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis (12,14,55,139). Their predicted mass (deduced from nucleotide sequence analysis) is approximately 20 kd smaller than the size estimated from SDS-PAGE. The NPs of filoviruses can be divided into a hydrophobic N-terminal half, which contains all the cysteine residues, and a hydrophilic C-terminal half, which contains most of the proline residues and is extremely acidic (192,193). The acidic nature of the C-terminal half of the NP, together with its phosphorylation, may account for the anomalous SDS-PAGE migration noted through reduction of the binding of negatively charged SDS molecules. Comparisons of the predicted amino acid sequences of the NPs of Marburg virus and the Zaire species of Ebola virus revealed a significant degree of homology in their N-terminal 400 residues, but not in the remaining C-terminal residues (193). This lack of homology has been noted in the approximately 100 C-terminal amino acids of paramyxovirus NPs (74), where this region of the NP is thought to interact with the matrix protein during virus assembly. It was determined that a span of about 130 residues in the central region of the NPs of filoviruses showed marked homology with paramyxoviruses and to a lesser extent rhabdoviruses (193). In another study, which compared the NPs of pneumonia virus of mice to those of other paramyxoviruses, rhaboviruses, and Ebola virus, it was suggested that the first 350 to 400 amino acids retained a similar structure that contains conserved regions that very likely have common functions (7). The N termini of the NPs of these viruses may function in the binding of genomic RNA, and the variable region may interact with other virus proteins, such as the matrix protein.

VP30

The VP30 protein is a minor nucleoprotein, and like the NP it is phosphorylated and tightly bound in the nucleocapsid (55,123). The N terminus is rich in basic amino acids (particularly arginine) and may be involved in binding to virus RNA or the acidic C terminus of NP.

Polymerase

As with other negative-strand RNA viruses, the RNA-dependent RNA polymerase, or L (large) protein, of filoviruses is the largest and least abundant virus protein in both the virion and infected cells. The predicted amino acid sequences of filovirus L proteins contain three regions in the N-terminal region and a putative ATP-binding motif in the C-terminal region that are conserved in the polymerases of paramyxoviruses and rhabdoviruses (148,218).

VP35

The position of the VP35 gene in the genome corresponds to that of the phosphoprotein genes of rhabdoviruses and paramyxoviruses, but there is no evidence that VP35 is phosphorylated. Nevertheless, it appears that this protein has an essential role as a cofactor in transcription and replication, similar to that of the P proteins of other NNS viruses (15,149,151)

Membrane-Associated Virion Proteins

GP

The GP is the sole structural protein making up the virion surface spikes that mediate virus entry into susceptible host cells through receptor binding. GP is the most studied of the filovirus proteins, not only for its importance in virus entry and pathogenesis but because it is a prime target for vaccine development. Research on filovirus GP has been facilitated through the use of recombinant DNA technology to permit biochemical and functional assays without the constraints of working with the infectious filovirus (38,101,200,206,217,228,229); research on other filovirus proteins has been similarly impacted.

Virion spikes are composed of homotrimers of the GP (65,199) anchored in the membrane in a type I orientation and are heavily glycosylated with both N-linked and O-linked glycans (13,62,83,197,199,215). The GPs of filoviruses exhibit a fairly high degree of homology in their N-terminal and C-terminal regions, whereas the middle third is highly variable (even between Ebola virus species). The central/variable region is hydrophilic, whereas the conserved regions appear to be more hydrophobic and contain the bulk of cysteine residues, most of which are conserved between Marburg virus and Ebola virus GPs. The variable region also contains most of the predicted N-linked carbohydrates and most if not all of the O-glycans. The addition of O-glycans confers an extended, mucin-like property to the variable region, and the decreased secondary structure may allow mutations to be more easily tolerated (198). Analysis of the GP carbohydrate composition has shown that Marburg virus isolates lack terminal sialic acid when grown in Vero E6 or MA-104 cells, unlike the GPs of Ebola viruses, which contain abundant a(2–6) and/or a(2–3) linked sialic acids (62). No neuraminidase activity has been found with any filovirus GP, and the differences seen in sialic acid addition may be caused by differences in targeting as they are directed though the trans Golgi apparatus.

The GPs of all filoviruses are posttranslationally cleaved by furin, a subtilisin/kexin-like convertase localized in the trans Golgi, and there are indications from studies of other viruses that cleavage can control pathogenicity (127). A precursor form (GP_0) is cleaved close to the end of the variable/mucin-like region, yielding GP_1 and GP_2 molecules (199,217,220). This endoproteolytic processing of the Ebola virus GP_0 was found to be dispensable, as virus entry proceeded despite mutation of the furin site (229), but cleavage is most likely required for efficient activity in the natural host. The location of the cleavage site is conserved in Ebola viruses, but the site itself shows some variability (199,217). The furin cleavage site for the Marburg virus GP is in the same region as the Ebola virus GP, but it is positioned more toward the N terminus (67 residues) (220). GP_1 and GP_2 are linked together by disulfide bonding to form a heterodimer (197,199,217), and mutational analysis has determined that a single cystine bridge links these molecules (200).

The virion peplomers are formed by trimers of the GP_1-GP_2 heterodimer and probably assemble in the endoplasmic reticulum. Crystallization and x-ray diffraction studies of portions of a recombinant fragment of GP_2 showed that trimerization occurs when heptad repeat sequences form coiled coils (141,224,225). These rod-shaped structures are similar in appearance to the HA_2 of influenza, the gp41/transmembrane (TM) glycoproteins of retroviruses, and SNAREs (37,141,225). Within GP_2 is a highly conserved sequence (26 residues long) overlapping a portion of the heptad repeats that was found to have a strong homology with oncogenic retrovirus TM glycoproteins (17,27,191,226). This protein sequence from retroviruses has been referred to as an immunosuppressive motif, based on its ability to inhibit T-cell proliferation and impair other immunologic responses *in vitro* (43,87,116), but this property has not been adequately evaluated for the related motifs present in filovirus GP_2 molecules.

Near the N terminus of GP_2 is a putative fusion peptide that is capable of inserting itself into membranes that contain phosphatidylinositol and resemble the lipid composition of hepatocyte plasma membranes (186). This fusion peptide is located in a position that is similar, with respect to the heptad repeats, to that of paramyxovirus, retrovirus, influenza virus, and coronavirus envelope glycoproteins (199). The fusion peptide is internally positioned and flanked by two conserved cysteines that are predicted to form a disulfide bond, and it is very similar to the TM of Rous sarcoma virus and avian leukosis virus (75,200). This peptide is believed to mediate the fusion of host and virus membranes as they are brought together by a conformational change in the peplomer during the entry process (101).

The GP_1 molecules in the peplomer are oriented with the hydrophilic and highly glycosylated C terminus projecting outward into the aqueous environment. This region presumably contains the major determinants for receptor recognition and binding (199,234), whereas the more hydrophobic N-terminal region of GP_1 connects this molecule to GP_2 by a single disulfide bond (200); the N-terminal region probably contributes to the formation of the stalk structure as well. Figure 6 shows a hypothetical model for the structure of the Ebola virus peplomer that is based on x-ray diffraction studies of crystallized GP_2 (141,225), results of site-directed mutagenesis of GP (200), and predictions based on similarities to retrovirus glycoproteins (75). The structure of the Marburg virus peplomer should be very similar. The predicted structure in Figure 6 lacks detailed information regarding GP_1, and x-ray diffraction data for this component will be difficult to obtain because of difficulties in crystallizing extensively glycosylated molecules.

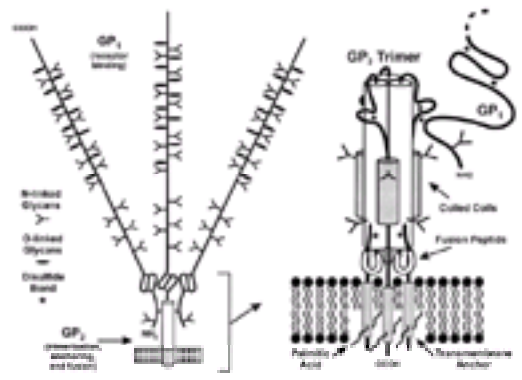


FIG. 6. Schematic representation of a proposed structure for the Ebola virus (Zaire species) surface peplomer (spike) predicted from genetic, biochemical, and x-ray diffraction studies (75,141,199,200,235). The *left* side of the figure shows the spike structure in which three molecules of GP_1 are covalently linked by a single disulfide bond to GP_2 molecules (Cys-53 is known and Cys-609 is predicted) arranged in a trimer and anchored in a lipid bilayer. The N-linked glycans shown attached to GP_1 are predicted from nucleotide sequence analysis (as are those of GP_2), but the number and position of all the O-linked glycans have been not been determined. The loops near the N-terminus of GP_1 formed by disulfide bonding are also predicted. At the *right* of the figure is shown a detailed depiction of the GP_2 trimer, which was generated from similarities to glycoproteins of retroviruses and from structural studies of crystallized, *Escherichia*

coli-expressed, partial sequences of GP_2.

VP40

The VP40 protein is believed to have a matrix protein function. This presumption is based on its being the most abundant protein in the virion, its somewhat hydrophobic profile, its release from purified virion nucleocapsids by nonionic detergents under low-salt conditions, and the position of its gene in the genome (55,61,121,191). By analogy with other matrix proteins, VP40 may facilitate virus budding by interacting with nucleocapsids and the plasma membrane (and at times with intracytoplasmic membranes, forming vacuoles) (185). Specific interactions of VP40 with membrane-associated GP and VP24 during the budding process may also occur. X-ray analysis of crystallized Ebola (Zaire) VP40 has been accomplished, and it should provide important insights into its function (51).

VP24

The VP24 is the third membrane-associated virus protein, and it has a decidedly hydrophobic profile. The role of this protein is unknown, but it may act as a minor matrix protein or function in the uncoating of the virion during infection.

Nonstructural Proteins

Secreted Glycoprotein and Delta Peptide

The SGP of Ebola viruses contains the same approximately 300 N-terminal amino acids as the GP, but the C terminus is unique (29 amino acids). Biochemical and antigenic analysis of SGP have shown that it is structurally distinct from GP (199,213). SGP forms homodimers that are linked in an antiparallel orientation by two disulfide bonds between the first and sixth cysteines on separate molecules. Dimer formation probably occurs in the endoplasmic reticulum. SGP is produced from a precursor molecule that, like GP_0, is cleaved by furin near the C terminus to release a short peptide (40 residues in length for the Zaire species) that contains O-linked glycans (no predicted N-glycans) and has been named delta peptide (214). Because delta peptide has only recently been described, no data on its biologic activity are currently available, but it is secreted from the cell and could have a role in pathogenesis. SDS-PAGE of the SGP shows that it migrates as a broad band containing at least three species, presumably caused by differences in processing of N-linked glycans. The function of SGP is unclear, but there is some evidence that it can bind to and inhibit the activation of human neutrophils (125,234). Because large amounts of SGP are present in the blood of acutely infected humans (195), it is plausible that SGP could contribute to the lack of inflammation seen in severe cases.

Virus Entry and Replication in Host Cells

The entry and replication of filoviruses follows the same paradigm established for rhabdoviruses and paramyxoviruses (5,128,221), and Figure 7 is a generalized depiction of the events. Infection of host cells is initiated by the recognition and attachment of surface peplomers (spikes) to receptor molecules found on the surfaces of host cells. This process of attachment may differ somewhat between Ebola and Marburg viruses, as retroviruses pseudotyped with GP have been shown to differ in their ability to infect host cells treated to alter receptor characteristics (38). An asialoglycoprotein receptor of hepatocytes has been shown to act as a receptor for Marburg virus (16), but it is likely that other receptors are also used by filoviruses. Following attachment, virions are presumed to enter the cell by a process of endocytosis, acidification of the endocytic vesicle, and fusion of virus and host membranes that releases the nucleocapsid into the cell cytoplasm, the site of replication (63,228). The negative-strand nature of these viruses makes transcription of the genome an obligatory event following release of the nucleocapsid into the cytoplasm. Polyadenylated, monocistronic mRNAs are synthesized from virus genes in a 3' to 5' direction (only encapsidated RNA can act as template). NP mRNA can be detected as early as 7 hours after infection, peaking at 18 hours (190). Translation of virus transcripts by the cellular machinery leads to a buildup of virus proteins (primarily NP), followed by a switch from transcription to replication. This switch results in the synthesis and encapsidation of full-length positive-sense RNA (antigenome), which serves as a template for the synthesis of full-length genomic RNA that is rapidly encapsidated. Depletion of irus capsid proteins causes a return to transcription, and eventually an equilibrium is established wherein transcription and replication are concurrent processes. Accumulated nucleocapsids in the cytoplasm can be detected as prominent inclusion bodies that can be easily visualized by light, immunofluorescent, and electron microscopy (129,157,235). When sufficient levels of negative-sense nucleocapsids and envelope-associated proteins are reached, a coalescing of these components occurs at the plasma membrane, or to a lesser extent at membranes forming intracellular vacuoles (59). Matrix protein is believed to facilitate the budding process by drawing the nucleocapsid tightly to the membrane, where it is enveloped and extruded from the cell as an infectious virion particle. Host cell protein synthesis is not shut down, but expression levels do diminish as the infection progresses.

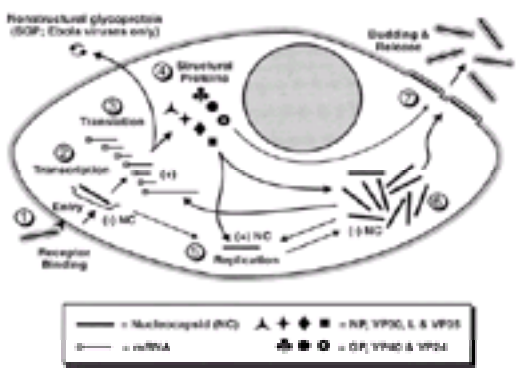


FIG. 7. Filovirus entry, replication, and release from a susceptible eukaryotic cell. The diagram shows stages of gene expression, genome replication, and virus assembly.

The growth of adapted strains of Marburg and Ebola viruses in cultured cells is striking. Intracytoplasmic vesiculation and mitochondrial swelling are followed by breakdown of organelles and terminal cytoplasmic rarification or condensation. Infection with the Reston or Côte d'Ivoire species of Ebola virus proceeds more slowly in tissue cultures, and cytopathic effects are usually less evident. Cytopathic changes can occur in concert with the accumulation of massive amounts of virus nucleocapsid material in intracytoplasmic inclusion bodies and large numbers of virions extracellularly.

Research directed at developing artificial replication systems for both Marburg and Ebola (Zaire) viruses, similar to those developed for paramyxoviruses and rhabdoviruses (180), will eventually enable the creation of infectious virus from DNA clones. T7 RNA polymerase-driven transcription of plasmids containing miniature versions of the Marburg and Ebola virus genomes (with a reporter gene) and encoding critical gene products (NP, L, VP35, and VP30) have been used to generate artificial nucleocapsids capable of being transcribed and replicated (149,151). The ultimate goal of such research will be the development of methodologies that enable specific engineering of filoviruses using recombinant DNA techniques. The ability to produce filoviruses with desired genetic modifications will provide an extremely powerful research tool to study replication, to dissect mechanisms of pathogenesis, and possibly to aid in vaccine development. However, concerns over the potential dangers and misuse of such systems have led to recommendations (and in some cases regulations) limiting the availability of the components, even to reputable institutions in which research is guided by the oversight of a biosafety committee.

CLINICAL FEATURES AND PATIENT MANAGEMENT

Filovirus infections in general are the most severe of the virus hemorrhagic fevers and much has been learned from close observations of acute human cases (29,71,76,100,142,169,171,174,196,231,232). The abrupt onset follows an incubation period of 4 to 10 days and is characterized by fever, chills, malaise, and myalgia. The subsequent signs and symptoms indicate multisystem involvement and include systemic (prostration), gastrointestinal (anorexia, nausea, vomiting, abdominal pain, diarrhea), respiratory (chest pain, shortness of breath, cough), vascular (conjunctival injection, postural hypotension, edema), and neurologic (headache, confusion, coma) manifestations. Bleeding is manifested as petechiae, ecchymoses, uncontrolled oozing from venipuncture sites, mucosal hemorrhages, and postmortem evidence of visceral hemorrhagic effusions. There is often a macropapular rash appearing around day 5; this is a valuable differential diagnostic feature and is usually followed by desquamation in survivors. Abdominal pain is sometimes associated with hyperamylasemia and true pancreatitis. In later stages, shock, convulsions, severe metabolic disturbances, and, in more than half the cases, diffuse coagulopathy supervene. Demise or improvement typically occurs around day 7 to 11, about the time the humoral antibody response is noted (Fig. 8) (132,142).

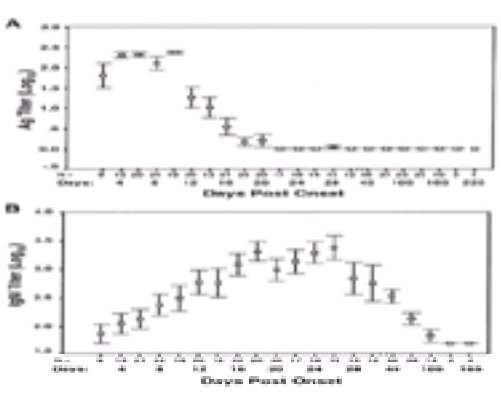


FIG. 8. Ebola Zaire antigen (**A**) and IgM (**B**) $\log_{10}$ titers from serum and blood of patients with laboratory-confirmed Ebola hemorrhagic fever. *Bars* represent 1 standard error of the mean for each time point. Time is shown in days after onset; time is divided into 2-day intervals up to day 30, and into 30-day intervals after 30 days. The numbers just under the horizontal axis are the number of cases in each time group.

The survivors have a prolonged convalescence with sequel including arthralgia, uveitis, psychosocial disturbances, and orchitis (142,184). Serologic studies have suggested that filoviruses of lower pathogenicity for humans (perhaps similar to the Reston species) may circulate in nature (11,111,114,133), and it was recently reported that asymptomatic infections of Ebola virus (Zaire species) occurred in the 1996 outbreak in Gabon (137). However, more extensive and controlled studies are required before such findings can be incorporated into the clinical understanding of human Ebola infections, especially those involving the Zaire species.

Clinical laboratory features of Ebola virus infection are less well documented than those for Marburg, but they appear to be similar. There is an early leucopenia and lymphopenia, subsequent neutrophilia, and marked thrombocytopenia, accompanied (in nonhuman primates) by abnormal platelet aggregation (67,69). Serum enzyme levels are elevated, typically with aspartate transaminase exceeding alanine transaminase, and with alkaline phosphatase and bilirubin levels normal or only moderately elevated. Later in the disease course, some patients may have biochemical and clinical jaundice.

The usual supportive measures for severely ill patients are indicated, including strategies more specific for those with

virus hemorrhagic fevers (167,172). There is no approved antiviral drug that is a useful treatment (ribavirin is ineffective) (97,167), nor are there other treatment modalities for filovirus patients. It is often surmised that improved supportive care will lead to an increase in survival, but there are no data on this subject. In fact, the very extensive virus involvement of the organs of these patients (235,236), the severe immunosuppressive lymphoid tissue lesions, and the lack of evidence of a humoral immune response in fatal cases taken together suggest that there may be a need for some method to limit virus replication before survival can be expected. Indeed, in 1996, a South African nurse infected from an Ebola patient received state-of-the-art care, yet ultimately died of the disease (179).

One approach to antiviral therapy has been the use of cellular S-adenosyl- L-homocysteine hydrolase inhibitors. These drugs are active *in vitro* and in mouse and nonhuman primate models (24,94). It is believed that these drugs act through the accumulation of S-adenosylhomocysteine and its selective inhibition of virus methylation reactions. Toxicity and pharmacokinetics are presently rate limiting in developing these drugs further.

Interferon (IFN)-a has shown relatively little *in vitro* (223) or *in vivo* (58,106) activity in experimental Ebola infections, even when given prophylactically. Patients have high levels of circulating IFN (211), and its use in postexposure prophylaxis would have the disadvantage of inducing fever and other side effects. A single report of treatment of a human infection with IFN and plasma is unconvincing although often cited as an example of the possible utility of these modalities (58).

Antibody therapy with simple convalescent plasma, which has been used successfully with Junin virus (172), seems to offer little in Ebola virus infections. Passive antibody therapy with polyclonal hyperimmune horse sera (19) is of only marginal utility in postexposure prophylaxis models in primates, requiring large volumes given within a very short time of inoculation (106,108,134). Several related approaches, however, may be useful. A human monoclonal antibody with neutralizing capacity was derived from bone marrow RNA libraries of convalescent Ebola patients by phage display, and it has shown protective capacity in the guinea pig model (143). In addition, murine monoclonal antibodies protective in the mouse model have been developed (227). These approaches offer hope for the development of postexposure treatments, but their usefulness will ultimately require validation using a primate model.

Less mainstream considerations include attempts to block the effects of the high levels of cytokines, a proposition that has proven to be difficult in septic shock and adult respiratory distress syndrome (211). During the 1995 Kikwit outbreak, seven of eight patients receiving whole blood transfusions from surviving patients lived (152). There were no controls and the transfusions were usually given to younger patients later in the epidemic and sufficiently late in their clinical course that some patients had already died—all factors that favor survival (188). Antibody is not a plausible candidate for an active ingredient in the transfused blood, but nevertheless the apparent success should prompt research into alternative approaches that might involve other soluble substances or cells in the transfused blood.

DIAGNOSIS

Clinical Diagnosis

Filovirus disease can be suspected in patients with a history of travel to an endemic area if they present with fever and constitutional symptoms. Rural travel, jungle or cave exposure, contact with sick persons, or contact with sick or dead primates all suggest need for additional concern. The laboratory should be used to exclude diseases such as malaria, leptospirosis, relapsing fever, shigellosis, rickettsial disease, and typhoid fever. Unless laboratory diagnosis is timely and accurate, empiric therapy for the other agents may be indicated. Malaria will be the most common potentially lethal confounder, but it must also be recognized that partially immune patients can be parasitemic without having malarial symptoms.

Laboratory Diagnosis

Ebola and Marburg Hemorrhagic Fever

The recent occurrence of Ebola (81,119,145) and Marburg hemorrhagic fever (230) outbreaks has offered an opportunity to utilize more recently developed diagnostic assays and technology on clinical materials collected from these patients. Patients in the acute stages of disease have high case fatality rates, and most of those who die do so without developing detectable antibodies. This emphasizes the diagnostic need for isolation of the virus or detection of its antigens or RNA.

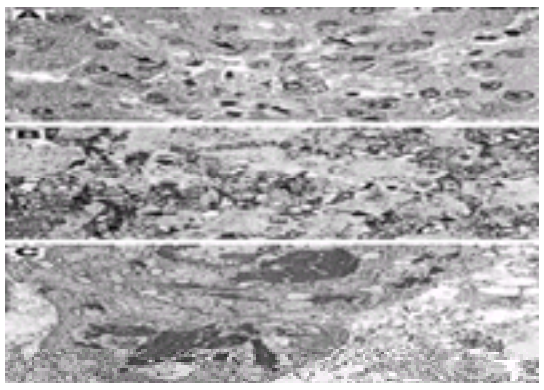
Before the diagnosis can be effectively accomplished, the specimens must be obtained and sent to a laboratory with the appropriate expertise and reagents. Because outbreaks have continued to include significant numbers of care givers who have direct contact with patients (6,72,119,179,231,232), those who collect samples need to take adequate barrier nursing precautions while obtaining the samples (34,122,164). Additionally, special care should be taken to avoid needle sticks and to dispose of the sharps in an appropriate manner so that others avoid needless exposure. Collection of specimens should be done with a mind toward sterility and prevention of cross-contamination of specimens; this has become particularly important in the day of ultrasensitive techniques such as reverse transcriptase–polymerase chain reaction (RT-PCR) amplification for diagnosis and characterization of the virus genome.

Although high-level containment is warranted because of the extreme hazard associated with handling the viruses, virus isolation remains relatively simple and sensitive: The virus grows well in a large variety of cell lines, although Vero or Vero E6 cells have been most used (161,165,209). The virus is relatively stable and some infectious virus may survive less than favorable handling and shipping; care should be taken to ensure the physical integrity for biosafety reasons and to maintain an adequate refrigerated or frozen state for biologic integrity of the sample to maximize diagnostic success. Clinical diagnostic criteria alone will not yield an etiologic diagnosis, particularly as the materials may be coming from relatively remote areas with relatively sparse clinical facilities.

An integrated approach to diagnosis is particularly useful when initial specimens from an outbreak or individual patients are being received. This includes attempts to isolate the virus, detect the virus antigens, and amplify the virus RNA by

RT-PCR with subsequent sequencing. Both antigen detection and the amplification of the virus RNA can be done on materials that have been rendered noninfectious. An efficient means of inactivating the virus for antigen detection (and serology) is the utilization of gamma irradiation from a cobalt-60 source (56). This allows the safe manipulation of the material outside of the containment laboratory, where work can be more expeditiously carried out. Likewise, the nucleic acid can be amplified by purification of the virus RNA from materials treated with guanidinium isothiocyanate, a chemical chaotrope that denatures the proteins of the virus and renders the sample noninfectious. Virus isolation, on the other hand, proceeds within the BSL4 laboratory where cytopathic effects from most of the virus strains can be recognized by microscopy and the virus identified by immunologic or electron microscopic means (see Fig. 1 and Fig. 2).

Detection of either Ebola or Marburg virus antigen by antigen-capture enzyme-linked immunosorbent assay (ELISA) has often been the most rapid means of diagnosing infections. The assay time is approximately 3 to 4 hours, and detection of antigen in the blood or serum of either nonhuman primates or patients in the acute course of the disease was very successful when compared to virus isolation or to RT-PCR for Ebola viruses (130,131,132 and 133,195). This assay was also useful in detection of virus antigen in frozen tissues (131). Filovirus antigens can also be detected by immunohistochemical (IHC) examination of formalin-fixed tissues by using specific polyclonal and monoclonal antibodies (Fig. 9). These methods have a unique role in cases in which archival tissues are the only specimens available for diagnostic testing. During the epidemic in Kikwit, Zaire, a novel and sensitive IHC test was developed at the Centers for Disease Control (CDC) in which skin biopsy specimens were used for the diagnosis of Ebola virus HF (82,138,236). Formalin-fixed biopsy specimens are not infectious, they may be taken in the most basic field conditions, and they may be sent without special precautions or refrigeration. This technique was useful for the diagnosis of fatal cases in subsequent Ebola outbreaks in Gabon (P. Rollin, personal communication, 1996) (236); lesser sensitivity was noted in the recent Marburg outbreak in the DRC (230).



P>**FIG. 9.** Pathologic features seen in the liver of an Ebola virus–infected human. **A:** Sinusoidal dilation and congestion and hepatocellular necrosis (H&E, ×250). Numerous filamentous intracellular Ebola viral inclusions (*arrows*) are seen in association with an area of coalescent hepatic necrosis. **B:** Ebola antigens are seen throughout tissue section in sinusoids, sinusoidal lining cells, and hepatocytes (*arrows*) (immunoalkaline phosphatase staining, naphthol fast red substrate with light hematoxylin counterstain, ×158). **C:** Electron micrograph of liver showing several large Ebola virus inclusions within infected hepatocytes (uranyl acetate and lead citrate stain, ×6,600). Abundant extracellular Ebola virus particles are also seen in hepatic sinusoids. Note variation in size and shape of viral particles associated with necrotic debris.

IgM-capture antibody ELISA proved useful in the diagnosis of recent infections in surviving patients (158). The IgG response of the patients was somewhat delayed and realistically could be expected only in the early convalescent sera of those patients who were destined to recover from their infections (132). The IgM antibodies detected by this test have been found to persist for only 2 to 3 months in macaques that were experimentally infected (133) and a similar relatively short period in surviving humans (132).

The use of RNA amplification by RT-PCR has added another rapid and sensitive tool for the diagnosis of Marburg or Ebola virus infections (189). The technique was first sparingly utilized during the 1989–1990 outbreak of the newly discovered Ebola Reston virus, but it has been used much more commonly in the recent human outbreaks in DRC in 1995 and in Gabon from 1994 to 1997, and in imported primates in Texas in 1996 and follow-up in the Philippines in 1996 (181,195). The RT-PCR should be combined with direct sequencing of the amplified product; this provides virus sequence information that enables phylogenetic comparison of the virus in a clinical specimen to other, previously recognized and characterized strains of the virus, and thus it can help determine the “molecular epidemiology” of an outbreak such as the multiple introductions in the series of Gabon outbreaks from 1994 to 1996 (82). Determination of sequence from amplified DNA product also mitigates one of the shortcomings of the technique: The high sensitivity of RT-PCR renders the technique prone to cross-contamination from other specimens or materials in the laboratory. If the sequence obtained from the clinical specimen matches that of control materials or previously recognized virus stains, skepticism is warranted. Precautions should be taken to perform RNA extractions in areas where chances of cross-contamination are minimized. We also recommend not reporting results on the basis of the initial PCR product but waiting until the sequence has been compared to that of other strains of the virus; the virologist should be satisfied that the product does not represent cross-contamination.

Retrospective Identification of Individuals Previously Infected with Ebola and Marburg Viruses by Testing for Virus Antibodies

Efforts to determine the prevalence of past filovirus infection with older generations of serologic tests, mostly the indirect fluorescent antibody test (IFAT) (113), have generally met with criticism because of the lack of specificity of the assay, as demonstrated when control groups with no exposure to filoviruses (32,169) were included. Individual filovirus antigens either tested with Western blot assay or expressed by molecular techniques have been developed for serologic tests to a limited degree, but using the assays for field testing with appropriate controls has been limited, leaving the applicability of these newer approaches largely unknown.

Experience with the IFAT during and following the 1989 Ebola Reston episode left us convinced of the lack of specificity of this assay for detecting past infections with filoviruses. This was evidenced by the finding of high prevalence among primate colonies containing only animals that had been bred in closed facilities for several generations without the occurrence of any disease recognizable as filovirus infection. Similarly, animal caretakers were found with moderate IFAT Ebola antibody prevalence, but there were also many individuals found to be positive among administrative personnel with little or no contact with the primates, and persons from the United States general population had an Ebola IFAT antibody prevalence of 2.7% (31). Even though the IgG ELISA test is more specific in nonhuman primates and North American human populations (133), background problems are encountered when testing populations from central African countries. There are many sera that nonspecifically bind to the noninfected antigen control, often to an extent that it is difficult to interpret the test. Adsorbing these sera with normal cell antigen does help but does not altogether obviate the difficulty.

Direct Visualization of Virus

Electron microscopy has been particularly useful in the diagnosis of filovirus infections (see Fig. 1 and Fig. 2). Negative-contrast electron microscopic examination of initial-passage cell culture supernates can be performed with safety after prefixation. Although the fine structure of virions is distorted, resolution of nucleocapsid structures within virions can establish the diagnosis (53,156,235). A similar approach may be tried on patient serum or plasma. For thin-section electron microscopic examination of patient tissues, any standard fixation (e.g., 1% glutaraldehyde in phosphate buffer), embedding (usually a rapid polymerization method), and staining system can be used. Most experience has been gained with examination of liver tissue, obtained at autopsy or via agonal biopsy. In several cases of Marburg and Ebola hemorrhagic fever, virions and uniquely structured inclusion bodies have been identified in liver tissue (153,173). Direct examination of body fluids has been valuable (80), but in inexperienced hands this can lead to a mistaken diagnosis if confirmatory methods are not available (118).

PATHOLOGY AND PATHOGENESIS

Infection in Experimental Animals

The pathology of Ebola virus infections has been extensively studied using tissues from outbreak cases and experimentally infected monkeys, guinea pigs, and mice. In general, the pathology of these models has a reasonable resemblance to that seen in humans (8,9,22,45,49,67,69,102,103,104,153,156,187,236,237). Of the animal models, the most realistic involves macaques, which undergo a course of infection after inhalation or injection that closely resembles the human disease (8,103). Other monkeys as well as guinea pigs serve as good experimental models, although a few passages in guinea pigs may be needed to adapt the viruses to that host (187). A mouse model has been developed for Ebola (Zaire species) (23), which is very advantageous given the abundance of reagents available for studying cell biology and immunology, and it also is useful for examining the genetic basis for the host responses to disease. However, the mouse model is probably the most distant from human infection, and data derived from these studies may not correlate with human disease and/or may be deficient in identifying certain processes.

In monkey models, the virulence of filoviruses is quite variable; a similar variability is seen in human infections (see later section, Epidemiology). Among Ebola viruses, the Zaire species is the most virulent and the Reston species is the least virulent (21,22,66,107). Infections with the Zaire species usually progress rapidly and are uniformly lethal 4 to 8 days after infection; as little as one infectious unit is required to cause disease. Naive animals can survive infections caused by the Sudan and Reston species, with times to death ranging from 7 to 14 days (21,22,66). Little is known regarding the virulence of the Côte d'Ivoire species in monkeys, but its slow growth in tissue culture tends to indicate a virulence similar to that of the Reston species. Monkeys can also survive infections with Marburg virus (112), and virulence appears comparable to that of the Sudan species of Ebola virus.

Pathology in Humans

The pathologic changes seen in patients dying with all filovirus infections are similar (52,53,153,154,235,236). Extensive necrosis is seen in parenchymal cells of many organs, including liver, spleen, kidney, and gonads; little infiltration is seen within infected tissues. The most characteristic histopathologic features are seen in the liver (see Fig. 9), where hepatocellular necrosis is widespread with intact, hyalinized, ghost-like cells often remaining in place amid large amounts of karyorrhectic debris. Often, extraordinary numbers of virions are present in this debris. Characteristic intracytoplasmic inclusion bodies (aggregates of viral nucleocapsid material) are present in intact hepatocytes. Light microscopic, electron microscopic, IHC, and *in situ* hybridization studies show concordance between tissue damage, the presence of viral antigens and nucleic acid, and sites of virus replication, suggesting that direct viral damage is one major element in the pathogenesis of the disease (see Fig. 9). Infection and damage to mononuclear phagocytes and endothelial cells are also characteristic of filovirus infections; Kupffer cells and capillary endothelium are major targets of infection. This is thought to play a critical role in the pathogenesis of filovirus infections through the secretion of physiologically active substances, including cytokines and other inflammatory mediators.

Significant injury to the microvasculature and increased endothelial permeability appear to be central to the pathogenesis of the shock and bleeding seen in filovirus infections. Spleen and lymph nodes show various degrees of lymphoid depletion with extensive vascular follicular necrosis and necrotic debris. These lymphoid lesions are also potentially very important in the outcome of infection, given the lack of an effective immune response in fatal cases (132) and the evidence for other forms of immunosuppression in surviving patients (117).

Pathogenesis of Disease

As noted earlier, cells of the mononuclear phagocytic system (macrophages and monocytes) have been implicated as sites of replication early in infection. Figure 10 is a schematic representation of the infection and spread of filovirus in

human and nonhuman primates. Filovirus infection of mononuclear phagocytes may also affect the signaling and processing of antigen, which could hamper the host's ability to mount a strong cell-mediated immune response. It has been proposed that sessile macrophages may act as vehicles for the transport of filovirus particles to specific organs (i.e., lung, liver, and spleen) (45,187,201,202), as they are highly susceptible to infection *in vitro* and they produce large amounts of infectious virions (59,98). Infected macrophages may also contribute to shock in fatal cases, as *in vitro* and animal studies have shown that cytokines, such as tumor necrosis factor (TNF)-a, are released (59,95,98); as noted later, elevated TNF-a levels are also seen in acute human cases of Ebola hemorrhagic fever (211). In a study of Marburg virus disease in guinea pigs (99), it was shown that treatment with anti-TNF-a serum on postinfection days 4 to 7 can prevent death (50% survival versus 100% mortality in controls), which is additional evidence for a possible role of cytokines in the disease process.

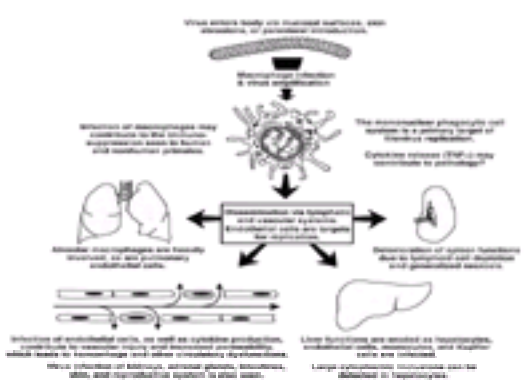


FIG. 10. Schematic illustration of the events and pathology associated with severe filovirus infections of human and nonhuman primates.

Elevated levels of cytokines [interleukin (IL)-2, IL-10, TNF-a, IFN-b, and IFN-g] have been detected in the serum of acutely infected patients who ultimately died of Ebola hemorrhagic fever during the 1995 Kikwit outbreak (211). High levels of cytokines may well contribute to the fatal outcome. Both Ebola and Marburg viruses grow well in human endothelial cells *in vitro* (86,87,203), which could account for the hemorrhaging and shock syndrome seen late in the disease. Infection of cultured human umbilical vein endothelial cells with Ebola Zaire have also shown that induction of immunomodulatory genes (MHC I family, IL-6, interferon regulatory factor-1, ICAM-1) and antivirus genes (2'-5'-oligoadenylate synthetase and PKR) by double-stranded RNA and interferons (IFN-a and IFN-g) is strongly inhibited by Ebola Zaire infection (85,86). Inhibition is selective, as induction of IL-6 and ICAM-1 by IL-1b is not inhibited (86). If these *in vitro* responses correspond to events *in vivo,* then infection of endothelial cells by Ebola virus would contribute to immunosuppression. These cells would be impaired in their ability to stimulate inflammation, cytotoxic T cell development, and humoral responses (via ICAM-1, MHC I, and IL-6, respectively), which could leave infected cells hidden from immune surveillance and clearance mechanisms.

In Ebola virus disease, it was initially speculated that SGP had a role as a decoy molecule that would help misdirect protective responses away from GP present on the surface of virions and infected cells. However, as their functional and structural properties came to light, it became evident that the roles of these two related glycoproteins differed dramatically. GP has been used to pseudotype murine retroviruses, and it directs these particles to cell types distinct from those recognized by SGP (234,235). Specifically, GP interacts preferentially with endothelial cells, though it may also bind to monocytes, hepatocytes, and other cell types. In contrast, SGP appears to bind to neutrophils through an interaction that involves the Fcg3 receptor, CD16 (125,234). Whether this interaction is direct or indirect remains uncertain, but it appears to affect neutrophil function, preventing the down-modulation of L-selectin in response to serum stimulation. SGP may also affect cell surface interactions of CD16 and other cell surface molecules that may affect cellular activation. Thus, it is likely that SGP inhibits the inflammatory response to infection, wheres GP promotes virus attachment and entry in endothelial cells, which are subsequently vulnerable to the virus's cytopathic effect.

It has been suspected that GP gene products have a major role in the pathogenesis of filovirus disease, partly because of the effects of the immunsuppressive motif found in GP_2, but also because of the potential to affect the functions of membrane-bound and/or soluble substances in the body (41,42,64,219). Recent studies have shown that the Ebola virus GP not only is capable of inhibiting T-cell proliferation in response to mitogen (41,42), but its expression in cultured human endothelial cells was observed to cause cytopathic effects (a decrease in aV integrin levels was noted), whereas expression of other virus proteins had little or no effect (234,235). This cytotoxic property was also demonstrated in explanted human and porcine blood vessels, resulting in endothelial cell loss and increased vascular injury (permeability). This toxicity was associated with the variable or mucin-like region, as deletion of this sequence abrogated the deleterious effect. It is expected that further characterization of the biologic activities of GP molecules will provide added support for their prominent role in pathogenesis.

Immunology and Protection

Filovirus infections of human and nonhuman primates are characterized by a lack of inflammation and the development of an undefined form of immunosuppression that inhibits the clearance of virus (171,196). In most fatal cases, antibodies against filovirus proteins are not detectable (see Fig. 8). In those cases that survive or where the disease is protracted, a slightly delayed humoral response is noted. In experimental animals, serum taken from convalescing patients or laboratory animals recovered from infection failed to protect consistently against disease, and antibody-mediated neutralization of virus replication in cell culture is inefficient and difficult to demonstrate (106,108,170,223). Because inflammation is lacking, cells of the mononuclear phagocytic system are infected, and humoral responses are inhibited, it appears that immune and inflammatory systems are both targets and potential responders to virus infection. However,

the role of specific lymphoid subsets and their possible mechanisms of immune protection are unclear.

Anecdotal reports that antibodies may protect against disease during outbreaks were contradicted by controlled studies in experimental primate models in which only minor delays in symptoms were seen following passive transfer of convalescent antibody, with no significant difference in overall survival (106,108). Recently, selected monoclonal antibodies derived from patients recovered from infection (143) were shown to neutralize Ebola virus infection in selected virus replication assays *in vitro*. Several classes of murine monoclonal antibodies directed against epitopes of the Ebola glycoprotein have since been identified, some of which provided protection against lethal challenge in a murine model of infection (227). These data suggested that antibodies may contribute to immune protection, but by themselves they are unlikely to be responsible for recovery or to confer solid protective immunity. The lack of a strong humoral response in fatal human and nonhuman primate infections, together with the inefficiency of antibody-mediated neutralization of virus, would suggest that a vigorous cell-mediated response is an important component in virus clearance (159). At the same time, it remains possible that selected, low-frequency antibodies can be found that may reverse the symptoms and outcome of infection and could prove therapeutically beneficial.

Attempts were made to develop killed vaccines to protect against filovirus disease using formalin-fixed or heat-inactivated virion preparations to immunize guinea pigs or nonhuman primates, and they were unsuccessful (1,39,96,99,140). Although humoral responses were generated in these animals, the preparations did not elicit strong protective immune responses. Encapsulation of killed, gamma-irradiated Ebola virions in liposomes containing lipid A has been shown to improve cytotoxic T-cell responses in mice (176), but protection against a lethal challenge was not examined. Traditional strategies of attenuating viruses for use as vaccines in humans would be unacceptable because of the potential for reversion back to a wild-type form. There is insufficient information on the pathogenicity of less virulent strains (such as the Reston subtype) passaged in human cells, and infection of monkeys appears to indicate that a strong cross-protective immunity does not develop (21,22,66), despite significant antigenic similarities among Ebola virus species.

Efforts to mimic natural infections through the use of recombinant DNA techniques to stimulate cytotoxic T-lymphocyte (CTL) responses via *de novo* synthesis of virus proteins (MHC class I restricted) have been very promising. Major progress in experimental vaccine development was reported first in the guinea pig, where it was possible to develop protective immunity against Ebola virus infection by genetic immunization (233). Immunization with plasmids encoding the virus NP, SGP, or GP elicited humoral and cellular immune responses against these gene products. In the case of NP, the response was primarily humoral in nature, whereas the exposure to SGP and GP revealed T-cell proliferative and cytotoxic responses. Although protection against lethal challenge was observed for each of these plasmids when animals were challenged within 1 month of the last immunization, long-lasting protection was observed only with GP or SGP. Protection correlated with antibody titer and antigen-specific T-cell responses to these gene products. These studies documented the feasibility of vaccination against Ebola virus infection and demonstrated that immunity can be developed to this pathogenic virus. Despite the correlation with antibody titer, it was not possible to provide protective immunity by passive transfer of immunoglobulin. Protection has also been observed in the murine model using plasmids expressing either NP or GP (210); immunity was long lived and CTL responses were induced.

An infectious replicon form of Venezuelan equine encephalitis (VEE) virus was also used in an attempt to induce protective immune responses against both Ebola and Marburg viruses. This system was used to immunize mice, guinea pigs, and monkeys (cynomolgus macaques) against the GP and NP of filoviruses (92,175), and it proved very effective in protecting animals against Marburg virus. For Ebola virus, protection has been induced in guinea pigs and mice, but monkeys have not been protected using the VEE replicon system. Immunization of guinea pigs with recombinant vaccinia viruses expressing Ebola virus GP has also shown some promise, but vaccinia viruses expressing NP, VP35, VP40, and SGP were ineffective in protecting against disease and death (84).

Vaccination with protein preparations also induces some protection against filovirus disease, as has been shown with a secreted form of the Marburg virus GP expressed in a baculovirus system (90,91). Partial protection against the homologous strain of Marburg virus was demonstrated, but this protection was much less effective against a genetically and antigenically disparate strain of Marburg virus (91).

The current trend in filovirus vaccine development is to use genetic immunization to prime the immune response and to boost with some other recombinant virus expression system. Genetic immunization is known to stimulate a strong cell-mediated immunity, which is critical to filovirus clearance and long-term protection, and boosting with a vectored system (VEE, vaccinia, adenovirus, or baculovirus system) stimulates a strong response that is not easily attained by continued DNA injections. It is anticipated that this approach will enable the stimulation of an immune response vigorous enough to protect nonhuman primates from lethal challenge.

Several recent reports provide insights into immunologic determinants of resistance and predictors of survival. Analysis of patients in outbreaks in northern Gabon in 1996 showed no difference in serum antigen levels between survivors and nonsurvivors during the first few days of illness, suggesting that host defense rather than early virus replication determines survival (4). A variety of immune responses were examined, including IFN-g levels in the serum, as well as T-cell responses measured using RT-PCR of mRNA. Patients who suffered from fatal infections showed a higher level of IFN-g protein initially, but mRNA levels for indicators of T-cell function such as CD3, CD8, perforin, or IFN-g fell. The decline in cytokine levels may reflect the loss of response of mononuclear cells such as seen late in sepsis (3), but the disappearance of T-cell receptor mRNA from blood suggests that actual cell death occurs. The presence of markers of apoptosis in the blood of patients who died, and decreases in the antiapoptotic molecule bcl-2 support the occurrence of widespread programmed cell death of T cells, as reported in lymphoid tissues of macaques infected with Ebola virus (77). Importantly, surviving patients developed more effective antibody responses to the virus nucleoprotein early and developed more sustained T-cell responses that persisted into the convalescent phase. These findings are consistent with other reports that markedly elevated levels of IFN-g, IFN-a, IL-2, IL-10, and TNF-a are associated with a poor prognosis during infection, as noted earlier (211).

There are also several similarities between human disease and relevant animal models of vaccine-induced protection. In

guinea pigs immunized with Ebola virus gene products, successful protection against lethal challenge was dependent on T cells. Both in humans and in guinea pig studies, the nucleoprotein elicited primarily a humoral response, whereas the GP antibody responses were relatively lower. The studies of natural infection in humans are thus consistent with those in rodent vaccination models. The development of antibody responses correlated with survival in the guinea pig and most likely reflected the successful T-cell responses required for antibody production, as has now been suggested in humans. It remains uncertain whether protection against early infection requires only T-cell immunity. More likely, innate immune or inflammatory responses are needed to more effectively contain virus replication. Although the humoral response is clearly not the major mechanism of recovery, it may have an ancillary role, as evidenced by the success of minor components of the polyclonal immune response to protect animals when they are rescued as monoclonal antibodies (227). Although the specific contributions of innate and acquired immunity in response to infection remain poorly understood, Ebola virus infection nonetheless serves as a model for the analysis of immunity to infectious causes of hemorrhagic fever and other virus infections. Further studies regarding its immunopathogenesis will help to define the pathophysiology of this disease and contribute to strategies for vaccine development.

EPIDEMIOLOGY

Marburg Virus

The first recognition of filoviruses and of filovirus disease occurred in 1967 when a physician in Marburg, Germany, noted a severe hemorrhagic illness among patients presenting to a local hospital. The primary patients had all handled monkey blood, tissues, or cell cultures traced to a shipment of African green (vervet) monkeys (*Cercopithecus aethiops*) exported from Uganda. Limited secondary spread was seen in contacts of the index cases in Marburg, Frankfort, and Belgrade, with the final toll being 26 primary and six secondary cases. The epidemic ceased with the euthanasia of the infected monkeys.

Between 1975 and 1987, three isolated index cases were identified among tourists or expatriates in sub-Saharan Africa, with no further transmission or only limited spread to a traveling companion or health care worker (76,112,198). In 1999, indigenous Marburg virus infection among native Africans in rural areas was first confirmed during a large and ongoing outbreak in Durba, northeastern region, DRC; those infected were mostly gold miners (230). Incidentally, this area has had multiple similar small outbreaks of a severe disease since 1994, with retrospective laboratory confirmation of Marburg infection in one survivor.

Ebola Virus, Zaire and Sudan Species

The first recognized outbreaks of Ebola hemorrhagic fever occurred simultaneously in northern DRC and southern Sudan in 1976, but they were caused by two distinct species (47,115,169,197). No index case was clearly identified in the Sudan outbreak, although initial cases originating in Nzara, Sudan, involved six workers at a cotton factory. The disease initially spread through nosocomial transmission, with the use of contaminated needles and lack of barrier nursing practices fueling the epidemic. The case fatality in the Sudan outbreak was 53%. The outbreak in DRC centered around a Belgian mission hospital in Yambuku, Equateur Region. During the 7-week period of the outbreak, 318 cases were identified with a case fatality of 88%. The single most significant factor in the spread of infection in the hospital was the reusing of syringes and needles, although secondary transmission to family members caused 45% of all recorded infections. The outbreak ended with closure of the hospital and quarantine of sick patients by local village practices.

The disease then vanished except for an isolated case in northwestern DRC in 1977 and a small outbreak in southern Sudan in 1979 associated with the same cotton factory in Nzara. Ebola hemorrhagic fever dramatically reemerged in DRC in 1995, when a total of 315 cases (81% case fatality rate) occurred over 7 months, centered in Kikwit but involving an additional 20 villages. The earliest documented patient was a charcoal worker, in whom the diagnosis was missed. For 3 months, the disease spread person to person in the community until it was amplified in the hospital setting and finally recognized.

Since then, three separate outbreaks caused by Ebola (Zaire species) were identified in Gabon over a 3-year period. In 1994, an outbreak of Ebola hemorrhagic fever occurred north of the provincial capital of Ogooue-Ivindo; it was retrospectively identified after originally being misdiagnosed as yellow fever. In January 1996, another outbreak, near the site of the 1994 outbreak, was linked to the butchering for consumption of a dead chimpanzee. In 1996–1997, there was a 7-month outbreak in Booue province, Gabon, involving 60 cases (case fatality, 75%) associated with eight generations of secondary cases and with secondary cases in the capital. A noteworthy case of secondary transmission involved the death of a nurse in Johannesburg who had been caring for a physician from Libreville, the capital of Gabon, with unsuspected Ebola hemorrhagic fever.

Most recently, the Sudan species has returned to biomedical attention in an epidemic beginning in Uganda in September 2000 and ending in January, 2001. More than 300 cases occurred with a case fatality of approximately 50%. The epidemic seems to have a single origin, with mechanisms of spread similar to that of prior outbreaks.

Ebola, Côte d'Ivoire Species

In 1994, a new Ebola species was isolated from an infected ethnologist in the Tai Forest reserve in Côte d'Ivoire. She had performed a necropsy on a chimpanzee from a troop that had lost several members to an illness later identified as Ebola (71,135). Later, a single seroconversion suggested a second surviving human case in nearby Liberia. This virus isolation extended the geographic distribution of known Ebola cases to include most of the African rain forest.

Ebola, Reston Species

In November 1989, an Ebola virus species with low or no pathogenicity for humans was recognized in a shipment of cynomolgus monkeys (*Macaca fascicularis*) housed at a quarantine facility in Reston, Virginia. These monkeys were

imported from the Philippines, and an unusually high mortality was observed in infected animals during quarantine. Simian hemorrhagic fever virus was also circulating in the facility (48), and efforts to culture this virus led to the detection of Ebola Reston (105). Four cases of acute filovirus infection were confirmed among the caretakers, none of whom experienced a clinical illness (166). Resumption of importation of monkeys has led to new outbreaks of monkey disease in the United States as late as 1996 (183) and Italy in 1992. Subsequent investigations have traced all the shipments except one to a single source in the Philippines. The mode of contamination of this exporter's holding compound has never been ascertained, although the virus either persisted or was reintroduced from wild-caught animals, resulting in continued movement of infected macaques. A few infected handlers were also identified by serologic methods without reports of severe illness or suspicious deaths among this cohort (31,32,35,89,145). Improved shipping, housing, and quarantine regulations have prevented human infections or hazard to the community in the United States (50).

Patterns of Filovirus Infections of Humans

The epidemiology of human infections in nature, besides the internationally recognized outbreaks, is unknown. However, the time elapsed between occurrence of the index cases and the recognition of the subsequent large outbreaks suggests that sporadic cases of unrecognized filovirus infections could readily pass unnoticed (109). The number of such identified clusters in this decade after a 20-year presumed hiatus may represent a combination of unidentified ecologic factors and increasing diagnostic interest in the wake of the Kikwit epidemic. Serologic surveys revealed antibody prevalence from 10.2% among gold panners in Gabon, to 9.3% among rural villagers in the DRC using ELISA-based assays. Ebola infection, potentially with nonpathogenic strains or strains of low pathogenicity, may be quite frequent in select rural African populations (81,111,133).

Whatever the source of the initial index case, person-to-person transmission is the means by which human filovirus outbreaks have been propagated. This generally involves intimate contact; secondary attack rates have not exceeded 10% to 15%, indicating that transmission is not efficient. However, this risk increases as a function of contact—for example, from 23% for family members sleeping in the same room as the patient, to 81% among persons providing active nursing care to a patient during the 1976 Sudan outbreak (6). The need for this intimacy is reflected in the relative paucity of infected children, who are less likely to be primary care givers for ill family members (54). Nosocomial transmission is a special problem and hospitals have often served as a source of disease amplification into the community and to health care workers. Health care providers pay a high price during the epidemics: A quarter of all cases during the 1995 Ebola Zaire outbreak were among health care workers. Extreme care should be taken with infected blood, secretions, excretions, tissues, and hospital materials and waste. Well-documented and surreptitious reuse of needles and syringes has also played a role in these outbreaks. And no person whose contact was exclusively parenteral during the 1976 Zaire outbreak survived. Sexual transmission has been reported with Marburg virus and appropriate precautions should be taken during 3 months of convalescence, based on isolation of filoviruses from semen, identification of virus RNA in genital secretions, and epidemiologic data (142,181,184).

Filoviruses are transmissible to nonhuman primates in the laboratory by aerosols (10,110), and virions have been identified in alveoli of infected monkeys (79,102,157,166). Furthermore, the outbreak of disease caused by the Reston species of Ebola virus among quarantined monkeys in 1989–1990 was strongly suspected to have been transmitted by droplets and perhaps small-particle aerosols (166). However, these animals were housed in a poorly ventilated building in which aerosols were likely to have been largely generated by handlers cleaning the facility. Aerosol transmission has not been unequivocally implicated in human outbreaks to date. Extremely efficient person-to-person transmission in the form of superspreaders, two individuals who may have been the source of infection for over 50 cases in the 1995 Ebola Zaire outbreak, has been recently reported (119). The mechanism of heightened transmission was not identified, although contact with the patient and/or cadaver was strongly implicated. In all episodes, isolation of patients and use of strict barrier nursing procedures, including protective clothing and respirators (special masks used for tuberculosis precautions provide a reasonably available source of protection), have been sufficient to rapidly interrupt transmission in the hospital. For community members, residual risks are present from cadavers, which should be handled accordingly (54).

Natural History of Filoviruses

The origin in nature and the natural history of Marburg and Ebola viruses remain a mystery despite a number of attempts to backtrack from human index cases in Africa or from monkey epidemics in Africa and the Philippines (25,46,136,155,182). Following the 1989–1990 Ebola Reston virus outbreak among monkeys imported from the Philippines to the United States, there was some speculation that nonhuman primates might be the primary reservoir. This would seem unlikely, given that filoviruses are highly pathogenic for nonhuman primates (66,70), and there has been no evidence for latent virus infection in these animals (68). It would seem that the viruses are zoonotic—transmitted to humans from ongoing life cycles in animals or arthropods (70,147). Lack of replication in arthropod cells or inoculated arthropods (208,223) argues against an arthropod intermediary for Ebola, but there are many possible arthropod hosts that have not been tested. The most extensive arthropod field surveys have failed to show any evidence for the presence of Ebola virus (178). Filoviruses share several biologic, ecologic, and virologic characteristics with Lassa virus, an arenavirus maintained by chronic infection of rodents in Africa: (a) the human infections caused by each are relatively insensitive to the antivirus effects of IFN, and in each cellular immunity is thought to play the dominant role in recovery, (b) each of the viruses has highly glycosylated peplomer proteins, possibly affecting immune surveillance and recognition, and (c) both viruses have a proclivity for infecting macrophages and induce immunosuppression after experimental infection in some hosts. The similarity of this overall profile suggests that filoviruses may establish persistent infection in a mammalian reservoir host(s).

Investigations of filovirus outbreaks in humans have provided circumstantial evidence that bats may be involved in the spread of filovirus disease. Two index Marburg virus cases were known to have visited the same bat-infested cave in Kenya, near the area in Uganda that produced the infected monkeys involved in the 1967 outbreak. Recently, many cases of Marburg virus disease in the northeast region of the DRC had some connection, directly or indirectly, with a bat-infested gold mine. Also, an index case in the first Ebola Sudan outbreak worked in a bat-infested factory building. Despite failure to detect virus in these environments, the possibility still exists that a transmission cycle among bats plays

some role in the natural history of filoviruses. This is supported by laboratory transmission studies, in which persistent infection of bats for up to 21 days following inoculation, with excretion in feces, has been documented (205). An extensive 3-month survey of native vertebrates following the 1995 outbreak in DRC, albeit months delayed from the purported index case, failed to identify Ebola-specific antibodies or a virus isolate in over 3,000 vertebrates sampled (136).

PREVENTION AND CONTROL

It is difficult to devise a strategy for primary prevention of human filovirus infections without knowing the reservoirs of the viruses in nature, without understanding what governs virus flow, and without knowing how human contact occurs. However, it is clear that the pattern of spread once the virus enters the primate population is by close contact. Thus, contact with sick or dead monkeys should be avoided. Humans with hemorrhagic illnesses should be taken to the hospital and nursed with barrier precautions. Methods to achieve barrier nursing, waste disposal, and other key elements inexpensively and practically in Africa have been devised, and a field-tested manual is available (138). One of the important elements is provision of sterile equipment for injections, a remarkable and tragic lack in Africa today.

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CHAPTER 41 Paramyxoviridae: The Viruses and Their Replication

Robert A. Lamb and Daniel Kolakofsky

The *Paramyxoviridae* include some of the great and ubiquitous disease-causing viruses of humans and animals, including one of the most infectious viruses known (measles), some of the most prevalent viruses known (measles, respiratory syncytial, parainfluenza viruses, and mumps virus), a virus that has been targeted by the World Health Organization for eradication (measles), viruses that have a major economic impact on poultry and animal rearing (Newcastle disease and rinderpest), and some newly discovered viruses and diseases (pinniped morbilliviruses, Hendra virus, and Nipah virus). The *Paramyxoviridae* are enveloped, negative-stranded RNA viruses that have special relationships to two other families of negative-strand RNA viruses, namely the *Orthomyxoviridae* (for the biologic properties of the envelope glycoproteins) and the *Rhabdoviridae* (for the similarity of organization of the nonsegmented genome and its expression). The *Paramyxoviridae* are defined by having a protein (F) that causes viral–cell membrane fusion at neutral pH. The genomic RNA of all negative-strand RNA viruses has to serve two functions: first as a template for synthesis of mRNAs, and second as a template for synthesis of the antigenome (+) strand. Negative-strand RNA viruses encode and package their own RNA polymerase (RNAP), but mRNAs are synthesized only after the virus has been uncoated in the infected cell. Viral replication occurs after synthesis of the mRNAs and requires the continuous synthesis of viral proteins. The newly synthesized antigenome (+) strand serves as the template for further copies of the (–) strand genomic RNA.

CLASSIFICATION

The family *Paramyxoviridae* has been reclassified in 2000 by the International Committee on the Taxonomy of Viruses into two subfamilies, the *Paramyxovirinae* and the *Pneumovirinae. Paramyxovirinae* contains three genera, Respirovirus, Rubulavirus, and Morbillivirus. *Pneumovirinae* contains the genera Pneumovirus and Metapneumovirus. The new classification is based on morphologic criteria, the organization of the genome, the biologic activities of the proteins, and the sequence relationship of the encoded proteins, now that most of the genome sequences have been obtained. The recently isolated Hendra virus, from horses and humans in Australia, Nipah virus, from pigs and humans in Malaysia, and a tree shrew (*Tupaia*) Paramyxovirus are presently unclassified members of the *Paramyxovirinae.* Newcastle disease virus (NDV) and other avian paramyxoviruses have been included in the Rubulavirus genus, primarily because of their nonconserved intergenic junctions and lack of a C-protein open reading frame (ORF), hallmarks specific to the Rubulavirus genus (see later, and Fig. 6). However, the organization of the NDV P gene and its mRNA editing profile resemble those found for species of the genera Morbillivirus and Respirovirus, but not for other species in the genus Rubulavirus. Thus, further consideration of the appropriate taxonomy of NDV and avian paramyxoviruses is required.

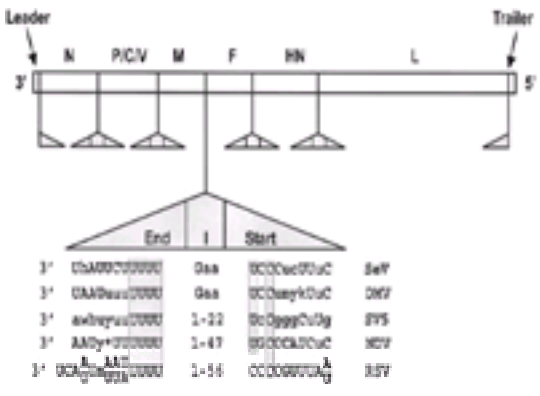

FIG. 6. Schematic diagram of a Paramyxovirus genome and the transcription end, intercistronic, and transcriptional start sequences. The positions of the extragenic 3'-terminal leader region and the 5'-terminal trailer region are shown. The conserved transcription regulatory sequences at the gene boundaries are indicated. These are sometimes known as E (end), I (intergenic), and S (start) sequences. The junctional sequences of representative viruses of each group, as (–) RNA, are shown. Nucleotides that are strictly conserved at each viral junctions are shown as *capital letters*; those that are mostly conserved (four of six junctions or better) are shown in *lower-case letters.* Other letters are used for less

conservation. *, includes nucleotide deletions.

The morphologic distinguishing feature among enveloped viruses for the subfamily *Paramyxovirinae* is the size and shape of the nucleocapsids (diameter of 18 nm, 1 μm in length, a pitch of 5.5 nm), which have a left-handed helical symmetry. The biologic criteria are (a) antigenic cross-reactivity between members of a genus and (b) the presence (Respirovirus and Rubulavirus) or absence (Morbillivirus) of neuraminidase activity. In addition, the different coding potentials of the P genes are considered and there is the presence of an extra gene (SH) in some rubulaviruses. The pneumoviruses can be distinguished from *Paramyxovirinae* morphologically, as they contain narrower nucleocapsids. In addition, the *Pneumovirinae* have major differences in the number of encoded proteins and an attachment protein that is very different from that of *Paramyxovirinae.* Examples of members of the three genera are shown in Table 1, and a phylogenetic majority-rule-consensus tree based on a combined cluster alignment of the nucleocapsid and phosphoprotein amino acid sequences is shown in Figure 1.

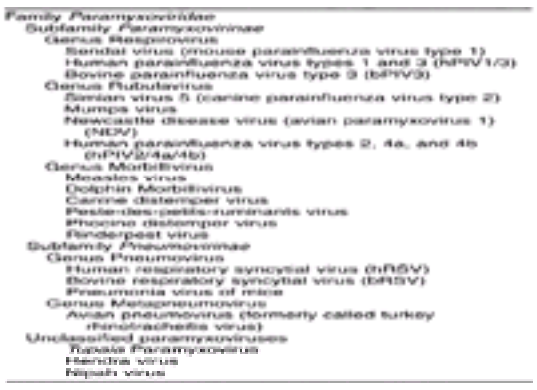

TABLE 1. *Examples of members of the family Paramyxoviridae*

FIG. 1. Evolutionary relationships of the *Paramyxoviridae*. Phylogenetic majority-rule consensus tree of the family *Paramyxoviridae* based on a combined cluster alignment of the nucleocapsid and phosphoprotein amino acid sequences of selected family members. Branch lengths represent relative phylogenetic distances according to maximum likelihood estimates based on the Dayhoff PAM matrix. Bootstrap support is 100% for all branching points, with the only exceptions being Hev (98%) and TPMV (77%). The following Paramyxovirus species are included in the cluster alignment (GenBank/EMBL/SwissProt accession numbers are given in parentheses): HeV (Hendra virus) (AF017149); TPMV (Tupaia Paramyxovirus) (AF079780); CDV (canine distemper virus) (P04865, P06940); PDV (phocine distemper virus) (P35944, P35939); DMV (dolphin Morbillivirus) (X75961, Z47758); MeV (measles virus) (Z66517); RPV (rinderpest virus) (Q03332, Q03335); NDV (Newcastle disease virus) (Z30084, Q06427); hPIV-4A (human parainfluenza virus 4A) (P17240, P22044); hPIV-4B (human parainfluenza virus 4B) (P17241, P21738); MuV (Mumps virus) (P21277, P19717); SV5 (simian parainfluenza virus 5) (AF052755); hPIV-2 (human parainfluenza virus 2) (P21737, P23056); SV41 (simian parainfluenza virus 41) (P27018, S60813); SeV (Sendai virus) (P04858, P04860); hPIV-1 (human parainfluenza virus 1) (P24304, P28054); hPIV-3 (human parainfluenza virus 3) (P06159, P06162); bPIV-3 (bovine parainfluenza virus 3) (P06161, P06163): oRSV (ovine respiratory syncytial virus) (Q83957, Q83956); bRSV (bovine respiratory syncytial virus) (P22677, P33454); hRSV (human respiratory syncytial virus) (P03418, P03421); PVM (pneumonia virus of mice) (P26589, U096449); TRTV (turkey rhinotracheitis virus) (U39295, U22110). Although not yet approved by the International Committee for the Taxonomy of Viruses, there is a wealth of data to suggest it is appropriate to remove NDV from the genus Rubulavirus and to designate it as belonging to a new avian genus of the *Paramyxoviridae*. (Adapted from ref. 276, with permission).

STRUCTURE AND REPLICATION STRATEGY OF THE*PARAMYXOVIRIDAE*

Paramyxoviruses contain nonsegmented, single-stranded RNA genomes of negative polarity, and they replicate entirely in the cytoplasm. Their genomes are 15 to 19 kB in length, and the genomes contain six to ten tandemly linked genes. A lipid envelope containing two surface glycoproteins (F and HN, or H or G), which mediate the entry and exit of the virus from its host cell, surrounds the virions. Inside the envelope lies a helical nucleocapsid core containing the RNA genome and the nucleocapsid (N), phospho- (P), and large (L) proteins, which initiate intracellular virus replication. Residing between the envelope and the core lies the viral matrix (M) protein that is important in virion architecture and that is released from the core during virus entry. In addition to the genes encoding structural proteins, paramyxoviruses contain “accessory” genes, which are found mostly as additional transcriptional units interspersed with the tandemly linked invariant genes. For the *Paramyxovirinae*, the accessory genes are found mostly as ORFs that overlap within the P gene transcriptional unit.

Intracellular replication of paramyxoviruses begins with the viral RNA-dependent RNAP (minimally, a homotetramer of P and a single L protein) transcribing the N-encapsidated genome RNA (N:RNA) into 5' capped and 3' polyadenylated mRNAs. The viral RNAP (vRNAP) begins all RNA synthesis at the 3' end of the genome, and it transcribes the genes into

mRNAs in a sequential (and polar) manner by terminating and reinitiating at each of the gene junctions. The junctions consist of a gene-end sequence, at which polyadenylation occurs by the reiterative copying of four to seven uridylates (followed by release of the mRNA), a short nontranscribed intergenic region, and a gene-start sequence that specifies capping as well as mRNA initiation. The RNAP occasionally fails to reinitiate the downstream mRNA at each junction, leading to the loss of transcription of further-downstream genes, and hence there is a gradient of mRNA synthesis that is inversely proportional to the distance of the gene from the 3' end of the genome. After primary transcription and translation, when sufficient amounts of unassembled N protein are present, viral RNA synthesis becomes coupled to the concomitant encapsidation of the nascent (+) RNA chain. Under these conditions, vRNAP ignores all the junctions (and editing sites), to produce an exact complementary antigenome chain, in a fully assembled nucleocapsid.

Virion Structure

The *Paramyxoviridae* contain a lipid bilayer envelope that is derived from the plasma membrane of the host cell in which the virus is grown (reviewed in ref. 43). *Paramyxoviridae* are generally spherical and 150 to 350 nm in diameter, but they can be pleiomorphic, and filamentous forms can be observed. Inserted into the envelope are glycoprotein spikes that extend about 8 to 12 nm from the surface of the membrane and that can be readily visualized by electron microscopy (EM). Inside the viral membrane is the nucleocapsid core (sometimes called the ribonucleoprotein core), which contains the 15,000- to 19,000-nucleotide, single-stranded RNA genome. Figure 2 is a highly stylized schematic diagram of the virions. F and HN are actually trimers and tetramers, respectively. No attempt has been made to represent the real abundance of F, HN, SH, or N subunits in the virion. The pleiomorphic nature of virion particles under EM is illustrated in Figure 3, and a comparison of the RNPs of influenza virus, rabies virus, and Sendai virus is shown in Figure 4.

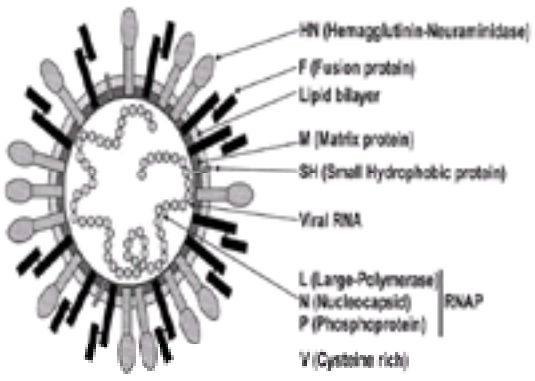

FIG. 2. Schematic diagram of a Paramyxovirus (not drawn to scale). The lipid bilayer is shown as the *gray concentric circle*, and underlying the lipid bilayer is the viral matrix protein shown as a *black concentric circle.* Inserted through the viral membrane are the hemagglutinin-neuraminidase (*HN*) attachment glycoprotein, and the (*F*) fusion glycoprotein. The relative abundance of HN and F is not illustrated by the diagram. The small integral membrane protein, SH, is found only in some rubulaviruses, such as SV5. The HN protein is thought to have a stalk region and a globular head, and the F protein consists of two sulfide-linked chains, F_1 and F_2. The HN protein is a tetramer and the F protein a trimer. Inside the virus is the negative-stranded virion RNA, which is encapsidated with the nucleocapsid protein (*N*). Associated with the nucleocapsid are the L and P proteins, and together this complex has RNA-dependent RNA transcriptase activity. For the rubulaviruses, the cysteine-rich protein V is found as an internal component of the virion, whereas for other members of the family, the V protein is found only in virus-infected cells. The nature of possible interactions between the cytoplasmic tails of the glycoprotein spikes and the matrix protein, and the interactions between the matrix protein and the nucleocapsid have not been fully elucidated and no attempt has been made to illustrate them.

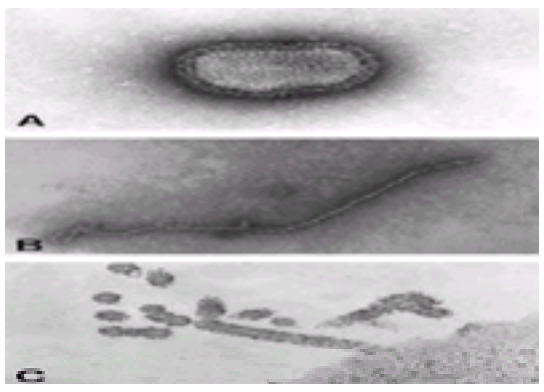

FIG. 3. Ultrastructure of SV5 virions revealed by negative staining. **A:** Negatively stained SV5 particle. The glycoprotein spikes on intact 150- to 300-nm virus particles can be observed (×226,280). **B:** Negatively stained SV5 nucleocapsid (×74,570). **C:** Budding SV5 virions particles from the surface of CV-1 cells. Colloidal gold staining of HN is shown (×24,700) (Micrographs courtesy of George Leser, Northwestern University).

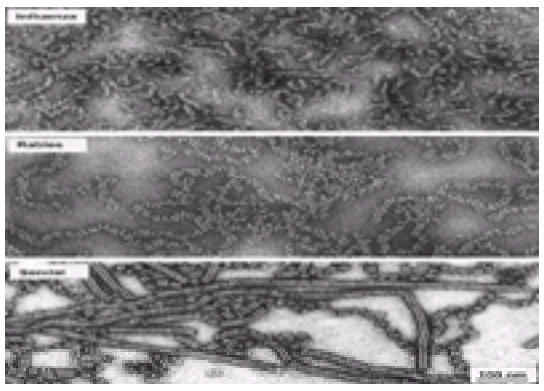

FIG. 4. Nucleocapsids of negative-strand RNA viruses. Electron micrographs of the nucleocapsids of three negative-strand RNA viruses, negatively stained with 1% sodium silicotungstate. *Top:* Ribonucleoprotein particles of influenza virus with a stoichiometry of 24 nucleotides per NP monomer. *Middle:* Nucleocapsids of rabies virus with a stoichiometry of nine nucleotides per N monomer. *Bottom:* Nucleocapsids of Sendai virus with a stoichiometry of six nucleotides per N monomer. All micrographs have the same magnification (bar = 100 nm). (Micrographs courtesy of Rob Ruigrok, European Molecular Biology Laboratory, Grenoble, France.)

The helical nucleocapsid, rather than the free genome RNA, is the template for all RNA synthesis. For Sendai virus, each nucleocapsid is composed of about 2,600 N, 300 P, and 50 L proteins (164). The N protein and genome RNA together form a core structure, to which the P and L proteins are attached. This nucleocapsid core is remarkably stable, as it withstands the high salt and gravity forces of cesium chloride density gradient centrifugation. By EM, the P and L proteins of nucleocapsids are not observed; they can be visualized only with the aid of antibodies (224). Holonucleocapsids (N:RNA plus P and L) have the capacity to transcribe mRNAs *in vitro*, presumably mimicking primary transcription in infected cells, and they are thought to be the minimal unit of infectivity.

When negatively stained preparations of Paramyxovirus nucleocapsids are viewed by EM, the most tightly coiled forms resemble the Tobamovirus tobacco mosaic virus (TMV)—that is, a relatively rigid coiled rod 18 nm in diameter, with a central hollow core of 4 nm and a helical pitch of about 5 nm (51, 52, 89). Unlike TMV, however, in which the nucleocapsid must disassemble so that its (+) RNA genome can function as a template, Paramyxovirus nucleocapsids function without disassembling; as far as we know, these nucleocapsids never disassemble naturally. This remarkable property is undoubtedly associated with the finding that, unlike TMV, these nucleocapsids are not rigid, although they become more rigid on trypsin treatment (194).

Sendai virus nucleocapsids exist in several distinct morphologic states at normal salt concentration (79, 113, 114). The most prevalent form in negatively stained preparations is the most tightly coiled one, with a helical pitch of 5.3 nm. Two other forms, one with a slightly larger pitch of 6.8 nm, and another with a much larger pitch of 37.5 nm, have also been noted. The fact that no structures of intermediate pitch have been found indicates that these are distinct states. It is thought that the template is copied without dissociation of N protein from the nucleocapsid, and the uncoiling of the nucleocapsid may be necessary for the polymerase to gain access to the RNA bases. It is possible that the viral polymerase traverses the nucleocapsid template by uncoiling the helix in front of it and recoiling it once the polymerase has passed a given position, much the same as cellular RNAP generates its template “bubble” in traversing dsDNA.

As expected, the diameter of the nucleocapsid decreases as the pitch increases and the nucleocapsid lengthens, and for Sendai virus the diameter is 3.5 nm less for the 6.8-nm form than for the 5.3-nm-pitch form. These latter values are very similar to those of Pneumovirus nucleocapsids, which also have a pitch of 7 nm. As discussed earlier, these differences in nucleocapsid morphology are used to distinguish the different *Paramyxoviridae*, but they probably relate mainly to the form that predominates in negatively stained preparations.

THE *PARAMYXOVIRIDAE* GENOMES AND THEIR ENCODED PROTEINS

Today we know the complete genome sequence for almost all members of the *Paramyxoviridae*, including Sendai virus, human parainfluenza virus 3 (hPIV-3), simian virus 5 (SV5), mumps virus, measles virus, canine distemper virus (CDV), and respiratory syncytial virus (RSV). (They are obtainable from the Web, at http://www.ncbi.nlm.nih.gov/.) The 15,000- to 19,000-nucleotide genomic RNA contains a 3' extracistronic region of about 50 nucleotides, known as the leader, and a 5' extracistronic region of 50 to 161 nucleotides, known as the trailer [or the (–) leader]. These control regions are essential for transcription and replication, and they flank the six genes (seven for certain rubulaviruses and eight to ten for pneumoviruses) (note that by the convention used for paramyxoviruses, the term *gene* refers to the genome sequence encoding a single mRNA, even if that mRNA contains more than one ORF and encodes more than one protein). The coding capacity of the genome of *Paramyxovirinae* is extended by the use of overlapping ORFs in the P gene. The gene order of a representative member of each subfamily is shown in Figure 5. At the beginning and end of each gene are conserved transcriptional control sequences that are copied into mRNA. Between the gene boundaries are intergenic regions (Fig. 6). These are precisely three nucleotides long for the respiroviruses and morbilliviruses but are quite variable in length for the rubulaviruses (1 to 47 nucleotides) and pneumoviruses (1 to 56 nucleotides) (see Fig. 6).

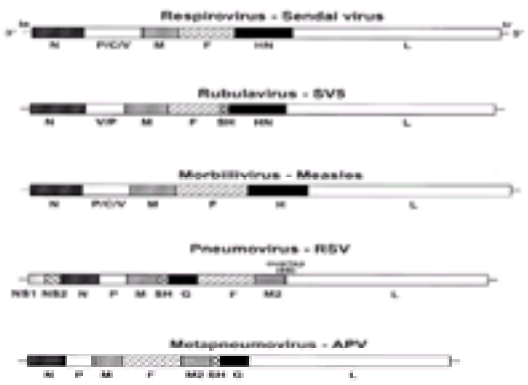

FIG. 5. Genetic map of a typical member of each genus of the *Paramyxoviridae*. The gene size is drawn to scale. Gene boundaries are shown by *vertical lines.* The hRSV L gene transcription overlaps that of the M2 gene by 68 nucleotides, and the bRSV overlap is 67 nucleotides, whereas PMV and APV do not have an overlap.

The Nucleocapsid Protein

The nucleocapsid (N) protein serves several functions in virus replication, including encapsidation of the genome RNA into an RNase-resistant nucleocapsid (the template for RNA synthesis), association with the P-L polymerase during transcription and replication, and, most likely, interaction with the M protein during virus assembly. The intracellular concentration of unassembled N is also thought to be a major factor controlling the relative rates of transcription and replication from the genome templates, by analogy to studies made on the Rhabdovirus vesicular stomatitis virus (VSV) (13,14).

The nucleotide sequence of the N gene of paramyxoviruses predicts that the N proteins range from 489 to 553 amino acids [molecular weight ratio (M_r) 53,167 to 57,896], and, unexpectedly for a protein that interacts with RNA, the protein has a net charge of –7 to –12, with the exception of mumps virus (+2). A comparison of these protein sequences, coupled with data obtained from protease digestions of nucleocapsids, suggests that N contains two domains. The N-terminal 80% of the protein of about 500 residues is relatively well conserved among related viruses, whereas the C-terminal 20% is poorly conserved, although this domain always contains a highly charged and mostly negative region (58,208). This hypervariable C-terminus appears to be a tail extending from the surface of a globular N-terminal body, because the C-terminal sequences of the SV5 and Sendai virus N protein in nucleocapsids are hypersensitive to trypsin digestion, leaving a 48-kd N-terminal core (53,114,194). However, the overall structure of the nucleocapsid seen by EM, and the resistance of the genomic RNA within this structure to nuclease attack are mostly unchanged by trypsin treatment (151). These data indicate that the determinants for the helical nature of the nucleocapsid, as well as the RNA binding domains, must lie within the highly conserved N-terminal body. Extensive deletional analysis indicates the N-terminal region of Sendai virus N protein is required for nucleocapsid assembly (18). For N proteins, an invariant sequence F-X4-Y-X4-S-Y-A-M-G (where X is any residue) is found near the middle of the protein in a region predicted to be hydrophobic, and it could be involved in N:N contacts during assembly (190), but the presence of several conserved aromatic residues also suggests that it may be involved in RNA binding. In any event, N does not appear to be a classic RNA binding protein, in that it does not contain any previously recognized RNA-binding motifs, nor does N interact with RNAs after transfer of the N protein to nitrocellulose membranes and probing with RNA (Northwestern blots). The C-terminal tail, on the other hand, contains most of the N-protein phosphorylation sites and antigenic sites (133,239).

The structure–function relationship of the Sendai virus N protein was examined using a cDNA-encoded genome of a defective-interfering (DI) particle, whose intracellular replication was directed by N, P, and L protein expressed from cotransfected cDNAs (58) (see later section, Molecularly Engineered Genetics). The entire C-terminal tail of N was found to be dispensable for assembling an encapsidated, complementary copy of the template, whereas deletions anywhere within the N-terminal body of the protein eliminated this activity. Thus, the C-terminal tail is not essential for assembling the nascent chain and helping the polymerase to ignore the junctions. However, templates that were themselves assembled with tailless N proteins were unable to act as templates for new rounds of genome replication. The highly charged domain of the C-terminal tail appears to be essential for this function, as deletions downstream of this domain have little or no effect. The precise template function of the N-protein C-terminal tail is unclear, but it may be required for the transition between helical states, as nucleocapsids with tailless N protein appear more rigid in electron micrographs (194). The tail, moreover, appears to mediate P-protein binding to nucleocapsids, as binding of antibodies specific to the C-terminal tail leads to the release of the normally tightly bound P proteins, both for Sendai virus and hPIV-1 (239). Whether P binds directly to this C-terminal tail domain is unclear, but if so, it could participate in the helical transitions thought necessary for template function.

The P Gene and Its Encoded Proteins

For the *Paramyxovirinae*, the P gene represents an extraordinary example of a virus compacting as much genetic information as possible into a small gene. The P gene gives rise to a plethora of polypeptides by means of using overlapping reading frames on a single mRNA transcript, by a remarkable process of transcription known as RNA editing, or pseudotemplated addition of nucleotides. The consequence of the addition of pseudotemplated nucleotides to an mRNA is that there is a shift of a translational reading frame into an alternative reading frame, and hence a new protein is translated (Table 2). In this chapter, it will be considered that the P genes of the *Paramyxovirinae* are composed of a series of modules that are combined in various ways via mRNA editing, or as additional modules expressed via unusual translational initiation (16,59,103,169). An illogical sequence of letters (C, C', D, I, P, V, W, X, Y1, Y2, and Z) has been used to name the various Paramyxovirus P gene products. However, based on some new biochemical data and some limited functional data for Sendai virus, a new nomenclature system is being introduced in this chapter, to describe the

different P modules encoded by the P gene.

		Ribosomal choice				mRNA editing		
Paramyxovirus	Host	P	V	C′	C	D	V	P
Sendai	Mouse	+	-	+	+	-	+	-
hPIV1	Human	+	-	+	+	-	-	-
bPIV3	Cattle	+	-	-	+	+	+	-
hPIV2	Human	+	-	-	+	+	(+)	-
Mumps	Human	-	+	-	-	-	-	+
SV5	Dog	-	+	-	-	-	-	+
hPIV2 and hPIV4	Human	-	+	-	-	-	-	+
NDV	Fowl	+	-	-	-	-	+	-
Measles	Human	+	-	-	+	-	+	-
Canine distemper	Dog	+	-	-	+	-	+	-
Rinderpest	Cattle	+	-	-	+	-	+	-
Phocine distemper	Seals	+	-	-	+	-	+	-

TABLE 2. *Examples of expressible P gene open reading frames*

All viruses of the *Paramyxovirinae* (with the notable exception of hPIV-1) contain an editing site, at which G residues are inserted into the P mRNA in a programmed manner during its synthesis (Fig. 7, the dotted vertical line) (35,215,274,281). The number of G residues inserted varies between the different viruses and appears to be matched to the organization of their alternate ORFs. The editing site separates each P gene into two segments, and it generates three different mRNAs in which the three possible reading frames downstream of the editing site are fused to a unique upstream ORF (see Fig. 7). These mRNAs are translated into three polypeptides with a common N-terminal segment and different C-terminal segments, called P, V, and W/D/I. In respiroviruses and morbilliviruses, additional P-gene complexity is provided by the presence of two (overlapping) ORFs (designated P-amino 1 and P-amino 2) upstream of the editing site, only one of which (P-amino 1) extends to the editing site. For Sendai virus and hPIV-1, the ORF that terminates before the editing site (P-amino 2, or C ORF) can also code for a nested set of up to four polypeptides, because of the use of four ribosomal initiation sites. For rubulaviruses, there is a single ORF upstream of the editing site (designated P-amino 2), which may be the counterpart of the respiroviruses and morbilliviruses C genes (see later). The mechanisms of expression of the protein products derived from the P gene are described later.

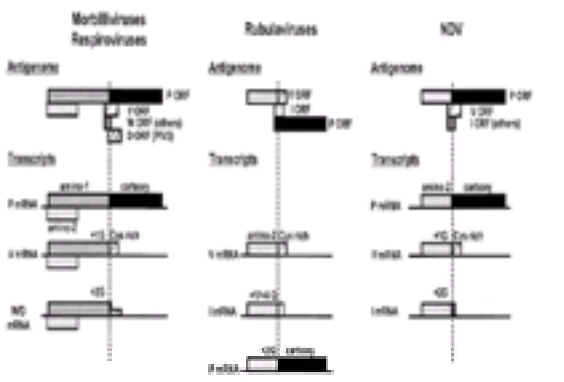

FIG. 7. Schematic diagram of the P gene of representative paramyxoviruses. For the respiroviruses and morbilliviruses, the mRNA for the P protein is transcribed faithfully (unedited) from the viral genome. Transcriptional RNA editing with the addition of one G nucleotide at the editing site produces an mRNA that encodes the V protein. Addition of two G nucleotides at the editing site produces an mRNA that encodes the W or D proteins (depending on the virus). The P mRNAs of the respiroviruses and morbilliviruses also contain the P-amino 2 (C protein) ORF at the 5' end of the mRNA independent of editing status (*light shading*). The unedited version of the Rubulavirus "P" mRNA encodes the V protein (*light shading*); the addition of two G nucleotides produces the P mRNA and the addition of one G produces the I mRNA. NDV follows the Respirovirus and Morbillivirus pattern, and its unedited mRNA is translated to yield the P protein, and RNA editing produces the V mRNA. The cysteine-rich domain of the V protein is indicated by *cross-hatching.* The RNA editing site, at which nontemplated nucleotides are added to the mRNAs is indicated by the *vertical dotted line.* As described in the text, the P gene is translated into a series of proteins that are thought to consist of a series of modules. P-amino 1 (*medium shading*) and P-amino 2 (*light shading*) are two modules upstream of the RNA editing site, and P-carboxy (*black shading*) is the P module downstream of the RNA editing site. Some sequence relatedness and limited functional studies have led to the speculation that P-amino 2 (C ORF) of Respirovirus is functionally related to the N-terminal module of the Rubulavirus P and V proteins. Thus, the P-amino 1 module is unique to the respiroviruses and morbilliviruses.

The Phosphoprotein

The P protein is the only P gene product that is essential for viral RNA synthesis (61), and it is involved in all its aspects. The Sendai virus P protein was first characterized as a homotrimer, but more recent biochemical evidence indicates the protein contains a coiled-coil domain that is tetrameric, and the entire protein is expected to be a tetramer (270). P protein is an essential component of the vRNAP and the nascent chain assembly complex (129), and additional P protein is also required during RNA synthesis (55). Although the L protein is thought to contain all vRNAP catalytic activities (222), L binds to the N:RNA template via the P proteins (129). This interaction between P and L requires a domain in P that maps to the C-terminal end of the coiled-coil oligomerization regions (Fig. 8) (62,238). This very C-terminal domain of P, which binds to the N:RNA, is predicted to form an a-helical bundle. These domains (all downstream of the editing

site) that fall in the region called P-carboxy are essential for viral RNA synthesis. For Sendai virus, the P-carboxy region is also sufficient for RNA synthesis, as this protein fragment by itself (residues 325 to 568) can substitute for intact P protein in all aspects of mRNA synthesis (55,63). Thus, the P-carboxy region represents the polymerase (pol) cofactor module. This region is relatively well conserved in predicted secondary structure for all viruses of the *Paramyxovirinae*, and all P proteins carry this essential module as the C-terminal segment of a fusion protein; this module is never naturally expressed by itself. For respiroviruses, morbilliviruses, and NDV, the P-carboxy pol-cofactor module is translated from the unedited mRNA (see Fig. 7); for the viruses in the genus Rubulavirus, the P-carboxy pol-cofactor module is translated from an mRNA with a 2G insertion (see Fig. 7). Although little is known about the RSV P protein, it too is predicted to contain a coiled-coil domain, and it interacts with itself in a yeast two-hybrid system (256). P protein oligomerization thus appears to be a general property of this virus family.

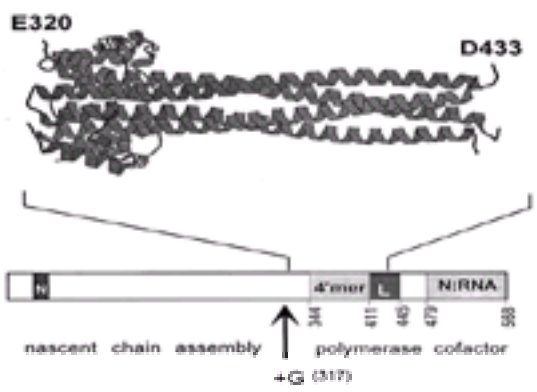

FIG. 8. Structure and function of the Sendai virus P protein. The P protein is shown below as a *horizontal bar.* Its known functional domains—namely, chaperoning unassembled N protein during the nascent chain assembly step of genome replication (N), self-assembly as a tetramer (4-mer), L protein binding site (L), and N:RNA binding site (N:RNA)—as determined by deletion analysis and discussed in the text, are indicated. Numbers refer to amino acid positions. The G insertion site (or editing site) in codon 317 used during mRNA synthesis, which separates the nascent chain assembly and polymerase cofactor domains of the P protein, is indicated. The core of the P protein homotetramer, a coiled coil of four parallel a-helices buttressed at their N-terminal ends by four short a-helical bundles (from E320 to D433), determined by x-ray crystallography (courtesy of Tabourieh and coworkers, EMBL, Grenoble, France, unpublished), is shown at the top of the figure.

For genome replication, in which RNA synthesis and assembly into nucleocapsids are coupled, at least for Sendai virus, one additional P-protein domain near the N-terminus (defined by deletion of residues 33 to 41) is also required (62). This domain apparently chaperones unassembled N protein during nascent chain assembly (assembly module), as the substrate for genome assembly is a P-N complex (129). The rest of the Sendai virus P protein is apparently dispensable for (unregulated) genome RNA synthesis and assembly, as a P protein in which residues 78 to 324 have been deleted is still quite active for mini-genome replication in transfected cells (62). The remaining P gene accessory proteins, like residues 78 to 324 of the Sendai virus P protein, are not required for the basic process of replicating viral genomes and expressing their mRNAs. Nevertheless, most of the accessory proteins play a role in regulating viral RNA synthesis, and/or in countering host cell defenses to viral infection. The properties and functions of the individual protein modules translated from the P gene are described next.

P Gene Modules (ORFs) Downstream of the Editing Site

Cysteine-Rich V ORF

The V ORF is found in the middle of the P gene of all three genera, and the V protein is expressed by all morbilliviruses and rubulaviruses, but only by some of the respiroviruses. In Sendai virus (281), bPIV-3 (219), the morbilliviruses (35), and NDV (258), the cysteine-rich V ORF is translated from an mRNA with a single G insertion, whereas in rubulaviruses the V protein is translated from the unedited mRNA (211,274). In either case, the resulting fusion protein is called V. The C-terminal portion of the V ORF is by far the best conserved of all the various P gene ORFs (Fig. 9), even though the V ORF is absent in hPIV-1 (184) and may not be expressed in hPIV-3 (76,93), both respiroviruses. There are seven perfectly conserved cysteine residues, and this domain binds two atoms of Zn^{2+} (176,212). The V domains of several (though not all) viruses specifically interact with the large subunit of the cellular damage-specific DNA-binding protein (UV-DDB) (175), but the consequences of this interaction and the function of V protein cysteine-rich domain remain to be elucidated. Like the P-carboxy pol-cofactor module, the cysteine-rich V ORF is found only as the C-terminal segment of a fusion protein; this module is never expressed by itself.

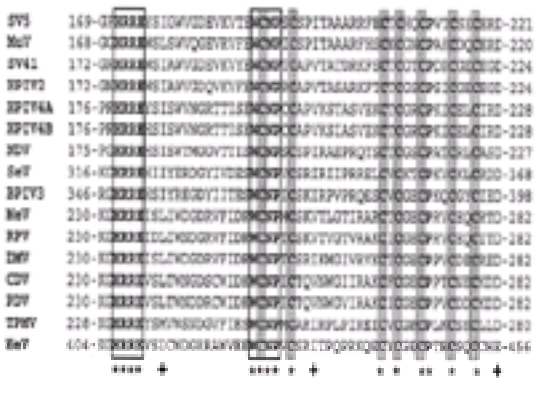

FIG. 9. Multiple amino acid sequence alignment of the conserved cysteine-rich C-terminal region of selected Paramyxovirus V proteins. *Numbers* indicate the amino acid position within the respective proteins. Conserved amino acid residues are shown in *boldface letters*, two areas of exceptionally high conservation are *boxed*, and the positions of the seven conserved cysteine residues are *shaded. Asterisks* and *plus symbols* below the alignment indicate identical and similar amino acid residues, respectively. The amino acid sequences of the Paramyxovirus species used in the alignment were from the P gene sequences indicated in Figure 1. (Adapted from ref. 276, with permission.)

The W/D/I ORF

The W/D ORF of respiroviruses and morbilliviruses is expressed from mRNAs with two inserted G residues (see Fig. 7). For most of these viruses, the insertion of two G residues into the mRNA is relatively rare and the ORF is closed by a stop codon shortly after the editing site. In these cases, it is referred to as the W ORF, and the ensuing P-amino-W fusion protein is called the W protein. The W ORF appears to serve mainly to truncate the P protein, producing essentially the N-terminal P-amino 1 assembly module of the P protein by itself. Despite the low abundance of +2G mRNAs in Sendai virus–infected cells (about 10% of the total P mRNA), the Sendai virus W protein can be easily detected (57). In two closely related respiroviruses (b-PIV-3 and hPIV-3) (92,243), the +2 ORF extends for 131 amino acids from the editing site (and is called D), and +2G mRNAs are not rare in h/bPIV-3-infected cells [it has been observed that mRNAs with one to six inserted G residues are almost equally abundant (93,219)]. The encoded P-amino 1-D fusion protein is called the D protein. The Rubulavirus protein in which the single upstream ORF (P-amino 2) is fused to the third possible (+1/+4) downstream ORF is also found in Rubulavirus-infected cells, and it is called I (119,211). However, because of constraints of the Rubulavirus mRNA editing mechanism, a single G is rarely inserted at this editing site, and the I protein is expressed from a +4G mRNA (211,274). Thus, the Rubulavirus P, V, and W/D/I proteins contain the N-terminal module of the P-ORF (P-amino 2), fused to, respectively, the P polymerase cofactor module (P-carboxy), the V ORF, and the W/D/I ORF (depending on the virus) in the remaining reading frame.

P Gene Modules (ORFs) Upstream of the Editing Site

Two types of P gene ORF are found upstream of the editing site, P-amino 1 and P-amino 2. The rationale for this nomenclature is based on the available data, and on speculation that P-amino 2 of Sendai virus (formerly called the nest set of proteins C', C, Y1, and Y2) have biochemical and sequence relationships to the N-terminal domain of the P protein of the rubulaviruses. Some limited data pertaining to the proteins of Sendai virus and SV5 involved in the interferon response to these viruses, also support the notion that the P-amino 2 domain of Sendai virus and the N-terminal P domain of SV5 are related in that they interact with different parts of the interferon-signalling pathway (see later). The P-amino 1 domain that is found only in respiroviruses and morbilliviruses is longer than P-amino 2 and is acidic in nature. P-amino 2, which is encoded by all P genes of the *Paramyxovirinae*, is shorter than P-amino 1 and is basic in nature.

P-Amino 1 Module

Unlike the downstream P-carboxy pol-cofactor module and the cysteine-rich V ORF module, whose conservation is evident across the entire virus subfamily, the ORF(s) upstream of the editing site are conserved only within each genus and can be very different between genera. For respiroviruses and morbilliviruses, the longer ORF (P-amino 1, with about 300 amino acids) that is fused to the downstream modules is relatively poorly conserved between genera, but they are all acidic in nature (pI of about 5.5). For Sendai virus, P-amino 1 contains a short region near the N-terminus (defined by deletion analysis) that is required to chaperone unassembled N protein as a P-N complex during the nascent chain assembly step of genome replication (62) (see Fig. 8). P-amino 1 also contains most of the serine and threonine phosphorylation sites of the P protein (23). The one or two major sites of each protein (and the respective kinases that catalyze the phosphorylation) are known for several viruses (66,136). For the respiroviruses, mutational analysis at these sites, within the context of an infectious cDNA and recovery of viable virus, has so far not been informative (134).

P-Amino 2, Including the C ORFs

As discussed earlier, two of the criteria used for considering the single Rubulavirus ORF (P-amino 2) with the C ORF of respiroviruses and morbilliviruses is that they are both about 170 amino acids and are basic (pI of about 10.5) and show a small degree of sequence relatedness. For respiroviruses and morbilliviruses, the C ORF overlaps the P-amino 1 module, and it is accessed by translational choice (Fig. 10). Morbilliviruses express one C protein (10) as do the newly isolated Hendra virus (301), Nipah virus (105a), and tree shrew Morbillivirus (276), whereas the respiroviruses, such as Sendai virus and hPIV-1, express four C-like proteins (C', C, Y1, and Y2) (60,99). The C protein ORFs of the respiroviruses and morbilliviruses are found at the same relative location on the P gene, and when their sequences are aligned there is clear conservation within each genus, but there is little conservation between these two genera. Although the NDV (avian Paramyxovirus) P gene does not contain a separate P-amino 2 ORF, the N-terminal domain of its P protein (before the editing site) is similar in size and overall charge to the P-amino 2 module and thus it may be a functional counterpart of the P-amino 2 (C) module. Similarly, for the rubulaviruses parts of the sequence of the V/P protein, shared N-terminal domain (P-amino 2) can be aligned with the Respirovirus P-amino 2 (C) protein sequence but not the Morbillivirus P-amino 2 (C) protein sequence (Fig. 11). Consistent with the different nature of the P protein N-terminal segments, the Respirovirus and Morbillivirus V proteins (P-amino 1 + V ORFs) are not found associated with viral nucleocapsids intracellularly or in virus particles (57,252), in contrast to the Rubulavirus V proteins (P-amino 2 + V ORFs), which are associated with intracellular nucleocapsids and found in virions (212,284). The various P-amino 2 ORFs may well serve different functions that are genus specific. Assuming that the various Paramyxovirus P proteins are functionally equivalent (N assembly module + pol-cofactor), the N assembly module would be contained within the P-amino 2 ORF of the Rubulavirus and NDV P protein.

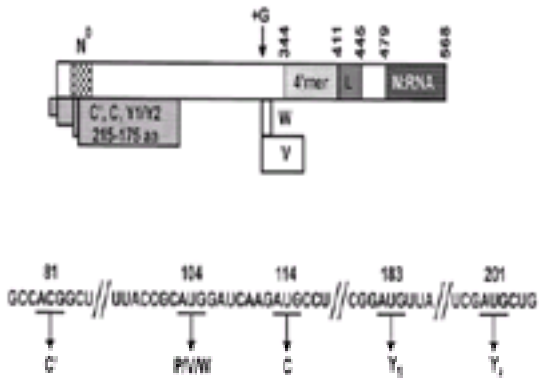

FIG. 10. Representation of the Sendai virus P mRNA to illustrate the use of multiple initiation codons. The nucleotide sequences surrounding the initiation codons for C', P, C, Y_1, and Y_2, all of which are derived from the C ORF, are indicated. The various functional domains of the P protein are also indicated. Note that the Y_1 and Y_2 initiation sites overlap the N assembly domain of P. The AUG codon at position 104 also starts the V and W proteins in the edited P gene mRNAs. All the initiation codons (the ACG for C8), except for the first one, are in a suboptimal context for initiation.

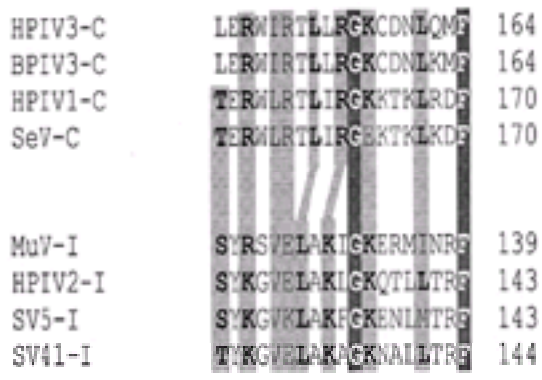

FIG. 11. A short stretch of sequence relatedness between the Respirovirus C proteins and P-amino 2 ORF of the Rubulavirus V/P/I proteins. Amino acid sequence relatedness of a region of the C proteins of hPIV-3, bPIV-3, hPIV-1, and Sendai virus (SeV) with the V/P/I proteins of mumps virus (MuV), hPIV-2, SV5, and SV41. The position of the phenylalanine whose mutation to serine in Sendai virus and hPIV-3 attenuates virulence, is shown on the *right.* Residues that are conserved by amino acid assignment and spacing within the segment are indicated by *black boxes*: residues that are semiconserved by assignment or spacing are indicated by *gray boxes.*

The Large Protein

The L protein is the least abundant of the structural proteins (about 50 copies per virion). The L gene is the most promoter-distal in the transcriptional map and thus the last to be transcribed. Its low abundance, its large size, and its localization to transcriptionally active viral cores suggested that it might be the viral polymerase. The L genes of most paramyxoviruses have now been sequenced and they are all of very similar length (about 2,200 amino acids), but there is little overall sequence homology outside the subfamily. There are, however, five short regions of high homology near the center of these proteins that are also conserved in the RNA-dependent RNAPs of other virus families (145,222). These regions are thought to represent structural features of a common, ancestral polymerase “fold” (222). Mutational analysis of the highly conserved residues of these homology regions indicates that these regions are essential for RNAP activity (257).

The P and L proteins form a complex, and both proteins are required for polymerase activity with N:RNA templates (61,105,129). The precise composition of the viral polymerase is unclear, but transcriptionally active nucleocapsids contain five to ten P proteins per L protein, and this complex can make mRNA *in vitro*, which is capped at its 5' end and also contains a poly(A) tail at the 3' end. Polyadenylation is thought to result from polymerase stuttering on a short stretch of U residues, but the capping step requires both guanylyl and methyl transferase activities. These latter activities are thought to be provided by the L protein, by analogy to VSV (see Chapter 38), and because similar reactions have not been described for cellular enzymes that operate in the cytoplasm. The Sendai virus L protein purified from virions was also found to phosphorylate the N and P proteins, and it has been suggested that L is the kinase (80) that has long been known to be associated with the viral core (160,235). However, the Sendai virus P and V proteins are phosphorylated when expressed in transfected cells in the absence of L (64), suggesting that phosphate addition to these proteins is the result of cellular kinases. In any event, the phosphorylation of these proteins appears to be a modification without an obvious function, as recombinant Sendai virus that lacks the major P protein phosphorylation site can be rescued and this virus grows normally both in tissue culture and in mice (134).

The Matrix Protein

The Paramyxovirus matrix (M) protein is the most abundant protein in the virion. The nucleotide sequence of the M gene and predicted amino acid sequence of many paramyxoviruses indicate that these proteins contain 341 to 375 residues (M_r about 38,500 to 42,500), are quite basic proteins (net charge at neutral pH of +14 to +17), and are somewhat hydrophobic, although there are no domains of sufficient length to span a lipid bilayer. In electron micrographs of virions, an electron-dense layer is observed underlying the viral lipid bilayer and this is thought to represent the location of this

protein. Fractionation studies of virions indicate that the M protein is peripherally associated with membranes and is not an intrinsic membrane protein. Reconstitution studies of purified M protein, and fractionation studies of infected cells indicate that the M protein can associate with membranes (84,162,200,245).

As a purified protein, the Sendai virus M protein can self-associate and form two-dimensional paracrystalline arrays (sheets and tubes) in low salt conditions (6,115,120), and there is a paracrystalline array of identical periodicity at the inner surface of the plasma membrane of infected cells when examined by EM after freeze-fracture (6). In addition, the M protein is also associated with nucleocapsids (261,299). The M protein probably contains amphipathic a-helices that insert themselves into the inner leaflet of the lipid bilayer, to coat this surface and organize its contacts with the helical nucleocapsid (15). Evidence that the M protein of Sendai virus interacts specifically with membranes expressing individually the F and HN glycoproteins has also been obtained, which implies that there is an interaction of the F and HN cytoplasmic tails with the M protein (246). Furthermore, genetically engineered recombinant measles virus and SV5 that lack glycoprotein cytoplasmic tails show a subcellular redistribution of the matrix protein (33,251). Thus, the M protein is considered to be the central organizer of viral morphogenesis, interacting with the cytoplasmic tails of the integral membrane proteins, the lipid bilayer, and the nucleocapsids. The self-association of M and its contact with the nucleocapsid may be the driving force in forming a budding virus particle (217). The relative abundance of basic residues in the M protein may reflect their importance in ionic interactions with the acidic N proteins.

Consistent with its central role in virus budding, M is often inactivated in persistent Paramyxovirus infections where budding fails to occur. For example, in subacute sclerosing panencephalitis (SSPE), a rare, progressive, and invariably fatal persistent measles virus infection of the brain, the M protein is either absent for a variety of reasons (reviewed in ref. 34) or, when present, is not associated with budding structures *in vivo* and is unable to bind to viral nucleocapsids *in vitro* (124,125). Although a genetically engineered recombinant measles virus that lacks an M protein has been obtained (32), it produces a four-log lower titer of released infectious particles than wild-type virus and remains mostly cell associated. Therefore it is reasonable to conclude that the M protein does play a very important function in virus assembly. Moreover, in model systems of persistent Sendai virus infection in culture in which the normally lytic infection is converted to a persistent one using DI particles, the change from a lytic to a persistent infection correlates mainly with M protein instability and an absence of budding structures (236).

The M protein of several paramyxoviruses is phosphorylated. For Sendai virus, a large proportion of the M protein is phosphorylated yet the M protein found in virions is not phosphorylated (163). However, a Sendai virus could be rescued from an infectious cDNA in which the single phosphorylation site in Sendai virus M protein had been eliminated (241). This M protein phosphorylation-minus mutant did not show an altered phenotype from wild-type virus in either cultured cells or mice. For the Rhabdovirus VSV, there is clear evidence that the VSV M protein that is associated with the RNP structure inhibits viral transcription (see Chapter 38). For *Paramyxovirinae*, there is no evidence that association of RNPs with M protein shuts down RNA synthesis in preparation for virus assembly, and this function may be carried out by another viral protein, such as the C protein.

Envelope Glycoproteins

All *Paramyxoviridae* possess two integral membrane proteins, and some rubulaviruses and all pneumoviruses encode a third integral membrane protein (Fig. 12 and Fig. 13). One glycoprotein (HN, H, or G) is involved in cell attachment and the other glycoprotein (F) in mediating pH-independent fusion of the viral envelope with the plasma membrane of the host cell. The assignment of specific biologic activities to individual glycoproteins was originally made on the basis of purification and reconstitution studies, mainly for the Sendai virus and SV5 proteins (248,250). For the respiroviruses and rubulaviruses, the attachment glycoprotein binds to cellular sialic acid–containing receptors, and these can be glycoproteins or glycolipids. The binding is probably of fairly low affinity but of sufficiently high avidity that these viruses agglutinate erythrocytes (hemagglutination). The attachment proteins of respiroviruses and rubulaviruses also have neuraminidase activity, and the proteins have been designated hemagglutinin-neuraminidase (HN). The Morbillivirus attachment protein (H) can cause agglutination of primate erythrocytes but lacks detectable neuraminidase or esterase activity. The restricted host range of measles virus for primate cells made it unlikely that sialic acid was the primary receptor for measles virus. It has been found that measles virus has a specific cellular receptor, the complement-binding protein known as either the membrane cofactor protein (MCP) or CD46 (74,201). The Pneumovirus RSV does not cause detectable hemagglutination, and the cellular receptor for RSV is not completely understood but it involves interactions with heparan sulfate, a glucosaminoglycan that is part of the extracellular matrix. Interestingly, the G protein of RSV can be deleted from the viral genome (146), and Sendai virus-like particles devoid of HN can infect cells via the asialoglycoprotein receptor (173). Both of these cases suggest the F protein may have a binding activity. After attachment of a *Paramyxoviridae* particle to the host-cell receptor, the viral envelope fuses with the host-cell plasma membrane, and the major viral protein involved in this process is the F glycoprotein.

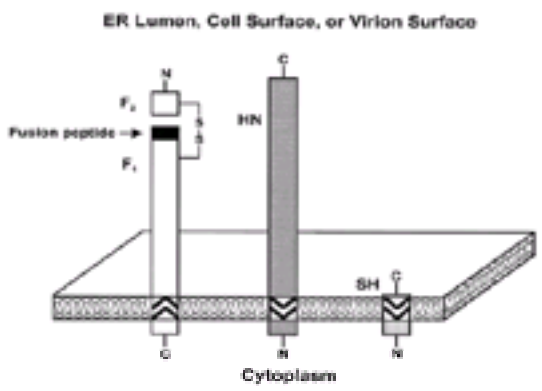

FIG. 12. Schematic diagram showing the orientation of Paramyxovirus integral membrane proteins. The F and HN protein orientations are shown. In *Paramyxovirnae*, only SV5 and mumps have an SH gene.

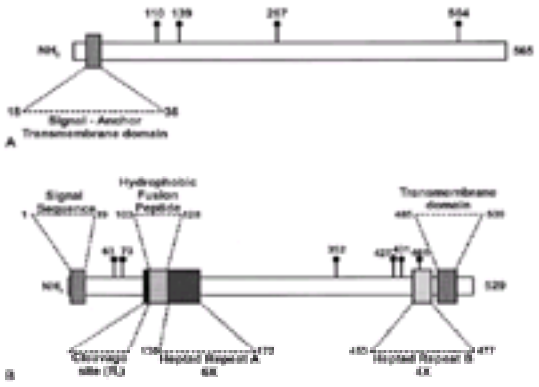

FIG. 13. Schematic diagram to show important domains and features of the Paramyxovirus glycoproteins. **A:** Hemagglutinin-neuraminidase attachment protein (based on the predicted sequence of the SV5 HN gene (121). The signal anchor transmembrane domain and the sites used for addition of N-linked carbohydrate (lollipops) (202) are indicated. **B:** Fusion protein (based on the predicted sequence of the SV5 F gene). The position of the signal sequence, the transmembrane domain, the cleavage site, the hydrophobic fusion peptide, and the heptad repeats A and B are indicated. The sites used for addition of N-linked carbohydrate (lollipops) (7) are indicated. R5 indicates the five arginine residues site for cleavage-activation. The heptad repeats A (six heptads) and B (four heptads) are shown.

Attachment Protein

The Respirovirus and Rubulavirus surface glycoprotein HN is a multifunctional protein as well as being the major antigenic determinant of the paramyxoviruses. HN is responsible for the adsorption of the virus to sialic acid–containing cell-surface molecules. In addition, HN mediates enzymatic cleavage of sialic acid (neuraminidase activity) from the surface of virions and the surface of infected cells. By analogy to the role of influenza virus neuraminidase (NA), it seems likely that the role of this neuraminidase activity is to prevent self-aggregation of viral particles during budding at the plasma membrane. These dual activities of HN can be modulated by halide ion concentration and pH (186). Whereas the halide ion concentration and pH of the extracellular environment is optimal for hemagglutination, Paramyxovirus neuraminidases have an acidic pH optimum (pH 4.8 to 5.5) suggesting that neuraminidase acts in the acidic *trans* Golgi network to remove sialic acid from the HN carbohydrate chains and from the F protein carbohydrate chains. In addition to the hemagglutinating and neuraminidase activities of HN, for most paramyxoviruses HN also has a fusion promoting activity—that is, coexpression of HN and F is required for cell–cell fusion to be observed (see later).

The nucleotide sequence of the HN gene is now known for every member of the *Paramyxovirinae*. The HN polypeptide chain ranges from 565 to 582 residues. For some strains of NDV, HN is synthesized as a biologically inactive precursor (HN_0), and 90 residues from the C-terminus are removed to activate the molecule (199,200). A schematic diagram of important features of the HN amino acid sequence is shown in Figure 13. The HN proteins are type II integral membrane proteins that span the membrane once. The HN protein contains a single hydrophobic domain, located close to the N-terminus, that acts as a combined signal and anchorage domain, targeting the nascent chain as it emerges from the ribosome to the membrane of the endoplasmic reticulum (ER) and ensuring the translocation of the polypeptide chain across the membrane, bringing about the stable anchoring of the protein in the lipid bilayer. This orientation of HN (see Fig. 12) is analogous to that of the influenza virus NA. Interestingly, all of the cellular enzymes that are resident in compartments of the exocytic pathway and that use carbohydrates as substrates are type II integral membrane proteins, suggesting evolution of all carbohydrate-modifying enzymes from a common progenitor.

The HN glycoproteins contain from four to six sites for the addition of N-linked carbohydrate chains. For the SV5 HN molecule, it has been shown that four sites are used (202). The Paramyxovirus HN glycoproteins have conserved cysteine, proline, and glycine residues between related paramyxoviruses, strongly suggesting a similarity of protein structure. HN forms an oligomer consisting of disulfide-linked homodimers that form a noncovalently linked tetramer. The oligomeric form of HN has been examined in detail by using bifunctional cross-linking reagents and sucrose density gradient analysis (179,193,203,237,275). Sendai virus HN molecules that were proteolytically removed from the surface of virions (at residue 131) retain enzymatic activity and with EM appeared as box-shaped molecules (10 × 10 nm) consisting of four subunits with fourfold symmetry (275). For the Paramyxovirus HN protein, the type II membrane orientation, the presence of a protease-accessible stalk that is not essential for enzymatic activity, and the existence of a tetrameric oligomeric form all parallel influenza virus NA. In addition, Paramyxovirus HN proteins contain a conserved sequence N-R-K-S-K-S that is similar to the known sialic acid binding site of influenza virus NA (280; reviewed in ref. 192). Computer modelling studies predicted that HN will have a b-sheet propeller motif similar to NA of influenza virus (50,166), and the recently obtained atomic structure of NDV HN bears out these predictions (54) (Fig. 14).

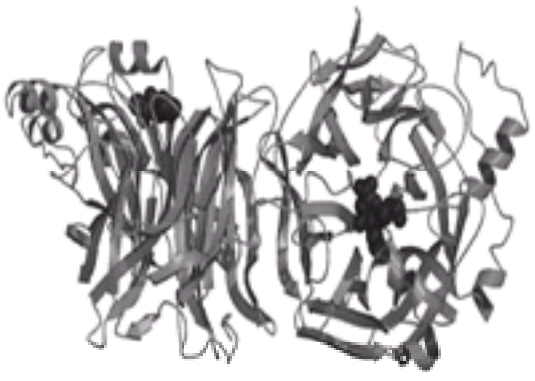

FIG. 14. The hemagglutinin-neuraminidase three-dimensional structure. Protease released, purified NDV HN was crystallized in the presence of the general sialidase/neuraminidase inhibitor 2-deoxy-2,3-dehydro-N-acetyl neuraminic acid (Neu5ac2n, or DANA). In the crystal, the molecule exists as a dimer whose parts are arranged such that the active sites are roughly at 90° to one another. The picture therefore shows a top and side view of each other monomer. The fold of each monomer is the characteristic b-propeller found in other neuraminidases, such as that of influenza virus. DANA is shown bound to the molecule. Structural studies have shown that the sialic acid binding (hemagglutinin) and sialic acid hydrolysis (neuraminidase) sites occupy the same location on the molecule. A structural difference was observed in HN crystallized alone or in complex with the receptor, suggesting a possible mechanism for transmission of a conformational change upon sialic acid binding that could trigger fusion through interactions of HN with the fusion protein. (Figure of the three-dimensional structure of the NDV HN provided by Susan Crennell, Toru Takimoto, Allen Portner, and Garry Taylor.)

For years, it was a matter of debate as to whether HN molecules contain combined or separate active sites for hemagglutinating and neuraminidase activities. Some evidence has been interpreted to indicate a single site that binds sialic acid tightly (hemagglutination) but hydrolyses the molecule slowly (neuraminidase) (249), whereas studies with temperature-sensitive mutants and monoclonal antibody inhibition of activities had been interpreted to suggest there are separate sites for the two activities (223,225). However, analysis of the atomic structure of the NDV HN protein suggests the presence of only a single sialic binding site (54).

The SV5 HN protein, upon being transported to the cell surface, is rapidly internalized by the clathrin-mediated endocytosis pathway (171). The signal for internalization is unusual as it is not located in the cytoplasmic tail but rather at the boundary of the transmembrane (TM) domain and the ectodomain (172). The reason for the internalization of SV5 HN, a process seemingly in conflict with virus assembly, is not known.

The Morbillivirus attachment protein (H) has extensive sequence relatedness and similarity with the HN glycoprotein of respiroviruses and rubulaviruses (reviewed in ref. 192). However, as discussed previously, the Morbillivirus H glycoprotein lacks detectable neuraminidase activity. Measles virus infection is restricted to cells derived from higher primates, and this observation eventually led to identification of the measles virus cellular receptor molecule as CD46, a complement cofactor (74,201), The measles virus receptor CD46 is a low-abundance cell surface protein, and in measles virus– infected cells the expression of CD46 is down-regulated. Therefore, CD46 is unlikely to cause virus aggregation during virus budding. Thus, measles virus, and morbilliviruses in general, may not need a neuraminidase activity in the manner in which other paramyxoviruses need a neuraminidase activity to act as a receptor-destroying enzyme to free the virus from the cell surface. The receptor for CDV and other morbilliviruses is not yet known.

The structure of the Pneumovirus attachment protein (G) is very different from the attachment protein of the *Paramyxovirinae*. The RSV G protein has neither hemagglutinating nor neuraminidase activity. The nucleotide sequence of the RSV G gene predicts that the protein is of about 289 to 299 amino acids (M_r 32,587), and it is a type II integral membrane protein with a single N-terminal hydrophobic signal/anchor domain (247,289). The G protein is found in virus-infected cells in both membrane-bound and proteolytically cleaved soluble forms. The distinguishing feature of the RSV G protein is the extent of its carbohydrate modification. On SDS polyacrylamide gel electrophoresis, the protein migrates with an apparent M_r of about 84,000 to 90,000, and the dramatic increase in molecular weight over that predicted for the polypeptide chain is because 8 to 12 kd is caused by the addition of N-linked carbohydrate (four potential addition sites) and 40 to 50 kd is caused by the addition of O-linked glycosylation (77 potential acceptor serine or threonine residues; 30% of total residues) (44,290, and references therein). Quite remarkably, it appears the RSV G protein is not essential for virus assembly or growth in tissue culture or animals, but it does confer a growth advantage. A virus that had been extensively passaged in cells was found to contain a spontaneous deletion of the G and SH genes (146), yet the virus replicated in Vero cells. In addition, the G gene has been deleted from recombinant virus recovered from an infectious cDNA clone (see Chapter 45). These finding suggest that RSV has an alternative mechanism for attachment to cells that does not involve G protein.

Fusion Protein

The Paramyxovirus fusion (F) proteins mediate viral penetration by fusion between the virion envelope and the host cell plasma membrane, and this fusion event occurs at neutral pH. The consequence of the fusion reaction is that the nucleocapsid is delivered to the cytoplasm. Later in infection, the F proteins expressed at the plasma membrane of infected cells can mediate fusion with neighboring cells to form syncytia (giant cell formation), a cytopathic effect that can lead to tissue necrosis *in vivo* and might also be a mechanism of virus spread.

Paramyxovirus fusion proteins are synthesized as an inactive precursor (F_0) that is cleaved by a host-cell protease. This releases the new N-terminus of F_1, thus forming the biologically active protein consisting of the disulfide-linked chains F_1 and F_2 (128,250). The Paramyxovirus F genes encode 540 to 580 residues (see Fig. 13). The F proteins are type I integral membrane proteins, which span the membrane once and contain at their N-terminus a cleavable signal sequence

that targets the nascent polypeptide chain synthesis to the membrane of the ER. At their C-termini, a hydrophobic stop-transfer domain (a TM domain) anchors the protein in the membrane, leaving a short cytoplasmic tail (about 20 to 40 residues). Comparison of the amino acid sequences of Paramyxovirus F proteins (reviewed in ref. 192) does not show overall major regions of sequence identity, but the similar placement of cysteine, glycine, and proline residues together with the overall hydrophobicity of the F proteins suggests a similar structure for all F proteins. The Respirovirus and Rubulavirus F_2 and F_1 subunits are glycosylated and there are a total of three to six potential sites for the addition of N-linked carbohydrate. For SV5 F protein, it is known that all four potential sites for addition of N-linked carbohydrate are used (7). The Morbillivirus F protein contains three sites in the F_2 subunit for N-linked carbohydrate addition, and all three sites are used, and there are no sites in F_1 for N-linked carbohydrate addition (2).

Protein sequencing studies of the F protein and nucleotide sequencing studies of the F genes have indicated that the N-terminal 25 residues of F_1 (fusion peptide) are extensively hydrophobic and this region of the F protein is highly conserved among F proteins of the *Paramyxovirinae* (up to 90% identity) (Fig. 15). The Paramyxovirus fusion peptides are thought to intercalate into target membranes, initiating the fusion process, and evidence for direct insertion into bilayers has been obtained using hydrophobic photoaffinity labeling probes (204). It has also been shown that the fusion peptides are sufficiently hydrophobic that they can act as a TM anchor domain to convert a formerly soluble protein to a membrane-bound form (210). The invariant nature of many of the residues of the fusion peptide between Paramyxovirus F proteins suggests a role more complex than preservation of hydrophobic residues required for a membrane-intercalating domain; neither signal sequences nor membrane anchorage domains show sequence conservation beyond their hydrophobic nature. It has been noted that if the fusion peptide is assumed to be an a-helix, then the invariant residues are located on one face of the helix (255). Interestingly, in a study on the conserved residues of the fusion peptide when the glycine residues at position 3, 7, or 12 were changed to alanine, and the altered F proteins expressed, a dramatic increase in syncytium formation was observed (130). Thus, the invariant amino acids in the fusion peptide may preserve a balance between high fusion activity and successful viral replication, as high fusion activity is deleterious to cell viability.

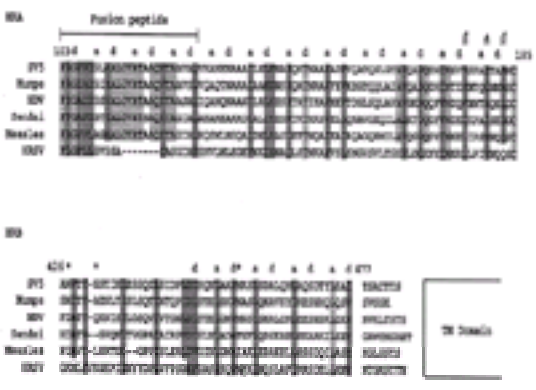

FIG. 15. Sequence alignment of the fusion peptide and the heptad repeats HRA and HRB of the SV5 F protein with that of other paramyxoviruses. Conserved residues among members of the *Paramyxovirinae* (i.e., not including hRSV) are *shaded light gray* and residues with similar properties (hydrophobic or charged) are *shaded dark gray.* Those residues that are also conserved in HRSV, which is evolutionarily distant from other paramyxoviruses, are indicated in the HRSV sequence with the appropriate shading. Letters immediately above the sequence indicate the predicted hydrophobic heptad repeat a and *d* residues. The predicted heptad is incorrect for residues 175 to 184, and the observed *a* and *d* residues in the atomic structure (see Fig. 16) are indicated above the predicted repeat and underlined. *Asterisks* indicate N-linked carbohydrate sites for the SV5 F protein. The SV5 sequence is numbered. NDV, Newcastle disease virus; HRSV, human respiratory syncytial virus. (Adapted from ref. 8, with permission.).

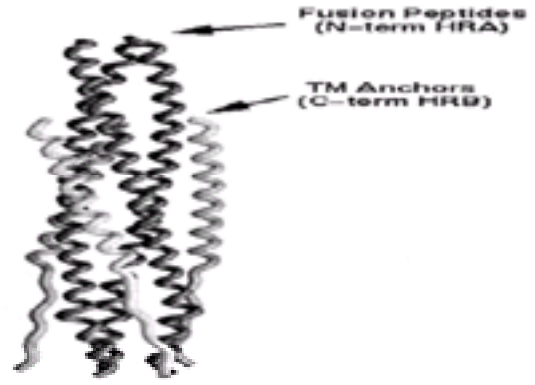

FIG. 16. The SV5 F1 core trimer structure. The complete SV5 F1 core trimer is shown. The central HRA (N-1) trimeric coiled coil is shown in *dark gray* and the buttressing antiparallel HRB (C-1) helices are shown in *light gray.* (Adapted from ref. 8, with permission.)

The fusion proteins of several widely disparate viruses share several important common features. The Paramyxovirus F protein, the human and simian immunodeficiency virus (HIV and SIV) gp160, Ebola virus GP, and influenza virus hemagglutinin (HA) protein all form homotrimers (26,39,85,237,296) that must be proteolytically cleaved to be biologically active (144,153,250). The resulting TM domain–containing subunits (Paramyxovirus F, F_1; HIV gp160, gp41; Ebola GP, GP2; influenza HA, HA_2), contain a hydrophobic fusion peptide at the new N-termini, which has been shown to insert into the target membrane during the fusion process (4,65,104,118). Additionally, two 4-3 heptad repeat regions are present in

each of these fusion proteins, one near the fusion peptide and one in close proximity to the TM domain (20,38). These regions are thought to be important in the fusion reaction, as mutations within these domains frequently lead to a fusion-deficient phenotype (29,42,240,254), and peptides corresponding to these regions have been demonstrated to block the fusion process (139,142,165,177,196,229,293,294,297,300).

The atomic structure of uncleaved and cleaved influenza virus HA are known (40,296). However, this is not the case for the intact glycoproteins HIV-1/SIV gp160, Ebola virus GP, or the Paramyxovirus F protein. However, biochemical, EM, nuclear magnetic resonance, and x-ray crystallographic analyses of most of HA_2 (22), portions of HIV-1/SIV gp41 (12,25,39,177,264,286,287), a portion of a retrovirus Env-TM domain (85), and most of Ebola virus GP2 (178,285) indicate that their heptad repeat regions show considerable similarity in that they all form trimeric coiled coils, with the N-terminal heptad repeat forming an interior, trimeric coiled coil surrounded by three antiparallel helices. This structure has been suggested to represent the final, most stable form of the protein, present either during or subsequent to membrane fusion, with peptide inhibitors most likely functioning by preventing formation of this core (12,177).

The Paramyxovirus F protein membrane proximal subunit (F_1) differs from that of HA_2, gp41, Env-TM, and GP2 because its ectodomain is much larger in size (383 residues versus 120 to 185 residues). F_1 contains two heptad repeat regions, one (HRA) adjacent to the fusion peptide and the other (HRB) adjacent to the TM domain (see Fig. 13). In contrast to the HA_2, gp41, Env-TM, and GP2 just described, where heptad repeats are separated only by small spacer regions, in paramyxoviruses about 250 residues separate the heptad repeat regions. Nonetheless, biochemical and EM analysis indicated that the Paramyxovirus heptad repeat peptides form a rod-shaped structure approximately 96 Å in length. Peptides (N-1) derived from HRA form a three-stranded a-helical coiled coil around which three peptides (C-1) derived from HRB are packed in an antiparallel manner. The N-terminus of N-1 and the C-terminus of C-1 are localized to the same end of the rod, placing the fusion peptides and TM domains close together (77,142). These biochemical data were confirmed by solving the crystal structure of the SV5 F_1 N-1/C-1 complex to 1.4 Å resolution (8). The 64 amino acids of N-1 fold into an 18-turn a-helix stretching the entire length of the coiled coil. The C-terminal 25 residues of C-1 form a seven-turn amphipathic a-helix, whereas amino acids 440 to 450 form an extended chain (Fig. 16 and Fig. 17).

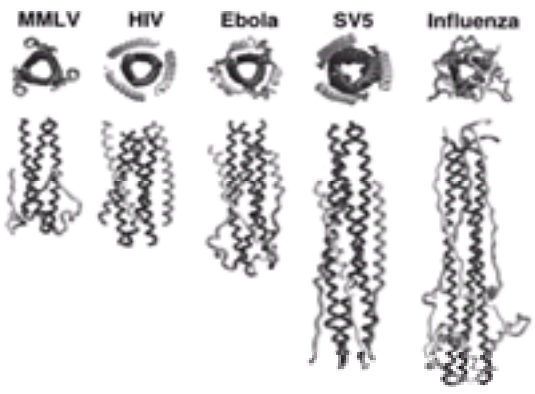

FIG. 17. Comparison of the SV5 F1 core trimer to other viral fusion protein structures. The proteins under comparison include the low-pH-induced influenza virus HA, $tBHA_2$, (22,41), HIV gp41 (39), murine Moloney leukemia virus Env-TM protein (85), and Ebola virus GP2 (285). The interior coiled coil is *dark gray* and the exterior polypeptide is *light gray.* Top and side views are shown for each molecule. (Adapted from ref. 8, with permission.)

Despite the similarity of core structure among these viral fusion proteins, there are important differences between the Paramyxovirus F proteins and other viral fusion proteins. Unlike influenza virus HA protein and HIV gp160, the Paramyxovirus F protein does not provide the primary binding role for the virus: Paramyxovirus primary binding to a target cell is mediated by the HN glycoprotein. However, as mentioned before, at least two *Paramyxoviridae* F proteins, those of Sendai and RSVs, appear to have attachment activity.

A large body of evidence suggests that viral fusion proteins undergo a conformational change to become fusion active (reviewed in ref. 117). For influenza virus HA, the cleaved HA (296) exists in a metastable conformation, trapped from achieving its lower-energy fusogenic conformation by a kinetic barrier. During virus uncoating in endosomes, the low pH environment of the endosomal lumen triggers a conformational change in HA, in which two separate a-helical regions in HA_2 (296) rearrange to form a single triple-stranded coiled coil (22,41). After this conformational change, the fusion peptide is relocated by about 100 Å to one end of the rod-shaped coiled coil (22): the basis of the “spring-loaded mechanism” of fusion (30). For HIV-1 gp120/gp41 and retrovirus Env-SU+Env-TM, binding to the viral receptor may induce the conformational change to release the fusion peptide (65,91,189). The trigger for the putative conformational changes of Paramyxovirus F proteins is unknown, although as described later for many paramyxoviruses there is a requirement for HN in mediating the fusion reaction (reviewed in ref. 161).

Although the Paramyxovirus F protein is the principal viral glycoprotein involved in virus–cell and cell–cell fusion, the emerging picture indicates the presence of a complex biologic machine. The SV5, measles virus, or RSV F cDNAs when expressed in mammalian cells cause syncytium formation (1,2,131,143,206,209,214), although it is important to note it is likely that many more cells express the F protein than are found in multinucleated cells (209). However, for many other paramyxoviruses including NDV, hPIV-2, hPIV-3, bPIV-3, mumps virus, and CDV (37,78,131,135,191,242,271,295,298) coexpression of F and HN (H for the morbilliviruses) is required for syncytium formation. Furthermore, there is a requirement that the homologous (i.e., of the same virus) HN, and not a heterologous HN, be coexpressed in the same cell (131,135,253). Thus, it has been suggested that a type-specific interaction occurs between the HN and F protein that is necessary for fusion to occur (135,161,253). A great deal of effort has been spent to map the regions of F and HN that interact. Mutations have been identified in the HN globular domain (68,188), stalk region (253,260,302), and TM anchor

(17,185) that decrease or abolish fusogenic activity with no effect on receptor recognition. Analysis of the fusion-promoting activity of chimeric HN molecules derived from different paramyxoviruses suggests that both the TM domain (with much of the stalk region) and parts of the globular head impart F specificity (70,265,278). Coimmunoprecipitation assays also indicate that F and HN can exist in a complex (259,298), and that for hPIV-3, F and HN undergo antibody-induced co-capping indicative of protein complex formation (298). Perhaps most intriguingly, a point mutation was found in the NDV HN globular head that abolishes both its receptor recognition and neuraminidase activity, and that also abolishes its ability to interact with F in coimmunoprecipitation assays (69). These latter data suggest that HN interacts with F only after binding its receptor.

A model that would rationalize the involvement of HN (or H) in fusion promotion is one in which the hypothesized conformational change in F to release the fusion peptide is highly regulated. For those *Paramyxovirinae* that require HN (or H) and F to be coexpressed to observe fusion, the first step would be the binding of HN (or H) to its receptor. On binding ligand, the HN (or H) protein would undergo its own conformational change, which in turn could trigger a conformational change in F to release the fusion peptide. In this way, F and HN operate as a coupled molecular scaffold to release and direct the fusion peptide to the target membrane (reviewed in ref. 161). Examination of mutants of SV5 F protein that require coexpression of HN to mediate fusion indicates that the HN function for F protein triggering can be supplanted by providing energy from raising the temperature, suggesting that the F–HN interaction provides energy to convert cleaved F from a metastable form to a fusogenic form (213).

Cleavage Activation

As discussed, the precursor F_0 molecule is biologically inactive and cleavage of F_0 to the disulfide linked subunits F_1 and F_2 activates the protein, rendering the molecule fusion-active and permitting viral infectivity. Thus, cleavage of F_0 is a candidate to be a key determinant for infectivity and pathogenicity, and for certain viruses this appears to be the case. Proteolytic activation of F_0 involves the sequential action of two enzymes, the host protease that cleaves at the carboxyl side of an arginine residue, and a host carboxypeptidase that removes the basic residues. The *Paramyxoviridae* can be divided into two groups: those that have F proteins with multiple basic residues at the cleavage site and those with F proteins that have a single basic residue at the cleavage site (Table 3). Cleavage of F proteins containing multiple basic residues at the cleavage site occurs intracellularly during transport of the protein through the *trans* Golgi network.

Sendai virus	G - V - P - Q - S - R ↓
hPIV1	D - N - P - Q - S - R ↓
hPIV3	D - P - **R** - T - **K** - **R** ↓
SV5	T - **R** - **R** - R - **R** - **R** ↓
Mumps	S - R - **R** - H - **K** - **R** ↓
NDV (virulent strain)	G - R - **R** - Q - **R/K** - **R** ↓
NDV (avirulent strain)	G/E - G - K/R - Q - G/S - R ↓
Measles	S - R - **R** - H - **K** - **R** ↓
RS V	K - K - **R** - K - **R** - **R** ↓

Consensus sequence for furin protease cleavage is

R - X - **R/K** - **R** ↓

Data from Hosaka et al. (125).

TABLE 3. *Amino acid sequences upstream of the F protein cleavage site of some members of the* Paramyxoviridae

Furin is a cellular protease localized to the *trans* Golgi network, and its sequence specificity for cleavage is R-X-K/R-R. The available evidence suggests that furin, a subtilisin-like endoprotease, is the (or one of the) protease(s) that cleaves F proteins intracellularly (207; reviewed in ref. 153).

Paramyxoviruses that have F proteins with single basic residues in the cleavage site (e.g., Sendai virus) are not usually cleaved when grown in tissue culture and, thus, only a single cycle of growth is obtained. However, the F_0 precursor that is expressed at the cell surface and incorporated into released virions can be cleavage-activated by the addition of exogenous protease (250), leading to multiple rounds of replication. Purification of a protease from the allantoic fluid of embryonated chicken eggs has indicated that the endoprotease responsible for Sendai virus activation is homologous to the blood clotting factor Xa, a member of the prothrombin family (100,102). A protease with a similar substrate specificity is secreted from Clara cells of the bronchial epithelium in rats and mice, and this enzyme is probably responsible for activating paramyxoviruses in the respiratory tract. For NDV, the nature of the cleavage site correlates with virulence of the virus. Those strains with multiple basic residues in the F_0 cleavage site are virulent and readily disseminate through the host, whereas those strains with F_0 molecules having single basic residues are avirulent and tend to be restricted to the respiratory tract where the necessary secreted protease can be found (199).

Other Envelope Proteins

The rubulaviruses SV5 and mumps virus both contain a small gene located between F and HN, designated SH (122,123). The SV5 SH protein is a 44-residue, type II integral membrane protein that is expressed at the plasma membrane and is packaged in virions (see Fig. 12). The functional role of SH in the replicative cycle of SV5 is unknown, and in tissue culture cells it can be deleted from an infectious cDNA and recombinant SV5 recovered (111). For mumps virus, although an mRNA transcript derived from the SH gene has been detected (81,82), identification of the SH protein was accomplished only recently (262).

Members of the *Pneumovirinae* also encode an SH protein. The RSV SH protein contains 64 amino acids and is expressed at the plasma membrane of RSV-infected cells as a type II integral membrane protein, and it is packaged in

virions (49,205). In RSV-infected cells, four SH-related polypeptide species have been identified: The M_r 4,800 species is thought to result from the initiation of protein synthesis at an internal AUG codon; the M_r 7,500 species is unglycosylated SH; the M_r 13,000 to 15,000 species is SH containing one high mannose N-linked carbohydrate chain; and the M_r 21,000 to 30,000 species is generated by the addition of polylactosaminoglycan to the N-linked carbohydrate chain (3,205). The SH gene was found to be deleted spontaneously from a virus passaged extensively *in vitro* (146), and it has been deleted from recombinant RSV (21) with only minor alterations in virus growth properties in tissue culture cells, or in the respiratory tract of mice or chimpanzees. Thus, the role of the SH protein in the RSV life cycle is not understood.

Pneumovirus M2 Gene

The RSV M2 gene contains two partially overlapping ORFs, designated M2-1 and M2-2, which give rise to two proteins, M2-1 (194 amino acids) and M2-2 (90 amino acids), respectively (47). The mechanism for translating the M2-2 ORF is not clear, but it may involve a ribosomal stop-restart mechanism analogous to that used for synthesis of the influenza B virus BM2 protein (132) (see Chapter 46). The M2-1 protein is an essential transcriptional elongation factor (45,87), and in its absence the polymerase does not transcribe beyond the NS1 and NS2 genes (87). The M2-1 gene also increases RNAP processivity across the gene junctions, attenuating transcriptional termination (87,106,107). The M2-2 gene is not essential for RSV growth, as it can be deleted from a recombinant RSV (11,140). However, the DM2-2 virus grows slowly in tissue culture and there is an increase in transcription and decrease in RNA replication (11,140), suggesting that M2-2 protein is involved in regulating transcription and RNA replication.

Pneumovirus NS1 and NS2 Genes

The RSV NS1 (139 amino acids) and NS2 (124 amino acids) are considered to be nonstructural proteins, although the difficulty in purifying virions from contaminating infected-cell debris for this poorly growing virus makes this assignment provisional. Neither protein is thought to be essential for virus growth in cultured cells or in chimpanzees, as the genes can be deleted from a recombinant RSV, but growth *in vitro* and *in vivo* is reduced substantially (19,273,292). In a minireplicon system, when NS1 was expressed, it was inhibitory to both transcription and replication (5), and expression of NS2 at high levels had a small inhibitory effect on transcription and replication (272). Thus, the role of these accessory proteins in the RSV life cycle remains to be fully understood.

STAGES OF REPLICATION

As far as is known, all aspects of the replication of *Paramyxoviridae* take place in the cytoplasm. An overview of the life cycle of the virus is shown schematically in Figure 18, and a diagram indicating the differences between transcription and replication is shown in Figure 19. Unlike in influenza viruses, mRNA synthesis in *Paramyxoviridae* is insensitive to DNA-intercalating drugs such as actinomycin D (reviewed in ref. 43), and the *Paramyxoviridae* can replicate in enucleated cells (221). In cell culture, single-cycle growth curves are generally of 14 to 30 hours duration, but they can be as short as 10 hours for virulent strains of NDV. The effect of viral replication on host macromolecular synthesis is quite variable, ranging from almost complete shutoff late in infection for NDV, to no obvious effect with SV5.

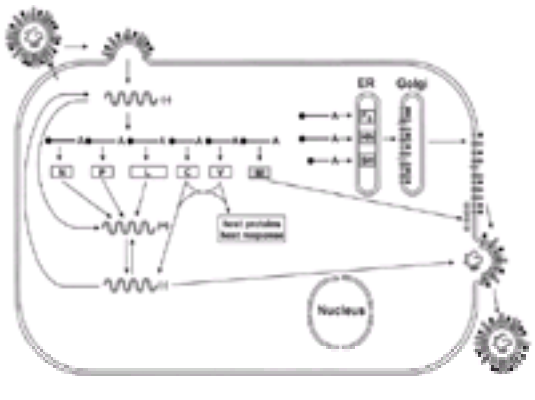

FIG. 18. Schematic representation of the life cycle of a Paramyxovirus. See text for details of the viral life cycle. The gradient of decreasing molar abundance of the mRNAs from N to L resulting from polar transcription is not illustrated [the 5' mRNA cap is indicated with a *filled circle* and 3' poly(A) tail by *(A)*]. Also not illustrated are the relative abundances of genomic nucleocapsid and antigenomic nucleocapsid (indicated with *wavy gray lines*, – and +, respectively). Secondary transcription by newly made genomic nucleocapsids also is not illustrated.

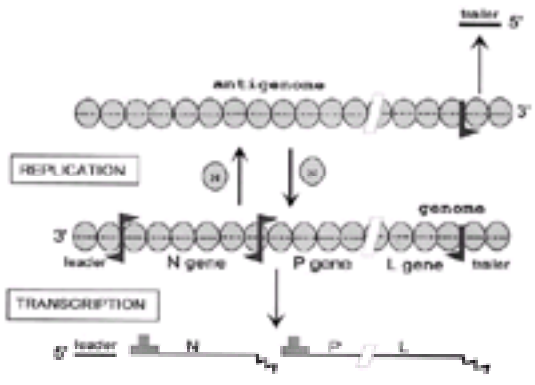

FIG. 19. Paramyxovirus RNA synthesis. Viral nucleocapsids, the templates for RNA synthesis, are shown as a linear array of N subunits (*ovals*), with *short vertical lines* and *arrowheads* indicating the gene junctions. The viral polymerase (P-L) transcribes the genome template, starting at its 3' end, to generate the (+) leader RNA and the successive capped

(*boxes*) and polyadenylated (*squiggly line*) mRNAs, by stopping and restarting at each junction. Once these primary transcripts have generated sufficient viral proteins, unassembled N (as a P-N complex) begins to assemble the nascent leader chain, and the coordinate assembly and synthesis of the RNA causes the polymerase to ignore the junctions, yielding the antigenome nucleocapsid. In this capacity, P acts as a chaperone to deliver N to the nascent RNA. The P-L polymerase can also initiate RNA synthesis at the 3' end of the antigenome in the absence of sufficient P-N, but only a (–) leader RNA (representing the 5' trailer of genome RNA) is made in this case. Note that genomic and antigenomic RNAs never appear as naked RNAs.

Virus Adsorption and Entry

For the respiroviruses and rubulaviruses, it has long been accepted that molecules containing sialic acid (sialoglycoconjugates) serve as cell surface receptors. This is based on the fact that sialidase of *Vibrio cholerae* acted as a “receptor-destroying enzyme” and protected the host cell from infection (reviewed in ref. 181). Sialic acid, the acyl derivative of neuraminic acid, is found both on glycoproteins and on lipids (sialoglycolipids or gangliosides). For Sendai virus, gangliosides function as both the attachment factor and the receptor for the virus (180,182,183). As described, the cellular receptor for the Morbillivirus measles virus is the cell surface protein CD46, and the cellular receptor for pneumoviruses, although not proven, seems to involve binding to glycosaminoglycans containing the disaccharide heparan sulfate and chondroitin sulfate B (88). Upon adsorption of the virus to the cellular receptor, the viral membrane fuses with the cellular plasma membrane at the neutral pH found at the cell surface, the consequence of which is the release into the cytoplasm of the helical nucleocapsids.

In the virus particle, the M protein shell is thought to make numerous contacts with the nucleocapsid. Upon fusion of the viral envelope with the cell plasma membrane and release of the nucleocapsid into the cytoplasm, a mechanism needs to exist to disrupt the M-N contacts. With influenza A virus, the factor that alters the equilibrium between self-assembly and disassembly is thought to be the difference in pH between the acidic uncoating compartment (endosomes) and the assembly site (plasma membrane). The driving force for Paramyxovirus uncoating is not known.

Viral RNA Synthesis

Viral Transcription (mRNA Synthesis)

All viral RNA synthesis begins at the very 3' end of the genome, and the cis-acting promoter sequences serve the dual function of initiating leader RNAs and antigenomes. The vRNAP for mRNA synthesis first has to transcribe the leader RNA before beginning mRNA synthesis at the N gene start signal (see Fig. 19). The recent discovery of two unexpected properties of the *Paramyxovirinae* has provided information on how vRNAP interacts with the genome 3' end to initiate RNA synthesis.

Requirement for Hexamer Genome Length for Efficient Genome Replication: The “Rule of Six”

Efficient replication of model mini-genomes in transfected cells requires that their total length be a multiple of six (Fig. 20) (27). For every Respirovirus or Rubulavirus examined to date using a mini-genome replication assay, RNA replication has been found to be much more efficient when the artificial genome was designed to be a multiple of six nucleotides. Out of 11 complete *Paramyxovirinae* genomic sequences determined to date, six were multiples of six, and it is likely that the deviation in the others will be found to reflect sequencing errors. Inefficient replication of non-hexamer-length genomes was not a result of the lack of encapsidation of the cDNA-expressed mini-genome but of the inability of vRNAP to initiate at the 3' end of these nucleocapsids. This hexamer rule is most likely related to the finding that each N subunit of the nucleocapsid is associated with precisely six nucleotides (79). The association of N monomers with nucleotide hexamers presumably begins with the first nucleotide at the 5' end of the nascent chain, and it continues by assembling six nucleotides at a time until the 3' end is reached, maintaining a precise hexamer arrangement (see Fig. 20). The efficiency of the 3'-end promoter then presumably depends on the position of the cis-acting sequences relative to the N subunits, and this is determined by the total number of nucleotides in the genome chain.

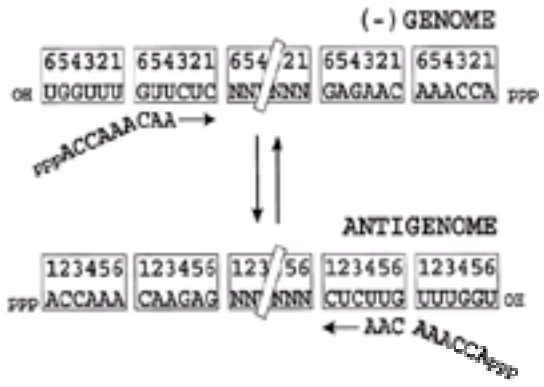

FIG. 20. Rule of six. A schematic representation of the rule of six for replicating the Sendai virus genome. Each *box* represents a single N-protein monomer associated with six nucleotides. ppp, 5' end; OH, 3' end.

Bipartite Nature of the Replication Promoter

The 3'-terminal 12 nucleotides of the genomes and antigenomes of each genus of the *Paramyxovirinae* are identical. This conservation at the termini highlights these two hexamers as a critical element of the replication promoter. The *Pneumovirinae* do not follow an integer rule (244); nevertheless, the extent of identity at the 3' termini of genomes and antigenomes is similar (187,228). For RSV, deletional and saturation mutagenesis has confirmed that the minimal genomic promoter is contained within the terminal 12 to 15 nucleotides of the 44-nucleotide-long leader region, although additional sequences downstream also enhanced RNA synthesis (see Chapter 45). For the *Paramyxovirinae*, it has not been possible to examine the limits of the replication promoters by deletion analysis (see later). Instead, sequence exchanges were made between the genomic and antigenomic promoters, which determines the relative replication efficiency of model mini-genomes (28). In this way, sequences that extended 30 nucleotides from the 3' end were found to affect Sendai virus replication efficiency.

Although sequences beyond the first 30 nucleotides of the Sendai virus genomic and antigenomic promoters could be exchanged freely (269), the deletion of any nucleotides downstream of the promoters, even if the hexamer rule was not violated, was highly deleterious (220,268). Similar results were found at the antigenomic promoter of SV5, where again sequence exchanges, but not deletions, downstream of the conserved 3'-end element were permitted (197). This suggested that a second promoter element lay downstream of the end element, and that the spacing between the two elements was critical for promoter function. This internal promoter element has been identified for both Sendai virus (268) and SV5 (197,198), and it consists of a single template C, or a 3'-GC dinucleotide, respectively, repeated three times with an internal spacing of precisely six nucleotides in the 13th, 14th, and 15th hexamer from the 3' end. The position of the two promoter elements is shown in Figure 21 in the context of the proposed structure of the Sendai virus nucleocapsid. As each turn of the nucleocapsid helix contains 13 N subunits, each associated with precisely six nucleotides (79), the terminal and internal promoter elements are aligned on the same face of the helix. Both promoter elements can then simultaneously interact with vRNAP to initiate RNA synthesis at the 3' end of the nucleocapsid. Morbilliviruses contain the same internal promoter element as respiroviruses, and all the members of the *Paramyxovirinae* probably contain bipartite replication promoters. In contrast, pneumoviruses do not appear to contain bipartite promoters, as RSV-based mini-genomes require only about 40 nucleotides from each genome end for efficient replication (see Chapter 45).

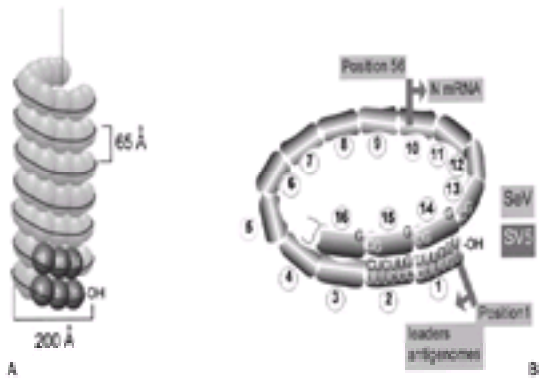

FIG. 21. Nucleocapsid structure, hexamer phasing of the nucleotide sequence, and the bipartite replication promoters of the *Paramyxovirinae*. **A:** A model of the Sendai virus nucleocapsid as an assembly of single N-protein subunits (*shaded spheres*), in the form of a left-handed helix with 13 subunits per turn, a pitch of 65 Å, and a hollow central core of 50 Å in diameter. The numbers refer to the position of each N subunit from the RNA 3' end. **B:** Expanded view of the first 16 N-protein subunits (drawn as *rounded rectangles*, numbered from the RNA 3' end) of the genome nucleocapsid. Positions refer to the nucleotide sequence relative to the RNA 3' (OH) end. The invariant elements of the bipartite genomic and antigenomic promoters of Sendai virus and SV5 are shown relative to the N subunits; each subunit contains precisely six nucleotides. The start sites for the leader RNA, antigenome RNA, and N mRNA are indicated with *arrows.* Note that both elements of the bipartite replication promoter are found on the same face of the helix, whereas N mRNA synthesis starts on the opposite face of the helix.

Primary Transcription

Early in virus infection, before the primary translation products have accumulated (or in the presence of drugs that inhibit protein synthesis at any tage of the infection), vRNAP is restricted to the production of leader RNAs and mRNAs. A leader RNA termination site has not been demonstrated by mutational analysis for any nonsegmented negative-strand RNA virus, and vRNAP that has initiated at the genome 3' end and reads through the leader–N junction may simply become nonprocessive and release its nascent chain heterogeneously within the N gene.

N mRNAs are invariably initiated opposite the second nucleotide of the 10th hexamer (see Fig. 21, genome map position 56) for the *Paramyxovirinae* (157). More than 90% of vRNAP that have initiated the Sendai virus N mRNA (and capped its 5' end) go on to finish the N mRNA (283). The processivity of this vRNAP during mRNA synthesis (transcription) is thus quite different from that which has initiated at the genome 3' end and reads through the leader–N junction in the process of RNA replication, which depends on concurrent assembly of the nascent chain for processivity. The vRNAP that has (re)initiated at position 56 in the process of transcription also responds to the cis-acting gene-end sequence at high frequency (see later), whereas during RNA replication these signals are ignored. mRNA 3' end formation and polyadenylation occurs by vRNAP reiteratively copying a short run of template uridylylates (four to seven long) within the gene-end sequence (see Fig. 6). This process leads to termination and release of the mRNA, and vRNAP is then free to move on to the next gene-start site and to reinitiate the next mRNA.

Initiation of a downstream mRNA depends on termination of the upstream mRNA consistent with a single vRNAP entry site at the genome 3' end. For respiroviruses and morbilliviruses, the next gene-start sequence is separated from the

gene-end sequence by precisely three conserved nucleotides (3'GAA; see Fig. 6). In contrast, for rubulaviruses, NDV, and pneumoviruses, the intergenic region is variable in length and sequence. Moreover, for RSV the L gene-start sequence is actually located upstream of the gene-end sequence of the upstream M2 gene (48). Thus, 90% of the transcripts that initiate the L mRNA terminate at the M2 gene-end sequence, generating aborted L mRNAs. The vRNAP is then free to scan the N:RNA in either direction for the L gene-start sequence, much as cellular RNAPs scan dsDNA in search of a promoter (86). The RNAP of *Rhabdoviridae* also scans the N:RNA for gene-start sequences after terminating an mRNA (see Chapter 38), and this scanning appears to be a general property of nonsegmented negative-strand RNA virus RNAPs following release of a completed mRNA.

The frequency with which vRNAP reinitiates the next mRNA at gene junctions is not perfect, and this leads to a gradient of mRNA abundance that decreases according to distance from the genome 3' end (36,127). This frequency can be regulated (at least in part) by the gene-start sequence, which is highly, but imperfectly, conserved. Reducing reinitiation at one junction leads to a coordinate reduction in the expression of all the downstream genes. Failure to terminate at a gene-end sequence, in contrast, leads to di- (or tri-) cistronic mRNAs in which only the upstream cistron is normally translated, because of the usual requirement that a eukaryotic ORF initiate near a 5' mRNA end. However, vRNAP that reads through a junction is spared falloff because of inefficient reinitiation. There are numerous examples of paramyxoviruses that specifically down-regulate the expression of particular enes, especially the F gene, by generating di-cistronic M-F mRNAs. In several cases, readthrough of this junction is associated with deviations from the consensus gene-end sequence; the most extreme example being the complete lack of an apparent gene-end signal in the M-F junction of SV41. For SV5, whose M gene-end sequence (3'-A**G**UUUCU$_4$) deviates from the consensus at a single position and contains the shortest U run (3'-A **A**NUUCU$_{4–7}$), lengthening the U run was without effect. However, converting the **G** five positions upstream of the U run to **A** restored vRNAP response to this gene-end sequence and prevented readthrough of this junction. However, analysis of genetically engineered recombinant Sendai virus and SV5, in which the gene termination, intercistronic region, and gene-start [EIS (see Fig. 6)] were switched for other EIS sequences, suggests that the simple model of transcriptional attenuation from the genome 3' end is not the only factor determining mRNA accumulation, particularly from the L mRNA (110,147).

RSV gene-end sequences are more diverse than their gene-start sequences (see Fig. 6). They operate at variable efficiency (see Chapter 45). This may be relevant to RSV infections, as the M2-1 protein not only increases vRNAP processivity between junctions during transcription, it also promotes readthrough of the junctions. M2-1 may play a role in fine-tuning the relative abundance of mono- and dicistronic mRNAs.

P Gene mRNA Editing

Most Paramyxovirus P genes contain a functional editing site at the start of the internal, overlapping V ORF (see Fig. 7). Transcription yields, in addition to faithful (unedited) mRNA, edited versions that contain variable numbers of inserted G residues. The number of G insertions that occur for each virus group mirrors their requirements to switch between the in-frame and *trans*-frame downstream ORFs (see Fig. 7). For the morbilliviruses, respiroviruses, and NDV, which require a single nucleotide insertion to shift to the V ORF from the genome-encoded P ORF, a single G is added as the predominant editing event (see Fig. 7). For the rubulaviruses, which require the insertion of two nucleotides to access the P-carboxy pol-cofactor domain of the P ORF from the genome-encoded V ORF, insertion of two Gs constitutes a high proportion of editing events. For bPIV-3 and hPIV-3, where both the V and D ORFs overlap the middle of the genome-encoded P ORF, one to six G residues are added at roughly equal frequency so that mRNAs encoding all three overlapping ORFs are transcribed.

As the paramyxoviruses replicate in the cytoplasm, they must provide enzymes for all aspects of their mRNA synthesis. Paramyxovirus vRNAPs polyadenylate their mRNAs by stuttering on a short run of template U residues (four to seven nucleotides long) at the end of each gene. By analogy to the polyadenylation mechanism, it was suggested that the G insertions at the specific site in the P gene (Fig. 22) would occur similarly by pseudotemplated transcription (35,138,274,281), and there is now strong experimental evidence that the insertions occur cotranscriptionally, by a stuttering mechanism (108,109,282). This mechanism of pseudotemplated transcription, outlined in Figure 23, would apply as well to mRNA polyadenylation if the mRNA–template hybrid is reduced to four base pairs (108).

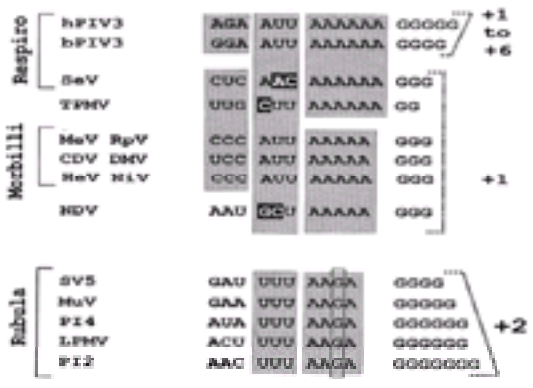

FIG. 22. Sequence relationships at the Paramyxovirus P gene editing sites. The sequences are written as (+) RNA, 5' to 3', and are grouped into the three current genera of the *Paramyxovirinae* except for NDV. Spaces have been introduced to emphasize the different elements of the sequence, and *shaded boxes* indicate sequence conservation. The short G run, which is expanded during mRNA editing, is shown on the right, together with the pattern of G insertions that occurs for each group. Note that the A run preceding the G run may be the only part of this cis-acting sequence that is strictly conserved according to genus. Also note that the second A residue upstream of the Rubulavirus G run is replaced by a G (highlighted with *rectangle*), which is thought to account for why most rubulaviruses insert a minimum of two G residues when stuttering begins (282).

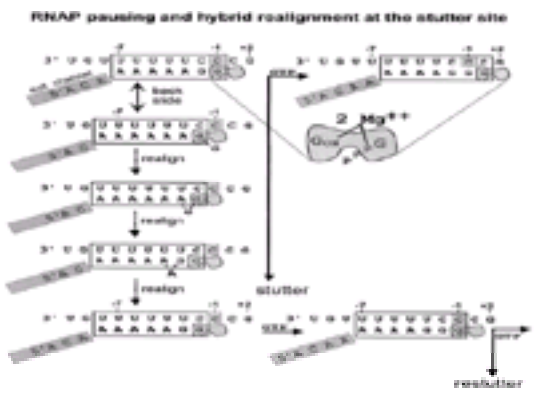

FIG. 23. A competitive kinetic model for Sendai virus RNAP stuttering elongation. The template and mRNA chains of the transcription elongation complex (TEC) at the editing site are shown schematically. The putative seven base-pair hybrid between the polypyrimidine tract of the (–) genome (top strand) and the polypurine run of the nascent mRNA chain (bottom strand) when the TEC is at the editing site is *boxed.* The NTP binding site (position +1) is empty. The nascent mRNA upstream (*left*) of the hybrid is proposed to enter an exit channel (*boxed in gray*) before reaching the surface of the RNAP, and this limits the length of the hybrid as TEC proceeds. The exit channel is thought to contain 10 nucleotides. The bipartite active site, in which the nascent mRNA 3' end (position –1) and the NTP a-phosphate are coordinated via two Mg^{++} ions is highlighted in *gray.* The TEC at the *top left* is at the editing site (middle template C, *boxed in gray*) and has just incorporated a strictly templated G (position –1). The TEC at the unique editing site presumably pauses because of backsliding of RNAP by one position along both the template and the mRNA chains, undoing the last base pair (bp) of the hybrid (and removing the mRNA 3' end from the active site) and reforming one bp upstream. RNAP at pause sites is envisaged as oscillating between the inactive backtracked alignment (*second line*) and the active alignment (*top line*). If a strictly templated GMP is the next nucleotide incorporated, RNAP moves past the unique stutter site and resumes normal elongation (*top right*). Alternatively, realignment of the hybrid also repositions the mRNA 3' end in the active site. Hybrid realignment when RNAP is in the backtracked state is initiated when the unpaired 3'G re-pairs with C-2 (*third line*), causing the penultimate G to bulge out. Realignment is completed on translocation of the single nucleotide bulge to the upstream side of the hybrid (*fourth line*), reforming a seven-bp hybrid that is nearly as stable as its predecessor (*fifth line*). The mRNA 3' G is now correctly repositioned in the active site, and nucleotide addition at this point leads to a single pseudotemplated G insertion, or stutter (lower-case g, *bottom right*). Having stuttered once, the transcription complex is back to where it started and has the same choices (second branchpoint, *bottom right*). Escape from stuttering occurs when TEC moves to a template position where hybrid realignment (stuttering) is no longer favored. Numbers above the genome sequence always indicate positions relative to the mRNA 3' end at the start of the stutter (*top left*, position –1).

Genome Replication

The (–) genome replicates via a full-length complementary copy called the antigenome, which, like the genome, is found only in assembled form. Compared to *Rhabdoviridae*, there are considerable amounts of antigenomes in Paramyxovirus-infected cells (10% to 40% of genome-sized RNAs for Sendai virus). Antigenomes can also represent 5% to 20% of the genome-sized RNAs in virus particles, as discrimination against antigenome nucleocapsids is poor during virus assembly (155,156,231). However, they contain no ORFs of any note and no mRNAs are known to be transcribed from them. The sole function of antigenomes is thought to be as an intermediate in genome replication, although the short trailer RNAs expressed from the antigenome 3' ends (see Fig. 19) may also play a role in preventing the host cell from undergoing programmed cell death (98).

A schematic diagram of the essential differences between transcription and replication is shown in Figure 19. After translation of the primary transcripts and accumulation of the viral proteins, antigenome synthesis begins. Here, ostensibly the same vRNAP (until then engaged solely in mRNA synthesis) copies the same template, but it now ignores all the junctional signals (and editing sites) and synthesizes an exact, complementary copy. Transcription and replication were first distinguished by finding that, when infected cells are treated with drugs that inhibit protein synthesis, mRNA synthesis continued normally but genome synthesis was lost very quickly (232). As genome synthesis and encapsidation appear to occur concomitantly, this requirement for ongoing protein synthesis has been associated with the continued supply of unassembled N necessary for genome encapsidation. The manner in which the coupling of genome assembly and synthesis leads to vRNAP ignoring the gene junctions and editing signals remains unclear (reviewed in ref. 158).

This coupling of genome assembly and synthesis also leads to a self-regulatory system for controlling the relative levels of transcription and replication. For VSV, the site for the initiation of antigenome encapsidation has been mapped to the first 14 nucleotides of the leader sequence, and for paramyxoviruses a similar situation appears to apply. In measles virus–infected cells, a rare polyadenylated transcript representing the leader and N gene sequences fused together is found, but only as a nucleocapsid, whereas the N mRNA is found only in a nonassembled form (31). As the leader sequences contain the encapsidation site, the leader must be separated from the body of the first mRNA (by termination and reinitiation at the first junction) to prevent the first mRNA from ending up in an assembled and untranslatable form. The proposed self-regulatory mechanism would then be quite straightforward; when unassembled N is limiting, vRNAP is preferentially engaged in mRNA synthesis, raising the intracellular levels of all the viral proteins, including unassembled N. When unassembled N levels are sufficient, some vRNAP would be switched to replication, thereby lowering the levels of unassembled N, as each initiation of encapsidation would commit about 2,600 N monomers to finish the assembled genome chain (154). The rare measles virus leader-N mRNA (31), described previously, was presumably made by vRNAP that began the synthesis of an antigenome nucleocapsid but nevertheless polyadenylated and terminated the chain at the second junction. The assembly and synthesis steps can then sometimes become uncoupled, possibly because assembly of the nascent chain does not keep pace with vRNAP movement as it approaches a junction. Whether

the level of unassembled N is the only mechanism that controls the relative levels of Sendai virus transcription versus RNA replication remains unclear. For RSV, however, there is increasing evidence that other viral gene products (e.g., the M2-2 protein) may also play a role (see preceding and Chapter 45).

Genome synthesis from antigenome templates is thought to take place in a fashion similar to that of antigenome synthesis, in that the promoter at the 3' end of the antigenome is also always “on,” and short trailer [also called (–) leader] RNAs are made from this region independently of ongoing protein synthesis (see Fig. 19). However, in contrast to synthesis from genome templates, there are no reinitiation sites on the antigenome template, so termination of the trailer RNAs in the absence of sufficient unassembled N protein serves only to recycle vRNAP. Under conditions of sufficient intracellular concentrations of unassembled N (and perhaps other viral proteins), encapsidation of the nascent trailer chain would quickly begin and this would prevent termination and lead to the synthesis on an encapsidated minus-strand genome.

Virion Assembly and Release

Like all other events in the life cycle of replication of paramyxoviruses, the intracellular site of nucleocapsid assembly is the cytoplasm. The nucleocapsids are thought to be assembled in two steps: first, association of free N subunits with the genome or template RNA to form the helical RNP structure, and second, the association of the P-L protein complex (152). By analogy to the mechanism of assembly of TMV nucleocapsid, which uses a defined nucleation site for the association of the first coat protein subunit with the RNA, and the observation that the Paramyxovirus mRNAs are not encapsidated (in contrast to antigenomes), it has been assumed that the (+) leader (5' end of the antigenome) and (–) trailer (5' end of the genome) regions contain specific sequences for initiating encapsidation (14).

The assembly of the second part of the virus, the envelope, is at the cell surface. In polarized epithelial cells, the *Paramyxovirinae* bud only from the apical surface. The viral integral membrane proteins are synthesized in the ER and undergo a step-wise conformational maturation before transport through the secretory pathway. The mechanisms of viral glycoprotein folding, oligomeric assembly, and sorting have been extensively characterized. Folding and conformational maturation of glycoproteins are not spontaneous events that occur in the cell. Rather, they are assisted by numerous folding enzymes and molecular chaperones. Viral integral membrane proteins, particularly HA and NA of influenza A virus, the G protein of VSV, and the HN protein of SV5 and Sendai virus, have been used extensively as paradigms of cellular glycoproteins, and much of the current understanding of the process occurring in the exocytotic pathway has been learned from studying these proteins (reviewed in refs. 73,116). Only correctly folded and assembled proteins are generally transported out of the ER. In the Golgi apparatus, the carbohydrate chains may be modified from the high-mannose to the complex form, and for those F proteins with multiple basic cleavage sites, cleavage occurs in the trans Golgi network. Finally, the glycoproteins are transported to the plasma membrane.

The mechanism by which the virus particle is assembled at the plasma membrane is unknown. As discussed, the viral M proteins are thought to play a major role in bringing the assembled ribonucleoprotein core to the appropriate patch on the plasma membrane to form a budding virion (Fig. 24). The protein–protein interactions involved in assembling a virion presumably must be specific, as cellular membrane proteins are largely excluded from the virions. It is presumed that the glycoprotein cytoplasmic tails make important contacts with the M protein, which, in turn, associates with the nucleocapsid. Genetically engineered recombinant measles virus and SV5 that lack glycoprotein cytoplasmic tails are defective in assembly and there is a relocalization of the subcellular distribution of the matrix protein (33,251). For Sendai virus HN protein, the cytoplasmic tail sequence SYWST has been shown to be important for incorporation into virions (263). Those *Paramyxovirinae* that have neuraminidase activity contain glycoproteins that lack sialic modification of their carbohydrate chains, and it is thought that the HN neuraminidase activity serves the same purpose as NA in influenza virus, to prevent self-binding and to prevent reattachment to the infected cell.

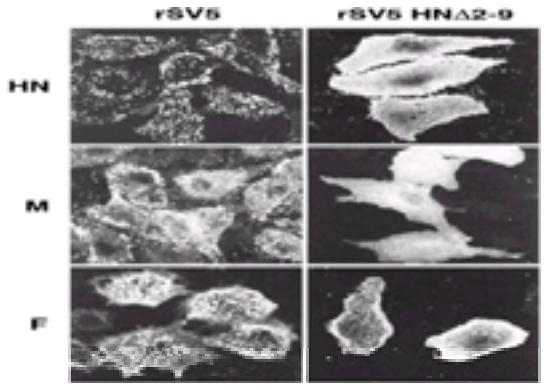

FIG. 24. Altered localization of viral proteins in cells infected with recombinant SV5 expressing an HN protein with a truncated cytoplasmic tail. In wild-type recombinant SV5 (rSV5)-infected cells, HN was found in highly localized patches on the cell surface, and it is likely that these patches are the sites of budding virus. In contrast, in cells infected with rSV5 HND2–9, a recombinant SV5 lacking residues two to nine of the HN cytoplasmic tail, a striking redistribution of the HN staining pattern was observed, with HN being distributed all across the cell surface. Concomitant with the altered HN staining pattern in cells infected with rSV5 HND2–9, a redistribution of the M protein straining pattern was observed. The similarity of HN and M staining patterns suggests that targeting of M protein into presumptive budding sites at the cell surface depends on the presence of an HN cytoplasmic tail. (Adapted from ref. 251, with permission.)

MOLECULARLY ENGINEERED GENETICS (REVERSE GENETICS)

The study of viruses and their interactions with host cells and organisms has benefited greatly from the ability to engineer

specific mutations into viral genomes, a technique known as reverse genetics (288). For RNA viruses, genome manipulation of the positive-sense RNA bacteriophage Qb was the first to be performed (90,266). The negative-stranded RNA viruses, in contrast to positive-sense RNA viruses, require that the virion RNA be assembled into an active transcriptase–replicase complex for the genome to initiate virus replication. Nonetheless, techniques to manipulate the genomes of nonsegmented negative-strand RNA viruses have now been developed (reviewed in ref. 230). The development of the system originally proved quite frustrating. A number of laboratories studying a number of different rhabdoviruses and paramyxoviruses, worked over a period of several years to establish the methods of reconstructing functional nucleocapsids from transfected cells. The concept of replicating mini-genomes using support plasmids providing N, P, and L proteins in *trans* was key to the development of the technology. This culminated in the successful recovery of infectious rabies virus in 1994, followed several months later by VSV, and several months later by Sendai, hRSV, and measles virus.

Rabies virus was rescued when plasmids encoding L, P, and N protein, as well as a plasmid containing the viral antigenome, all under control of the bacteriophage T7 RNA polymerase promoter, were transfected into cells infected with a recombinant vaccinia virus expressing the bacteriophage T7 RNA polymerase protein (vac-T7). For example, the following viruses have been rescued: VSV (170,291), measles virus (227), hRSV (45,141), Sendai virus (97,150,174), rinderpest virus (9), hPIV-3 (75,126), SV5 (112), NDV (218,233), and bRSV (19). A schematic diagram showing the general scheme for rescue is illustrated for SV5 in Figure 25. Some refinements to the original technique have been made, such as the use of stably transfected cell lines expressing the bacteriophage T7 RNA polymerase (in lieu of vac-T7 infection), or one or more of the viral proteins required for genome replication (19,227).

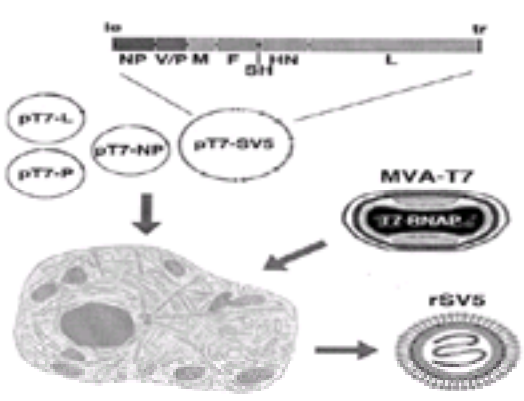

FIG. 25. Schematic diagram for generating infectious SV5 from cloned cDNA. Nonsegmented negative-strand virus rescue involves the transfection of plasmids encoding the viral P, N, and L proteins (and sometimes other viral proteins, depending on the virus), as well as the viral antigenome, all under control of the T7 promoter. The bacteriophage T7 RNA polymerase is provided either by infection with a vaccinia virus expressing T7 polymerase (in this case, modified vaccinia virus Ankara, MVA-T7) or by transfecting into cell lines that stably express the protein. pT7-SV5 contains a complete copy of the SV5 genome (15,246 nucleotides) and is flanked at one end by a bacteriophage T7 RNA polymerase (T7 RNAP) promoter and at the other end by a hepatitis delta virus ribozyme and T7 transcriptional terminator. The plasmids pT7-L, pT7-P, and pT7-N each contain the cDNA for the SV5 L, P, and N proteins, respectively, under the control of T7 RNAP promoters, such that mRNA transcripts encoding L, P, and N can be transcribed using T7 RNAP. A549 cells (a human cell line that is permissive for SV5 and can be efficiently transfected) were infected with MVA-T7 and transfected with pT7-SV5 and the three support plasmids pT7-L, pT7-P, and pT7-N. (Adapted from ref. 112, with permission.)

VIRAL ACCESSORY GENES AND THEIR INTERACTIONS WITH THE HOST

In all studies on reconstituting intracellular virus replication in transfected cells from model mini-genomes, only the P (as defined above), L, and N proteins were found to be strictly required to initiate genome expression and amplification. However, for RSV, coexpression of the M2-1 protein increases vRNAP processivity during transcription (46,87,107). For all the other accessory proteins, their initial coexpression was either without effect or it inhibited mini-genome replication. For the Sendai virus C protein, inhibition of replication was so severe that C coexpression from the P-protein support plasmid had to be suppressed to recover infectious virus (24). Despite these neutral or negative effects on genome replication and expression in transfected cells, reverse genetics has shown that the accessory genes also exert positive effects during natural infections of cells in culture, or in animals as a result of interaction with the host, such as countering the interferon system (see later).

Respirovirus and Morbillivirus V, W, and D Proteins

Infectious measles virus or Sendai virus engineered to have inactive editing sites (i.e., by introduction of mutations at the editing site, so that these viruses cannot express their V and W proteins) are viable, and they grow in cell culture slightly more rapidly than wild-type virus (67,148,252), consistent with an inhibitory role for V and W in genome amplification (56). However, an important step toward understanding the role of the V protein in the Sendai virus life cycle was made when it was found that, although the Sendai virus–edit minus mutant grew perfectly well in cell culture, it was avirulent for mice (148). In contrast to wild-type virus, Sendai virus–edit minus could not sustain its infection of the respiratory tract and was quickly cleared from the animal. Given that mice could restrict Sendai virus–edit minus replication so quickly, it was suggested that V functioned to counteract some aspect of the animal's innate immune system (148).

To examine whether this avirulence was caused by the loss of V, or W, or both proteins, Sendai virus–Vstop mutant, in which the V ORF is closed and expresses a W-like protein in place of V, was characterized (67,149). In 3-week-old outbred mice, Sendai virus–Vstop was almost as attenuated for virulence as Sendai virus–edit minus. In inbred mice, however, Sendai virus–Vstop was almost as virulent as wild-type Sendai virus, whereas Sendai virus–edit minus

remained avirulent. Thus, the W protein can mostly replace the V protein for counteracting host defenses in the inbred mouse strain. The W protein, however, cannot replace the V protein in the more robust outbred animals, where the cysteine-rich domain of the V protein is clearly required for virulence. The finding that the cysteine-rich domain of V protein is not essential for virulence in some mice may be relevant for hPIV-1, which does not contain a V ORF or an editing site (184). The hPIV-1 may express low levels of a W-like protein by other means, such as ribosomal frameshifting. For hPIV-3, two or more (depending on the strain) stop codons are present between the editing site and the almost perfectly conserved cysteine-rich domain, and a V-like protein has not been demonstrated biochemically in hPIV-3 infections. Nevertheless, the introduction of stop codons in both the V and D ORFs simultaneously (but not individually) attenuated hPIV-3 replication *in vivo* (76). Thus, a hPIV-3 V-like protein may be expressed at low levels after all, by some mechanism other than editing, and may contribute to viral virulence along with the D protein.

Measles virus–edit minus mutant is also attenuated compared to wild-type virus in mice with severe combined immunodeficiency disease (SCID) that have been engrafted with human thymocyte/liver as a substrate for measles virus replication. Measles virus–edit minus mutant also grows to lower titers in the lungs of cotton rats (277,279). Measles virus–edit minus is also slightly less virulent in transgenic mice that express the human CD46 receptor (195,216). Thus, Morbillivirus V/W proteins may also function in countering host defenses. On the other hand, rubulaviruses that cannot express their V protein have not been reported. It may be that the Rubulavirus V protein has a significant role in intracellular virus replication. Precisely what aspect of innate immunity the V, W, or D proteins affect remains unclear. Neither natural killer cells nor the interferon system seems to be involved for Sendai virus (96,101).

C Proteins and Rubulavirus V Proteins

Measles viruses that cannot express their single C protein are viable, and they replicate in cell culture in a manner similar to wild-type viruses (226). The hPIV-3 also expresses a single C protein, but hPIV-3-C-minus growth is clearly restricted in cell culture (76). Sendai virus, in contrast, expresses four “C” proteins (C', C, Y_1, and Y_2), and recombinant Sendai virus (rSeV), which cannot express any of their C proteins, is at the limit of recovery from DNA and grows to titers that are four logs lower than wild-type virus (159,167). The number of expressed C proteins, and their relative importance for the intracellular replication of each virus, is thus quite different, with the caveat that the “wild-type” measles virus is a cell culture–adapted mutant.

The Sendai virus C' and C proteins (but not Y_1 and Y_2) are promoter-specific inhibitors of viral RNA synthesis (24,267). Consistent with this, infections with rSeV that cannot express C' or C individually (by mutating their ribosomal start codons) accumulate more viral RNAs than wild-type rSeV infection, and cytopathic effects are seen earlier (167). However, the simultaneous absence of both C' and C paradoxically delays viral RNA accumulation and cytopathic effects, and these viruses form “pin-hole” plaques. Thus, in addition to their negative effects on viral RNA synthesis (which apparently operate later in infection when these essentially nonstructural proteins have accumulated), C' and C apparently also provide a positive function without which the onset of viral RNA accumulation intracellularly is delayed. This positive function of C is not seen when a recombinant Sendai virus infection is launched from plasmids in transfected cells. Here, the early events in the infectious process have been bypassed (by jump-starting viral replication from DNA), and only the later inhibitory effects are detected. Presumably either C' or C (but not Y_1 or Y_2) can supply this early required function in cell culture infections. Viruses unable to express either C' or C individually would have this function provided by the remaining protein and would not show a debilitated phenotype.

Mutation of residue phenylalanine 170 of the Sendai virus C protein to serine (by classic or reverse genetics) strongly reduces virulence in mice. The C protein F170S mutation was discovered as one of two mutations (the other in the L gene) in a highly virulent virus strain (Sendai virus strain M; LD_{50} of 40) that became avirulent (Sendai virus variant MVC; LD_{50} of >800,000) on adaptation to LLC-MK2 cells (137). Recombinant Sendai virus carrying the C protein F170S mutation in an otherwise wild-type background is also avirulent in mice (95), and introduction of this mutation into hPIV-3 (C protein F164S) similarly attenuated virulence *in vivo* (76). Although rSeV-C protein F170S was avirulent, it grew normally in the mouse respiratory tract at first. However, like Sendai virus–edit minus, virus was then quickly cleared. The rapidity of the antiviral response in immunologically naive mice again suggested that some aspect of innate immunity is involved. One such function has recently been identified. Infection of interferon (IFN)-competent cells with Sendai virus, or SV5, does not induce an IFN-mediated antiviral state. These infections do not affect the secretion of IFN but somehow interfere with its receptor-mediated signaling, as they prevent added IFN from inducing an antiviral state (71,96). For Sendai virus, the C gene is clearly involved, as Sendai virus-C protein F170S infection does not interdict IFN signaling (96), and C protein expression independent of virus infection prevents added IFN from activating IFN-responsive promoters (94). Consistent with this latter finding, Sendai virus-(C-minus) infection was avirulent, whereas Sendai virus-(C'-minus) was found to be as virulent for mice as wild-type virus, even though this virus overexpresses Y_1 and Y_2. Thus, the AUG_{114}-initiated C protein is specifically required for virulence in mice, or to interfere with IFN signaling (96,168). For SV5, however, it is the V protein that functions to interdict IFN signaling (71), and the SV5 V protein interferes with IFN signalling by causing the proteasomal degradation of the transcription factor, STAT-1 (72). Given that the IFN system is the first line of defense against viral infections, it is possible that the Morbillivirus C proteins (83,195,216) or one of the Pneumovirus accessory proteins, also functions to counteract the IFN-mediated cellular antiviral response.

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CHAPTER 42 Parainfluenza Viruses

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HISTORY

The parainfluenza viruses include important respiratory tract pathogens of infants and children, as well as important viruses of mammals and birds. These medium-sized (150- to 200-nm) enveloped viruses have a nonsegmented negative-strand RNA genome and belong to the family *Paramyxoviridae* of the order *Mononegavirales*, the nonsegmented negative-strand RNA viruses (240). The parainfluenza viruses that infect humans were first recognized following the application of tissue culture and hemadsorption techniques to the study of pediatric respiratory tract disease (24,28,137). The four human parainfluenza viruses (hPIV1 to hPIV4) were first recovered during the period of 1956 to 1960. They were recognized by cytopathic effects that developed in infected tissue cultures (hPIV2) or by the hemadsorption reaction in which guinea pig red blood cells become adsorbed to an infected tissue culture monolayer (24,28,137). The hPIV1, 2, and 3 were initially isolated from infants and children with lower respiratory tract disease, and hPIV4 was recovered from children and young adults with minor upper respiratory tract illness. Soon after their discovery, these viruses were shown to be a major cause of croup (hPIV1, 2, and 3) as well as pneumonia and bronchiolitis (hPIV3) (27,29,30,90,159,228). As a group, hPIV1 to 3 are second only to respiratory syncytial virus (RSV) as a cause of serious respiratory tract disease in infants and children (208).

Before the first human virus strains were isolated, a parainfluenza virus had been recovered from mice inoculated with an autopsy specimen from an infant with respiratory disease (173). This agent, Sendai virus, was later shown to be indigenous to mice and not a human pathogen. Its antigenic relationship to hPIV1 led to some confusion when it was used in serologic studies of patients with acute respiratory disease. Ultimately, Sendai virus was classified as the murine counterpart of hPIV1. Animal counterparts were subsequently identified for other human parainfluenza viruses. Simian virus 5 (SV5), a common contaminant of primary monkey kidney tissue cultures and a cause of croup in dogs, was shown to be a counterpart of hPIV2, whereas a bovine shipping fever virus was classified as the bovine counterpart of hPIV3 and designated bPIV3 (1,26,45). Simian virus 41 (SV41) also was isolated as a contaminant of primary monkey kidney cell culture and appears to be even more closely related to hPIV2 than is SV5 (314). In addition, nine distinct serotypes of avian parainfluenza virus are now recognized, of which Newcastle disease virus (NDV) is the prototype of serotype 1 (3).

The parainfluenza viruses share biophysical and morphologic properties and are unambiguously related on the basis of their gene map as well as nucleotide and amino acid sequence. They also are related antigenically, except that, of the avian viruses, only NDV and possibly avian parainfluenza virus type 2 exhibit antigenic relatedness to the other parainfluenza viruses (3,129,167,174,314,315).

Newly available methods of reverse genetics (see section Genetics and Reverse Genetics, later) have made it possible to recover complete infectious recombinant virus entirely from cDNA for a number of parainfluenza viruses, namely Sendai virus, hPIV3, bPIV3, SV5, and NDV (70,86,109,115,233). In addition, helper-dependent minireplicon systems have been developed for a number of parainfluenza viruses, providing an additional tool for molecular biologic studies. Application of the technology of reverse genetics to these viruses has revolutionized studies of their molecular biology and pathogenesis and has facilitated vaccine development.

Several of the parainfluenza viruses, namely Sendai virus, SV5, and NDV, have been used extensively in studies that have defined many of the basic biochemical and molecular biologic properties of the paramyxoviruses. This information is summarized in Chapter 41; the present chapter will provide additional detail and focus on biologic and medical aspects of the human parainfluenza viruses.

INFECTIOUS AGENT

Classification

The parainfluenza viruses are classified in the subfamily *Paramyxovirinae*, family *Paramyxoviridae* (Chapter 41), order *Mononegavirales* (the nonsegmented negative-strand RNA viruses, referred to as mononegaviruses) (240). This order contains four families: *Bornaviridae* (a family that is somewhat distinct and is not considered further here), *Rhabdoviridae*, *Filoviridae*, and *Paramyxoviridae*. The last three families share a number of properties: (a) the negative-sense RNA genome is single-stranded; (b) the genome is present in the form of a nucleocapsid, tightly bound along its entire length by the major nucleocapsid protein; (c) the virally encoded polymerase is packaged in the virion; (d) genome transcription is sequential and yields predominately nonoverlapping subgenomic mRNAs; (e) the replicative cycle is cytoplasmic; and (f) progeny virions bud at the plasma membrane. In *Paramyxoviridae*, the fusion activity is segregated on a separate, additional surface glycoprotein, the fusion (F) protein. F is active at physiologic pH and mediates viral penetration at the plasma membrane (175).

The parainfluenza viruses comprise two of the three genera of the subfamily *Paramyxovirinae*, namely Respirovirus and Rubulavirus. Mumps virus (Chapter 43) also is a Rubulavirus, but historically it is not considered to be a parainfluenza virus because its disease is not based in the respiratory tract. The other genus of the subfamily *Paramyxovirinae* is Morbillivirus (measles virus, Chapter 44). Morbilliviruses are related to the parainfluenza viruses on the basis of gene map and certain nucleotide and amino acid sequences but are distinct antigenically and in the disease they produce. The paramyxovirus family contains a second subfamily, namely the *Pneumovirinae*, represented by RSV (Chapter 45). Pneumoviruses, which are more distant relatives with limited amino acid sequence relatedness, are not related antigenically, but they share the property of being important agents of respiratory tract disease.

The segregation of the parainfluenza viruses between the Respirovirus (hPIV1 and hPIV3) and Rubulavirus (hPIV2 and hPIV4) genera is based on a number of distinguishing features (Table 1 and Fig. 1 and Fig. 2): (a) antigenic relatedness and amino acid sequence identities are greater within than between genera; (b) there are genus-specific differences in proteins, such as the presence of one or more C proteins in respiroviruses but none in rubulaviruses, the presence of an additional gene encoding the small hydrophobic SH protein in some rubulaviruses but not in respiroviruses, and a smaller P protein in rubulaviruses (see Table 1); (c) conserved sequence motifs at the 3' and 5' ends of the genome (illustrated for hPIV3 in Fig. 1) and at the ends of each gene are more highly conserved within than between genera; (d) intergenic regions of respiroviruses are conserved trinucleotides, whereas those of rubulaviruses are variable in length and not conserved (see Fig. 2); and (e) the details of RNA editing differ between the two genera (see Table 1). The rubulaviruses have more diversity in genome organization and in nucleotide and amino acid sequences than do the respiroviruses. The hPIV4 has been divided into two antigenic subgroups, A and B, based on antigenic differences detected by hemadsorption-inhibition (HI) and monoclonal antibody reactivity (169). Sequence analysis shows that these two subgroups are very closely related: For example, the NP, P, and M proteins are 93% to 97% identical between hPIV4A and B, whereas they are 33% to 49% identical between hPIV4A/B and hPIV2, SV5, or mumps virus (226).

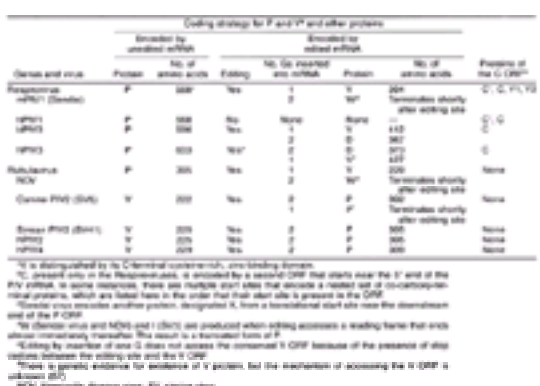

TABLE 1. *Coding strategy of the P/V mRNA of the parainfluenza viruses (PIVs) of the Respirovirus and Rubulavirus genera*

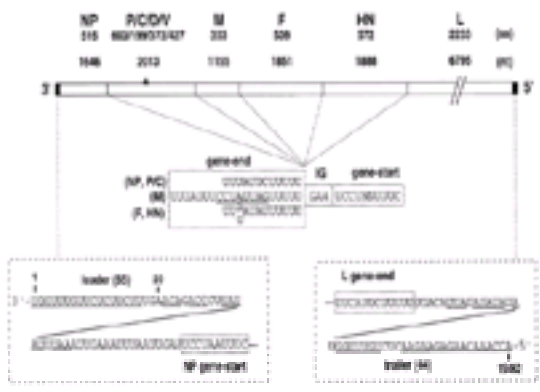

FIG. 1. Map of the RNA genome (3' to 5', negative sense) of hPIV3 (JS strain). Each *open box* represents a separate encoded polyadenylated mRNA. Above each box are the mRNA length [exclusive of poly(A) or editing] in nucleotides (*nt*), the length(s) of the encoded protein(s) in amino acids (*aa*), and the protein name(s); slashes indicate that more than one proteins are encoded by a single mRNA. The sequences of the extracistronic 55-nucleotide 3'-leader and 44-nucleotide 5'-trailer regions and adjoining NP gene-start or L gene-end transcription signals, respectively, are shown below each box. Terminal complementarity between the leader and trailer regions is indicated by *underlining.* Note that

there is an additional element, not shown here, that is conserved between the 3' ends of the genome and antigenome: namely, three conserved G residues at positions 79, 85, and 91 spaced at hexamer intervals (294,295) The gene-end, intergenic (*IG*), and gene-start sequences at the gene junctions are shown: The eight nonconsensus nucleotides that interrupt the M gene-end sequence are *underlined.* The intergenic trinucleotide is italicized, as are the last three nucleotides of the leader region and the first three nucleotides of the trailer region, which for hPIV3 and a number of other paramyxoviruses resemble intergenic trinucleotides. The editing site (nucleotides 2504 to 2508 in the complete hPIV3 sequence) is marked with an *asterisk.* (For additional review, see refs. 7,72,84,279,280,283,285.)

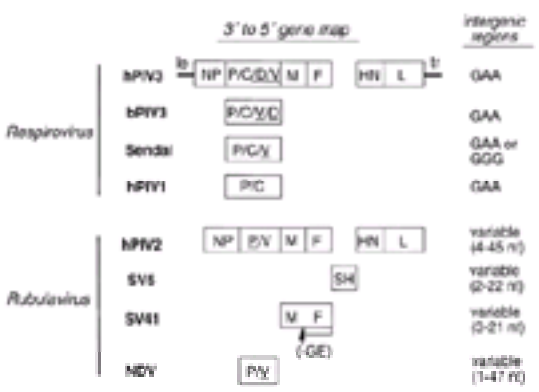

FIG. 2. Comparison of parainfluenza virus gene maps (3' to 5', negative-sense genome RNA). Encoded mRNAs are shown (*boxes*). Complete maps (not to scale) are shown for hPIV3 (Respirovirus) and hPIV2 (Rubulavirus). *le* indicates 3'-leader and *tr* indicates 5'-trailer. Gaps have been introduced to optimize alignment of mRNAs encoding analogous proteins. Gene map features are shown for the other members of each genus only if they differ from the prototype of the genus. For example, the other respiroviruses differ from hPIV3 in the proteins encoded by the P mRNA; SV5 differs from hPIV2 in having the additional SH gene; and SV41 differs in lacking a gene-end signal for the M gene (*-GE*) and in synthesizing two mRNAs from this region, an M-F readthrough mRNA and an F mRNA. The gene map of hPIV4 is very similar to that of hPIV2 and is not shown. For the P mRNAs, the protein products of ORFs fused by editing are *underlined.* The C, C', Y1, Y2, X, W, and I proteins encoded by the P mRNAs of some viruses are not shown (see Table 2). (For additional review, see refs. 61,109,154,155 and 156,171,219,262,269,270,279,285,288).

NDV is the only parainfluenza virus whose taxonomic classification within *Paramyxovirinae* is unclear. On the basis of its general gene map and other features, NDV appears to be a Rubulavirus and currently is so classified: It lacks a C protein, has a smaller P protein (albeit not as small as those of the other rubulaviruses), and has variable intergenic regions (see Table 1 and Fig. 2). However, in some other ways it resembles a Respirovirus, such as features of its RNA editing (Table 1) and closer nucleotide sequence identity at the conserved genomic termini. On the basis of overall nucleotide sequence identity, NDV does not segregate with either Respirovirus or Rubula virus and thus may represent a distinct genus (61). Amino acid sequence alignments of the various proteins generally are consistent with NDV being a somewhat distinct member of the Rubulavirus genus or, alternatively, being classified as a separate genus. NDV and the avian parainfluenza viruses are now being considered for possible reclassification as a new fourth genus within subfamily *Paramyxovirinae*.

The term *parainfluenza* was originally coined because some of its disease symptoms are influenza-like and because the virus particle, like that of influenza virus, has hemagglutination and neuraminidase activities. Indeed, information on glycoprotein processing and activities, host immunity, and viral pathogenesis of the influenza viruses can often serve as a general model for the parainfluenza viruses. However, the parainfluenza viruses and influenza viruses (the latter belonging to the family *Orthomyxoviridae*) differ in many ways. In contrast to parainfluenza viruses, influenza viruses have a segmented negative-strand RNA genome, transcribe and replicate in the nucleus, have splicing and cap-snatching activities, and lack nucleotide or amino acid sequence relatedness with parainfluenza viruses except for certain widely conserved polymerase protein amino acid sequence motifs (234). The surface glycoprotein primary and tertiary structure of influenza viruses exhibits similarities to that of parainfluenza viruses, and this hints of a distant common origin (40).

Virion Morphology and Activities

Parainfluenza viruses are pleomorphic and range in average diameter from 150 to 200 nm. Virions are bounded by a lipid bilayer membrane bearing spikelike surface projections composed of the tetrameric hemagglutinin-neuraminidase (HN) or trimeric F glycoprotein (162). These surface glycoproteins are anchored in the plasma membrane of the infected cell or in the viral envelope by a hydrophobic transmembrane domain, with most of the protein oriented extracellularly and leaving a small tail at the inner face of the membrane. This tail is thought to play a role in virus assembly through its interaction with matrix (M) protein, which, in turn, interacts with intracellular nucleocapsids (170,232,263). F protein alone is sufficient for virion assembly (287).

The surface glycoproteins execute a number of biologic activities. The hemagglutination activity of the HN protein mediates adsorption of virus to the host cell to initiate infection. This activity can be measured experimentally by the agglutination of erythrocytes by virus in suspension (hemagglutination) or by the adsorption of erythrocytes to infected cell monolayers expressing HN (hemadsorption). Late in infection, the neuraminidase activity of HN cleaves its sialic acid receptor to release progeny virions. This activity can be quantitated using sialic acid derivatives as substrates in a colorimetric or fluorimetric assay. The F protein mediates fusion between the viral envelope and the host cell plasma

membrane, an activity which can be measured *in vitro* by lysis of erythrocytes.

The RNA genome is contained within a helical nucleocapsid whose morphology is conferred by the tight association of the genome with about 2,600 molecules of the nucleocapsid protein, NP (162,176). Interestingly, the nucleotide length of genomes of respiroviruses and rubulaviruses is an even multiple of six. Mutational studies with recombinant viruses or helper-dependent mini-genomes recovered from cDNA indicated that this feature is necessary for efficient RNA replication (21,166). Called the “rule of six,” this requirement is thought to reflect a nucleocapsid organization in which each NP monomer associates with six nucleotides. It might represent a requirement for proper spacing of cis-acting RNA signals relative to the NP monomer or the genomic termini (166) (see later section Genome and Encoded mRNAs and Proteins). This rule appears to be more strict for some parainfluenza viruses than for others (211). Morbilliviruses obey the rule of six, but the pneumoviruses and rhabdoviruses do not. For parainfluenza viruses, as for all nonsegmented negative-strand RNA viruses, the genome and the antigenome (which is its positive-sense replicative intermediate) are found only in nucleocapsids and never as free RNA. Indeed, the synthesis of the genome or antigenome is tightly linked to concurrent encapsidation of the nascent RNA (325).

The nucleocapsid also contains about 300 copies of the phosphoprotein P and about 40 copies of the major polymerase subunit L (71,176). Within the virion, the nucleocapsid is wound about itself; however, when it is released by disruption of virus, it assumes a linear, flexible conformation that is approximately 1,000 nm in length (42). Nucleocapsids have been observed in several distinct morphologic states that are thought to have functional significance (71). The nucleocapsid has an RNA-dependent RNA polymerase that consists of the P and L proteins in association with NP-bound genome (105). Purified virions can be activated for cell-free transcription by disruption of the envelope with detergent, and the virion-associated polymerase contains all the activities required to transcribe the viral genome in its entirety into mRNAs. Cell-free transcription is stimulated by the addition of host-cell cytoskeletal proteins, such as tubulin in the case of Sendai virus (202) and actin in the case of hPIV3 (60). The nucleocapsids of parainfluenza viruses and other paramyxoviruses associate with the cellular cytoskeleton during synthetic and maturation steps, and it may be that the interplay between viral and cytoskeletal components is integral to viral RNA synthetic processes.

Paramyxoviruses mature by budding from the surface of infected cells at membrane sites where the viral glycoproteins and M protein have accumulated. During the process of budding, the nucleocapsid is enveloped within the extruding virus particle. Virions contain predominantly genomic (negative-strand) RNA nucleocapsids (162). Depending on the virus and cell type, a varying, but small, proportion of virions contain antigenomic genomes. Hence, at least in such cell types, there does not appear to be a mechanism for stringent selective packaging of the genomic nucleocapsid.

Genome, Encoded mRNAs, and Proteins

The Respirovirus or Rubulavirus genome is a single strand of negative-sense RNA of about 15,000 nucleotides (nts) in length (Table 2). The map of the genome of hPIV3 (see Fig. 1), the most extensively characterized human Respirovirus, is compared to other respiroviruses and the rubulaviruses in Fig. 2. Viruses of the two genera encode six nonoverlapping polyadenylated mRNAs, except for SV5 which encodes seven. Five of these mRNAs each encode a single structural protein, namely, NP, M, F, HN, or L. The additional mRNA of SV5 encodes the small hydrophobic SH protein (see Fig. 2).

Virus	Genome length	GenBank accession no.	Reference
Sendai virus	15,384	M30202 M29343	21, 269, 270
hPIV2	15,649[a]	X57559	154, 155, 171
SV5	15,246	AF052755	169
SV41	15,450	X64275	156, 219
hPIV3	15,462	Z11575	7, 285
bPIV3	15,480	D84095 AF178654 AF178655	7, 282, 288
NDV	15,186	AF077761	61

[a] 15,648 nucleotides does not conform to the rule of six (21) and is likely to be either 15,642 or 15,648 nucleotides.

SV, simian virus; NDV, Newcastle disease virus.

TABLE 2. *The length and accession number of the complete sequence of the genomes of various parainfluenza viruses (PIVs)*

The remaining mRNA, the P/V mRNA, has a complex coding strategy with considerable variability between the genera and among individual viruses. The P and V proteins are encoded by alternative forms of the mRNA produced by the novel mechanism of RNA editing described later. In Respirovirus and NDV, the exact-copy mRNA encodes P and the edited version encodes V, whereas in all rubulaviruses except NDV, the situation is the converse (see Table 1). The Respirovirus P/V mRNA contains a second 5'-proximal open reading frame (ORF) that overlaps that of P and encodes one or more C proteins. Rubulaviruses do not have this ORF.

Cis-acting sequences in parainfluenza virus genomes have been identified both by nucleotide sequence conservation within and between viruses and on the basis of direct mutational analysis using reverse genetics (see later section Genetics and Reverse Genetics). As will be described later, the cis-acting elements of parainfluenza viruses are relatively small and circumscribed. They consist of (a) a dispartite promoter contained within the 3'-terminal 90–96 nucleotides of the genome and antigenome, (b) transcription signals 10 and 13 nucleotides long at the upstream and downstream ends, respectively, of each gene, and (c) an RNA editing motif in the P/V gene. In the respiroviruses, the intergenic trinucleotide might also be a cis-acting signal. Thus, cis-acting signals occupy only slightly more than 2% of the genome. The first 13 to 14 nucleotides at the 3' end of the parainfluenza virus genome are highly conserved within and between the Respirovirus and Rubulavirus genera and are obvious candidates to be part of the promoter (61). Also,

within each Respirovirus and Rubulavirus, there is extensive sequence identity between the 3' ends of the genome and antigenome (the latter is encoded by the 5' end of the genome), which also suggests that a conserved promoter sequence is present at each end of the molecule. For example, hPIV3 has 85% identity between the 3'-terminal 38 or 39 nucleotides of the genome and antigenome (see Fig. 1), and this sequence was conserved even in PIV3 genomes that had become highly mutated during a 29-month persistent infection of cells in culture (210). These conserved sequences confer complementarity between the 3' and 5' ends of genomic RNA, and they have the potential to form a short panhandle. Such panhandles are important in RNA synthesis by influenza virus. However, studies with minireplicons indicate that panhandles are unlikely to play a role in the function of the parainfluenza viral genome or antigenome (295). Thus, these conserved ends most likely do not interact, but each contains a conserved promoter. Furthermore, it is thought that encapsidation of the genome and antigenome occurs cosynthetically and initiates at a sequence near the 5' end of the nascent molecule. Thus, the complement of each promoter, representing the 5' end of the nascent RNA, might contain an encapsidation signal.

To date, mutational analysis of promoter sequences is incomplete and has focused on sequences involved in RNA replication. Nonetheless, a common model has emerged. For Sendai virus, the genome promoter appears to consist of two elements, one contained within the 3'-terminal 31 nucleotides of the leader region, and a second contained within nucleotides 79 to 96, a region located in the leader proximal nontranslated region of the NP gene (294,295). The element represented by the first 31 positions probably involves the dense concentration of highly conserved nucleotides mentioned previously, whereas the element in nucleotides 79 to 96 appears to consist of three key C residues at positions 79, 85, and 91 (in the negative-sense 3' to 5' sequence). Interestingly, if the genome sequence is considered to consist of hexamers, each determined by association with an NP monomer according to the rule of six mentioned previously (21), the spacing of these key C residues corresponds with the first nucleotide of hexamers 14, 15, and 16 (numbered from the 3' end). Thus, the rule of six might operate to maintain certain cis-acting signals in the correct position relative to higher-order nucleocapsid structure (166). Inspection of the sequence at the 3' end of the antigenome suggests that it contains the same promoter elements, and thus the genomic and antigenomic promoters are likely to be very similar. A similar picture, differing only in details of sequence and spacing, emerged from analysis of the replication promoter of the Rubulavirus SV5 (212). Sequence alignments suggest that Sendai virus promoter is conserved among respiroviruses, whereas the slightly different version described for SV5 is conserved among rubulaviruses (212).

The sequences involved in the initiation of transcription have not been defined. It is likely that the main elements of the transcription and replication promoters involve the same nucleotides, although there also may be some differences in the cis-acting signals involved in initiating these two processes. Interestingly, a recombinant Sendai virus was made in which the first 42 nucleotides of the 55-nucleotide 3' leader end of the genome were replaced with the corresponding nucleotides of the antigenome. The resulting virus was viable and was competent for transcription and RNA replication, although it differed in some biologic properties (86). This showed that the antigenomic promoter, like the genomic promoter, is able to direct transcription as well as replication, even though in the natural situation it directs only replication.

The polymerase transcribes the parainfluenza virus genome in a linear, sequential manner guided by short conserved signals at the gene termini: a gene-start signal of approximately 10 nucleotides at the upstream end, which encodes the 5' end of the mRNA, and a gene-end signal of approximately 13 nucleotides at the downstream end, which directs polyadenylation and termination of the subgenomic mRNA (61). The polymerase remains template bound, and it reinitiates synthesis at the adjacent downstream gene. This mode of transcription is typical for mononegaviruses. The mRNAs are capped and polyadenylated. Both of these activities are directed by the viral polymerase. The gene-start and gene-end signals tend to be highly conserved among the genes of a given virus, and thay are also conserved between related viruses. The gene-start and gene-end signals of hPIV3 are illustrated in Figure 1. Note that the gene-end signal terminates with a run of U residues, which encodes the poly(A) tail of the mRNA by stuttering.

For all mononegaviruses, there is a gradient of transcription in which promoter-proximal genes are expressed more efficiently than promoter-distal genes. This is because of polymerase falloff during sequential transcription, which probably reflects an inherent inefficiency in resuming transcription at each gene junction. This is one of the major factors in the regulation of gene expression by parainfluenza viruses and mononegaviruses in general. A second major factor is that the large L gene is expressed much less efficiently than would be expected based on its position as the last gene in the map. It was demonstrated directly with recombinant SV5 that this was not the result of some deficiency in the signals at the HN-L junction, and thus it probably reflects polymerase falloff within this very long gene or sensitivity of this large mRNA to degradation (107).

Differences in transcription signals can influence gene expression. For example, there are several examples of parainfluenza viruses in which the efficiency of the M gene-end signal in directing termination is reduced because of sequence differences. In each case, this is associated with an increase in the synthesis of a readthrough of the M and F genes and a decrease in the expression of monocistronic M and F mRNAs. The M gene-end signal of hPIV3 contains an apparent eight-nucleotide insertion (see Fig. 1) (279). In SV5, a single nucleotide difference in the M gene-end signal reduces its efficiency (244). In SV41, the M gene-end signal is lacking altogether (see Fig. 2): M is expressed solely as an M-F readthrough mRNA. Interestingly, the F gene of SV41 also is expressed as a monocistronic mRNA by initiation at its gene-start signal. The manner by which this occurs is unknown, but it is speculated that some polymerases might fail to initiate at the M gene-start signal and would then be free to slide along the M gene and initiate at the F gene-start signal. The common feature of these three examples is a reduction in the synthesis of monocistronic F mRNA, which most likely is a mechanism for reducing the synthesis of this protein. One might speculate that reduced synthesis of this fusogenic factor might prolong cell viability and thereby enhance virus production. Regulation at the gene junctions was also studied with complete infectious recombinant SV5, in which the naturally occurring HN-L gene junction was replaced by each of the other naturally occurring junctions (107). This strategy evaluated the efficiency with which each junction executed the stop and restart steps of transcription. The ratios of HN to L varied over only a fourfold range. However, this was sufficient to significantly alter plaque size and the kinetics of growth of the recombinant viruses.

The paramyxoviruses of the two genera differ with regard to intergenic sequences (see Fig. 2). The genes of respiroviruses are separated by the trinucleotide GAA (or GGG) (269,270,279), which is highly conserved both within

respiroviruses and between respiroviruses and morbilliviruses. This high degree of conservation suggests that these trinucleotides, flanked on either side by the gene-end and gene-start sequences, might be a cis-acting transcription signal. In addition, an intergenic-like trinucleotide is found at the end of the leader region and the beginning of the trailer region (Fig. 1). In contrast, the intergenic regions of rubulaviruses are quite variable in sequence and length, ranging from 1 to 45 nucleotides (see Fig. 2). For SV5, a virus that has variable intergenic regions, studies in a minireplicon system indicated that alteration of the sequences in the intergenic region can influence the efficiency of termination of the upstream gene and reinitiation at the downstream gene (243). There was no evidence of a specific cis-acting signal, and intergenic length did not appear critical, but it appeared that sequence composition influenced the efficiency of termination and reinitiation.

Respiroviruses and rubulaviruses execute RNA “editing,” a feature that is also seen with morbilliviruses and filoviruses. This involves the cotranscriptional insertion of one or several G residues into the nascent P/V mRNA by stuttering of the viral polymerase at a specific site in the template midway in the P/V gene. An array of mRNAs is produced: They include the unedited, exact-copy form as well as species with one, two, or more inserted G residues. The details of editing differ between the respiroviruses and rubulaviruses, with NDV following the pattern of the respiroviruses (see Table 1). In respiroviruses (and NDV), the exact-copy mRNA encodes the P protein. Also, individual viruses can differ in the distribution of added G residues. The Respirovirus consensus editing site is 3'-UUUUUCCC (negative sense), although the nine nucleotides that lie immediately upstream of this motif also influence editing (106). The addition of a single G nucleotide (or any multiple of 3, plus 1) shifts the reading frame to access an internal V ORF, resulting in the synthesis of the V protein containing a characteristic cysteine-rich motif. In contrast, for rubulaviruses other than NDV, the situation is the converse, with the exact-copy mRNA encoding the V protein. The consensus editing site is 3'-AAAUUCUCCC. The insertion of two G residues (or any multiple of 3, plus 2) shifts the reading frame to access the internal P ORF, resulting in the synthesis of the P protein.

Additional proteins also can be made by editing. For example, for both hPIV3 and bPIV3, the insertion of two G residues accesses a second internal ORF and results in a chimeric protein called D protein. No other paramyxovirus has a D ORF. In other respiroviruses, the addition of two G residues accesses an ORF that quickly terminates and results in a protein called W, which essentially is a truncated P protein. In rubulaviruses, the addition of one G residue results in a similar truncated P protein that is called I.

There are two respiroviruses that do not conform to the pattern just described for expressing V. hPIV1 does not appear to edit, and its V ORF is interrupted by numerous stop codons (255). Thus, hPIV1 does not make a V protein. HPIV3 has an intact, conserved V ORF, but it is separated from the editing site by several stop codons (7). This makes it unlikely that an hPIV3 V protein could be translated efficiently from an edited mRNA, suggesting that it might be accessed by another mechanism, as there is genetic evidence that an hPIV3 V protein is made (67).

The spectrum of proteins encoded by the P/V mRNA of respiroviruses is increased by the presence of C ORF, which in some cases is accessed by more than one translational start codon (see Table 1).

Properties of the Proteins

All parainfluenza viruses encode the following six proteins: the two surface glycoproteins HN and F, the three nucleocapsid-associated proteins NP, P, and L, and the nonglycosylated internal protein M. These six proteins are essential for multicycle virus growth, and each is a component of the virion. The respiroviruses and rubulaviruses encode one or more additional proteins that are not essential for growth in cell culture and therefore are referred to as accessory proteins, namely V, D, W, I, X, C, and SH. These accessory proteins presumably reflect adaptation of each virus to its particular host, although the relatively wide distribution of proteins such as V and C suggests that they provide several functions useful to the virus.

Nucleocapsid Protein

The major NP is 509 to 557 amino acids in length and the amino acid sequence is relatively well conserved. The NP encapsidates genomic and antigenomic RNA, with each NP monomer associating with six nucleotides (this corresponds to 2577 and 2564 molecules of NP per nucleocapsid for hPIV3 and Sendai virus, respectively). RNA replication is dependent on cosynthetic encapsidation of the nascent RNA by NP. This protein can participate in nucleocapsid formation only when it is available in a soluble complex with P; if expressed on its own, NP self-assembles and is not available to form authentic nucleocapsids (17, 117). The N-terminal 75% of the molecule is the more highly conserved part. It is involved in forming the soluble complex with P as well as in subsequently associating with other NP monomers and with RNA to form the nucleocapsid (17, 56). The more variable C-terminal 25% of the molecule is not required to form the nucleocapsid but is essential for it to function as a template. This variable tail mediates association between the nucleocapsid and P (16).

Phosphoprotein

The P protein is an integral part of the transcriptase/replicase, but its exact functions in these processes have not been defined (14). The P protein is not highly conserved within a genus and not at all between genera. The P protein of respiroviruses (568 to 603 amino acids) is larger than that of rubulaviruses (392 to 399 amino acids) (see Table 1). In both genera, P forms a homooligomer by noncovalent association (Sendai virus) or disulfide bonds (some strains of NDV). Recent evidence indicates that this is a tetramer. The P protein consists of conserved amino- and carboxy-terminal halves separated by a divergent spacer that spans the editing site, and this division segregates the protein into two functional modules (55, 280, 305). The P protein is so named because it is phosphorylated, mainly at one or two serine residues in the N-proximal module (18, 19). Whether this serves a structural or regulatory role, or both, remains to be determined. Through its N-proximal module, P associates with soluble NP, which maintains NP in a soluble form that can participate in nucleocapsid formation (58, 117). The C-proximal module (a) contains the trimerization site (54), (b) mediates binding to the NP-RNA nucleocapsid (53, 257), and (c) mediates binding with soluble L to form the polymerase

complex (117). Thus, the N-proximal module is a chaperone for soluble NP, and the C-proximal module is essential for RNA synthesis (53). Approximately 300 copies of P are packaged in the Sendai virus particle.

C Protein

The C protein, found only in respiroviruses, is an abundant small basic protein that is not well conserved, is associated with the nucleocapsid, binds the L protein, and is packaged in small amounts in the virus particle (118,332). The C protein has been shown to inhibit transcription and RNA replication, an effect that acts mainly at the genomic promoter and might reflect a role for C in regulating recognition of cis-acting signals (20,118,293). The Sendai virus C ORF is initiated at an ACG codon to yield the C' protein, and initiation also can occur at each of the next three ATG codons to yield C, Y1, and Y2 proteins, in that order, from the 5' end (see Table 1). Also, initiation of Y1 and Y2 translational start sites occurs through a novel ribosomal shunt mechanism that does not require AUG codons (177). In comparison, hPIV1 encodes C' and C proteins and possibly Y1 protein, with C' arising from a GUG initiation codon. hPIV3 and bPIV3 encode only one C protein.

Ablation of expression of all of the C-related proteins of Sendai virus and the single C protein of hPIV3 strongly attenuated the replication of both viruses *in vitro* and *in vivo*, and point mutations in C also decreased replication *in vivo* (67,133,172). Ablation of Sendai virus C, but not C', was highly attenuating (172,177). That there would be an apparent functional difference between these two proteins was surprising, because C' contains all the amino acids of C, differing only by nine additional amino acids at its N-terminus.

Although the role that the C protein(s) plays in augmenting replication of virus *in vitro* and *in vivo* has not been defined, it might interfere with the host's innate immunity. Sendai virus C proteins interfere with the establishment of an interferon alpha- or beta-induced antiviral state, in part by inhibiting the production of the interferon-induced Stat1 transactivator protein (85,96). The Sendai virus C protein increases apoptosis when expressed intracellularly (132). The mechanism responsible for these two activities of the C proteins remains to be elucidated.

V, W, I, and D Proteins

The V protein consists of the upstream module of P attached to the cysteine-rich, conserved, carboxy-terminal V module. The V protein of the Rubulavirus SV5 is a component of the nucleocapsid and is packaged in the virion (229). In contrast, the Respirovirus Sendai virus V protein appears to be nonstructural. The V protein of Sendai virus binds soluble NP but not NP-RNA, as would be expected for a protein containing the upstream module of P. The Sendai virus V protein inhibits RNA replication, perhaps because it competes with the formation of NP-P complexes needed for encapsidation (119). The V protein of some, but not all, parainfluenza viruses also binds to the cellular damage-specific DNA binding protein, although the significance of this is unknown (181).

The W (Respirovirus) and I (Rubulavirus) proteins result when RNA editing shifts into a reading frame that terminates shortly downstream, and thus W and I essentially consist of the upstream module of P. W protein binds to soluble NP and inhibits RNA replication.

The D protein, which is found only in hPIV3 and bPIV3, is a fusion protein of the P (241 to 242 amino acids) and D (125 to 131 amino acids) ORFs (see Table 1). D is expressed from the P gene by the process of transcriptional editing in which two nontemplated G residues are added to the P mRNA at the RNA editing site.

The V protein of Sendai virus, like the C protein, is important in virus growth and pathogenesis *in vivo*. A series of recombinant Sendai viruses were constructed in which expression of V and W was ablated by inactivation of the editing site, or in which the expression of V was ablated separately by interrupting its ORF. Ablation of expression of V and W together produced viruses that were highly restricted in virus replication and in production of disease in mice (63,151,152). The effect of ablation of only V was less drastic, and a virus lacking V was not as attenuated as one lacking both V and W. This suggests that both V and W play an important role in supporting robust virus replication *in vivo*. Recombinant hPIV3 viruses were constructed in which the D or V ORFs were interrupted individually or together by introduction of translational stop codons and were assayed for replication in hamsters and primates. Silencing either ORF alone did not have an effect on replication *in vitro* or *in vivo*, but silencing both ORFs reduced replication *in vivo* by a modest but significant amount. This provides genetic evidence that both the D and V proteins are produced by hPIV3 and that they play a role in replication *in vivo*, although they clearly are not essential for replication (67).

The V protein also interferes with host innate immunity. This has been directly confirmed for SV5, where the V protein was shown to interfere with the establishment of an interferon-mediated antiviral state at the level of signal transduction (65). This indicates that different accessory proteins, namely, the V protein of the rubulaviruses and the C proteins of the respiroviruses, each function to counteract the antiviral activities of the interferon system.

X Protein

A small abundant nonstructural protein of unknown function called X, which has been identified only for Sendai virus, is generated by translational initiation at an AUG near the downstream end of the P ORF and contains the carboxy-terminal 95 amino acids of P (57).

Large (L) Protein

The L protein is a large, nonabundant protein that is the major polymerase subunit. It is a multifunctional protein responsible for catalytic steps of RNA synthesis, capping and methylation. The N-terminal half of L contains blocks of highly conserved amino acids that are thought to be functional domains. The N-terminal half of the molecule also is essential for the formation of a complex with P (225). Approximately 20 to 40 copies of L are present in the Sendai virus

particle.

Matrix Protein

The matrix protein (M) is a well-conserved, nonglycosylated protein that is located on the inner face of the virion envelope. M is thought to drive virion morphogenesis and mediate association between the nucleocapsid and the envelope. For example, intracellular expression of the M protein of hPIV1 alone can induce the budding and release of empty virus-like particles. Expression of NP and M together yields extracellular particles containing nucleocapsid-like structures (47). The Sendai virion contains approximately 3,000 copies of M.

Small Hydrophobic Protein

Among the parainfluenza viruses, only SV5 encodes an SH protein, although a comparable SH gene is found in mumps and RSV. SH is a transmembrane virion envelope protein in which the C-terminus comprises a very small ectodomain. The SH protein of SV5 can be deleted without any effect on virus growth *in vitro*, virion morphology, or fusion activity (108).

F Glycoprotein

The fusion (F) glycoprotein (Fig. 3) mediates penetration of the host cell by fusion of the viral envelope to the plasma membrane. Late in infection, when newly synthesized F glycoprotein has accumulated on the surface of the infected cell, it also can mediate fusion with contiguous uninfected cells, leading to (a) the formation of a syncytium and (b) spread of infection in the local area by this recruitment mechanism. Syncytium formation is a prominent feature of the cytopathic effect produced by viruses of the two genera in cell culture. The SV5 F protein can direct syncytium formation when expressed alone by a recombinant vector (120), but for all the respiroviruses and the other rubulaviruses studied to date, syncytium formation is insignificant unless the HN protein is coexpressed (175,197,267,268) (see later and Chapter 41). This suggests that in most instances, viral penetration requires coordinate attachment and fusion activities. Although it is thought that parainfluenza viruses attach to the host cell via sialic acid receptors, Sendai virus can also enter cells via an alternative pathway that involves binding of cleaved F_0 to the hepatocyte-specific asialoglycoprotein receptor. The significance of this alternative mechanism in pathogenesis of infection *in vivo* is not known (11).

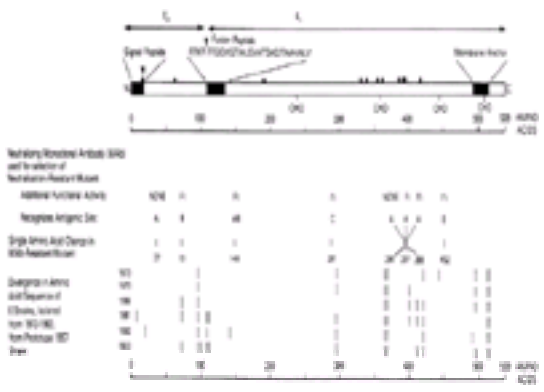

FIG. 3. Linear structure and antigenic organization of the hPIV3 fusion protein (prototype strain 47885, 1957). · denotes cysteine, *FI* denotes fusion-inhibition, and | denotes the site of amino acid difference. (From ref. 35, with permission.)

The F proteins of hPIV3 and other parainfluenza viruses are very similar in size (529 to 565 amino acids) and share a moderate level of sequence identity that is greater within the same genus, 63% for respiroviruses and 25% to 51% for rubulaviruses, than between genera, 16% to 21%. Among the different F proteins, there are two to six potential acceptor sites for N-linked carbohydrates whose number and location are moderately conserved within but not between the two genera. In optimal sequence alignments, nine of the ten cysteines present in the mature hPIV3 F protein are conserved in other viruses of the two genera. Also, proline and glycine residues, which are important determinants of folding, tend to be well conserved. Sequence alignments and structure predictions of the F proteins of the respiroviruses and rubulaviruses, as well as of the morbilliviruses and pneumoviruses, suggest that there are common features of secondary and higher-order structure (23,82,198) (Chapter 41).

The F protein is a typical type I glycoprotein (see Fig. 3). The N-terminus has a hydrophobic signal peptide of approximately 18 amino acids that mediates insertion into the rough endoplasmic reticulum during synthesis and is cleaved by signal peptidase (12). There is a second hydrophobic region near the carboxy terminus (amino acids 494 to 516 in hPIV3, the location being similar in other viruses of the two genera) that functions to anchor the protein in the membrane, and the carboxy-terminal 23 amino acids constitute the cytoplasmic tail of the F protein. The F protein alone appears to be sufficient to mediate association with the M protein and the nucleocapsid during virion morphogenesis (287). As is typical for membrane proteins, F is synthesized as an inactive precursor, F_0, in the rough endoplasmic reticulum, where the following modifications occur: glycosylation, cleavage of the signal peptide, folding, disulfide bond formation and rearrangement, and assembly into noncovalently associated homooligomers, which are probably trimers (191,198,199,201,256,266). The oligomer, which represents a single virion spike, is then transported through the exocytic pathway to the plasma membrane.

F_0 is converted into the fusogenic form following cleavage by a trypsin-like host endoprotease to yield two subunits, F_2 (amino acids 19 to 109 in hPIV3) and F_1 (residues 110 to 539 in hPIV3), which remain linked by a disulfide bond. The site of cleavage has been confirmed by direct sequence analysis of the amino terminus of F_1 of both hPIV3 and Sendai virus

(89,283). The cysteine residues of Sendai that link the two subunits are residues 70 (the only cysteine in F_2) and 199 (the N-terminal-most in F_1). Cleavage of F_0 is a prerequisite for virus infectivity, an important determinant of tissue tropism and pathogenesis. Cleavage results in a conformational change in the molecule, as assayed by changes in reactivity with monoclonal antibodies, ultraviolet circular dichroism spectrum, and detergent binding (123,165,318).

The F_1 amino terminus created by cleavage is an internal hydrophobic region of the F_0 protein called the fusion peptide (amino acids 110 to 135 in hPIV3, its location being similar in the F proteins of other paramyxoviruses). This is the most highly conserved sequence in the F protein, having exact conservation among viruses of the two genera at 12 of the first 26 positions (Chapter 41). Four lines of evidence support the view that the fusion peptide is important in fusion and probably functions by direct insertion into the target membrane. First, the high degree of sequence conservation of the fusion peptide is consistent with its having a direct functional role, and its hydrophobicity and alpha helical character are also suggestive of membrane insertion. Second, cleavage, which liberates the fusion peptide as the amino terminus of F_1, is accompanied by a conformational rearrangement of the molecule and an increase in its hydrophobicity (123,165,318). This suggests that cleavage activation is linked to the liberation of the hydrophobic fusion peptide. Third, synthetic peptides mimicking the amino terminus of F_1 inhibit fusion by Sendai virus, SV5, and the morbilliviruses measles and canine distemper viruses (253,254). Fourth, site-specific mutations that should increase the alpha helical nature of the fusion peptide of SV5 augmented its fusogenicity (120), a result that is fully compatible with the proposal that the fusion peptide is directly involved in membrane penetration.

Not surprisingly, other regions of F_1 also appear to play a critical role in the fusion process. For example, three neutralization-resistant mutants of Sendai virus selected by different F-specific neutralizing, fusion-inhibiting monoclonal antibodies did not have a mutation within the fusion peptide (238). Instead, each mutant sustained an amino acid substitution distant from this region, namely, at amino acid residue 399. One of the monoclonal antibodies bound a synthetic peptide whose sequence spanned the mutation site, confirming that it was part of the epitope. These findings suggest that a region distant from the fusion peptide in the linear sequence of the molecule either plays an important role in positioning the fusion peptide correctly or becomes located close to it in the mature folded molecule so that fusion can be inhibited directly by an antibody that binds to the distant sequence around amino acid 399. A model for F protein function has been described (175) that shows its similarities to the penetration proteins of other viruses such as influenza virus. It is now thought that two regions containing heptad repeats, one of which abuts the hydrophobic fusion peptide and the other located adjacent to the transmembrane domain, form a stable trimer of heterodimers that is required for proper folding of the mature fusion protein structure (140). This heptad repeat structure is actively being studied as a target for action of antiviral compounds.

The F proteins of the two genera fall into two groups with regard to cleavage phenotype (Table 3). The F_0 proteins of most of these viruses, including hPIV3, bPIV3, hPIV2, SV5, and virulent strains of NDV, are readily cleaved in most cell types. Cleavage is mediated by intracellular protease(s) in the Golgi compartment or trans-Golgi network of the exocytic pathway (259). This protease appears to be nearly ubiquitous, conferring a broad host range, although different cells can vary in the efficiency with which cleavage occurs, and a few cell types appear to lack the necessary protease (241). The protease (or one of several possible proteases) responsible for activation has been presumptively identified as furin, a calcium-dependent serine protease that is a component of the constitutive exocytic pathway. This is supported by the observations that cleavage of the F protein of virulent NDV does not occur in furin-defective cells (220) and that cleavage can be mediated by a recombinant vaccinia virus expressing furin cDNA in these cells (95,222). Furin cleaves on the carboxy-terminal side of the motif Arg/Lys-X-Arg/Lys-Arg, which is consistent with the cleavage sites of most of the intracellularly cleaved F_0 proteins. Following cleavage, the basic residues of the cleavage site are removed by further proteolysis (165).

	Six amino acids immediately preceding the fusion peptide					
Virus	-6[a]	-5	-4	-3	-2	-1
Sendai (murine PIV1)[a]	Gly	Val	Pro	Gln	Ser	Arg↓
hPIV1[a]	Asp	Asn	Pro	Gln	Ser	Arg↓
NDV (several avirulent strains)[a]	Gly	Gly	Arg	Gln	Gln	Arg↓
	Glu		Lys		Ser	
NDV (12 virulent strains)[b]	Gly	Arg	Arg	Gln	Arg	Arg↓
					Lys	
hPIV3 (prototype strain and four clinical isolates)[b]	Asp	Pro	Arg	Thr	Arg	Arg↓
					Lys	
hPIV3 (three clinical isolates)[b]	Asp	Pro	Arg	Thr	Glu[c]	Arg↓
hPIV2 (prototype strain)	Thr	Thr	Arg	Gln	Lys	Arg↓
hPIV2 (nine clinical isolates with host-range restriction in vitro)[b]	Thr	Thr	Arg	Gln	Glu[c]	Arg↓
	Ala	Pro				
SV5 (canine PIV2)[b]	Thr	Arg	Arg	Arg	Arg	Arg↓

[a]Cleaved extracellularly by secretory protease
[b]Cleaved intracellularly by protease of the exocytic pathway
[c]Indicated residue in clinical isolate abolishes furin motif of prototype
[d]Position relative to the cleavage site indicated by ↓
Source: From refs. 8,13,37,93,193,195,210,283,288,310,311,324

TABLE 3. *Amino acid sequences of the cleavage peptides of parainfluenza (PIV) F_0 proteins*

In contrast, the F_0 proteins of Sendai virus, hPIV1, and the so-called avirulent (less pathogenic) strains of NDV are insensitive to intracellular proteases. The cleavage site of their F_0 protein consists of a single arginine residue preceding the fusion peptide, with the avirulent strains of NDV having a second arginine or lysine at position –4 (see Table 3) (13,93,310,311). A secretory endoprotease, which appears to have a primary role in activating Sendai virus and avirulent NDV *in ovo*, has been purified from eggs and identified as blood clotting factor Xa, a calcium-dependent serine protease that is secreted into the egg's allantoic and amniotic fluids (97,158,218). It cleaves on the carboxy-terminal side of arginine in the motif Glu/Gln-Gly/Ser/Thr-Arg. An activating secretory protease with a similar activity, named tryptase Clara in reference to the bronchiolar epithelium cells that produce it, has been isolated from rat lung (158,261,304). Sendai virus infection was shown to stimulate secretion of tryptase Clara (157). These same proteases are responsible for cleavage of the hemagglutinin protein of most strains of influenza A virus in most cell lines. In tissues that lack the appropriate secreted protease, infectious virus will undergo only a single round of replication because the parainfluenza

virions produced contain only F_0 and thus are noninfectious. They can be rendered infectious if F_0 is cleaved with exogenous trypsin. Analysis of Sendai virus mutants confirmed the critical role of the cleavage site in activation; mutants in which the arginine residue at the cleavage site had been replaced with isoleucine lost the ability to be activated efficiently by trypsin (131). That cleavage occurs extracellularly *in vivo* was confirmed for Sendai virus using an antipeptide antiserum specific for the cleavage region of F_0: It blocked cleavage sterically but had no effect on the activity of already-cleaved F protein. When administered *in ovo* or into the respiratory tract of rats, the antibody inhibited the production of infectious virus (301).

The presence of a furin-type cleavage site is not obligatory for intracellular cleavage. A Sendai virus mutant with the cleavage site Pro-Lys was readily cleaved intracellularly in many cell types. Other Sendai virus mutants, selected by passage *in vitro* in the absence of trypsin, gained the ability to be cleaved intracellularly as a result of amino acid changes at or near the cleavage site that did not involve arginine or lysine and included the loss of a nearby acceptor site for N-linked sugar in F_1 (299). Interestingly, sequence analysis of clinical isolates of hPIV3 revealed variation in the cleavage site sequence. Specifically, four of seven isolates similar to the prototype strain had the sequence Arg-Thr-Lys/Arg-Arg, which is consistent with furin cleavage, whereas three other isolates had the sequence Arg-Thr-Glu-Arg in which Glu at position –2 ablated the furin motif (Table 3) (322). Nonetheless, the latter strains were fully competent for replication and the production of infectious virus *in vitro* and in the respiratory tract of rhesus monkeys. This indicates that a complete furin motif is not necessarily obligatory for efficient replication *in vivo*. Also, several clinical isolates of hPIV2 showed differences in the cleavage sequence. Whereas the prototype strain has the furin motif sequence Arg-Gln-Lys-Arg, the sequence of a number of clinical isolates was Thr/Pro-Arg-Gln-Glu-Arg, which is very similar to the Glu substitution pattern described previously for hPIV3 (see Table 3). In this case, restricted growth *in vitro* was observed in simian Vero cells but not in primary cultures of primate cells. This suggests that additional unknown proteases are involved in processing the F protein of human parainfluenza viruses in primate cells. The structural requirements for cleavage and virulence can now be addressed using reverse genetics to systematically modify the cleavage site.

The efficiency with which intracellular cleavage occurs can vary among different F_0 proteins, presumably because of the subtle structural differences mentioned. Reduced cleavability is associated with reduced host range *in vitro*, presumably because of differences in protease availability among different cell types. For example, an avirulent NDV strain that had a site that should be compatible with efficient cleavage by furin (Gly-Lys-Gln-Arg-Arg) was cleaved in bovine kidney tissue culture cells but not in canine kidney cells, baby hamster kidney cells, or primary chick embryo cells (241). Thus, there is a broad gradient of “cleavability,” determined largely but not exclusively by the cleavage site sequence, which can confer differences in cell tropism *in vitro* and *in vivo*.

HN Glycoprotein

The hemagglutinin-neuraminidase glycoprotein (Fig. 4) mediates the first step in infection, namely virus adsorption, by attachment to cellular surface receptors that are sialic acid–containing glycoproteins or gangliosides (184,185 and 186). This activity is responsible for the ability of the virus to agglutinate erythrocytes, which is employed in serodiagnosis and experimental laboratory studies. HN also functions late in infection to cause enzymatic cleavage of sialic acid (neuraminic acid) residues on the virus and on the infected cell surface that must be removed to prevent self-aggregation of virus particles during release from infected cells and to prevent their reattachment to the infected-cell surface. Among the mononegaviruses, only the respiroviruses and rubulaviruses have a neuraminidase.

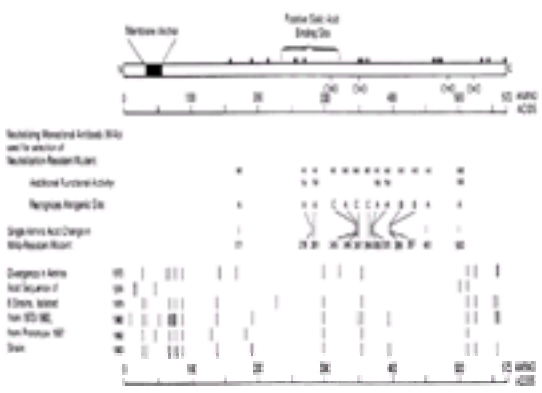

FIG. 4. Linear structure and antigenic organization of hPIV3 hemagglutinin-neuraminidase protein (prototype strain 47885, 1957). Putative sialic acid binding site refers to neuraminidase domain. · denotes cysteine; *HI* denotes hemagglutination-inhibition; *NI* denotes neuraminidase-inhibition; | denotes site of amino acid difference. (From ref. 35, with permission.)

The dual functions of HN appear to be modulated by halide ion concentration and pH (194). Hemagglutination activity appears to be favored by the halide ion concentration and pH of the extracellular environment, consistent with the role of HN in binding to extracellular receptors, whereas neuraminidase activity is optimal at lower pH and halide ion concentration, consistent with the role of HN in stripping sialic acid from newly formed viral and host-cell glycoproteins during transport to the cell surface. The depletion of sialic acid residues on cell surface proteins is thought to facilitate release of newly budded virus.

For almost all respiroviruses and rubulaviruses examined to date, the HN protein also participates in fusion (175,197,267,268) (Chapter 41). For example, syncytium formation by cells expressing the F protein is greatly augmented by coexpression of its cognate HN. The participation of HN might simply involve its attachment function, perhaps by positioning the target membrane at a precisely defined optimal distance, or a more direct role which remains

to be defined.

The hPIV3 HN protein (see Fig. 4) is typical for the viruses of the two genera. It is 572 amino acids in length, compared to 565 to 582 amino acids for the other viruses. It has four potential sites for addition of N-linked carbohydrate side chains; other viruses of the genera have five to ten sites (counting only those that are located in the extracellular domain and whose sequences are compatible with utilization), whose approximate locations tend to be similar. The amino acid sequence of HN is moderately conserved, with hPIV3 having 77%, 48%, 49%, 22%, 23%, 22%, or 23% identity, with the HN proteins of bPIV3, hPIV1, Sendai virus, hPIV4A, mumps virus, NDV, or SV5, respectively. The locations of most of the cysteine residues (of which there are 14 in the HN of hPIV3) are highly conserved in optimal sequence alignments, as are many of the proline and glycine residues. This suggests that these surface glycoproteins have a similar tertiary structure.

Amino acid sequence comparisons of the HN proteins of the two genera indicate that they share regions of linear, segmented sequence identity (40,82,139,154,198). The sequence, Asn-Arg-Lys-Ser-Cys-Ser (residues 252 to 257 in hPIV3), is conserved exactly among the other HN proteins (35,40,139). This motif is located within a 90-amino-acid region (residues 234 to 323 in hPIV3) that has been proposed to represent part of the sialic acid binding site of the neuraminidase domain (35,40,139). The predicted secondary structure of this region is similar to that identified as the sialic acid binding site of influenza A viral neuraminidase by x-ray crystallography (41). Consistent with this observation, substitutions introduced into the NDV HN cDNA involving the Asn, Arg, Lys, or the N-proximal Ser greatly decreased the neuraminidase activity of the expressed protein, and some of the substitutions reduced reactivity with monoclonal antibodies that inhibited neuraminidase activity (197). In contrast, a second conserved sequence in NDV (corresponding to positions 408 to 413 in hPIV3) was shown to be important in maturation: Substitutions did not affect its biologic functions but frequently interfered with folding (267).

The HN protein is anchored in the membrane by a hydrophobic region of about 25 amino acids in length that is located near the amino terminus. This serves as a noncleaved signal sequence that both mediates insertion into membrane and anchors the protein, leaving the amino-terminal 31 amino acids (in the case of LPIV3) cytoplasmic and the remaining amino acids extracellular (type II orientation). Folding and oligomerization into tetramers occur concurrently within the rough endoplasmic reticulum (39,191,198,201). For some (e.g., most NDV strains) but not all (e.g., hPIV3) viruses of the two genera, the oligomeric monomers are stabilized by intermolecular disulfide bonds.

The higher-order structure of the HN protein has been studied in greatest detail for Sendai virus. The HN tetramer, which presumably represents a single virion surface spike, appears to contain several domains: the cytoplasmic tail, the transmembrane anchor, a stalk that is sensitive to trypsin cleavage, and a globular head that represents most of the extracellular domain and contains most of the conserved amino acids. The globular head retains all of the biologic activities and the major antigenic sites, and the stalk probably is a support structure. When visualized by electron microscopy, solubilized Sendai virus HN tetramers (prepared by proteolytic removal of the N-terminal 131 residues) have a box-shaped head (10×10 nm) in which the four identical subunits exhibit fourfold symmetry (306). Interestingly, the shape and dimensions of the box-shaped head of HN are similar to those of the head of the influenza A virus neuraminidase tetramer. Based on this general similarity in organization and on the identification in the HN proteins of conserved amino acids corresponding to active-site-residues in the influenza virus neuraminidase, it is thought that the three-dimensional structures of the two proteins are broadly similar (40,139).

The HN protein of some avirulent strains of NDV is synthesized as a longer precursor, HN_0, in which the functions of the hemagglutinin and neuraminidase are inactive (215). Activation requires an endoproteolytic cleavage, which results in the loss of a small, 9-kd glycopeptide from the carboxy terminus and, as with F_0, a change in conformation (165,215,265,318). Like the F_0 protein of these strains, HN_0 is resistant to cleavage in most cell types and presumably is cleaved by extracellular secretory proteases, but unlike F_0 it does not have a marked preference for trypsin and can be activated *in vitro* by a variety of proteases (215). Sequence analysis suggests that HN_0 is a longer (616 amino acids), ancestral version of the shorter (571 or 577 amino acids), noncleaved HN proteins of virulent strains. The shorter ORFs of the virulent strains might have arisen by the introduction of translational stop codons into an ancestral 616-codon ORF. This was suggested by the finding that the ORFs of the virulent strains retained the apparent relic of an in-frame C-terminal extension beyond the nonsense codon(s) terminating the current ORF. The introduction of an appropriately spaced nonsense codon into an HN_0 ancestor would yield a shorter form that does not require activation. Consistent with this idea, most of the stop codons of the shorter forms line up with potential Arg cleavage sites in HN_0 (196). A counterpart to HN_0 has not been described for any other paramyxovirus, although the sequence of the HN gene of hPIV4 has been interpreted as containing a relic of such an extension (9).

Antigenic Composition and Determinants

Antibodies against most or all of the different major species of parainfluenza virus proteins can be detected in postinfection sera from animals and humans. However, the HN and F proteins are the only antigens that have been shown to induce antibodies that neutralize infectivity as measured by inhibition of plaque formation, hemadsorption, or cytopathic effects in cell culture. *In vivo*, the parenteral administration of polyclonal or monoclonal antibodies specific to Sendai HN or F mediates resistance to challenge with Sendai virus (223,224,246). Infection of rodents with vaccinia virus recombinants expressing the HN or F glycoprotein of hPIV3 or SV5 induces a high level of resistance in the lungs to challenge with the respective virus (231,282). Similarly, immunization with purified hPIV3 HN and F glycoproteins induces a high level of immunity in cotton rats, guinea pigs, and hamsters (4,74,246,247). The ability of the hPIV3 HN and F glycoproteins to induce a protective response is unaffected by deglycosylation (73). In contrast to the strong protective immunity induced by HN and F glycoproteins, immunity to the other proteins is a weaker immunity of short duration (290).

Antibodies to HN can be measured by HI and neuraminidase-inhibition (NI) assays, which are based on the ability of the antibodies to block these activities of purified virions *in vitro*. Antibodies specific to the F protein can also be measured by inhibition of syncytium formation (fusion inhibition) in cell culture, as well as by inhibition of hemolysis of erythrocytes

(hemolysis) by purified virions *in vitro*. However, HN activity, in addition to F activity, is required for efficient hemolysis and syncytium formation by most respiroviruses and rubulaviruses, so that HN-specific antibodies also inhibit these activities (168). In addition, antibodies to each of the major virus proteins can be measured by immunoprecipitation (129).

The antigenic and functional organization of the HN of hPIV3, Sendai virus, and NDV have been defined in some detail through (a) a study of the interaction of monoclonal antibodies with virus and (b) sequence analysis of spontaneous antigenic mutants resistant to neutralization by the monoclonal antibody used for their selection. Initially, a panel of 19 monoclonal antibodies was used to analyze the hPIV3 HN, presently the best understood of the human Respirovirus attachment glycoproteins (36). A topologic map of hPIV3 antigenic sites and an operational map of the epitopes within each site were derived from competitive-binding assays of pairwise combinations of antibodies (321). Six antigenic sites were defined; five of the sites (A, B, D, E, and F) did not overlap, whereas site C overlapped sites A and B. Similar analysis of Sendai virus and NDV identified four antigenic sites on the HN of each virus (127,183,217,223,237,335).

Three of the hPIV3 HN sites (A, B, and C) are of particular interest because they react with monoclonal antibodies that neutralize virus and inhibit hemagglutination (36,322). Additional information concerning the antigenic structure of the hPIV3 HN has been provided by analysis of neutralization-resistant mutants selected with these neutralizing monoclonal antibodies. The reactivity pattern of a panel of hPIV3 HN monoclonal antibodies with the mutants, as well as a collection of 38 hPIV3 strains recovered from 1957 to 1983, indicated that the six antigenic sites contain at least 14 distinct epitopes (35,36). Eleven of these epitopes are involved in neutralization; seven are present in site A, two in site B, and two in site C. Neutralizing and HI monoclonal antibodies directed at five of the site A epitopes also inhibit neuraminidase activity, although several Sendai virus HN monoclonal antibodies were described that inhibit neuraminidase activity but not hemagglutination. Thus, the sites involved in the two activities probably overlap substantially but are not exactly identical (183,235,237).

The neutralization epitopes of hPIV3 HN have been provisionally located on the molecule by sequence analysis of 13 neutralization-resistant mutants selected with monoclonal antibodies to site A, B, or C (see Fig. 4) (36). Each of the mutants sustained a single amino acid substitution that was located predominantly in the C-terminal half of the molecule. Different monoclonal antibodies directed to the same site (and in two instances to the same epitope) selected mutations widely separated on the HN molecule, suggesting that these sites and their epitopes are formed by juxtaposition of distant regions during folding of the molecule into its mature configuration (36,322). Similar observations have been made for Sendai virus HN (307). Consistent with this interpretation is the observation that boiling and reduction of hPIV3 or NDV HN markedly reduces its reactivity with a panel of monoclonal antibodies (35,127). NDV monoclonal antibodies to four distinct antigenic sites also bind very weakly to nascent HN molecules (191,217), and neutralization epitopes could not be mimicked by synthetic peptides (112), providing additional evidence that folding is required for the formation of antigenic sites. Many of the antigenic sites defined by the murine monoclonal antibodies for hPIV3 HN (and F) are recognized by antibodies in postinfection human sera (322).

The antigenic and functional organization of parainfluenza virus F glycoproteins have also been elucidated using the strategy employed for HN. Competitive-binding assay of 26 hPIV3 F-specific monoclonal antibodies identified seven nonoverlapping antigenic sites (A to F) and one site (AB) that bridged sites A and B (35). Fourteen neutralization epitopes were identified within sites A, B, C, and AB. The remaining sites each contain a single epitope that is not involved in neutralization, whereas site A also contains a single nonneutralization epitope.

Sequence analysis of antigenic mutants of hPIV3 selected by F-specific monoclonal antibodies that neutralize virus, and in some instances inhibit fusion, has provided a provisional localization of epitopes involved in these two antibody functions (see Fig. 3) (35,319). Neutralization epitopes in site A or B contain amino acid residues that are derived from both the F_1 and F_2 subunits of the molecule, suggesting that distant regions of the linear protein are folded into close proximity to create the mature antigenic sites (see Fig. 3). Consistent with this interpretation is the finding that NDV F monoclonal antibodies that neutralize virus and inhibit fusion activity fail to react with (a) nascent F or (b) mature F that has been boiled and reduced (309). The single amino acid substitution mutations that occur in fusion-inhibition-resistant hPIV3 mutants do not involve the fusion peptide (residues 110 to 135). Instead, the site of mutation is located upstream in F_2 (amino acid 73) or distantly downstream in F_1 (amino acid 287, 397, or 398) (see Fig. 3) (35). Similar observations have been made for Sendai virus and NDV F glycoproteins (238,309). The hPIV3 F antigenic site B that induces fusion-inhibiting antibodies appears to be highly conformational because a site B monoclonal neutralizing antibody selected two independent fusion-inhibition escape mutations that were widely separated on the linear protein, residues 73 (F_2) and 452 (F_1).

Early studies of sera from infants infected with hPIV1, 2, or 3 indicated that related antigenic determinants are present on their HN proteins even though these viruses are considered to be distinct serotypes (44). These relationships among the human PIV types serve to confound serologic diagnosis and seroepidemiology. Usually, the primary response to parainfluenza virus HN is relatively specific for the infecting virus serotype, whereas the response to subsequent infection by a heterotypic virus of the group (or a booster response to the same serotype) is more cross-reactive. hPIV1 is most closely related to its murine counterpart, Sendai virus, with which it shares antigenic determinants on F, NP, and M as well as HN (100,129,168). hPIV2 is also most closely related to its canine counterpart, SV5, through shared antigens on HN, F, NP, P, and M (154). hPIV3 and bPIV3 exhibit the closest antigenic relationship of any human paramyxovirus and its animal virus counterpart (1). For example, these viruses differ only fourfold in reciprocal HI tests. Their proximity, when analyzed by HI (or neutralization as well), can be explained by the fact that the viruses share 2 of the 11 neutralization epitopes present on HN (36). Three of the 14 neutralization epitopes on F are also shared (319).

Although hPIV1, 2, and 3 each appear to be monotypic, some degree of polymorphism within a serotype undoubtedly exists. This has been demonstrated most convincingly for hPIV3. Analysis of 38 hPIV3 strains recovered over a 26-year interval in widely separated locations (United States and Australia) indicated that 6 of the 11 neutralization epitopes on HN, as well as 3 of 14 such epitopes on F, were completely conserved among all strains (35,36). Two of the three neutralization sites on HN, as well as two of four such sites on F, had one or more epitopes that were completely conserved. The observed variation in HN and F neutralization epitopes did not involve progressive accumulation of

changes with time because variation detected in early isolates was not consistently conserved in later strains. Rather, it appears that the heterogeneity that exists results from random mutations that are not subject to strong immunologic selective pressure (35). Selective pressure of preexisting immunity to hPIV3 is probably much less than that observed for influenza A viruses because hPIV3 can reinfect previously infected individuals with high efficiency, thus allowing the virus to survive in nature without a pressing need for variation in protective viral antigenic sites. Sequence analysis of hPIV1, hPIV2, and NDV has documented the apparent progressive accumulation of sequence differences, resulting in distinct "lineages," but antigenic changes have been noncumulative and nonprogressive, and they do not correlate with the genetic lineages (111,113,114,168,245,260).

Infection of mice with Sendai virus induces development of major histocompatibility class I–restricted, CD8+ cytotoxic T lymphocytes (CTLs) that are associated with resistance to reinfection (122,149,150). In one inbred strain, CTLs from Sendai-infected animals lysed target cells infected with a vaccinia virus recombinant expressing NP, but not those expressing the F, HN, M, or P/C protein (150). Thus, in this particular strain and among these proteins, only NP was a significant target antigen. Assay of synthetic peptides spanning the entire NP sequence identified residues 321 to 336 as the single immunodominant epitope (150). Immunization of mice with a peptide bearing this epitope induced levels of CTL equivalent to those induced by infection with Sendai virus, and it induced a high level of resistance to virus challenge (150). It should be noted that the CTL response and the immunity it provides is usually transient, whereas immunity provided by virus-specific antibodies is considerably more durable (290). It is likely that other mouse haplotypes recognize different or additional CTL epitopes. The roles of viral proteins in inducing other components of cellular immunity remains to be explored.

Normal adult humans tested for memory CD8+ CTL against hPIV1 demonstrated strong responses to HN, P, and NP (and fragments of NP as well as the previously mentioned peptide-borne epitope), a weak response to M, and an insignificant response to F (59). In the same study, donor CTLs that had been stimulated *in vitro* with hPIV1 showed high reactivity with the closely related Sendai virus and, remarkably, lower but clearly demonstrable reactivity against hPIV3. Memory CD4+ CTLs also have been detected in humans (277), although CD4+ and CTLs were not a significant part of the response of normal mice following primary infection (122).

Propagation and Assay in Cell Culture

The human parainfluenza viruses grow well in primary simian or human kidney cell cultures, which allows efficient recovery of these viruses from clinical specimens (25,26,28,31). LLC-MK2, a rhesus kidney heteroploid cell line, and NCI-H292, a human lung carcinoma line, have also been shown to be efficient for recovery and growth of the human parainfluenza viruses (22,78). In these cells, trypsin (1 to 2 µg/mL) is added to the medium to recover hPIV1, but this is not necessary for LPIV2 or hPIV3 (22,78). Virus infection of tissue culture can be monitored either by hemadsorption or by immunofluorescence staining. hPIV2 and hPIV3, but not hPIV1, produce a cytopathic effect that is characterized by syncytium formation, particularly in heteroploid cell lines. hPIV3 can readily be quantitated by plaque assay or by limiting dilution and direct observation of cytopathology, whereas plaque production or growth following limiting dilution by the other human parainfluenza viruses usually is visualized by hemadsorption or immunostaining. Typical yields in tissue culture for hPIV1, 2, 3, 4A, and 4B are $10^{6.5}$, $10^{6.5}$, $10^{7.8}$, $10^{5.7}$, and $10^{3.7}$ tissue culture infectious dose$_{50}$ ($TCID_{50}$) per milliliter of medium, respectively (22).

Nature of Cell Injury

The nature of cell injury *in vivo* is not fully understood and seems to pursue different pathways for different parainfluenza viruses. Some viruses, particularly SV5, can cause a persistent, productive infection in primary cell culture that does not kill cells or shut off cellular RNA or protein synthesis (33,116). In contrast, syncytium formation leading to cell death is a prominent feature of infection of heteroploid cell cultures with hPIV2 or 3 (24,25,29,43). A third situation has been observed for Sendai virus, which induces programmed cell death (87,313). Interestingly, a recombinant Sendai virus was constructed that serendipitously did not induce apoptosis, and this mutant readily established persistent infection *in vitro* (87). It has been proposed that the response of the host cell membrane to a virus of either genus plays a role in determining whether that virus initiates a moderate infection, as in primary monkey cells, or induces formation of syncytia leading to cell death, as occurs in heteroploid cells (116). Syncytium formation is thought to be a major cause of cell death when tissue cultures are infected with hPIV3. Syncytium formation following infection can be blocked by treatment of the cell monolayer with neuraminidase, indicating that fusion by hPIV3 is highly dependent on its attachment function. Blocking cell fusion prevents the development of cytopathic effects and leads to the establishment of a persistent infection (200). On the other hand, in humans, the pathology of fatal parainfluenza virus disease usually does not include giant-cell formation unless the patient has a severe defect in T-lymphocyte function (2,62,135) or is profoundly immunosuppressed (326). A possible role for apoptosis as an important cause of cell death *in vivo* remains to be investigated.

Infection in Experimental Animals and Other Laboratory Hosts

Hamsters are very sensitive to infection by hPIV1, 2, or 3 (44,45,48,290). Infection is, however, usually asymptomatic, and pulmonary pathology is minimal or undetectable. Guinea pigs, cotton rats, and ferrets also undergo a silent infection with these viruses. Chimpanzees and a variety of monkeys can be infected with hPIV1-3, but only hPIV3 causes symptomatic illness that occurs in both chimpanzees and African green monkeys (34,69). The absence of significant disease manifestations in animals other than chimpanzees and the mild disease observed in the latter host species reflects reduced replication of the virus in these experimental animal models.

Some strains of hPIV1, 2, or 3 replicate in the embryonated chicken egg, whereas others do not (29). To date, eggs appear to be less sensitive for isolation of virus from patients than are monkey kidney cell cultures. Primary isolation of a parainfluenza virus in embryonated eggs has not been reported, with the exception of Sendai virus and three strains of hPIV2.

hPIV1, 2, and 3 multiply slowly after inoculation into the amniotic cavity of 7- to 8-day-old embryonated eggs; to obtain maximal titers, incubation at 34° to 36°C for 5 to 7 days is required (29). hPIV3 grows only in the amniotic cavity, whereas one strain of hPIV2 and several strains of hPIV1 have been adapted to the allantoic cavity of the egg. Adaptation of primary isolates of Sendai virus to eggs or tissue culture cells is often accompanied by reduction in virulence for mice.

GENETICS AND REVERSE GENETICS

Genetics

As a group, RNA viruses exhibit a high frequency of mutation attributable to the lack of a 3'-5' exonuclease “proofreading” mechanism in their RNA polymerase (284). This favors the development of genetically heterogeneous virus populations. For hPIV3, the frequency of a spontaneous mutant resistant to neutralization by an individual monoclonal antibody used for its selection has been estimated to be $10^{-4.6}$ to $10^{-7.3}$; for example, in one study, 10 of 13 mutants occurred at an estimated frequency of $10^{-6.0}$ or greater (321). Because the detection of such mutants is subject to the requirement that they be viable and be able to produce a plaque, the actual misincorporation rate is probably higher. For vesicular stomatitis virus (family *Rhabdoviridae*), the misincorporation frequency has been estimated experimentally to be 10^{-3} to 10^{-4} (284).

The error-prone nature of RNA synthesis provides the potential for rapid evolution, and indeed, viruses of the two genera readily “evolve” under selective pressure *in vitro*. However, these viruses appear to evolve very slowly in nature. Sequence analysis of NDV strains isolated over a 50-year period identified what appears to be a progressive accumulation of nucleotide changes, but this was not accompanied by progressive antigenic change (260). The hPIV3 F and HN glycoproteins have undergone little variation (1.5% to 2.4%) during the four decades since the first strain was recovered from humans (27,28,36). Variation has not been progressive, and the differences have been significantly less than those described for the H1 hemagglutinin of influenza A virus that had undergone antigenic drift over a 7- to 8-year interval (250).

The various viral proteins exhibit different rates of change as assayed by the extent of divergence between related viruses. For example, divergence between hPIV3 and Sendai virus is greatest for the C and P proteins (77% and 75% differences in amino acid sequence, respectively), less for F and HN glycoproteins (59% and 53% differences, respectively), and least for the NP, M, and L proteins (41%, 39%, and 38% differences, respectively) (72,83,280,283). Similarly, divergence of human and bovine PIV3 proteins is greatest for the D ORF and least for the M protein (7). Relationships among the viruses based on amino acid sequence divergence are consistent with those determined by serologic assays, though the latter method is less sensitive in revealing relatedness (94,168).

Extensive studies of nonsegmented negative-strand RNA viruses have failed to demonstrate homologous recombination (98,310,330). However, one form of recombination that involves polymerase jumping does occur. When copying the genome or antigenome RNA strand, the polymerase can disengage from the template and, with the nascent strand still attached, reinitiate elsewhere on the same or a different template and resume synthesis. This is the origin of the defective interfering (DI) genomes, which contain large internal deletions eliminating most of the viral genome as a result of the polymerase jump. DI genomes cannot supply the necessary viral proteins to support their own replication, but they can be propagated when virus is passaged at a high multiplicity of infection, conditions under which the nondefective virus supports DI genomes in trans. Because they compete for viral proteins, DI RNAs typically interfere with the replication of nondefective genomes (see Chapter 41 for a more complete description).

Initially, genetic analysis of respiroviruses and rubulaviruses was performed by chemical mutagenesis of infectious virus followed by identification of mutants that exhibited an identifiable property such as temperature sensitivity. Like other nonsegmented negative-strand RNA viruses, certain pairs of mutants bearing distinct lesions exhibited complementation during coinfection. Before the genetic map of Sendai virus had been established by sequence analysis, characterization of 10 conditional lethal, temperature-sensitive (ts) mutants suggested the existence of seven complementation groups (236). However, the exact number of viral genes defined by these complementation groups could have been less than seven because of the possible occurrence of intracistronic complementation. In most instances, later sequence analyses of these and other paramyxovirus mutants did not provide unambiguous identification of the site of mutation that specified the observed mutant phenotype. In these instances, it was difficult to differentiate such mutations from incidental silent mutations that also occurred during passage. The lack of homologous recombination was another obstacle to genetic analysis. Therefore, it was necessary to develop a method for introducing defined site-specific changes into the viral genome. This was accomplished by the recovery of infectious virus from viral cDNA.

Reverse Genetics

Recently, methods for producing infectious recombinant virus from cDNA have been developed for a number of parainfluenza viruses, including Sendai virus, hPIV3, bPIV3, SV5, and NDV (70,86,109,115,233). In the case of the parainfluenza viruses, this involves cotransfecting cells with plasmids encoding a complete genome or antigenome RNA and the NP, P, and L proteins. Expression of the plasmids is driven by bacteriophage T7 RNA polymerase, which can be supplied by a vaccinia virus recombinant or by constitutive expression in an engineered cell line. These viral components self-assemble into a nucleocapsid, which then launches a productive infection. The recovery of infectious virus typically is not very efficient, probably reflecting inefficiency of nucleocapsid self-assembly under these conditions. In natural infection, the assembly of nucleocapsids is thought to involve tight coordination between RNA synthesis and concurrent encapsidation, and it had been generally assumed that preformed genomic or antigenomic RNA would be unable to assemble into a nucleocapsid. However, this was disproved by the demonstration that Sendai virus genomic or antigenomic RNA synthesized *in vitro* could be transfected into cells expressing NP, P, and L and recovered in infectious recombinant virus (153). Whether genomic or antigenomic RNA is introduced by transfection or expressed from a plasmid, recovery of infectious virus is reliable, and, once recovered, recombinant virus can be propagated in the same

fashion as biologically derived virus.

In a second type of reverse genetics system, the cDNA-encoded genome or antigenome RNA is replaced by a minireplicon, which typically is a synthetic version of a naturally occurring DI RNA or is a truncated version of genome or antigenome RNA in which the viral genes have been deleted and replaced by one or more marker genes such as chloramphenicol acetyl transferase or luciferase. When complemented by NP, P, and L supplied by either coinfecting standard virus or transfected plasmids (21), the minireplicon is encapsidated and undergoes efficient RNA replication and transcription, which typically is monitored by Northern blot hybridization. A minireplicon system has advantages for certain studies because it is more amenable to mutational analysis and experimental manipulation *in vitro* than is complete recombinant virus.

The ability to insert predetermined changes into infectious virus makes it possible for the first time to manipulate mononegaviruses in a directed fashion, thus revolutionizing studies of molecular genetics and pathogenesis as well as efforts to develop new or better vaccines. For example, this technologic advance made possible the identification of the rule of six and permitted detailed characterization of cis-acting RNA signals (see earlier section, Genome and Encoded mRNAs and Proteins). It also made it possible to demonstrate that parainfluenza viruses can accommodate and express a foreign gene without loss of viability when the gene is placed under the control of gene-start and gene-stop signals and inserted into the viral gene order. This very likely will have important applications in vaccine development (see later section, Prevention and Control). Chimeric recombinant hPIV3 has been constructed in which the HN and F proteins were replaced with those of PIV1, or in which the NP protein was replaced with that of bPIV3 (6,291). These chimeras might also prove useful in vaccine development (see later section, Prevention and Control).

In other studies, the function of certain viral proteins has been investigated by interfering with their expression by silencing translational start sites, introducing stop codons, altering the editing site, or deleting the gene altogether. These studies showed that the accessory proteins of respiroviruses and rubulaviruses, namely the C, V, D, and W proteins, contribute to efficient replication *in vitro*, *in vivo*, or both (see earlier section, Properties of the Proteins).

PATHOGENESIS AND PATHOLOGY

The hPIVs replicate in the epithelial cells that line the respiratory tract, causing rhinitis, pharyngitis, laryngitis, tracheobronchitis, bronchiolitis, and pneumonia. Early during parainfluenza virus infection, the mucous membranes of the nose and throat are involved. Obstruction of the paranasal sinuses and eustachian tube may also occur and lead to sinusitis and otitis media. Many patients with mild disease may have limited involvement of the bronchi as well. n more extensive infections there is a tendency for hPIV1 and 2 to involve the larynx and upper trachea, resulting in the croup syndrome; such infections may extend also to the lower trachea and bronchi, with accumulation of inspissated mucus and resultant atelectasis and pneumonia (Table 4) (205,227,228). When hPIV3 produces severe disease, infection of the small air passages is likely, with the development of bronchopneumonia, bronchiolitis, and/or bronchitis (see Table 4) (227,228,328).

Illness	Number tested	Patients with evidence of infection with virus indicated (%)							
		RSV	Para 3	Para 1	Para 2	Any Para[a]	Flu A H3N2[a]	Flu A[a] H3N2	Flu B
Pneumonia	1,161–1,742	25.0	11.2	3.5	1.6	14.4	3.5	5.4	1.0
Bronchiolitis	873–1,186	43.1	9.4	2.4	1.1	10.9	0.9	2.5	0.4
Croup	586–776	9.0	18.3	23.3	12.2	41.4	7.7	24.1	1.9
Pharyngitis/bronchitis	896–1,337	10.6	11.0	3.7	2.0	14.7	2.0	4.6	0.9
Total respiratory	3,520–5,104	23.3	11.5	6.0	3.2	17.9	3.2	7.1	1.0
Inpatient control	1,237–2,155	5.4	5.0	1.9	1.2	7.5	0.5	0.9	0.5

[a]Infection documented by virus isolation and/or a complement-fixing antibody response.
[b]Studies performed at Children's Hospital National Medical Center from 1957 to 1976. Data from ref. 160 for RSV; ref. 161 for influenza; ref. 228 for PIV.
[c]Tested from 1957 to 1968.
[d]Tested from 1968 to 1976.

TABLE 4. *Infections*[a] *caused by parainfluenza, influenza, or repiratory syncytial virus in pediatric inpatientsl*[b]

An intensive longitudinal study of infants and children in a semiclosed nursery indicated that 80% of individuals undergoing primary infection with hPIV3 developed a febrile illness; in one third of the illnesses, there was involvement of the lower respiratory tract, resulting in either pneumonia or bronchitis (29). When infants and children were studied less intensively in a family setting, the estimate for lower respiratory tract involvement during primary hPIV3 infection was 13% (92). Longitudinal nursery studies also indicated that one half of initial hPIV1 infections and two thirds of initial hPIV2 infections produced a febrile illness (29). Lower respiratory tract involvement also occurs commonly during primary hPIV1 infection; about 25% of primary infections produce bronchitis or pneumonia (92). Severe acute laryngotracheobronchitis (croup), which is the most dramatic and serious manifestation of initial parainfluenza virus infection is noted in only 2% to 3% of primary hPIV1 or 2 infections (29,91).

Until recently, little was known about the mechanisms responsible for localization of infection within the respiratory tract and the severity of human parainfluenza virus disease. This has changed because comparative information has become available from studies of animal viruses such as Sendai virus and NDV in tissue culture and embryonated eggs, as well as in their natural hosts. In addition, evaluation of live attenuated virus vaccine candidates in nonhuman primates and clinical trials has provided new insights. The extent of virus replication appears to be a major factor in pathogenesis. For example, studies of candidate live attenuated hPIV3 vaccines in experimental animals and clinical trials indicate that a mutation that reduces virus replication *in vitro* always reduces virus replication and disease symptoms *in vivo*. A second important factor in pathogenesis is the location of infection (i.e., organ tropism). Studies on tropism indicate that (a) receptors for virus attachment are widely distributed and probably do not represent a major determinant, (b) the cleavage

phenotype of the F_0 protein and tissue-specific differences in protease availability appear to be major determinants, and (c) the cellular site of budding can be an important determinant. Other viral determinants include interference with the host's innate immunity mechanisms (e.g., with its interferon response as described). The host's adaptive immunologic response plays a role in restricting the virus to the respiratory tract and in controlling and eliminating infection, and it also very likely contributes to disease manifestations.

Because the major cell receptors for parainfluenza viruses are sialic acid–containing surface macromolecules of wide distribution, they are considered unlikely to be important determinants of tissue tropism. *In vitro*, these viruses commonly infect cell lines derived from various tissue types and nonhost species with considerable efficiency. This is consistent with a ubiquitous distribution of appropriate sialic acid receptors, though there is the caveat that immortalized cell lines might not be the best predictors of host range. For example, the pneumotropism of Sendai virus is not the result of an insufficiency of receptors on other tissues, as evidenced by the ability of the virus to initiate a single round of replication efficiently in explanted slices of various organs (130,300,302). Growth of mouse-passaged Sendai virus in eggs rapidly selected for one of two specific single amino acid substitutions in HN, which in either instance altered hemagglutinin and neuraminidase substrate specificities, and this appears to be important for adaptation to chick cells (134). However, this shift in receptor specificity did not alter the pathogenesis of these viruses in mice, which was interpreted as support for the view that HN is not a major host-range determinant.

In contrast, cleavage-activation of the F_0 precursor (and, for those viruses where it occurs, the HN_0 precursor) is a major determinant of tropism in animal models and in some instances in natural infection (213,214). As described, F_0 proteins exhibit a spectrum of cleavage phenotypes, ranging from that of Sendai virus, which is insensitive to intracellular cleavage and depends on an exogenous trypsin-like protease for cleavage, to those exhibited by some of the virulent NDV strains, which are readily cleaved intracellularly in most cell types. Naturally occurring strains of NDV that have poorly cleaved F_0 proteins (and, where they occur, HN_0 proteins) exhibit reduced virulence and in some instances have been used as live attenuated vaccine virus strains. In eggs, these attenuated viruses replicate productively only in the epithelial cells of the allantoic and amniotic cavities, where the factor Xa protease required for cleavage has been shown to be localized (218); in birds, such viruses are restricted to the epithelial lining of the respiratory or intestinal tracts. In contrast, NDV strains that have a readily cleaved F_0 protein can spread and replicate in many organs of the embryo or bird with concomitantly increased virulence. Synthesis of the HN protein as an inactive HN_0 precursor appears to confer attenuation in addition to that associated with poorly cleaved F_0 protein (215,260). This initial correlation between cleavability and tissue localization and virulence was described with the caveat that there were many other sequence differences in F and other proteins between the avirulent and virulent strains that also might have contributed to differences in virulence. For example, an additional difference in almost all of the avirulent strains was the substitution of leucine for phenylalanine at the amino terminus of the F_1 subunit (“the fusion peptide”) (93,310,311). Most recently, a study performed with recombinant NDV confirmed the importance of the cleavage site of F_0 in pathogenesis. Specifically, a recombinant in which the cleavage site was of the poorly cleaved variety and was followed by leucine was confirmed to be avirulent, whereas another recombinant that was identical except that the cleavage site contained the furin motif and was followed by phenylalanine was virulent (233).

The role of F_0 cleavage in localization and virulence was also demonstrated in studies involving various mutants of Sendai virus with altered protease-activation phenotypes. Sendai virus, like the avirulent strains of NDV, is restricted in replication *in ovo* to the allantoic and amniotic membranes and, in its natural host, the mouse, to the lumenal epithelium of the respiratory tract (261,298). Mutants that sustained a single amino acid change at the cleavage site, conferring the altered phenotype of cleavage activation by chymotrypsin or elastase and resistance to cleavage by trypsin, failed to replicate productively *in ovo* even at the usual sites unless the appropriate unnatural protease was added to the allantoic fluid (125,131,264). Similarly, trypsin-resistant mutants that were grown *in vitro* and activated with the appropriate unnatural protease could execute only a single cycle of replication (yielding nonactivated, noninfectious virus) when inoculated intranasally in mice (299). This indicated that the presence of the appropriate protease was important in localization of infection. Interestingly, one elastase-activated, trypsin-resistant mutant differed from the others and from the wild type in that it gained the ability to be cleaved in Madin Darby bovine kidney (MDBK) cells, apparently by utilizing a host intracellular elastase-like protease (299). This illustrates that the potential exists for a change in host range that results from a change in protease specificity.

Analysis of the Sendai virus protease-activation mutant F_1-R and its derivatives confirmed that cleavability is important for the pantropic phenotype and identified another important determinant of this phenotype, namely, the cellular site at which budding occurs. In contrast to wild-type Sendai virus, the F_1-R mutant was shown to be readily cleaved in most cell cultures as a result of a mutation at the cleavage site and ablation of a nearby carbohydrate side chain, and it was pantropic in mice. The mutant also had an altered budding phenotype, budding in a bipolar manner rather than exclusively from the apical membrane as is the case with the wild-type parent. This altered budding phenotype was associated with two amino acid substitutions in the M protein (297,300). Whether high cleavability and bipolar budding were both required for the pantropic phenotype was addressed using (a) a series of revertants that had regained the wild-type cleavage phenotype (as a result of reversion at the cleavage site) but had retained bipolar budding (297) and (b) additional protease-activation mutants that had an F protein that had acquired the ability to be readily cleaved intracellularly (as a result of mutations at or near the cleavage site) but that retained the wild-type apical budding phenotype (300). Neither intracellular cleavability nor bipolar budding alone was sufficient to confer pantropism when mice were infected intranasally. However, intracellular cleavability alone was sufficient if the virus was administered systemically. Thus, bipolar budding appears to mediate dissemination from the respiratory tract, presumably by delivering virus into subepithelial tissues and blood. Intracellular cleavability allows subsequent productive infection of additional organs (188). Also, basolateral budding was shown to be required for fusion with neighboring cells (303). However, caution should be exercised in extrapolating these findings. For example, the site of intracellular budding does not seem to have the same significance for a Morbillivirus, measles virus, which buds apically but typically establishes a systemic infection (249).

It is tempting to speculate that an interplay between susceptibility of viral F_0 to cleavage and the capacity of host cells to

effect intracellular cleavage or elaborate the appropriate extracellular protease may be involved in localization and intensity of parainfluenza virus infection within the human respiratory tract. hPIV1 has a cleavage site identical to that of Sendai virus and requires trypsin for the production of infectious virus *in vitro* (193), whereas hPIV3 has a cleavage site consistent with intracellular processing. Thus, the fact that hPIV3 has a greater predilection to cause pneumonia than does hPIV1 might be caused in part by its possession of an F_0 protein that can be cleaved in many cell types (78,193). The significance of the heterogeneity of the cleavage site of hPIV2 and hPIV3 clinical isolates to their pathogenesis was considered earlier in the section, Properties of the Proteins.

Comparison of naturally occurring strains of Sendai virus or NDV, or studies in which virus was attenuated by passage in cell culture, also provided evidence of large differences in virulence that could not be accounted for by the cleavage phenotypes of F or HN (38,258,333). Thus, other viral determinants of pathogenesis remain to be identified. This can now be addressed by gene exchanges between different viruses using reverse genetics.

The accessory proteins of parainfluenza viruses also can affect virus growth and pathogenesis, and they appear to do so by interfering with the host's innate immune response as well as host cell biology (see earlier section, Properties of the Proteins).

Host immunity also can be an important factor in localization of virus infection. Whereas Sendai virus pneumotropism may be determined by budding site and protease availability as described, that of hPIV3 appears to be influenced significantly by host immunity. This was demonstrated experimentally by studies in which immunosuppression of hPIV3-infected cotton rats resulted in dissemination of the virus and productive infection in other organs (239). Similarly, in children with severe combined immunodeficiency disease, systemic, fatal infection with hPIV3 has occurred, with dissemination to the liver, myocardium, and cerebrospinal fluid, as well as lungs (75,80). The rare isolation of hPIV3 from cerebrospinal fluid associated with aseptic meningitis in infants and children (49), as well as the association of hPIV3 with parotitis (52), also indicates that these viruses can occasionally disseminate from the respiratory tract in humans.

A possible role of the immune response in pathogenesis is also suggested by the observation that infants and children who develop parainfluenza virus croup produce local, virus-specific IgE antibodies earlier and in larger amount than patients of comparable age with parainfluenza virus upper respiratory tract illness (327). Also, histamine is detected in nasopharyngeal secretions of croup patients more often than in secretions from patients with upper respiratory tract disease caused by the same virus (327). Based on these observations, it was proposed that more rapid and increased production of parainfluenza virus IgE antibodies mediates histamine release in the trachea and the subglottic region, which in turn produces the symptoms of croup (327). It is not clear whether a more pronounced virus-specific IgE response plays a role in pathogenesis of croup or whether it merely reflects more extensive production of viral antigens and consequent increased antibody response during severe disease. Cell-mediated immune response to parainfluenza viral antigens, as well as parainfluenza virus–specific IgE antibody response, has also been reported to be greater among infants with parainfluenza virus bronchiolitis than among infected infants who developed only upper respiratory illness (327). These observations have been interpreted as evidence for a role of immunologic factors in parainfluenza virus bronchiolitis, but the caveat cited before also applies. Infection of epithelial cells with hPIV2 was reported to increase adhesion of, and concomitant cell damage by, neutrophils, suggesting another potential factor in immune-mediated pathology (308).

Parainfluenza viruses have been used in a number of animal models to study airway responsiveness to various stimuli following infection. In the guinea pig, which undergoes a brief, self-limited, and asymptomatic bronchiolitis after intratracheal inoculation of bPIV3, hyperresponsiveness to both histamine and a choline receptor agonist, arecholine, was seen from day 4 until day 16 after infection (76). The effect lasted through the full recovery of the epithelium but was accompanied by airway hypercellularity and depletion of mucosal mast cells. Similar responses to SV5 have been observed in beagle puppies (180).

Older children or adolescents who had severe croup as infants or young children often exhibit bronchial hyperactivity following exercise or inhalation of methylcholine (100,182). It is not known whether heightened airway reactivity is a preexisting condition that contributes to the pathogenesis of croup or whether inflammatory damage during croup produces a prolonged state of increased reactivity. Clearly, an enhanced IgE and histamine response would assume greater importance if preexisting heightened airway reactivity, especially in the trachea and subglottic region, played a role in pathogenesis of croup.

Severe respiratory tract disease caused by hPIV 1, 2, or 3 generally occurs in the first 3 to 5 years of life. In addition, reinfection with hPIV3 less frequently involves the lower respiratory tract (92). These findings suggest that primary infection confers on the host a relative resistance to subsequent severe parainfluenza virus illness.

Viremia is probably an uncommon event during parainfluenza virus infection in humans (99). Nevertheless, several observations suggest that it may occur more often than we suspect. There is one report of hPIV3 viremia in humans (99) and during experimental infection in hamsters (136). There are also several case reports of meningitis in which hPIV3 was recovered from the cerebrospinal fluid (49). It should also be noted that the SV5 commonly spreads systemically in monkeys to distant sites such as the kidneys (26).

Microscopic pathology is not well defined, as very few fatal cases of parainfluenza virus disease have been studied. Syncytium formation was observed in the lungs of two infants with severe immune deficiency who died of hPIV3 pneumonia; however, giant-cell formation is not a feature of fatal parainfluenza virus disease occurring in immunocompetent individuals (2,62,66).

CLINICAL FEATURES

In children, the most common type of illness consists of rhinitis, pharyngitis, and bronchitis, usually with fever (227,228). The most common initial symptoms are cough, hoarseness, and fever. The cough may be croupy, but respiratory distress

is not present. Approximately three fourths of such ill children have a temperature above 100°F; fever usually lasts 2 to 3 days. Coarse breath sounds, rhonchi, erythema of the pharyngeal mucous membranes, and rhinitis are the characteristic physical findings. Cervical adenopathy is uncommon (228). Otitis media is seen frequently in PIV infections, and virus is present in the middle ear fluids of patients (110).

When croup develops, the initial symptoms of rhinitis, pharyngitis, fever, and cough progress. After several days, the cough worsens and becomes brassy, “seal-like,” or barking, and stridor ensues. Most children recover after 24 to 48 hours; in some children, however, air hunger develops, with cyanosis, sternal and intercostal retractions, and progressive airway obstruction. The anteroposterior (AP) radiograph of the neck (which should be obtained under carefully controlled medical supervision, if at all) shows glottic and subglottic narrowing (the “steeple sign”) and differentiates this disease from epiglottitis.

When bronchiolitis or pneumonia develops, fever persists and the cough progresses and becomes somewhat productive. It is accompanied by wheezing, tachypnea, retractions, and, in severe cases, cyanosis. The chest radiograph shows interstitial or perihilar infiltrates and air trapping. In some patients, a combined bronchopneumonia–croup syndrome occurs. There are also case reports of children who have developed severe pulmonary disease resembling the adult respiratory distress syndrome following parainfluenza virus infection (121).

As reviewed later, infection of immunocompromised patients is usually prolonged and may be severe, with a mortality of 30% in adults infected during the period immediately following bone marrow transplantation (329). This indicates that parainfluenza viruses are inherently virulent and do not require an immune response to cause severe disease.

DIAGNOSIS

Differential

In croup, other viral causes must be considered. Influenza A virus is a common cause of croup but is usually confined to epidemic periods (see Table 4). RSV is also occasionally a cause of croup. Measles virus, particularly in developing nations, may also produce severe laryngeal inflammation. Hemophilus influenzae type B causes epiglottitis, which presents the most important differential diagnosis, although with the advent of widespread immunization this has become a rarity in the United States. Epiglottitis is usually of sudden onset, without a prodrome of rhinitis, hoarseness, or cough, and is accompanied by high fever, leukocytosis, a “toxic” appearance, and drooling. The AP x-ray of the neck is characteristic, as is the cherry-red enlargement of the epiglottis, which should be examined only as part of the intubation procedure or when preparations for nasotracheal intubation have been made. Another bacterial infection that must be differentiated from viral croup is bacterial tracheitis. This disease, described first in 1979 (138), is usually caused by *Staphylococcus aureus* and may be a complication of viral croup rather than a primary bacterial infection. Intubation is frequently necessary, as well as antistaphylococcal antibiotic therapy. In bronchiolitis or pneumonia of infancy, other viruses (particularly RSV) and *Chlamydia trachomatis* are the most frequent etiologic agents to be considered. Pneumonia in older children is more commonly caused by *Mycoplasma pneumoniae.*

Laboratory

Presumptive diagnosis of parainfluenza virus infection can be made based on young age, history, clinical findings, and relation to known or characteristic prevalence of virus in the community. Definitive diagnosis, however, requires recovery of the virus from appropriate specimens taken from the respiratory tract, identification of viral antigens in respiratory tract secretions by immunofluorescence or another form of immunoassay, or detection of viral RNA in nasal secretions by reverse transcription followed by the polymerase chain reaction (29,46,88). One method for virus isolation involves spin-enhanced cultures, in which virus is detected after 2 to 7 days of culture by immunofluorescent staining with monoclonal antibodies (178,221). Serodiagnosis by HI, complement fixation (CF), or neutralization can establish that infection with a member of the parainfluenza virus group has occurred, but frequent heterotypic responses make specific diagnosis by serology difficult (25,31).

TREATMENT

Symptomatic treatment of croup usually includes humidification of air by ultrasonic nebulizer and periodic inhalation of racemic epinephrine. Large doses of systemic corticosteroids (0.3 mg/kg of dexamethasone) are considered to prevent or shorten the period of intubation in severe croup (141), and recent evidence favors the use of inhaled steroids even in more moderate disease (163).

Specific antiviral treatment is not available. However, ribavirin exhibits efficacy *in vitro*, and it may be that this drug will prove to be efficacious when delivered by small-particle aerosol, as in the treatment of influenza A virus or RSV illness (164,271).

EPIDEMIOLOGY

Morbidity, Mortality, Age

Types 1 to 4 parainfluenza viruses have a wide geographic distribution. The first three types have been identified in most areas where appropriate tissue culture and hemadsorption techniques have been applied to the study of childhood respiratory tract diseases. Thus far, hPIV4 subtypes 4A and 4B, which are more difficult to recover in tissue culture, have been isolated in fewer areas, but serologic studies suggest that they also are relatively ubiquitous.

Each of the four parainfluenza virus types can cause acute respiratory tract disease in humans. This etiologic relationship is indicated by two observations. First, each of the virus types has been recovered significantly more often

from patients with respiratory diseases than from individuals free of such illness (27,29,30,159,227). Second, types 1, 2, 3, and 4A viruses each have produced upper respiratory tract infection and illness when administered to adult volunteers (143,312,316,317).

The parainfluenza viruses as a group are exceeded only by RSV as an important cause of viral lower respiratory tract disease in young children, and like RSV they commonly reinfect older children and adults to produce upper respiratory tract disease. Reinfection of adults, as well as children, with the parainfluenza viruses has been recognized on a number of occasions, particularly with hPIV3 and hPIV1, and disease leading to hospitalization can occur in adults (187). Although the frequency of reinfection is not known, it is probable that most individuals have repeated experience with hPIV1, 2, and 3. In a series of three outbreaks of hPIV3 infection in a semiclosed nursery population, it was observed that 17% of the children infected during one outbreak were reinfected during a subsequent outbreak, although the interval between the first and last outbreaks was only 9 months (29). Reinfection of preschool children living at home also occurs with high frequency (92). Illness usually occurs less often and is less severe during reinfection than during primary infection.

In a longitudinal study of families with young children, the overall rate of parainfluenza virus infection as estimated by serology (HI and CF) was 44.4 infections per 100 person-years (77). Type-specific infection rates could not be estimated because of the high frequency of heterotypic serologic response. Parainfluenza virus infection rate varied from 59% for children 2 to 5 years of age to 40% for adults. Spread of virus within infected families was extensive, as indicated by serologic studies; following an index infection, 64% of family members developed a serum HI antibody response. Parainfluenza virus was introduced into the family primarily by preschool children (77).

There is considerable diversity in both epidemiologic and clinical manifestations of infections caused by the parainfluenza viruses, and this diversity does not always sort on taxonomic lines. Thus, although they are in the same genus (see Table 1), hPIV1 is the principal cause of croup in children, whereas the principal serious illness caused by hPIV3 is pneumonia or bronchiolitis, and hPIV3 is second only to RSV as a cause of viral pneumonia and bronchiolitis in infants less than 6 months of age (see Table 4) (27,29,30,64,227). Likewise, although they belong to different genera, hPIV2 closely resembles hPIV1 in clinical manifestations, but serious illness occurs less frequently. Infections with hPIV4 are detected infrequently, and associated illnesses are usually mild.

The parainfluenza viruses are most important as respiratory tract pathogens during infancy and childhood. In this age group, hPIV1, 2, and 3 cause a spectrum of effects ranging from inapparent infection to life-threatening lower respiratory tract disease. Studies in different parts of the world indicate that hPIV1, 2, and 3 are associated with approximately 41% of severe croup. hPIV1 is the most important of the parainfluenza viruses causing croup. In addition to croup, hPIV1, 2, and 3 are also responsible for a smaller but appreciable proportion of other acute respiratory tract diseases of infancy and early childhood.

Parainfluenza infection generally occurs very early in life. Serologic surveys indicate that at least 60% of children have been infected with hPIV3 by 2 years of age and that approximately 80% have been infected by 4 years of age (227,228). When infants and children are studied longitudinally, it is apparent that infection with hPIV3 occurs even more commonly during infancy and early childhood than is indicated by serologic survey. During a longitudinal study of 121 infants and young children, the incidence of infection was approximately 67 per 100 per year during the first 2 years of life (92). Infection with hPIV1 or 2 generally occurs somewhat later, but by 5 years of age a majority of children have been infected with hPIV2, and over 75% have been infected with hPIV1 (227,228). hPIV3 often causes illness during the first months of life, at a time when infants still possess serum neutralizing antibodies derived from their mother (251). In contrast, in young infants, maternally derived antibodies appear to prevent both infection and severe disease with either hPIV1 or 2. Severe illness caused by hPIV1 or 2 is rather rare under 4 months of age (27,29,90). After 4 months, there is a rise in the number of cases of croup and other lower respiratory tract diseases caused by hPIV1 or 2. This high incidence continues until approximately 6 years of age. After a child reaches school age, there is a much lower incidence of lower respiratory tract disease caused by hPIV1 or 2, and the occurrence of lower respiratory tract illness in individuals infected with either virus during adolescence or adult life is unusual, although this does occur on occasion (187). This reduction in infection and disease is caused by protective immunity from previous infection(s).

From 1957 to 1961, hPIV1 appeared to be endemic; infection occurred sporadically and without a definite seasonal pattern (27,29). Beginning in 1962, a different pattern developed, in which sharp outbreaks of hPIV1 occurred every 2 years in the autumn of even-numbered years. In contrast, hPIV2 caused outbreaks in the autumn of odd-numbered years (5,29,91). At present, hPIV1 and 2 epidemics are synchronous, occurring during the fall of odd-numbered years. For many years, hPIV3 exhibited an endemic pattern, with infection occurring in all seasons of the year. Within this endemic pattern, small outbreaks occurred, but there was no predictable periodicity. Within the past 10 years, there has been a shift toward yearly spring epidemics of hPIV3 infection. In the United Kingdom, hPIV3 is seen most frequently in the summer. The seasonality of hPIV1 and 2 is reflected in the seasonality of croup (64). In infants and children in the United States, the peak incidence of croup is always in the autumn, with a smaller peak in the cold months of the winter reflecting influenza and RSV as causes of the syndrome.

The parainfluenza viruses are troublesome as causes of infection in the hospital, particularly hPIV3 (204). Children who are admitted to the hospital for nonrespiratory tract illness are often infected during their hospital stay, and this is commonly associated with the development of serious lower respiratory tract disease. Nucleic acid sequence analysis has been used to analyze hospital outbreaks, and it is clear that, although outbreaks caused by a single genotypically uniform strain occur, much of the problem is sporadic spread of multiple strains within the hospital or community (145). Illness in hospital staff can occur and may be a factor in spread (272).

As mentioned earlier, there is no evidence that variation in virulence of human parainfluenza viruses plays a significant role in the epidemiology of these agents, possibly because the appropriate studies have not been performed. However, naturally circulating strains of NDV, a closely related avian Paramyxovirus, exhibit a wide spectrum of virulence for its

natural avian hosts (216,310).

There is no evidence that animal strains of PIV1 (Sendai virus) or 3 (bPIV3) infect humans in nature. Occasional reports have described the recovery of the SV5 from human specimens, but none of these observations have been confirmed. A Rubulavirus-like virus isolated 20 years ago from monkeys showed the greatest sequence and antigenic relatedness to NDV and avian parainfluenza type 2 viruses, suggesting that cross-species transmission and adaptation had occurred (174).

A number of parainfluenza viruses readily establish persistent infection in cell culture (33,87,116,200). It is possible that persistence also occurs *in vivo*, perhaps accounting for the prevalence of parainfluenza viruses in their natural host population (203). However, this is unlikely, at least for the human parainfluenza viruses. These infections remain largely localized in the respiratory tract and induce potent systemic and local humoral and cellular immune responses. It seems unlikely that the relatively small genomes of these viruses encode mechanisms to evade immune recognition and clearance by adaptive immunity, although, as mentioned before, certain animal parainfluenza viruses do appear to encode proteins that interfere with innate immunity. Instead, it is the high rate of reinfection and subclinical infection, rather than viral persistence, that maintains the virus in the population. This reflects the difficulty of maintaining solid long-term immune protection in the upper respiratory tract, caused by (a) the transient nature of local secretory IgA and cytotoxic T cells and (b) the inefficiency of transudation of serum antibodies to the lumen of the respiratory tract, particularly the upper respiratory tract. The possibility that human parainfluenza viruses might block responses to interferon, suggested in recent studies with Sendai virus and SV5, would be an additional factor aiding reinfection.

Spread and Infectivity

Transmission of parainfluenza viruses is by direct person-to-person contact or by large-droplet spread; however, the viruses do not persist long in the environment (9). The high rate of infection early in life, coupled with the frequency of reinfection, suggests that these viruses spread readily from person to person. There is evidence from studies in adult volunteers that the infectious dose of hPIV1 is small. For example, in one study, two thirds of adults who possessed a moderately high level of preexisting serum-neutralizing antibodies became infected and developed a “common cold–like” illness following intranasal instillation of 80 $TCID_{50}$ of hPIV1 (278). Also, reinfected individuals appear to be infectious. hPIV3 appears to be the most efficient of the parainfluenza viruses in its ability to spread from person to person (29). hPIV3 generally infects all susceptible individuals in a semiclosed population (such as a nursery) in a relatively short time. In contrast, hPIV1 and 2 appear to be less effective in this regard, infecting 40% to 69% of susceptible individuals in semiclosed populations (29,142). There is no evidence that infection is transmitted from humans to animals or vice versa.

During experimental infection of adult volunteers, the interval between administration of hPIV1, 2, or 3 and onset of upper respiratory tract symptoms ranges from 3 to 6 days (Fig. 5) (143,209,278,316,317). The incubation period in pediatric infections has not been defined; however, information is available concerning the interval between exposure to hPIV3 and the subsequent initial shedding of this virus. In several institutional outbreaks of infection, this interval was 2 to 4 days (29).

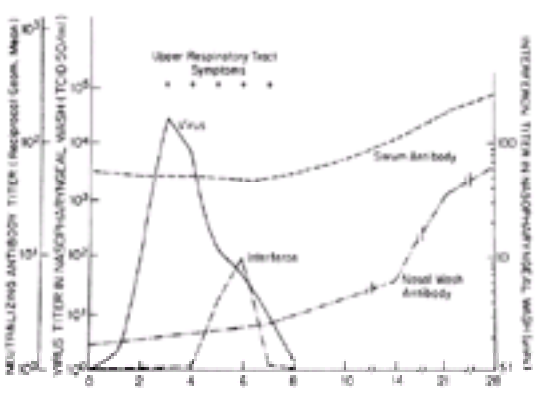

FIG. 5. Experimental infection of adults with wild-type human parainfluenza type 1 virus (*hPIV1*). Adult volunteers, all of whom had been infected previously with PIV1 under natural conditions, were administered 10^5 $TCID_{50}$ of hPIV1 by the intranasal route. *Abscissa* indicates days after inoculation (209).

The interval during which an individual infected with a parainfluenza virus can infect another person is not known. Longitudinal studies indicate that hPIV3 is usually shed from the oropharynx for 3 to 10 days (median of 8 days) during initial infection, whereas during reinfection the virus is detected during a shorter interval (29). On occasion, infants and young children may shed hPIV3 for as long as 3 to 4 weeks (79). Prolonged shedding of hPIV3 has also been observed occasionally in adults with underlying chronic lower respiratory tract disease (99). It is not clear whether chronic damage to the respiratory tract is responsible for this unusual manifestation of adult reinfection.

Immunity

Most studies of immunity to parainfluenza viruses have focused on adaptive immune responses, and in particular on secretory and serum antibodies against the virus. As described later, these are the major correlates of resistance to infection and reinfection. The contribution of innate immunity has received less attention, although parainfluenza viruses such as Sendai virus have long been known to be very efficient inducers of alpha and beta interferons. The ability of several accessory proteins of animal parainfluenza viruses to interfere with the antiviral activities of interferon was described earlier (in the section, Properties of the Proteins). The importance of interferon in parainfluenza virus infections in humans has not been defined. However, it is likely that inhibition of the interferon-induced antiviral state may

play a role in virulence as suggested by the attenuation of hPIV3 for monkeys that resulted from ablation of C protein function (67,275). A significant proportion (30%) of young patients with hPIV1 infection develop a detectable interferon response (geometric mean titer of 23.5 units) during the acute stage of illness (101). This frequency is somewhat less than that observed during influenza A virus infection of children (55%) but is decidedly greater than that observed during RSV infection of infancy and childhood (4%). If human parainfluenza viruses also interfere with the antiviral activity of interferon in their natural host, then the induction of interferon by these viruses would not necessarily be protective.

In a longitudinal study of hPIV3 outbreaks in infants and young children resident in a semiclosed nursery, preexisting serum-neutralizing antibodies correlated with resistance to infection and illness (29). However, resistance associated with serum-neutralizing antibodies was only partial. One third of infants and children with a high serum antibody level became infected. Although reinfection occurred, a partial effect of immunity from previous infection was indicated by a shorter period of virus shedding compared to that observed during primary infection. Moderate levels of serum antibody were not associated with complete protection against febrile illness, as such illness occurred approximately 40% as often as during first infection. A somewhat greater reduction of lower respiratory disease was observed during second infection with hPIV3 during a longitudinal family study (92).

Infants also appear to be partially protected from infection and disease by hPIV1 and hPIV2 serum antibodies. This protective relationship is suggested by the relative sparing of young infants from hPIV1 and 2 infection and associated disease at a time when they possess serum antibodies passively acquired from their mother. Also, the risk of infection with hPIV3 during the first 4 months of life is inversely related, in the aggregate, to the level of neutralizing antibodies present in cord serum at birth (92). However, the protective effect of passive immunity to hPIV3 is less than that observed for hPIV1 and 2, as some infants with a moderately high level of maternally derived serum antibodies become infected with hPIV3 and develop illness (92).

The ability of serum-neutralizing antibodies to confer resistance to pulmonary replication by parainfluenza viruses has been well documented in experimental animals, where parenteral administration of polyclonal or monoclonal virus-neutralizing antibodies conferred a high level of resistance to virus replication in the lower respiratory tract and a lower level of resistance in the upper respiratory tract (223,224,239,246). In addition, these studies demonstrated that significant resistance in the respiratory tract requires a high serum antibody titer. This reflects the absence of a specific mechanism for transporting serum IgG to the mucosal surface of the lumen of the respiratory tract. The transport of IgG by nonspecific transudation is driven by high serum titer and is highly inefficient.

Experimental infection of adults possessing varying levels of local nasal secretory IgA-neutralizing antibodies and a relatively high level of serum-neutralizing antibodies indicated that resistance to hPIV1 or 2 infection and upper respiratory disease was mainly a function of mucosal antibodies (278,312). Similar information is not available for hPIV3, nor is information available concerning the immunologic determinants of resistance to parainfluenza virus disease of the lower respiratory tract in adults. Also, the efficacy of IgA in mediating resistance has been demonstrated in studies in experimental animals. IgA and IgG were equally effective in providing resistance to Sendai virus challenge in mice when administered directly into the respiratory tract (190). However, in the natural situation, IgA has the advantage of being specifically transported through the epithelium to the surface of the lumen as well as being able to neutralize virus within infected epithelial cells (189). The central role of respiratory tract IgA was demonstrated unambiguously for influenza virus in mice, where resistance to influenza virus replication in the respiratory tract of previously infected immune mice was ablated by topical administration of anti-IgA antibodies (247,252). Comparable findings would be expected for the parainfluenza viruses. Studies in hamsters showed that preparations of hPIV3 glycoproteins induced a higher level of local IgA and a higher level of resistance to intranasal challenge when administered intranasally rather than subcutaneously, illustrating the importance of direct stimulation of local immunity (247).

Infection with hPIV3 stimulates the development of serum antibodies to most of the viral proteins, but only antibodies to the two viral surface glycoproteins, HN and F, have been shown to have virus-neutralizing activity *in vitro* or *in vivo* (148). Primary infection usually induces a greater antibody response to the HN protein (322). Reinfection is often required to stimulate a high level of serum antibodies to the F protein. Initial studies with SV5 in tissue culture suggested that antibodies to F might be more important than those to HN because they could both neutralize virus infectivity and prevent cell-to-cell spread of infection by cell fusion, whereas HN-specific antibodies could only neutralize virus infectivity (195). However, it is now recognized that most other parainfluenza viruses require both HN and F for efficient syncytium formation, and antibodies against HN interfere with F activity in this situation (128,237). Furthermore, evaluation of vaccinia virus recombinants expressing the hPIV3 HN or F glycoprotein, as well as purified baculovirus-produced HN and F glycoproteins in cotton rats, indicated that HN induced more effective immunity than did F (15,282).

Adults respond to reinfection by developing an increase in local nasal secretory IgA antibodies that neutralize virus infectivity (see Fig. 5) (278). In contrast, young infants undergoing primary infection produce local nasal IgA antibodies that neutralize virus infectivity poorly or not at all (334) and exhibit a decreased serum antibody response to the HN glycoprotein (144). Production of nonfunctional local antibodies, as well as a poor serum F and HN antibody response, may explain why reinfection (especially with hPIV3) occurs with considerable frequency during infancy and early childhood.

The importance of CTLs in recovery from human parainfluenza virus infection has not been formally established. Nevertheless, it is likely that this form of cell-mediated immunity is important in the recovery process because hPIV3 infection of infants or children with profound T-cell deficiency can lead to fatal giant cell pneumonia (62). Memory CTLs of both CD4+ and CD8+ subsets specific for hPIV1 have been isolated from normal adult donors, although their contribution to resistance to reinfection is not known (59,277).

The importance of class I and class II major histocompatibility complex–restricted, virus-specific T cells in recovery from pulmonary Sendai virus infection was demonstrated during a study in which mice, the putative natural host, were challenged by the intranasal route (122,149,150). Intranasal infection resulted in a rapid increase in pulmonary CD8+ CTLs, whose appearance coincided with the decrease in titer of pulmonary virus and resolution of infection (122). However, possible participation of other effectors such as secretory or serum antibodies was not defined. Immunization of

mice with a peptide bearing a major epitope for CD8+ CTLs induced a high level of resistance to virus challenge (150). Mice that had a mutant nonresponder class I restriction element failed to develop a virus-specific CD8+ CTL response upon infection, whereas serum antibody, delayed-type hypersensitivity, and natural-killer-cell responses were unimpaired. These mice cleared infection but Sendai virus had an LD_{50} that was tenfold lower than that for wild-type congenic mice. The LD_{50} for congenic nude mice that lacked all subsets of T cells but produced natural killer cells was 300-fold lower than that determined in wild-type mice (149). The survival time of infected nude mice could be increased (from 19 days) by passive administration of cloned Sendai virus-specific CD8+ cells (to 25 days), or CD4+ cells (to 34 days), or both (>100 days). In contrast, interleukin-2 alone did not provide significant prolongation (149). In a situation in which infection was not lethal, removal of CD8+ cells, either through antibody depletion or disruption of the b2-microglobulin gene, resulted in delayed viral clearance and 20% mortality (122), whereas antibody-mediated depletion of CD4+ cells inhibited antibody production but delayed viral clearance only marginally with no mortality. Removal of CD4+ cells by antibody depletion did not affect the development of the CD8+ CTL response, but removal of both subsets resulted in a failure to clear the virus and 100% mortality (122). Pulmonary virus-specific CD8+ cells exhibited efficient cell-killing activity *in vitro*, suggesting that this is a major effector function *in vivo*. CD4+ cells isolated from normal Sendai virus–infected mice did not have significant *in vitro* cell-killing activity, nor did they seem to have a critical role in promoting the CD8+ response (122). It seems likely that a major antiviral activity of CD4+ cells is to provide T-cell help for antibody-producing B cells (122,149).

Consistent with this, children and adults with various forms of immunodeficiency, usually involving both the cellular and humoral arms of the immune system, may develop particularly severe illness and can shed virus for a prolonged period (62,75,80,242). Pneumonia caused by a parainfluenza virus occurring during recovery from bone marrow transplantation carries a mortality of 30% (329), and children who are immunodeficient as a consequence of human immunodeficiency virus infection may shed parainfluenza viruses for months to years (192).

In summary, it is useful to distinguish between elements of immunity that are critical in clearing an infection, and those important in conferring resistance to reinfection. Innate immunity presumably functions in both situations. During resolution of infection, antibodies and CTLs play important independent roles. With respect to prevention and resolution of reinfection, the protection mediated by CTLs and secretory antibodies is relatively short-lived, whereas serum virus-neutralizing antibodies mediate long-term resistance, especially in the lower respiratory tract.

PREVENTION AND CONTROL

Initially, inactivated parainfluenza virus vaccines prepared from infected embryonated eggs or primary monkey kidney cell culture were developed for hPIV1, 2, and 3 and shown to be immunogenic in children, as indicated by the development of serum HI and neutralizing antibodies. Unfortunately, parenteral administration of these vaccines failed to induce resistance to parainfluenza virus disease (32,81). Perhaps this is not surprising in view of the importance of local secretory IgA antibody in resistance and the relative ineffectiveness of parenterally administered vaccines in stimulating this response. Selective inactivation of F glycoprotein by formalin may also have contributed to the ineffectiveness of inactivated vaccines, as suggested by the observation that formalin selectively destroys the antigenicity of the F glycoprotein of the related mumps virus. More recently, hPIV3 adsorbed onto immunogenicity-enhancing microcarriers was shown to protect hamsters against subsequent virus challenge when immunization was performed by the intraperitoneal route, but it was poorly protective when given by the more relevant intranasal route (248). An efficient method of immunization against parainfluenza virus disease using a killed virus vaccine remains to be developed.

Subunit vaccines have also been evaluated, namely, preparations of the hPIV3 HN and F glycoproteins isolated from purified hPIV3 or from insect cells infected with recombinant baculoviruses expressing the HN or F protein or a chimeric form of HN and F encoded by an engineered cDNA (15,102,179,246,320). The glycoproteins expressed individually in baculovirus did not appear to differ significantly from native antigens, based on their reactivity with panels of monoclonal antibodies representing the major neutralization epitopes. Parenteral immunization of cotton rats with these viral glycoproteins induced satisfactory levels of neutralizing, HI, and FI antibodies, and the immunized animals were highly resistant to intranasal challenge with hPIV3. Also, intranasal administration of the purified glycoproteins was effective in inducing a local IgA response (247). To date, none of the aforementioned strategies has been validated in nonhuman primates or in clinical trials. In a number of studies, antigens of a variety of human viruses that were highly immunogenic in small experimental animals, proved to be much less immunogenic in chimpanzees or humans (206). Also, evaluation of vaccines in a host such as the cotton rat, which is semipermissive for hPIV3 replication and thus easier to “protect,” can yield artifactually encouraging results (206). Experience gained during the development of RSV vaccines suggests that immunogenicity observed in small rodents provides an overly optimistic prediction of the response of humans to the same material. In addition, subunit vaccines have decreased immunogenicity in immunologically naive subjects, and their immunogenicity can be suppressed by passively acquired antibodies, both of which suggest decreased usefulness of such vaccines in the first 6 months of life (207). In any case, subunit vaccines might prove to be useful for boosting the response to a prior live attenuated virus vaccine, or the immunogenicity of such a vaccine might be improved through the use of novel adjuvants or methods of administration (74).

Another approach to immunization involves construction of viable viral recombinants that express parainfluenza virus protective antigens for use as live vaccines. This would have the advantage that antigens would be expressed *in situ* and thus should be conformationally unperturbed. This approach was evaluated by constructing vaccinia virus recombinants expressing the hPIV3 F or HN glycoprotein (282). Both glycoproteins appeared to be expressed in authentic, fully immunogenic form. Infection of cotton rats with either vaccinia virus recombinant, or of patas monkeys with both recombinants, induced a high level of PIV3-specific serum neutralizing antibodies, and the immunized animals developed significant resistance to challenge with hPIV3 (281,282). However, subsequent studies with replication-defective vaccinia virus recombinants, which are more appropriate for use in humans because of their greater margin of safety, were less encouraging (68,331). Replication-defective vaccinia virus recombinants that expressed the PIV3 HN or F glycoprotein were administered to rhesus monkeys by three routes simultaneously—intranasal, intratracheal, and intramuscular. Monkeys immunized in this manner were resistant to subsequent challenge with wild-type PIV3. Unfortunately, this protective effect was partially abrogated when monkeys were infused with PIV3-specific simian antibodies the day before

immunization. Significantly, this suppressive effect of a physiologic amount of passive antibodies was not observed when monkeys were immunized intranasally with a live attenuated PIV3 vaccine candidate. Suppression of immune response by maternally derived antibodies is an important issue because almost all young infants to be immunized would possess such antibodies.

Another approach to immunoprophylaxis involves development of live attenuated vaccine virus mutants suitable for administration by the intranasal route. Previous efforts at development of parainfluenza and RSV vaccines suggested that live attenuated, intranasally administered vaccine viruses represented the most effective strategy for prevention of viral lower respiratory tract infection in experimental animals. This would offer the advantage of direct stimulation of local respiratory tract immunity by all of the protective viral antigenic sites in their most authentic form. Importantly, live attenuated viruses are protective in the presence of passively acquired antibodies (68).

A novel approach to the development of live parainfluenza virus vaccines that has not been evaluated in humans as yet is illustrated by the “protease-activation” mutants of Sendai virus described previously. These mutants were selected for cleavage by elastase or chymotrypsin and, concurrently, resistance to cleavage by trypsin (124,296). Intranasal instillation of a moderate amount of mutant virus (about 10^6 plaque-forming units) induced serum virus-specific antibodies in mice as well as significant resistance to subsequent challenge with wild-type virus (124,296). Additional mutations in the cleavage site region may be required before this approach can be considered feasible for the human parainfluenza viruses, because a Sendai virus mutant with an amino acid substitution at the cleavage site (residue 116) and residue 109 was genetically unstable in mice (124).

Development of one of the initial candidate attenuated hPIV3 vaccines employed the oldest successful strategy for formulation of a live virus vaccine. First used by Jenner more than 200 years ago, this strategy for protection against a virus virulent for humans relies on immunization with an antigenically related animal virus not virulent for humans. bPIV3 and hPIV3 are approximately 25% related by cross-neutralization (1). Their HN glycoproteins share two neutralization epitopes in antigenic site A, and three neutralization epitopes are shared on their F glycoproteins (35,36,321). The two shared epitopes on HN, as well as two of the three shared epitopes on F, are completely conserved among human PIV3 isolates, whereas the third shared epitope on F is very highly conserved (97%). bPIV3 was immunogenic in cotton rats and squirrel monkeys and induced a significant level of resistance to challenge with hPIV3 (323). In addition, bPIV3 was shown to be 100- to 1,000-fold more restricted in replication in the respiratory tract of rhesus monkeys and chimpanzees than hPIV3, indicating a partial host-range restriction that would be expected to be associated with reduced virulence in humans (323). However, strains of bPIV3 grew well enough *in vivo* to induce a serum neutralizing antibody response that should, based on previous experience, provide resistance to hPIV3 infection. In clinical trials, bPIV3 was administered intranasally to 50 infants and children, 2 to 60 months of age (144,146). The candidate vaccine was well tolerated and appeared to be satisfactorily attenuated. Of seronegative children who received 10^4 or 10^5 $TCID_{50}$, 85% became infected and shed virus that retained its attenuation phenotype when tested for level of viral replication in rhesus monkeys. The candidate vaccine elicited a mean serum HI antibody titer for bPIV3 of 1:40, and for hPIV3 of 1:16, in 92% and 61% of the seronegative vaccinees, respectively. These encouraging results in seronegative infants and children indicated that bPIV3 was safe, infectious, and stable with regard to its attenuation phenotype. Furthermore, the candidate vaccine induced a level of protective antibodies that should provide significant resistance to hPIV3 infection (34,146,206). A second “Jennerian” parainfluenza vaccine candidate was recently described. Sendai virus (murine PIV1) was highly immunogenic when administered intranasally to African green monkeys, and immunized animals were completely resistant to replication of a second dose of the candidate vaccine or to subsequent challenge with hPIV1 (126).

A second type of live attenuated hPIV3 vaccine was developed by passaging a wild-type virus in cell culture at suboptimal temperature (20° or 22°C) (10,50,51). A promising vaccine candidate was identified after 45 cold-passages. This *cp*45 mutant exhibited three interesting properties: (a) cold adaptation (*ca*) (i.e., an ability to replicate efficiently at 20°C, a temperature restrictive for wild-type PIV3); (b) temperature sensitivity (*ts*) (i.e., a decreased ability to produce plaques at the high end of the normal temperature range); and (c) reduction in level of replication (“attenuation” or *att*) in hamsters, monkeys, and humans (104,147). This mutant was considered to be a promising vaccine candidate because its *ca* and *ts* phenotypes were stable after replication in cell culture, and it induced significant resistance in hamsters and monkeys to subsequent challenge with wild-type hPIV3 (104).

Evaluation of the *cp*45 mutant in rhesus monkeys showed that it was attenuated as a consequence of a mixture of *ts* and non-*ts* mutations (104). Subsequent evaluation in chimpanzees confirmed its satisfactory level of attenuation (103). Significantly, the mutant was still able to induce a high level of resistance to wild-type virus challenge. Although *cp*45 exhibited a partial loss of the *ts* phenotype during replication in chimpanzees, such partial revertants remained satisfactorily attenuated when tested in additional chimpanzees. This illustrates the importance of having multiple attenuating mutations, some of which are non-*ts* in nature, to maximize stability of the attenuation phenotype. Preliminary clinical evaluation of the attenuated *cp*45 mutant in seronegative infants and small children suggested that this candidate vaccine strain was suitably infectious and attenuated, as well as moderately immunogenic (147). In addition, each of 135 virus isolates from vaccinees retained the *ca* and *ts* phenotype.

The production and characterization of live attenuated parainfluenza virus vaccines have been greatly facilitated by the recent development of reverse genetics. For example, existing attenuated viruses can be sequenced and their relevant mutations identified by their introduction into recombinant wild-type virus. This makes it possible to differentiate attenuating mutations from incidental mutations. Novel mutations, such as those affecting the cleavage site of the F protein or genomic RNA regulatory sequences, can also be introduced. Attenuating mutations identified in these ways can then be introduced in various combinations into wild-type virus or existing attenuated mutants to produce a graded series of genetically defined vaccine viruses.

These approaches have been and are currently being used to characterize the *cp*45 hPIV3 vaccine candidate, as well as to construct derivatives of *cp*45 that might also be useful for immunization. The *cp*45 differs from hPIV3 wild-type parent (strain JS) by 20 mutations (Fig. 6) (275,286). Five of these changes are located in coding regions but are not thought to be significant because the amino acid sequence is not altered and they do not involve cis-acting signals. The remaining

15 changes include four nucleotide substitutions in the 3' leader region, one nucleotide substitution in the NP gene-start signal, and 10 amino acid substitutions distributed among six viral proteins, namely NP, C, M, F, HN, and L (see Fig. 6). These mutations, either singly or in combination, were introduced into full-length, wild-type viral cDNA and the resulting viable mutants were then analyzed to determine their *ca*, *ts*, and *att* phenotypes (275). Multiple mutations throughout the hPIV3 genome were shown to contribute to the *ca*, *ts*, and *att* phenotypes. The three L gene mutations were tested separately or together (see Fig. 6). Each of these L mutations contributed individually to the *ts* phenotype and to growth restriction in hamsters (i.e., the *att* phenotype) but did not specify the *ca* phenotype either separately or together (274). The triple L gene mutation recombinant virus was as attenuated in hamsters as the *cp*45 mutant, indicating that the L gene mutations make a major contribution to the attenuation exhibited by the *cp*45 vaccine virus. Two other proteins, namely C and F, were also identified as sites in which amino acid substitution could independently specify attenuation. It is likely that the multiplicity of mutations responsible for the *ts*, *ca*, and *att* phenotypes of *cp*45 is responsible for the observed stability of these phenotypes during replication *in vivo*. Improvement of *cp*45 hPIV3 could also be achieved by addition of other mutations imported from attenuated mutants of related paramyxoviruses of the same genus, another genus, or another subfamily. An example of the latter approach was the small incremental increase in attenuation of *cp*45 hPIV3 that was achieved by importation of an L protein mutation from the Pneumovirus RSV mutant into an analogous site of the hPIV3 mutant (273).

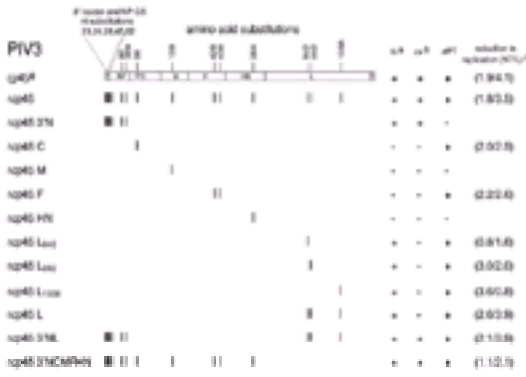

FIG. 6. Contribution of 15 nucleotide (*nt*) or amino acid (*aa*) point mutations to three laboratory phenotypes exhibited by the *cp*45 hPIV3 JS strain candidate live attenuated vaccine virus. The genome position of each nucleotide substitution in the 3' leader and the N gene-start (*GS*) signal or each amino acid substitution in the NP, C, M, F, HN, and L proteins is indicated by a *vertical line*. Each line represents the relative position of the indicated mutation in the biologically derived *cp*45 mutant or in a cDNA recombinant mutant constructed to bear that mutation. **a:** Temperature sensitivity (*ts*): A virus is defined as exhibiting the *ts* phenotype if plaque formation at 40° is more than 100-fold reduced compared to wild-type (*wt*) rhPIV3 at 40°C. **b:** Cold-adaptation (*ca*) is defined as a greater than 10-fold increase in replication at 20°C compared to that of wild-type HPIV3 at 20°C. **c:** Attenuation (*att*): A virus is defined as exhibiting the *att* phenotype when its replication in lungs or nasal turbinates of hamsters is reduced by at least 10-fold compared to wild-type rhPIV3. **d:** Reduction (log_{10}) in virus replication in the respiratory tract of hamsters compared to wild-type hPIV3 (*NT*, nasal turbinates; *L*, lungs). **e:** *cp*45 is a biologically derived virus. All the other viruses are mutant hPIV3 (JS strain) recombinants derived from cDNA.

The feasibility of increasing the breadth of immunity conferred by the *cp*45 hPIV3 vaccine candidate has been investigated by constructing a viable chimeric recombinant virus in which the F and HN ORFs of wild-type hPIV3 were replaced by the corresponding sequences of wild-type hPIV1 (291). The resulting hPIV3-1 chimera replicated as efficiently *in vitro* and *in vivo* (hamsters) as its hPIV1 parent and induced effective immunity to hPIV1. That the hPIV3-1 virus was fully viable *in vitro* and *in vivo* was remarkable, given the divergence in F and HN amino acid sequence between the two serotypes. For example, the cytoplasmic and transmembrane domains of F and HN share less than 20% amino acid identity between hPIV3 and hPIV1. Because these regions are generally thought to interact with internal proteins such as M, it might have been expected that a swap of the complete glycoproteins would have reduced viability (289), but this was not the case. Introduction of the attenuating mutations of *cp*45 into hPIV3-1 yielded a chimeric virus that was satisfactorily attenuated and highly immunogenic in experimental animals (276,292). Interestingly, the immunity induced by hPIV3-1 had two components: (a) a moderate level of resistance to hPIV3 challenge, which appeared to be mediated by cellular immunity specific for internal hPIV3 proteins and which was short-lived (290), and (b) a high level of resistance to hPIV1 challenge that was durable and was mediated by antibodies to hPIV1 HN and F (290). The finding that the hPIV3-specific response induced by the antigenic chimera was short lived suggests that reimmunization with the same hPIV3 vector bearing other antigenic determinants, such as the HN and F proteins of hPIV2, should be feasible (290). Thus, immunization against hPIV1, 2, and 3 might be achieved using antigenic chimeras constructed on the hPIV3 *cp*45 backbone.

Parainfluenza viruses also have been evaluated as a vector for added foreign genes. In this strategy, the foreign gene is placed under the control of gene-start and gene-end parainfluenza virus transcription signals and inserted into the gene order. Because of the polarity of transcription, placement in a more promoter-proximal location would provide a higher level of expression. For example, gp160 of HIV was inserted into the Sendai virus genome at a promoter-proximal location and recovered as a recombinant virus, which then expressed an additional mRNA encoding the foreign protein (336). This turns out to be a surprisingly efficient expression strategy, and foreign inserts in mononegaviruses are remarkably stable. Also, this strategy can be used to produce foreign proteins *in vitro* for use as subunit vaccines. Alternatively, the recombinant parainfluenza viruses expressing the selected foreign protein can be used to deliver the foreign antigen by infection. For example, recombinant hPIV3 has been constructed that expresses the HN or F protein of hPIV2 or the HA protein of measles virus as an additional gene (unpublished observations). The capacity for accepting additional foreign sequence in a viable recombinant is quite large and is still being explored: More than 3,900 nucleotides can be added to the hPIV3 genome with little effect on replication *in vitro* and with only a small reduction in replication in hamsters (unpublished observations). The use of viruses such as hPIV3 as vectors has several advantages. The

intranasal route of immunization is highly immunogenic. It also partly abrogates the suppressive effects of serum antibodies, and hence parainfluenza viruses can circumvent the inhibiting effects of maternal antibodies in young vaccinees as well as of preexisting serum antibodies in older vaccinees. An appropriate level of attenuation of the vector can then be achieved by reverse genetics. The existence of three serotypes of human PIV expands the range of vectors. The fact that essentially all adults and older children are seropositive for the human parainfluenza viruses and hence protected against serious disease means that the use of such a vector would not pose a biosafety risk to the general population.

An additional application of reverse genetics to parainfluenza virus vaccine development involves construction of novel attenuation mutations that do not exist in nature. One example involves Sendai virus or hPIV3 C or V protein knock-out mutations that attenuate virus growth *in vivo* (see earlier section, Properties of the Proteins). A second example involves the transfer of attenuating point mutations in related viruses such as RSV or Sendai to the corresponding sequences in hPIV3 (67,273). Another example involves the construction of virus chimeras that contain a mixture of internal protein genes of hPIV3 and bPIV3. In one construct, the NP ORF of hPIV3 was replaced by that of bPIV3. The resulting virus was fully viable *in vitro*, but in monkeys it exhibited the host-range restriction typical of bPIV3 (6).

In summary, reverse genetics provides a method for identifying the basis of attenuation of existing attenuated virus vaccine candidates. It also can produce new attenuation mutations. Desirable mutations in different viruses can be combined through a cDNA intermediate, something that is not possible in nature because of the inability of the mononegaviruses to undergo homologous recombination. This makes it possible to incrementally adjust the attenuation phenotype of a recombinant vaccine strain to accommodate information that is obtained during clinical evaluation. Finally, the ability to bring together the antigenic determinants of one virus and the attenuating internal proteins of another virus provides the flexibility needed to produce multivalent vaccines in a timely manner.

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CHAPTER 43 Mumps Virus

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HISTORY

In the 5th century B.C., Hippocrates described a mild epidemic illness associated with nonsuppurative swelling near the ears and, variably, with painful swelling of one or both testes. The name *mumps* may derive from an old English verb that means to grimace, grin, or mumble (185). Hamilton, a physician of the late 18th century, is credited as being the first to associate central nervous system (CNS) involvement with mumps in his description of the neuropathology of a fatal case (87).

A number of investigations suggested that there was a filterable, transmissible agent responsible for mumps (62,79,81,107). The demonstration that mumps virus could be isolated and propagated in embryonated eggs (86) enabled the demonstration of the hemagglutinating (139), hemolytic (164), and neuraminidase (33) properties of the virus. The introduction of tissue culture as a practical alternative for the propagation and study of the virus (89) further advanced the molecular understanding of mumps virus and the epidemiology and pathogenesis of mumps.

An attenuated mumps virus vaccine was introduced in the United States in 1967 (162). This resulted in a steady decline in the incidence of reported mumps cases, from 76 per 100,000 population in 1968 to the current low level of less than one reported case per 100,000 population (37,39). However, the widespread use of mumps vaccines remains relatively restricted to more affluent countries. Therefore, mumps is likely to remain a common worldwide human viral infection for some time.

INFECTIOUS AGENT

Mumps virus is a member of the genus Paramyxovirus in the family *Paramyxoviridae* (see also Chapter 41).

Propagation and Assay in Cell Culture

Mumps virus infects various primary and established mammalian cell lines *in vitro*, including BSC-1, Vero, MDBK, and HeLa. Mumps virus can also be propagated in the allantoic cavity of embryonic chickens and in chicken embryo cells *in vitro*. The presence of mumps virus is normally detected by the presence of syncytia followed by lysis of infected monolayers. Cytopathic effects of mumps virus in culture vary considerably among isolates and host-cell systems. Cell-to-cell fusion produces massive syncytia composed of large homogeneous masses of cytoplasm enclosing numerous nuclei (91) (Fig. 1). Such cell fusion requires expression of mumps virus glycoproteins HN and F (216). The presence of serine at position 195 of the fusion glycoprotein, F, seems to be obligatory for the appearance of significant syncytium formation (217). Other cytopathic effects of various mumps virus strains include spindle formation, cytoplasmic vacuolization and inclusions, and cell pyknosis and lysis; in some instances, there is little evident morphologic change (84,151). The type of cytopathic effect produced does not appear to correlate reliably with the *in vivo* behavior of the virus.

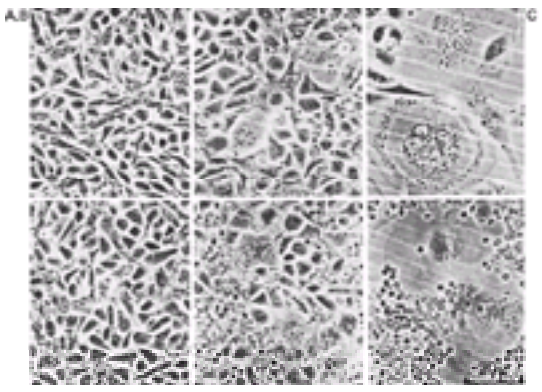

FIG. 1. Comparative cytopathic effects of avirulent O'Take (**B**) and neurovirulent Kilham (**C**) experimental mumps virus strains are illustrated in phase photomicrographs of BSC-1 cells taken 6 days after omparable low multiplicity or mock (**A**) infection. The monolayers of the *lower row* were hemadsorbed with guinea pig red blood cells. Occasional diploid cells are seen following the O'Take infection. This contrasts markedly with the syncytial-type cytopathology of the Kilham infection.

Because not all mumps virus strains produce cytopathic effects *in vitro*, mumps virus replication can be verified by hemadsorption (guinea-pig red blood cells), normally within 3 to 6 days of inoculation (201). Mumps virus–specific antiserum is then used in hemadsorption-inhibition (HI), plaque reduction neutralization (PRN) assays, and direct or indirect immunofluorescence assays. In addition, genome amplification by reverse transcriptase polymerase chain reaction (RT-PCR) and sequencing can be performed to identify unequivocally the isolate (22).

Some noncytolytic mumps virus infections are the result of persistent infection *in vitro* (90,154,224,235), and these may be instructive regarding naturally occurring, persistent Paramyxovirus infections. Factors that may contribute to mumps virus persistence *in vitro* include interferon (IFN) production, noncytopathic mutant selection, and the generation of defective interfering particles containing subgenomic RNA (70,151). Persistent replication *in vitro* has been associated with poor induction of IFN-induced 2',5'-oligoadenylate synthetase, linked to a decrease in STAT-1a (257). However, the relative contributions of these factors *in vivo*, in the presence of a host immune response, are not known.

Biologic Characteristics

Virion Morphology

Mumps virions are markedly pleomorphic (100 to 600 nm), and roughly spherical with an outer membrane that envelops an inner, coiled helical structure (Fig. 2). However, filamentous and bizarre forms are common even when virions are fixed *in situ* and examined in resin-embedded sections (Fig. 3). Multiploid mumps virions whose size and heterogeneity increase with multiplicity of infection have been reported. The envelope is studded with projections formed by the viral glycoproteins that rise 12 to 15 nm from the virion surface. The coiled inner structure or nucleocapsid is a ribonucleoprotein complex that appears to be a hollow tube with a unit length of approximately 1 µm, a diameter of 17 nm, and a central core of 5 nm (99,100,153). Full-length genomic RNA is an unsegmented, single-stranded macromolecule of negative polarity that contains about 15.3 kilobases (3).

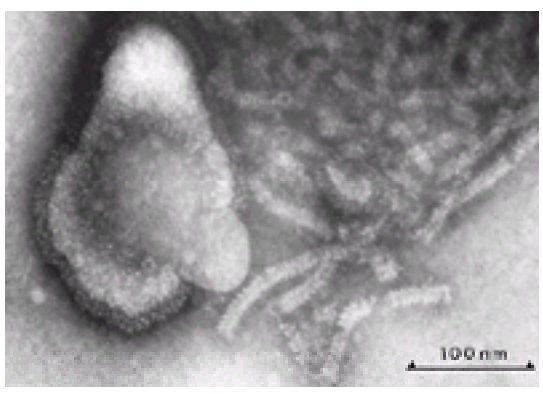

FIG. 2. Mumps virus preparation negatively stained with phosphotungstic acid, showing a typical virion studded with fine surface projections that represent the viral glycoprotein spikes. The membrane of the lower right portion of the virion has burst, allowing the escape of nucleocapsids, seen as scattered linear structures to the right of the field, that exhibit a typical herringbone pattern.

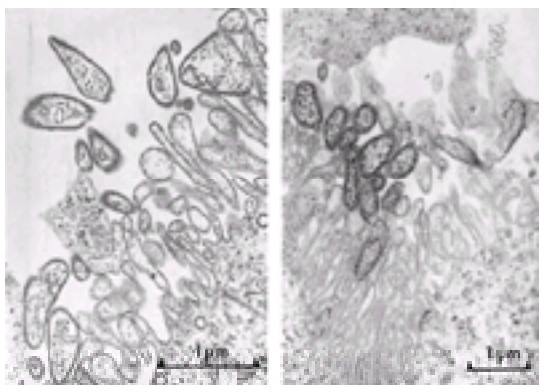

FIG. 3. Left: Pleomorphic mumps virions budding from a choroid plexus epithelial cell. **Right:** A subjacent section processed for postembedding localization of the major nucleocapsid-associated protein using monoclonal anti-NP antibody. The NP is localized by punctate, electron-dense reaction products below the surface membranes of the cell.

Key Virus Antigens

Many investigators have contributed to the delineation of the structural polypeptides of mumps virus (94,101,106,157,158,166,174,175,200). The virus contains six major structural proteins: nucleocapsid-associated protein (NP), phosphoprotein (P), and polymerase protein (L) are associated with the nucleocapsid; membrane or matrix (M) protein; and two glycoproteins, a hemagglutinin-neuraminidase (HN) and a fusion (F) molecule (Table 1). All mumps virus genes have been sequenced (55,57,58,129,237,239,250). The gene order for mumps virus is 3-*NP-P-M-F-SH-HN-L*-5 (56). There is a nontranscribed leader sequence of 55 nucleotides (215) and a trailing sequence of 24 nucleotides (170) that share inverse complementarity at their extremes. Consensus intragenic start (3'-U C C GG U N CU U U UC U) and stop (3'-A UA AU U C AU$_{6-7}$) sequences are separated by intergenic sequences of one to seven nucleotides (56). Mono-, di-, and polycistronic transcripts of these genes are variably found in mumps virus–infected cells (2).

Gene	mRNA[b]	Amino acids	MW in kd[c] Predicted	Observed	Biologic activity
NP	1,845	540	61.4	68–73	Major NC-associated protein; protects genomic RNA from cellular proteases
P	1,212	391	41.6	45–47	NC-associated phosphoprotein; forms part of the transcriptase complex
M	1,253	375	41.6	37–42	Membrane-associated protein; involved in NC alignment
F	1,721	538	58.8	65–74	Surface glycoprotein with fusion activity; mature form is a disulfide-linked complex of F$_1$ (48–51 kd) and F$_2$ (10–16 kd)
SH	310	57	6.7	6	Hydrophobic membrane-associated protein
HN	1,887	582	64	74–80	Surface glycoprotein with hemagglutinin-neuraminidase activity; disulfide-linked oligomer in native state
L	6,925	2,201	250.6	180–200	NC-associated protein, contributes to RNA-dependent RNA polymerase

[a]Composite properties from multiple studies; see text for specific references.
[b]Number of nucleotides in mRNA not including the polyadenylation tract.
[c]Approximate molecular weight in kd given first as predicted from the nucleic acid sequence and then as observed by gel electrophoresis.
NC, nucleocapsid; MW, molecular weight.

TABLE 1. *Mumps virus genes and gene product*[a]

Both mumps virus glycoproteins are membrane bound (106). The larger glycoprotein, HN, has both hemagglutinating and neuraminidase activities (106). In the native state, the HN protein is a disulfide-bonded oligomer. The F protein is composed of two disulfide-linked glycopolypeptides (F_1 and F_2) of unequal size (94,157,166,200,238,240). Both glycoproteins are acetylated (157). The HN glycoprotein mediates the adsorption of virus to host cells, and monospecific antibody against HN inhibits virus infectivity (33,173). The F glycoprotein mediates the fusion of lipid membranes at neutral pH; thus, F is required for the penetration of the viral nucleocapsid into the host cell and for hemolysis (174). Expression of both HN and F are required for efficient cell-to-cell fusion (216).

In contrast to the case for several other paramyxoviruses, the failure of some mumps virus strains to induce syncytia *in vitro* is not caused by incomplete processing of the F glycoprotein (157). However, differences in neuraminidase enzyme function of the nonfusing and fusing strains have been reported (158,240). For example, conversion from a nonfusing to a syncytium-forming phenotype was correlated with the substitution of isoleucine for threonine at HN residue 181 (236).

As deduced from the nucleic acid sequence, the HN protein consists of 582 amino acids and it has a molecular weight of about 64 kd when unglycosylated, with minor variations in predicted mass between mumps virus strains (134,237). HN appears to be held in the lipid bilayer by the hydrophobic domain of 19 residues near the amino terminal, a domain that probably also serves as a signal sequence in a manner similar to other Paramyxovirus HNs. There are nine potential N-linked glycosylation sites on HN, five of which are most likely used. The *F* gene encodes a protein of 538 amino acids with a calculated molecular weight of about 58.8 kd (57,239). At the extreme amino terminus of F_2, there is a hydrophobic signal sequence of 19 residues that is not present in the mature protein (239). The molecule is anchored into the bilayer by a stretch of 30 hydrophobic amino acids located near its carboxyl terminus. The active forms of the F protein (F_1 and F_2) are produced by cleavage of the immature protein (F_0) by a host-cell protease. This protease recognizes a sequence of five basic amino acids (Arg-Arg-His-Lys-Arg) at the carboxyl terminus of the F_2 protein. The fate of this segment after cleavage is unknown. A sole cysteine in F_2 then provides the only disulfide link with the membrane-bound F_1. The hydrophobic F_1 amino terminus is highly conserved and similar to other Paramyxovirus F_1 terminal domains. This hydrophobic domain of F_1 is presumed to be the functionally important region for fusion, initiating the fusion process by

intercalation with the lipid bilayer of target cell cytoplasmic membrane. Depending on the virus strain, there are six or seven potential glycosylation sites on F, five on F_1, and one or two on F_2 (239). Both the HN and F of mumps virus bear homology to simian virus 5 (SV5) and Newcastle disease virus (NDV). The greatest overall homology, both by sequence comparison and immunologic cross-reactivity, is with parainfluenza virus types 2 and 4 (119,126).

Mumps virus contains another membrane-associated protein called the membrane or matrix (M) protein, which is not glycosylated (106,174). M protein plays a critical role in the assembly of paramyxoviruses by mediating the alignment of the nucleocapsid under regions of virus-modified host cell membrane before the budding of the virion (149). As deduced from nucleic acid sequence data, the M protein is composed of 375 amino acids and has an estimated mass of about 41.6 kd. Its primary structure is conserved among mumps virus strains (53,57) and exhibits considerable sequence homology with that of other viruses such as NDV.

Nucleocapsids isolated from mumps virions contain the major structural protein NP and lesser amounts of P and a high-molecular-weight polypeptide, L (174). There are two closely related forms of the NP that resolve by electrophoresis. Both forms of NP are phosphorylated, and the extent of phosphorylation may affect the electrophoretic mobility of the proteins (166). Alternatively, the heterogeneity of NP may reflect limited proteolytic degradation of the protein. It is known that NP is readily susceptible to such degradation, as evidenced by the multiple NP-related polypeptide species observed when nucleocapsids are isolated from infected cells (150,200). Based on the nucleic acid sequence of the mRNA, the NP protein should contain 549 amino acids and have an estimated molecular weight of about 61.3 kd (215). This is 8 to 10 kd less than that usually observed by gel electrophoresis (150,174), even after *in vitro* transcription from cDNA constructs (215). The primary structure of NP is well conserved among phenotypically divergent mumps virus strains. Mumps virus NP is most homologous to human parainfluenza virus types 2 and 4B (215).

The virion possesses an RNA-dependent RNA polymerase or transcriptase (120), most likely involving both P and L. The P protein of mumps virus has been identified by its internal location in the virion, size, and association with nucleocapsid (150). Consistent with what is known of other paramyxoviruses, the P protein is phosphorylated (166). Three distinct mRNAs derive from the *P* gene: (a) a faithful transcript of the *P* gene virion RNA (vRNA) that encodes NS2 (also referred to as I), (b) P, and (c) NS1. The latter two are produced after RNA editing of vRNA by guanosine insertion at a G-rich region of the *P* gene (178,213). P has a predicted mass of 41.6 kd (54,213). The cysteine-rich domain of NS1 may be involved in transcription/replication of the vRNA (178). The accuracy of insertion of the nontemplated G may vary with the mumps virus strain (58). By electrophoresis, these species (P, NS1, and NS2) have apparent molecular weights of 45 kd, 28 kd, and 19 kd (94).

There is a large, virus-specific, about-200-kd polypeptide both in disrupted mumps virions and in mumps virus–infected cells (94,101,151,157). Associated with the viral nucleocapsid structure as part of the transcriptase complex, this protein is considered the L protein. The *L* gene contains 6,925 nucleotides and encodes a polyprotein of 2,261 residues with a predicted molecular weight of 256.6 kd (170). Motifs within the predicted L primary structure are consistent with the presence of an active polymerase catalytic site, an RNA template contact region, and an ATP binding site (170).

The remaining mumps gene encodes a small hydrophobic (SH) protein (56,57). The SH mRNA is found in mumps virus–infected cells and encodes a 57-amino-acid molecule. SH has an amino terminal stretch of 25 residues that could readily associate with membranes (55). Recently, detection of the SH protein was reported in the endoplasmic reticulum and perinuclear Golgi of infected cells using antisera prepared against SH oliogopeptides (214). However, the inability to identify SH in the Enders mumps virus strain indicates that the SH gene product is not required for viral replication.

Serologic Determinants

Two mumps virus antigens, S and V, are largely composed of the NP and HN proteins, respectively (106). Using polyclonal antisera in PRN assays, a broad cross-reactivity to HN from a variety of mumps strains has been demonstrated (254). Interestingly, although some type-specific anti-HN neutralizing antibody has been reported, there was no correlation found between specific changes in nucleic acid sequence with type-specific antibodies (176).

P>Monospecific polyclonal and monoclonal antibodies that react only with the glycoproteins have localized the red blood cell adsorption and neutralization sites to domains on the HN molecule that are distinct from those with neuraminidase activity (174,200,247). Similarly, the hemolytic activity of the virus resides with the F glycoprotein (174) and is distinct from red cell adsorption activity. Virus-specific antisera and mumps virus anti-NP monoclonal antibodies serologic cross-reactivity is highest between mumps virus and human parainfluenza viruses (105).

Infection in Experimental Animals

Host Range

The only natural host of mumps virus is man. Experimentally induced mumps infections have been reported in several species, most significantly, monkeys (138), adult and suckling hamsters (172), suckling mice (123), neonatal and suckling rats (187,191), and developing chick embryos (86).

Experimental Animal Models

Inoculation of mumps virus into suckling hamsters produces neurologic disease reminiscent of some features of human infection. The neuroadapted Kilham mumps virus strain, originally isolated from human breast milk, produces a severe meningoencephalitis and hydrocephalus in suckling hamsters on intracerebral inoculation (172,211). Developmental brain defects, such as a Chiari type I cerebellar malformation, have also been identified, possibly linked to the increased intracranial pressure in the setting of rapid postnatal growth of the cerebellar cortex (212).

In the 1950s, there was a report of experimental infection of young rats (187). Now there is renewed interest in the rat

experimental model, examining its value for predicting the human neurovirulence risk from mumps virus vaccine strains (191). In addition, unique evidence of neurodevelopmental damage, such as cerebellar hypoplasia, has been reported in the mumps virus–infected neonatal rat.

PATHOGENESIS AND PATHOLOGY

Virus Entry, Primary Replication, Spread

Susceptible individuals in close, but not necessarily intimate, contact are required to establish an epidemic (79). Transmissibility after nasal or buccal mucosal inoculation of virus (107) suggests that natural infection is initiated by droplet spread. During the 18-day incubation period from exposure to first clinical symptoms, the virus multiplies in the upper respiratory mucosa, spreads to draining lymph nodes, and then disseminates via a transient plasma viremia (120).

Virus is shed in saliva for as long as 6 days before the onset of parotitis (93). Termination of viral excretion in saliva correlates with the local appearance of virus-specific secretory IgA, as early as 5 days after disease onset (41). Virus-specific IgM antibodies are also present early in saliva (181). Thus, patients with mumps are capable of spreading virus by the respiratory route over a 7- to 10-day interval. Plasma viremia disappears coincident with the development of mumps virus–specific humoral antibody, which can be detected in serum as early as 11 days after induced apparent or inapparent infection of humans (93). Mumps virus preferentially infects activated human T lymphocytes (63). Animal models suggest that circulating infected lymphocytes provide a means for the spread of virus in the face of mounting humoral immunity (246).

Cell and Tissue Tropism

Parotid Gland

Initial clinical symptoms usually relate to infection of the parotid gland, but viral involvement of this gland is neither a primary nor an obligate step in the infection (121). Virus infects the ductal epithelium, producing intracytoplasmic inclusions (viral nucleocapsids) and viral antigens, with eventual desquamation of involved cells (147,242). Periductal interstitial edema and a local inflammatory reaction primarily involving lymphocytes and macrophages are also seen (242). Swelling, inflammation, and tissue damage in the parotid gland may produce elevation of serum and urine amylase levels (199).

Kidney

Virus also frequently disseminates to the kidneys, where epithelial cells of the distal tubules, calyces, and ureters appear to be primary sites of virus replication (242). Viruria, as an expression of renal involvement, can be detected in most patients, sometimes for as long as 14 days after the onset of clinical symptoms (226,228). Mild abnormalities of renal function have been described but are usually of little clinical importance (226). Although virus dissemination to, and replication in, the parotid gland and kidney can occur simultaneously, replication in renal tissue is more prolonged and continues well beyond the appearance of neutralizing antibody in serum.

Central Nervous System

The CNS is a common site of mumps virus dissemination with or without associated signs of parotid gland involvement. Virus is readily recovered from human cerebrospinal fluid (CSF) during the early phases of mumps meningoencephalitis (121). Using CSF pleocytosis as a marker of viral invasion of the brain, at least half of all infections are associated with replication of virus within the CNS (29). Symptomatic mumps meningoencephalitis is significantly more common in males than in females (19).

Viral invasion of the CNS presumably occurs across the choroid plexus (29). Blood-borne infected mononuclear cells may cross the fenestrated endothelium of the choroid plexus stroma and serve as a source for subsequent infection of the choroidal epithelium. However, direct passage of plasma virions across the endothelium of the choroid plexus to infect choroidal epithelium has not been excluded. Maturation of virus from the ventricular surfaces of choroidal cells provides progeny virions that are widely distributed through ventricular pathways and the subarachnoid space by CSF (Fig. 4).

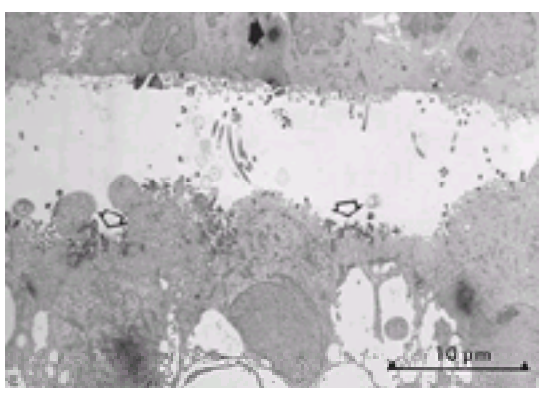

FIG. 4. Postembedding electron-microscopic immunocytochemical staining of a region of the lateral ventricle of a hamster on the fifth day following intraperitoneal infection with the Kilham strain of mumps virus. Anti-NP monoclonal antibody was used as the primary reagent. The reaction product defines nucleocapsids located below the cytoplasmic membranes at sites of virion budding along both the ependymal and choroidal (*unfilled arrows*) surfaces. Intracytoplasmic nucleocapsid inclusions are also demarcated by the reaction product in both choroid plexus and individual ependymal

(*filled arrow*) cells.

Although the specific virus genomic regions linked to mumps virus neurotropism and neurovirulence are not known, some nucleic acid sequences have been found to be associated with nervous system disease. In Japan, wild-type viruses that lacked a *Bam*HI restriction cleavage site in the *P* gene, compared to viruses that contained this cleavage site, were disproportionately associated with symptomatic meningitis (193). The presence of A in base position 1,081 of the *HN* gene is found in wild-type viruses (83), and this sequence has been proposed to be associated with increased neurovirulence of live, attenuated vaccine viruses (26). In contrast, a G in the same position was not associated with CNS disease (6). However, other sites on the mumps virus genome very likely contribute to neurovirulence [e.g., nucleotide positions 1,470 and 1,570 (amino acid positions 464 and 498, respectively) in the *HN* gene] (6).

Neurologic manifestations during mumps are usually those of meningeal infection (e.g., headache, fever, and stiff neck). Infection of the lining of the ventricles (ependymal cells) is also seen. Virus can penetrate brain parenchyma by contiguous spread from infected ependymal cells that line the ventricular cavities of the brain. However, frank encephalitis (e.g., infection of neurons in brain parenchyma leading to obtundation and other neurologic deficits) is less common. Once within neurons, virus most likely spreads along neuronal pathways, as reported in subhuman primates (138). In addition, the direct effects of mumps virus infection on neural cells may be compounded, in rare instances, by a virus-associated autoimmune attack on CNS myelin sheaths (i.e., autoimmune-mediated postviral encephalitis).

Typical CNS pathology of mumps virus encephalitis includes perivascular infiltration with mononuclear cells, scattered foci of neuronophagia, microglial proliferation, and perivascular demyelination (52,219). When seen, the selective periventricular myelin loss with relative sparing of axons is typical of parainfectious autoimmune encephalitis.

Rarely is mumps CNS infection fatal. However, a few fatalities have been reported, both in immunocompromised and in previously healthy individuals (10). Most cases of mumps meningitis resolve without sequelae. However, electroencephalographic changes, ataxia, and behavioral disturbances may take months to resolve following mumps meningoencephalitis (128). Permanent and ongoing neurologic damage may occur, such as obstructive hydrocephalus (169), deafness (142), myelitis (13), cerebellar ataxia (45,102), and, more rarely, problems from a variety of other neurologic syndromes. Hydrocephalus develops days to years after initial mumps virus infection (169). Narrowing or obliteration of the aqueduct of Sylvius, presumably the result of desquamation of infected ependymal cells (110) and/or cross-linking of ependymal cell processes by the agglutinating properties of mumps virions (244). Blockage of the aqueduct restricts the flow of CSF to normal adsorptive sites over the cerebral convexities and results in progressive enlargement of the lateral and third ventricles. Hydrocephalus can lead to progressively worsening headaches, mental status changes, and gait abnormalities. Deafness is probably the result of direct damage to the cochlea and, to a lesser extent, cochlear neurons (142).

Persistent CNS Infection

The cell-associated-virus persistence in the CNS extends well beyond the establishment of specific immune defenses (231). Persistence of inflammatory cells (pleocytosis) and oligoclonal immunoglobulin (bands) in the CSF indicate continued antigenic stimulation by persistent mumps virus (143). Such persistence, on rare occasions, has been associated with CNS diseases such as chronic encephalitis resulting in intellectual deterioration and seizures (231).

Gonads

Symptomatic gonadal involvement occurs primarily in postpubertal men and is rarely documented in women (14). Ovary infection may be underappreciated, because unless a pelvic exam is performed abdominal or pelvic pain from inflamed ovaries might be attributed to pancreatic infection. It is unclear why orchitis is less often seen in prepubertal boys. Virus has been isolated from testicular biopsies of the affected gland within the first 4 days of symptoms (17), strongly suggesting that symptomatic gonadal involvement reflects local virus replication and inflammation. The seminiferous tubules may be the primary site of viral replication, with local lymphocytic infiltration and edema of interstitial tissues.

Pancreas

Pancreatic involvement is usually expressed as mild epigastric pain, but severe hemorrhagic pancreatitis (61) and transient exocrine function abnormalities (49) have been reported. The mumps virus infects human pancreatic beta cells *in vitro* (232), and this occurs after experimental infection of hamsters (246). Viral infections have been considered a possible precipitating event leading to the onset of about one third of all cases of juvenile-onset or type I insulin-dependent diabetes mellitus (IDDM). Mumps may account for up to 8% to 22% of the preceding viral illnesses (125). Whether mumps virus causes IDDM is still unclear (48). In some cases of IDDM, autoantibodies to insulin or pancreatic islet cell antigens have been detected (189). Some epidemiologic studies (72) have reported an association between mumps and IDDM, but other studies have not (198). There is no association between mumps and type II diabetes (144).

Heart

Myocardial invasion occurs frequently in mumps, as indicated by electrocardiographic abnormalities (8). Although it is seldom symptomatic, there may be an interstitial lymphocytic myocarditis and mild pericarditis following mumps replication in cells of the myocardium and pericardium (30). Mumps virus myocarditis may lead to a rare but serious sequela of endocardial fibroelastosis (167). In contrast, mumps virus does not cause inclusion-body skeletal muscle myositis (258).

Mild to moderately severe mono- or polyarticular and, often, migratory arthritis has rarely been associated with mumps (80). Mumps virus can not be isolated from joint fluids or synovial tissues, and there is no evidence for significant immune complex deposition.

Fetus/Neonate

Mumps may produce a fetal wastage when contracted by the mother during the first trimester of pregnancy, occurring after blood-borne infection of the placenta, with or without subsequent spread of virus to involve fetal tissues directly (203,256). Mumps virus has been isolated from fetal tissues following spontaneous first-trimester abortion on the 4th day of maternal mumps (134) and from tissues derived from a planned therapeutic abortion of seronegative women 1 week after vaccination with live attenuated mumps virus (253). A proliferative necrotizing villitis with decidual cells containing intracytoplasmic inclusions has been described in the products of spontaneous and induced abortions (73).
Late-gestation intrauterine infection was reported in an infant born to a mother who developed mumps more than 4 weeks before delivery, diagnosed by RT-PCR testing of the infant's cord blood cells (249). This infant developed severe pulmonary symptoms, including hypertension and hemorrhage. Mumps virus is excreted in breast milk (122), but few cases of perinatal mumps have been described. Perinatal infection was suggested by virus recovery from an 8-day-old infant with upper respiratory symptoms whose mother developed mumps 4 days before parturition (112,135).

There appears to be a somewhat different mode of pathogenesis of mumps in newborns. In the first year or two of life, infants may have only pulmonary involvement without associated evidence of parotitis (112,249). Split immunologic recognition in the infants can follow maternal parotitis, resulting in mumps-specific, cell-mediated immune responses without concomitant humoral antibody response (1,206,222).

Immune Response

The host's immune response to mumps virus is complex but can be superficially divided into cellular and humoral responses.

Cell-Mediated Immune Response

In vitro lymphocyte-proliferative responses to mumps virus antigens are readily measured in seropositive individuals (103). Cell-mediated responses to previously recognized antigens may be diminished during and for up to 4 weeks following human infection with attenuated mumps vaccine virus (133) and, most likely, with natural mumps as well.

CD8+ and gamma-delta cytotoxic T lymphocytes have been demonstrated in blood and/or CSF following infection with wild-type or vaccine mumps virus strains (16,42,64,131). Mononuclear cell inflammation in mumps virus–infected tissues is well developed at the time of onset of clinical disease, suggesting that specific cellular responses develop during the incubation period. The presence of Ia+ T lymphocytes and specifically sensitized cells in both the blood and the CSF of some patients with active mumps meningitis supports this view (69). After immunization, peak immune responses are found 2 to 3 weeks later (42). Circulating cytotoxic T-cell responses restricted to autologous infected target cells are found within the first few weeks of mumps meningitis. Furthermore, variability in the host's cellular immune response to virus-infected cells might influence the clinical expression of the disease and contribute to the varied clinical expression of mumps.

Despite the importance of the cellular response in the recovery from most human viral diseases, there is little evidence to imply that the development of cellular immunity is crucial for resolution of mumps. However, some contribution of altered immune surveillance on the course of human mumps is suggested, for example, by a report of persistence of mumps virus antigens in lymph nodes and splenic tissues of patients with Hodgkin's disease (223).

Humorally Mediated Immune Response

The development of the complement fixation (CF) test using mumps virus antigen allowed the demonstration of (a) virus-specific antibody as a marker of prior mumps virus infection, (b) mumps cases without or prior to parotitis, and (c) correlation of seropositivity with resistance to clinical mumps (68,106,148). HI, PRN, enzyme-linked immunosorbant assays (ELISA), or radioimmunoassays (RIA) have largely supplanted the use of CF in the laboratory and in clinical studies of mumps humoral immunity (88,159,188,195). ELISA is valuable for rapid screening of large numbers of samples and typically shows more than 90% correlation with PRN tests (88). However, in a portion of the postvaccination sera, results for ELISA and PRN do not correlate (184). Thus, while the ELISA has proven quite sensitive, it appears less specific than the PRN, in part possibly because of cross-reacting antibody directed against parainfluenza viruses.

Mumps virus–specific IgM-class responses usually exceed IgG-class responses early in the infection and wane within the first 2 to 6 months (18,225). Virus-specific IgM-class antibody responses thus appear to be the most sensitive serologic indicator of acute or very recent infection and have the advantage of showing little cross-reactivity with related antigens of the parainfluenza viruses (159). Virus-specific IgG-class antibody responses do not reach maximal values until about the 3rd week of clinical disease (225), but they probably persist indefinitely. Using immunoprecipitation techniques, IgG-class antibodies that recognize HN, F, NP, and P are consistently produced following natural infections; a detectable IgG-class antibody response to M is unusual (Wolinsky and Salmi, unpublished data). The major IgG response is composed of immunoglobulin of the IgG1 subclass, with a minor contribution from IgG3 (194), suggestive of a T-helper-class type I response.

The role of humoral antibody in arresting the infection cannot be underestimated. Seropositive patients challenged intravenously with up to 10^9 plaque-forming units of mumps virus showed only transient fever, and no symptoms of reinfection developed even though an anamnestic rise in antibody titer was seen 4 to 5 days later (171). Animals are protected from otherwise lethal viral challenge by neutralizing monoclonal antibody administered several days after

primary virus inoculation (247). The development of HI and PRN antibody in the hamster model correlates well with the fall in peak virus titers in infected organs. However, in hamsters, viral antigens continue to be expressed, and clinical CNS disease actually occurs after virus titers have fallen considerably (246).

Neurovirulence

Development of a reliable *in vitro* or *in vivo* assay for understanding and predicting the pathogenesis of mumps virus strain-specific variations in neurovirulence for the human nervous system has proven a difficult and, as yet, unmet goal. *In vitro*, there are variable and contradicting reports of links between cytopathology and neurovirulence (152,191,244,246). *In vivo* model systems, such as rodents and primates, have shown variable neurotropism (i.e., ability to enter the nervous system) and neurovirulence (i.e., ability to damage the nervous system) profiles among various mumps virus strains (146,147,152,191,192,245,246). However, whereas some report correlation of wild-type strain neurovirulence in animal models with appropriate rankings of human neurovirulence in the clinical setting, other authors suggest no predictive value of the animal model for human mumps-related CNS disease. Moreover, model systems capable of reliably discriminating the subtle neurovirulence differences among attenuated vaccine strains are even more difficult to achieve. Development of an animal model that can predict the relative neurovirulence of mumps virus strains for human CNS is still an important goal. Such a model will aid in the identification of changes in virus genomic sequences responsible for neurovirulent phenotypes and development of sophisticated preclinical neurovirulence testing of candidate vaccines.

An association has been reported between specific sequences in viral genes and modulation of some of the neurovirulent phenotypes of the mumps virus strains. After *in vitro* selective pressure with HN-specific monoclonal antibodies, escape mutant strains show significantly reduced neurovirulence for hamsters *in vivo* (146). Analysis of these variants at the genomic level suggests that substitution of a cysteine for an arginine at position 360, within a postulated monoclonal antibody neutralization epitope of HN, results in attenuation (130). Subsequent studies on the HN of neurovirulent vaccine strains suggest that nucleic acid position 1,081, amino acid 335, is also associated with neurovirulence phenotype (26). The nucleotide A (Lys-335) in this position has been found in mumps virus vaccine and wild-type strains associated with CNS disease (26,83), whereas the presence of nucleotide G in this position (Glu-335) has been associated with neuroattenuated phenotype (6). In addition, the loss of a *Bam*HI restriction cleavage site in the *P* gene has also been associated with a neurovirulent phenotype in vaccine and wild-type virus strains (193). Thus, although limited information is currently available, data suggest that genomic sequences associated with neurovirulence phenotype are most likely located in multiple areas of the genome and involve more than one virus gene.

EPIDEMIOLOGY

Age

In an unvaccinated population, immunity to mumps is usually acquired between the ages of 5 and 14 years, with maximal acquisition of humoral antibody occurring between 4 and 7 years (7). In an urban population in 1978, 95% of adult women had serologic evidence of immunity, with a lower prevalence of immunity to mumps seen in rural communities. Premature infants may be particularly susceptible to mumps virus infection (77). At birth, 55% of infants born at less than 32 weeks gestation were found to be seronegative for mumps, although only 3% of their mothers were seronegative. By 3 months of age, 100% of the premature infants were seronegative for mumps virus, suggesting these infants may be susceptible to mumps well before 1 year of age.

Morbidity and Mortality

The major morbidity from mumps primarily results from complications of meningitis, encephalitis, and orchitis. These occur as age- and sex-specific hazards, with peak risk in postpubertal boys (7). Before World War II, mumps was an important cause of disability among troops and was 3 times more common than measles, the next most frequent communicable disease affecting military strength (79). Subsequent vaccine use has eliminated mumps as an important military concern. With the case fatality ratio for mumps between 1.6 and 3.8 per 10,000 (36), and with 0.5% to 2.3% of all mumps encephalitis cases being fatal (162), it is apparent that most fatal mumps cases have CNS manifestations.

Origin and Spread of Epidemics

Mumps virus, like measles virus, requires a population of about 200,000 people to sustain it. Such population densities were first achieved some four to five millennia ago (20), so it follows that mumps virus first evolved about 5,000 years ago. Presently, mumps is a geographically unrestricted disease except for its absence among a few remote tribes or isolated small island populations. In modern-day urban populations and in the absence of immunization, mumps occurs endemically, with peak incidence rates in the winter and spring months (162). The clustering of cases presumably reflects increased contact rates among susceptible children, usually attributed to the gathering of children at school (145). Mumps outbreaks are still reported even among highly vaccinated populations in developed countries (15,24,40,95). In Switzerland, after a low activity of mumps from 1986 to 1991, several outbreaks were reported in 1992 indicating an incidence of almost 200 cases per 100,000 population (75). Although primary vaccine failure (i.e., individuals who failed to develop immunity following vaccination) probably accounts for most mumps cases in vaccinated populations, there is also evidence of secondary vaccine failures (i.e., previously immune, vaccinated individuals with waning antimumps immune protection over time). For example, persons vaccinated more than 3 to 5 years before these outbreaks were more likely to contract mumps, consistent with waning immunity over time (4,24,95). Fortunately, secondary vaccine failure was rare in individuals who had received two doses of vaccine (95).

Prevalence and Seroepidemiology

Data on the incidence of mumps are complicated by the fact that approximately 30% of all mumps virus infections are subclinical (140,148). Furthermore, only one quarter of all cases are seen by a clinician, and, of these, as few as 14% are

reported to public health agencies (140). Annual incidence rates vary, with peaks every 2 to 7 years. Three-year peak cycles have been observed in England (7).

The highest incidence reported in the Unied States since 1922 was almost 250 per 100,000 population in 1941. In 1968, when mumps vaccine was introduced, the yearly incidence was 76 per 100,000 population, and it has steadily declined to just over 600 cases—that is, less than one case per 100,000 population in the United States (Fig. 5) (39).

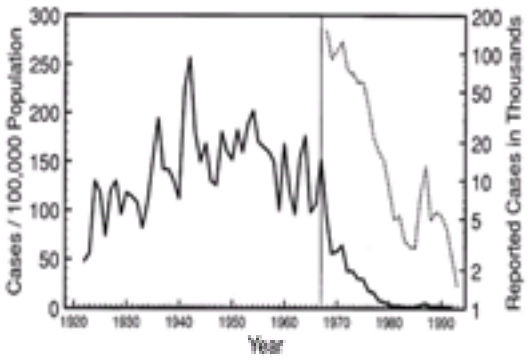

FIG. 5. Incidence of mumps in the United States from 1922, and annual reported cases reported from 1968 (37,44). The vaccine was first licensed for general usage in 1967.

Among 2- to 5-year-old children, mumps virus is a frequent cause of subclinical and symptomatic upper respiratory tract infections without parotitis (46). Parotitis occurs in only approximately 20% of all mumps infections in children under age 2. Furthermore, preschool-aged children were an important source of virus introduced into families with recent newborns (46).

In the outbreak of mumps among Eskimos on the St. Lawrence Island, where mumps was previously unknown, the epidemic evolved over 27 weeks from the time of induction of the signal case. After the epidemic, 87% of susceptible islanders had developed mumps CF antibody. Serologically defined inapparent infections were most common at the extremes of life (below age 4 and above age 60). Analysis of clinical symptoms obtained by history showed that 95% of all cases had parotitis, with 5% having orchitis without parotitis. The incidence of both mastitis and orchitis was highest among the postpubertal population.

Mumps virus was the most common cause of encephalitis in the United States until 1975 (38). In unvaccinated populations, mumps accounts for up to half of all encephalitis cases (186). In an epidemic setting, 11% of clinical mumps cases had CNS disease, 90% with meningitis and 10% with meningoencephalitis (183). Severe mumps meningoencephalitis is most common between the ages of 5 and 9 years, with a clear male predominance in all age groups (19). In adults (e.g., male military recruits), approximately 4% have symptomatic CNS involvement (156).

Once established, natural immunity to mumps is long-lived. Reinfection is distinctly uncommon. For example, reports of apparent symptomatic reinfection in individuals with preexistent CF antibody (183) may reflect known cross-reactivities of antibodies to other paramyxoviruses with mumps virus CF antigens (46).

New Epidemiologic Approaches

The most exciting molecular epidemiology advance has been RT-PCR to identify individual virus strains to group virus by related sequence. The SH gene sequence has also been used to characterize mumps virus isolates for clinical diagnosis, outbreak description, and confirmation of vaccine-induced adverse events, and to identify new virus strains (2,3,117,132,177,208,255). Although there is no uniform format for genotype groupings (118), a scheme of SH genotype categories A to F_1 has been proposed (220). In addition to geographic localization of some genotypes, there have been reported associations between the genotype clusters of mumps virus and propensity for CNS disease expression. For example, genotype A is associated with parotitis but rarely meningitis, whereas genotypes C and D are associated with meningitis, with and without parotitis (220). Final correlation of genotype group with clinical or geographic associations awaits international confirmation and agreement.

Other mumps genes have been used for strain identification. Based on restriction fragment length polymorphism, some vaccine strains can be distinguished from wild-type mumps virus strains (26,117,251). The sequence of the F gene, amplified by RT-PCR directly from clinical samples, has been reported to be useful in identifying circulating wild-type mumps virus strains (82). In contrast, the M gene sequence appears to be highly conserved between strains and of less use for strain identification (5).

CLINICAL FEATURES

The clinical features of mumps reflect the pathogenesis of the infection, as reviewed here. The incubation period is about 18 days (93,107), but approximately one third of all mumps virus infections occur without recognized symptoms (148,183). Moderate fever is present at the onset of disease, with defervescence a few days later. The feature most characteristic of mumps is salivary gland swelling (95% of all symptomatic cases), particularly the parotid glands (90% bilateral involvement) (183). Submaxillary gland enlargement and involvement of the sublingual glands also occurs. Enlargement of individual glands usually is maximal within 48 hours, at which time pain becomes most prominent, and may persist for a week or more (92). Virus is present in the saliva for several days before the onset of clinical disease

(91) and for up to 5 days later (41). The virus can be detected in urine for several weeks after the onset of mumps (228).

Various organs can be symptomatically involved during mumps. These include the testes, CNS, epididymis, prostate, ovary, liver, pancreas, spleen, thyroid, kidneys, labyrinth, eyes, thymus, heart, mammary glands, lungs, bone marrow, and joints. They are generally affected after parotitis, but their involvement may be clinically evident before, during, or even in the absence of parotid enlargement.

After mumps virus infection, about one quarter of all males develop orchitis, more frequently unilateral, and more often after puberty (183). Swelling and associated pain in the involved testes follow a time course similar to that of the parotid gland. Mumps orchitis often leads to testicular atrophy of the involved and, rarely, the clinically uninvolved gland (14). Atrophy after mumps orchitis is rarely implicated as a cause of male sterility (202). About 15% of females complain of breast swelling and tenderness. Like orchitis in men, the incidence of mastitis doubles among postpubertal women (183). Nausea and vomiting with or without epigastric or left-upper-quadrant pain are frequent features of mumps and occurred in about half of all cases during the St. Lawrence Island epidemic (183). It is unclear whether these symptoms reflect involvement of pancreas or other viscera. Some of these generalized features of the illness may reflect the effects of circulating IFN, which can be found in serum early in the clinical course of mumps (165,233).

Approximately half of mumps cases develop pleocytosis. Asymptomatic CNS involvement is common, since mumps meningoencephalitis occurs only in 10% to 20% of clinical cases (29,183,205). Mumps meningitis may precede parotitis, but it typically develops 5 days following parotitis. However, 50% of individuals with mumps meningoencephalitis do not have detectable salivary gland enlargement (111,121).

Diagnosis

The onset of mumps meningitis is usually marked by fever, and vomiting, neck stiffness, headache, and lethargy are quite common (9). Drowsiness and lethargy appear more commonly in younger patients, and meningeal signs are more readily detected in older patients. Seizures occur in 20% to 30% of patients with CNS symptoms (9,76).

In other childhood viral diseases such as measles, the electroencephalogram (EEG) is frequently abnormal even in the absence of CNS symptoms (109). In contrast, in mumps the EEG is usually normal, even when clinical seizures occur (76). However, in some deeply obtunded patients, diffuse slowing with increased voltage is seen.

About one third of all patients with CNS symptoms have either meningoencephalitis or pure encephalitis (209). Frank delirium and focal neurologic deficits are uncommon (111,162). Patients with mumps meningitis and meningoencephalitis without focal deficits, prolonged or recurrent focal seizures, or papilledema have marked clinical improvement of neurologic function within 2 to 4 days. Even with profound obtundation, patients recover with few sequelae (9,29,32).

Differential

When parotitis is present, the clinical diagnosis of mumps is straightforward, even if the disease is not epidemic. Nonetheless, other causes of parotitis should be considered: starch ingestion, iodine sensitivity, thiazide ingestion, tumors, Sjögren's syndrome, sarcoidosis, suppurative infections (including atypical mycobacteria), and other viruses such as parainfluenza types 1 and 3, influenza A, coxsackie, and lymphocytic choriomeningitis viruses. Without parotitis or with inconspicuous salivary gland enlargement, symptoms of other visceral organ or CNS involvement may predominate, and thus laboratory confirmation of the diagnosis is required, even during an epidemic.

Laboratory

Laboratory diagnosis presently rests on infectious viral isolation, appropriate serologic studies of acute and convalescent sera, or recovery of viral genome by RT-PCR. Virus isolation from saliva is most successful during the first 4 to 5 days of symptoms (42). Virus is recovered from urine for up to 2 weeks after symptoms begin (227), especially if care is taken to first clear the urine of debris, then concentrate the virus by ultracentrifugation (228). In patients with meningitis, the CSF can be expected to yield virus in, at best, about half of samples taken within 8 to 9 days of the onset of CNS disease (121,248). Samples are preferably plated fresh or held at –70°C. Monkey kidney primary or cell lines may be used for isolation, although other continuous cell lines are nearly comparable in sensitivity (182). Not all primary mumps virus isolates show characteristic syncytial cytopathic effects. Therefore, hemadsorption techniques should be applied to all cultures before they are discarded as negative. Immunofluorescence techniques not only provide direct confirmation of the isolation of a hemagglutinating virus in the culture but also, with appropriate reagents, specify the isolate as mumps virus (137). Amplification and sequencing of gene segments (e.g., SH) from cell extracts of primary isolates can provide useful epidemiologic data and virus strain identification (67). Combinations of techniques, such as amplifying live virus in clinical samples via Vero cell co-cultivation, followed by RT-PCR amplification of virus genome, can provide a sensitivity of 1 to 20 units of infectious virus per milliliter (22).

When performed on the same day, comparison of mumps-specific antibody titers from serum taken at the onset of clinical disease and a convalescent serum sample obtained 2 to 4 weeks later usually shows the anticipated rise in CF, HI, PRN, or hemolysis-in-gel (HIG) assays (68,85). Determination of virus-specific IgM and IgG levels on a single acute-phase serum provides an alternative when paired sera are not available (159,225). The current standard for relatively specific, rapid, and cost-effective serologic diagnosis is ELISA (43,88,141).

Examination of the CSF of patients with mumps virus infection shows abnormalities in the majority of cases, beginning with the onset of CNS disease (12). Cell counts peak around the third day of neurologic symptoms, averaging 250/mm ^{3} and, may exceed 1,000/mm^3 (121,143,248). The pleocytosis then wanes gradually over several weeks (69,243), up to as long as a year (230). The CSF is usually under normal pressure (32) and the protein content in the CSF is elevated in 60% to 70% of all cases (69,121). Protein levels up to 100 mg/dL can be seen; on occasion, they exceed 700 mg/dL. This appears to reflect damage to the blood–brain barrier, as indicated by elevated albumin indices that do not normalize for

several weeks to months after the onset of CNS symptoms (143). The CSF glucose content is modestly depressed to 17% to 41% of the serum value in 6% to 29% of all cases (51,111,243). About one third of all patients have evidence of intrathecal IgG synthesis and demonstrable oligoclonal immunoglobulins during the first week of CNS symptoms, and this oligoclonal antibody may persist for over 2 years (69). Over one half show increased IgG indices by the third month following mumps meningitis (69,143,231). Using sensitive ELISA or imprint immunofixation assays, up to 90% of patients with acute mumps meningitis can be shown to produce virus-specific IgG-class antibodies within the CNS compartment (143,225,231), and one half show mumps virus–specific IgM (65). In many instances of mumps meningitis, diagnosis can be confirmed by elevated CSF/serum antibody ratios from a serum sample set taken during illness (165,225), as mumps antibodies are uncommon in the CSF during other CNS infections (50).

Of all cases of mumps with symptoms severe enough to warrant hospitalization, less than 10% can be expected to show transient electrocardiograph abnormalities consistent with myocardial damage. These usually consist of ST-segment depression, but patients can show evidence of atrioventricular conduction defects, including complete heart block (8). Seldom is the cardiac involvement acutely symptomatic. However, mumps virus myocarditis can be linked to serious long-term sequelae, such as endocardial fibroelastosis (167).

TREATMENT

Treatment of mumps and its various complications is generally symptomatic. In a controlled study, adult men presenting with parotitis were alternatively given either an intramuscular injection of 20 mL of gammaglobulin prepared from human convalescent serum or simply confined to a hospital for routine, nonspecific symptomatic therapy (74). Orchitis developed in 4 of 51 antibody-treated and 14 of 51 symptomatically treated patients ($p < .01$ by chi square). No protective effect was seen with gammaglobulin obtained from a normal donor serum pool (74). This study suggests that immunotherapy with high-titer polyvalent or monoclonal antibody preparations could be useful in selected cases but should be used very early in the course of the illness. Recently, experimental treatment of orchitis with IFN-a-2B has been reported (35).

PREVENTION AND CONTROL

Control measures for mumps must be primarily directed at prevention. The high incidence of asymptomatic infection, the relatively long period of virus shedding before clinical symptoms emerge, and the infectivity of nonparotitis cases render futile any patient quarantine procedures that might be tried to stem an epidemic. The mass use of hyperimmune gammaglobulin can reduce the incidence of mumps in a community if administered very early in an outbreak (47), but such an approach has generally not been effective in clinical practice (190). Therefore, prevention through an effective vaccination program remains the only rational approach to the control of mumps.

Vaccination

Two general types of mumps virus vaccines have been used. The vast majority of mumps vaccinations are with live attenuated mumps virus preparations. Except for specific indications, killed mumps virus antigens have a more restricted value and have been largely supplanted by the more effective live attenuated vaccines.

Killed Virus Vaccine

Killed mumps virus vaccines have a limited role, as serologic evidence of immunity may wane without periodic booster injections (185). Serologic responses following immunization with either killed or live attenuated vaccines are similar (113). It is likely that the formalin-inactivated mumps vaccine can induce antibody to the HN glycoprotein, but it does not induce a response to certain F-glycoprotein configurations (168). However, most inactivated vaccine recipients generated antibody to denatured F protein (113). Furthermore, induced antibody appears to be limited to the IgG class, with no IgM class responses detected by RIA following immunization with killed vaccine (104). Finally, cell-mediated immune responses following killed vaccine appear to be delayed compared to those seen after natural infection (104).

The killed vaccine does provide effective, if not durable, protection and does not appear to predispose to later atypical mumps (180). It therefore may provide an alternative for vaccination of immunocompromised, susceptible patients. Furthermore, the rather extensive experience with inactivated mumps virus vaccine suggests that molecularly engineered component vaccines may be effective in stimulating a protective immune response to mumps virus.

Live Attenuated Virus Vaccines

The use of live attenuated mumps virus vaccines has caused a remarkable decline in the incidence of mumps and related sequelae of wild-type infection. Several different strains of live attenuated mumps virus vaccine are in international use at this time, and new vaccines are continuing to be evaluated and licensed. Many vaccines are now known to be mixtures of several related strains, including Jeryl Lynn, Urabe Am9, and Leningrad 3 (3,23,26). In general, the efficacy of mumps vaccines is good relative to their profile of safety and adverse event induction. However, at least one mumps virus vaccine strain was withdrawn from use in several countries because of an unacceptable incidence of associated meningitis (26,27).

Continuous serial passage to select for attenuation is the strategy used to develop mumps vaccines (59,93,252). At present, there is no clear distinguishing marker for attenuation of mumps virus strains apart from the failure of a passaged isolate to produce clinical symptoms in man. In addition, because of demonstrated and theoretical considerations about spread of virus to placenta and fetal tissues, administration of vaccines containing live mumps virus during pregnancy is not advised (185).

Jeryl Lynn/RIT 4385

The Jeryl Lynn B strain of mumps originated as an egg allantoic fluid isolate (34). This isolate was attenuated first by 17 serial passages in embryonated hens' eggs and chick embryo cell cultures. The Jeryl Lynn vaccine is a mixture of at least two virus strains (3,28). A predominant strain (JL5) was identified along with a second strain (JL2) in a ratio of 5:1, respectively. Recently, a new mumps vaccine (RIT 4385) derived from the JL5 component of Jeryl Lynn strain has been licensed in Europe (204).

Vaccination of susceptible children with a single subcutaneous inoculation of Jeryl Lynn vaccine does not produce serious clinical symptoms, and vaccine virus cannot be isolated from blood, urine, or saliva (60). However, some dissemination of Jeryl Lynn vaccine virus must occur, as virus has been isolated from placental tissues of two of three seronegative women immunized with attenuated live virus vaccine 7 to 10 days prior to planned induced abortion (253).

Urabe Am9

The Urabe Am9 vaccine was developed in Japan and is widely used in North America (not the United States), Europe, and Japan, proving to be an effective, immunogenic vaccine. However, recently Urabe Am9 was withdrawn from many markets because of an unacceptably high incidence of CNS adverse events (e.g., meningitis) (27,66,67,221). It is not surprising, therefore, that this vaccine appears to disseminate more widely in the vaccinee than does Jeryl Lynn. Aseptic meningitis with vaccine virus recovered from CSF complicates from 1 in 1,000 to 1 in 11,000 vaccinations with Urabe strain–containing vaccines (160). Symptoms of meningitis appear within 4 weeks of vaccination (155).

Virus recovered from several Urabe vaccinees with meningitis possessed the wild-type substitution of A for G in nucleotide position 1,081 of the *HN* gene (26). Brown and Wright also reported that the Urabe Am9 vaccine was a mixture of viruses with A or G in base position 1,081. This G to A change resulted in a Glu to Lys change at amino acid 335. Interestingly, amino acid 335 may be an important neutralization epitope (6). These data suggested that postvaccine meningitis might be associated with a virus with the wild-type sequence at position 1,081 in the *HN* gene. Furthermore, it has been suggested that this virus, capable of replication in the CNS, was one of the preexisting components of the vaccine, rather than a reversion to wild type (28). It is important to note, however, that other evidence suggests that genomic differences at other sites also contribute to the neurovirulent phenotype (6,28).

Rubini

A live attenuated virus vaccine developed from a wild type isolated in Switzerland, the Rubini strain sequence bears similarity to the Enders strain and was originally licensed based on studies showing high immunogenicity rates. Following mumps outbreaks in Switzerland and Singapore, recent reports suggest that the Rubini strain has a lower immunogenicity profile (75), possibly suggestive of primary vaccine failure (78) or waning immunity with time (4). Additional studies are needed to resolve these issues.

Serologic Response

Neutralizing antibody is usually present within 2 weeks of vaccination (60), and a serologic response to vaccination is anticipated for over 90% of vaccinees (31,88,97). While antibody titers after vaccination develop more slowly and are lower than those following natural infection, protection from natural mumps appears complete (31,60), and neutralizing antibody has been shown to persist for more than 19 years (185). Combined immunization with live attenuated measles, mumps, and rubella virus vaccines does not adversely affect either the serologic response to mumps virus or the apparent long-term protection from mumps (185,241).

Adverse Events

Millions of doses of Jeryl Lynn mumps virus–containing vaccine have been distributed in the United States, with few serious adverse effects noted (96). Most reported complications such as rash, pruritus, and purpura have been allergic in nature and are both uncommon and usually mild and self-limited (38). Approaches for the immunization of egg-allergic children have been detailed (136). A comprehensive survey of the literature by the Vaccine Safety Committee of the Institute of Medicine failed to find adequate information to establish or reject a causal relationship between the administration of Jeryl Lynn mumps virus–containing vaccines and the development of encephalopathy, encephalitis, residual seizure disorders, optic neuritis, transverse myelitis, Guillain-Barré Syndrome, IDDM, or sterility due to orchitis (for extensive reviews, see refs. 21,207).

Based on information from countries in which multiple mumps virus vaccines are licensed and utilized, there are some mumps virus strain-specific differences in adverse events. Whereas both Urabe and Jeryl Lynn vaccine strains have been associated with parotitis, with an incidence of 0.7% (11), Jeryl Lynn vaccine has not been associated causally with significant CNS disease. In contrast, in Japan at least 311 meningitis cases were reported among 630,157 recipients of the Urabe Am9 vaccine, with 96 cases being confirmed by vaccine virus isolated from the CSF (210). Urabe vaccine–associated meningoencephalitis was also documented in other countries, such as Canada and England (11,98,160). The majority of the affected vaccinees became ill within 15 to 21 days after vaccination. There was a male predominance and the meningitis was reported to be mild with no sequelae noted. Interestingly, there are suggestions of variable rates of postvaccination meningitis with Urabe vaccines from different Japanese manufacturers, raising a question of manufacturing consistency (124).

Other adverse events include orchitis and transmission of vaccine virus to siblings following Urabe vaccination (210). Although deafness, IDDM, pancreatitis, autism spectrum disorder, and inflammatory bowel disease (114,163,234) have been reported following mumps vaccination, the most current evidence does not support a causal association with the vaccine (11,179,218).

Clinical Use

The generally accepted practice is to vaccinate all children with trivalent measles-mumps-rubella vaccine between 12 and 15 months of age to avoid the presence of passively transferred maternal antibody (196). However, between 15% and 19% of children vaccinated at age 12 to 18 months may be seronegative by PRN 4 years after vaccination (161). Concerns raised by this and other studies led to the demonstration of the benefits and adoption of the two-dose regimen (25). The doses are administered at least 1 month apart; typically, children receive the first dose of vaccine between 12 and 15 months of age, and the second dose before entering elementary school at 4 to 5 years of age (38). Data suggest that administering the second dose at 11 to 13 years appears to be equally effective (38,108). Vaccination in the face of recent chemotherapy or radiation therapy is unlikely to induce adequate serologic responses and should be delayed for at least 3 months following such treatment (185). Studies of mumps virus–containing vaccines in nonimmunocompromised human immunodeficiency virus–infected patients, primarily in the form of trivalent mumps, measles, and rubella vaccines, have not documented serious or unusual adverse effects; however, in these patients the clinician needs to consider the risk–benefit ratio of vaccination and consult the current recommendations of the Advisory Committee on Immunization Practices (38). Vaccination as early as 9 months of age may be equally as effective, particularly with infants born to vaccinated mothers who have not experienced wild-type infection (115,197).

At one point, a transient reversal in the marked trend for reduced numbers of reported cases of mumps in the United States created considerable concern. A detailed analysis suggested that the observed relative increase of incidence is a reflection of the pool of susceptible children, teenagers, and young adults who were not immunized aggressively for mumps (15,24,40,44,95,116,205), especially during the first decade following introduction of the vaccine. Interestingly, theoretical models of the effects of mass vaccination programs suggest that the average age of disease can be anticipated to rise with the level of induced immunity, with the additional paradoxical result of increased relative risk of serious consequences of mumps infection (e.g., orchitis in susceptible postpubertal males) (7).

Although mumps and its complications are usually mild, most analyses of theoretical cost–benefit ratios favor an intensive vaccination program for affluent, developed nations (71,116,122,127,205).

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CHAPTER 44 Measles Virus

Diane E. Griffin

Measles is a highly contagious disease characterized by a prodromal illness of fever, coryza, cough, and conjunctivitis, followed by the appearance of a generalized maculopapular rash. Introduction of measles into virgin populations and endemic transmission in populations with inadequate medical care are associated with high mortality. Despite the development of a successful live attenuated vaccine, measles remains a major cause of mortality in children, particularly in developing countries, and a cause of continuing outbreaks in industrialized nations.

HISTORY

Measles is a relatively new disease of humans and probably evolved from an animal Morbillivirus. Phylogenetically, measles virus (MV) most closely resembles rinderpest virus (RV), a pathogen of cattle (461) (Fig. 1), and it is postulated that MV evolved in an environment where cattle and humans lived in close proximity (413,458). Because large numbers of people are required to generate sufficient susceptible individuals to maintain measles in a population, measles probably evolved in the early centers of civilization in the Middle East where populations attained sufficient densities to maintain transmission (413).

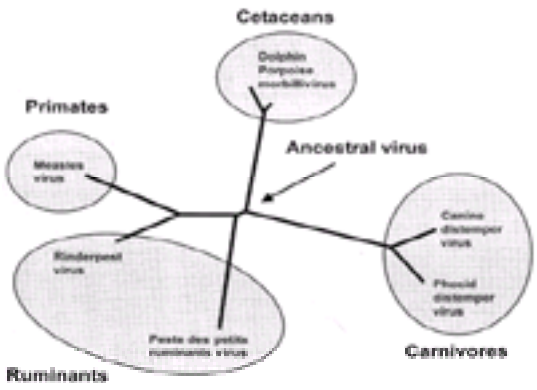

FIG. 1. Genetic relationships between morbilliviruses based on comparison of the nucleotide sequences of the N genes. The tree was derived using PHLIP, DNADIST and FITCH programs. The branch lengths are proportional to the mutational differences between the viruses and the hypothetical common ancestor that existed at the nodes in the tree. (From ref. 43, with permission.)

Abu Becr, an Arab physician known as Rhazes of Baghdad, is generally credited with distinguishing smallpox from measles in the 9th century. He dated its first description to the 6th century. Rhazes referred to measles as *hasbah*, “eruption” in Arabic, and regarded it as a modification of smallpox (281,517). One distinction noted was that “anxiety of mind, sick qualms and heaviness of heart, oppress more in the measles than in the smallpox” (517). Repeated epidemics of illnesses characterized by a rash are recorded in European and Far Eastern populations between A.D. 1 and 1200 (413). It appears that measles spread across the Pyrenees into France with the Saracen invasion of the 8th century (534). Repeated epidemics identified as measles are not recorded until the 11th and 12th centuries, and it is first

mentioned as a childhood disease in 1224 (413).

In the European literature, the name applied was “morbilli,” derived from the Italian “little diseases” to distinguish it from plague, *il morbo*, but morbilli included several exanthemata. Sanvages in 1763 defined morbilli as measles but called it rubeola [derived from the Spanish (534)], leading to a common confusion with rubella that persists to the present.

Introduction of measles into previously unexposed populations has been associated with high morbidity and mortality (67,129,534). Epidemics of rash illnesses were associated with episodes of depopulation in China, India, and the Mediterranean region. There was 26% mortality associated with introduction of measles into the Fiji Islands in 1875 (281,534). Approximately 56 million people died as a result of European exploration of the New World, largely because of the introduction of Old World diseases, notably smallpox and measles, into native Amerindian populations. Decreases in population are likely to have facilitated the transfer of Spanish culture to South America (413).

Many of the basic principles of measles epidemiology and infection were elucidated by the studies of Peter Panum, a young Danish physician who was sent to the Faroe Islands in 1846 during a large measles epidemic (481). Panum deduced the highly contagious nature of the disease, the 14-day incubation period, and the lifelong immunity present in older residents, and he postulated a respiratory route of transmission.

Complications of measles were first described in the 18th century. In 1790 James Lucas, an English surgeon, described the first case of postmeasles encephalomyelitis in a young woman who developed paraparesis as the rash was fading (384). Medical textbooks of the 19th century associated measles with the exacerbation of tuberculosis, and in 1908, while working at a tuberculosis hospital in Vienna, von Pirquet recorded the disappearance of delayed-type hypersensitivity skin test responses to tuberculin during a measles outbreak (641).

Subacute sclerosing panencephalitis (SSPE) was first described in 1933 by Dawson in a 16-year-old boy with progressive neurologic deterioration (147). Histologic examination of the brain showed eosinophilic intranuclear and intracytoplasmic inclusions in neurons and glial cells (147). Reports of paramyxovirus-like particles in the inclusions (616) were followed rapidly by documentation of elevated MV antibody in serum and cerebrospinal fluid (CSF), staining of the inclusions with antibody to MV (131), and culture of MV from SSPE brains (486).

INFECTIOUS AGENT

In 1757, measles was formally shown to be caused by an “infectious” agent when the Scottish physician Francis Home, attempting immunization, transmitted the disease to naive individuals using blood taken from measles patients during the early stages of the rash (285). In 1905, Hektoen transmitted disease to volunteers with blood “free of bacteria” taken from measles cases in the acute stage (270). In 1911, Goldberger and Anderson transmitted measles to monkeys with filtered respiratory tract secretions from measles patients and successfully passaged disease from one monkey to another (226).

Propagation and Assay in Cell Culture

Primary Isolation

The virus was first grown in tissue culture in 1954 by Enders and Peebles, who inoculated primary human kidney cells with the blood of David Edmonston, a child with measles (177). Isolates were also made from peripheral blood leukocytes or respiratory secretions inoculated onto primary monkey kidney cells (173,177). Because of problems with animal use and possible contamination of primary cells with simian viruses, continuous monkey kidney cell lines (e.g., Vero, CV-1) subsequently replaced primary kidney cell cultures for MV isolation (589). However, isolation of wild-type strains is most often successful using an Epstein-Barr virus–transformed marmoset B lymphocyte line, B95-8 (342), or human cord blood leukocytes (201). Generally, the first observable sign of virus growth is cell–cell fusion and syncytia formation (Fig. 2).

FIG. 2. Typical cytopathic effects of syncytium formation associated with measles virus replication in (**A**) Vero cells and (**B**) lung tissue of a monkey with giant cell pneumonia. (Courtesy of W. Bellini.)

Laboratory Propagation and Assay

Once MV has been isolated in culture, it can often be adapted to replicate in a variety of cells in culture. For instance, MV has been grown in primary human cells, in human cell lines, and in primary monkey kidney and testis cells, as well as monkey cell lines (173).

The process of adaptation of MV to foreign hosts, such as the chick embryo and canine and bovine kidney cells, has led successfully to the development of live attenuated vaccine strains (332,333,423,568). Mouse cell lines are generally nonpermissive, except for neuroblastoma lines where there is restricted synthesis of MV proteins and a low yield of infectious virus (386,503).

Most experimental work is done with tissue culture–adapted strains, so virus stocks are generally grown in Vero cells using a low multiplicity of infection to avoid accumulation of defective interfering particles (123). These cell lines are most useful for titration of adapted virus by plaque formation (9) using an agar or methylcellulose overlay. The virus replicates slowly, and 3 to 5 days of culture are needed before plaques become visible. Longer periods may be required to reach

the endpoint of a titration. Heat shock may increase the yield of infectious virus (482). Stocks of nonadapted strains may be assayed by syncytium formation in B95-8 or human cord blood cells. Identification of infectious foci is optimized by using immunocytochemical techniques to detect MV-infected cells.

Biologic Characteristics

Genetically, the morbilliviruses form two groups of viruses related either to canine distemper virus (CDV) or to RV (see Fig. 1). Morbilliviruses are distinct from other paramyxoviruses in that they form intranuclear inclusion bodies (Fig. 3). Virions are pleomorphic and range in size from 100 to 300 nm. The envelope carries surface projections with a length of 9 to 15 nm, which are composed of the viral transmembrane hemagglutinin (H) and fusion (F) glycoproteins (Fig. 4). The nucleocapsid (total length of 1.2 µm) is packed within the envelope in the form of a symmetrical coil (385). Virions may also contain actin from the cellular cytoskeleton, which is involved in the final steps of budding from the plasma membrane (79).

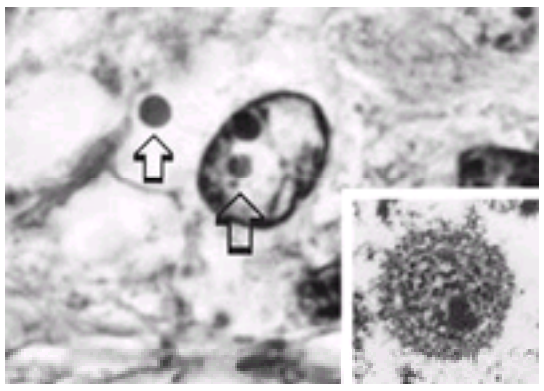

FIG. 3. Morbillivirus inclusion body formation within a canine distemper virus-infected astrocyte. By light microscopy, cytoplasmic inclusions (*small arrow*) are discrete and intensely eosinophilic, representing dense aggregates of viral nucleocapsid. The most common expression of intranuclear inclusions is an eosinophilic circular structure (*large arrow*). The ultrastructural basis for this intranuclear inclusion is the complex nuclear body (*inset*), a nucleolar derivative containing N protein. (From ref. 463, with permission.)

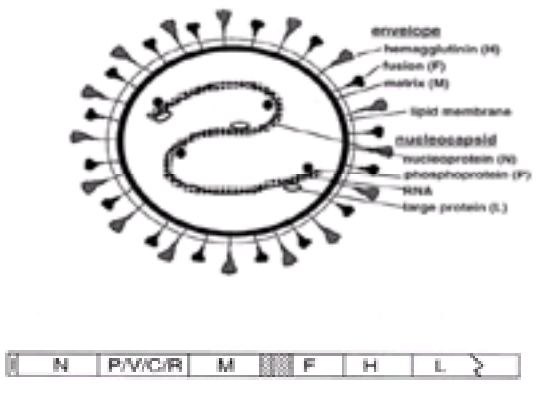

FIG. 4. Schematic diagram of measles virus and the genome and gene order. I is the leader and P/V/C/R is a multicistronic gene encoding P, V, C, and R proteins. The *shaded region* between M and F contains approximately 1,000 nucleotides of untranslated RNA.

Proteins

The proteins of MV are listed in Table 1.

Protein	Amino acids (N)	Apparent size (kd)	Infected cell	Virion	Suggested function and special features
Nucleoprotein (N)	525	60	Cytoplasm	Nucleocapsid	Most abundant of viral proteins; closely associated with full-length + and − sense RNA to form helical capsids; phosphorylated
Phosphoprotein (P)	507	70	Cytoplasm	Nucleocapsid	More abundant in infected cells than virions; likely involvement in regulation of transcription and replication; complex formation with N and L; phosphorylated
C	186	20	Cytoplasm and nucleus	Not present	Read from the same transcript as P, but a different reading frame; downregulates transcription?
V	299	45	Cytoplasm	Not present	Read from an "edited" transcript of P gene; phosphorylated; contains cell-CCCH tetrakus; function unknown
Matrix or membrane (M)	335	39	Inner leaflet of plasma membrane, cytoplasm (perinuclear inclusions)	Inner surface of membrane	Virion assembly and budding; inhibits transcription?
Fusion (F)	550	59	ER, Golgi, and plasma membrane	Transmembrane dupe N surface protein	Glycosylated; membrane fusion, virus entry, and hemolysis activity in conjunction with H; 50 kd inactive precursor F_0, mature sits F_1 and F_2
Hemagglutinin (H)	617	76	ER, Golgi, and plasma membrane	Transmembrane dupe N surface protein	Receptor binding, hemagglutination activity, disulfide-linked dimer, glycosylated
Large (L)	2,183	250	Cytoplasm	Nucleocapsid	Catalytic component of viral polymerase; least abundant protein; RNA transcriptase and replication, complex formed with P?

TABLE 1. *Characteristics of the Measles Virus Proteins*

Nucleocapsid Protein

The nucleocapsid (N) mRNA is the first transcribed from the genome, and the N protein is the most abundant of the viral proteins. N (525 amino acids) is synthesized on free ribosomes and folded in the cytoplasm (230), and it plays a central role in the replication of viral genomic RNA. N is phosphorylated, and this modification appears to regulate function. Free N is phosphorylated on serine, whereas nucleocapsid-associated N is phosphorylated on serine and threonine and, in persistently infected cells, also on tyrosine (231,569). N appears as a 60-kd band on polyacrylamide gels, but it is often cleaved by cellular proteases during extraction to 45- and 41-kd products (236). N can self-assemble but usually surrounds viral genomic and antigenomic RNAs that possess the leader sequence (108) to form the nucleocapsid structures (627). This structure is the required template for both replication and transcription and, in conjunction with the P and L proteins, comprises the ribonucleoprotein complex (510). N can be transported to the nucleus but is usually retained in the cytoplasm by binding to P (298). Amino acid sequence conservation is strong in the N-terminal portion of the protein that is required for self-assembly into nucleocapsids or interaction with P (38,230,592) (Fig. 5). There is variability in the region between residues 130 and 150 (542) and in the C-terminal 125 residues (37,117,541,542,615) that are located on the outside of the nucleocapsid structure (87). Analysis of the variable C terminus of N has been useful for identification of genotypes and analysis of the molecular epidemiology of measles (542,615).

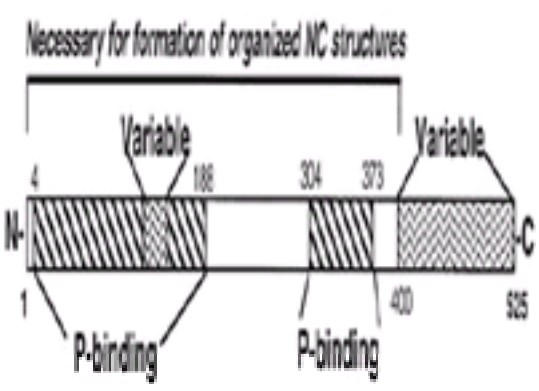

FIG. 5. The nucleocapsid (*N*) protein. Diagram showing the regions of N required for binding to the P protein and regions of sequence variability between strains.

P, C, V, and R Proteins

The P (phospho) protein (507 amino acids) is activated by phosphorylation and binds to N, L (large), and RNA to form the replicase complex (526). Serine/threonine phosphorylation of P is carried out by cellular kinases, primarily casein kinase II (144). Although the 72-kd P protein is abundant in the infected cell, only small amounts are present in the packaged virus. The N terminus is acidic and highly phosphorylated, and it binds to L, whereas the region between amino acids 204 and 321 contains the domain that binds to N (298) (Fig. 6). The P protein oligomerizes and interacts with L and N through the unique C-terminal portion of the protein (136,264,377) and modulates assembly of nucleocapsids (592).

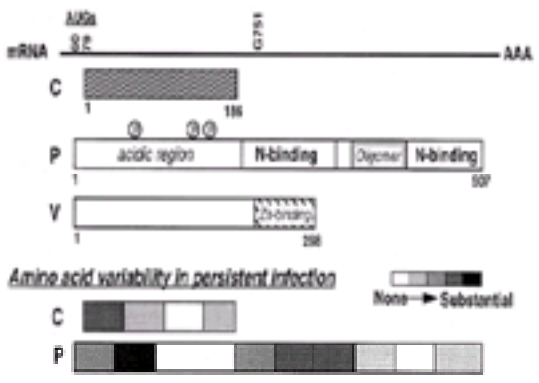

FIG. 6. The phospho (*P*), C, V, and R proteins. Diagram of the mRNAs and encoded proteins. P and C are initiated at AUGs in different reading frames. V is translated from an edited RNA with a nontemplated G at nucleotide 751. R is the truncated form of P that is produced by ribosomal frame shifting to access the V open reading frame 24 nucleotides upstream of the V stop codon.

The P gene of MV, like many members of the *Paramyxoviridae*, encodes several proteins in addition to P. The C protein is encoded by the same mRNA, but it is translated using an initiator methionine codon 19 nucleotides downstream from that for P and an overlapping reading frame (57). In contrast, the V protein shares the initiator methionine and the amino-terminal 231 amino acids of the P protein. RNA editing to add an extra non-template-directed guanosine (G) residue at position 751 following three genomically encoded Gs results in a different carboxyl terminus. The last 276 amino acids normally encoded by the P mRNA are replaced with a cysteine-rich domain of 68 amino acids (113) that has zinc-binding properties (375). Both V and C interact with cellular proteins (377). V is phosphorylated, diffusely distributed in the cytoplasm of infected cells, and affects N–P interaction (229,620,649). A fourth protein R is a truncated form of P that is produced by ribosomal frameshifting, which accesses the V open reading frame 24 nucleotides upstream of the V

stop codon (376). The C and V proteins play a role in the regulation of transcription and replication, possibly by modulating the intracellular environment for replication (377).

Neither C nor V is necessary for MV replication in Vero cells (500,563), but deletion of C decreases MV replication in peripheral blood mononuclear cells and epithelial cells of the thymus, suggesting an important *in vivo* role for the protein (180,632). In glioblastoma cells, absence of V decreases the amount of virus released and increases intracellular accumulation of viral proteins and RNAs. Overexpression of V does not alter replication in Vero cells but decreases cytopathic effect, whereas in glioblastoma cells virus production is decreased (563,620). *In vivo*, absence of V delays and prolongs MV replication in human thymic epithelial cells, whereas overexpression is associated with more rapid replication (632). Absence or overexpression of V decreases MV replication in the lungs of cotton rats (620).

Matrix Protein

The envelope of the virion consists of the matrix (M) protein (335 amino acids) and the two transmembrane glycoproteins F and H. M is a basic protein with several conserved hydrophobic regions (33,56) (Fig. 7). The mRNAs for Morbillivirus M proteins contain approximately 400 nucleotides of noncoding sequence at the 3' end of unknown function (56). In infected cells, the M protein is associated with nucleocapsids and with the inner layer of the plasma membrane (278). M appears to interact with the intracytoplasmic regions of one or both transmembrane glycoproteins, perhaps stabilizing or organizing the membrane environment in preparation for budding of virions (626). Deletion of M increases cell-to-cell fusion and decreases production of infectious virus (110). When bound to the viral ribonucleoprotein complex, M inhibits transcription (603). The ability to bind nucleocapsids and inhibit RNA transcription is often defective in M proteins from viruses that cause persistent infection (277,603).

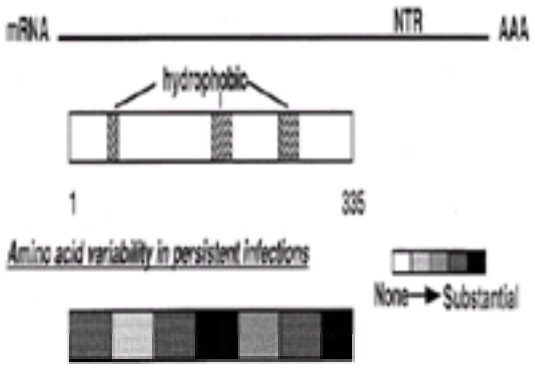

FIG. 7. The matrix (*M*) protein. Diagrams of the mRNA with its long 3' nontranslated region (*NTR*) and the protein showing the conserved hydrophobic regions. The M protein from persistent infections has frequent amino acid substitutions across the entire length. (Portions adapted from refs. 112 and 117, with permission.)

Fusion Protein

The fusion (F) protein is a highly conserved type I transmembrane glycoprotein synthesized as an inactive precursor protein (F_0) of about 60 kd. The mRNAs for Morbillivirus F_0 proteins contain unusually long (460 to 585 nucleotides), G-C–rich, 5'-nontranslated regions (NTRs) that are predicted to have extensive secondary structure and are followed by clusters of three to four AUGs (Fig. 8) (186,418,519). The 5' NTR influences the choice of AUG and enhances translation of functional F protein (109,186,674). There is a 28-residue putative signal sequence. After translation, F is glycosylated, tetramerized, and transported to the plasma membrane. In polarized epithelial cells, this transport is primarily to the basolateral surface (73,674). F_0 is cleaved by furin in the trans-Golgi to yield the 41-kd (F_1) and 18-kd (F_2) disulfide-linked activated F protein (80,261,652) (see Fig. 8). The cleavage site consists of five basic amino acids (residues 108 to 112: Arg-Arg-His-Lys-Arg). Arg 112 is a critical determinant of cleavage, as mutation results in a reduced rate of F transport to the cell surface, aberrant cleavage, and abolition of fusogenic activity necessary for infection (14,652).

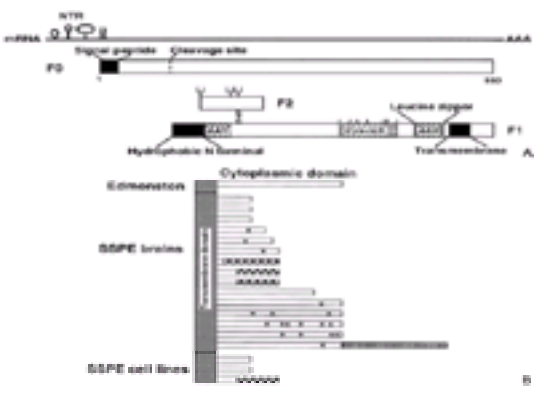

FIG. 8. Diagram of the fusion (*F*) protein. **A:** The mRNA with its long 5' nontranslated region (*NTR*) and proteins. F_0 is an inactive precursor that is cleaved into the active disulfide-linked F_1 and F_2 proteins. **B:** The truncations, point mutations (*x*), and altered reading frames ( ) found in the cytoplasmic portions of F_1 from persistent infections. (Portions adapted from refs. 558 and 60, with permission.)

Glycosylation is necessary for proteolytic processing and transport to the cell surface (555). F_2 has all the predicted N-linked glycosylation sites (residues 29, 61, and 67). Mutation of any of these asparagines, particularly Asn-29 or -61, decreases transport to the cell surface and impairs proteolytic cleavage, stability, and the fusion capacity of F, presumably by altering F_2 conformation (15,293).

F_1 contains a highly conserved stretch of hydrophobic amino acid residues at the new amino terminus. This region is postulated to play an important role in the fusion process (519). Oligopeptides identical to or nearly identical to this hydrophobic segment of F_1 inhibit fusion activity (520). However, mutants of MV resistant to the fusion inhibitory effect of these oligopeptides do not have predicted amino acid alterations in this hydrophobic region but rather in the cysteine-rich region postulated to be important for interaction with H (299). Additionally, there are two predicted amphipathic a-helices: one adjacent to the putative fusion peptide and another that contains a leucine zipper motif N-terminal to the transmembrane region. Mutagenesis of the leucines in the zipper region adversely affects fusion (88), and a synthetic peptide representing this region inhibits fusion (97,358).

Fusion requires the expression of H as well as F (665). Production of chimeric CDV-MV proteins has identified a cysteine-rich region (residues 337 to 381) within F_1 that is important for interaction with H (664). The 33-residue cytoplasmic domain is frequently altered in viruses associated with persistent infection (60,117,558) (see Fig. 8). Mutations include premature stop codons, missense mutations, altered reading frames, and nonconservative amino acid substitutions. These changes interfere with virus envelope assembly and budding and increase cell-to-cell fusion (111,116).

Hemagglutinin Protein

The hemagglutinin (H) protein (617 amino acids) is the receptor-binding and hemagglutinating (HA) protein and is an important determinant of Morbillivirus cellular tropism (597). It is a type II transmembrane glycoprotein and resides on the surfaces of infected cells and virions as a disulfide-linked homodimer (13,261). H acts in conjunction with F during fusion and entry (116,665). Residue 98 and the region between residues 244 and 250 have been implicated in functional cooperation of H with F (203). H is folded and dimerized in the endoplasmic reticulum (ER) and has 13 strongly conserved cysteines, all in the ectodomain. Cleavage studies indicate that cysteine residues at 139 and 154 are responsible for intermolecular disulfide bonding of monomeric H glycoproteins (554).

Modeling of the three-dimensional structure suggests that the N terminus forms a stem supporting and contributing to a b-propeller structure with a sialic acid binding site analogous to the HN proteins of other paramyxoviruses (Fig. 9) (359). Previously unsuspected neuraminidase activity can be demonstrated for RV and peste des petits ruminants virus but not MV (359). Mutagenesis studies suggest that cysteine residues at 287, 300, 381, 394, 494, 579, and 583 are important for oligomerization and folding (296). The structural model is consistent with disulfide bonds between cysteines at 287 and 300, 381 and 386, 394 and 494, and 570 and 579 (359). Neutralizing epitopes are located on the top loops of the b-propeller. The membrane cofactor protein (CD46) binding site maps primarily to a large loop between sheets 4 and 5, on the side of the b-propeller and away from the tetramerization site (J. P. M. Langedijk, unpublished) (see Fig. 9).

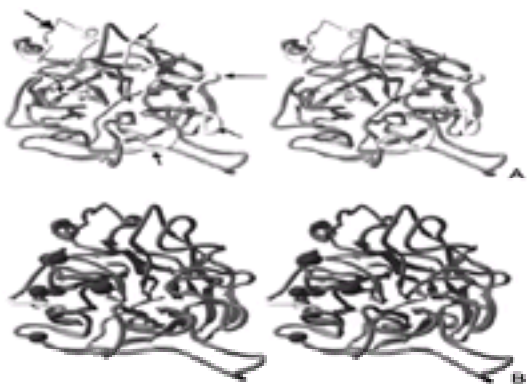

FIG. 9. Stereo ribbon diagram of the hemagglutinin (*H*) protein. **A:** The location of linear epitopes (*white ribbons marked by arrows*) according to peptide binding studies (235,236,237,238,239,240,241,242,243,244 and 245,276,277,278,279,280,281,282,283,284 and 285,309,310,311,312,313,314,315,316,317 and 318,368,369,370,371,372,373,374,375,376,377,378,379,380,381,382,383,384,385,386,387,388,389,390,391,392,393,394, 395 and 396,442,443,444,445,446,447,448,449,450 and 451, and 587,588,589,590,591,592,593,594,595 and 596) (391,392). The major antigenic site (367) is indicated with a *thick arrow.* **B:** Regions involved in CD46 binding according to peptide inhibition studies (483) indicated as *white ribbon* (468,469,470,471,472,473,474,475,476,477,478,479,480,481,482,483,484,485,486 and 487), and according to mutation studies (300,483,580) indicated as *dark spheres* (473,474,475,476 and 477,481,493,495,506, and 546). (From ref. 359, with permission.)

The H protein of the well-studied Edmonston strain of MV has five predicted N-linked glycosylation sites, and they are clustered at positions 168, 187, 200, 215, and 238 (13). The first four of these sites are used (116,294). Glycosylation at 215 is heterogeneous, which may account for the two types of H often observed on polyacrylamide gels (467). More recent MV isolates have an additional glycosylation site at residue 416, and this correlates with a loss of HA activity (548). Glycosylation is necessary for proper folding, antigenicity, dimerization, and export of H from the Golgi (294). Studies on H processing indicate that the rate of intracellular transport is relatively slow, taking approximately 30 minutes for oligomerization and an hour to reach the medial Golgi (295). In polarized epithelial cells, H is transported to both the

apical and basolateral membranes. H proteins associated with persistent infections often have mutations that affect glycosylation, oligomerization, and intracellular transport (116).

Hemagglutination of monkey erythrocytes is indicative of binding to CD46 and is dependent on the C-terminal 18 amino acids (70) of H and the regions involving residues 451 and 481 (483). Tyrosine at 481 allows binding to CD46, whereas asparagine does not (292).

Large Protein

The large (L) protein (2183 amino acids) is highly conserved and contains the Gly-Asp-Asn-Gln motif common to the RNA polymerases of negative-strand viruses (584). L protein is present in small quantities in the infected cell, interacts with and functions in association with P, and is part of the viral nucleocapsid in the cell and in the virion (594). The P binding domain has been mapped to the N-terminal 408 amino acids (288).

Cellular Receptor

The cellular receptor for wild-type MV is generally accepted to be a glycoprotein present on human and monkey cells that interacts with the H protein. Candidate receptors or coreceptors include 20-kd and 30.6-kd proteins on Vero cells identified with an antiidiotypic antiserum (346), a substance P–binding protein on neurons (263), and moesin (a membrane-organizing extension spike protein) on murine cell lines (166), subsequently shown to affect syncytium formation but not to act as a receptor (156). The only glycoprotein that clearly has been identified as a cellular receptor for MV, by demonstrating the ability to confer MV binding and replication to murine cells, is membrane cofactor protein, or CD46 (158,446,461). This receptor was identified with a monoclonal antibody that blocked Edmonston infection of HeLa cells (447) and reacted with CD46 (446). Additional evidence for the role of CD46 as receptor was provided by studies with anti-CD46 antibodies and hybrid cell lines containing human chromosome 1, the location of the CD46 gene (158).

CD46 is a widely distributed human complement regulatory protein expressed preferentially on the apical surface of polarized epithelial cells (73). It normally binds and acts as a cofactor in the proteolytic inactivation of C3b/C4b by factor I (378). Monkeys have a CD46 homolog, but such a protein has not been identified in mice (378,447,449). Multiple species of mRNA are produced by alternative splicing of CD46 transcripts. All code for proteins that contain an identical N-terminal signal peptide, four short consensus repeats (SCR) or complement-control protein domains, a transmembrane region, and an anchor (Fig. 10). Isoforms differ in the length and composition of an extracellular serine/threonine/proline (STP) domain near the transmembrane segment and in having one of two alternative cytoplasmic tails (323,544). Four isoforms are common on human cells in different tissues and all can serve as receptors for MV (218,397). SCRs 1 and 2 interact with the H protein to bind MV (436), and SCRs 2, 3, and 4 bind C3b/C4b (312). The N-linked carbohydrates on SCR-2, but not the O-linked carbohydrates on the STP domains, are important for MV binding (389,390). The H binding site involves one planar face of SCR-1 and SCR-2 (85,107,291,396,436). Increasing the distance between the MV binding domain and the transmembrane domain increases binding but decreases virus fusion (86). MV infection of cells or expression of the H protein alone of some, but not all, strains of MV leads to down-regulation of CD46 by rapid internalization from the cell surface (351,447). This alters susceptibility of the cell to complement-mediated lysis (559). In persistently infected cells, CD46 down-regulation is accomplished through a mechanism that recognizes a membrane-proximal Tyr-X-X-Leu motif in the cytoplasmic domain (675). Interestingly, CD46 is also down-regulated by the H proteins of morbilliviruses such as RV that do not use CD46 as a receptor (212). In macrophages, the cytoplasmic tail of CD46 is associated with intracellular kinases (671), and cross-linking of CD46 can alter macrophage function (279,328). Down-regulation is necessary for major histocompatibility complex (MHC) class II processing and presentation of H (525). Amino acids at positions 451 and 481 of H are important determinants of CD46 down-regulation (45).

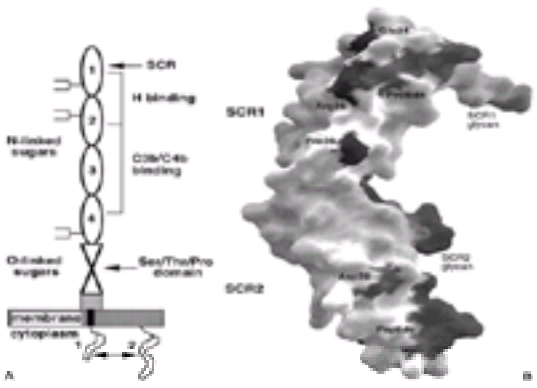

FIG. 10. The measles virus receptor membrane cofactor protein or CD46. **A:** In the diagram, the extracellular N-terminal portion contains four short consensus repeats (SCRs) with three predicted sites for N-linked glycosylation. SCR-1 is absent in CD46 from New World monkeys, and SCR-2 is mutated in baboons (290). The Ser/Thr/Pro domain near the membrane is a site for O-linked glycosylation. The C-terminus has two different cytoplasmic tails. (Adapted from ref. 378, with permission.) **B:** Molecular structure of the two N-terminal SCRs derived from the x-ray crystal structure. Regions involved in MV binding are highlighted. (From ref. 107, with permission.)

It is likely that MV entry into cells requires interaction of the virion with more than one cell surface protein. The existence of a coreceptor is suggested by a number of observations: (a) attachment to the cell occurs through H, but penetration requires F as well (128); (20) small hydrophobic peptides bind to cells and block virus penetration without affecting binding (521); and (c) L cells expressing CD46 are not susceptible to MV infection, but other murine cells are (397,446).

MV receptors in addition to CD46 may be used preferentially by some strains of MV (46,89,612). Marmosets are

susceptible to measles, and marmoset B cells are more sensitive than Vero cells for isolation of wild-type strains of MV. However, marmoset CD46 lacks the SCR-1 domain and does not render Chinese hamster ovary cells susceptible to infection with wild-type MV (437), and antibody to CD46 does not block infection of B95 cells with wild-type MV strains (292). The H gene of wild-type, but not vaccine, strains renders B95, but not HeLa or COS cells, susceptible to fusion (612). Similar observations have been made with wild-type viruses isolated on human B-cell lines (46). Ability to bind CD46 is determined, at least in part, by the amino acid at residue 481 of H (292). Recently, CD_w 150 (SLAM), a surface protein expressed on T, B, and dendritic cells, has been identified as an additional measles receptor (613a). Further characterization of wild-type strains isolated in different ways will be necessary to determine the receptor used *in vivo*.

Hemagglutination and Hemadsorption

Measles virus binds to the erythrocytes of Old World monkeys and most efficiently agglutinates red blood cells from *Cercopithecus aethiops* (African green), patas, and rhesus macaques. This is consistent with the tissue distribution of the CD46 molecule, which is not present on human red blood cells, and with the molecular characteristics of primate CD46 (290,378). Erythrocytes from other primates hemagglutinate poorly. In baboons, this is the result of a mutation in SCR-2, and in New World monkeys to an absence of SCR -1 (290). HA occurs optimally at physiologic pH and 37°C, and cells productively infected with some strains of MV can be identified by their ability to adsorb monkey erythrocytes (hemadsorption). Both HA and hemadsorption are properties of the H glycoprotein. Many wild-type MV isolates have little HA activity or require a high salt concentration. HA is improved by adaptation to growth in Vero cells (580). Many of the low HA viruses encode an H protein with an asparagine at residue 481 and/or an additional glycosylation site at residue 416 (292,549). Salt-dependent HA may be determined by the amino acid at residue 117 of the H protein (523).

Fusion and Syncytium Formation

Hemagglutination is followed within a few hours by lysis of the agglutinated erythrocytes (348). The biologic activities of hemolysis and cell fusion are properties of F, but they require coexpression of H (15,116,128,609,665). Cell-to-cell fusion occurs at neutral pH; therefore, syncytium formation occurs in infected cells *in vitro* and in infected tissues *in vivo* (177) (see Fig. 2). Syncytium formation is affected by cytochalasin B and by the presence of the actin filament–plasma membrane cross-linker moesin, suggesting a role for the cytoskeleton in fusion (53,156). Several regions of the activated F protein are important for fusion activity (see previous discussion of F).

Cytopathic Effects

Measles virus replication in cell culture monolayers results in cytopathic changes of three varieties: multinucleated giant cells (syncytia), altered cell shape, and inclusion bodies (178). Fusion of infected cells with uninfected cells may produce syncytia with 50 or more nuclei bounded by a single cytoplasmic membrane (see Fig. 2). Nuclei in the center of the syncytia have marginated chromatin (177,577) and are undergoing apoptotic cell death (182) leading to plaque formation *in vitro*. Infected cells may also change from a normal polygonal shape to a stellate, dendritic, or spindle-shaped appearance with increased refractility to light. This type of “strand-forming” cytopathic effect appears after several passages (423,570) and may be related to the production of defective interfering particles (412).

Both spindle-shaped cells and syncytial cells may contain intracytoplasmic and intranuclear inclusion bodies (see Fig. 3). Cytoplasmic inclusions are generally larger than nuclear inclusions and contain RNA encapsidated with N protein. Cytoplasmic inclusions are decorated with P and M protein, producing “fuzzy” or “granular” nucleocapsids (78,527) (Fig. 11). In CDV-infected cells, cytoplasmic nucleocapsids of two densities can be distinguished. Biologically active light nucleocapsids contain a 70-kd heat-shock protein, whereas biologically inert dense nucleocapsids do not (466).

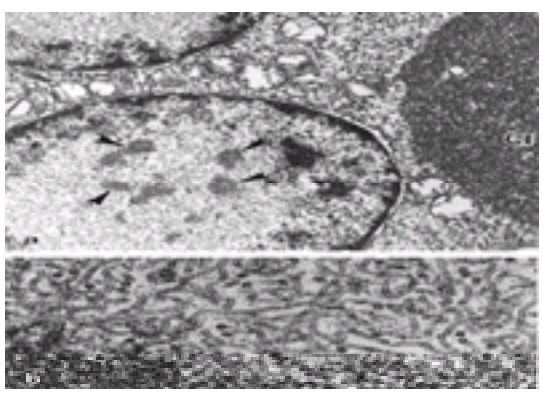

FIG. 11. Electron micrographs of MV inclusion bodies. **A:** Large cytoplasmic inclusion (*CI*) in the brain of a hamster infected with an SSPE strain of MV; the nuclei on the left contain free or aggregated smooth nucleocapsids (*arrowheads*). **B:** Detailed structure of nuclear nucleocapsids in SSPE-infected cells. (From ref. 162, with permission.)

Intranuclear Cowdry type A inclusion bodies are characteristic of morbillivirus infections and occur late in infection (504), but there is no evidence to suggest that the nucleus plays a role in replication. The frequency of development of nuclear inclusions varies with the virus strain (443,454). Electron microscopic examination of CDV-infected cells has shown that intranuclear inclusion bodies are usually complex nuclear bodies derived from nucleoli (463) (see Fig. 3). These nuclear bodies contain N and a cellular heat-shock protein associated with translocation of N from the cytoplasm to the nucleus (465). Similar nuclear bodies have been described in MV infection (478). MV N when expressed alone migrates to the nucleus (127,299,593), where it can assemble into nucleocapsids (199) that lack P, M, and viral RNA (464) and appear “smooth” by electron microscopy (78) (see Fig. 11). Coexpression and binding of P to assembled nucleocapsids is associated with cytoplasmic retention (298). It has been postulated that late in infection the amount of P protein may be

limiting, allowing N to move into the nucleus without encapsidation of viral RNA (79,298).

Antigenic Composition and Strain Variation

Measles virus has long been considered an antigenically stable monotypic virus. Antisera from individuals infected decades ago retain the ability to neutralize more current wild-type strains of MV, and vice versa. Nevertheless, variability, primarily in the N and H genes, has been recognized by nucleotide sequence analysis (523,540,542,551,615) and monoclonal antibody reactivity (62,223,579,611).

The N proteins of wild-type viruses exhibit antigenic heterogeneity in the C-terminal region (87,223), and N genes differ by up to 7% within the C-terminal 456 nucleotides (524,542,615) (see Fig. 5). The H gene nucleotide sequence is quite variable, particularly between residues 167 and 241, where the five potential N-linked glycosylation sites are located (540). The observed rate of mutation of H in virus circulating in defined geographic locations is low, estimated at 5×10^{-4} per year for a given nucleotide (523), whereas the rate of mutation during growth *in vitro* is higher, estimated at 9×10^{-5} per replication for a nucleotide (566).

Strains examined to date can be separated into eight different clades and at least 15 different genotypes (Fig. 12). Some are co-circulating, while others may be extinct (see Fig. 12). Few differences have been described among MV vaccine strains regardless of the geographic origin of the parent virus, suggesting that MV globally was quite similar when most of the parent strains were isolated (541).

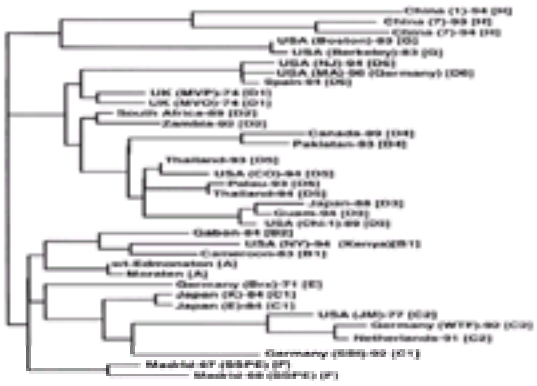

FIG. 12. Evolutionary relationships of wild-type and vaccine strains of measles virus. The phylogenetic tree is based on the complete sequence of the N gene and shows representatives of the 15 recognized genotypes designated in *square brackets* (19). Vaccines are represented by the Moraten strain. (Courtesy of P. Rota and W. Bellini.)

PATHOGENESIS AND PATHOLOGY

Classic Measles

Measles is typically a childhood infection of humans spread by the respiratory route. Disease is characterized by a latent period of 10 to 14 days and a 2- to 3-day prodrome of fever, coryza, cough, and conjunctivitis, followed by the appearance of a characteristic maculopapular rash. The onset of the rash coincides with the appearance of the immune response and initiation of virus clearance (Fig. 13). Recovery is accompanied by lifelong immunity to reinfection (481). Monkeys that have been exposed to infected humans or experimentally infected with wild-type strains of MV develop a similar disease (635), and much of our more detailed understanding of pathogenesis and sites of virus replication come from studies of nonhuman primates.

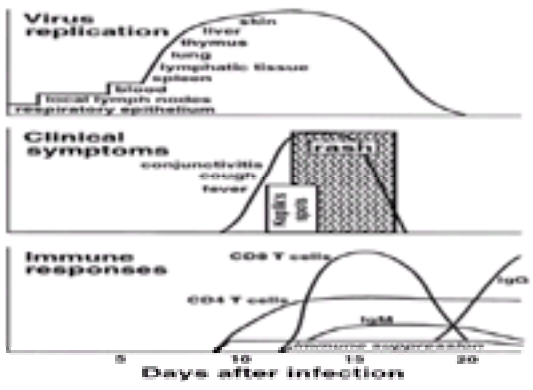

FIG. 13. Pathogenesis of measles. Virus is spread by the respiratory route; replication begins in the respiratory epithelium and spreads to monocyte/macrophages, endothelial cells, and epithelial cells in the blood, thymus, spleen, lymph nodes, liver, skin, and lung and to the conjunctivae and the mucosal surfaces of the gastrointestinal, respiratory, and genitourinary tracts. The rash appears at the time of the virus-specific immune response. Clearance of virus is approximately coincident with fading of the rash.

Entry and Sites of Primary Replication

Virus is spread by aerosol and enters by the respiratory route. Initial infection is established in the respiratory tract with virus replication in tracheal and bronchial epithelial cells (550,577). From the respiratory tract there is extension to local lymphatic tissues, perhaps carried by pulmonary macrophages or dendritic cells (326,573). MV replication in lymphoid tissue results in the appearance of lymphoid or reticuloendothelial giant cells first described by Warthin (651) and Finkeldey (195). Typically these are large cells of up to 100 µm or more in diameter that may contain 100 nuclei aggregated near the center. Inclusion bodies are not generally present. Warthin-Finkeldey cells tend to be located at the periphery of germinal centers and in submucosal lymphoid tissue.

Spread

Amplification of virus in regional lymph nodes results in the appearance of a viremia and spread of virus through the blood to infect a variety of organs. Numerous studies have documented a cell-associated viremia, present prior to and at the time of the rash (201,306,543). Only rarely has a plasma viremia been detected and then only prior to the appearance of neutralizing antibody (487), but viral RNA can be detected in plasma by reverse transcriptase polymerase chain reaction (RT-PCR). The primary cell infected in blood is the monocyte (184). Infection of monocytes and macrophage cell lines by some strains of MV increases the expression of LFA-1, an adhesion molecule that promotes adherence to endothelial cells and may contribute to virus dissemination (29,301). Leukocytes other than monocytes can be infected *in vitro* and may be infected *in vivo* as well (477). The viremia is accompanied by leukopenia (31,573).

Target Tissues and Cells

Lymphoid organs and tissues (e.g., thymus, spleen, lymph nodes, appendix, and tonsils) are prominent sites of virus replication (253,473,550) as is evidenced by the increasing numbers of giant cells in lymphatic tissue prior to the appearance of the rash (195,266,571,598,651). In the spleen, macrophage-rich areas are primary sites of MV replication. In the thymus, epithelial cells are infected (425), leading to thymocyte apoptosis and a prolonged decrease in the size of the thymic cortex. Other lymphoid tissues recover promptly (659). MV also spreads to numerous other organs, including the skin, conjunctivae, kidney, lung, gastrointestinal tract, respiratory mucosa, genital mucosa, and liver. In these various sites, the virus replicates primarily in endothelial cells, epithelial cells, and/or monocytes and macrophages (184,215,253,335,425,473,602,605). Endothelial cell infection is accompanied by vascular dilatation, increased vascular permeability, mononuclear cell infiltration, and infection of surrounding tissue (150).

The histopathology of the measles rash suggests that the initial event is the infection of dermal endothelial cells (71,338). This is followed by spread of infection into the overlying epidermis, with infection of keratinocytes in the stratum granulosum leading to focal keratosis and edema (605). Epithelial giant cells form and a perivascular mononuclear infiltrate accumulates (150,187). Koplik's spots found on the oral mucosa are pathologically similar to the rash and involve the submucous glands (150).

Pathologic examination of other tissues shows multinucleated epithelial giant cells that resemble those formed in tissue culture (see Fig. 2). Epithelial giant cells are prominent at the onset of the rash and are readily demonstrated in nasal secretions and the conjunctivae during the prodrome and first days of the rash (49,370,453,556). MV-infected epithelial cells are also shed into the urine (370,380).

Immune Responses

The immune responses to MV are important for clearance of virus and recovery from infection and are directly responsible for several of the clinical manifestations of measles. Appreciation for the roles of various components of the immune response in recovery from infection has been gained from “experiments of nature,” in which the outcome of MV infection in patients with selective deficiencies of immunologic function has been documented (232). In general, patients with hypogammaglobulinemia recover, whereas individuals with severe congenital or acquired deficits in cellular immune responses or combined deficits in cellular and humoral responses are prone to developing progressive disease.

Nonspecific host responses may contribute to control of virus replication during the incubation period, but the onset of clinically apparent disease coincides with the appearance of MV-specific immune responses. There is marked activation of the immune system (Fig. 14), which is coincident with the appearance of immune suppression. Immune suppression and immune activation continue for many weeks after apparent recovery.

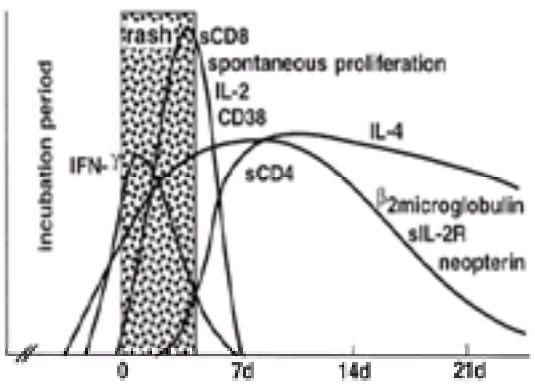

FIG. 14. Appearance of circulating cytokines during uncomplicated measles. The patterns suggest early activation of CD8 and type 1 CD4 T-cells followed by prolonged activation of type 2 CD4 T cells.

Early Nonspecific Responses

Measles virus infection of cells *in vitro* induces production of interferon (IFN)-a and IFN-b, which decrease MV replication and increase expression of MHC class I antigen on infected cells (154,315). Increased levels of IFN are present in serum 8 to 11 days after measles immunization (491), but elevated plasma levels of biologically active IFN have not been documented during natural infection (247,583). Natural killer (NK) cells constitute another potentially important early defense mechanism, but studies of NK activity indicate that NK cell function is actually lower than normal during measles (247). These studies have been performed using samples from humans collected at the time of the rash and do not exclude induction of and roles for IFN and NK cells at earlier stages of infection.

Antibody

Antibodies are first detectable when the rash appears (51,235). The isotype of MV-specific antibody is initially IgM, and in mice, IgM production depends on T cells (189). Later, the isotype switches primarily, but not exclusively, to IgG1 and IgG4 (172,403). IgG is initially of low avidity, but this increases steadily over several months 193). IgA, IgM, and IgG antibodies to MV are found in secretions (83,490). Antibodies are eventually produced to most viral proteins (235,456,623) (Fig. 15).

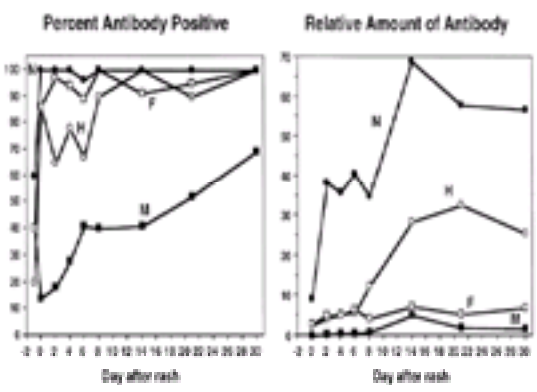

FIG. 15. Production of antibody to measles virus proteins during natural infection. **A:** Percent of individuals positive for antibody to each protein. **B:** Relative amounts of antibody to each protein. Antibody was measured by immunoprecipitation. (Adapted from ref. 235, with permission.)

The most abundant and most rapidly produced antibody is to N (235,456), and this is the primary reactivity measured in the complement fixation (CF) test (456). Because of the abundance of anti-N antibody, absence of this antibody is a reliable indicator of seronegativity. The M protein elicits only small amounts of antibody except in atypical measles (235,387).

Antibody to F is measured by inhibition of MV hemolysis of monkey erythrocytes [hemolysin inhibition (HLI)], by immunoprecipitation of F or specific enzyme immunoassay (50,456,457,674). Antibodies to F contribute to virus neutralization, probably by preventing fusion of the virus membrane with the cell membrane at the time of virus entry (395). Two regions of F induce neutralizing antibodies: one includes amino acid 73 and the other includes amino acids 388 to 402 (26,188,395). Human sera recognize linear epitopes in six to seven regions spread over much of the F protein (662), and these linear B-cell epitopes are frequently close to T-cell epitopes (434). Monoclonal antibodies (MAbs) to F may cross-react with cellular heat-shock proteins (578).

Antibody to H is measured by inhibition of the agglutination of monkey erythrocytes by MV [hemagglutination inhibition (HAI)], but these antibodies also may inhibit hemolysis and fusion (105,224,456,459). Antibodies to H can also be measured by immunoprecipitation (457) or specific enzyme immunoassays (50,674), and they are the primary antibodies measured by neutralization of virus infectivity (224,225). Four to six neutralizing epitopes have been mapped by competitive binding of MAbs and analysis of different strains and escape mutants (105,297,300,366,579). Human convalescent sera show reactivity to linear epitopes, as well as epitopes dependent on conformation and glycosylation (294,435). One of the major conformational epitopes has been localized to the region between amino acids 368 and 396 (366). In the proposed propeller model of H, essentially all of these epitopes are predicted to be on an exposed surface on top of the molecule (see Fig. 9) (359).

Antibody can protect from MV infection, may contribute to recovery from infection, and may play a role in establishing persistent infection (1,8,355,507). There is *in vivo* and *in vitro* evidence for a role in recovery. Antibody-dependent cell-mediated cytotoxicity (ADCC) correlates temporally with cessation of cell-associated viremia (202). Antibody binding to infected cells alters intracellular virus replication and may contribute to control of infection (206,207,228,562). Failure to mount an adequate antibody response carries a poor prognosis (657). The role of antibody in protection from infection is discussed in the section Immunization, and the role in establishing persistence is discussed in the section Persistent Infection.

Cellular Immunity

The ability to recover from measles was postulated by Burnet to be an indication of the adequacy of T-lymphocyte-mediated immune responses (91). There is abundant evidence that CD8 T cells are activated and expanded in response to infection. MV-specific, human leukocyte antigen (HLA)-restricted, CD8 cytotoxic T lymphocytes

(CTLs) (318,353,588,633) and proliferating CD8 T cells (646) with evidence of clonal expansion (426) are detectable in blood at the time of the rash, and in bronchoalveolar lavage fluid during pneumonitis (439). Cultures of peripheral blood mononuclear cells with autologous MV-infected cells or peptide-pulsed cells after recovery expand CD8 T cells with cytolytic activity demonstrating that CD8 T-cell memory is established by infection (318,319,444,633). Soluble CD8 and b2 microglobulin (a component of the MHC class I molecule that presents antigen to CD8 cells) are increased in plasma (245,247) (see Fig. 14). MV infection of cells *in vitro* up-regulates MHC class I expression through production of IFN-b (154). MV antigens that induce CD8+ cytotoxic T cells include the N, P, H, and F proteins (276,318,637).

CD4 T cells are also activated in response to MV infection. *In vitro*, binding of H to CD46 targets virion proteins to an endosomal compartment for efficient presentation by MHC class II proteins (219). CD4 T cells proliferate during the rash (646), although absolute numbers of circulating cells often decreases (374). Soluble CD4 becomes elevated in plasma and remains so for several weeks after recovery (244) (see Fig. 14). Classic CD4 T-cell responses, such as MV-specific proliferation and lymphokine production, are stimulated during measles (633). Lymphocyte proliferation can be induced by most MV proteins in immune individuals and is usually MHC class II restricted (58,433,492,537,637). Cytokines produced by CD4 T cells determine their function. Type 1 CD4 T cells produce IFN-g, which activates macrophages, and interleukin (IL)-2, which promotes T-cell proliferation. These are the primary mediators of classic delayed-type hypersensitivity. Type 2 CD4 T cells produce IL-4, IL-5, IL-10, and IL-13, which are important for B-cell growth and differentiation and for macrophage deactivation. In measles, IFN-g, neopterin (a product of activated macrophages), and soluble IL-2 receptor rise during the prodrome, prior to the appearance of the rash (245,246). This is followed by elevation of IL-2, soluble CD4, and soluble CD8 at the time of the rash (245,248). As the rash fades, IL-4 and IL-5 increase, and this elevation persists in some individuals for weeks (248). This pattern of cytokine production suggests early activation of CD8 (IFN-g) and type 1 (IFN-g and IL-2) CD4 T cells during the rash, followed by activation of type 2 (IL-4, IL-5) CD4 T cells during recovery.

Longevity of the Immune Response

Epidemiologic studies have documented that long-term protection from reinfection does not require reexposure (481). Immunologic memory includes both continued production of antibody (68) and circulation of MV-specific T cells (307,633,672). There is no evidence for persistence of latent virus, although intermittent reports of detection of MV RNA in the blood and tissues of normal individuals (251,560) make this a possibility. Extensive replication of MV in lymphoid tissue may maximize the interaction of viral antigen with antigen-retaining follicular dendritic cells in germinal centers, leading to long-term antibody production.

Immune Suppression

There is *in vivo* and *in vitro* evidence of immune suppression during measles (Fig. 16). Immune suppression is probably a multifactorial process and is likely to underlie the increased susceptibility to other infections characteristic of measles. *In vivo*, delayed-type hypersensitivity skin test responses to recall antigens, such as tuberculin, are suppressed prior to the rash and for several weeks after apparent recovery from infection (595,610,641,656). Production of antibody and cellular immune responses to new antigens is impaired (132). Reactivation of tuberculosis (129) and remission of immunologically mediated diseases such as nephrotic syndrome, juvenile rheumatoid arthritis, and idiopathic thrombocytopenic purpura (371,372,677) have been reported to follow measles. *In vitro*, lymphoproliferative responses to mitogens are suppressed and lymphokine production is abnormal (23,282,647). Analysis of the *in vitro* abnormalities suggests defects in the responses of both monocytes and lymphocytes.

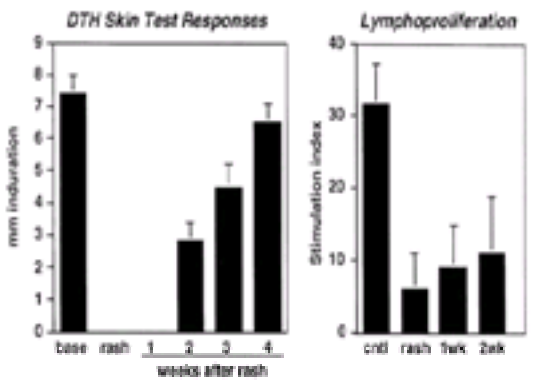

FIG. 16. Immunosuppression during measles. Changes during measles in tuberculin-induced delayed-type hypersensitivity (DTH) skin test responses in children with measles who had previously received immunization against tuberculosis and changes in proliferation of peripheral blood mononuclear cells to the mitogen phytohemagglutinin. (Data from refs. 610 and 282, with permission.)

Monocytes are infected during measles (184), and monocyte function is abnormal, with low levels of tumor necrosis factor (TNF)-a production during both acute and convalescent phases of the disease (241). Decreased TNF-a is observed when macrophage-lineage cells are infected with MV *in vitro* and monocyte production of IL-12 is suppressed by cross-linking the MV receptor CD46 (328,363).

Circulating T cells are decreased in number in most individuals during the acute phase of measles, and profound lymphocytopenia is associated with HLA-AW32 (134,143,656,660). Both normal and abnormal proportions of CD4 and CD8 T cells have been reported during measles, and it is likely that the numbers of circulating T cells are rapidly changing during the acute phases of infection (24,142,242,374,646). A greater decrease in CD4 T cells has been associated with malnutrition and more severe disease (337). Infection of thymic epithelial cells induces terminal

differentiation of these cells and apoptosis of thymocytes that are not infected. These events may have long-term consequences for cellular immune function (30,630).

One of the reasons for low T-cell proliferation to mitogens *in vitro*, especially during convalescence, is inadequate production of IL-2. Addition of recombinant IL-2 to measles lymphocyte cultures supports spontaneous proliferation and improves mitogen responses (242,645). When *in vitro* production of other cytokines is assessed, IFN-g is low to normal and IL-4 is high compared to controls (244,647), suggesting that type 2 CD4 T cells are preferentially activated. *In vitro* neutralization of IL-4 improves proliferation (244). The suppressive effects of IL-4 on type 1 CD4 T cells and macrophages may contribute to suppression of delayed-type hypersensitivity and macrophage activation during measles.

In vitro infection of leukocytes and leukocyte cell lines with the Edmonston strain of MV induces abnormalities of B-cell, T-cell, macrophage, and dendritic cell function, which may be relevant to immune suppression during measles. Resting lymphocytes and dendritic cells produce little virus unless activated (205,565). Virus production by macrophages decreases with cellular maturation (205,272,601). T-cell proliferation to mitogens and soluble antigens (405,590,683) and cytotoxic function (106), as well as B-cell production of immunoglobulin (106,406,619), are suppressed by infection. T and B lymphocytes infected *in vitro* can be activated by mitogen, but they proliferate poorly because entry into S phase and progression through the cell cycle is impaired (405,445). Failure of MV-infected T cells to proliferate is linked to poor responses to IL-2 and somatostatin (54,350). Dendritic cells lose the ability to stimulate an allogeneic response and undergo apoptosis when co-cultured with T cells (205,249). Monocytes and dendritic cells exposed to MV have impaired production of IL-12, a cytokine necessary for induction of type 1 CD4 T cells (205,328). In monocytes, increased nitric oxide and suppressed IL-12 production are the result of MV interaction with CD46 (279,328). Likewise, B-cell production of IgE is stimulated by CD46 cross-linking in the presence of IL-4 (310). Other studies suggest that CD46-independent interactions of H and F with lymphocytes and soluble factors produced by infected T cells inhibit T-cell proliferation and contribute to immune suppression (208,249,450,557).

Autoimmunity

An autoimmune demyelinating disease, postinfectious encephalomyelitis or acute disseminated encephalo-myelitis (ADEM), is an important complication of measles (422) (Fig. 17; see Table 2) and is associated with an immune response to myelin basic protein (322) similar to that seen in animals with experimental autoimmune encephalomyelitis. The similarity of these diseases and lack of MV in the brain have led to the current understanding that ADEM is an autoimmune disease (322,344) induced by measles.

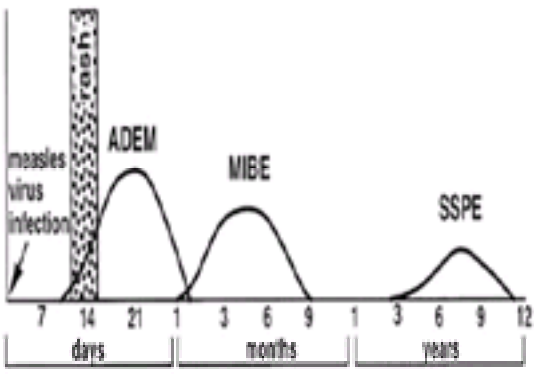

FIG. 17. Time after infection of the three neurologic complications of measles: infections or acute disseminated encephalomyelitis (*ADEM*), measles inclusion body encephalitis (*MIBE*), and subacute sclerosing panencephalitis (*SSPE*).

Disease	Host	Typical age of infection	Measles virus in brain	Incidence	Pathology	Time course
Acute disseminated encephalomyelitis (ADEM)	Normal	>2 yrs	No	1:1000 cases	Inflammation, demyelination	Monophasic, weeks
Measles inclusion body encephalitis (MIBE)	Immunosuppressed	Any	Yes	?	Inclusion bodies	Progressive, months
Subacute sclerosing panencephalitis (SSPE)	Normal	<2 yrs	Yes	1:100 cases	Inclusion bodies, inflammation	Progressive, years

TABLE 2. *Neurologic complications of Measles*

The mechanism of induction of autoimmune disease during measles is not clear. Hypotheses have included altered presentation of myelin antigens resulting from MV infection of oligodendrocytes (344), molecular mimicry of myelin antigens by MV (316), and dysregulation of immune responses (282). There is little evidence for MV infection of cells in the nervous system (217,322,425). The possibility of molecular mimicry with cross-reactivity between myelin basic proteins and MV protein has been explored using computer searches and studies of T-cell and antibody cross-reactivity. Sequence analyses have revealed areas of homology (316), but the biologic relevance of these potential cross-reactions is not known. Neither cross-reactive antibodies nor cross-reactive T-cell clones have been identified in humans or rats

with demyelinating disease induced in conjunction with MV infection (367). Genetic susceptibility to ADEM has been postulated, but the number of patients studied has been insufficient to clearly identify a link between MHC antigens or other genetic markers and disease (240,361).

Studies of immune regulation have shown that patients with encephalomyelitis differ from patients with uncomplicated disease by having a distinct pattern of immunologic abnormalities. IgE is more persistently elevated and soluble IL-2 receptor is lower in patients with encephalitis (239,245). The timing of autoimmune encephalomyelitis and the occurrence of a pathologically similar disease as a complication of treatment with IL-2 (233) suggest that immune activation and dysregulation during measles may play a role in allowing activation and expansion of autoreactive lymphocytes.

Release

Epidemiologic data suggest that infected individuals become infectious for nonimmune contacts a few days before the onset of the rash. At this time, virus can be cultured from the mucous membranes of the nasopharynx, conjunctivae, and mouth (173), suggesting that the respiratory tract is the site of virus release.

Virulence

Strains of MV can clearly differ in virulence, as attenuated vaccine strains cause little disease in humans and wild-type strains cause measles. Sequences of these strains have been compared but phenotypes have not yet been correlated with genotypes (608). A lack of animal models for assessing virulence has handicapped attempts to determine the molecular basis for changes in virulence. Monkeys (rhesus or cynomolgus macaques) develop a rash illness similar to measles in humans and are probably the most relevant model system for assessing virulence. Limited studies have shown that adaptation of wild-type MV to growth in Vero cells selects for a virus that no longer causes a rash in monkeys. This attenuation is associated with predicted amino acid changes in the *P* and *L* proteins, but the specific changes responsible for decreased virulence are not yet known (607).

A second model system that has been used to assess virulence is the study of replication of MV in human cells implanted into immunodeficient mice with severe combined immunodeficiency disease (SCID). In thymic implants, wild-type viruses grow more rapidly and to a higher titer, and they cause more extensive thymocyte apoptosis than attenuated strains (30,631). In human mononuclear cell implants, wild-type strains grow better than vaccine strains (304). Again, the regions of the genome and the specific amino acid substitutions associated with these changes in replication and tissue destruction have not yet been defined. However, the availability of a reverse genetic system for MV now makes manipulation of the genome and these more definitive experiments possible (501).

Persistent Infection

Measles virus can establish persistent infection of cells *in vitro* and *in vivo*. A number of cell lines persistently infected with MV have been established, and neurologic disease can be caused by persistent infection of neurons and glial cells.

Persistently Infected Cells in Culture

Replication of MV usually causes death of cells in culture. The elements determining persistent noncytopathic versus lytic infection are not completely understood, but properties of both the infected cell and the infecting virus contribute. Methods used to establish persistently infected cell lines include (a) passage of virus at high multiplicity with generation of defective interfering particles (522,658), (b) passage of infected cells in the presence of antibody (546), (c) cultivation of cells surviving lytic infection (192,546), and (d) co-cultivation of cells with MV-infected brain cells from patients with SSPE or from persistently infected animals (416).

Persistent noncytopathic infection is most easily established in neuronal cells (421,503), but it has also been established in lymphoid, epithelial, and glial cell lines (192,383,416,546) and is associated with down-regulation of CD46. Identified cellular factors that affect the ability of MV to establish and maintain persistent infection are expression of heat-shock proteins, IFN-inducible proteins, and possibly RNA-binding proteins (362,421,564). Increased levels of cyclic adenosine monophosphate inhibit virus replication (528) and may correlate with the restriction of MV replication associated with neuronal differentiation (421). Cellular protein synthesis is relatively unaffected by MV infection, but specific cellular proteins (e.g., cell surface receptors) and functional responses (e.g., signal transduction, expression of transcription factors) may be altered, particularly in neural cells (48,197,252,613).

Strains of MV differ in their ability to establish persistent nonlytic infection in the same host cell (192). Replication of variants likely to cause persistent infection is often temperature sensitive (234,503). Persistent MV infection is usually accompanied by a marked decrease in the amount of infectious virus released and by intranuclear and intracytoplasmic accumulation of nucleocapsids with decreased evidence of virus-induced cytopathic effect compared to lytic infection (546). Some cell lines produce no infectious virus (92): Persistent infection is apparently maintained by passage of encapsidated viral RNA to daughter cells during cell division and therefore may not require production of infectious virus. The ability to transfer infection from persistently infected cells to uninfected cells is postulated to be an important mechanism for spread of infection in SSPE and has been demonstrated in organ cultures of neural tissue (576).

Limited expression of viral proteins on the surface of persistently infected cells has led to the suggestion that defects in synthesis of viral envelope proteins may be an important component of persistent infection (416,545,678). Defects in glycoprotein expression may in part result from limited mRNA production associated with steep transcriptional gradients (115,510) and an increase in bicistronic messages (115,303). However, mutations in these genes also may lead to proteins with altered expression or function. In persistently infected HeLa cells, M was rapidly degraded and H was not properly processed in the ER (678).

Subacute Sclerosing Panencephalitis

Much is known and has been written about SSPE, but the pathogenesis remains poorly understood. SSPE is a rare (approximately 1 in 1 million) late complication of measles that is most likely to occur if the original MV infection occurred at a young age (151,314). The route of virus entry into the central nervous system (CNS) is unknown, but infection of cerebral endothelial cells is one possibility (183,340). The incubation period is usually 7 to 10 years (424) (see Fig. 17). Extensive sequence analysis of viral RNA from various parts of the brain in SSPE suggest that the virus in the CNS is clonal (34), implying that virus entered the brain at one time (presumably during the original acute infection) and then gradually spread throughout the nervous system.

Studies of Brain and Brain-Derived Cell Lines

At the time that neurologic symptoms occur, neurons and glia contain nuclear and cytoplasmic viral inclusion bodies (275), antibody responses to virus are vigorous and evident both in serum and CSF (82,622), and there is an extensive mononuclear inflammatory reaction in the CNS (148,478). Both white matter and gray matter are affected (148). Virus replication is defective and when virus can be recovered it is only by co-cultivation or explantation of brain tissue (128,486). Brain tissue from patients with SSPE contains defective interfering particles that may contribute to persistence (585). CD46 is absent and no virus is seen budding from the surface of infected cells (161,462). Nuclear inclusions are filled with “smooth” nucleocapsids (161,275). The cytoplasm contains “fuzzy” nucleocapsids that extend into neuronal processes, suggesting that virus can spread within the CNS by synaptic transmission of the ribonucleoprotein from cell to cell (311,484). The observation that strains of MV isolated from patients with SSPE are more likely than standard strains of MV to cause neurologic disease after intracerebral inoculation into small animals (468) suggests that the virus has adapted to growth in neural tissue.

Viruses capable of production of infectious virus *in vitro* are infrequently recovered from SSPE patients, but extensive sequence analysis of viral RNAs from tissue suggests that SSPE viruses are genetically distinguishable from those that cause acute measles, especially in the viral genes encoding the M, F, and H proteins (33,35,117,558,669). Studies of brain have revealed differences in the relative amounts of the various viral mRNAs and proteins (35,365,585), in the antigenicity of viral proteins (485), and in RNA sequences coding for viral proteins (35,117) between SSPE viruses and wild-type or vaccine strains of MV. It is not known for certain whether these differences represent unique features of the original infecting virus, selection for growth in the CNS, or selection for growth in the presence of a vigorous antibody response.

In general, expression of M protein is low (153,255,365). This may be the result of a lack of synthesis of M (596) or an instability of the synthesized protein (575). A variety of defects have been encountered in the mRNA encoding M extracted from SSPE brain (117). Mutations occur throughout the gene (see Fig. 6), and the frequent U to C sequence changes noted in some viruses suggest mutation of double-stranded RNA in persistently infected cells by adenosine deaminase (biased or adenosine/inosine hypermutation) (112,169,670), although the role of this enzyme has been questioned (287). M transcripts often lack initiator AUGs necessary for expression (603). When expressed, M proteins may have defects in binding to viral nucleocapsids (277) and in down-regulating transcription (603). In one study, M protein of the SSPE strain remained in the cytosol, whereas the M protein from a related wild-type strain was bound to nucleocapsid at the plasma membrane (278). Defects in M are so common in SSPE that M is likely to be dispensable for virus growth and spread in the CNS, and it is possible that abrogation of the function of M is necessary to establish persistent CNS infection.

Aberrant M protein was believed to be the hallmark of persistent MV infections in SSPE patients (255). However, further investigations of SSPE viral genomes and proteins have demonstrated that alterations in any of the MV envelope proteins that in some way interfere with assembly and budding of infectious virus can be associated with persistent infection and SSPE (35,116,117,558). H proteins are often defective in intracellular transport and protein–protein interactions important for cell–cell fusion (116). Truncations, mutations, and deletions in the cytoplasmic domain of F are almost universal (see Fig. 8). Truncations do not affect fusion but may interfere with virus budding (116,558).

Immune Responses

The possibility that the development of SSPE represents a defect in the immune response to MV has led to investigations of humoral and cellular immune responses to MV and other antigens. The antibody response to MV is accentuated with significant production of MV-specific antibody by plasma cells residing in the CNS. This antibody appears in CSF, leading to elevations in the level of CSF immunoglobulin, much of which is MV specific (138,181,621). Antibody produced in the CNS is of restricted heterogeneity, leading to the appearance of oligoclonal immunoglobulin bands on electrophoretic analysis of CSF (168,638). Antibodies against the N and P proteins present in the ribonucleoprotein complex are particularly abundant, and antibody against the M protein is particularly deficient (153,255). The antibody produced is capable of mediating complement-dependent lysis of infected cells (470), but it is ineffective in clearing virus from the CNS.

Antibody to MV has been postulated to play a role in establishing persistent CNS infection either through alteration of the induction of the primary immune response at the time of infection (507), through production of immune complexes (591), or through modulation of infection once virus is in the nervous system (206,325). Antibody can cap viral antigens from the cell surface, producing infected cells that are refractory to immune-mediated lysis (325). Antibody may also contribute to establishment of persistent infection by suppression of P and M protein production (207).

Experiments in small mammals have shown that treatment with antibody after intracerebral infection with neuroadapted strains of MV attenuates acute disease but increases the incidence of subacute or chronic encephalitis associated with persistent virus infection (506). Cases of SSPE have been associated with passive transfer of immune globulin, and

persistent infection has been induced experimentally by passive transfer of antibody (507).

Studies of cellular immunity have been less extensive. MV-specific lymphoproliferation is similar to that of healthy seropositive individuals but MV-specific cytotoxic T-cell activity is decreased (152). General measures of cellular immunity, such as skin test responses to various recall antigens and proliferative responses to mitogens, are normal (33). The mononuclear inflammatory response in the brain includes CD4 and CD8 T cells, as well as monocytes and Ig-secreting B cells (181,442). Class I and class II MHC expression is increased in brain and b2 microglobulin, soluble IL-2 receptor, and soluble CD8 are increased in CSF (414). Thus, there is no evidence for a global defect in immune responses, but there is evidence that these immune responses are ineffective in clearing virus from the CNS.

Other Chronic Diseases

In addition to having a clear role in SSPE, MV also has been implicated in the pathogenesis of Paget's disease (47,511), tosclerosis (25,411), chronic active hepatitis (529), achalasia (324), multiple sclerosis (4,16), and Crohn's disease (642). For most of these diseases, an etiologic link to measles is controversial (20,61,419), and for none has a definitive role for MV been identified.

Veterinary Correlates

Canine Distemper

Canine distemper was first described by Carré in 1905 as an infectious disease of young dogs associated with gastroenteritis, pneumonitis, conjunctivitis, and encephalomyelitis (100). Canine distemper was shown to be of viral etiology in 1926 (357), and the virus was isolated in primary canine kidney cells in 1959 (530). Canine distemper is a severe disease of canines, raccoons, ferrets, and seals, with many similarities to measles, but direct infection of the CNS and neurologic disease is more common. CDV has a broader host range than many morbilliviruses and it appears to be expanding (260). Recently, CDV has spread to large felines in the wild in Africa (533) and in zoos in North America (260). CDV is spread by aerosol and the disease is characterized by fever, coryza, conjunctivitis, gastroenteritis, and pneumonitis (164,165). CDV appears first in bronchial lymph nodes and tonsils, followed by a cell-associated viremia. Infection spreads to epithelial tissues and enters the brain by infecting endothelial cells or by infiltration of infected monocytes (21,32). Like measles, canine distemper is associated with profound short- and long-term immune suppression, and much of the mortality is caused by secondary infections (121,347). CDV replicates in CD4 T cells (313) and is cleared in most animals with the appearance of antibody to envelope proteins and lymphocyte-mediated cytotoxicity for infected cells (21,22,347,349).

Dogs, ferrets, and lions may develop neurologic complications characterized by gait abnormalities and seizures (164,165). Neurologic disease is most common in young dogs (348) and can occur either early after infection or 3 to 4 weeks later associated with infection of oligodendrocytes and demyelination (72,373). Persistence of virus in neurons and epidermal cells of the feet (21) lead to diseases known as old dog encephalitis and hard pad disease (21). Some cases of old dog encephalitis have defective virus with intranuclear inclusions in the CNS coexisting with high levels of antiviral antibody, features in common with SSPE (309,373). In other cases, virus can be isolated without co-cultivation, and the disease cannot be clearly distinguished from late-onset encephalitis (309).

Rinderpest and Peste des Petits Ruminants

Rinderpest was first recognized as a distinct clinical entity causing severe gastroenteritis in cattle during a 4th century European epizootic (493). The causal agent of this disease was reported to be filterable in 1902 (5) and was isolated in bovine embryonic kidney cells in 1957 (494). Sequence analysis suggests the existence of a second bovine morbillivirus that causes persistent infection (41).

Rinderpest is a highly contagious disease of ruminants and swine characterized by inflammation, hemorrhage, necrosis, and erosion of the gastrointestinal tract (5). RV replicates in lymphocytes and macrophages, leading to lymphoid necrosis (604), and in epithelial cells of the gastrointestinal, respiratory, and urinary tracts, and in endocrine and exocrine glands, but not in the brain or spinal cord (666). It is an important veterinary pathogen. Vaccine and virulent strains of RV have been completely sequenced and the virus has been rescued from cDNA (40,42).

Peste des petits ruminants causes a disease of sheep and goats in Africa, Asia, the Middle East, and India (574). The disease was first discovered in West Africa in 1942 and is characterized by erosive stomatitis, pneumonitis, and diarrhea (429). The etiologic agent is most closely related to RV (see Fig. 1) (220).

Morbillivirus Infections of Aquatic Mammals

In 1987, an epizootic of respiratory disease with high mortality caused by secondary infections occurred in seals in the Baltic and North Seas (640). Serology suggested infection with a morbillivirus related to CDV, and the causal agent, phocine distemper virus, was isolated in 1988 (336). This disease is characterized by fever, nasal and ocular discharge, and severe respiratory, gastrointestinal, and CNS symptoms. The primary disease is complicated by a variety of secondary viral, bacterial, and parasitic infections, suggesting the type of immune suppression seen in measles and canine distemper (475). Phocine distemper virus is most closely related to CDV (see Fig. 1) (75,137). Similar epizootics of severe respiratory disease were subsequently recognized in porpoises along the Irish Coast (409), in dolphins in the Mediterranean Sea (157), and in monk seals off the coast of West Africa (476). Distinct but related morbilliviruses were isolated from these animals (see Fig. 1) (44,157,409,476,639). Cetaceans infected with either porpoise or dolphin morbillivirus develop pneumonia and encephalitis with virus demonstrable in lung, brain, spleen, and urinary bladder.

Experimental Infection in Animals

Nonhuman primates can be infected naturally or experimentally with MV, and they provide the only experimental model for the pathogenesis of this disease in humans. Small laboratory mammals generally are not susceptible to wild-type strains of MV, although limited replication has been reported in the respiratory tract of cotton rats (673) and in transgenic mice expressing CD46 (432). Several neurotropic rodent-adapted strains of MV have been developed by repeated intracerebral passage, but these strains do not produce acute disease similar to that seen in humans but rather serve as models for neurologic diseases such as SSPE.

Primates

Natural MV infection occurs only in humans. However, a number of species of monkeys are susceptible to measles and can contract the disease by the respiratory route through contact with humans (364,515,635,655). Monkeys can also be infected experimentally by the intranasal, intratracheal, or subcutaneous routes of inoculation (31,71,635). Disease is generally similar to that seen in humans, and deaths are often caused by secondary infections (515). Experimentally infected rhesus and cynomolgus macaques have served as animal models for studies of the pathogenesis of human measles, immune suppression, and vaccine-induced protection from measles, and for analysis of virus virulence (31,71,175,342,407,634,635 and 636,680). Variations in susceptibility of different primate species may be linked to polymorphisms in the MV receptor CD46 and availability of other MV receptors used by wild-type strains of virus (292). Neurologic disease can be induced by intracerebral inoculation of cells infected with neuroadapted strains of MV (8).

Marmosets and tamarins are also susceptible to natural and experimental MV infection by the respiratory route, but they develop a fulminant disease with high mortality characterized by interstitial pneumonitis, gastroenterocolitis, and bacteremia (11,364,381). Infection with vaccine strains produces little disease (381). Intracerebral inoculation with the most virulent strains of MV produces encephalitis, but attenuated strains do not (10).

Hamsters

Of all small mammals, hamsters appear to be the most susceptible to infection with MV. Wild-type, vaccine, and SSPE strains of MV replicate, or can be adapted to replicate, in the brains of newborn hamsters (93,102,317). IFN-resistant subpopulations of viral stocks grow preferentially (102). Hamsters can also be infected using cells persistently infected with MV (600). Newborn animals develop hyperirritability leading to death within a few days. Brains from these young animals show a generalized giant cell encephalitis with intranuclear and intracytoplasmic inclusion bodies (see Fig. 11) and a polymorphonuclear inflammatory response (643). Older animals develop myoclonus with infection of cortical, thalamic, and hippocampal neurons without giant cell formation (600,643). Ependymal infection may lead to hydrocephalus (317). Cell-free virus can be easily recovered from the brains of newborn, but not older, hamsters (96). For some MV strains (e.g., HBS), there is evidence of replication outside the CNS (103) and dissemination of virus by infected monocytes (410). Demyelination is uncommon (643), but some hamsters inoculated as newborns with the LEC SSPE strain develop myelitis 5 to 50 weeks after infection (101).

Mice

Several strains of MV have been adapted to grow in the brains of newborn mice (93,99,308,404). The most extensively studied viruses have been the mouse-adapted Edmonston (MAED) (308) and the hamster neurotropic (HNT) strains (93). In newborn mice, these viruses grow well and cause fatal disease associated with lethargy and wasting within 10 days after intracerebral inoculation. Histopathology and immunocytochemical staining show neuronal and glial infection and giant cell encephalitis (243,382,448). By electron microscopy, nucleocapsid material is seen in neurons and glia (274). Virus is generally cell associated but can be assayed by plaque formation in human or primate cells (653). Inoculation by peripheral routes does not produce detectable disease or infection (448).

Infection of 4- to 6-week-old mice by the intracerebral route with MAED, HNT, or SSPE strains of MV results in a nonproductive infection of neurons in which viral proteins are produced without infectious virus (243). Infected neurons are primarily in the rhinencephalon, limbic system, and striatum without mononuclear cell infiltration, giant cells, or inclusions. The MV-antigen-positive neurons have only smooth nucleocapsid structures and no immunocytochemically detectable H, F, or M proteins, suggesting that viral replication is defective at a stage prior to assembly of virions (274,382,536). The immune response plays no identifiable role in these age-related differences in disease. It appears that maturation of CNS cells converts the production of MV from the infectious form in the newborn mouse to a primarily defective form in the 4-week-old mouse (243,274,536). Weanling mice develop severe disease consisting of hyperexcitation and seizures (243,382,508). This clinical disease is associated with production of excitotoxic amino acids and selective bilateral destruction of pyramidal cells in the hippocampus (18,382), which can be prevented by administration of N-methyl-D-aspartate-receptor antagonists (18).

The genetic background of the mouse influences the disease induced. Mouse strains vary in the amount of virus necessary to induce disease and in the rapidity with which age-dependent resistance develops; C3H/He mice are the most susceptible and SJL the most resistant (448). Weanling BALB/c mice develop fatal acute noninflammatory encephalopathy (382,508), whereas SJL mice develop little acute disease but often succumb to a chronic encephalitis associated with limbic system inflammation (508). C57BL/6 mice that survive acute disease develop chronic neurologic disease weeks to months later (122). Mice with immune deficiencies are more susceptible than immunocompetent mice (194,628).

Recently, a number of investigators have produced a variety of strains of mice engineered to provide better systems for studying measles pathogenesis. Mice with SCID have been grafted with human peripheral blood mononuclear cells (304,619) or implanted with human fetal thymus and liver, which mature to form thymic tissue (30). In thymic implants, MV shows strain-specific differences in virus growth and thymocyte apoptosis that have been useful for assessing virulence and determinants of replication *in vivo* (30,631,632). Immunologically normal mice with transplanted human leukocytes develop graft-versus-host disease and can be infected with MV (304). Transgenic mice expressing human CD46 have

been produced using genomic DNA or cDNAs with promoters designed to provide generalized or tissue-specific expression of the CD46 transgene. In general, infectious virus cannot be recovered from the mice, but evidence of MV replication is greater than in control nontransgenic mice and is enhanced by the absence of IFN (74,289,432,505). These studies have demonstrated that blocks to MV replication in mice are at multiple levels, not solely at the level of the CD46 receptor.

Ferrets

Intracerebral inoculation of ferrets with cells infected with certain SSPE strains of MV causes acute encephalitis (330,617). Subacute or chronic encephalitis is established if animals are immunized prior to intracerebral injection with infected cells (617,618). Pathologic and clinical features in animals with chronic disease resemble SSPE with cerebral inflammation, gliosis and intranuclear inclusion bodies, electroencephalographic changes, bound immunoglobulin in brain, and high titers of antibody to MV in serum and CSF (84,330,617,618).

Rats

The HNT strain can infect laboratory rats after intracerebral inoculation. The CAM strain, adapted to rat brain by serial passage, causes fatal encephalitis after intracerebral inoculation into newborn rats (366). Neurovirulence may be related to amino acid changes in the H glycoprotein (366). The type of disease produced in older rats is determined by the strain of rat. In Brown Norway rats, only 10% to 20% develop acute encephalitis, and no late disease develops in survivors. In Lewis rats, 80% to 90% develop acute fatal encephalitis, and the remainder develop subacute disease 4 to 6 weeks after infection. Infectious, cell-free virus can be recovered during acute, but not subacute, disease (368). Transgenic expression of human CD46 does not increase the permissiveness of rats for MV infection (451). Cotton rats (*Sigmodon hispidus*) can be infected by the respiratory route and develop evidence of immune suppression (450).

Rats have been used extensively to study the role of the immune response in MV-associated neurologic disease. Acute encephalitis in newborn rats can be modulated by passive transfer of neutralizing antibody to the H protein. Such antibody treatment results in a late onset of disease due to MV that has escaped neutralization by MAb (366). Prior immunization with the H, F, or N protein is protective for adult Lewis rats, and both CD4 and CD8 T cells participate in protection from CNS infection (388,513). Features of subacute disease that are similar to SSPE include an inability to recover virus by routine methods, restricted expression of MV envelope protein mRNAs in the brain and intrathecal synthesis of oligoclonal MV-specific antibody (368,561). Demyelination is not a feature of subacute encephalitis, but demyelinating disease can be more easily induced in previously infected animals (369).

EPIDEMIOLOGY

Classic Measles

In the Absence of Widespread Immunization

The study of island populations has been important in establishing many of the principles of measles epidemiology. The pioneering concepts were formulated by Panum in his observations of the transmissibility, incubation period, and lifelong immunity of measles during the 1846 epidemic in the Faroe Islands (481). These concepts were furthered by the 1883 observations of Hirsch, who noted that an epidemic ends when there are no more susceptible individuals (281).

Measles is one of the most infectious of communicable diseases. It is estimated that 76% of household exposures of susceptible persons leads to measles (286) and that the reproductive number (R_0) or average number of secondary cases produced by an infectious individual in a totally susceptible population, is 15 to 20 (427). In temperate climates, measles is more frequent in the winter and early spring, perhaps related to increased virus survival at low relative humidities (262). Transmission is most efficient through direct exposure to an infected individual, but MV can survive for hours in respiratory droplets, and direct contact is not required (76,171). Individuals are most infectious from 4 to 5 days before through 4 days after the appearance of the rash (76,129).

There is no animal reservoir or evidence of latent or epidemiologically significant persistent infection. Therefore, maintenance of MV in a population requires a continuous supply of susceptible individuals. Because older members of a community are immune through previous exposure to the virus, endemic measles is primarily a disease of childhood. If the population is too small to establish endemic transmission, the virus cannot be maintained (64).

Mathematical calculations and studies of islands and cities with populations of different sizes have shown a requirement for a population of 250,000 to 500,000 to establish measles as an endemic disease (63,334). This is approximately the population of the earliest urban civilizations in ancient Sumer around 3000 B.C., where measles is postulated to have emerged.

In large population centers, measles is endemic with occasional epidemics as the number of susceptible individuals increases. The frequency of epidemics is determined by the numbers of susceptible and immune individuals, the duration of infectiousness, patterns of population mixing, and the contributions of chaos in the system (334,472). The size of the population is also a primary determinant of the age of seroconversion (65). In developing countries with large populations, high birth rates lead to infection at an early age. The average age of infection is earlier in urban than in rural areas in both developed and developing countries (141). In small isolated populations, measles epidemics are controlled by extinction and chance reintroduction of the virus, which leads to infection at older ages on average (129,518).

Effects of Immunization

Immunization has altered the epidemiology of measles by reducing the susceptible individuals in the population, causing

an increase in the average age at infection and a lengthening of the interepidemic period (141). In countries with high rates of immunization, the average age for measles is increased, because herd immunity reduces transmission and indirectly protects children from infection (65). Very young infants are protected from measles (and from response to vaccine) by maternal antibody. The duration of protective antibody in the infant depends on the level of maternal antibody, a primary determinant of the level of antibody in the infant at birth. The source of maternal immunity (vaccine or natural measles) and gestational age at birth are determinants of the amount of antibody passively transferred, and, therefore, the length of time required for initial levels of antibody to decay to the point that an infant will become susceptible to measles (394,480). When outbreaks occur in areas of sustained high vaccine coverage, an increasingly large portion of the cases will be in older individuals who are susceptible because of primary or secondary vaccine failure.

Morbidity and Mortality

Severity of measles in a population is determined by a number of factors. Mortality is highest at the extremes of age, in those with low socioeconomic status, and in those without access to medical care (129,155,532,552). Mortality is also increased in malnourished individuals (39) and in secondary cases in a household, presumably the result of exposure to a higher dose of infecting virus (2,345). High mortality is common when the virus is introduced into virgin populations (129). Susceptibility of these populations is not completely understood, but genetic homogeneity of the populations and infection of many older individuals may be contributing factors (67,129).

Molecular Epidemiology

Sequencing of the H gene or the variable C-terminal portion of the N gene has identified several different lineages of MV that have characteristic temporal and geographic distributions (19,59,524). Some are localized to specific regions of the world and some are extinct, but most are widely distributed. Currently eight distinct clades with several genotypes are recognized (see Fig. 12) (19). Assembly of this increasingly large database of MV genotypes has aided in the identification of measles transmission pathways. Identification of the source of the MV causing disease will become increasingly important as control programs are implemented and identification of cases as imported or indigenous is necessary (59).

Subacute Sclerosing Panencephalitis

Subacute sclerosing panencephalitis occurs preferentially in boys from rural areas with a history of measles at an early age (257,314,424,681). The mean time to onset of SSPE after measles is 7 years (see Fig. 17) (424). There is no clustering of cases to suggest that the virus causing the initial infection is different from the virus causing uncomplicated disease. Several studies have identified exposure to birds as a risk factor, but the relationship to SSPE has not yet been explained (257). The incidence of SSPE has decreased dramatically since introduction of measles immunization, and there is no evidence that the vaccine virus can induce SSPE (424,681).

CLINICAL FEATURES

Classic Measles and Its Complications

Measles has an incubation period of 10 to 14 days, spanning the time from exposure to the appearance of clinical disease (see Fig. 13). The first prodromal symptoms of measles are fever, malaise, and anorexia, followed by cough, coryza, and conjunctivitis. The prodrome lasts 2 to 3 days, and during this time small, bright-red spots with a bluish white speck at the center, Koplik's spots, may become visible on the buccal mucosa, providing early diagnostic evidence of measles (343). The prodrome ends when the rash appears. Characteristically, the rash is maculopapular and appears first on the face and behind the ears and then spreads in a centrifugal fashion to the trunk and extremities. It begins to fade 3 to 4 days after it appears. Generalized involvement of lymphoid tissue may result in lymphadenopathy, mild splenomegaly, and appendicitis (215,335). In uncomplicated measles, clinical recovery begins soon after the appearance of the rash.

Respiratory Disease

The prominent respiratory symptoms are manifestations of diffuse mucosal inflammation in response to widespread infection of epithelial cells. Interstitial pneumonitis resulting from MV replication and inflammation in the lower respiratory tract is common in uncomplicated disease and frequently is detectable only by radiography or by measuring the alveolar–arterial oxygen gradient (238,273,335). Pneumonitis is more likely to be clinically severe during pregnancy (12,167). Symptomatic giant cell pneumonia (see Fig. 2) is seen primarily in immunocompromised individuals (176,200,401,502). Most of the severe pneumonia that complicates measles and leads to chronic pulmonary disease is caused by secondary bacterial and viral infections (52,238,329,430,499). Other common respiratory complications caused by secondary infections are otitis media and laryngotracheobronchitis (26,52,538). In addition to increasing susceptibility to new infections, previous viral and bacterial infections may be reactivated (129,474,599).

Gastrointestinal Disease

Measles virus replication in the liver, particularly in bile duct epithelium, is common in all ages, but clinically evident hepatitis is most frequent in adults (28,216,222,668). Diarrhea is a common complication of measles, particularly in young patients requiring hospitalization (26,237). Many epithelial surfaces are infected with MV and this may lead directly to gastrointestinal symptoms (428). However, diarrhea is frequently associated with secondary bacterial and protozoal infections (237,682). This complication often compounds the borderline nutritional status of young children in developing countries (163,256).

Myocardial Disease

Electrocardiographic abnormalities, including prolongation of the P-R interval, ST-segment, and T wave changes, can be detected in 20% to 30% of children with uncomplicated measles (227). However, symptomatic cardiac disease is uncommon and is most often related to transient conduction abnormalities. Disease may be more severe in immunocompromised individuals, and a fatal case of arteritis has been reported (606). In autopsy studies, myocardial and pericardial lesions are usually attributable to systemic bacterial infections.

Neurologic Disease

There is little evidence that the brain parenchyma is an important target tissue for MV replication during acute disease in immunologically normal individuals (173,425). However, apparently uncomplicated measles is frequently accompanied by a CSF pleocytosis (259,469) and changes in the electroencephalogram (221,259), suggesting the possibility of CNS infection. The occasional appearance of slowly progressive neurologic disease [measles inclusion body encephalitis (MIBE)] in immunosuppressed individuals (438) and the occurrence of late neurologic disease (SSPE) in immunologically normal individuals infected at a young age attests to the ability of MV occasionally to enter the brain and replicate in neurons and glial cells.

ADEM complicates 1 in 1,000 cases of measles, mostly in individuals over the age of 2 years (422). It usually occurs within 2 weeks after the onset of the rash (see Fig. 17) (379). Clinically, the disease is characterized by an abrupt onset of fever and obtundation, accompanied by seizures and multifocal neurologic signs. The majority of surviving patients have neurologic sequelae (322,370). Pathologic studies show that this is a perivenular demyelinating disease, not an inclusion body encephalitis like MIBE and SSPE (Table 2) (379). The pattern of demyelination suggests that it is immunologically mediated (217). There is little evidence for virus in the brain either as assessed directly by virus isolation or by techniques to detect viral antigen or RNA (217,425), or as assessed indirectly by the appearance of MV-specific antibody or MV-induced IFN in the CSF (322,360). There is, however, the induction of an autoimmune response to myelin basic protein (322).

Eye Disease

Measles is considered to be an important cause of childhood blindness. In India, 50% of children with measles had coarse punctate keratitis (512). In many parts of Africa, most childhood blindness is caused by corneal lesions. In areas of vitamin A deficiency, the two problems are synergistic and corneal ulceration resembling keratomalacia is a frequent complication of measles (553).

Determinants of Morbidity and Mortality

Predictors of poor outcome are severe lymphopenia (133) and poor antibody response (657). Measles modified, but not prevented, by previous immunization is associated with lower fever and decreased communicability (170). Mortality associated with measles is most common at the extremes of age and is rarely the result of uncontrolled replication of MV, except in immunocompromised individuals (39,52,425,552). There is high mortality in poor populations, particularly among children under 5 years of age with average case fatality rates in developing countries estimated at 2% to 6% (141). Risk factors for increased mortality are underlying illness, low socioeconomic status, and lack of access to medical care (155,532,552). Malnutrition increases mortality due to measles, and measles in turn increases malnutrition (163,428,552). Malnutrition affects immune responses to MV, impairs virus clearance, and may lead to lower rates of diagnosis (142,159,498). Vitamin A is a particularly important nutrient influencing the outcome of measles (39). Low serum retinol is common in severe measles because of either prior dietary deficiency or impaired mobilization from hepatic stores (77,95).

Atypical Measles

A more severe form of measles, with unusual clinical features, was seen in recipients of an inactivated measles vaccine used in the United States and Europe in the mid 1960s. Atypical measles differs from typical measles by having higher and more prolonged fever, unusual skin lesions, and severe pneumonitis (118,211,441). The rash often is accompanied by evidence of hemorrhage or vesiculation and begins on the extremities and spreads to the trunk. Pneumonitis is associated with distinct nodular parenchymal lesions and hilar adenopathy (238,679). Abdominal pain, hepatic dysfunction, headache, eosinophilia, and edema are also described. Cases of atypical measles have been reported 2 to 13 years after administration of the inactivated vaccine (254,654) and still may be occurring (200). Administration of live virus vaccine after two to three doses of killed vaccine did not eliminate subsequent susceptibility to atypical measles (124) and was often associated with severe local reactions (94,352).

Hypotheses about the pathogenesis of atypical measles included an abnormally intense cellular immune response (210), an inability of the inactivated vaccine to induce local respiratory tract immunity (55), and a lack of production of F antibody, which allowed virus to spread from cell to cell despite the development of anti-H antibody (417,455). In the rhesus macaque model of atypical measles, the formalin-inactivated vaccine induced transient neutralizing and fusion-inhibiting antibodies, but no CTL response. Subsequent infection with MV caused immune complex deposition, vasculitis, and an increase in eosinophils in blood and lungs. Antibody to most proteins appeared earlier than in unimmunized monkeys, suggesting previous priming for a nonprotective type 2 CD4 T-cell and antibody response that resulted in immune complex–mediated disease (496).

Measles in the Immunocompromised Host

Children with hypogammaglobulinemia appear to recover uneventfully from MV infection, whereas those with deficiencies in cellular immune responses may not (232). Two types of progressive disease are recognized in these individuals, giant

cell pneumonia and MIBE, often in the absence of the typical rash or other characteristic features of measles (90,176,401,586).

Giant cell pneumonia was originally described by Hecht in 1910 (269) and is characterized by increasing respiratory insufficiency beginning 2 to 3 weeks after a history of exposure to measles. Giant cell pneumonia is most frequently seen in children with congenital or acquired immunodeficiency but also has been reported in immunosuppressed adults (408). Virus can be isolated from lung tissue (176) and bronchial and nasopharyngeal washings (440). Pathology shows multinucleated epithelial alveolar and bronchiolar giant cells with intranuclear and intracytoplasmic inclusion bodies (23). There is often evidence of systemic disease, with giant cells visualized in multiple organs (23,176).

MIBE occasionally accompanies giant cell pneumonia (327) but is more often present as an isolated primary manifestation of progressive MV infection in immunocompromised individuals (see Table 2). Neurologic disease usually becomes evident 1 to 6 months after exposure to measles (see Fig. 17). Behavioral abnormalities progress to myoclonus, focal seizures, confusion, and coma (438,667). Retinopathy is occasionally present (258). Pathology shows gliosis with inclusions in glial cells and neurons. Giant-cell formation is unusual and little inflammation is present (7). MV can occasionally be recovered from brain, but the virus present is usually defective, as it is in SSPE (7,36,535,667). Viral nucleocapsids without budding particles are seen intracellularly by electron microscopy (7,535). The mRNAs for genes encoding the H, M, and F envelope proteins are reduced, and these proteins are often undetectable (36,114). MIBE may be fundamentally similar to SSPE, but clinical symptoms are manifested earlier after infection and are more rapidly progressive in the absence of an immune response (535).

Subacute Sclerosing Panencephalitis

Subacute sclerosing panencephalitis most commonly occurs in individuals under the age of 20 who present many years after measles, with mental deterioration accompanied by alterations in personality. This is followed by the appearance of myoclonus, characteristic electroencephalographic changes, and progressive neurologic deterioration marked by optic atrophy and akinetic mutism. Death occurs within months to years after onset of the disease (204). Antibody to MV is elevated in both serum and CSF, and inclusions are present in glial cells and neurons (275).

DIAGNOSIS

The classical clinical features of measles—fever, erythematous maculopapular rash, coryza, cough, conjunctivitis, and Koplik's spots—are generally sufficient to make the diagnosis, especially in the setting of a community outbreak. However, not all of these signs and symptoms may be present in measles, and many are shared with other diseases (393). The Centers for Disease Control and Prevention clinical case definition for measles includes: (a) a generalized maculopapular rash of 3 days or more, (b) fever of 38.3°C (101°F), and (c) one of the following: cough, coryza, or conjunctivitis (119). Disease manifestations are particularly likely to be modified, with absence of fever and/or rash, in very young infants, immunocompromised patients, malnourished children, and previously immunized individuals (170,393,498). The differential diagnosis of this clinical picture includes scarlet fever, rubella, parvovirus B19, human herpesvirus-6, human herpesvirus-7, meningococcemia, Kawasaki disease, toxic shock syndrome, dengue, and other causes of rash and fever (69,83,145).

Laboratory diagnostic procedures consist of isolation of virus, direct detection of the virus, viral RNA or viral antigens in secretions, detection of IgM, or low avidity IgG antibody by enzyme immunoassay (EIA) or documentation of seroconversion using HAI, CF, virus neutralization, or IgG-specific EIA on serum taken during the acute and convalescent phases.

Detection of Virus

Virus can be cultured from peripheral blood mononuclear cells, respiratory secretions, conjunctival swabs, and urine, but virus recovery is difficult and rarely used as the sole means of diagnosing acute disease. Cell lines vary in their susceptibility to infection by wild-type isolates, with primary monkey kidney cells, human cord blood leukocytes, and a marmoset B-cell line (B95-8 or B95a cells) being most sensitive (201,342).

Epithelial cells from the nasopharynx, buccal mucosa, conjunctivae, or urine can be used for direct cytologic examination for giant cells and inclusions and for antigen detection (370,589). Generally, the most useful antibody is one directed to N since this protein is most abundant in the infected cell and N antibody reactivity is retained using a wide variety of fixation methods (589). Direct examination for virus is of particular importance for diagnosis in immunocompromised individuals in whom antibody responses may not be present. The detection of MV RNA by RT-PCR using primers targeted to highly conserved regions of the N, M, or F genes has been successfully applied to a variety of clinical samples (184,321,438,582).

Detection of Antibody

The clinical diagnosis of measles is most often confirmed by serology. Samples ideally consist of acute and convalescent serum pairs, but detection of MV-specific IgM in serum or saliva or low avidity IgG in serum is diagnostic and may require only a single sample (83,193,625). IgM antibody appears at the time of the rash and can be detected by 3 days and up to 4 weeks after the onset of the rash in most individuals (271,539). MV-specific IgG peaks approximately 2 weeks after the onset of rash and gradually increases in avidity. EIA allows differential detection of IgM and IgG and is widely used because of its convenience. Plates may be coated with virus or with recombinant MV proteins (81,302). For detection of IgM, use of either the antibody capture method or preremoval of interfering IgG from serum is required for reliable results (624).

The HAI test detects antibody primarily to H and correlates well with the neutralization test (66). The major limitations of the HAI test are the requirement for fresh sensitive monkey erythrocytes, the difficulty of producing sufficient antigen for a

large number of tests, and the possible presence of nonspecific HA inhibitors in serum. The CF test has been used to determine measles immunity (51). These titers are less stable over time, and the test is more difficult to perform and is not in common use currently. The virus plaque reduction neutralization assay remains the gold standard against which other tests are measured. It is more sensitive than HAI or EIA tests (9), and it provides the best correlate for protection from infection (125) and therefore the best measure of response to immunization (1,8).

PREVENTION AND CONTROL

Treatment

There is no standard antiviral treatment for measles. Ribavirin inhibits MV replication *in vitro* (581) and may reduce the severity of symptoms (62). Immunocompromised patients with pneumonitis have shown no clear evidence of improvement after treatment with aerosolized ribavirin, but case reports suggest that intravenous ribavirin may be more efficacious (200,438).

Administration of high doses of vitamin A during acute measles decreases morbidity and mortality even in the absence of clinical evidence of vitamin A deficiency (39,135). In areas of vitamin A deficiency and xerophthalmia, supplementation prevents blindness caused by measles-induced corneal destruction. The World Health Organization (WHO) recommends high-dose vitamin A supplementation for all children with measles in countries where the fatality rate is 1% or more (661).

Numerous therapeutic agents, including amantadine, IFN, isoprinosine, ribavirin, and transfer factor, have been used for the treatment of SSPE. Evaluation of the efficacy of any of these regimens is difficult because the disease is rare, the course is variable, reports are anecdotal, and the benefits are at best short term.

Immunization

History

The earliest attempts at vaccination against measles (by Home in 1749) were based on the principles of variolation (285) with the reasoning that introduction of disease through the skin would lessen the effects on the lung. “Morbillization” was in general unsuccessful (270). Subsequent approaches between 1920 and 1940 designed to inactivate or attenuate the virus by culture in chick embryos also met with limited success (398). The isolation of MV in tissue culture opened the way for a more concerted approach to vaccine development using the Edmonston strain of virus (177). Killed virus vaccines were developed using formalin and TWEEN-ether for viral inactivation (650). Simultaneously, attenuated vaccines based on adaptation of MV to growth in chick cells were developed both in the United States and Russia (332,489). Attenuated live virus vaccine is widely used and has dramatically decreased the incidence of measles in all countries in which it has been effectively delivered (Fig. 18).

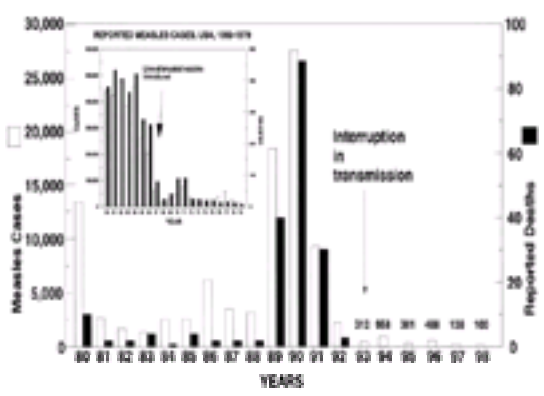

FIG. 18. Reported measles cases and deaths in the United States from 1960 to 1998. *Insert* shows data from 1960 to 1979. *Arrow* indicates documented interruption of indigenous transmission of measles in the United States. (From Centers for Disease Control and Prevention, with permission.)

The immune responses necessary for protection have not been completely defined. Successful live attenuated virus vaccines have been developed for measles (331), rinderpest (495), and canine distemper (531). These vaccines induce both neutralizing antibody and cellular immune responses (479,644). Antibody is sufficient for protection because infants are protected by maternal antibody (8,66). Passive transfer of immune serum can modify or interfere with measles immunization (583a) and partially protects children from measles after exposure. The nature and specificity of the protective antibodies have not been identified, but data from animals immunized with recombinant vaccines suggest that antibody to either H or F is neutralizing and protective (160,676).

Inactivated Vaccines

The inactivated vaccine was alum precipitated and used in a three-dose regimen (104,650). Recipients of these inactivated vaccines developed moderate levels of neutralizing and HAI antibodies and low levels of CF antibody (104,179,190). The vaccine was protective when exposure to measles occurred within several months after immunization (190,209). However, antibody titers declined rapidly, recipients again became susceptible to measles (179,209,509), and when infected they developed the more severe disease, atypical measles (441,509), as discussed previously.

Attenuated Live Virus Vaccines

The first attenuated live measles vaccine was developed by adaptation of the Edmonston strain of MV to chick embryos and subsequently to chick embryo fibroblasts after passage in primary renal and amnion cells to produce the Edmonston B virus (174,333). Inoculation of this virus produced no detectable viremia, and no spread to the respiratory tract or clinical symptoms in monkeys, but it did induce antibody and protected from subsequent challenge. This vaccine was efficacious (356) and licensed in 1963, but it was reactogenic, producing fever and rash in a large proportion of immunized children (331). These reactions could be reduced by giving antibody to MV simultaneously (514). Further passage of the Edmonston B virus in chick embryo fibroblasts by Schwarz produced a more attenuated virus that was licensed in 1965 (567). The Schwarz vaccine currently serves as the standard measles vaccine in much of the World. The Moraten strain used in the United States was licensed in 1968 and is closely related to Schwarz (541). Other Edmonston-derived vaccine strains (e.g., Zagreb, AIK-C) and attenuated strains developed independently of the Edmonston-derived vaccines using similar strategies (e.g., CAM, Leningrad-16, Shanghai-191) are also successful vaccines (280,320,489). The lyophilized vaccine is relatively stable, but the reconstituted vaccine rapidly loses titer at room temperature (415).

The recommended age of immunization has varied from 6 to 15 months and remains a subject of discussion (140,149). The probability of seroconversion and the antibody levels induced are determined by the level of persisting MV-specific maternal antibody and age of the infant (140,213,214). Therefore, the recommended age of immunization is determined by a balance between the optimal age for seroconversion and the probability of acquiring measles before that age. Both parameters show regional variation (140,394,400). In areas where measles remains prevalent, measles immunization is routinely performed at 9 months, whereas in areas with little measles, immunization is often at 12 to 15 months of age. Respiratory routes of immunization have been advocated for younger infants (547) but have proven either unsuccessful or impractical, although they may hold promise for use in older children (139).

Genetic polymorphisms in immune-response genes also affect the likelihood of seroconversion (267,268). The role of common childhood illnesses on seroconversion is unclear (354,420). Any potential decrease in seroconversion must be balanced against the loss of the opportunity for immunization and the consequent risk of the child's acquiring measles. Overall, the efficacy of a single dose of measles vaccine in infancy is estimated at 80% to 95% (250,587).

Similar compromises must be considered with respect to immunizing individuals with human immunodeficiency virus (HIV) infection (431). Progressive fatal infection has been occasionally associated with measles vaccine in severely immunocompromised children and adults. Overall, measles vaccine has been well tolerated in HIV-seropositive children and adults, but the seroconversion rate is lower. Because of the potential severity of measles in these individuals, the vaccine is recommended for routine administration to infants without respect to HIV serostatus in most countries, but it is not recommended for those with known low CD4 T-cell counts.

Administration of standard doses of live attenuated MV vaccine results in transient lymphopenia (196), suppression of delayed-type hypersensitivity skin test responses to recall antigens (196,684), decreases in antigen- and mitogen-stimulated proliferation of lymphocytes (196,283,305,684), and altered cytokine production (283,305,645). Many of these changes occur independent of serologic status (684) and are qualitatively similar to the changes that occur during measles. No evidence has been presented that this alteration of immune responses is clinically important. Adverse reactions are few and consist primarily of allergic reactions.

The dose of MV routinely used for immunization is between 10^3 and 10^4 plaque-forming units. When 10- to 100-fold-higher doses have been used, seroconversion in younger infants (4 to 6 months of age) is improved. However, subsequent follow-up of children receiving high titered vaccines in countries with high childhood mortality showed an increased mortality in girls over the subsequent 2 to 3 years (284,341). Mortality was less than in children who received no measles immunization and was not caused by measles, but rather by a relative increase in the incidence of other infections (3). The pathogenesis of delayed increased mortality after high titered vaccine is not understood but occurred primarily in those who developed a rash after immunization, and it may be related to long-term suppression of immune responses similar to that induced by measles (572,648).

The immune response to the live attenuated vaccine is similar to that induced by natural disease. However, the duration of vaccine-induced immunity appears to be more variable. In general, levels of antibody are lower after immunization than after recovery from natural disease, and these levels decay with time (130,339). Secondary vaccine failure rates have been estimated to be approximately 5% at 10 to 15 years after immunization (402), but they are probably lower when immunization is given after 12 months (17). A second dose of vaccine is recommended to provide an opportunity to immunize persons who missed or did not respond to the first dose (6,141,497). It is not clear that a second dose increases levels of antibody in individuals with waning immunity (130,399). The two-dose strategy has been credited with elimination of indigenous measles in Finland and the United States (120,488).

Experimental Vaccines

Development of new vaccines has been hampered by an incomplete understanding of protective immunity and of the enhanced disease after inactivated vaccine. Nevertheless, a number of experimental vaccines have been developed. The primary motivation for development of a new vaccine is to increase thermostability and to provide a vaccine that can be given to young infants. Immunization with the H, F, and/or N protein expressed in poxvirus, adenovirus, or Bacille Calmette-Guerin (BCG) can protect mice against challenge with MV (191,198,614,663). DNA, peptides, and MV proteins delivered with a variety of carriers can induce cellular and humoral immunity in mice (98,185,265,674). Monkeys are the most relevant challenge model, and initial studies have shown some protection against severe disease with BCG-expressed N (680). Immune stimulating complexes (Iscoms) prepared with MV envelope proteins can induce protective immunity in the presence of passively transferred antibody (634).

Prospects for Eradication

Theoretically, measles is an ideal virus for eradication through immunization: There is only one serotype, most cases are clinically identifiable, there is no animal reservoir, and a vaccine is available (516). In 1979, this was identified as a goal in the United States (516) but only recently has endemic transmission in the United States been interrupted (120). In 1989, the WHO/UNICEF Joint Committee on Health Policy declared control as the first step toward eradication (146). The highly communicable nature of the virus requires continued maintenance of very high immunization coverage to prevent outbreaks, with at least 98% antibody seropositivity needed for herd immunity (65). The role of asymptomatic infection of partially immune individuals in maintaining transmission is unclear (143). Global programs, such as the Expanded Program for Immunization, have increased vaccine coverage worldwide and have resulted in significant decreases in measles and measles mortality (141,471). However, delivery remains difficult in many areas because of civil strife, the need to maintain a cold chain for the vaccine, and the continued inability to immunize young infants. Measles remains a common infection in many parts of the world. Current strategies for eradication focus on delivery of vaccine to large numbers of individuals in a short period of time through mass campaigns (452). Eradication remains a worthy but difficult goal.

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CHAPTER 45 Respiratory Syncytial Virus

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INTRODUCTION

Human respiratory syncytial virus (RSV) is an enveloped, nonsegmented negative-strand RNA virus. RSV was first isolated in 1956 from a symptomatic laboratory chimpanzee during an outbreak of illness resembling the common cold (282). Shortly thereafter, the same virus was recovered from infants with respiratory illness, and serologic studies indicated that infection in infants and children was common (69). RSV quickly became recognized as the most important viral agent of serious respiratory tract disease in the pediatric population worldwide (14,135,142,197,232,292,320). RSV is receiving increasing recognition as an important agent of disease in the elderly and in bone marrow transplant recipients (108,115,178,416,419) as well as in the general adult population (298).

RSV lacks effective antiviral therapy or an approved vaccine, although effective passive immunoprophylaxis involving administration of RSV-neutralizing antibodies is now available for high-risk individuals (17,153,216). Worldwide, RSV remains one of the pathogens deemed most important for vaccine development (1,157). Its poor growth *in vitro* and instability and the lack of convenient animal models for authentic disease hamper studies with RSV.

CLASSIFICATION

RSV is classified in family *Paramyxoviridae,* order *Mononegavirales* (the nonsegmented negative-strand RNA viruses or mononegaviruses). Members of *Paramyxoviridae* have the following features: (a) the genome is a single strand of negative-sense RNA found exclusively in an RNAse-resistant helical nucleocapsid that also contains the viral polymerase; (b) the genome is transcribed by a sequential stop–restart mode, in which the polymerase is guided by cis-acting signals and produces subgenomic mRNAs; (c) the viral replicative cycle is cytoplasmic; (d) progeny virions acquire a lipid envelope by budding at the plasma membrane; and (e) entry into the host cell is by cell-surface fusion. Members of *Paramyxoviridae* have six genes in common (Fig. 1), encoding the RNA-binding N protein of the nucleocapsid, the nucleocapsid phosphoprotein P, the major polymerase subunit L, the nonglycosylated matrix protein M, an attachment glycoprotein G, H, or HN, and the fusion glycoprotein F. Individual *Paramyxoviridae* members have one to five additional genes that typically encode accessory proteins that enhance growth but usually are not essential.

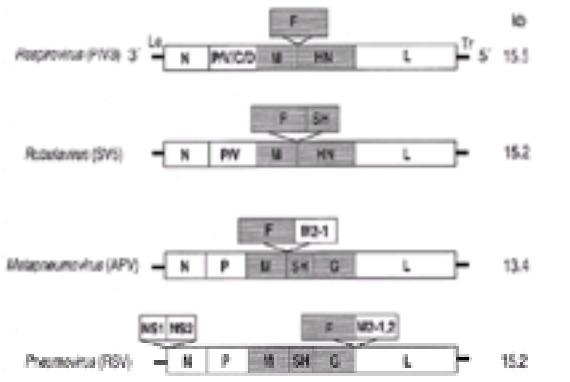

FIG. 1. Comparison of the gene maps of respiratory syncytial virus (RSV) and selected members of family *Paramyxoviridae.* Viruses: human parainfluenza virus type 3 (PIV3) and simian virus 5 (SV5) represent the respirovirus and rubulavirus genera of the Paramyxovirus subfamily, respectively; avian pneumovirus (APV) and RSV represent the

Metapneumovirus and Pneumovirus genera of the Pneumovirus subfamilies, respectively. The gene maps are drawn as 3' to 5' negative-sense genomic RNAs. Each rectangle indicates an encoded mRNA, with the encoded protein(s) identified. The envelope-associated proteins are shaded. Le and Tr, extragenic leader and trailer regions. The gene overlap between the RSV M2 and L genes is not indicated. This comparison illustrates common proteins (N, P, M, F, HN or G, and L) and species-specific proteins (NS1, NS2, SH, M2-1, M2-2), and the gene order difference between genera Pneumovirus and Metapneumovirus. Note the resemblance of the gene orders of APV and SV5.

Paramyxoviridae contains two subfamilies: *Paramyxovirinae,* which includes Sendai, measles, mumps, and Newcastle disease viruses, simian virus 5 (SV5), the human parainfluenza viruses, and others; and *Pneumovirinae,* which is represented by RSV. These subfamilies are referred to hereafter as the paramyxoviruses and the pneumoviruses. Pneumoviruses are distinguished from paramyxoviruses because (a) they are only distantly related by amino acid sequence, sharing low but significant relatedness in F and L proteins and possibly a domain in the N protein (27,65,340,366,371); (b) pneumoviruses encode eight or 10 mRNAs compared with six or seven for paramyxoviruses; (c) pneumoviruses encode additional proteins not found in paramyxoviruses: NS1, NS2, M2-1, and M2-2; (d) pneumoviruses lack RNA editing and do not follow the “rule of six” (see Chapter 41) (355); (e) pneumoviruses lack the V, D, and C proteins found in various paramyxoviruses; and (f) pneumoviruses have an unusual, mucin-like G attachment protein that is distinct structurally from the hemagglutinin–neuraminidase (HN) or hemagglutinin (H) protein of paramyxoviruses.

Pneumovirinae has two genera (see Fig. 1). Genus Pneumovirus contains human RSV as well as bovine, caprine, and ovine RSVs and pneumonia virus of mice (PVM). The second genus, Metapneumovirus, consists of avian pneumovirus (APV, formerly called turkey rhinotracheitis virus). APV differs from the RSV/PVM group on the basis of sequence divergence, the absence of NS1 and NS2 genes, and the difference in gene order for the F and M2 genes.

INFECTIOUS AGENT

Virion

The RSV virion (Fig. 2) consists of a nucleocapsid contained within a lipid envelope. When visualized by negative staining and electron microscopy, virions can appear as irregular spherical particles that range in diameter from 150 to 300 nm, similar to paramyxoviruses. Virion preparations can include a high content of filamentous forms that are 60 to 100 nm in diameter and up to 10 μm in length (Fig. 3) (37,45,347). When RSV virions are irradiated with ultraviolet (UV) light, infectivity is lost with single-hit kinetics (102). Thus regardless of structure, each infectious particle contains a single functional copy of the genome.

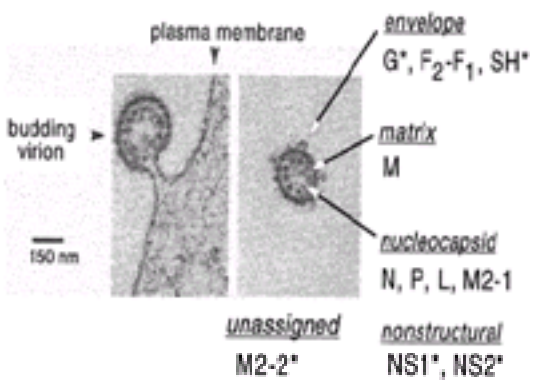

FIG. 2. Electron photomicrographs of a budding (**left**) and free (**right**) virion (from ref. 224) (original magnification, ´132,000), and virion locations of the 11 viral proteins. Note that the NS1, NS2, SH, M2-2, and G genes can each be deleted from viable recombinant virus (*asterisks*) and on that basis encode nonessential accessory proteins. From refs. 36,44,49,382,384, and 420.

FIG. 3. Electron photomicrograph of a cross section of a respiratory syncytial virus (RSV)-infected HeLa cell (from ref. 224, with permission) (original magnification, ´12,800). The changes due to infection include a large cytoplasmic inclusion (*C*), budding particles (*B*), and filamentous particles (*FP*). Microvilli (*arrows*) are not due to the infection.

The envelope consists of a lipid bilayer that is derived from the host plasma membrane. It contains three virally encoded transmembrane surface glycoproteins, the attachment protein G, the fusion protein F, and the small hydrophobic SH protein. There also is a matrix M protein that is thought to form a layer on the inner envelope face. The viral glycoproteins are organized separately into virion “spikes,” which are visualized as short (11–20 nm), closely spaced (intervals of 6 to 10 nm) surface projections. The RSV nucleocapsid is a symmetrical helix similar in appearance to that of the paramyxoviruses, except that the helical diameter is 12 to 15 nm rather than 18 nm.

Pneumoviruses lack a neuraminidase. A hemagglutinin has been reported for a single pneumovirus, involving the G protein of PVM (252). RSV has four nucleocapsid proteins packaged in the virion: the major nucleocapsid protein N, the phosphoprotein P, the antitermination factor M2-1, and the large polymerase subunit L. Polymerase activity has not yet been identified in purified virions, although partially purified nucleocapsids isolated from infected cells direct efficient transcription of the entire genome (23,199).

Proteins

Eleven RSV genes and their encoded proteins have been identified (see Fig. 2 and Table 1), accounting for all of the significant open reading frames (ORFs).

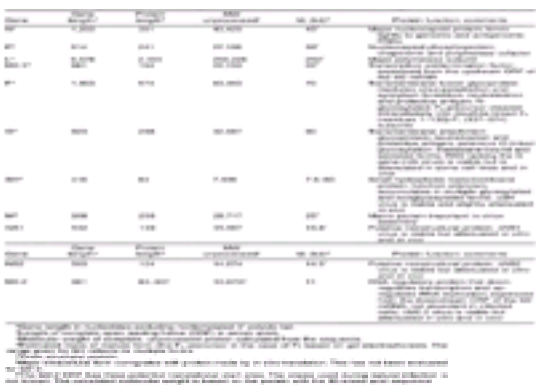

TABLE 1. *RSV genes and their encoded proteins*

Fusion F Protein

The RSV F protein (Fig. 4) directs viral penetration by fusion between the virion envelope and the host cell plasma membrane, delivering the nucleocapsid to the cytoplasm. Later in infection, F protein expressed on the cell surface can mediate fusion with neighboring cells to form syncytia. This is a prominent cytopathic effect and probably is an additional mechanism of viral spread. The pneumovirus and paramyxovirus F proteins are of similar length, are processed similarly, have generally similar structural features, and have a low but significant level of global sequence relatedness (82,366). Like the other F proteins, RSV F appears to form trimers (54). A detailed discussion of their structure and function is provided elsewhere (Chapter 41) (54,66,254).

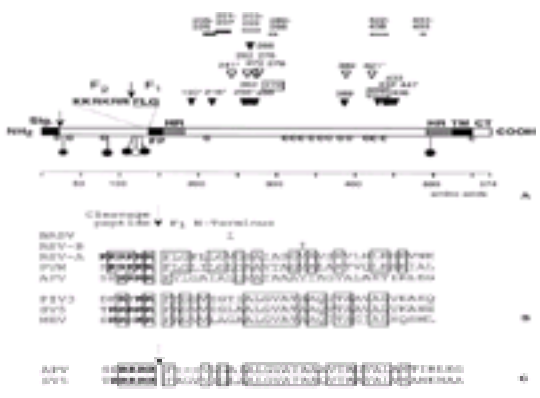

FIG. 4. Structure and antigenic map of the respiratory syncytial virus (RSV) F protein. Hydrophobic domains are *solid:* Sig., signal peptide; FP, fusion peptide; TM, transmembrane anchor. Heptad repeats (HR) are *shaded* (254). *Arrows* identify the cleavage site for the signal peptide and for generation of the F_1 and F_2 subunits: the expanded sequence shows the cleavage peptide (KKRKRR) and the first three amino acids of the FP of F_1 (*FLG*). Cysteine residues (*c*); potential acceptor sites for N-linked carbohydrate that are conserved (*stalk with solid circle*) or not conserved (*stalk with open circle*) among different RSV strains (211). Antigenic map (254): *arrowheads,* amino acid positions of point mutations determined from sequence analysis of monoclonal antibody–resistant mutants (MARMs) of the A2 (*open arrowheads*) (31,99) and Long (*solid arrowheads*) (15,254) strains. Monoclonal antibodies (MAbs) assigned to the following antigenic sites selected the following changes in strain A2: site A, single changes at positions 262, 272, 275, or 276; site B, 389; and site C, double mutations (*asterisks*) at positions 241/421. Position 266 (gray arrow) was identified in a strain A2 MARM selected using a recombinant human antibody fragment (99). MAbs assigned to the following antigenic sites selected the following changes in the Long strain: site I, 389; site II, single mutations at position 262, 268, or 272 or double mutations at 190/258 or 216/262 (*asterisks*); site IV, 429; site V, single changes at 432, 433 or 447; site VI, 436. Sites A and B correspond to II and I, respectively. Many of the site A MAbs have high fusion-inhibition titers, and many of the site A MARMs have a small plaque phenotype (31). Numbers are boxed in cases in which the selecting MAb was shown to be reactive with a short synthetic peptide (of wt sequence) spanning the position. Synthetic peptides (at top): *open boxes*, peptides that reacted with RSV-specific polyclonal antisera and/or MAbs; *solid boxes,* peptides that in

addition induced RSV-neutralizing antibodies (15,93,254,392) **B:** Comparison of the sequences of the cleavage peptide and N-terminus of the F_1 subunit of several pneumoviruses: bovine RSV (BRSV), RSV subgroup A (RSV-A), RSV subgroup B (RSV-B), PVM, and APV, and several paramyxoviruses [human parainfluenza virus type 3 (PIV3), simian virus 5 (SV5), and measles virus (MeV)]. For BRSV and RSV-B, only the assignments that differ from those of RSV-A are shown. *Boxes,* Amino acid assignments conserved among two or more of the indicated pneumoviruses or paramyxoviruses. The basic residues of the cleavage peptide are in *bold*; the cleavage site is indicated with an *arrow,* and the hydrophobic fusion peptide at the N-terminus of the F_1 subunit is *underlined.* **C:** Comparison of the sequences of the cleavage peptide and N-terminus of the F_1 subunit of APV and SV5, with gaps introduced to emphasize amino acid sequence relatedness

The F protein of RSV, like that of the paramyxoviruses, is synthesized as the precursor F_0 that is cleaved by cellular protease in the trans-Golgi compartment or network to yield the disulfide-linked heterodimer F_2–F_1. The cleavage site of the RSV F_0 precursor consists of six tandem Arg and Lys residues, and thus contains the cleavage motif for the cellular furin protease (Arg/Lys-X-Arg/Lys-Arg). The presumption that RSV F_0 is activated by this ubiquitous protease suggests that cleavability is not a limiting factor in RSV tropism or pathogenesis, in contrast to the well-known models of Sendai virus and certain strains of Newcastle disease virus (NDV). Cleavage liberates the hydrophobic “fusion peptide” at the N-terminus of the F_1 subunit, which is thought to be directly involved in membrane insertion. The amino acid sequence of the fusion peptide is not well conserved between paramyxoviruses versus most pneumoviruses (see Fig. 4B), suggesting that its function does not depend on a specific sequence. Somewhat surprisingly, however, the fusion peptide of APV shows considerable similarity to that of SV5 (Fig. 4C; see Animal Pneumoviruses, later).

Expression of RSV F by a recombinant simian virus 40 or a recombinant vesicular stomatitis virus resulted in syncytium formation in cell culture, and the latter virus gained the ability to enter cells by surface fusion (223,309). Thus RSV F can mediate entry and syncytium formation independent of the other RSV proteins. This differs from the situation with most paramyxoviruses, for which both F and its cognate HN attachment protein are necessary for syncytium formation. Although the F protein can mediate syncytium formation by itself, studies with plasmid-expressed proteins in cell culture indicate that coexpression of the G and SH proteins increased the efficiency of syncytium formation (186).

Attachment G Protein

The G glycoprotein (Fig. 5) was identified as the major RSV attachment protein because antibodies specific to G blocked the binding of virions to HeLa cells, whereas antibodies to F prevented fusion but not binding (248,401). Human RSV G is 289 to 299 amino acids in length, depending on the strain, which is only half the size of the HN or H attachment proteins of other paramyxoviruses (572–617 amino acids). The G protein lacks sequence relatedness or structural similarity with the paramyxovirus HN or H proteins, and it is difficult to imagine that they share a common ancestor (214,417). Whereas the HN and H glycoproteins consist of a folded globular head supported on a stalk, the pneumovirus G protein is a heavily glycosylated nonglobular species whose ectodomain has similarities with mucins (see later).

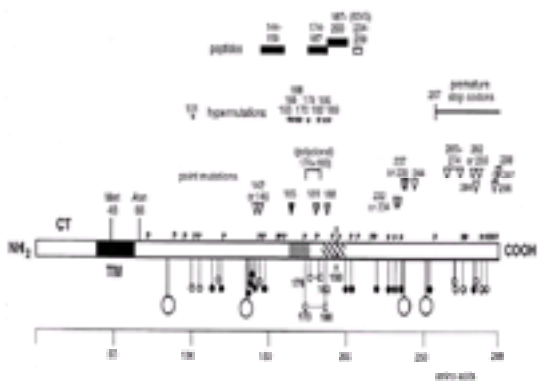

FIG. 5. Structure and antigenic map of the respiratory syncytial virus (RSV) G protein, based on strains A2, Long, and Mon/3/88 of subgroup A. TM, hydrophobic signal anchor (*solid*); CT, cytoplasmic tail. The segment of amino acid sequence that is conserved exactly between the subgroups (amino acids 164–176) is *shaded* (214). A proposed heparin binding site (amino acids 184–198) is *hatched* (123). Potential acceptor sites for carbohydrate (strain A2): serine (*stalks with small circles*) and threonine (*stalks with small solid circles*) residues predicted by sequence context (180) to be acceptor sites for O-glycosylation; potential acceptor sites for N-linked sugars (*stalks with large circles*). (P) Proline residues; (C) conserved cysteine residues, with disulfide bonding indicated (147). Met-48 is the translational start site for the secreted form (348); Asn-66 is its N-terminus after proteolytic trimming (348). The *dotted vertical line* at position 193 marks the site of the largest frame shift that gives rise to viable virus (274); hence the downstream portion of G is nonessential. Antigenic map as determined by sequence analysis of monoclonal antibody–resistant mutants (MARMs) or reactivity of MAbs with synthetic peptides (261,274): Four types of mutations were found in the MARMs: point mutations, premature stop codons, hypermutations, and frame shifts. Point mutations: certain MARMs selected with MAbs to conserved (*solid triangles*), group-specific (*shaded triangles*), or strain-specific (*open triangles*) (see the text) epitopes contained one (or two) amino acid substitutions identified by sequence position. Sometimes resistance to a given MAb was conferred by either of two closely spaced substitutions (i.e., 142 or 145) or two coordinate substitutions (265 + 274). Polyclonal monkey serum selected MARMs containing substitutions at positions 174 and 183 (375). Premature stop codons: certain MARMs, all representing strain-specific MAbs, contained mutations creating termination codons at or after position 257. Hypermutations: mutations were identified in nine different MARMs selected with four different group-specific or conserved MAbs, and those that were present in two or more MARMs are indicated with *asterisks* and identified by sequence position (261, 274, 399). Frame-shifts: MAb 63/G, representing a conserved epitope, selected MARMs containing various frame shifts (not shown, see the text) and bound to a synthetic peptide containing amino

acids 204–209. Synthetic peptides (top): peptide 187–200 induced RSV-specific neutralizing antibodies in rabbits (P.L.C., unpublished data); peptide 144–159 induced RSV-specific neutralizing antibodies in mice but was not protective; peptide 174–187 reacted with RSV-specific polyclonal antisera and MAbs and induced resistance in mice to RSV infection in the absence of detectable RSV-neutralizing serum antibodies (305,391). Peptide 204–209 bound to MAb 63/G, which selected MARMs containing frame-shifts. Note: the amino acid positions of threonine and serine residues depicted here as having O-linked side chains are threonine: 113, 119, 137, 138, 139, 141, 147, 199, 203, 219, 227, 231, 235, 253, and 282; serine: 100, 105, 117, 144, 269, 270, 275, 283, 287, 290.

G is a type II transmembrane glycoprotein with a single hydrophobic region near the N-terminal end (amino acids 38–63 in strain A2) that serves as both an uncleaved signal peptide and a membrane anchor, leaving the C-terminal two thirds of the molecule oriented externally. The G protein also is synthesized as a secreted form that arises from translational initiation at the second AUG in the ORF (amino acid 48), which lies within the signal/anchor (190,348). This truncated species apparently is then trimmed proteolytically, because amino acid sequence analysis of the secreted protein showed that it lacks the N-terminal 65 amino acids of the complete protein, and thus lacks the entire signal/anchor. There also was evidence of a second form lacking the N-terminal 74 amino acids. Secreted G was reported to compose 80% of the G protein released from infected cells 24h post infection. The significance of the secreted form is unclear, although it might function as a decoy to trap RSV-neutralizing antibody, and might influence the immune response (see Antigenic Determinants).

The G protein is heavily glycosylated with N-linked and, especially, O-linked sugars (418) and is palmitylated. Its polypeptide backbone has an M_r of approximately 32 kd. The addition of N-linked sugars increases the M_r to 45 kd. This corresponds to four N-linked side chains, which account for all of the potential acceptor sites in strain A2. The addition of N-linked sugars occurs cotranslationally, and G then assembles into homo-oligomers that probably are trimers or tetramers. The addition of O-linked sugars occurs later in the trans-Golgi compartment or trans-Golgi network, and thus the sugars presumably are added to the exterior surface of the oligomer. Mature G protein migrates in gel electrophoresis as a broad, seemingly heterogeneous band with an M_r of 80 to 90 kd, although less-glycosylated forms of M_r 45 to 80 kd can accumulate in substantial quantity.

The ectodomain of the G protein has a high content of serine and threonine residues (33–35.5%, compared with a data-base average of 13%), which are potential acceptor sites for O-linked sugars. The G ectodomain also has a high content of proline residues (10–13%, compared with a data-base average of 5%), which could contribute to an extended, unfolded secondary structure. These features of amino acid composition, together with the high content of O-linked sugars, also are characteristic of mucins. Mucins are host glycoproteins that form a protective barrier on the respiratory, gastrointestinal, and reproductive tracts.

An engineered F–G chimeric protein expressed by a recombinant baculovirus in insect cells was shown to contain 24 to 25 O-linked side chains of monosaccharides (*N*-acetylgalactosamine) or disaccharides (galactose-b-1-3-*N*-acetylgalactosamine) (410). This probably is a reasonable estimate for the approximate number of side chains in authentic G protein expressed in mammalian cells, although the composition of the side chains in mammalian cells cannot be deduced from the baculovirus material because of differences in carbohydrate processing in insect and mammalian cells. There are more than 70 serine and threonine residues in the G ectodomain, and it is not known which of these are acceptor sites for O-linked sugars. In mucins, O-linked side chains are added to serine or, preferably, threonine residues in regions of exposed and extended secondary structure with a general lack of hydrophobic and bulky residues (180). Figure 5 shows the positions of the 25 sites in the strain A2 G protein that are predicted to be the most likely to contain O-linked sugars.

The extent of G glycosylation varies among different cell lines (134), which probably reflects the existence of a number of *N*-acetyl-galactosaminyl transferases with overlapping but different substrate specificities and tissue distributions. These differences in G-protein glycosylation were shown to be associated with differences in antigenicity. It also is likely that, from molecule to molecule, there is variability in occupancy of O-linked acceptor sites and in the extent of side chain processing. This is suggested by the diffuse appearance of the G protein in gel electrophoresis. The presence of a heterogeneous sheath of host-specified sugars might partially shield this viral antigen from immune recognition.

Among the proteins, G stands out as having a high degree of antigenic and sequence diversity among strains (see Antigenic Subgroups and Diversity, and Table 3). The diversity occurs mainly in the G ectodomain, which has only 44% amino acid identity between the two subgroups, compared with 83% for the transmembrane and cytoplasmic domains. The amino acid sequence positions of potential N-linked and O-linked glycosylation sites also are poorly conserved, although the general locations of the latter are similar. Locations of proline residues are moderately conserved, and the overall hydrophobicity profile is well conserved, suggesting a general conservation of a higher-order organization (214).

Protein	RSV B1[a]	BRSV[b]	PVM[c]	APV[d]
NS1	87	69	16[e]	-[g]
NS2	92	84	19[e]	-[g]
N	96	93	60	41
P	91	81	33	29
M	91	89	41	38
SH	76	38	19[e]	13[f,h]
G	53	30	14[f]	16[f,i]
F	89	81	39	38
M2-1	92	80	41	41
L	93	84	NA[j]	43

[a]Ref. 79, and references therein.
[b]Ref. 227.
[c]Ref. 44.
[d]Ref. 338 and references therein.
[e]Refs. 339, 340, and references therein.
[f]<20% is considered to be insignificant global identity.
[g]APV does not have NS1 and NS2 genes.
[h]The APV SH protein is 2.7 times longer than RSV SH.
[i]The APV and PVM G proteins are ~30% longer than that of RSV and lack the conserved cystine noose (but they are not more highly related to each other, and share only 15% identity).
[j]NA, Not available.

TABLE 3. *Amino acid sequence identity (percentage) between RSV strain A2[a] (subgroup A) and the indicated pneumovirus*

However, one segment of the G protein is highly conserved in human RSV: residues 164 to 176, located midway in the ectodomain. This conserved domain, which is relatively devoid of potential acceptor sites for carbohydrate, overlaps four closely spaced, exactly conserved cysteine residues (positions 173, 176, 182, and 186) and coincides with a tight turn in the predicted secondary structure (214,244). Disulfide linkages occur between Cys^{173} and Cys^{186}, and between Cys^{176} and Cys^{182} (147), resulting in a disulfide-linked tight turn or cystine noose. Several mutant viruses that were selected for resistance to neutralization by certain G-specific monoclonal antibodies (MAbs; Antigenic Determinants) contained substitution of the cysteine residue at position 176, 182 or 186, or at both 182 and 186, showing that these particular cysteine residues are dispensable for virus replication and that either one, or both, of the two disulfide bonds similarly are dispensable (261,274,399). This conserved region is an obvious candidate to be important in attachment activity. In addition, amino acids 184 through 198, a segment that overlaps the cysteine-rich region, was suggested to be a heparin-binding site involved in attachment to cellular glycosaminoglycans (GAGs) (123).

Remarkably, the G protein is not essential for virion assembly and propagation *in vitro* or *in vivo,* although in most cases it enhances viral growth. A biologically derived virus designated B1cp-52, which was recovered after extensive Vero cell passage of the B1 strain of subgroup B, contains a spontaneous deletion of the G and SH genes (227). This virus replicated efficiently in Vero cells, to an intermediate level in cotton rats, and at a very low level in humans. This restricted growth *in vivo* likely is due to the absence of G rather than SH, because deletion of the latter gene alone from recombinant RSV had little effect on replication *in vitro* and *in vivo* (see later) (49,420). Recombinant RSV from which the G gene alone had been deleted (DG virus) has a host-range restriction: it grows efficiently on Vero cells but inefficiently on HEp-2 cells (M. N. Teng and P.L. Collins, unpublished data). One interpretation is that RSV has an alternate, G-independent mechanism of attachment whose efficiency is cell dependent.

Small Hydrophobic SH Protein

SH is a short integral membrane protein that is anchored by a hydrophobic signal-anchor sequence (amino acids 14 to 41) with the C-terminus oriented extracellularly (83). A comparable SH is found in SV5 and mumps virus but not in any other mononegavirus. Intracellularly, SH accumulates in several different forms: SH_0 is an abundant, full-length, nonglycosylated species (M_r 7,500); SH_g (M_r 13,000 to 15,000) contains in addition a single N-linked carbohydrate side chain; SH_p (M_r 21,000 to 60,000 or more) has an N-linked side chain that has been further modified by the addition of polylactosaminoglycan; and SH_t (M_r 4,800) is a nonglycosylated species that is derived from initiation at the second methionyl codon (Met-23) in the ORF. All forms associate into oligomers, which appear to be at least pentamers on the basis of chemical cross-linking (83). SH_0 and SH_p are the predominant forms in virions. This complex processing pathway is conserved among human and bovine strains, but the significance of the multiple forms and the polylactosamine is unknown.

Recombinant RSV from which the SH gene has been deleted (DSH virus) is fully viable in cell culture (49). Indeed, it formed larger plaques and had a modest growth advantage in certain cell lines, which might reflect the shorter genome length or smaller number of genes to transcribe. The DSH virus was fully competent for syncytium formation *in vitro. In vivo,* it was attenuated in the upper, but not the lower, respiratory tract of mice, and was slightly attenuated at both sites in chimpanzees (49,420). Thus, remarkably, the absence of the SH protein has little apparent consequence for infectivity *in vitro* or *in vivo.*

The function of the SH protein remains unknown. The SH protein had essentially no effect on the efficiency of formation and passage of RSV virus-like particles generated in cell culture from plasmid-expressed components (382). Syncytium formation using plasmid-expressed G, F, and SH was the most efficient when all three proteins were present (186), suggesting that SH enhanced the function(s) of one or both of the other two glycoproteins. Expression of the SH protein in bacteria increased cell permeability to small-molecular-weight compounds, an activity that seemed to be more specific than simply a consequence of the protein's being membrane associated (324). This suggested that the SH protein forms membrane channels, perhaps analogous to the M2 protein of influenza A virus. However, it is unclear what role such an activity would play for RSV.

Matrix Protein M

The RSV M protein, a nonglycosylated inner virion protein, is smaller than its paramyxovirus counterparts (256 amino acids vs. 335–375 amino acids) and lacks unambiguous sequence relatedness with these other M proteins. The M proteins do have the common feature of a hydrophobic domain in the C-terminal half of the molecule, which might mediate interaction with membrane.

The M protein of mononegaviruses is thought to have two general functions: rendering the nucleocapsid transcriptionally inactive before packaging, and mediating association of the nucleocapsid with the nascent envelope. In a reconstituted minigenome system (see Genetics and Reverse Genetics), coexpression of the RSV M protein did not inhibit RNA replication or transcription (unpublished data), suggesting that it does not function in this manner. However, M was confirmed to play an important role in the formation of virus-like particles (382).

Nucleocapsid/Polymerase Proteins N, P, M2-1, and L

The RSV N, P and L proteins copurify with nucleocapsids and are present in dense cytoplasmic inclusion bodies that are

thought to contain nucleocapsids (see Fig. 3) (133). RSV N (391 amino acids) is smaller than its paramyxovirus counterparts (489–553 amino acids). Sequence alignments of RSV N with the N-terminal 400 amino acids of the N proteins of other mononegaviruses identified three segments of similar sequence and predicted secondary structure (27). The N protein binds tightly to genomic and antigenomic RNA to form an RNAse-resistant nucleocapsid.

The RSV P protein (241 amino acids) is the major phosphorylated species. It is smaller than its paramyxovirus counterparts (391–602 amino acids). Like other mononegavirus P proteins, RSV P appears to function as a chaperonin for soluble N. This is illustrated by the observations that N expressed alone forms large aggregates and is inactive in encapsidating minigenome RNA, whereas N and P expressed together are sufficient for minigenome encapsidation (18,122). In addition, soluble N and P form stable complexes that can be immunoprecipitated.

Approximately 80% of the phosphorylation of P in RSV-infected cells involves Ser-232, and almost all the remainder involves Ser-116, Ser-117, Ser-119, and Ser-237 (25,356). Ser-232 and Ser-237 are in consensus motifs for cellular caseine kinase 2 and can be phosphorylated by this enzyme *in vitro.* However, this kinase alone did not reproduce exactly the *in vivo* labeling pattern, indicating that other kinases or factors are involved (25). Most of the phosphate of P was not essential for intracellular viral transcription and replication in a minigenome system (396). However, bacterially expressed P protein was inactive for transcription in a reconstituted *in vitro* system unless it was phosphorylated first (25). This illustrates that P also functions as a polymerase cofactor and must be phosphorylated to be functional. Polymerase reconstituted with unphosphorylated P protein produced a series of short oligonucleotide transcripts from the 3' end of the genome (106), suggesting that one of the functions of phosphorylated P is to convert newly initiated polymerase into a stable complex.

The RSV L protein is very similar in size to its rhabdovirus and paramyxovirus counterparts and contains six discrete segments that contain highly conserved residues and presumably represent polymerase motifs (371). RSV L is thought to be a close functional counterpart to the L proteins of paramyxoviruses and other mononegaviruses.

The M2-1 protein is encoded by the 5'-proximal ORF of the M2 mRNA (81). M2-1 was initially thought to be a second matrix protein but is now known to be a transcription antitermination factor that is essential for viral viability (77,80,120,181,182). Consistent with this nucleocapsid function, M2-1 can bind to the N protein, and intracellular M2-1 colocalizes with N and P in the previously mentioned cytoplasmic inclusion bodies (133). M2-1 contains a cysteine–histidine zinc finger motif near its amino-terminus that is essential to its antitermination function (183). Gel electrophoresis of M2-1 from RSV-infected cells revealed at least two electrophoretically distinct species, and the one migrating more slowly was phosphorylated (183).

N, P, and L are the viral proteins necessary and sufficient for RNA replication (80,154,433). N, P, and L also can direct transcription, but full processivity requires in addition the M2-1 protein. On a minigenome mimicking the 3' end of the genome, polymerase in the absence of M2-1 could efficiently transcribe only the NS1 and NS2 genes (120). When the M2-1 protein was coexpressed, transcription of NS1 and NS2 increased severalfold, and transcription of downstream genes occurred. M2-1 also increases the frequency of readthrough at gene-end (GE) transcription termination signals, and hence has a site-specific antitermination activity (120,181,182). Paramyxoviruses do not have a counterpart to M2-1, although the filovirus VP30 might be similar.

Nonstructural Proteins NS1 and NS2

NS1 and NS2 are detected in only trace amounts in preparations of purified virions and hence are considered nonstructural. This assignment is provisional because the RSV virion is difficult to purify and analyze. The NS1 protein has been shown to coprecipitate with the M protein (110), whereas the NS2 protein colocalizes in cells with the N protein (413) but does not coprecipitate with any viral protein (110). The NS2 protein is unstable, with a half-life in infected cells of approximately 30 minutes (110). Both proteins have been reported to form homo-oligomers (110).

In a minireplicon system complemented by plasmid-supplied support proteins, expression of NS1 was strongly inhibitory to transcription and RNA replication (18). NS2 also was somewhat inhibitory to both processes, but only at relatively high levels of expressed protein. This suggested that NS1, at least, might serve some role in RNA synthesis. NS1 and NS2, singly or together, do not appear to have an important role in the assembly or passage of virus-like particles (382).

Deletion of NS1 or NS2 alone from recombinant RSV attenuated virus growth *in vitro* and *in vivo,* and deletion of both together had an additive effect (44,383,384,420). Thus these proteins enhance growth but are not essential. Viruses lacking either or both NS1 and NS2 appear to be somewhat delayed in the accumulation of viral RNA and protein, but their roles in the viral replicative cycle remain to be defined. The V and C accessory proteins of certain paramyxoviruses have recently been shown to be pathogenesis factors *in vivo* (see Chapter 41), and also have been shown to function as antagonists of the interferon system. This raises the possibility that NS1, NS2, or any other of the RSV accessory proteins might have similar roles, or might interact with the host in some other manner. However, interferon effects alone cannot account entirely for the reduced replication of the DNS1, DNS2, or DNS1NS2 viruses *in vitro*, because this phenotype is seen in Vero cells, which lack the interferon a and b structural genes.

M2-2 Regulatory Factor

The M2 mRNA has two ORFs: the M2-1 ORF is 5'-proximal (nucleotides 10–594) in the mRNA, and the M2-2 ORF is downstream (nucleotides 563–835) and slightly overlaps the first (81). The two ORFs encode separate proteins, the 22-kd M2-1 protein and the 11-kd M2-2 protein (3,81). The M2-2 ORF is expressed by a translational termination–reinitiation mechanism. Specifically, ribosomes that complete the translation of the M2-1 ORF can reinitiate at the M2-2 ORF at any one of three (for strain A2) closely spaced AUG codons located slightly upsteam of the M2-1 termination codon (3). The abundance of the M2-2 protein in infected cells is low but increases throughout infection (2). Expression of the M2-2 gene can be ablated in recombinant virus, indicating that it is not an essential gene (36,207). The DM2-2 virus grew more slowly in HEp-2 cells than did wild-type (wt) virus, although it eventually reached a titer that was

reduced only 10-fold. Transcription by the DM2-2 virus was increased, and RNA replication was reduced compared with that of wt virus, indicating that M2-2 has a role in regulating RNA synthesis (see Replicative Cycle, later). When virus-like particles were assembled from plasmid-expressed RNA and protein, coexpression of M2-2 stimulated the production of virus-like particles severalfold (382). It is possible that, by inhibiting transcription, M2-2 depletes the genome of nascent RNAs in preparation for packaging into virions. A comparable function has been ascribed to the matrix M protein in other mononegaviruses. The DM2-2 virus was very attenuated in rodents and chimpanzees, although the virus did replicate and stimulated a strongly protective immune response (207,384).

Viral RNAs and Cis-Acting Elements

Table 2 lists the pneumoviruses for which complete genomic RNA sequences are available. The map of RSV genomic RNA is shown in Fig. 6. Antigenomic RNA, the intermediate in RNA replication, is an exact complementary copy of genomic RNA. Genomic and antigenomic RNAs are neither capped nor polyadenylated, and both are tightly associated with the N protein to form RNAse-resistant nucleocapsids. Most (90%) of genomic RNA is accounted for by the ORFs for the 11 major proteins, and less than 3% constitutes the cis-acting signals described later.

Virus	Genome length	GenBank/ accession no.	Reference
RSV strain A2 (subgroup A)	15,222[a]	M74568	79
RSV strain S2 (subgroup A)	15,150	J138402	366
RSV strain B1 (subgroup B)	15,225	AF013254	337
BRSV strain A51908	15,140[a]	AF092942	44
APV	13,370	Not available[b]	369

[a] The sequence has been confirmed to be correct by recovery of infectious recombinant virus.
[b] A complete assembled APV sequence was not available.

TABLE 2. *Sequences of pneumovirus genomic RNA*

FIG. 6. Respiratory syncytial virus (RSV) genomic RNA (strain A2). **A:** Map of RSV genomic RNA, 3' to 5' (not to scale). The *large boxes* underneath show details of the structures of the gene junctions and 68-nt gene overlap. Nucleotide lengths are indicated. In the gene-start and gene-end signals, in cases of more than one possible nucleotide assignment at a given position, the more frequent is given uppermost. **B:** Sequences of the 3' ends of the genomic and antigenomic RNAs. *Double dots,* nucleotide identity. *Asterisk,* position 4, where substitution of C in place of G increases replication and improves recovery of recombinant virus (79,208).

RSV encodes 10 major subgenomic mRNAs. Each contains a single major ORF except for M2, which contains the M2-1 and M2-2 ORFs, as already mentioned. The RSV mRNAs are capped at the 5' end with the structure $m^7G(5')ppp(5')Gp$ (24) and are polyadenylated at the 3' end. Both modifications are thought to be mediated by the viral polymerase. Transcription also generates readthrough mRNAs because of inefficient termination at the various genes. These readthrough mRNAs are exact copies of two or more adjacent genes and their intervening intergenic regions and account for approximately 10% of total mRNA. Only the upstream ORF of a readthrough mRNA would be expected to be expressed efficiently, given the general inefficiency of eukaryotic ribosomes for initiation at internal ORFs, and so readthough has the effect of reducing the amount of translatable mRNA of each downstream gene represented in the particular readthrough mRNA.

Each gene begins with a 10-nucleotide gene-start (GS) signal 3'-CCCCGUUUA(U/C) that is highly conserved except for the L gene, which has the somewhat divergent signal 3'-CCC**U**GUUU**U**A (differences underlined). These differences do not appear to have any functional significance (240). Synthesis of each mRNA initiates at the first nucleotide of the signal. Each gene terminates with a semiconserved 12- to 13-nucleotide gene-end (GE) signal (see Fig. 6A) that directs polyadenylation and release of the completed mRNA (78,240,241). Studies with model minireplicons and recombinant viruses indicate that the GS and GE signals are independent, circumscribed signals that are largely independent of the nature of the adjacent sequences (47,48,240,241).

The first nine RSV genes are separated by intergenic regions that range in length from one to 56 nucleotides among the available sequenced strains (78,210). The intergenic regions lack consensus sequence motifs or any obvious features of secondary structure and are not well conserved among strains, although regions that are short (such as N/P) or long

(such as F/M2) in the prototype A2 strain tend to be so in other strains. The last two RSV genes, M2 and L, overlap by 68 nucleotides (84,119). Thus the GS signal of the L gene is located inside of, rather than after, the M2 gene. By this arrangement, the 68-nucleotide overlap sequence is transcribed twice, once during synthesis of the M2 mRNA, and a second time during the synthesis of the L mRNA. Overlapping genes in nonsegmented negative-strand viruses have also now been described for filoviruses, Borna disease virus, and certain rhabdoviruses.

The 3' ends of genomic and antigenomic RNA are 81% identical for the first 24 to 26 nucleotides, after which relatedness is insignificant (see Fig. 6B). This appears to represent the major element of a conserved promoter (121). Analysis of the promoter requirements in genomic RNA showed that the sequence required for efficient RNA replication (synthesis of antigenome) was contained within the first 25 nt, whereas transcription required the first 15 nucleotides as well as the GS signal of the first gene (121) (R. Fearns and P. Collins, unpublished data). The spacing of the GS signal relative to the 3' end was important, implying that initiation of transcription involves simultaneous contact between the 3'-terminal sequence and the GS signal (121).

REPLICATIVE CYCLE

Attachment and Entry

Recent studies showed that the RSV G protein binds to certain cellular GAGs, which are long unbranched polysaccharides of the extracellular matrix that consist of repeating disaccharide subunits (123,176). GAGs have been shown to be involved in interactions between cells and various viruses and other pathogens. The RSV G protein appears to bind to GAGs containing the disaccharides heparan sulfate and chondroitin sulfate B. On the one hand, this might occur by electrostatic interaction between the negatively charged GAGs and basic residues in G (123), but other work suggests that a specific GAG component, the monosaccharide iduronic acid, is important, implying a greater specificity for binding (176). It is possible that the interaction between G and cellular GAGs is the attachment step, or it might be an initial contact that is followed by a second binding step involving one or more of the three RSV surface proteins. As mentioned earlier, the biologically derived B1 cp-52 virus that lacks the G and SH genes, or recombinant RSV that lacks the G gene, can grow efficiently in Vero cells and, to a lesser extent, *in vivo.* This implies that that the remaining surface protein, F, can mediate attachment and might represent either an alternative mechanism or a second step. Although G is dispensable *in vitro,* it clearly is important for efficient infection *in vivo.*

RSV enters the cell by fusion at the plasma membrane rather than by endocytosis (369). In cell culture, the initiation of fusion by adsorbed virions appeared to be a slow step but, once started, the process was rapid (19,247). RSV fusion is inhibited below 18°C, and is efficient over a pH range of 5.5 to 8.5 (369).

The RSV F protein was recently shown to bind to the cellular protein RhoA, a small guanosine triphosphatase (GTPase) of the Ras superfamily (321,322). This interaction involves amino acids 146 to 155 in F and amino acids 67 to 110 in RhoA. A peptide containing part of this RhoA domain inhibited RSV replication *in vitro* and in the respiratory tract of mice (322), presumably by saturating the F protein of the virus. These findings suggested that RhoA might function as a receptor for the F protein, although protein-binding studies and inhibition of viral infectivity with a synthetic peptide should be interpreted with caution. Furthermore, although RhoA can associate with the inner face of the plasma membrane, it probably is not exposed extracellularly, and it is unclear how it could function as a viral receptor. Alternatively, an association between F and RhoA might be part of viral penetration. RhoA also might play a role in RSV infection through its signaling function. RhoA controls a wide array of cellular activities, including reorganization of the cytoskeleton and changes in cell morphology, adhesion, contractile responses, proliferation, and gene expression. Thus RhoA has the potential to trigger a variety of cellular responses of potential importance to virus infection, pathogenicity, and immunobiology.

All events in the RSV replicative cycle occur in the cytoplasm without nuclear involvement, and the virus can grow in enucleated cells. RSV infection results in the formation of dense cytoplasmic inclusions that appear to consist of nucleocapsids (see Fig. 3).

RNA Synthesis and Regulation

RSV transcription and replication have general similarities with the simpler prototype mononegaviruses Sendai virus and vesicular stomatitis virus (see Chapter 38 and Chapter 41). Important distinctions are that, as noted earlier, RSV transcription involves the M2-1 antitermination protein, and regulation of RNA synthesis involves the M2-2 protein and possibly the NS1 protein. RSV transcription also requires the cellular proteins actin and profilin (50,51).

UV mapping experiments confirmed that the RSV genes are transcribed by a sequential fashion from the 3' end (102). Studies with minireplicons confirmed that the polymerase does not enter the genome at internal genes (119,241).

As with all mononegaviruses, there is a polar transcription gradient such that promoter-proximal genes are transcribed more frequently than are downstream genes (85). This is the major control of gene expression. The efficiency of transcriptional stop–restart appears to vary modestly among the various RSV gene junctions, providing a second level of regulation of gene expression (181,240). Somewhat unexpectedly, this does not seem to be due to the heterogeneous intergenic regions, because they were functionally indistinguishable in a minigenome system, and deletion of the intergenic region altogether also did not affect the efficiency of transcription stop–restart (239). The conserved nature of the GS signals makes them unlikely to account for gene-specific differences in expression. The GE signals are more diverse and could be responsible for the gene-specific differences. Indeed, the NS1 and NS2 GE signals were shown to be less efficient than the others for termination and production of monocistronic mRNA (240). The basis of the effects of the different gene junctions on termination, readthrough, and reinitiation remains to be characterized fully. Gene length also appears to play a role in gene expression, because the very large L mRNA is much less abundant than the others (85). This difference might occur at the level of synthesis or stability of this large mRNA.

Minigenome experiments suggested that the M2-1 protein can reduce the gradient of transcription in a concentration-dependent fashion, increasing the efficiency of expression of downstream genes. This could occur because of its role in promoting processive transcription, as well as its role in increasing transcription across gene junctions (120,181,182). However, it is not clear whether putative regulation occurs during virus infection.

The overlap between the M2 and L genes mentioned earlier places the M2 GE signal within the L gene, 57 to 68 nucleotides from its start (84). Analysis of mRNAs synthesized during RSV infection showed that, consistent with the function of a GE signal, approximately 90% of the L gene transcripts terminate at this site to produce a short polyadenylated RNA containing the first 68 nucleotides of the L mRNA. The synthesis of full-length L mRNA thus depends on transcriptional readthrough. This illustrates one instance in which this presumed polymerase "error," readthrough at a GE signal, is necessary for the synthesis of an essential mRNA. It remains to be determined whether the efficiency of synthesis of full-length L can be increased by an increased level of M2-1 protein. Mutational analysis of a minireplicon confirmed that the overlapped gene is transcribed by sequential transcription (rather than independent polymerase entry) (119). These studies showed that, to access the L GS signal, the polymerase must first transcribe the M2 gene in its entirety and arrive at the M2 GE signal. It apparently then backtracks to the L GS signal. Indeed, the polymerase was found to be capable of reinitiating at a nearby GS signal inserted either upstream or downstream, indicating that it can scan in either direction. In the naturally occurring overlap, only the GE and GS signals appeared to be essential. The ability of the polymerase to backtrack also explains why L gene transcription is not strongly attenuated by the overlap: prematurely terminated polymerase can recycle back to the L GS signal.

Antigenomic RNA, the positive-sense replicative intermediate, is 10- to 20-fold less abundant intracellularly than genomic RNA. The two RNAs are present in virion preparations in this same proportion, implying a lack of discrimination in packaging. The synthesis of genomic and antigenomic RNAs is dependent on ongoing protein synthesis (200), probably reflecting a need for cosynthetic encapsidation. For paramyxoviruses and rhabdoviruses, cosynthetic encapsidation is thought to mediate a switch from transcription to RNA replication. However, studies with a minigenome system suggest that RSV transcription and RNA replication are not controlled by the intracellular levels of N and P protein (122). Recombinant DM2-2 virus exhibited an increase in transcription and decrease in RNA replication (36). Relative to the level of genomic RNA template, transcription was increased seven- to 18-fold. It was suggested that the intracellular concentration of M2-2 mediates a switch from transcription to RNA replication. When M2-2 is low early in infection, transcription occurs at a high level. As the level of M2-2 increases, transcription is inhibited, and replication is favored. This would be consistent with the requirements for virion formation: the early period of transcription results in high levels of viral mRNAs that are stable and continue to be translated, and the shift to replication provides progeny genome for packaging. Thus RSV is proposed to have temporal regulation of RNA synthesis involving a switch from transcription to replication mediated by a downstream product of transcription.

The expression of certain RSV genes might also be controlled at the level of translation. For example, the SH mRNA is in a suboptimal context for ribosome initiation. In addition, the first AUG codon in the G mRNA initiates a short ORF that overlaps the one for the G protein and might have the role of diverting ribosomes and down-regulating G synthesis.

RSV mRNAs and proteins can first be detected intracellularly by 4 to 6 hours after infection. The synthesis of ORF peaks at approximately 12 to 16 hours after infection and then levels off, whereas synthesis of proteins peaks at approximately 18 to 20 hours. Progeny virus can be detected as early as 8 to 10 hours after infection and reaches a peak after 24 hours. RSV has little effect on cellular macromolecular synthesis: cellular DNA and RNA synthesis diminish beginning after 11 hours after infection and are approximately halved by 18 hours, whereas protein synthesis is little affected (249). The amount of RSV macromolecular synthesis is much less than that of some of the prototype RNA viruses such as vesicular stomatitis virus but nonetheless is considerable. At the peak of protein synthesis, the major viral species can readily be detected by pulse labeling and direct electrophoretic analysis. Under optimal conditions, the more abundant viral proteins can be detected faintly among the electrophoretic pattern of total cell proteins by direct protein staining.

Virion Morphogenesis

Video microscopy showed that RSV virion maturation occurs within circumscribed regions on the cell surface, which might reflect a clustering of virus components or a dependence of budding on localized subcellular structures (19). Budding appeared to be the reverse of penetration, implying a common mechanism of membrane flow. Nascent virions also could be observed on occasion to fuse back with the host cell. Budding into intracellular vesicles also has been reported (16). The efficient replication *in vitro* of the B1cp-52 virus, lacking the G and SH genes, and of the DSH and DG recombinant viruses, indicates that F is the only viral transmembrane envelope protein required for efficient virion formation. Virus budding in polarized cells occurs at the apical surface (347), which *in vivo* might reduce systemic dissemination of the virus from the epithelium of the respiratory tract. Disruption of cellular actin filaments drastically reduced the production of infectious virus, implying a role for the cytoskeleton in assembly (50).

GENETICS AND REVERSE GENETICS

The frequency of isolation of MAb-resistant mutants (MARMs), selected with various MAbs specific to the F protein, was estimated to be 10^{-4} to 10^{-6}, which is similar to that observed with other RNA viruses (31). The actual mutation rate likely is higher, because lethal mutations would not have been recovered. Other information on the stability of RSV *in vitro* was obtained from studies designed to produce live-attenuated vaccines. In one study, 52 passages of strain A2 at progressively lower temperatures resulted in a cold-passaged (cp) RSV that had acquired only five point mutations (128,424). Detailed characterization of six temperature-sensitive (ts) attenuated mutants generated by chemical mutagenesis and biologic selection identified in each a single unique point mutation responsible for the ts and attenuation phenotype. One mutant also contained a single additional amino acid change that did not contribute to the ts or attenuation phenotype, and hence the introduction of incidental mutations into infectious virus was surprisingly rare (86,124,221,222,421,422).

The spontaneous deletion of the SH and G genes during *in vitro* passage, resulting in the B1 cp-52 virus, has already been mentioned (227). Interestingly, there was evidence for multiple independent deletion events in the virus population involving these two genes. Deletion of SH and G during this particular passage series presumably could be accommodated because neither gene is required for efficient growth in Vero cells, as mentioned previously. This propensity to delete G is puzzling because studies with recombinant RSV showed that inserts of foreign sequence are surprisingly stable, as has been found with other mononegaviruses (47,48). Point mutations accumulate in inserted foreign sequence at a low rate over multiple cycles of replication, but there was no evidence of deletion, and functional foreign protein was expressed by most recombinant RSV plaque isolates even after multiple passages.

Other types of unusual mutations also have been observed in RSV subjected to strong selective pressure *in vitro.* For example, multiple point mutations have been described in the NS2 or G genes that apparently were acquired by biased hypermutation (261,383). The finding that strain variation involves a disproportionately high frequency of A to G changes has been suggested to be evidence of hypermutation in nature (263). Frame shifting can occur at several A-rich sequences in the downstream half of the G gene, which accesses an alternative ORF. This has been detected by analysis of cloned cDNAs of viral RNA from infected cells (57), and also was identified in certain MARMs isolated *in vitro* (261) (see Antigenic Determinants). A single natural isolate had suggestive evidence of having sustained a frame shift (376), suggesting that this can occur in nature but is not common. The alternative ORF that is accessed by frame shifting is not thought to encode a functional domain. Other MARMs contained premature stop codons in the downstream end of the G ORF, and this might also occur in nature because certain natural isolates contain small C-terminal deletions (58,376). It is likely that these unusual types of mutations can be found in G because its structure is relatively tolerant of mutation.

Homologous recombination is not known to occur. Thus far, defective-interfering RSV genomes similar to those of other model mononegaviruses such as Sendai and vesicular stomatitis viruses have not been identified by molecular analysis, although there is indirect evidence for their existence (389).

Studies with RSV and mononegaviruses in general have been revolutionized by “reverse genetics,” by which complete infectious recombinant virus can be recovered from cDNA. The production of recombinant RSV requires the intracellular coexpression, from transfected plasmids, of antigenomic RNA and the N, P, L, and M2-1 support proteins (44,77,79,208). The requirement for M2-1 as a fourth support protein distinguishes RSV from the paramyxoviruses (77). This defined for the first time a correct sequence for wt RSV, and provided the means to introduce predetermined changes into infectious virus via the cDNA intermediate.

A second type of reverse genetic system involves minireplicons, which are short cDNA-encoded versions of genomic or antigenomic RNA in which some or all of the viral genes have been deleted and replaced with one or more genes encoding marker proteins such as bacterial chloramphenicol acetyl transferase or luciferase. Minireplicons usually do not contain the genes necessary for their own replication and must be complemented by viral proteins provided by cotransfected plasmids or by coinfecting standard RSV. They are useful because it is easier to perform extensive mutational and biochemical analyses with a short replicon containing only one or a few genes instead of the complete genome. The fact that the minireplicon and support proteins are encoded by separate plasmids also means that each component can be manipulated separately without affecting the synthesis of the others.

ANTIGENIC SUBGROUPS AND DIVERSITY

RSV exists as a single serotype but has two antigenic subgroups, A and B. Antigenic dimorphism among RSV strains was recognized initially by a three- to four-fold reciprocal difference in neutralization between the A2 and 18537 strains, now known to represent the two subgroups (74). Subsequent analysis of strains recovered over the past 30 years using murine MAbs indicated that isolates resembled either the A2 strain (subgroup A) or the 18537 strain (subgroup B) (10,11,192,286). Antigenic dimorphism is most evident with MAbs against the G glycoprotein. The antigenic relatedness between the two subgroups also was investigated by analysis of postinfection polyclonal antibody responses in cotton rats and infants by enzyme-linked immunosorbent assay (ELISA) with purified F or G glycoproteins. This indicated that the two subgroups are 25% related antigenically overall, with the F proteins being 50% related and the G proteins 1% to 7% related (191,213). Subgroup A strains are often thought to be associated with more severe disease (402), but in some studies, subgroup B strains appeared more virulent (198). Subgroup B viruses also replicate less well in mice and chimpanzees. The existence of antigenic heterogeneity might be a factor in the ability of RSV to reinfect (see Epidemiology).

The complete genomic RNA sequences of strains A2 and B1 of subgroups A and B have 81% nucleotide identity. As might be expected, cis-acting signals such as the genomic termini and the GS and GE signals tend to be highly conserved (90–100% nucleotide identity), whereas intergenic and noncoding gene regions are relatively more divergent (32–50% nucleotide identity) (210). The different proteins vary considerably in the extent of sequence divergence between the subgroups (Table 3). Divergence is greatest in the ectodomains of the SH and G proteins, which retain only 50% and 44% sequence identity between subgroups. The global nature of the sequence differences indicates that the two subgroups represent two lines of divergent evolution, rather than being variants that differ only at a few major antigenic sites. Within subgroups, the percentage nucleotide and amino acid identity between viruses is much higher, but otherwise follows the same pattern with regard to regions of relatively greater or lesser divergence (214,386). Within a subgroup, amino acid sequence divergence in the G protein can be as high as 20% between strains (132,376). Distinct lineages within each subgroup have been identified and shown to cocirculate during epidemics and to have broad geographic distribution (56,58,75,132,263).

During the past three decades, the two subgroups have been relatively stable antigenically and have cocirculated in most yearly epidemics studied, with subgroup A usually predominating (75,175,192,284,394). Analysis of 18 subgroup A strains and five subgroup B strains recovered from geographically diverse regions over a period of 30 years using F-specific murine MAbs that defined four antigenic sites with a total of 16 separate neutralization epitopes indicated that

seven epitopes were conserved in all but one of the strains (31). An additional unique epitope that is defined by a human RSV-neutralizing antibody Fab selected from a phage-display library also was conserved in every one of 19 RSV isolates recovered over a 30-year period from diverse locations (99). These observations indicate that subgroup divergence in protective antigenic regions on the F glycoprotein has been neither extensive nor rapid. It did not appear that mutations that confer escape from host immunity accumulated with time. Thus, if the RSV F protein undergoes antigenic drift, it does so on a much longer time scale than is the case for influenza A virus.

The G protein gene also has been examined for evidence of progressive antigenic changes. The G ectodomain is unusual because a high percentage (51–58%) of the nucleotide differences in its coding sequence within or between the two subgroup strains result in amino acid sequence changes, compared with values of 10% to 22% observed for other RSV proteins (212,263). Whereas synonymous nucleotide changes (silent at the amino acid level) in the G gene were evenly distributed, the nonsynonymous ones were clustered in the variable regions of the ectodomain (274). Considering that (a) G is one of the major protective antigens, (b) its amino acid diversity is accompanied by antigenic diversity, and (c) the genetic diversity occurs mainly in the ectodomain, this suggests that selective immune pressure operates in nature. Although immune pressure would be expected to operate similarly against F, the other major protective antigen, it likely is more evident with G because of its apparent tolerance for amino acid substitution. In contrast, the folding, processing, and function of F appear to be much more sensitive to amino acid substitutions (9). Analysis of viral isolates obtained over successive epidemics or over many years provided evidence suggestive of progressive amino acid changes in G that was associated with differences in reactivity with MAbs (58,132). This suggests that the G protein gradually accumulates antigenic differences over time, but the process is neither rapid nor extensive. Interpretation of these data is complicated by the large natural genetic and antigenic variation in the G protein and the complex pattern of G epitopes. It should be remembered that the two subgroups exhibit only a three- or four-fold difference in cross-neutralization, and relatively minor antigenic changes in G might not have a significant effect on cross-protection *in vivo*.

ANIMAL PNEUMOVIRUSES

Bovine RSV (BRSV) is a respiratory pathogen of cattle and is one of the etiologic agents of shipping fever, an economically important livestock disease. RSV and BRSV have an overlapping host range in cultured cells, with the RSV growing better in primate cells and BRSV replicating more efficiently in bovine cells (45). BRSV grows much less efficiently than human RSV in cotton rats, owl monkeys, and chimpanzees (45,325). Antibodies against BRSV or RSV recognize the N, P, M, and F proteins of the heterologous virus (21). In one study, nine of 16 F-specific murine MAbs raised against RSV had significant neutralizing activity against a bovine strain, and pooled human serum with high neutralizing activity against human RSV also efficiently neutralized BRSV (31). Antigenic analyses with MAbs suggest that BRSV has two antigenic subgroups (21,129,336).

BRSV has the same gene order as RSV, and their complete nucleotide sequences share 72.8% identity. Proteins that are relatively more, or less, conserved between subgroups A and B exhibit the same pattern between RSV and BRSV (see Table 3). The 13-amino-acid sequence in the G protein extracellular domain conserved between the two human RSV subgroups is identical for only six positions between RSV and BRSV, although the four cysteine residues and their spacing are conserved (245). BRSV and RSV GS signals and the gene overlap are nearly identical, whereas GE signals and intergenic regions are more variable.

Recombinant BRSV has been recovered from cDNA using a strategy similar to that for human RSV (44). As was the case with human RSV, BRSV NS2 was found to be a nonessential gene whose deletion attenuated the virus *in vitro.* Replacement of the 45-nt leader region of BRSV with the 44-nt leader of human RSV (82% identity) had no effect on replication *in vitro,* providing evidence of the interchangeability of cis-acting signals (44).

Ovine RSV and caprine RSV also have been isolated. The available sequences indicate that caprine RSV is very similar to BRSV, whereas ovine RSV is more distinct; for example, its G protein has 62% and 21% to 29% identity with G of BRSV and human RSV, respectively (4,257). It is thought that ovine RSV and BRSV represent two branches of ruminant RSV, with caprine RSV being grouped with BRSV (4,94).

PVM is a respiratory pathogen whose natural host appears to be the mouse and other rodents. PVM has the same gene order as RSV, although it lacks the gene overlap (64). In addition, its P mRNA contains a second ORF that is expressed as a 137-amino-acid protein and thus represents a twelfth gene not found in the other pneumoviruses (26). The N and P proteins of RSV and PVM cross-react serologically (252). Sequences have been reported for all of the PVM genes except for L (see Table 3).

APV, previously called turkey rhinotracheitis virus, causes respiratory tract disease in turkeys, chickens, and other birds (301). It is the sole representative of genus Metapneumovirus. The genomic RNA of strain CVL14/1 has been sequenced completely (see Table 2 and Table 3). Compared with RSV, APV lacks the NS1 and NS2 genes, the genome is 1.8 kb shorter, and the F-M2 genes precede the SH-G genes in APV, whereas they follow them in RSV (see Fig. 1) (251,339,432). APV has an M2-2 gene, although it might not be expressed (2). If not, APV has eight genes. The percentage amino acid identities for the proteins of APV with their counterparts from human RSV strain A2 are summarized in Table 3. Comparison between PVM and APV is not shown, but in general, these two viruses are as divergent from each other as each is from RSV. Two antigenic subgroups of APV are somewhat more divergent than are the two human RSV subgroups (220). Apart from the presence of the M2-1 gene, the gene map of APV resembles that of the rubulavirus SV5 (see Fig. 1). In addition, its fusion peptide resembles that of SV5 (see Fig. 4C). The significance of this is unclear, but it might mean that APV is an evolutionary link between the rubulaviruses and the pneumoviruses.

BRSV and PVM have the potential to serve as useful models for human RSV. Unfortunately, both grow less well *in vitro* than does human RSV. Unlike RSV, strains of PVM have been identified that differ considerably in virulence, probably because of rapid attenuation of the virus during passage in cell culture (92, 338).

PROPAGATION *IN VITRO*

RSV grows in a fairly wide variety of human and animal cells. Fresh RSV isolates do not appear to require adaptation to cell culture, and conversely, laboratory strains are virulent in chimpanzees and humans. The cell line considered to be the most efficient for virus isolation and propagation is HEp-2, which originally was from an epithelial carcinoma of the larynx but now appears to have been contaminated with and outgrown by HeLa cells, an epithelium-like cell from a cervical adenocarcinoma, which nonetheless is a good substrate for RSV. The A549 line, derived from a type II alveolar epithelial lung carcinoma, also is commonly used. Human RSV grows with reasonable efficiency in bovine cells, and even can be propagated in baby hamster kidney cells, albeit with reduced efficiency. Cell lines can lose their ability to support the growth and cytopathic effect of RSV during serial propagation in culture, necessitating frequent monitoring. Calcium and glutamine in the culture medium are important for replication and cytopathic effect (258,358). The need for calcium probably reflects the calcium dependence of the furin protease.

Up to 90% of infectious progeny RSV in cultured cells remains cell associated (19,247), reflecting either a defect in the final stage of budding or an association with cell-surface molecules as might occur in the absence of a receptor-destroying activity analogous to a neuraminidase. Much of the cell-associated virus can be released by sonication or agitation. Ten plaque-forming units per cell is a typical yield. Virion preparations commonly contain large amounts of cellular membranous material. A large fraction of released virions appear to be empty and therefore presumably noninfectious (20). The particle/plaque-forming unit ratio of two preparations of RSV grown on WI-38 human diploid lung cells was estimated to be $10^{3.5}$:1 to $10^{4.5}$:1 (52), indicative of a very high content of noninfectious particles. Surprisingly, 99% of infectivity of a preparation of BRSV was retained by a 0.45-μm filter, and all infectivity was retained by a 0.22-μm filter (317), suggesting that most of the infectious particles produced *in vitro* are aggregates or filaments. RSV rapidly loses infectivity during storage, handling, and purification: this can be partly overcome by including stabilizing agents such as buffer, sucrose, and magnesium sulfate (155), although the last agent can be toxic to cell culture and animals. Heterogeneity and lability make it difficult to concentrate RSV without considerable loss of infectivity.

Infectious virus is quantitated by plaque assay under methylcellulose or by limiting dilution. RSV plaques develop somewhat slowly, and methods have been developed for their more rapid and efficient detection by immunofluorescence or immunostaining (60,74,388).

INFECTION OF EXPERIMENTAL ANIMALS

A wide range of animal species, including cotton rats, mice, ferrets, guinea pigs, hamsters, marmosets, lambs, and a number of nonhuman primates, can be infected by administration of RSV directly into the respiratory tract (33,53,187,362). The chimpanzee alone resembles the human in being highly susceptible to infection by contact, in supporting moderate to high levels of virus replication, and in developing mild signs of disease symptoms resembling those of mild disease in humans (33), but there are severe limitations on their use because of low availability and high rate of seropositivity. The most widely used small experimental animals are cotton rats and mice. Inbred strains of mice can vary 100-fold in permissiveness for replication. The BALB/c mouse is one of the more permissive strains, but is somewhat less permissive than cotton rats, particularly for subgroup B strains (97). Infection of rodents results in a low to moderate level of virus replication that peaks on day 4 and is quickly cleared. *In situ* hybridization of lung tissue from cotton rats at the peak of virus replication showed that only scattered cells were infected (293). Rodents do not exhibit overt respiratory tract disease, although pulmonary histopathologic changes and respiratory signs that resemble mild bronchiolitis have been noted. Under some conditions, particularly with high-titered RSV inoculum, infected mice exhibit weight loss, changes in pulmonary function, and ruffled fur that are considered to be indicators of disease (53,395). The mouse model has the advantage of a wide array of immunologic reagents and genetically manipulated strains, but both the mouse and the cotton rat share the disadvantage of the uncertainty in extrapolating findings from a semipermissive inbred animal to the human. The evaluation of vaccines or antiviral agents in experimental animals in which RSV replication is semipermissive can yield overly optimistic results.

EPIDEMIOLOGY

Hallmarks of RSV are (a) it is the single most important cause of hospitalization for viral respiratory tract disease in infants and young children worldwide, (b) it infects the very young infant and even the neonate (170) despite the presence of transplacentally derived RSV-specific maternal serum immunoglobulin G (IgG), (c) reinfection is common, albeit with reduced disease and (d) it produces large yearly epidemics. Bronchiolitis or pneumonia occurs most frequently between ages 6 weeks and 9 months, and the peak incidence of lower respiratory tract disease is between ages 2 and 7 months (135,197,320). This corresponds with diminishing titer of maternally derived antibodies. The risk of serious RSV disease is increased by prematurity, young age, chronic cardiac or lung disease, immunodeficiency or immunosuppression, and family history of allergic disease. However, approximately three fourths of hospitalizations for RSV disease occur in infants and children who were previously healthy (404).

RSV is a major cause of serious pediatric respiratory tract disease. In a 13-year surveillance of infants and young children in Washington, DC, RSV infection was detected in 43% of those hospitalized with a diagnosis of bronchiolitis and in 25% of those with pneumonia (Fig. 7, and see Table 4 in Chapter 42) (232,292). Only 11% of those hospitalized with bronchitis and 10% of those with croup had RSV infection. Overall, RSV infection was detected in 23.3% of infants and children hospitalized for any respiratory tract disease, compared with 5.4% of those seen for nonrespiratory illness during this interval. A prospective survey of lower respiratory tract illness in children followed up from birth identified RSV in 39.5% of all episodes (197). In a 10-year study in Rochester, New York, RSV was associated with 50% to 80% of hospitalizations for bronchiolitis and 30% to 60% of those for pneumonia during November through April, the time of RSV epidemics (see later) (158). In the United States, RSV was recently estimated to be responsible for 73,400 to 126,300 hospitalizations annually for bronchiolitis and pneumonia alone among children younger than 1 year (359). These typical findings clearly show that RSV is the major cause of bronchiolitis, and one of the major causes of pneumonia, during the

first few years of life.

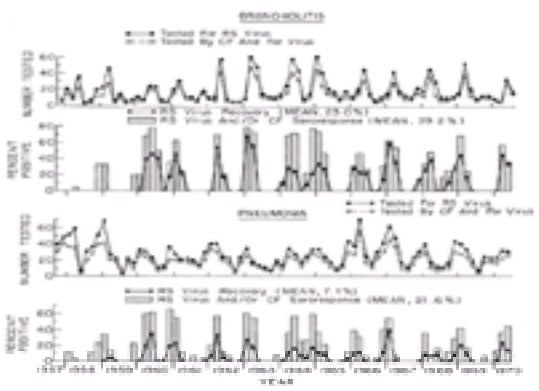

FIG. 7. Documented respiratory syncytial virus (RSV) infection in patients hospitalized with bronchiolitis or pneumonia, by 2-month intervals from October 1957, shortly after the first isolation of RSV from humans, to June 1970 (232). Serum samples were collected on admission and 3 weeks later and assayed for complement-fixing (CF) RSV-specific antibodies. Oropharyngeal swabs were collected and assayed for RSV recovery. The frequency of successful RSV isolation increased beginning in 1960 because of improved cell culture. These methods likely underreport the incidence of RSV infection: the CF antibody assay can be obscured by RSV-specific maternally derived antibodies, and virus isolation can be unsuccessful because of virus instability or insensitivity of the cell culture. Nonetheless, RSV infection was documented in 43% of patients with bronchiolitis and 25% with pneumonia. Note that the timing of the peak of the RSV epidemic can vary, sometimes occurring in the spring (1960 and 1964), and other times with a peak from November to February (1960/1961 winter).

Infection and reinfection with RSV are frequent during the first few years of life. Serologic surveys have shown that approximately one half of infants who are at risk during an RSV epidemic become infected (145,320,364). In certain settings, such as day care centers, the attack rate approaches 100% during epidemics (189). In one prospective study, the infection rate for RSV was 68.8 per 100 during the first year of life and 82.6 per 100 during the second year. All children were infected by age 24 months, and half experienced two infections. The risk of reinfection decreased to 33.3 per 100 by age 48 months (145). In a day care center, 98% of RSV-naive infants and young children were infected when exposed to RSV during an outbreak, and the frequency of reinfection during two subsequent biennial epidemics was 74% and 65%, respectively (189). Reinfection of adults also is common, particularly under conditions in which exposure to virus is heavy, and it is likely that RSV contributes considerably to respiratory tract illness in this age group (298). Several studies have shown that hospital staff on pediatric wards have an infection rate of 25% to 50% during RSV epidemics (151,163,170,297). RSV is introduced into families primarily by a young school-age child, after which infection spreads to older siblings and adults with high frequency. In one prospective study, approximately 40% of all family members older than 1 year were infected after the introduction of RSV (167). Reinfection at all ages is usually symptomatic.

Rates of hospitalization of infants for RSV disease have varied somewhat with the setting, with several developed countries having rates of one in 100 to one in 200 infections during the first year of life (73,232,315). The hospitalization rate was higher (one in 70) for heavily industrialized areas and lower socioeconomic groups (144,145). Hospitalization in middle- and high-income families is much less frequent, occurring in only one in 1,000 infections (143). Overall, the rate of hospitalization for RSV disease in developed countries is 1 to 20 per 1,000 infants younger than 1 year, with 9% of patients requiring mechanical ventilation (144,228,402). Very high rates of hospitalization for RSV can occur in certain populations, such as the rate of 53 to 249 per 1,000 infants younger than 1 year reported for Alaskan natives (228). In the U.S., RSV was estimated to be responsible for annual hospitalization costs of $300,000,000 in 1985 dollars (273).

Mortality due to RSV infection is not common in developed countries. There are no accurate determinations of the overall death rate, but a survey from the mid 1970s estimated the fatality rate at 0.5% to 2.5% of hospitalized children with RSV infection (73). In 1985, RSV was estimated to be responsible for 4,500 deaths annually in the U.S. (273), but this probably is an overestimate (359). With the advent of more effective modern intensive care, more recent estimates are as low as 0.3% to 1.0% of hospitalized children (281,300). Analysis of respiratory deaths in infants and young children reported to the Centers for Disease Control in Atlanta during the years 1975 to 1984 indicated that RSV was the virus most frequently reported in infants of ages 1 to 5 months, and influenza was the one most frequently reported in children of ages 24 to 59 months (12).

Morbidity and mortality from RSV infection is highest in infants with underlying illness. Infants with chronic lung disease (previously called bronchopulmonary dysplasia) are at high risk for prolonged, severe, or fatal illness (152). Mortality in infants with congenital cardiac or pulmonary disease was approximately 40% in the 1970s but has now declined to 3% to 4% of hospitalized patients because of improved supportive treatment (12,126). RSV infection is associated with a mortality rate of 20% to 67% in children and adults with immunodeficiency or immunosuppression. Bone marrow transplant patients are among the most severely immunocompromised and have the highest risk of fatal disease (12,108,287,353,419).

There is growing evidence that RSV is a pathogen of considerable importance in the elderly, whether infection is acquired during an outbreak in a nursing home or in the hospital or sporadically in the community (114,178,278). In one study, RSV accounted for 27% of acute respiratory illness in institutionalized elderly (115), and an overall attack rate of 40% was described (278). In another study, RSV was associated with 10% of hospitalizations of elderly for acute cardiopulmonary or influenza-like disease, with a death rate of 10%, and thus was equivalent in importance to influenza A virus in this setting (114). The clinical manifestations of the two viruses were similar, although the length of stay and

use of antibiotics and therapy for bronchospasm were somewhat greater in the case of RSV. The immunologic correlates of the susceptibility of the elderly to RSV disease are not clear, but do not appear to involve a reduced humoral response to infection (117).

RSV has a worldwide distribution. Wherever appropriate studies have been performed, RSV has been found to be the major pediatric viral respiratory tract pathogen. Serious lower respiratory tract disease occurs 30% more commonly in male than in female subjects (320); in urban centers in the U.S., the various forms of lower respiratory disease associated with RSV infection are seen more frequently in nonblack infants (320). Hospitalization for RSV disease is required more frequently for children from families with lower socioeconomic status (73,142,364).

RSV has a clear seasonality in temperate zones of the world. In urban centers, epidemics occur yearly in the late fall, winter, or spring but not during the summer (see Fig. 7) (232,285,425). In the Northern Hemisphere, the virus is rarely isolated during August or September, although isolation of RSV throughout the year was reported in the southeastern U.S. (177). Each RSV epidemic lasts approximately 5 months, with 40% of infections occurring during the peak month in the temporal center of the outbreak (39). The peak may occur as early as December or as late as June in the Northern Hemisphere. Most outbreaks peak in February or March. It is not know why the virus disappears in the spring, nor from where it reemerges in the fall and winter. In tropical or subtropical areas, epidemics usually occur during the rainy season (59) and have also been reported during gatherings such as for religious festivals (195).

A number of studies indicate that subgroup A and B viruses can alternate in prevalence during successive years (283,323,406). One interpretation is that reinfection with the heterologous subgroup is favored because it can partially evade previously induced immunity. Consistent with this view, a subgroup shift was not evident when the initial infection occurred in children younger than 6 months, a time when immune responses are reduced because of maternal antibodies and immunologic immaturity (406). However, as indicated earlier, the two subgroups differ only three- to four-fold based on neutralization with convalescent serum, which is about the same as that seen between successive antigenic variants within an influenza A virus subtype. Furthermore, many reinfections involve virus of the same subgroup as the previous infection (283). Thus antigenic dimorphism probably contributes to the high incidence of initial reinfection during early childhood, but its effect appears to be modest.

RSV is spread by infected respiratory secretions. The major mode of spread appears to be by large droplets or through fomite contamination rather than through droplet nuclei or small-particle aerosols (159,162). Spread requires either close contact with infected individuals or contamination of the hands with fomites from surfaces to which infected respiratory secretions have been introduced by mechanical means and subsequent contact between fingers and nasal or conjunctival mucosa (self-inoculation). When adult volunteers were inoculated with cell culture–grown virus, infection was easily accomplished if virus was introduced either onto the conjunctival sac or into the nose but not into the mouth (164).

RSV is a major cause of nosocomial infection (151,163). The rate of hospital-acquired infection for infants and children during an RSV season was reported to range from 26% to 47% in newborn units and from 20% to 40% for older children. Hospital staff appear to play a major role in nosocomial spread of RSV infection. The likelihood that an individual infant or child will acquire nosocomial RSV infection increases with the duration of stay and the number of individuals housed in his or her room (163).

Attempts to control such outbreaks through the use of gowns and masks have been unsuccessful. However, recent studies suggest that nosocomial spread in nurseries and pediatric wards can be reduced by a combination of measures: (a) limitation of visitors, (b) active surveillance for RSV infection among patients and new admissions, (c) cohorting of RSV-infected patients and their caregivers, and (d) institution of various barriers to virus spread. Precautions that appear to be effective include strict observance of hand washing and the use of gloves and gowns, with some possible additional protection when eye–nose goggles are worn. These measures are thought to reduce passive transfer of RSV-containing respiratory secretions to patients and also are thought to protect the staff from becoming infected by self-inoculation onto the conjunctiva or nasal mucosa (131,151,255,297).

CLINICAL FEATURES

In the normal infant who encounters RSV for the first time at age 6 weeks to 9 months, RSV infection usually causes upper respiratory symptoms. In 25% to 40% of such infections, however, the respiratory tract below the larynx also becomes involved (145,225). Bronchiolitis and pneumonia are the primary manifestations of lower respiratory tract disease, and probably represent a continuum of increasing severity of disease. Asymptomatic infection in this young age group is probably uncommon. In those infants in whom lower respiratory tract signs develop, there is a prodromal phase of rhinorrhea sometimes accompanied by a decrease in appetite. Cough may appear simultaneously, but it occurs more often after an interval of 1 to 3 days. At that time, there also may be sneezing and a low-grade fever. Soon after the cough has developed, the child may begin to wheeze; if the disease is mild, the symptoms may not progress beyond this stage. Examination usually shows moderate tachypnea, diffuse rhonchi, fine rales, and wheezes. There is profuse rhinorrhea, intermittent fever, and frequently otitis media (185). At this stage the chest radiograph is usually normal. In most instances, uneventful recovery occurs after an illness of 7 to 12 days.

In more severe cases, the coughing and wheezing progress, and the child becomes dyspneic. Hyperexpansion of the chest is evident, and there may be intercostal and subcostal retractions. The child refuses feedings. Severe tachypnea is common, even in the absence of visible cyanosis; and in advanced disease, as the child tires and hypoxia becomes more extreme, listlessness and respiratory failure occur. The chest radiograph often shows a combination of air trapping (hyperexpansion) and peribronchial thickening or interstitial pneumonia. Segmental or lobar consolidation, usually involving the right upper lobe, also is occasionally seen (343). Pleural effusion is rare. Late bacterial superinfection is unusual (173) and is probably less frequent than simultaneous bacterial–viral coinfection (318,385). Severe lower respiratory tract illness is most common in patients younger than 9 months. Bronchiolitis may occur in older infants and children, but beyond the age of 18 months, it is usually seen in children with recurrent wheezing who carry the diagnosis

of asthma.

Many infants who require hospitalization are hypoxemic on admission (169) and remain so for a prolonged period (up to several weeks), even though recovery has ensued. This hypoxemia is probably due to an abnormally low ventilation/perfusion ratio. Hypercarbia has been observed in some studies but not in others (169).

In infants with underlying cardiac or respiratory disease, the progression of symptoms may be rapid. This is particularly true of those with cyanotic congenital heart disease or chronic lung disease (152,272). In these instances, respiratory failure requiring intubation and ventilation may develop on the second or third day of illness. In children younger than 2 years with cystic fibrosis, the consequence of RSV infection may be prolonged respiratory morbidity (193).

In the newborn infant, most RSV infections produce only upper respiratory symptoms (41,170), reflecting the protective effects of maternal RSV-specific serum antibodies against disease in the lower respiratory tract. Bronchiolitis is rare, and severe infection is more often characterized by lethargy, irritability, and fever or temperature instability than by specific respiratory signs (170). In infants who were born prematurely, and sometimes in normal infants younger than 6 weeks, apneic spells may occur in response to RSV infection (271). This is often seen even in the presence of minimal respiratory signs and may be the predominant symptom bringing the infant to medical attention. Such apneic spells, although often recurrent during the acute infection, are usually self-limited and rarely cause neurologic or systemic damage. Exceptions to this occur, however, and such episodes should be an indication for hospitalization and careful medical supervision.

RSV has been detected in the lungs of a percentage of children dying suddenly and unexpectedly (sudden infant death syndrome, SIDS). However, there is no statistically significant association between RSV and SIDS, and the detection of RSV in some SIDS cases likely reflects its prevalence as well as the peak incidence of both RSV infection and SIDS in winter months (100).

Abnormalities in pulmonary function appear to be common after RSV bronchiolitis or pneumonia (or disease due to other respiratory viruses) and may persist for 7 to 10 years or even into adulthood (63,218,229,259,265). One investigation of 18 infants who had been hospitalized for bronchiolitis (half of which was due to RSV) identified only one individual with normal pulmonary function 1 year later (373). Ten had increased airway resistance, two had increased thoracic gas volumes, and five had both abnormalities. Another study of 23 children 10 years after bronchiolitis found that although all were symptom free (a criterion for admission to the study), 20 had some measurable physiologic abnormality of lung function or arterial blood gases (230). Recurrent wheezing may be common after RSV bronchiolitis or pneumonia and has been reported in 10% to 50% of former patients (63,337,363), but it is rarely of great severity. A large case–control study of 200 children hospitalized for bronchiolitis or pneumonia (half of which was due to RSV) showed that 7 years later, there was a significant tendency toward decreased pulmonary function, recurrent cough, wheezing, school absenteeism, asthma, and bronchitis in both of the previously hospitalized groups (279). Postexercise or pharmacologically induced bronchial lability is also increased, despite an absence of symptoms (156,363); furthermore, long-term hypoxia has been identified in several studies (168,230). The pulmonary function deficit is partially responsive to bronchodilators, indicating a bronchospastic component (63).

It remains unclear whether infection with RSV (or other respiratory viruses) causes subsequent abnormal pulmonary function, or whether the abnormalities observed existed before infection and indeed contributed to disease. Both possibilities might occur. A cause-and-effect relationship between RSV infection and subsequent long-term pulmonary abnormalities has not been demonstrated. The development of an effective RSV vaccine or highly effective antiviral agent for general use would help resolve this question. One study implicated RSV infection as a risk factor in the development of asthma (361), but other studies suggest that viral infections are not a major factor in the development of allergic airway disease (259,372,431). There is increasing support for the view that severe RSV infection with subsequent chronic or recurrent respiratory tract sequelae can be due to preexisting deficiencies. For example, Martinez et al. (260) measured pulmonary function in infants at birth or shortly thereafter and found a strong correlation between prior reduced function and the development of bronchiolitis with wheezing on RSV infection. This correlation persisted in children in whom at least one further lower respiratory infection developed during the first 3 years of life (260). A comparable independent study yielded similar conclusions (431). In another study, elevated peripheral blood eosinophil counts during RSV bronchiolitis were generally predictive of wheezing at age 7 years, implying the contribution of a preexisting immunologic anomaly (107). It is likely that respiratory disease and recurrent abnormalities in children are due to multiple factors including prior underlying anatomic or functional abnormality that might predispose to initial and recurrent acute bronchiolitis; a tendency to asthma or airway hyperreactivity that might predispose to recurrent wheezing; and infection, by RSV or other airway pathogens, as well as other environmental factors such as family smoking, that might trigger episodes of lower respiratory dysfunction and aggravate long-term or recurrent airway pathology. Most pulmonary abnormalities associated with prior respiratory tract infection resolve by age 8 to 12 years.

Acute RSV infections are common in normal adults, particularly in medical personnel or in individuals caring for small children (163,167). Such episodes are usually characterized by rhinorrhea, pharyngitis, cough, bronchitis, constitutional symptoms of headache and fatigue, and fever. Disease usually lasts about 5 days but may be more prolonged. In older persons, particularly those with underlying disease, severe pneumonia may occur, leading to hospitalization and the development of adult respiratory distress syndrome (ARDS), requiring intubation and ventilation.

RSV infection in severely immunocompromised individuals usually starts with an upper respiratory tract infection for 5 or 6 days, with symptoms similar to those of a normal host. There is a higher incidence of lower respiratory tract disease, and infection progresses to pneumonia in more than 50% of subjects, with patients manifesting sinusitis, cough, wheezing and dyspnea, and giant cell pneumonitis (416,419). The mortality rate associated with RSV pneumonia in adults with profound immunosuppression due to leukemia or bone marrow transplant can be as high as 80% to 100% (419).

DIAGNOSIS

Differential Diagnosis

The differential diagnosis of RSV infection in infants younger than 6 months must include all other causes of acute lower respiratory tract illness in this age group. This includes parainfluenza viruses, particularly parainfluenza virus type 3, adenoviruses, influenza viruses, rhinoviruses, and enteroviruses. In infants younger than 4 months, this also includes *Chlamydia trachomatis* (32). *Chlamydia trachomatis* causes interstitial pneumonia with cough and, in some cases, wheezing, although fever is usually absent in this syndrome. In immunodeficient children, *Pneumocystis carinii* infection must be considered. Differentiation from bacterial pneumonia may at times be difficult, although wheezing is usually not present, and infants with bacterial pneumonia may also have systemic illness associated with bacteremia.

In the newborn, other causes of listlessness, lethargy, and apnea include bacterial infections and metabolic diseases. In older infants, infections with RSV must be differentiated from other causes of mild respiratory tract infection as well as causes of acute bronchospastic disease, including inhalation of environmental allergens and aspiration of a foreign body.

In adults, RSV infection should be suspected in any older patient (with or without underlying cardiopulmonary disease) who has acute bronchitis or pneumonia accompanied by low-grade fever, particularly when such an event occurs as part of a cluster of cases in the winter season.

Presumptive diagnosis of RSV infection in infants can often be made on the basis of the clinical syndrome combined with the time of year and other epidemiologic features. It is common to observe that many members of the family have respiratory illness at the time of, or just preceding, an episode of RSV bronchiolitis or pneumonia in an infant. Usually the infant has far more serious illness than other members of the family (167).

Laboratory Diagnosis

Definitive diagnosis depends on the laboratory. The gold standard is isolation of virus in cell culture. Specimens are best obtained by aspiration or gentle washing out of nasal or nasopharyngeal secretions or by nasal swab (22,266). The lability of the virus requires that samples be kept cold without freeze–thawing and be inoculated as quickly as possible into cultured cells that have been confirmed to be sensitive to RSV. The development of RSV cytopathology requires 3 to 7 days or more, but detection can be made more rapid and efficient by immunofluorescence or immunostaining and the use of shell vial cultures (60,219,388,407).

Early diagnosis is advantageous because it allows implementation of measures to limit spread, such as in a hospital setting or nursing home, and early implementation of antiviral therapy, which should increase its effectiveness. Rapid methods involve the detection of viral antigen or nucleic acid in secretions collected as described earlier (219,407). In the direct immunofluorescent assay (DFA), exfoliated cells are reacted with fluorescent RSV-specific antibody and examined by microscopy. In the enzyme immunoassay (EIA), the sample containing respiratory secretions is incubated briefly with RSV-specific antibodies that are immobilized on plastic, and antigen thus captured is detected by reaction with a second, enzyme-linked RSV-specific antibody. The two methods are similar in sensitivity, but DFA has the advantage that the pattern of immunofluorescence can be evaluated, providing additional confirmation of specificity. DFA also requires specialized equipment (fluorescent microscope) and greater expertise. More recently, assays based on reverse transcription and polymerase chain reaction are commercially available. These can equal or exceed the sensitivity of cell-culture or antigen-based assays, and the inclusion of appropriate additional primers allows simultaneous assay for multiple pathogens (109,118,378).

Serologic methods are used less frequently. In older children and adults, an increase in complement-fixing (CF), neutralizing, or ELISA serum antibodies is a fairly sensitive index of reinfection with RSV. Asymptomatic infections are usually undetectable serologically. Serologic tests in infants are less sensitive, particularly in those younger than 4 months, with ELISA being the most sensitive assay (344). Serologic assays have been improved using ELISA with purified viral proteins or synthetic peptides (242,429), permitting the sensitive identification of immunoglobulin isotype or subclasses of RSV-specific antibodies.

PATHOLOGY AND PATHOGENESIS

The incubation period from time of infection to onset of illness for RSV is 4 to 5 days (209,225). Virus replication initiates in the nasopharynx, commonly reaching a titer of 10^4 to 10^6 $TCID_{50}$ (50% tissue culture infectious dose) per milliliter of nasal secretion in young infants (160,161) and a somewhat lower titer in experimentally infected adult volunteers (128,276). In infants hospitalized with RSV disease, virus titer usually is at a level of $10^{3.5}$ to 10^5 $TCID_{50}$ per milliliter and decreases gradually during hospitalization (161,163,171,270,407). Some infants continue to shed virus for as long as 3 weeks after initial hospitalization. In one series of studies, clinical recovery often occurred despite the persistence of viral shedding from the upper respiratory tract (160,161), although in other studies, clinical recovery was usually coincident with the end of virus shedding (269,270). It is generally thought that there is a positive correlation between the level of virus replication and the severity of disease.

RSV replicates primarily in the superficial layer of the respiratory epithelium (136,302). It also infects macrophages and monocytes and appears to interfere with some of their functions (105). Viral spread from the upper to the lower respiratory tract likely involves aspiration of secretions or spread via the respiratory epithelium. The virus also can spread from cell to cell by fusion without emergence into the extracellular fluid, although the importance of this mechanism *in vivo* is unclear. Lower tract signs usually appear 1 to 3 days after the onset of rhinorrhea, and it is assumed that this reflects the timing of viral spread into the bronchi and bronchioles. The titer of virus in the lower respiratory tract is not known, but based on studies in chimpanzees, it likely is similar to that in the upper respiratory

tract.

Although RSV is pneumotropic, it is not strictly limited to that site. In several cases of fatal RSV infection of severely immunocompromised infants or adults, virus did spread to other organs, including kidney, liver, and myocardium, although this spread was not thought to be the cause of death (125,277). Similar findings have been made in immunosuppressed experimental animals (215,426). Although viremia has not been described during infection of normal infants and children, RSV antigens have been detected in circulating mononuclear leukocytes of such individuals (105), and viral genomic RNA and mRNA have been detected in cells from arterial blood, but not in cerebrospinal fluid (306).

The pathology of severe RSV infection in infants is similar to that of bronchiolitis and pneumonia resulting from other respiratory viruses. During bronchiolitis, there is necrosis and proliferation of the bronchiolar epithelium and destruction of ciliated epithelial cells (1,302). A peribronchiolar infiltrate of lymphocytes, plasma cells, and macrophages develops, with migration of the lymphocytes among the mucosal epithelial cells. Submucosal and adventitial tissues become edematous, and secretion of mucus is excessive, which combines with cell debris and inflammatory cells to obstruct the bronchioles and alveoli, causing either collapse or emphysema of distal portions of the airway. In those instances in which pneumonia occurs, the interalveolar walls thicken as a result of mononuclear cell infiltration, and the alveolar spaces may fill with fluid. There is usually a patchy appearance of these pathologic changes, even though disease may be widespread. Immunostaining identified virus-infected cells in the bronchial, bronchiolar, and alveolar epithelium, and identified numerous syncytial cells (302). RSV antigen can be relatively abundant in lower respiratory tract disease, although the staining is usually focal, and in some cases of fatal RSV bronchiolitis, antigen was present only in small amounts (136,302). There is little information about the microscopic pathology of milder cases of bronchiolitis or pneumonia resulting from RSV infection. It is assumed that, in most instances, infection is predominantly in the small bronchioles, with inflammation of the peribronchiolar tissues and, to some extent, the interstitium.

The relative lack of direct information on the development of pathology in the infected human infant has been supplemented by studies of experimental or natural infection of calves and lambs with BRSV (42,43,264,427). Experimental administration of BRSV usually involved a large inoculum and sometimes multiple doses. Under these conditions, significant disease is observed by 3 to 7 days in all animals. At necropsy, gross lesions were observed with consolidated and emphysematous areas involving up to 70% of the affected lobe or complete lung (264,427). In the bronchiolar epithelium, the virus replicated in both ciliated and nonciliated cells, with syncytium formation, necrosis, desquamation, and considerable ciliary damage. Loss of ciliated and nonciliated cells and the degeneration of the epithelium also were observed in the nose, trachea, and main bronchi. In the alveolar epithelium, both type I and type II pneumocytes were infected, with the latter seeming to be a more important target. Lymphocytes and neutrophils accumulated in the subepithelial zone, and bronchiolar and alveolar lumina were filled with an exudate containing mucus, desquamated epithelial cells, neutrophils, lymphocytes, and macrophages. Necrosis, syncytium formation, hyperplasia, and hypertrophy also led to considerable occlusion of bronchiolar and alveolar lumina. Neutrophils were frequently found in association with infected cells, suggesting that they are important in the early inflammatory response. Virus-induced lesions resolved by 10 to 30 days after infection.

Direct virus-mediated cytopathology is an important component of RSV pathogenesis. RSV infection results in considerable damage to the epithelium and, importantly, to the bronchiolar ciliary apparatus (390). This interferes with the removal of mucus and cell debris, facilitating the collection of exudate in the bronchioles and alveoli. An important factor contributing to the frequency of severe lower respiratory tract disease in the young infant is the small diameter of the bronchioles, which obstruct easily in the face of necrosis and edema (196). As described later, it also is likely that host immunity contributes to RSV disease. However, RSV is an inherently pathogenic virus, and severe RSV disease is not dependent on the adaptive immune response. For example, RSV replicates to an increased level and causes severe pulmonary pathology in immunosuppressed cotton rats (426). Severely immunocompromised bone marrow transplant recipients also are at high risk for fatal RSV disease.

The mechanism of RSV-mediated cell death is not known and probably involves an accumulation of damage resulting from viral infection, with one possible example being perturbation of the cell membranes due to the expression of the highly fusogenic F protein. RSV infection of A549 cells *in vitro* induced the transcription factor IRF-1, which controls an apoptotic pathway (377). However, only a modest increase in apoptosis was observed in one study (306), and not at all in another study (377). As mentioned previously, RSV does not appear to interfere specifically with host-cell macromolecular synthesis.

The temporal concurrence of maternally derived, RSV-specific serum antibodies in young infants and the peak incidence of severe RSV disease initially suggested that RSV antibodies might mediate an immunopathologic reaction (268). For example, antigen–antibody complexes have the potential to induce injury by triggering the classic or alternate complement pathways or by activating granulocytes (113). Possible effects due to IgE are discussed later. However, although some contribution to pathology cannot be ruled out, several lines of evidence indicate that by far the major effect of RSV-specific antibodies is protective rather than pathogenic: (a) there is a relative sparing of serious RSV disease during the first 5 to 6 weeks of life, when maternally derived antibody titers are highest; (b) some prematurely born infants develop bronchiolitis in the absence of measurable acute-phase RSV-neutralizing antibodies; (c) there is an association between low umbilical cord blood RSV antibody titer and serious RSV disease; and (d) parenteral administration of RSV-neutralizing antibodies to experimental animals and, more recently, to high-risk infants provides protection rather than enhancement of RSV infection or disease.

It has been suggested that RSV-specific IgE is involved in the pathogenesis of RSV disease. In some studies, the level of free IgE in secretions from infants during convalescence from RSV infection was higher in individuals with asthma and wheezing and was correlated with the severity of hypoxia during acute disease (46,415). Moreover, increased levels of histamine and leukotrienes were detected in secretions of patients with bronchiolitis or RSV-induced asthma but not in those of patients with either RSV pneumonia or upper respiratory illness (397). These soluble factors are mediators of bronchoconstriction and presumably were released during the interaction of IgE, RSV, and mast cells in the respiratory epithelium. However, other studies did not find convincing evidence of increased IgE expression or mast cell

degranulation (111,387), and thus a general role for IgE in RSV pathogenesis remains uncertain.

The inflammatory response that is initiated by RSV infection (see Immunity) contributes to some pathologic manifestations of RSV disease. For example, granulocytes that are recruited into RSV-infected airways by proinflammatory chemokines can mediate direct destruction of virus-infected cells (405), which helps resolve infection but also damages tissue. Granulocytes also release degradative enzymes and, in the case of eosinophils and basophils, soluble effectors such as leukotrienes. Analysis of bronchial lavage and nasopharyngeal secretions of infants hospitalized with RSV bronchiolitis identified neutrophils as the predominant leukocyte (76% in bronchial lavage and 93% in nasopharyngeal secretions), with lymphocytes (9% and 5%) and monocytes (10% and 2%) being less abundant, and eosinophils being detected only in bronchial lavage (1%) (112). It should be noted that this method samples cells in the lumen and not in the entire lung and that prevalence of a specific cell type does not necessarily indicate its contribution to pathogenesis. Another study demonstrated elevated levels of eosinophil cationic protein, a cytotoxic protein found in eosinophil granules, in nasopharyngeal secretions of infants with RSV bronchiolitis (137). The local inflammatory response likely contributes to edema in the RSV-infected lung.

Studies in mice provided evidence that RSV-specific $CD8^+$ major histocompatibility complex (MHC) class I–restricted, $CD8^+$ cytotoxic T cells (CTLs) may play a role in the pathogenesis of RSV disease. Passively transferred, *in vitro*–stimulated RSV-specific $CD8^+$ CTLs can clear virus from the lungs of persistently infected, gamma-irradiated mice (61). However, under these experimental conditions, acute pulmonary damage occurs, and affected mice often die. Similarly, $CD8^+$ CTLs or CTL clones accelerate clearance of RSV from the lungs of infected immunocompetent mice, but clearance is accompanied by acute, and sometimes fatal, pulmonary disease. In mice treated from birth with anti-mu antibody to deplete B lymphocytes, the cellular immune response mediated both viral clearance and disease (148). Studies using mice in which $CD4^+$ or $CD8^+$ cells were selectively immunodepleted indicated that both subsets contributed to recovery as well as to a pathologic reaction, but that $CD8^+$ cells were more potent in both functions (149). These observations suggest that a balance exists between the protective and disease-producing effects of $CD8^+$ CTLs.

Another factor suggested to play a role in RSV pathogenesis is the level of stimulation of the Th1 and Th2 subsets of $CD4^+$ T lymphocytes, which are distinguished by increased expression of interferon-g versus interleukin 4 (IL-4) and IL-5, respectively. Immunization of BALB/c mice with a vaccinia virus recombinant expressing the G protein, or with formalin-treated RSV, induces a $CD4^+$ T-cell response with an increased Th2 component (5,150). Subsequent challenge with RSV results in enhanced pulmonary histopathology and eosinophilia, a response that could be ablated by depletion of either $CD4^+$ T cells or the Th2 cytokines IL-4 and IL-10 before challenge (89,91). A Th2-biased $CD4^+$ T-cell response might augment disease by chemotaxis and activation of leukocytes such as eosinophils, as suggested by the eosinophilia observed in the BALB/c mouse (357), or by other mechanisms such as IL-4–mediated class switching to IgE production and enhanced airway mucus production (76). These findings have clear relevance to situations in which G is expressed alone, such as in a subunit vaccine, and this paradigm also is relevant to the enhanced RSV disease observed with a formalin-inactivated RSV vaccine that was evaluated in the 1960s (see Vaccines later). However, it probably is less relevant in a natural infection in which Th2 $CD4^+$ T cells appear to be down-regulated by natural killer (NK) cells and $CD8^+$ T cells (201,203,367) Another weakness of this paradigm is that “disease” in this mouse model depends on a prior sensitization step that does not have a counterpart in the natural RSV disease. On the contrary, natural RSV disease is most severe in RSV-naive (unsensitized) infants and is rapid rather than delayed in its onset, and reinfection is associated with reduced rather than enhanced disease. However, some features of the immature immune system are intriguing with regard to a possible role of Th2 $CD4^+$ T cells in RSV pathogenesis. For example, the $CD4^+$ T-cell response in neonates and infants is transiently biased toward a Th2 response, and the young infant has a reduced CTL response (71,342). RSV-specific peripheral blood mononuclear cells from infants hospitalized with RSV disease exhibited a Th2-biased recall response (352), whereas cells from somewhat older children and adults secreted predominantly Th1 cytokines (13). Whether this is significant for RSV disease and immunology remains to be determined.

Mice with a targeted disruption of surfactant A, a collectin, were shown to have a reduced ability to restrict RSV, which could be restored by exogenous surfactant (246). Collectins are sugar-binding proteins secreted by type II epithelial cells of the respiratory tract that opsonize virus and facilitate phagocytosis by macrophages. Surfactant A appears to act by specifically binding to the F protein (141). Infants with severe RSV disease were found to have a reduction in surfactant concentration and function, although whether this is an effect or a cause (a predisposing host factor) of serious disease is not known (101,231).

The genetic background of the host is likely to be an important determinant in pathogenesis. This is evident from the differences in RSV replication and extent of pulmonary pathology exhibited by different strains of inbred mice (202,368). It is reasonable to suppose that, in the young infant, numerous factors ranging from surfactant expression to predisposition to reactive airway disease help determine whether an infection progresses to serious disease.

ANTIGENIC DETERMINANTS

The F and G glycoproteins are the major protective antigens of RSV. Immunization with either or both proteins, whether by RSV infection, a recombinant vector, or purified protein, confers long-lasting (although frequently incomplete) resistance to reinfection and lower respiratory tract disease that is mediated by neutralizing antibodies.

B Lymphocytes

Postinfection sera of humans or experimental animals contain antibodies against the various virion structural proteins. However, only antibodies against the F or G glycoprotein neutralize infectivity *in vitro* and confer resistance to RSV infection when transferred passively to experimental animals (379,403). Studies in which rodents were immunized with a series of recombinant vaccinia viruses, each of which expressed an individual RSV protein, confirmed that only the F and G proteins induce a significant RSV-neutralizing antibody response as well as a long-lived protective immune response

(87).

The antigenicity and immunogenicity of the F protein is highly dependent on its folded structure (254). A large fraction of F-specific MAbs efficiently neutralize infectivity, many inhibit fusion, and many are reactive with both antigenic subgroups and with BRSV. Competitive binding between MAbs identified at least four major antigenic sites on the F protein, and epitopes within these sites were defined by analysis of naturally occurring antigenic variants and of MARMs. Sequence analysis of MARMs showed that escape from neutralization by each of the various MAbs involved one or two amino acid substitution(s) each (see Fig. 4A) (15,31,99). Each position thus identified either constitutes part of the respective epitope or is important to its structure. All of the changes lie within the F_1 subunit, and none involves the fusion peptide directly, likely because this hydrophobic sequence usually is not accessible. In some cases, the view that the identified residue is part of the epitope was confirmed by reactivity of the MAb with a corresponding synthetic peptide, providing a second method for antigenic mapping (15,93,253,254,392). Certain F-specific peptides also have been shown to induce neutralizing antibodies in experimental animals (93,392). However, it is likely that most of the neutralization epitopes on F are conformational in nature and cannot be mimicked efficiently by synthetic segments. The repertoire of F-specific antibodies induced by RSV infection in humans has been assayed by recombinant cDNA cloning of lgG Fab fragments, expression, and phage display. Approximately half of the Recombinant antibodies that neutralized infectivity efficiently were specific for fully mature F protein on the cell surface and virions. The other half of the antibodies had low neutralizing activity and appeared to be specific for intracellular F protein, and thus were probably raised against immature F protein released from lysed cells (354).

In contrast to F, MAbs against the G protein react with denatured protein on Western blot, and thus reactivity is not highly dependent on a properly folded structure (261,274). Individual MAbs against G generally are not efficient in neutralizing infectivity, although neutralization can be achieved with mixtures of MAbs (262) or with G-specific polyclonal antiserum (213). Because of the considerable antigenic diversity of G, it is more useful to describe G-specific MAbs on the basis of reactivity with natural strains. The MAbs fall into three groups: (a) a small number react with viruses from both antigenic subgroups and thus recognize “conserved” epitopes; (b) a larger number react broadly within but not between subgroups and are “group specific”; and (c) a yet larger number react with some strains but not others within a subgroup and are “strain specific” (261). The conserved and group-specific MAbs often react with nonglycosylated G, whereas strain-specific MAbs often react only with glycosylated G (261).

Sequence analysis of MARMs selected with G-specific MAbs revealed an interesting variety of mutations, summarized in Fig. 5 (261,274). Some of the MARMs selected with strain-specific MAbs each contained one or two closely spaced amino acid substitutions located mostly after amino acid 232. Others contained premature stop codons that truncated the protein by up to 42 amino acids. Thus epitopes for the strain-specific MAbs mapped to residues near the C-terminus of the molecule. Synthetic peptides containing sequence from the C-terminal 84 amino acids reacted with convalescent human sera from primary infection and, interestingly, altering the peptides to include point mutations found in nature reduced antigenicity (55). However, vector-expressed G protein lacking the C-terminal 68 amino acids was as immunogenic and protective as the complete G, suggesting that this region is not needed for a protective response (310).

Epitopes for the group-specific and conserved MAbs involve the central region of the molecule containing the conserved domain and flanking cystine noose (amino acids 164–186). A few of the MARMs that were selected with group-specific or conserved MAbs, or with nonhuman primate antiserum, had one or two point mutations in this region (261,375). A number of MARMs contained multiple amino acid substitutions that likely arose from biased hypermutation: as many as 20 A-to-G transitions and up to 13 amino acid changes were involved, and many changes fell within the conserved/cystine noose domain (261). Another “conserved” MAb selected MARMs that each contained a frame-shift mutation. These MARMs each contained a single nucleotide deletion in either of two runs of As that shifted the reading frame at codon 193 or 205 into an alternative ORF. Thus the mutations had the effect of replacing as many as 106 amino acids from the C-terminus of G with as many as 81 amino acids encoded by the alternative frame. This MAb reacted with a synthetic peptide containing amino acids 204 to 209, thereby mapping a major element of its epitope. Synthetic peptides containing sequence from the central region of the G ectodomain have been shown to react with MAbs, and one that represents amino acids 174 to 187 was protective in mice and calves (28,29,392). Similarly, several peptides spanning amino acids 152 to 204 reacted with MAbs and induced resistance to RSV in mice (327), confirming the importance of this central region in immunogenicity and antigenicity.

T Lymphocytes

In seropositive humans, the N, SH, F, M, M2, and NS2 proteins have been shown to stimulate RSV-specific memory $CD8^+$ CTLs (70). There was no apparent association between MHC haplotype and protein specificity. In BALB/c mice, the F, N, and M2-1 have been shown to induce primary and memory $CD8^+$ CTL responses, and the response to M2-1 was specific to MHC haplotype $H\text{-}2K^d$ (237,313). In particular, infection with a vaccinia recombinant expressing a peptide containing amino acids 82 to 90 of M2-1 induced levels of RSV-specific $CD8^+$ CTLs in BALB/c mice comparable to those induced by RSV infection (236). In contrast, although G is one of the two major protective antigens, a significant G-specific MHC class I–restricted CTL response has not been detected in humans or experimental animals.

The RSV proteins differ in their pattern of priming $CD4^+$ T lymphocytes in the BALB/c mouse. F protein expressed from a recombinant vaccinia virus induced a $CD4^+$ T-cell response that was biased toward the Th1 subset, whereas the G protein induced a Th2-biased response (5). In the mouse model, increased sensitization of Th2 $CD4^+$ T cells followed by challenge with RSV results in an immunopathologic response marked by pulmonary eosinophilia. The effect observed with complete G could be mimicked by a synthetic peptide containing amino acids 184 to 198 of the G protein or by a vaccinia virus recombinant expressing that sequence (365,380).

One interpretation is that the G protein, and the 184 to 198 G peptide in particular, contain one or more epitopes that preferentially stimulate Th2 $CD4^+$ T cells. However, other studies indicated that the $CD4^+$ T-cell response is controlled by factors that are not epitope specific. First, the form of the antigen was found to be important, with soluble proteins favoring a Th2-biased response. For example, the secreted form of the G protein was observed to give an enhanced

stimulation of Th2 CD4$^+$ T cells, and F protein that had been engineered to be secreted was associated with a shift toward a Th2-biased response (35,217). Second, the concurrent stimulation of RSV-specific CD8$^+$ T cells was shown to down-regulate the Th2 CD4$^+$ T-cell response, probably through secreted lymphokines such as interferon-g. For example, when the strong CD8$^+$ T-cell epitope described earlier for the M2-1 protein was coexpressed with G, there was a marked down-regulation of the immunopathogenic response (367). Conversely, expression of the F protein in a CD8$^+$ T cell–deficient mouse primed for an eosinophilic response on subsequent RSV challenge (201,367). This might explain why soluble antigens often induce a Th2-biased CD4$^+$ T-cell response: such antigens typically are inefficient in stimulating CD8$^+$ T cells. The Th2-biased CD4$^+$ T cell response associated with the G protein also is consistent with the inability of G to induce a significant CD8$^+$ T-cell response. Third, the route of immunization was important: administration of vaccinia-G by the intradermal route primed for eosinophilia, whereas the intraperitoneal route did not (34). The basis for this difference is not understood, but indicates that local immune factors are important. Finally, the genetic background of the host is important (202), particularly elements of the genetic background that lie outside of the MHC locus (368). These elements have not been defined, but could include genes such as those involved in the local inflammatory response or cytokine network. Although the host and viral determinants of the CD4$^+$ T-cell response to RSV are complex, these studies provide the unifying concepts that CD8$^+$ T cells are important in regulating the RSV-specific CD4$^+$ T-cell response, and that the form, composition, and mode of administration of an RSV vaccine can greatly influence the immune response.

IMMUNITY

The adaptive immune system has the primary role in recovery from RSV infection and resistance to reinfection (95,289). For example, immunodeficient children fail to clear RSV and shed virus for many months rather than the typical interval of 1 to 3 weeks (67,172). Severely immunocompromised adults, such as bone marrow transplant recipients or individuals with leukemia, have a very high incidence of RSV infection leading to serious disease and death.

In the BALB/c mouse, pulmonary NK cells appear in the first few days after RSV infection, followed in turn by pulmonary CD8$^+$ CTLs and secretory and systemic antibody. In addition to being direct effectors against virus-infected cells, NK and CD8$^+$ T cells can modulate the immune response by secretion of lymphokines, especially interferon-g (201,203,367). Clearance of virus was temporally associated with the increase in CD8$^+$ CTLs (8). Athymic nude mice shed virus persistently, which could be cleared by adoptive transfer of primed T cells (62). Studies in which the CD4$^+$ and CD8$^+$ T-lymphocyte subsets were depleted individually or together showed that both are important in clearing a primary infection (149). Studies in which mice were depleted of B lymphocytes showed that RSV-specific antibodies are not required for clearance of virus during a primary infection, but are very important in restricting replication and disease on reinfection (148). Passive administration of antibodies to RSV-infected cotton rats or primates at the height of infection greatly reduced virus replication, showing that antibodies can be effective in restricting a primary infection (187,330). However, CD8$^+$ CTLs appear to be required to eliminate virus completely (316). The relatively minor role played by antibodies in clearing primary infection in the mouse model might reflect the rapid clearance of a semipermissive infection and the relatively slower kinetics of appearance of antibody compared with cellular immunity. In human infants, where infection is more robust and longer in duration, the decrease in virus shedding in nasal secretions was associated with the appearance of RSV-specific IgA antibodies (269). RSV-specific cytotoxic cells, thought to be CD8$^+$ T lymphocytes, have been detected in peripheral blood mononuclear cells from infants with RSV disease (71,204). It is reasonable to assume that, as in experimental animals, they are important in resolving RSV infection in humans. Thus both cellular and humoral immunity contribute to resolving RSV infection.

With regard to resistance to reinfection, immunization of BALB/c mice with recombinant vaccinia viruses that each express an individual RSV protein showed that F and G are the only proteins that induce RSV-neutralizing antibodies and long-lived resistance to challenge RSV replication (87). Immunization with antigens that stimulated RSV-specific CD8$^+$ CTLs but not neutralizing antibodies (the M2-1 and N proteins) conferred resistance to subsequent infection, but this was short lived and had largely waned by 28 days (87,90,130,236). Thus antibodies mediate long-term resistance to reinfection, whereas memory CD8$^+$ CTLs induced by primary infection did not augment resistance to reinfection, at least not in the BALB/c mouse.

RSV infection does not induce significant levels of type I interferons (a and b) in nasal secretions (165,166,267), although in some cases, low or moderate levels have been reported (299). The low and inconsistent detection of interferon is in contrast to parainfluenza and influenza virus infections. *In vitro,* RSV typically does not induce interferons and is not sensitive to their antiviral effects, although more recent work indicated that RSV can induce interferon-b (17,72,138,346). Thus it appears that type I interferons, or at least interferon-a, play a minor role in restricting RSV replication *in vivo.* Whether RSV expresses an interferon antagonist, such as have been described for influenza and parainfluenza viruses, is not yet known.

Newborn term infants typically possess transplacentally derived maternal RSV-specific serum IgG (344). This appears to provide a relative sparing of incidence and severity of RSV disease during the first 5 to 6 weeks of life, when the titer of passive antibodies is highest. The protective effect observed during this period is inversely proportional to passive antibody titer (144,170,197,320). Breast-feeding might also be a source of passive protection against RSV disease, although its impact is not great and remains controversial (127,197).

Serum and secretory antibodies to both the F and G proteins are produced to high titer in response to RSV infection by most older infants and young children in whom maternal antibodies had declined to a low level (68,269,290,344,412). However, the response of 1- to 8-month-old infants to RSV infection is blunted, with fewer infants mounting a response, and the levels of F and G antibodies achieved are only 15% to 25% those of older subjects. The diminished antibody response of young infants reflects two factors, immunologic immaturity and a suppressive effect of maternally transmitted antibodies (290,291). In experimental animals, passive transfer of RSV antibodies decreased the frequency and magnitude of the antibody response to F and G proteins expressed by vaccinia recombinants and also decreased the neutralizing activity of the antibody that was induced (291). Animals manifesting this diminished antibody response

exhibited decreased resistance to challenge with RSV. Additional information on the antibody response of infants to RSV infection has come from clinical trials of live-attenuated RSV vaccines (429). These studies showed that (a) the predominant antigen recognized by the 1- to 2-month-old infant was the G glycoprotein, with 82% of the vaccinees responding to this glycoprotein compared with 40% to F; (b) the response was best measured by using reagents to detect RSV-specific serum IgA antibodies; (c) a serum IgG response to F and G was readily detected in RSV-naive 6- to 24-month-old children but was rarely detected in 1- to 2-month-old infants, in part because this response was masked by the presence of maternally derived IgG antibodies; (d) RSV-specific IgA antibodies were detected infrequently in nasal washes; and (e) infection of the 1- to 2-month-old infant induced nearly complete resistance to replication of a second dose of vaccine, which correlated directly with the previously mentioned RSV G protein–specific serum IgA response. Thus the very young human infant appears to be capable of mounting a protective immune response to RSV, and this appears to be focused on the G protein.

Secretory antibodies play an important role in resistance to reinfection, although the effect is not long-lasting after primary infection. The secretory antibodies produced by infants in response to RSV infections often fail to neutralize the virus *in vitro* (269), and this defect may be at least partially responsible for the incompleteness of natural immunity after infection during infancy and early childhood. As individuals grow older, however, multiple reinfections induce higher levels of IgA antibody and a more sustained secretory immune response (189,414). Studies of experimental infection of adult volunteers with wt RSV have shown that immunity to infection correlates better with the level of nasal RSV-neutralizing IgA antibodies than with serum RSV-neutralizing antibodies (276,411). Nevertheless, secretory IgA antibodies are only partially protective, because volunteers could still be infected and become symptomatic even when such antibodies were present at a low to moderate level.

Serum RSV-neutralizing antibodies persist at higher titer for a much longer duration than do secretory IgA antibodies. However, serum IgG antibodies gain access to the respiratory tract primarily through the relatively inefficient process of passive transudation (289). The relative inefficiency with which serum antibodies protect the respiratory tract was demonstrated in cotton rats, where the therapeutic effect of RSV-neutralizing serum-derived antibodies was 160-fold greater when administered topically versus parenterally (332,334). In addition, serum antibodies are less efficient in conferring protection in the upper respiratory tract compared with the lower respiratory tract. Serum antibodies in the respiratory tract neutralize RSV independent of complement or the Fc portion of the molecule (331). The ability of serum antibodies to confer effective resistance to RSV is indicated by the observation that the susceptibility to RSV of staff caring for pediatric inpatients correlates inversely with RSV-specific serum antibody level (297). This point also is demonstrated by the effectiveness of parenterally administered antibodies in RSV immunoprophylaxis of high-risk infants (see Prevention and Control). In contrast to the inefficiency with which serum antibodies conferred resistance in the nasopharynx of cotton rats, infection of this region by RSV provided a high level of resistance to reinfection. Thus both local immune mechanisms and serum antibodies contribute to protection against reinfection, and serum antibodies appear to be the longer lived but less efficient component.

RSV infection of the epithelial cells and macrophages of the respiratory tract induces the transcriptional activation of a network of cellular genes involved in inflammation, immune responses, and apoptosis (139,377). This appears to be a generalized mucosal response to infection or injury, and it is not yet known whether there are unique aspects in the response to RSV in addition to the poor induction of type I interferons. RSV infection of epithelial cells and macrophages results in the induction or increased production of overlapping sets of proinflammatory lymphokines, including IL-6, IL-8, RANTES (regulated on activation, normal T-cell expressed and secreted), tumor necrosis factor a (TNF-a), and others (311,319,390,393). Other molecules that increase in response to RSV infection include cell adhesion molecules that can mediate adherence of infiltrating leukocytes to infected epithelial cells, MHC molecules that can enhance the adaptive immune response, and Fas (CD95) that can sensitize RSV-infected cells to apoptosis mediated by cytotoxic T cells (138,307,370).

Lymphokines can have direct antiviral activities. Pretreatment of cells *in vitro* with TNF-a inhibited RSV replication, and mice that had been depleted of TNF-a and subsequently infected with RSV exhibited an increase in the magnitude and length of disease symptoms (304). In BALB/c mice, pulmonary expression of interferon-g from a DNA vector reduced RSV replication (238). Lymphokines also are important in attracting and activating specific leukocyte subsets, including macrophages, neutrophils, eosinophils, T lymphocytes, and NK cells. These cells can help resolve infection but in some cases can damage tissue, either by direct attack on infected cells or through the release of additional lymphokines and degradative enzymes (205,405). In particular, eosinophil degranulation releases the vasoactive compounds histamine and leukotriene C4, which are potential mediators of bronchospasm (312). Two of the granular enzymes, eosinophil cationic protein and eosinophil-derived neurotoxin, are RNAses that have direct, but weak, antiviral activity (103). Soluble mediators also can augment the inflammatory response and can have other effects such as stimulating mucus secretion.

Analysis of nasal wash and tracheal aspirates from RSV-infected individuals confirmed the presence of elevated levels of inflammatory cytokines and chemokines including IL-8, RANTES, MIP-1 a, IL-6, TNF-a, IL-1b, and others (30,184,360). Leukocyte numbers in respiratory washes were increased substantially. In summary, the inflammatory response contributes to controlling RSV infection through elaboration of antiviral molecules and recruitment of effector cells and also contributes to stimulating and regulating the adaptive immune response.

RSV is unusual in its ability to infect in the presence of moderate titers of maternal antibodies and to readily reinfect persons of all ages, with some infections occurring within a short interval after the previous infection (174,189). This is in contrast to other viruses of the respiratory tract such as influenza virus and rhinoviruses. For example, although influenza viruses and RSV alike infect respiratory tract epithelium, influenza virus infection provides very strong long-term immunity, whereas immunity to RSV is much less complete. Reinfection with influenza viruses is largely the result of antigenic variation in the hemagglutinin and neuraminidase protective antigens, whereas reinfection with RSV does not require antigenic drift. The reasons for the difference in duration of immunity between RSV and influenza viruses, and the ability of RSV to readily cause symptomatic reinfection, remain undefined but might involve the following factors. First, RSV is a highly infectious and transmissible virus capable of infecting almost all susceptible subjects in close contact with an infected individual (174,189). Second, the course of infection is relatively rapid, which likely outpaces the secondary immune response, as exemplified by the lack of effectiveness of memory $CD8^+$ CTLs in protecting against

reinfection (87,90,236). Third, the tropism of the virus for the epithelium of the respiratory tract allows it to replicate at a site in which the major mediator of resistance, the secretory IgA response, is relatively short lived and protection by serum IgG is relatively inefficient. However, most of these factors are shared by influenza and RSV. Fourth, RSV exists as two antigenic subgroups, and there is considerable antigenic diversity even within subgroups, especially in the G protein. This antigenic diversity might be particularly important in the young infant, which appears to respond more strongly to the G protein than to the F protein (429). Fifth, the G protein is an unusual antigen that, perhaps because of the many sugar side chains and unusual nonglobular structure, induces antibodies that are poorly neutralizing on an individual basis. Sixth, the secreted form of G might act as an antibody decoy and also might preferentially induce an immunopathogenic component of the $CD4^+$ T-cell response. Seventh, RSV infection of monocytes and macrophages might impair certain of their functions, and the production of IL-1 inhibitor and IL-10 could dampen the overall immune response (319). Finally, specific RSV gene products might interfere with the immune response. For example, the cysteine noose of the G protein has structural similarity to a subdomain of the TNF receptor and might be a TNF antagonist (243).

Host-specific factors also contribute to the ability of RSV to reinfect. As mentioned earlier, secretory and systemic antibody responses in the very young are reduced because of immunologic immaturity and the immunosuppressive effects of maternal antibodies. Cell-mediated immunity also might be diminished in the very young infant (71). Hence failure to achieve effective protection against the first reinfection could reflect this diminished primary immune response. Although protection is incomplete and is often overcome under natural conditions, cumulative immunity from repeated reinfections protects older children and adults against more serious forms of infection, particularly disease involving the lower respiratory tract.

TREATMENT

Treatment of severe RSV disease of the lower respiratory tract requires considerable supportive care: mechanical removal of secretions, proper positioning of the infant, administration of humidified oxygen, and, in the most severe cases, respiratory assistance (for recent reviews, see 206,235,266,275, and 349). The use of bronchodilators is controversial but may be beneficial in older infants if wheezing is an important symptom. Corticosteroids are commonly used as antiinflammatory therapy, although there is a general lack of data demonstrating efficacy. Improvements in supportive care have clearly made a major impact on mortality from severe bronchiolitis or pneumonia. Although this is difficult to document precisely, it is illustrated by the improvement in the mortality rate for infants and children with congenital heart disease and pulmonary hypertension: a decrease from 44% to from 3% to 4% (126,300). Advances in general pediatric intensive care may well be similarly responsible for the overall low mortality rate for children hospitalized with RSV disease (152,272,281,300,374).

The nucleoside analog ribavirin (1-b- D-ribofuranosyl-1,2,4-triazole-3-carboxamide; Virazole) was approved in 1986 for treatment of RSV infection (12,349). Ribavirin has antiviral activity in cell culture and in cotton rats, although its mode of action remains unclear. Clinically, it is administered as a small-particle aerosol using a tent, mask, or mechanical ventilator, delivered for 12 to 18 hours daily for a period of 3 to 7 days. Alternatively, it can be administered at a higher dose for 2 hours 3 times daily. When delivered as an aerosol, it appears to have little or no acute systemic toxicity and is not associated with long-term adverse effects, although there are concerns over possible hazard to caregivers and family. Initial trials indicated a modest but consistent beneficial effect on both virus shedding and clinical illness (12,275). Conversely, evidence is lacking that ribavirin decreases mortality, the duration of hospitalization, or the need for supportive therapies or that it improves long-term pulmonary function (12,341). In some studies there were trends suggesting that therapy was beneficial, although not statistically significant. The American Academy of Pediatrics has changed its recommendation that ribavirin “should be used” to “may be considered” for RSV-infected infants and children with congenital heart disease, chronic lung disease, immunosuppressive disease, prematurity, or severe disease (12). In such cases, treatment should be initiated as promptly as possible (as soon as the diagnosis of RSV infection is proven or strongly suspected). Studies are now in progress for high-risk groups such as bone marrow transplant recipients in which ribavirin is given by the aerosol or intravenous routes, or both, with or without RSV-neutralizing antibodies (see later), and anecdotal reports indicate improved benefit.

The success of parenterally administered RSV-neutralizing antibodies for RSV immunoprophylaxis of high-risk infants and young children (see Passive Immunoprophylaxis, later) suggested that RSV-antibodies also might be effective as antiviral therapy against established infection. The passive parenteral infusion of serum-derived RSV-neutralizing antibodies into cotton rats or primates at the peak of RSV infection strongly reduced virus replication without evidence of any immunopathologic effect (187,332). Direct administration of RSV-neutralizing antibodies into the respiratory tract, rather than by parenteral inoculation, also was effective in reducing pulmonary virus replication, and a comparable therapeutic effect was achieved by using a 160-fold smaller quantity of antibodies (330). Intravenous infusion of RSV-neutralizing antibodies into previously healthy or high-risk infants and children hospitalized for RSV disease substantially reduced the level of virus shedding, to levels comparable to those achieved with ribavirin, but produced little or no improvement in clinical outcome (188,256,350,351). Initial studies of the aerosol route of administration provided similar findings (345), but additional evaluation is needed. As indicated earlier, combination therapy with antibodies and ribavirin also is being evaluated.

A number of other candidate anti-RSV therapeutic agents are under preclinical evaluation (104). In some cases, efficacy *in vitro* or in small experimental animals was reported to equal or exceed that of ribavirin. However, experience with ribavirin and antibody therapy, as described earlier, indicates that achieving a substantial reduction in virus replication in an infant or small child experiencing serious RSV disease does not necessarily improve the clinical outcome, perhaps because at that late time, pulmonary pathology cannot be effectively countered simply by reducing viral replication (as with ribavirin) or neutralizing free virus (as with antibodies). Other experimental therapeutic approaches include intratracheal administration of surfactant (398) or modification of the production of inflammatory cytokines and chemokines (38). Topically administered interferon is generally considered to be ineffective (194). Vitamin A therapy, which reduces morbidity and mortality associated with measles virus, did not appear to benefit RSV-infected children (40).

PREVENTION AND CONTROL

Passive Immunoprophylaxis

Infusion of RSV-neutralizing antibodies into experimental animals confers substantial resistance in the respiratory tract to a subsequent RSV challenge (187,333). These findings led to development of a successful strategy for passive immunoprophylaxis of high-risk individuals (17,275). RSV intravenous immunoglobulin (RSV-IVIG, or RespiGam) consists of fractionated human IgG prepared from sera prescreened for high RSV-neutralizing activity. RSV-IVIG is administered to infants and children at high risk for serious RSV disease in monthly intravenous infusions of 750 mg/kg, given for 4 or 5 months during the RSV season. Given prophylactically, RSV-IVIG reduced the frequency of hospitalization and duration of stay by approximately 55%, and decreased the number of days spent in intensive care by 97% (12,153). However, there is as yet no evidence that mortality due to RSV was reduced (280). More recently, an RSV-neutralizing, F-specific murine MAb was humanized by recombinant methods by transferring its complementarity-determining regions onto a human IgG1 backbone, resulting in the antibody called Synagis (previously referred to as MEDI-493 or palivizumab) (14,216). The original murine MAb, designated 1129 (31), efficiently neutralizes almost all members of RSV antigenic subgroups A and B. Its epitope involves amino acid 275 of the F protein (99). Synagis is 50- to 100-fold more effective on a weight basis in neutralization than is RSV-IVIG, and thus the total amount of immunoglobulin administered could be reduced. This permits administration by a monthly intramuscular injection (15 mg/kg) rather than an intravenous infusion that takes several hours. Its efficacy was comparable to that of RSV-IVIG. This strategy is now in use for immunoprophylaxis of premature infants or those with chronic lung disease (14). Passive immunoprophylaxis also is beginning to be evaluated in bone marrow transplant recipients.

Vaccines

Because the peak incidence of serious RSV disease is at age 2 to 7 months (197,320), a pediatric RSV vaccine must be capable of stimulating effective resistance before the second month of life. Obstacles to successful immunization during the first month of life include immunologic immaturity and the immunosuppressive effects of maternally derived serum RSV-neutralizing antibodies (71,291,429). The second major target population for an RSV vaccine is the elderly, in whom it has been projected that an RSV vaccine would be cost effective (140,178). Efforts to develop a vaccine for either target group include live-attenuated and subunit RSV candidates. Live-attenuated vaccines that are satisfactory for use in RSV-naive infants likely would be overattenuated for the elderly (146).

A vaccine consisting of formalin-inactivated, concentrated RSV was evaluated in infants and children in the mid-1960s. This vaccine induced an immune response that was not protective and, on subsequent natural infection, increased the frequency and severity of RSV disease (226,233). This appeared to be a more frequent and more severe form of the natural disease, rather than an altered disease, as was associated with a formalin-treated measles virus vaccine (329).

A number of factors likely contributed to the lack of protection. Because the formalin-inactivated vaccine was administered parenterally, it probably failed to induce appreciable local respiratory tract RSV-specific secretory IgA antibodies. In addition, retrospective analysis showed that the vaccinees had an altered, weakened systemic antibody response compared with that induced by natural infection (294,296). Specifically, vaccinees developed a high titer of serum antibodies, particularly to the F glycoprotein, but these were not effective in neutralizing RSV. It is likely that the formalin treatment denatured protective epitopes on the F and G proteins. The absence of strong local and systemic RSV-neutralizing antibody responses left vaccinees susceptible to subsequent natural infection, whereupon the expression of viral antigens initiated an immunopathologic response. Critical to this understanding was the development of experimental animal models in which immunization with formalin-inactivated RSV vaccine, followed by intranasal challenge with RSV, results in enhanced pulmonary histopathology, a surrogate of the enhanced disease observed in the vaccinees (150,314,335,367,409). In cotton rats, passive transfer of serum from animals immunized with formalin-RSV, followed by RSV challenge, did not result in disease enhancement (88). This indicated that the immunopathogenic effect was not mediated by antibodies induced by the vaccine, but rather was mediated by immune cells.

Peripheral blood lymphocytes from vaccinees exhibited an exaggerated proliferative response to RSV antigens *in vitro* compared with that observed after natural RSV infection (234). Reagents were not then available to identify the specific lymphocyte subset involved. More recent experiments in mice and cotton rats indicated that the inactivated vaccine induced an increased level of virus-specific $CD4^+$ T lymphocytes but did not induce RSV-specific $CD8^+$ T lymphocytes, which are important antiviral effectors and regulators of the cellular immune response to RSV (367). Furthermore, immunization with inactivated RSV induced a greater stimulation of the Th2 $CD4^+$ T-cell subset than did infection with RSV (150,314,409). As already mentioned, this immunopathogenic response could be abrogated by depletion of $CD4^+$ T cells or the Th2 cytokines IL-4 and IL-10 before challenge (89,91). It is not clear whether an increased number of sensitized $CD4^+$ T cells, or the disproportionate stimulation of the Th2 subset, or both, was most important in the immunopathologic response. In any case, sensitization by formalin-RSV resulted in exaggerated proliferation and activation of RSV-specific memory $CD4^+$ T cells on subsequent infection. The detailed mechanism of the immunopathology remains speculative, but might involve increased cellular infiltration and secretion of cytokines and chemokines in the infected lungs, which in turn would recruit other inflammatory cells in an exaggerated response.

Also the serum RSV-neutralizing antibody response of vaccinees to RSV infection was observed to be significantly reduced compared with age-matched, unvaccinated controls (294). This diminished antibody response would be expected to retard resolution of infection, thereby increasing severity of disease. Vaccinees continued to exhibit an exaggerated *in vitro* lymphocyte-proliferation response to RSV antigens even after a subsequent natural infection, suggestive of a lingering imbalance in the cellular immune response (234). However, enhancement of RSV disease was not observed during subsequent episodes of reinfection.

A number of other vaccine strategies are being investigated, including synthetic peptides (28); recombinantly derived viral antigen (308,328), “DNA vaccines” (250); replication-defective recombinant vaccinia virus (430); recombinant

vesicular stomatitis virus (223); live-attenuated RSV (see later); and a protein-subunit vaccine based on F and G protein purified from RSV-infected cells (381,400). The subunit vaccines have focused on the F and G glycoproteins based on their identification as the major protective antigens. So far, clinical trials have been reported only for live-attenuated RSV and the infected-cell protein vaccine.

Protein subunit vaccines can induce disease enhancement in experimental animals resembling that described earlier for the formalin-treated vaccine (179,295,303), although this was not observed in another study (328). The nonreplicating nature of these vaccines likely will not induce the strong $CD8^+$ T-cell response that appears to be important for regulating cellular immunity to RSV antigens (203,367). It may be that unique features of certain of these recombinant proteins (328) or the use of various adjuvants (381) will overcome this limitation and make them suitable as pediatric vaccines. In addition, RSV protein vaccines may find use in seropositive children and the elderly, where the safety issue of primary immune priming is obviated because of previous natural infection (116,326,408). Maternal immunization is another potential use for a protein vaccine (288).

A vaccine based on the intranasal administration of live-attenuated RSV has the advantage of mimicking natural infection, which has never been shown to prime for enhanced disease (408). In addition, in experimental animals, the topical route of administration induces strong local and systemic immune responses and is partially resistant to the immunosuppressive effects of passively transferred maternal antibodies (98). A live-attenuated RSV vaccine readily replicated and immunized 1- to 2-month-old infants despite the presence of maternal antibodies, which indicates the feasibility for the use of this type of vaccine in the target population (429).

Studies beginning in the 1960s produced a series of attenuated, immunogenic RSV cp or ts mutants, but these proved to be underattenuated, overattenuated, or genetically unstable (128,428). It became clear that it would be difficult to produce a genetically stable virus with the appropriate balance between attenuation and immunogenicity. It was then decided to use the cp-RSV virus (128) as the substrate for the introduction of ts mutations by chemical mutagenesis. Cp-RSV, which is not ts, had been shown to be attenuated in chimpanzees, adults, and older RSV-naive infants, but was insufficiently attenuated in RSV-naive younger children. Mutant derivatives of cp-RSV exhibited varying degrees of temperature sensitivity and restriction of virus replication *in vivo.* Two of the more restricted mutants were subjected to another cycle of chemical mutagenesis, which yielded mutants that were more ts and more restricted in replication in mice and chimpanzees (96). Mutants were obtained that exhibited complete or almost complete restriction of virus replication in the lower respiratory tract of chimpanzees, stability of the ts phenotype, and moderate immunogenicity, and they induced almost complete protection against infection by wt RSV. A similar strategy also was pursued by another research group (386). A number of these vaccine candidates have been evaluated clinically, with successive testing in seropositive adults, seropositive children, RSV-naive children, and RSV-naive infants of age 1 to 2 months, the latter being the target population. Only the most attenuated of the biologically derived vaccine viruses, one designated cpts248/404, progressed to the final group (429). This virus was immunogenic and protective against a second vaccine dose, but induced mild nasal congestion, indicating the need for a small amount of additional attenuation (429).

The ability to recover infectious recombinant RSV from cDNA has greatly facilitated the characterization and further development of live-attenuated viruses (79,86,208). For example, cp-RSV and six of its ts derivatives were sequenced and their attenuating mutations identified by their insertion, individually and in combination, into the wt recombinant RSV. The majority of attenuating mutations was found to involve single amino acid substitutions in the L protein; interestingly, however, the most attenuating mutation involved a nucleotide substitution in the M2 GS signal (86,221,421). It was then possible to recreate biologically derived viruses such as cpts248/404 and attenuate them further by the addition of one or more attenuating mutations from a different virus (422). Deletion of the NS1, NS2, SH, or M2-2 gene, individually or in combination, also represents another type of attenuating mutation, one that should be particularly refractory to reversion (36,384,420).

It also is possible to expedite the development of a subgroup B-specific RSV vaccine by using reverse genetics. The G and F glycoproteins of several attenuated subgroup A vaccine candidates were replaced by their subgroup B counterparts, resulting in a panel of live-attenuated vaccine candidates bearing the subgroup B antigenic determinants (423). Alternatively, the subgroup B G gene can be inserted into a subgroup A virus as an extra gene creating a single virus with dual specificity (208).

BRSV has been considered as a live “Jennerian” vaccine for use in humans based on its antigenic similarity with human RSV, especially in the F protein, and its attenuation due to host range restriction in primates. Unfortunately, BRSV does not replicate efficiently in chimpanzees, and as a consequence, does not induce resistance to RSV challenge (45). The genes of recombinant BRSV are now being systematically replaced by their counterparts from human RSV to develop a chimeric virus bearing the antigenic determinants of human RSV in a backbone that is attenuated by one or more BRSV genes (45).

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CHAPTER 46 *Orthomyxoviridae:* The Viruses and Their Replication

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The *Orthomyxoviridae* are enveloped viruses with a segmented single-stranded RNA genome that has been termed "*negative stranded*" because the viral messenger RNAs (mRNAs) are transcribed from the viral RNA segments; by convention, mRNA is positive stranded. The genomic RNA of negative-stranded RNA viruses has to serve two functions: first as a template for synthesis of mRNAs and second as a template for synthesis of the antigenome positive strand. Negative-stranded RNA viruses encode and package their own RNA-dependent RNA transcriptase, but mRNAs are only synthesized once the virus has been uncoated in the infected cell. Viral replication occurs after synthesis of the mRNAs and requires synthesis of viral proteins. The newly synthesized antigenome positive strand (for influenza virus, often termed *template RNA* or *complementary RNA* [cRNA]) serves as the template for further copies of the negative-stranded genomic RNA.

Among the RNA viruses, influenza virus is special in that all of its RNA synthesis—transcription and replication—takes place in the nucleus of the infected cell. The nucleus provides the environment for the synthesis of influenza virus mRNAs by an unusual process: initiation requires m^7GpppX^m-containing capped primers that are generated from a subset of host cell RNAs by an influenza virus–encoded cap-dependent endonuclease. In addition to stealing the capped 5' end of host mRNAs, influenza virus uses another aspect of the host cell nuclear function, namely the splicing machinery. The mRNAs synthesized by influenza virus and by one other virus, bornavirus (see Chapter 51), provide the only known examples of splicing of RNA that is not transcribed from DNA by RNA polymerase II.

In recent years, a remarkable catalog of mechanisms has been identified by which eukaryotic cells have expanded their genome-coding capacity beyond the linear array of nucleotide sequences usually thought to encode a protein. Such coding strategies create diversity by increasing the number of proteins encoded and, in addition, provide a means by which the expression of these proteins can be regulated. Eukaryotic viruses have usually provided the prototypic example of molecular mechanisms that serve to maximize coding potentials. Indeed, the compactness of the viral genome may be an important factor contributing to the success of a virus as a cellular parasite. The influenza viruses provide some remarkable examples of genome diversity: spliced mRNAs and overlapping reading frames, bicistronic mRNAs and overlapping reading frames, and coupled translation of tandem cistrons. The variety of mechanisms used for the synthesis of proteins by influenza virus provides a paradigm of successful exploitation of a genome.

CLASSIFICATION

The family *Orthomyxoviridae* (from the Greek *orthos,* meaning "standard, correct," and *myxa*, meaning "mucus") contains four genera as defined in the seventh report of the International Committee for the Taxonomy of Viruses: influenza A, B, and C viruses, and thogotovirus (sometimes called influenza D viruses). Influenza viruses were named *Orthomyxoviridae* because of their ability to bind to mucus (hemagglutination) and to distinguish them from another family of enveloped negative-stranded RNA viruses (*Paramyxoviridae*). Influenza is from the Italian form of Latin *influentia*, meaning "epidemic," originally used because epidemics were thought to be due to astrological or other occult influences.

The influenza A, B, and C viruses can be distinguished on the basis of antigenic differences between their nucleocapsid (NP) and matrix (M) proteins. Influenza A viruses are further divided into subtypes based on the antigenic nature of their hemagglutinin (HA) and neuraminidase (NA) glycoproteins. Other important characteristics that distinguish influenza A, B, and C viruses are as follows:

1. Influenza A viruses infect naturally a wide variety of avian species, humans, and several other mammalian species, including swine and horses. Influenza B virus appears to infect naturally only humans, and influenza C virus has been isolated mainly from humans but also from swine in China.
2. The surface glycoproteins of influenza A viruses (HA and NA) exhibit much greater amino acid sequence variability

than their counterparts in the influenza B viruses. Influenza C virus has only a single multifunctional glycoprotein, the hemagglutinin-esterase-fusion protein (HEF).

3. As discussed later, there are morphologic features that distinguish influenza A and B viruses from influenza C viruses.
4. Although influenza A, B, and C viruses possess similar proteins, each virus type has distinct mechanisms for encoding proteins.
5. Influenza A and B viruses each contain eight distinct RNA segments, whereas influenza C viruses contain seven RNA segments.

Little is known about the genus *Thogotovirus,* which comprises tickborne viruses that are structural and genetically related to influenza A, B, and C viruses (190) and contain a single glycoprotein (218). Because of space limitations and the need to discuss basic principles, these viruses are not considered further.

VIRION STRUCTURE

The *Orthomyxoviridae* are composed of about 1% RNA, 70% protein, 20% lipid, and 5% to 8% carbohydrate (reviewed in 51). The lipid envelope of influenza viruses is derived from the plasma membrane of the host cell in which the virus is grown (reviewed in 51). Influenza A and B virions are morphologically indistinguishable, whereas influenza C virions can be distinguished from the other genera because the glycoprotein spike is organized into orderly hexagonal arrays (51). Influenza A or B virions grown in eggs or in tissue culture cells have a fairly regular appearance in the electron microscope, with particles that are 80 to 120 nm in diameter. In contrast, virus strains isolated from humans or animals and propagated by single passage in culture exhibit greater heterogeneity and pleomorphism. The morphologic characteristics of influenza A viruses are a genetic trait (153) but also depend on the cell type in which the virus is grown (285). Although the genes that specify spherical morphology have not be identified, the trait segregates independently of the HA and NA proteins (155).

The most striking feature of the influenza A virion is a layer of about 500 spikes radiating outward (10 to 14 nm) from the lipid envelope. These spikes are of two types: rod-shaped spikes of HA and mushroom-shaped spikes of NA. The ratio of HA to NA varies but is usually 4:1 to 5:1. Influenza A, B, and C viruses also encode another integral membrane protein, the M_2, NB, and CM2 proteins, respectively. Biochemical evidence indicates these proteins are only present in a few copies in virions (13,24,337,395). The viral matrix protein (M_1) is thought to underlie the lipid bilayer and to associate with the ribonucleoprotein core of the virus. M_1 is the most abundant virion protein (Table 1). There is no evidence for the existence of a stable lipid-free “core” structure other than the ribonucleoprotein (RNP).

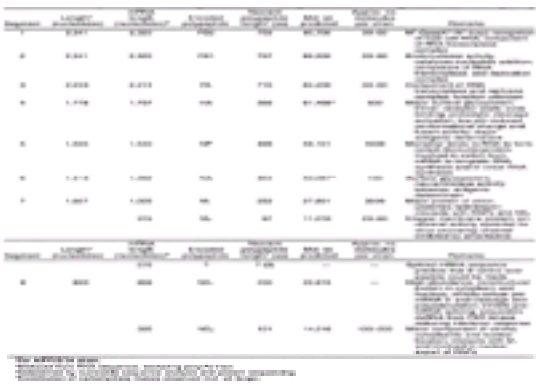

TABLE 1. *Influenza A virus genome RNA segments and coding assignments*

Inside the virus, observable by thin-sectioning of virus or by disrupting particles, are the RNP structures, which can be separated into different size classes (68) and contain eight different segments of single-stranded RNA. (Fig. 1 presents a schematic diagram of virion, and Fig. 2 shows electron microscopy photographs). The RNPs have the appearance of flexible rods (272); depending on the salt concentration during sample preparation or method of staining, the flexibility of the RNPs appears to vary (52,109,272). The RNP strands often exhibit loops on one end and a periodicity of alternating major and minor grooves, suggesting that the structure is formed by a strand that is folded back on itself and then coiled on itself to form a type of twin-stranded helix. Larger helical structures, interpreted to be formed by the orderly aggregation of the smaller segments, have occasionally been seen in partially disrupted virions (5,220). These structures might represent ordered complexes of the eight separate RNPs.

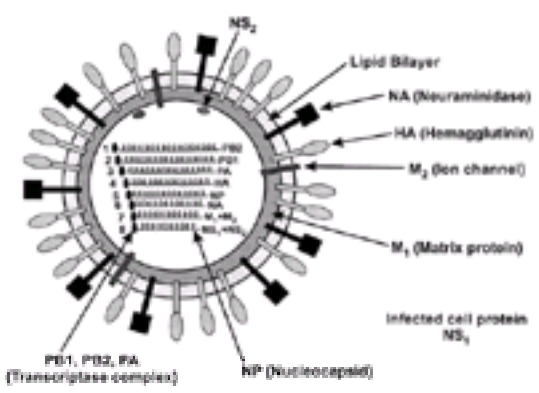

FIG. 1. A schematic diagram of the structure of the influenza A virus particle. Three types of integral membrane protein

—hemagglutinin (HA), neuraminidase (NA), and small amounts of the M_2 ion channel protein—are inserted through the lipid bilayer of the viral membrane. The virion matrix protein M_1 is thought to underlie the lipid bilayer but also to interact with the helical ribonucleoproteins (RNPs). Within the envelope are eight segments of single-stranded genome RNA (ranging from 2341 to 890 nucleotides) contained in the form of an RNP. Associated with the RNPs are small amounts of the transcriptase complex, consisting of the proteins PB1, PB2, and PA. The coding assignments of the eight RNA segments are also illustrated. RNA segments 7 and 8 each code for more than one protein (M_1 and M_2, and NS_1 and NS_2, respectively). NS_1 is found only in infected cells and is not thought to be a structural component of the virus, but small amounts of NS_2 are present in purified virions.

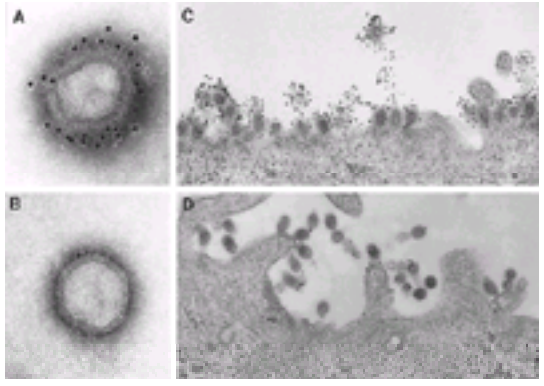

FIG. 2. Electron micrographs of purified influenza virus virions and virions budding from the surface of MDCK cells. **A:** Influenza A/Udorn/72 virus negatively stained with hemagglutinin (HA) decorated with 10-nm gold. **B:** Influenza A/Udorn/72 virus negatively stained with M_2 decorated with 10-nm gold. **A** and **B,** Final magnification ×227,500. **C:** Thin section of an influenza A/Udorn/72 virus-infected MDCK cell with HA decorated with 10-nm gold. **D:** Thin section of an influenza A/Udorn/72 virus–infected MDCK cell with HA decorated with 10-nm gold. **C** and **D,** Final magnification ×58,000. (Data from George Leser, Northwestern University.)

The RNPs consist of four protein species and RNA. NP is the predominant protein subunit of the nucleocapsid and coats the RNA: about 20 nucleotides per NP subunit. Associated with the RNPs is the RNA-dependent RNA polymerase complex consisting of the three P (polymerase) proteins, PB1, PB2, and PA, which are present at only 30 to 60 copies per virion (133,176). The RNA in the RNP complex remains sensitive to digestion by RNAase, and the RNA can be displaced by polyvinylsulfate (reviewed in 171). This suggests that the structure of the influenza virus RNP is very different from that of the RNAase-insensitive *Paramyxoviridae* RNP (see Chapter 41). The polymerase proteins in the RNP complex carry out the cap-binding, endonuclease, RNA synthesis and polyadenylation reactions described later (194,265,266,267 and 268).

The NS_2 protein (named as the second protein encoded by RNA segment 8) is present at 130 to 200 molecules per virion and forms an association with the M_1 protein (282,388), which is thought to be an essential interaction in the virus life cycle for export of the RNP complex from the nucleus (236) (see later). It has been suggested that NS_2 be renamed NEP for nuclear export protein (236).

INFLUENZA VIRUS GENOME ORGANIZATION AND ENCODED PROTEINS

Influenza A and B viruses each contain eight segments of single-stranded RNA (ssRNA), and influenza C viruses contain seven segments of ssRNA (influenza C viruses lack an *NA* gene). The early evidence for a segmented genome of influenza viruses has been extensively reviewed (172). The major step forward in understanding the structure of the influenza virus genome came from the electrophoretic separation of the virion RNAs on polyacrylamide gels (10,214,246,247,271,283,284). The assignment of specific RNA segments to virus polypeptides was made in three ways:

1. One method depended on the apparent differences between strains in the electrophoretic mobility of the RNA segments on polyacrylamide gels and the ability to distinguish proteins between strains, either by immunologic methods for HA and NA or by differences in the gel mobility of polypeptides. Reassortants were prepared between two parental strains, with comparisons made of the mobilities of the RNA segments and polypeptides between the parental types and each recombinant. From the analyses, gene assignments were made (reviewed in 244).
2. A hybridization strategy was dependent on base-sequence homologies between corresponding RNA segments of different strains and temperature-sensitive (ts) mutants (302).
3. A direct method of hybrid arrest of translation of individual influenza virus mRNAs using purified vRNA segments (173,174).

The gene assignment for influenza A virus is as follows: RNA segment 1 codes for PB2, 2 for PB1, 3 for PA, 4 for HA, 5 for NP, 6 for NA, 7 for M_1 and M_2, and 8 for NS_1 and NS_2 (see Table 1). The complete nucleotide sequence of influenza A virus PR/8/34, and many RNA segments of other subtypes, was obtained by 1982 (reviewed in 171); today, the complete sequence of many influenza A and B viruses and one influenza C virus has been obtained. The PR/8/34 strain of influenza A virus contains 13,588 nucleotides. In this chapter, for space considerations, no attempt has been made to cite the original publication of the several hundred influenza virus nucleotide sequences obtained and deposited in the EMBL/GenBank database.

The Polymerase Proteins and Their Genes

The three largest RNA segments encode the PB1, PB2, and PA proteins (apparent M_r 96,000, 87,000, and 85,000, respectively) (133,176). Because of anomalous migration of influenza virus RNA segments on gels, the proteins encoded by RNA segments 1 and 3 from different subtypes are different. Therefore, the proteins are named after their behavior on isoelectric focusing gels: two P proteins were found to be basic (PB1 and PB2) and one acidic (PA) (125,343,383). RNA segments 1 and 2 are both 2,341 nucleotides in length and code for proteins of 759 amino acids (PB2) and 757 amino acids (PB1), respectively; PB1 and PB2 are basic proteins at pH 6.5, with a net charge of +28. Because the sizes of the two polypeptides PB1 and PB2 are very similar, the ability to separate them on polyacrylamide gel electrophoresis must be due to factors such as differential binding of SDS. RNA segment 3 is 2,233 nucleotides in length and codes for a protein of 716 amino acids that has a charge of –13.5 at pH 6.5. The three P proteins form a complex in the cytoplasm and nucleus of cells that is largely resistant to disruption by normal immunoprecipitation buffers and the complex sediments on sucrose gradients at 11S to 22S (63). After synthesis in the cytoplasm, the P proteins are transported to the nucleus, possibly as a complex. However, expression of the individual P proteins from cDNA has shown that each P protein migrates to the nucleus and each contains a karyophilic signal (1,139,324). A description of the known functions of the PB2, PB1, and PA proteins in RNA synthesis is presented later.

Influenza B and C viruses also encode three P proteins that show extensive sequence identity and homology to those of influenza A virus, and it is presumed these proteins have similar roles and properties such as those of influenza A virus.

The Nucleocapsid Protein and Its Gene

NP is the major structural protein that interacts with the RNA segments to form RNP. NP is also one of the type-specific antigens; that is, it is distinct for each of influenza A, B, and C viruses. NP is also the major target of cross-reactive cytotoxic T lymphocytes generated against all influenza virus subtypes in mice and humans (reviewed in 391). It is encoded by RNA segment 5 of influenza A virus and is 1,565 nucleotides in length. NP contains 498 amino acids with a predicted M_r of 56,101, and the protein is rich in arginine residues and has a net positive charge of +14 at pH 6.5 (384). Unlike what might be expected for a protein that interacts with the acidic phosphate residues of RNA, however, there are no clusters of basic residues. This implies that many regions of the NP molecule may contribute to RNA bindings. After synthesis in the cytoplasm, NP molecules are transported to the nucleus. Nuclear targeting of NP is an intrinsic property of the protein (199,295). Two nuclear localization signals (NLS) have been localized to NP: residues 198 to 216 (360) and residues 1 to 38 (230,355). Thus, NP may interact with several members of the importin family, soluble protein receptors involved in nuclear import. NP residues 327 to 345, originally thought to be an NLS (59), are now thought to be cytoplasmic retention signal (64). Late in virus-infected cells, NP of influenza A virus is cleaved from a 56-kd protein to a 53-kd protein by a cellular caspase, but it is not known whether NP cleavage is merely a marker for the onset of apoptosis in infected cells or has a specific function in virus–host interaction (400).

Influenza B virus NP contains 560 amino acids and is 47% homologous to influenza A virus NP, and the influenza C virus NP protein contains 565 amino acids and has several regions of homology with the NP of influenza A and B viruses (reviewed in 171).

The Hemagglutinin Protein and Its Gene

Introduction

The hemagglutinin was originally named because of the ability of the virus to agglutinate erythrocytes (116,213) by attachment to specific sialic acid–containing receptors. HA has three known roles during the influenza virus replicative cycle:

1. HA binds to sialic acid–containing receptors on the cell surface, bringing about the attachment of a virus particle to the cell.
2. HA is responsible for penetration of the virus into the cell cytoplasm by mediating the fusion of the membrane of the endocytosed virus particle with the endosomal membrane, the consequence of which is the viral nucleocapsids are released into the cytoplasm.
3. HA is the major antigen of the virus against which neutralizing antibodies are produced, and influenza virus epidemics are associated with changes in its antigenic structure.

HA is encoded by RNA segment 4 and is synthesized on membrane-bound ribososmes and translocated into the lumen of the endoplasmic reticulum (ER) of infected cells as a single polypeptide HA_0 (M_r about 76,000). The HA spike glycoprotein is a homotrimer (375,380) of noncovalently linked monomers. Each HA polypeptide chain (H3 subtype) consists of an ectodomain of 512 residues, a carboxyl-terminal proximal transmembrane domain of 27 residues, and a cytoplasmic tail of 10 residues (347). HA is cotranslationally modified by the addition of up to seven oligosaccharide chains added to the ectodomain, and three palmitate residues are added via a thioether linkage to the three C-terminal proximal cysteine residues (223,224,301). Depending on the virus strain, host cell type, and growth conditions, HA exists either in an uncleaved precursor form (HA_0) or in a cleaved form consisting of two disulfide-linked chains (HA_1, M_r about 47,000; and HA_2, M_r about 29,000). Cleavage is a prerequisite for the virus to be infectious and hence is a crucial determinant in pathogenicity and in the spread of infection (reviewed in 329). The newly liberated N-terminus of HA_2 (fusion peptide) is hydrophobic, is highly conserved among HAs of different influenza virus strains, and is an essential participant in HA fusion activity.

RNA Segment 4: Gene Structure and Hemagglutinin Amino Acid Sequence

The first gene of influenza virus to have its nucleotide sequence determined was HA (274). Since then, the nucleotide

sequence of RNA segment 4 of all 15 known HA antigenic subtypes and many variants within a subtype have been determined. RNA segment 4 ranges from 1,742 to 1,778 nucleotides and encodes a polypeptide of 562 to 566 residues. The HA_1 chain is from 319 to 326 residues, and HA_2 is from 221 to 222 residues. Depending on the HA subtype, the number of residues lost on proteolytic cleavage between HA_1 and HA_2 ranges from one to six residues. The deduced amino acid sequence of the influenza B/Lee/40 HA has 24% homology to HA_1 and 39% homology to HA_2 of A/PR/8/34, suggesting a close evolutionary relationship between influenza A and B virus HAs (169,170).

Three-Dimensional Structure of Hemagglutinin

Although intact HA "spikes" can be isolated from purified influenza virions and infected cells by detergent solubilization of membranes, when the detergent is removed, the hydrophobic transmembrane domains aggregate and HA "rosettes" form. However, bromelain treatment of virus releases an antigenically and structurally intact trimeric ectodomain of HA (BHA), which is water soluble and contains all of HA_1 and the first 175 of the 221 residues of HA_2 (23,376). BHA of influenza virus A/Aichi/68 H3 subtype crystallized, and from x-ray diffraction data, the three-dimensional structure of both cleaved and uncleaved HA was determined (39,376,380) (Fig. 3).

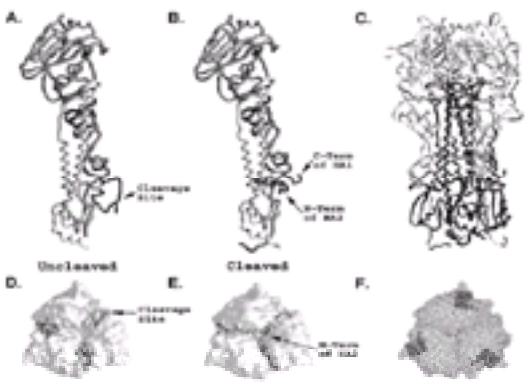

FIG. 3. Schematic diagram of the three-dimensional structure of the uncleaved hemagglutinin (HA) trimer and the cleaved HA trimer as determined from x-ray diffraction data. **A:** Side view of uncleaved HA monomer. **B:** Side view of cleaved HA monomer. **C:** Side view of cleaved HA trimer. **D:** Top down view of uncleaved HA trimer. **E:** Top down view of cleaved HA trimer. For **D** and **E,** shown in *dark gray* are the residues of the C-terminus of HA_1 and the N-terminus of HA_2 that change their position on cleavage. Note that the fusion peptide on cleavage becomes burried in the interior of the trimer. **F:** Top down view of the HA trimer. The residues that make contact with the HA receptor, sialic acid, are shown in *dark gray.* This figure was produced using Protein Data Bank (http://www.rcsb.org/pdb/) coordinate files 1HGF, 1HA0 and 1HGG.

HA_0 and cleaved HA_1 + HA_2 are superimposable in structure except for 19 residues, in a sequence bracketing the cleavage site. The HA trimer extends 135 Å from the membrane and is composed of two predominant regions: (1) a long fibrous stem extending 76 Å from the membrane, which forms a triple-stranded coiled-coil of a-helices and is derived from HA_2 residues; and (2) the globular head of the molecule, which is derived from HA_1 residues and consists predominantly of an antiparallel b-sheet (see Fig. 3). In uncleaved HA_0, eight residues that immediately surround the cleavage site, including five hydrophobic residues of HA_2, project away from the molecules into aqueous solvent. HA_0 contains a deep cavity, which is partially filled by the C-terminus of HA_1 and which swings out of the way on cleavage. This permits filling of the cavity by residues 1 to 10 of the hydrophobic fusion peptide (the newly generated N-terminus of HA_2). Cleaved HA contains the fusion peptides buried in the subunit interfaces of the stem of the trimer (see Fig. 3) and is presumed to exist in a metastable form. Uncleaved HA cannot undergo the low-pH–induced refolding events associated with HA-mediated membrane fusion. It is not known whether it is the positioning of the fusion peptide in the cavity that sets the trigger or whether the covalent bond that links HA_1 to HA_2 restricts the movement of the fusion peptide. However, the localized structural rearrangement on cleavage causes nonpolar residues of the fusion peptide to bury ionizable residues located in the cavity, residues that have been implicated in the low-pH–induced refolding event (39).

The HA receptor-binding site is a pocket located on each subunit at the distal end of the molecule (see Fig. 3). The pocket is inaccessible to antibody, and the residues forming the pocket (Tyr-98, Trp-153, His-183, Glu-190, Leu-194) are largely conserved among subtypes (380; reviewed in 321 and 370). Confirmation that the pocket is the receptor-binding site was obtained by isolating HAs with single–amino acid substitutions at residue 226 that conveyed an altered specificity for sialic acid linked to galactose by either a2,6 or a2,3 linkages (288,367). Because the receptor-binding specificities differ among human trachea (a2,6), avian intestine (a2,3), and pig tracheae (a2,3 and a2,6) (134), the number of types of HA molecules that can transfer successfully from one species to another may be restricted.

The three-dimensional structure of HA indicates that five of the six carbohydrate chains on HA_1 and one carbohydrate chain on HA_2 lie on the lateral surface. The only exception is one carbohydrate chain on HA_1, which appears to stabilize the oligomeric contacts between globular units at the top of the structure (380). Although there is no known required function for carbohydrate on the native HA molecule, addition of carbohydrate is needed for the correct folding of HA in the ER (reviewed in 66).

Five antigenic sites have been mapped on the three-dimensional structure of HA by determining the location of amino acid changes in HAs of natural influenza virus isolates and in HAs of antigenic variants selected by growth in the presence of monoclonal antibodies (376). The regions of antigenic variation cover much of the surface of the globular head of HA, including residues around the antibody-inaccessible receptor-binding pocket. Monoclonal antibodies to each

of the five antigenic sites neutralize the infectivity of the virus.

The Low-pH–Induced Hemagglutinin Conformational Change

Exposure of HA to low pH (299,371) triggers a progression of irreversible conformational changes that ultimately lead to fusion of the viral and cellular membranes. The low pH found in endosomal compartments is the physiologically relevant trigger to release the hydrophobic fusion peptides. However, heat and urea treatments can also induce this change (36,293). Thus, native (pH neutral) cleaved HA is considered to be in a metastable state, and the low-pH–induced form is in a more stable form.

The HA low-pH–induced conformational change, in which HA_1 remains largely unchanged but HA_2 undergoes a refolding event, has been studied extensively. Low pH treatment of HA makes the protein susceptible to digestion by several proteases (58,65,318), and the single disulfide bond that links the HA_1 and HA_2 subunits becomes exposed (97). Furthermore, new antigenic epitopes are revealed with the concomitant loss of others (57,363,372). Despite these changes, x-ray crystallographic analysis indicated that the monomeric HA_1 globular head is virtually unaltered in structure after low-pH treatment (15). Nonetheless, rational engineering of cysteine residues into the head of HA_1 indicates that partial dissociation of the most distal region of the trimer occurs (90).

The x-ray crystallographic structure of proteolytic fragments of the low-pH–induced form of HA has been determined (33,40) (Fig. 4). In part, the structure fits with a model predicted for the low-pH–induced form (37). The x-ray structure shows that a protein refolding event occurs in which the fusion peptide is relocated more than 100 Å toward the target membrane (see Fig. 4). This refolding can be seen by comparison of the neutral-pH form with the low-pH–induced form. In the neutral-pH form, HA_2 residues 76 to 126 form a long a-helix, residues 56 to 75 form an extended loop, and residues 38 to 55 form a short a-helix (380). In comparison, in the low-pH–induced conformation, HA_2 residues 40 to 105 form a triple-stranded a-helical coiled-coil extending 100 Å. The N-terminal coiled-coil is terminated by a four-residue (HA_2 residues 34 to 37), threefold annular N cap structure that prevents the a-helix from propagating further toward the fusion peptide. Residues 106 to 112, which in the neutral structure are a-helical, uncoil to form an extended loop; the membrane proximal region of HA_2 swings up to pack against the long coiled-coil, running antiparallel to the N-terminal a-helix, packing in the groove between a-helices in the coiled-coil (see Fig. 4). The N- and C-terminal residues of HA_2 form an extensive set of interactions that fix the N-terminal and C-terminal residues of the molecule together at one extreme end of a thermally stable rodlike structure (33,40). The refolding event that moves the fusion peptide toward the target membrane is an essential step in bringing the two bilayers together. The mechanism by which two bilayers of two opposing membranes are fused together is not known, but some important observations have been made:

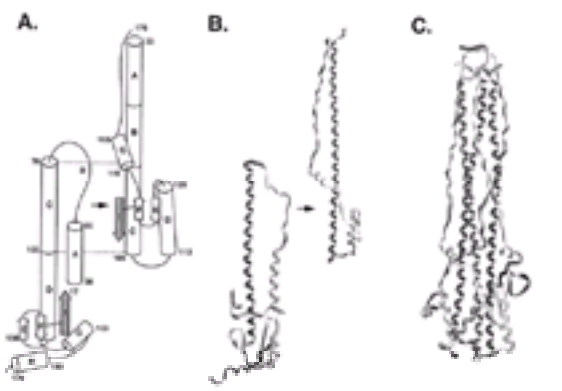

FIG. 4. The three-dimensional structure of the influenza virus HA after low-pH-induced protein refolding. **A:** Schematic diagram drawn from analysis of the x-ray crystallographic data of a proteolytic fragment of HA ($TBHA_2$) in the low pH-induced conformation. Only a monomer is shown. The long a-helix, extending 100 Å from near the proteolytic cleavage site at HA_2 residue 38 to HA_2 residue 105, participates in forming a triple-stranded coiled-coil in the trimer. In addition, the membrane-proximal portion of HA_2 swings up to pack against this long coiled-coil; residues HA_2 106 to 112, helical in the neutral pH structure, form an extended loop. (Redrawn from data of refs. 33 and 40). **B:** The atomic structure of a proteolytic fragment of the low-pH–induced form of HA_2 monomer. **C:** The atomic structure of a proteolytic fragment of the low-pH–induced form of HA_2 trimer. (This figure was produced using coordinate files available from Protein Data Bank coordinate file 1HTM and http://www.crystal.harvard.edu/wiley/coordinate_files.html.)

1. The conformational change in HA has to occur at the right time and in the right place because premature release of the HA fusion peptide causes oligomers to aggregate (294; reviewed in 319), and the refolding of HA_2 to its final and energetically most stable form is irreversible (40).
2. The fusion peptide can intercalate into lipid bilayers (reviewed in 30).
3. Several trimers are required to form a competent fusion pore (72).
4. HA requires its transmembrane domain for complete fusion to occur. A genetically engineered HA anchored to membranes with a glycosyl phosphatidylinositol anchor promotes hemifusion but not complete fusion (150).
5. It has been hypothesized that the free energy released on refolding of HA_2 may be used in the process of membrane fusion (40).

Cleavage Activation of Hemagglutinin and Its Correlation with Pathogenicity

As discussed previously, cleavage of the HA_0 precursor to HA_1 and HA_2 is a prerequisite for the conformational change to the low pH form and hence is a prerequisite for virus infectivity (158,187). Those HAs that contain the sequence

R-X-K/R-R in the connecting peptide are cleaved intracellularly by furin, a protease resident in the trans-Golgi network (TGN) (330; reviewed in 157). Those HAs with a single arginine residue in the connecting peptide (including all three known antigenic subtypes of human influenza) are not cleaved when grown in tissue culture cells (except for strain A/WSN/33; see later), but these HAs can be cleaved by addition of exogenous trypsin. When grown in embryonated eggs, influenza viruses with HA that have a single arginine residue at the cleavage site are cleaved, probably by a factor Xa–like protease (93). After cleavage, the basic residues are lost from the cleavage site, suggesting that two enzymes, the protease and an exopeptidase of the carboxypeptidase B type, are involved in activation of HA (67,87).

It was proposed that the cleavage site would correlate with the virulence of the virus and that virulent strains would contain the furin recognition motif, whereas the avirulent strains would contain only a single arginine residue (18,148); there is experimental support for this notion derived from work done with avian influenza viruses (reviewed in 157 and 329). However, there are complexities to this simple correlation: some avirulent H5 influenza virus strains have a furin recognition site. These avirulent H5 virus HAs contain an oligosaccharide chain positioned near the cleavage site, which is thought to alter accessibility of a protease to the cleavage site, whereas the virulent H5 virus HAs lack this oligosaccharide (146,147).

Among human influenza viruses, only A/WSN/33 (H1N1) can replicate in a variety of cultured cells without addition of trypsin (44), and genetic studies indicate that WSN NA is essential for WSN HA cleavage (303). It has been determined that WSN NA binds and sequesters the serum protease precursor plasminogen (92), a protease previously implicated in HA cleavage in tissue culture cells (189). Presumably, the binding of plasminogen by NA leads to a higher local concentration of the protease precursor. The structural basis for WSN NA binding plasminogen is thought to be the presence of a C-terminal lysine residue, and the absence of an oligosaccharide side chain found on all other N1 subtype NA molecules (92,196).

Influenza C Virus Glycoprotein, HEF

Influenza C virus contains only a single glycoprotein that has three activities: hemagglutinin, esterase, and fusion (HEF). The HEF protein has receptor-binding activity for 9- *O*-acetyl-*N*-acetylneuraminic acid, a fusion activity, and a receptor-destroying activity, which is a neuraminate- *O*-acetyl esterase (and not a neuraminidase) (113,348). The atomic structure of a protease-released form of HEF has been determined (289) (Fig. 5). Although HEF and HA have only about 12% sequence identity, the overall structure and details of the protein folds are quite similar. HEF is a polypeptide of 655 residues and consists of three domains: (1) an elongated stem active in membrane fusion that is closely related to the structure of HA_2 of influenza A virus, (2) a receptor-destroying esterase domain, and (3) a receptor-binding domain. The receptor-binding domain is inserted into a loop of the (esterase) domain, and the esterase domain is inserted into a loop in the stem. The esterase domain of HEF is related in structure to the esterase of *Streptomyces scabies* and a brain acetylhydrolase. HEF is cleaved by a trypsin-like protease (with a concomitant gain of low-pH–induced fusion activity and infectivity) to two disulfide-linked subunits (111,239). The structure of HEF is such that it can be anticipated that HEF will undergo a low-pH–induced conformational change similar to that known to occur for HA. The principal difference is that, unlike HA, the hydrophobic fusion peptide of HEF is not at the immediate N-terminus but instead is located six residues from the N-terminus of HEF. The hydrophobic fusion peptide tucks into the trimer, and the six N-terminal residues of HEF loop back out.

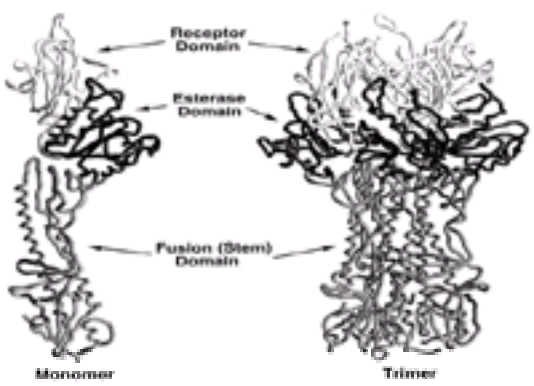

FIG. 5. The structure of the influenza C virus HEF glycoprotein. (**Left:** monomer. **Right:** trimer. Fusion, esterase, and receptor domains are indicated. (This figure was derived from atomic coordinates available from http://www.crystal.harvard.edu/wiley/coordinate_files.html .)

At one time, it was thought possible that intracellular transport of HEF might require the expression of a second viral protein. However, expression of biologically active HEF of influenza C virus strains C/Ann Arbor/1/50 and C/Taylor/1223/47 indicated their cell surface expression did not require coexpression of another viral protein (257).

The Neuraminidase and Its Gene

Introduction

The NA integral membrane protein is the second subtype-specific glycoprotein of influenza A and B viruses. The NA is a homotetramer (M_r about 220,000) containing a head domain that is enzymatically active and a stalk region that is attached to the membrane. NA is important both for its biologic activity in removing sialic acid from glycoproteins and as a major antigenic determinant that undergoes antigenic variation. Neuraminidase (acylneuraminyl hydrolase, EC 3.2.1.18) catalyzes the cleavage of the a-ketosidic linkage between a terminal sialic acid and an adjacent D-galactose or D-galactosamine (94). The role of NA in the influenza virus life cycle is still unclear. Influenza viruses containing a ts

mutation in NA aggregate at the surface of infected cells at the nonpermissive temperature, suggesting that one function of NA is the removal of sialic acid from HA, NA, and the cell surface (248). NA may also permit transport of the virus through the mucin layer present in the respiratory tract, enabling the virus to find its way to the target epithelial cells. Some avian neuraminidases (N1–3, N5–9; N4 not tested) also have a receptor binding site that causes hemagglutination, although the role of this receptor binding function in the influenza virus life cycle is not clear (103,160,186).

RNA Segment 6: Gene Structure and NA Amino Acid Sequence

The nucleotide sequence of all nine NA subtypes has been obtained (reviewed in 47 and 171). For the A/PR/8/34 subtype, RNA segment 6 is 1,413 nucleotides in length and encodes a polypeptide of 453 residues. The NA polypeptide contains only one hydrophobic domain sufficient to span a lipid bilayer, which is located near its N-terminus (residues 7 to 35). This domain acts as a combined uncleaved signal and anchor domain that both targets NA to the membrane of the ER and brings about its stable attachment in the membrane. Thus, NA is oriented with its N-terminus in the cytoplasm, and it is a prototype class II integral membrane protein (Fig. 6). There are five sites for the potential addition of N-linked carbohydrate.

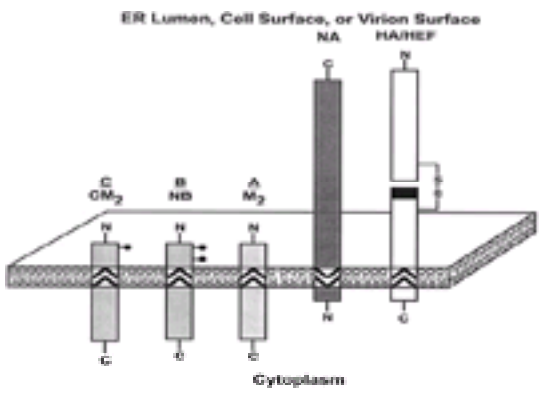

FIG. 6. Schematic representation of the orientation of the influenza A, B and C virus integral membrane proteins HA (HEF), NA, M_2, NB, and CM2.

Comparison of NA sequences among the nine subtypes of influenza A virus and influenza B virus reveals varying degrees of homology for the cytoplasmic tail, transmembrane (TM) domain, stalk, the head overall, and the catalytic active site. The cytoplasmic tail N-Met-Asn-Pro-Asn-Gly-Lys is conserved among influenza A subtypes (perhaps for a role in viral assembly; see later) but not between type A and B viruses. The TM domains of the A virus subtypes and B viruses share only the common property of hydrophobicity and not sequence homology. The stalks show a great deal of variability even between influenza A virus subtypes both in amino acid sequence and in length (62 to 82 residues, and the A/WS/33 stalk is 24 residues). The head domain shows extensive homology (42% to 57%) between influenza A virus subtypes and some homology (29%) between the type A and B viruses.

Influenza B virus RNA segment 6, in addition to containing the open reading frame (ORF) which encodes NA (466 residues), contains a second ORF of 100 residues (NB) that overlaps the NA reading frame by 292 nucleotides (309). The initiation codon used to initiate NA is the second AUG initiation codon from the 5' end of the mRNA. The NB initiation codon is the first AUG codon from the 5' end of the mRNA, and it is separated from the second AUG codon by 4 nucleotides (309). NB and NA proteins accumulate in virus-infected cells in a 0.6:1 ratio, suggesting that about 60% of ribosome preinitiation complexes scanning the NB/NA mRNA do not initiate protein synthesis at the first AUG codon but continue scanning to the second downstream AUG codon (378).

Three-Dimensional Structure of Neuraminidase

NA molecules lacking their TM domain and part of the stalk region can be obtained by pronase digestion. The isolated heads of the molecules retain enzymatic activity and antigenic properties. The protease-released neuraminidases of A/Tokyo/3/67, A/RI/5+/57, A/Tern/Australia/75, and B/Beijing/87, among others, have been crystallized, and from x-ray diffraction data, the three-dimensional structure has been determined to high resolution (19,34,48,49,345) (Fig. 7). The box-shaped head (100 Å × 100 Å × 60 Å) has circular fourfold symmetry stabilized in part by calcium ions believed to be bound on the fourfold axis of symmetry among a cluster of eight acidic groups. Each monomer of NA folds into six topographically identical, four-stranded, antiparallel b-sheets arranged in the manner of the blades of a propeller (345). The amino acids involved in the active site of NA were identified based on locating the substrate sialic acid in the molecule and obtaining an electron density difference map (49). The substrate-binding site was identified as a large pocket on the surface of each subunit rimmed by charged residues and consisting of nine acidic and six basic residues that are conserved between influenza A and B viruses. Mutagenesis of these conserved residues indicate that changes in these sites result in a loss of enzymatic activity (193).

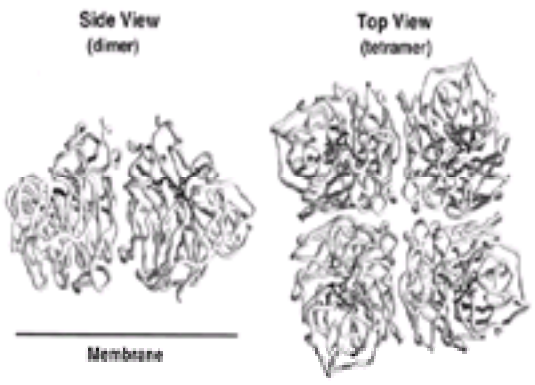

FIG. 7. Structure of the influenza A virus neuraminidase. **Left:** Side view of NA dimer. **Right:** Top down view of NA tetramer. (This figure was produced using Protein Data Bank coordinate file 7NN9.)

The HA activity reported for the N9 subtype of NA is associated with a second sialic acid (Neu5Ac)-binding site on the surface of NA separate from the enzyme active site (362). The x-ray structure of a complex of Neu5Ac with N9 NA indicates Neu5Ac binds in the second site in the chair conformation in a similar way to which Neu5Ac binds to HA (344).

In a manner analogous to that done for HA, antigenic sites were mapped on NA by analysis of changes in naturally existing strains and in variants selected with monoclonal antibodies. Four antigenic sites were identified in influenza A virus NA, each consisting of multiple epitopes (364), and these cluster into the distal surface loops that connect the various strands of b-sheet. Note that antibodies to NA are not neutralizing but rather inhibit plaque size enlargement when incorporated into the overlay medium (154).

The Influenza B Virus NB Glycoprotein

The NB ORF on influenza B virus RNA segment 6 encodes the NB glycoprotein (Fig. 8). NB is an integral membrane protein that is abundantly expressed at the plasma membrane of influenza B virus–infected cells (308), and genetic and biochemical data have indicated that NB is oriented in membranes with an 18-residue N-terminal ectodomain, a 22-residue TM domain, and a 60-residue cytoplasmic tail (377). NB lacks a cleavable signal sequence but instead contains a single hydrophobic domain that is thought both to target NB to membranes and to anchor NB in a stable manner. The N-terminal ectodomain of NB contains two N-linked carbohydrate chains attached to residues 3 and 7 (377). These carbohydrate chains are further modified by the addition of a number of repeating units of galactose b1®4-*N*-acetylglucosamine b1®3 (Gal b1®4-GlcNAc b1®3) attached to a (mannose) $_3$ (GlcNAc)$_2$ core oligosaccharide, known as *polylactosaminoglycan modification* (377,379). Originally, NB was thought to be lacking from virions, but recent analysis of virions, after endo-b-galactosidase digestion to remove polylactosaminoglycan, suggests that NB is a component of virions present at about 100 copies per virion (13,24). The function of NB in the influenza virus life cycle is not known. The influenza B virus NB glycoprotein has the same uncommon membrane orientation (type III integral membrane protein) as the influenza A virus M_2 protein encoded by RNA segment 7 (see later and Fig. 6). The influenza A virus M_2 protein has ion channel activity, but influenza B virus lacks a direct M_2 counterpart. However, because the replication strategies of the influenza type A and B viruses are similar, there has been considerable speculation that the influenza B virus NB protein may have ion channel activity (reviewed in 184). NB has been expressed in bacteria and the purified NB protein incorporated into planar bilayers. Electrophysiologic recordings from these preparations indicated an ion channel activity (335). However, the lack of a selective inhibitor makes it difficult to assign definitively an ion channel activity to NB.

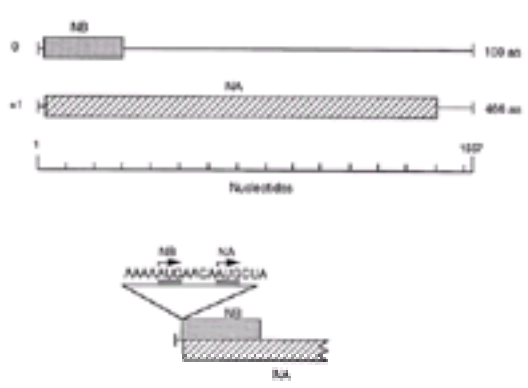

FIG. 8. Schematic representation of open reading frames (ORFs) in influenza B virus RNA segment 6. The NB and NA overlapping reading frames are shown. **Lower section:** Nucleotide sequences surrounding the AUG codons used to initiate the synthesis of NB and NA glycoproteins. (Drawn from data in ref. 308, with permission.)

RNA Segment 7 of Influenza Virus

Introduction

RNA segment 7 of influenza A virus encodes two known proteins, the matrix protein M_1, which lies inside the lipid envelope and constitutes the most abundant polypeptide in the virion, and the M_2 protein, which is a minor component of

virions and has ion channel activity. Three mRNA transcripts have been identified that are derived from influenza A virus RNA segment 7: a colinear transcript encoding M_1 protein, a spliced mRNA encoding the M_2 protein, and an alternatively spliced mRNA ($mRNA_3$), which has the potential to encode a 9–amino acid peptide, but it has not been identified in virus-infected cells. Influenza B virus RNA segment 7 encodes two polypeptides using tandem cistrons: the matrix protein M_1 and the 109-residue BM_2 protein of unknown function. The equivalent RNA segment in influenza C virus (RNA segment 6) is transcribed into a colinear mRNA that encodes the p42 protein. A portion of the colinear transcripts are spliced to form the mRNA encoding the matrix protein. The p42 precursor protein is translated from the colinear mRNA and subsequently processed to yield p31 and CM2 proteins.

RNA Segment 7 Gene Structure and Encoded Proteins

Influenza A Virus

RNA segment 7 of influenza A virus encodes two known proteins, M_1 and M_2. The gene is 1,027 nucleotides in chain length and has one large ORF of 252 residues that encodes the M_1 protein (2,180,382) (Fig. 9). A colinear transcript mRNA encodes the M_1 protein, whereas the M_2 protein is encoded by a spliced mRNA (183). The M_2 mRNA contains a 51-nucleotide virus-specific leader sequence, a 689-nucleotide intron, and a 271-nucleotide [excluding poly(A) tail] body region. The leader sequence of the M_2 mRNA body region encodes 88 residues in the +1 reading frame and overlaps the M_1 protein by 14 residues. A second, alternatively spliced mRNA ($mRNA_3$) has also been identified; it has a 5' leader sequence of 11 virus-specific nucleotides and shares the same 3' splice site as the M_2 mRNA. No evidence has been obtained to indicate that $mRNA_3$ is translated, but if it is, it would yield a 9-residue peptide identical to the C-terminus of the M_1 protein (183).

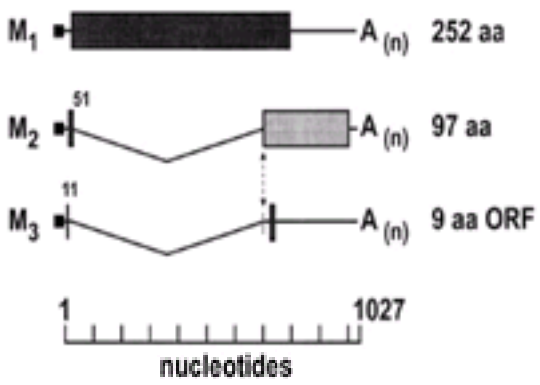

FIG. 9. Schematic diagram of the influenza A virus M_1, M_2, and $mRNA_3$ and their coding regions. Thin lines at the 5'- and 3'-termini of the mRNAs represent untranslated regions. *Shaded* or *hatched areas* represent coding regions in 0 or +1 reading frames, respectively. The introns are shown by the V-shaped lines. Rectangles at 5' ends of the mRNAs represent heterogeneous nucleotides derived from cellular RNAs that are covalently linked to viral sequences. No evidence has yet been obtained that $mRNA_3$ is translated *in vivo.* (Adapted from ref. 182, with permission.)

The M_1 protein contains 252 residues (M_r, 27,801) and is a type-specific antigen of influenza virus, and comparison of its predicted amino acid sequence among influenza A virus subtypes indicates it is highly conserved (reviewed in 171).

Influenza B Virus

RNA segment 7 of influenza B virus encodes two known proteins, M_1 and BM_2. The gene is 1,191 nucleotides in length and contains two ORFs (Fig. 10). The first ORF in the 0 reading frame begins at AUG codon nucleotides 25 to 27 and continues to a termination codon at nucleotides 769 to 771. This ORF encodes the 248–amino acid M_1 protein, 63 residues of which are identical to those of the influenza A virus M_1 protein (27). A second ORF in the +2 frame, and overlapping the M_1 protein ORF by 86 residues, has a coding capacity of 195 residues and is designated BM_2 (27,126). A polypeptide, BM_2, derived from the BM_2 ORF, was identified in cells infected with influenza B virus by using an antisera generated to a b-galactosidase–BM_2 ORF fusion protein (126). The BM_2 protein appears to be a soluble and cytoplasmically located protein of unknown function. A mutational analysis indicates that the BM_2 protein initiation codon overlaps with the termination codon of the M_1 protein in a translational stop-and-start pentanucleotide UAAUG, and that expression of the BM_2 protein requires termination of M_1 synthesis adjacent to the 5' end of the BM_2 coding region; thus, termination of translation and the reinitiation event are tightly coupled (126) (see Fig. 10). In prokaryotes, coupled tandem cistrons with the termination codon of one gene overlapping the initiation codon for a downstream gene is a common situation for coordinating regulated bacterial genes (e.g., *trp* operon; reviewed in 234); however, it is uncommon in eukaryotes.

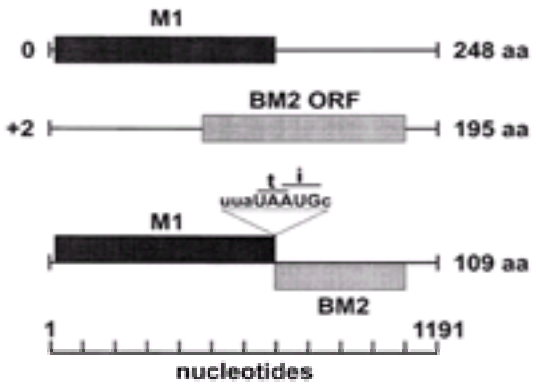

FIG. 10. Schematic representation of the open reading frames (ORFs) in influenza B virus RNA segment 7. **Top two lines:** The ORF encoding the M_1 protein contains 248 residues, and the BM_2 ORF consists of 195 residues (27). **Third line:** the extent of the BM_2 ORF used to translate the BM_2 protein found in influenza B virus–infected cells. The pentanucleotide at which M_1 translation termination (*t; UAA*) and BM_2 translation initiation (*i; AUG*) is shown in capital letters. (Data from ref. 126, with permission).

Influenza C Virus

RNA segment 6 of influenza C virus contains 1,180 nucleotides and contains a single ORF of 374 residues (259,386) (Fig. 11). This RNA segment gives rise to two mRNA species. One is a colinear mRNA that contains a 374–amino acid residue ORF (seg 6 ORF) that is translated to yield the precursor protein, p42. Synthesis of the p42 protein has been detected in influenza C virus–infected cells (122). Proteolytic cleavage of p42 at an internal signal peptidase cleavage site gives rise to the p31 and CM2 proteins (124,259). The second mRNA species that results from splicing of the colinear mRNA (386) encodes the matrix (CM1) protein, consisting of the N-terminal 242 residues of the seg 6 ORF followed by a stop codon introduced through mRNA splicing. The N-terminal product of p42 cleavage, p31, is identical in amino acid sequence to the CM1 protein except for the presence of 17 amino acids at its C-terminus that are mostly hydrophobic. The p31 protein binds tightly to lipid membranes with properties more like those of the integral membrane protein CM2 than those of the peripheral membrane protein CM1, and it is rapidly degraded in virus-infected cells (259,260).

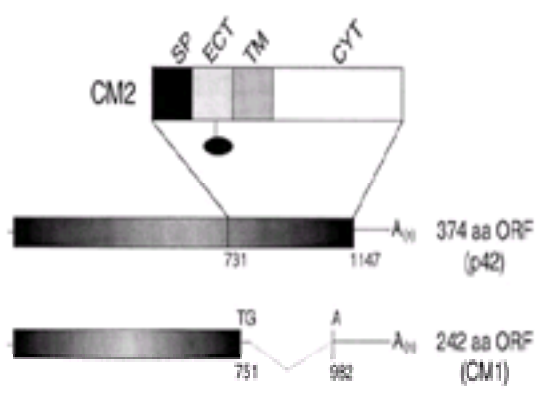

FIG. 11. The CM2 glycoprotein is a translation product of the full-length, colinear mRNA derived from influenza C virus RNA segment 6. Schematic diagram depicting the mRNAs derived from influenza C virus RNA segment 6 and their encoded proteins. The CM2 protein is processed from p42, and CM2 has been shown to contain a cleaved signal peptide (*SP*), an amino-terminal extracellular domain (*Ect*) modified by addition of N-linked carbohydrate (*black oval*), a hydrophobic transmembrane domain (*TM*), and an intracellular cytoplasmic tail (*Cyt*). (Data from ref. 259, with permission.)

The influenza C virus CM2 protein is a small glycosylated integral membrane protein (115 residues) that spans the membrane once. The CM2 protein forms disulfide-linked dimers and tetramers and is oriented in membranes in a NoutCin orientation. CM2 contains a single site for N-linked carbohydrate addition, which is frequently further modified with lactosaminoglycans. The CM2 protein is abundantly expressed at the cell surface of virus-infected and cDNA-transfected cells and is incorporated into influenza C virions (123,258) (see Fig. 6).

The Matrix Protein

It is now virtually dogma that the matrix protein underlies the viral lipid envelope and provides rigidity to the membrane. In addition, it is widely believed that the M_1 protein interacts with the cytoplasmic tails of the HA, NA, and M_2 proteins and also interacts with the RNP. However, experimental evidence for these very plausible interactions has been difficult to obtain. Solubility properties of purified M_1 protein (i.e., soluble in chloroform-methanol and 0.5 M KCl) are consistent with the protein being a peripheral membrane protein. An interaction between M_1 and lipid has been shown *in vivo* using light-activated cross-linking and *in vitro* using purified M_1 protein and liposomes (reviewed in 171). Contacts between the M_1 protein and the RNPs are suggested by finding that purified RNPs often contain M_1 protein; and by using immunoelectron microscopy, it has been found that the RNPs are heavily labeled with M_1 antibody (221). Purified M_1 protein inhibits transcription *in vitro* (390,402), and if M_1 protein is not dissociated from the RNPs *in vivo,* the RNPs fail to be transported to the nucleus (208). At late times after infection, transport of the M_1 protein into the nucleus is required

for exit of the newly assembled RNPs from the nucleus (208) (see later). An interaction between the M_1 protein and RNA has been demonstrated by using filter-binding assays and blotting procedures (351,390). An NLS has been mapped to residues 101 to 105 (RKLKR) (389).

The x-ray crystal structure of the N-terminal portion of the M_1 protein (residues 2 to 158) at low pH has been determined (304) (Fig. 12). Under these conditions the M_1 protein fragment forms a dimer related by a noncrystallographic twofold axis at pH 4.0. Each monomer consists of nine a-helices, eight loop regions, and no b-strands (304). A highly positively charged region (10 residues from each monomer) on the dimer surface is well positioned to bind RNA, whereas the hydrophobic surface opposite the RNA-binding region is hypothesized to be involved in membrane interactions. It is possible that the hydrophobic surface could be buried or exposed after a conformational change (304). The M_1 protein contains a zinc-binding motif (Cys-Cys-His-His type, residues 148 to 162 of influenza virus A/PR/8/34 M_1 protein). However, the x-ray crystallographic structure indicates this sequence does not form a functional zinc finger (304).

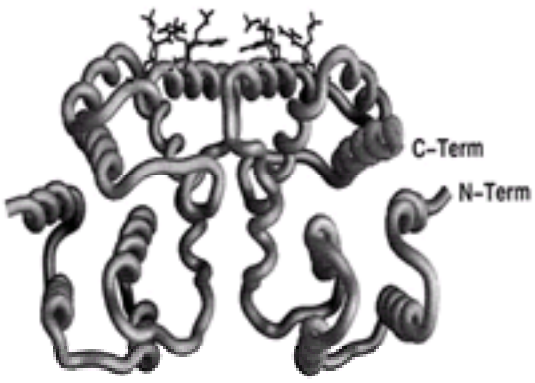

FIG. 12. Three-dimensional structure of the influenza A virus matrix protein. The structure shown is a dimer of M_1 residues 1 to 158. The 9 helical regions in each monomer are clearly visible, and the 10 positively charged residues at the top of the structure that are thought to interact with RNA are shown. (This figure was produced using Protein Data Bank coordinate file 1AA7.)

An interaction between M_1 and the M_2 protein has been suggested based on the observation that virus growth restriction by anti-M_2 antibodies can be overcome by mutations in M_1 (394) (see later). Finally, an interaction has been observed between M_1 and the NS_2 protein found in purified virions (388).

The M_2 Protein and Its Ion Channel Activity

As discussed previously, the M_2 integral membrane protein (97 amino acids) is encoded by a spliced mRNA derived from genome RNA segment 7 (173,183). The M_2 protein is abundantly expressed at the plasma membrane of virus-infected cells but is greatly underrepresented in virions because only a few (on average, 20 to 60) molecules are incorporated into virus particles (185,395,396). In polarized cells, the M_2 protein is expressed at the apical cell surface (128), the surface at which influenza virus particles bud. The M_2 protein spans the membrane once; by using domain-specific antibodies and specific proteolysis, it has been shown that M_2 protein is orientated such that it has 24 N-terminal extracellular residues, a 19-residue TM domain, and a 54-residue cytoplasmic tail (185). The presence of an N-terminal extracellular domain in the absence of a cleavable signal sequence indicates that the M_2 protein is a model type III integral membrane protein that is dependent on the signal recognition particle for cotranslational insertion into the ER membrane (130) (see Fig. 6). The M_2 protein cytoplasmic domain is posttranslationally modified by phosphorylation on a serine (residue 64) (119) and palmitoylation on cysteine (residue 50) (119,333,346), but neither posttranslational modification is required for the function of the M_2 ion channel (38,119,340).

The native form of the M_2 protein is minimally a homotetramer consisting of either a pair of disulfide-linked dimers or disulfide-linked tetramers (9,117,298,334) that form a left-handed four-helix bundle with an interhelix tilt of 20 to 30 degrees (161,262) (Fig. 13).

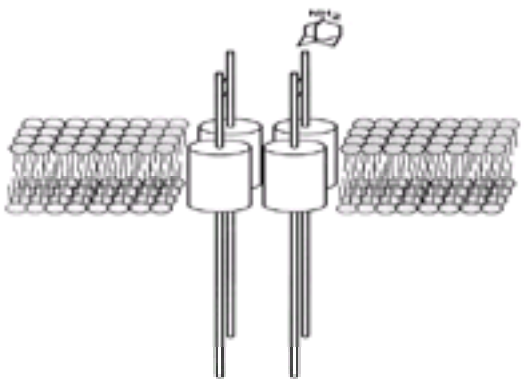

FIG. 13. Schematic diagram of the influenza virus M_2 protein ion channel in a membrane. The influenza virus M_2 protein is a disulfide-linked homotetramer with each chain consisting of 97 amino acid residues with 24 residues exposed extracellularly, a 19-residue transmembrane domain, and a 54-residue cytoplasmic tail. The disulfide bonds can form between the same subunit partners, as shown; otherwise, after the first bond is made, the second disulfide can link to

another partner to form the fully disulfide-linked tetramer. The M_2 protein has a pH-activated ion channel activity that conducts protons. The channel is specifically blocked by the antiviral drug amantadine hydrochloride. The pore of the channel has been shown to be the transmembrane domain of the M_2 protein. The ion channel activity is essential for the uncoating of influenza virus in endosomal compartments in the infected cell. (Drawn from data in ref. 185, with permission.)

The M_2 protein TM domain was deduced to be the target of the anti–influenza virus drug amantadine. Amantadine (1-aminoadamantane hydrochloride) displays a specific anti–influenza A virus action (60) and has been used in the prophylaxis and treatment of influenza A virus infections (reviewed in 243). Mutants that were resistant to amantadine were found to contain amino acid changes in the M_2 protein TM domain (108).

From studies on the effect of amantadine on virus replication (see later), it was hypothesized that the M_2 protein functions as an ion channel that permits ions to enter the virion during uncoating and also to act as an ion channel that modulates the pH of the Golgi apparatus (334; reviewed in 104).

It has been established that the M_2 protein has ion channel activity by experiments in which the M_2 protein was expressed in oocytes of *Xenopus laevis* and in mammalian cells, and measuring membrane currents. This M_2 ion channel activity is blocked by amantadine and is regulated by changes in pH. The channel is activated at the lowered pH found in endosomes and the TGN (43,118,263,352,353 and 354). The ion selectivity of the M_2 ion channel activity indicated the M_2 channel is highly specific for H^+ ions (43,219).

RNA Segment 8 of Influenza Virus: Unspliced and Spliced mRNAs Encode the NS_1 and NS_2 Proteins

RNA segment 8 of influenza A and B viruses and the smallest RNA segment of influenza C virus (RNA 7) encode two proteins, NS_1 and NS_2. NS_1 is encoded by a colinear mRNA transcript, whereas NS_2 is encoded by a spliced mRNA. The finding of a spliced NS_2 mRNA was the first evidence for splicing with an RNA virus that does not use a DNA intermediate in its replication.

RNA Segment 8 Gene Structure and Encoded Proteins

RNA segment 8 of influenza A virus is 890 nucleotides in length and contains a large ORF encoding the NS_1 protein (M_r about 26,000), ranging from 202 to 237 residues depending on the virus. In addition to the NS_1 protein, genetic and biochemical evidence has shown that RNA segment 8 encodes a second protein, NS_2 (M_r about 14,000) and that NS_2 is translated from a small mRNA (350 nucleotides) (131,132,174,177,178). Nucleotide sequencing studies indicated that the NS_1 mRNA is unspliced, directly encoding the NS_1 protein, whereas the NS_2 mRNA contains a 473-nucleotide intron. NS_1 and NS_2 share a 56-nucleotide leader sequence that contains the AUG codon used for initiation of protein synthesis. Consequently, the NS_1 and NS_2 proteins share 9 N-terminal amino acids before the intron; translation of the body of the NS_2 mRNA then continues in the +1 ORF, which overlaps the NS_1 frame by 70 residues (181). The arrangement of the NS_1 and NS_2 mRNAs and their ORFs is shown in Figure 14.

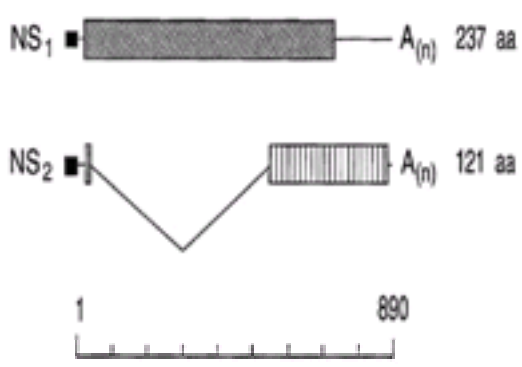

FIG. 14. Model for the arrangement of the influenza A virus NS_1 and NS_2 mRNAs and their coding regions. Thin lines at the 5'- and 3'-termini of the mRNAs represent untranslated regions. *Shaded* or *hatched areas* represent coding regions in 0 or +1 reading frames, respectively. The intron is shown by the *V-shaped line.* Rectangles at the 5' ends of the mRNAs represent heterogeneous nucleotides derived from cellular RNAs that are covalently linked to viral sequences. (Adapted from ref. 177, with permission.)

RNA segment 8 of influenza B virus is 1,096 nucleotides in length and contains a large ORF of 281 residues encoding NS_1 (281 residues; M_r, 32,026). An analogous arrangement of unspliced and spliced mRNA transcripts is found to encode NS_1 and NS_2, as described previously for influenza A virus. The NS_2 mRNA has a 5' leader sequence of 75 nucleotides, which is shared with the NS_1 mRNA, and contains the AUG codon used for initiation of protein synthesis, such that 10 N-terminal residues are shared between NS_1 and NS_2. The 350-nucleotide body region of the NS_2 mRNA encodes in the +1 reading frame 112 residues, overlapping the NS_1 ORF by 52 residues (25,26).

RNA segment 7 of influenza C virus (the equivalent of RNA segment 8 for influenza A and B viruses) contains 934 nucleotides. The NS_1 protein contains 286 residues (225). The NS_2 protein (122 residues) is encoded by a spliced mRNA: 62 N-terminal residues are shared by NS_1 and NS_2, and after the splice junction, translation continues in the +1 ORF for 59 residues. The second ORF of influenza virus RNA segment 7 is completely overlapped by that of the NS_1 protein (226).

The NS_1 Protein

The NS_1 protein (M_r about 26,000) is expressed in large amounts in influenza virus–infected, cells but it has not been detected in virions, hence the designation NS for *nonstructural* (166,188,317). NS_1 is found in infected cells in the nucleus and is associated with polysomes (50,166,168,188). The NLS within NS_1 has been mapped, and it has been found that the protein contains two separate signals (residues 34 to 38 and within residues 203 to 237) (99). The NS_1 protein also contains a nuclear export signal (NES), a short leucine-rich sequence that mediates the nuclear export of proteins (reviewed in 91). The NS_1 NES has been mapped to residues 138 to 147 (198).

Where the NS_1 and NS_2 ORFs overlap (in NS_1 proteins of 237 residues), the amino acid sequences indicate that NS_2 is conserved at the expense of NS_1 (reviewed in 171). These observations support the original suggestion (385) that NS_1 and NS_2 may have been colinear on the vRNA but not overlapping and that read-through of a termination codon at the C-terminus of NS_1 allowed the NS_1 protein to become longer.

NS_1 protein is a multifunctional protein, and many of its functions in the influenza virus life cycle have been determined as described later.

The NS_2 Protein

The NS_2 protein (M_r about 11,000), originally thought to be nonstructural, is now known to exist in virions (130 to 200 molecules on average) and to form an association with the M_1 protein (282,388). The subcellular localization of NS_2 has been indicated to be nuclear (98) and cytoplasmic (324). The NS_2 protein has an NES sequence, and its role in the export of RNPs out of the nucleus is discussed later.

STAGES OF REPLICATION OF INFLUENZA VIRUSES

Virus Adsorption, Entry, and Uncoating

Influenza viruses bind to sialic acid residues present on cell surface glycoproteins or glycolipids through the receptor-binding site in the distal tip of the HA molecules (see Three-Dimensional Structure of Hemagglutinin, earlier). Different influenza viruses have different specificities for sialic acid linked to galactose by a2,3 or a2,6 linkages, and this is dependent on specific residues in the HA receptor-binding pocket (367) (see Fig. 3). Carbohydrate chains of avian intestine contain predominantly sialic acid linked to galactose by a2,3 linkages, pig trachea contain sialic acid linked to galactose by a2,3 and a2,6 linkages, and human trachea carbohydrate chains contain predominantly sialic acid linked to galactose by a2,6 linkages (134). The specificity of HA for sialic acid in a2,3 or a2,6 linkages to galactose is a key determinant in restricting the transfer of influenza virus directly from avian species to humans without mutations in the HA sialic acid–binding site occurring (see Chapter 47). Although the interaction of HA with sialic acid is of fairly low affinity, a high avidity of the virus for cell surfaces is probably achieved by multiple low-affinity interactions.

Influenza viruses enter cells by receptor-mediated endocytosis, the high-capacity endocytic activity by which components of the medium are internalized in clathrin-coated membrane-bound vesicles, formed by invagination of specialized coated-pit domains of the plasma membrane. After internalization, the clathrin coat is removed, and vesicles fuse with endosomes (a series of organelles of increasingly acidic pH), beginning with mildly acidic primary endosomes and progressing to late endosomes. The acidification of endosomes is brought about by H^+-ATPases. The uncoating of influenza virions in endosomes is dependent on the acidic pH of this compartment, and agents such as acidotropic weak bases (ammonium chloride, chloroquine) and carboxylic ionophores (monesin and nigericin) raise the pH of endosomes and block influenza virus uncoating (211). However, these acidotropic weak bases do not have a specific anti–influenza virus effect: raising the intraluminal pH of endosomes blocks the uncoating of most viruses that enter cells by the endocytic pathway (reviewed in 207). For the influenza virus RNPs to penetrate into the cytosol, they have to cross the membrane of the virion and that of endosomes. This is accomplished by HA-mediated fusion of the viral membrane with the cellular membrane (see The Low-pH–Induced Hemagglutinin Conformational Change, earlier) (Fig. 15 and Fig. 16). The precise time and location of penetration depends on the pH dependence of the transition of HA to its low-pH–induced form. For human strains such as A/Japan/305/57, the transition to the HA low-pH form occurs at pH 5.3, which is found in late endosomes and occurs with a half-life of 20 to 35 minutes after virus entry (328).

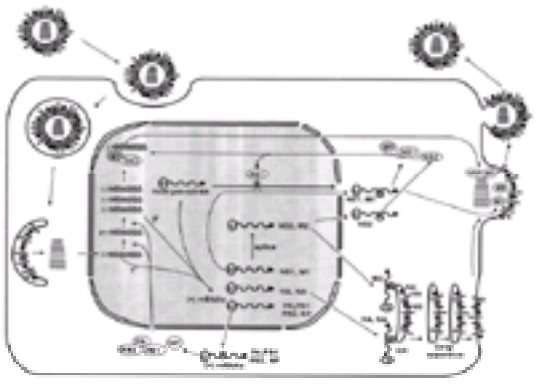

FIG. 15. Schematic diagram of the life cycle of influenza virus. See text for details of the model.

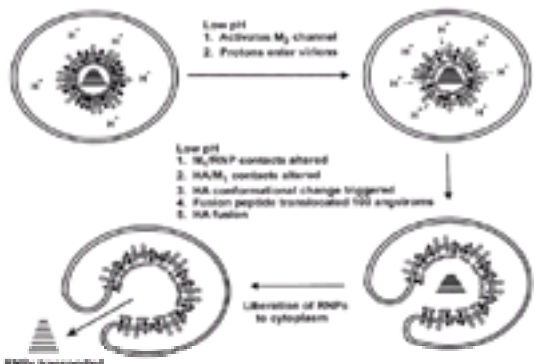

FIG. 16. Schematic diagram of the proposed role of the M_2 ion channel activity in virus entry. The M_2 ion channel activity is thought to facilitate the flow of protons from the lumen of the endosome into the virion interior, bringing about dissociation of protein–protein interactions between the HA cytoplasmic tail and M_1, M_1, and lipid or RNPs and M_1 from the RNPs.

Studies with the antiviral drug amantadine led to the hypothesis that the M_2 ion channel activity is essential for the uncoating process. For all influenza A virus strains, the amantadine block to viral replication occurs at an early stage in the virus life cycle between the steps of virus penetration and uncoating (32,320). In the presence of the drug, the influenza virus M_1 protein fails to dissociate from the RNPs, and transport of the RNPs to the nucleus is blocked (208). Once a virion particle has been endocytosed, the low-pH–activated ion channel activity of the virion-associated M_2 protein (118,263,354) permits the flow of ions from the endosome to the virion interior, to disrupt protein–protein interactions and free the RNPs from the M_1 protein (see Fig. 16) (reviewed in 104,110, and 179). Support for this idea comes from detergent solubilization studies that demonstrate that the interaction between M_1 and the RNPs can be disrupted by mildly acidic pH (397,399). An attractive feature of this hypothesis for virus uncoating is that the low pH of endosomes where uncoating occurs, in contrast to the neutral pH at the plasma membrane where assembly occurs, would push the equilibrium of the disassembly–assembly process in favor of uncoating. Another consequence of the M_2 protein ion channel activity in virions during the uncoating process may be to prepare HA for the fusion process. It has been found that fusion of influenza virus with liposomes is slowed in the presence of amantadine, and it was suggested that intraviral low pH facilitates influenza virus fusion, possibly by weakening interactions of the cytoplasmic tail of HA with the M_1 protein or RNPs (28,369).

Microinjection and immunoelectron microscopy experiments indicate that the RNPs enter the nucleus through the nuclear pore as an intact RNP after the M_1 protein has been dissociated from the RNP. All four proteins that are in the RNP (NP, PB1, PB2, and PA) contain NLS, and the RNPs are actively transported into the nucleus (reviewed in 374). The M_1 protein that has dissociated from the RNP, or newly synthesized M_1 protein, may enter the nucleus by passive diffusion because it is below the 50-kd cutoff for energy-dependent selective nuclear uptake mechanisms, but as discussed previously, a putative NLS in the M_1 protein has been identified. An examination of the fate of the proteins of the infecting virion indicates that whereas HA is degraded in lysosomes (half-life, 120 minutes), more than 30% to 35% of M_1 and NP persists for many hours (209).

Messenger RNA Synthesis and Replication of Virion RNA

Distinctive Feature of Influenza Virus mRNA Synthesis

Unlike other RNA viruses, influenza virus mRNA synthesis has a unique dependence on host cell nuclear function. One early observation was that a-amanatin, at concentrations that specifically inhibit the cellular DNA-dependent RNA polymerase II, inhibits influenza virus mRNA synthesis in infected cells, but not the virus mRNA synthesis that is catalyzed *in vitro* by the virion-associated transcriptase (175,205,325).

The discovery of the role of this host nuclear function came from studies of the virion-associated polymerase (reviewed in 163). The important observation was that this enzyme is incapable of initiating viral mRNA synthesis *in vitro* unless a primer is supplied. The primers are capped (m^7GpppX^m-containing) RNA fragments derived by cleavage of host cell RNA polymerase II transcripts. Hence, influenza virus scavenges host cell RNA polymerase II transcripts to supply the primers

for its own mRNA synthesis. A cap-dependent endonuclease that is intrinsic to the influenza virus polymerase cleaves capped RNAs 10 to 13 nucleotides from their 5' ends, preferentially at a purine residue (266) (Fig. 17). The resulting capped fragments serve as primers for the initiation of viral mRNA synthesis. Priming does not require hydrogen bonding between the capped primer fragments and the 12-nucleotide sequence that is found at the 3' ends of all eight virion (vRNA) segments of influenza A viruses (165). Rather, priming requires the presence of a 5'-methylated cap structure (20,265,266). Transcription is initiated by the incorporation of a G residue onto the 3' end of the resulting fragments, directed by the penultimate C residue of the vRNAs (20,21,114,164,265,266). This process has been called *cap snatching*.

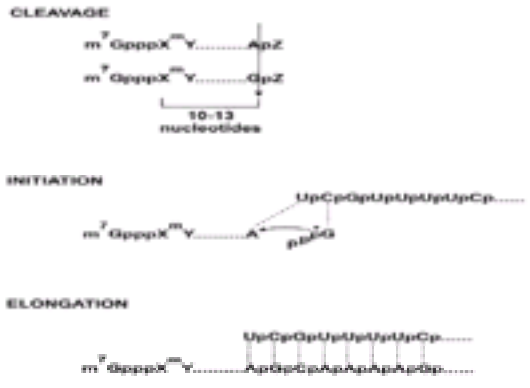

FIG. 17. Influenza virus messenger RNA (mRNA) synthesis requires initiation by capped RNA primers cleaved from cellular nuclear RNAs (see text for details).

Overview of mRNA Synthesis and Virion RNA Replication

Most of our knowledge concerning influenza virus gene expression and RNA replication has been gained from studying influenza A viruses. After priming with 5' capped host-derived mRNA fragments, the mRNA chains are elongated, copying the template up to a point at which a stretch of uridine residues is reached, 15 to 22 nucleotides before the 5' ends of the vRNAs. At this uridine (u) stretch, which ranges in length from 4 to 7 residues in the vRNA segments, transcription terminates, and polyadenylate residues [poly(A)] are added to the mRNAs (105,286) (Fig. 18). Termination occurs apparently as a result of stuttering or reiterative copying of the stretch of U residues, thereby adding a poly(A) tail to the 3' ends of the viral mRNAs (203,273,286). For replication to occur, an alternative type of transcription is required that results in the production of full-length copies of the vRNAs. The full-length transcripts, or *template RNAs,* are initiated without a primer and are not terminated at the poly(A) site used during mRNA synthesis (105,106 and 107). The second step in replication is the copying of the template RNAs into vRNAs (163). This synthesis also occurs without a primer because the vRNAs contain 5' triphosphorylated ends (393). The three types of virus-specific RNAs—mRNAs, template RNAs, and vRNAs—are all synthesized in the nucleus (305,306,323). In the nucleus, the viral mRNAs undergo at least some of the same processing steps as cellular RNA precursors. Internal adenosine residues of influenza virus mRNAs are methylated (167,227), and two of the viral mRNAs are spliced.

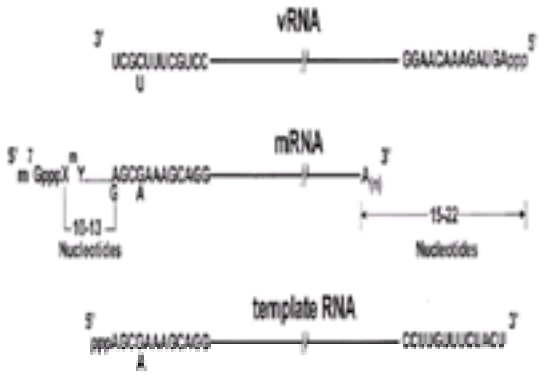

FIG. 18. Schematic diagram to illustrate the differences between influenza virus virion RNA (vRNA) segments, mRNAs, and full-length cRNA or template RNA. The conserved 12 nucleotides at the 3' end and 13 nucleotides at the 5' end of each influenza A virus vRNA segment are indicated. The mRNAs contain an m^7GpppN^m cap structure and, on average, 10 to 13 nucleotides derived from a subset of host cell RNAs (see Fig. 17 and text). Polyadenylation of the mRNAs occurs at a site 15 to 22 nucleotides before the 5' end of the vRNA segment. The template RNA contains at its 5'-terminus pppA, and it is a complete copy of the vRNA segment. Depending on the RNA segment, there is a U/A or C/G at position 4.

Viral mRNA Synthesis

The initial information about the mechanism of viral mRNA synthesis was obtained from *in vitro* studies of the polymerase complex isolated from virions by detergent disruption. The nucleocapsids consist of the individual vRNAs associated with four viral proteins: the NP protein and the three P proteins (PB1, PB2, and PA) (133,343). The P proteins catalyze viral mRNA synthesis, and some of their roles were determined by analyses of the *in vitro* reaction catalyzed by virion nucleocapsids. By using ultraviolet (UV) light–induced cross-linking, it was shown that the three P proteins exist in the form of a complex and that the PB2 protein in this complex recognizes and binds to the cap of the primer RNA

(16,22,343). Cap recognition by PB2 was verified by analysis of the *in vitro* transcription activity of the nucleocapsids derived from PB2 ts mutants (342). The UV–cross-linking experiments also indicated that the PB1 protein most likely catalyzes nucleotide addition (22). No specific role for PA in viral mRNA synthesis has been found.

New insights into the mechanism of mRNA synthesis have been provided by experiments in which viral polymerase complexes are formed using recombinant gene expression, specifically vaccinia virus vectors encoding the three P proteins. When cells are co-infected with three vaccinia viruses encoding the PB2, PB1, and PA proteins, the polymerase complexes that are assembled are inactive in all *in vitro* assays designed to measure the various activities of the polymerase. The polymerase complexes acquire cap-binding and endonuclease activities only when influenza vRNA is present (45,100,194,341). First, binding of the common 5'-terminal sequence of the vRNA segments (AGUAGAAACAAG) activates the capped RNA-binding activity of the PB2 protein. Subsequent binding of the common 3' terminal sequence of the vRNA segments (UCGU/CUUUCGUCC) activates endonuclease activity, thereby enabling the polymerase complex to catalyze capped RNA-primed mRNA synthesis. Consequently, vRNA molecules function not only as templates for mRNA synthesis but also as essential cofactors that activate the catalytic functions of the polymerase that produce capped RNA primers. By means of this control mechanism, the production of primers is activated only when the template for viral mRNA synthesis, namely vRNA, is present.

By using these recombinant polymerase complexes, the sequences of five active sites of the viral polymerase have been identified (194,194a) (Fig. 19). As indicated previously, in the absence of vRNA, the complex of the three P proteins is enzymatically inactive. However, the PB1 subunit of these complexes contains a functional binding site for the 5'-terminal sequence of vRNA. This binding site is composed of an amino acid sequence that is centered around two essential arginine residues at positions 571 and 572. This amino acid sequence, which is conserved in all sequenced influenza A virus PB1 proteins, does not exhibit significant homology to any sequence in the database.

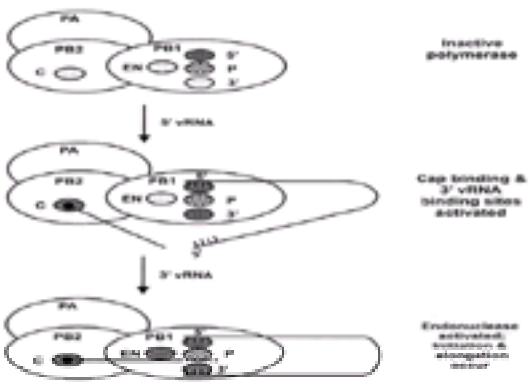

FIG. 19. Activation of the catalytic functions of the influenza virus polymerase by specific vRNA sequences. This model is described in the text. 5', the binding site for the 5' end of vRNA; 3', the binding site for the 3' end of vRNA; C, the binding site for capped RNA; EN, endonuclease active site; P, polymerase active site. *Light gray* denotes an inactive site, and *dark gray* denotes an active site. The P site is shown with interior dots because it is not known whether it is constitutively active or requires activation. The 5' cap structure of the capped RNA is denoted by a *black circle.* (Modified from ref. 194, with permission.)

As a result of binding the 5'-terminal sequence of vRNA, two new functions of the polymerase are activated:

1. An allosteric change occurs in PB2 to activate the cap-binding activity (45,100,194,194a,341), and the region of PB2 important in the process is a tryptophan-rich domain, thought to be analogous to the cap-binding domain in the eIF4E translation initiation factor (206,212).
2. An allosteric change occurs in PB1 to activate the RNA-binding activity specific for the 3'-terminal sequence of vRNA. The region of PB1 involved in this activity is located in a sequence 300 amino acids distant from the PB1 sequence that binds the 5'-terminal sequence of vRNA (194).

When the 3'-terminal sequence of vRNA subsequently binds to PB1, a second allosteric alteration causes the activation of the enzymatic activity that endonucleolytically cleaves capped mRNAs 10 to 13 nucleotides from their 5' end sequences (45,100,194,341). The endonuclease active site, which is in the PB1 protein subunit, contains at least three essential acidic amino acids, comparable to the active sites of other enzymes that cut polynucleotides to produce 3'-OH ends, including RNase H and polynucleotidyl transferases, such as retroviral integrases and bacteriophage Mμ transposase (387).

The capped RNA fragments that are produced by the endonuclease serve as primers for the initiation of viral mRNA synthesis. Nucleotide addition is catalyzed by the PB1 protein, which contains the four conserved sequence motifs of RNA-dependent RNA polymerases (14,269). Mutagenesis experiments established that a core sequence of one of these motifs, namely the SDD sequence at amino acids 444 to 446 of the PB1 protein, is part of the active site for nucleotide addition (14). Thus, the PB1 protein plays a central role in the catalytic activity of the viral polymerase, both in the activation of the enzyme activities that produce capped RNA primers and in the subsequent catalysis of RNA chain elongation.

A key feature of this system is the requirement that the 5' and 3' ends of vRNA bind to the PB1 protein sequentially as ssRNA sequences that are not hydrogen-bonded to each other. These results are not consistent with the original “panhandle” hypothesis, which postulated that the 5' and 3' ends of vRNA, which exhibit considerable complementarity, are hydrogen-bonded to each and that this extensively double-stranded structure binds to the viral polymerase and functions as the “promoter” for the initiation of viral mRNA synthesis (120,127). The existence of such a panhandle

structure requires that the viral polymerase possess a helicase activity that is capable of melting out this structure so that the polymerase can access and copy the bases at the 3' end of the vRNA. However, when the 5' and 3' ends of vRNA are in the form of a hydrogen-bonded structure, the addition of this RNA species to the functional polymerase complexes formed using vaccinia virus vectors does not activate either cap-binding or endonuclease activity (341), indicating that these polymerase complexes lack the requisite helicase activity. Other experiments have also led to modifications of the original “panhandle” model; for example, in the “RNA-fork” model, most of the 5'- and 3'-terminal bases are not hydrogen-bonded to each other (81). Finally, biochemical experiments have shown that the 5' and 3' ends of vRNA molecules in the activated nucleocapsids that are isolated from influenza virions are not juxtaposed unless the polymerase is present (159).

The model for the initiation and subsequent elongation of influenza virus mRNA chains suggests a mechanism for the polyadenylation and termination of synthesis that occur before the 5' end of the vRNA is reached (Fig. 20). It can be postulated that the specific binding of the 5' end of vRNA to the PB1 subunit of the polymerase persists throughout mRNA synthesis. Consequently, the vRNAs would be threaded through the polymerase complex, in a 3' to 5' direction, during mRNA synthesis. Eventually, the polymerase itself would act as a physical barrier to continuation of mRNA synthesis through its site of attachment. This block would result in the reiterative copying of the stretch of U residues in the vRNA template that is adjacent to the polymerase-binding site, thereby producing the poly(A) sequence followed by termination.

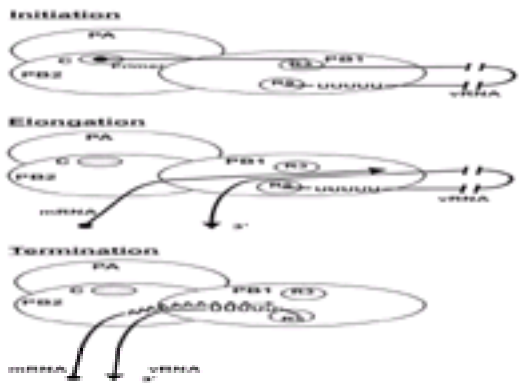

FIG. 20. Model for the elongation and termination of influenza virus mRNA chains. The specific binding of the 5' end of vRNA to the PB1 subunit of the polymerase persists throughout mRNA synthesis, so that the vRNA is threaded through the polymerase complex, in a 3' to 5' direction, during mRNA synthesis. R3 and R5 are the sites on PB1 to which the 3' and 5' ends of the vRNA bind. As a consequence of binding both the 3' and 5' ends of the RNAS, the polymerase itself acts as a physical barrier to continuation of mRNA synthesis, resulting in the reiterative copying of the 5- to 7-U residues that is adjacent to the polymerase-binding site, followed by termination.

Replication of Virion RNA

Replication of virion RNA occurs in two steps: the synthesis of template RNAs, the full-length copies of the vRNAs; and the copying of template RNAs into vRNAs (163). To switch from the synthesis of viral mRNAs to that of template RNAs, it is necessary to change from capped RNA-primed initiation to unprimed initiation and to prevent termination and polyadenylation (antitermination) at the poly(A) site, 15 to 22 nucleotides from the 5' ends of the vRNA templates, that is used during viral mRNA synthesis.

Antitermination requires NP proteins that are not associated with nucleocapsids (11), and this antitermination activity is found in the soluble fraction of infected cell nuclear extracts and can be specifically depleted using an antiserum directed against the NP protein (11). In addition, using a virus containing a ts mutation in the NP protein, template RNA, but not mRNA synthesis, is ts both *in vivo* and *in vitro* (215,306). The most likely explanation for these results is that the nonnucleocapsid NP protein molecules bind to the common 5' ends of the nascent transcripts (11). It seems possible that as additional NP protein molecules are added to the elongating RNAs, they bind to the sequence of 4 to 7 A residues in the mRNA that are copied from the stretch of U residues in the vRNA template. As a consequence, this mRNA sequence is prevented from slipping backward along the vRNA template, and reiterative copying of the stretch of U residues in the vRNA template would be blocked. Supporting data for this hypothesis are awaited, however, and it is not clear how this process would eliminate the physical barrier to the copying of the 5' ends of the vRNAs caused by the polymerase itself. This mechanism predicts that the number of NP protein molecules in infected cells regulates the levels of mRNA synthesis versus genome RNA replication because full-length template RNAs are synthesized only when NP protein molecules are present.

The next step in replication, copying the full-length template RNAs to produce negative-stranded vRNAs, also requires the addition of NP protein molecules to the elongating RNA molecules (306). No vRNA-sense RNAs of discrete sizes are made in the absence of these soluble NP molecules, indicating that elongation of vRNA chains most likely ceases at any time at which NP is not available. Consequently, the newly synthesized vRNAs are in the form of nucleocapsids that can be readily packaged into progeny virus particles.

As noted previously, the switch from mRNA synthesis to the synthesis of full-length template RNAs requires that the polymerase switch from capped RNA-primed initiation to unprimed initiation. The biochemical process by which this occurs is not known, but one hypothesis for this change in initiation is that the form of the polymerase that carries out vRNA replication differs from the one that carries out mRNA synthesis (163). According to this hypothesis, the mRNA synthesis polymerase produces only subgenomic, polyadenylated mRNAs, even in the presence of NP protein molecules. This polymerase requires the PB2 cap-binding protein to produce capped RNA primers, but apparently does

not require the PA protein. In contrast, the replication polymerase, which is present only in infected cells and not in virus particles, requires NP protein molecules to produce full-length template and vRNA strands. This provides an explanation for the fact that vRNA and template RNA are encapsidated with NP subunits, whereas mRNAs are not encapsidated with NP subunits (105,270; reviewed in 163). The replication polymerase initiates the unprimed synthesis of either full-length template RNAs or vRNAs, and presumably requires the PA protein, but not the PB2 cap-binding protein.

Nuclear Export of Ribonucleoproteins

The original model for vRNP nuclear export proposed that the M_1 protein promoted vRNP nuclear export by forming a vRNP-M_1 complex in the nucleus (209) with export occurring through the NES on M_1. Confirming evidence that M_1 is a mediator of vRNP nuclear export was provided when it was found that expressed M_1 can induce the export of nuclear-trapped vRNPs (31). However, vRNP export can occur even when the vast majority of M_1 is trapped in the nucleus by altered hyperphosphorylation (281,373). The M_1 protein lacks an NES, and evidence has been presented that the NES is provided by the NS_2 protein and that vRNP export is mediated by a vRNP-M_1-NS_2 protein complex (236,358) (Fig. 21). The NES in the NS_2 protein would overcome the NLS sequences in the NP and P proteins, a phenomenon that has been observed in other examples of nuclear export (91). The NS_2 protein, which has been shown to bind to the M1 protein (358), contains a leucine-rich region at residues 97 to 105, which is a putative NES for interacting with an exportin, a family of cellular proteins that mediate nuclear export (236); and microinjection of anti-NS^2 antiserum inhibits vRNP export in infected cells (236). There is also the possibility that the NLS sequences in the NP and P proteins are overidden by a different mechanism mediated by the tight binding of NP to actin (64). Thus, in the life cycle of influenza virus, RNPs from input virions are transported into the nucleus, where their transcription and replication occurs, and new RNPs are transported back from the nucleus to the cytoplasm. Thus, knowledge of the switch required for nuclear import and nuclear export of the RNPs is crucial to understanding viral replication.

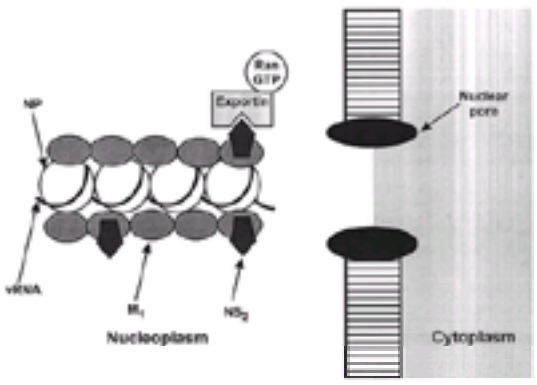

FIG. 21. An M_1-NS_2 protein complex mediates the nuclear export of vRNA-containing nucleocapsids. The current model for export of vRNPs from the nucleus is shown. A nuclear export signal in NS_2 interacts with a member of the cellular exportin family of proteins. The exportin is most likely associated in a complex with RAN-GTP protein to mediate export of the RNP–M_1–NS_2 protein complex from the nucleus. (Modified from ref. 236, with permission.)

Regulation of Viral Gene Expression in Infected Cells

Influenza virus infection can be divided into an early and late phase of gene expression. However, unlike DNA viruses, in which specific patterns of transcription occur before and after DNA replication, the phases of influenza virus gene expression entail quantitative changes of transcription of individual RNA segments as opposed to qualitative changes (176,305,316).

During the early phase, the synthesis of specific vRNAs, viral mRNAs, and viral proteins are coupled (106,305,323). The first event detected after primary transcription is the synthesis of template RNAs, presumably copied off the parental vRNAs. About equimolar amounts of each of the template RNAs are made. The peak rate of template RNA synthesis occurs early and then sharply declines. Specific template RNAs are then selectively transcribed into vRNAs. Specifically, the NS and NP vRNAs are preferentially synthesized early, whereas the synthesis of M segment vRNA is delayed. The rate of synthesis of a particular vRNA correlates with, and therefore most likely determines, the rate of synthesis of the corresponding mRNA and of its encoded protein. The NS_1 and NP mRNAs and proteins are preferentially synthesized at early times, whereas the synthesis of the M_1 mRNA and protein are delayed. Thus, the control of viral protein synthesis during the early phase is predominantly a direct consequence of the regulation of vRNA synthesis, that is, the selective copying of specific template RNAs into vRNAs.

During the late phase, the relationships between the syntheses of vRNAs, viral mRNAs, and viral proteins change dramatically (305). The synthesis of all the viral mRNAs reaches its peak rate at the beginning of the late phase, and the rate of synthesis of all the mRNAs then decreases precipitously. In contrast, the rate of synthesis of all the vRNAs remains at or near maximum during the second phase. Thus, vRNA and viral mRNA synthesis are not coupled during the second phase. In addition, viral mRNA and protein synthesis are not coupled because the synthesis of all the viral proteins continues at maximum levels during the second phase. Previously synthesized viral mRNAs undoubtedly direct viral protein synthesis during the second phase. The pattern of protein synthesis during the second phase differs from that in the first phase. During the second phase, two structural proteins, M_1 and HA, which are poorly synthesized in the first phase, are synthesized at high rates along with the other structural proteins.

As discussed previously, the synthesis of all three types of virus-specific RNAs occurs in the nucleus (163). The template

RNAs, which are synthesized only at early times, remain in the nucleus to direct vRNA synthesis throughout infection (305). In contrast, the vRNAs, which are in the form of nucleocapsids, are efficiently exported to the cytoplasm, particularly at later times (73,306).

A significant part of the replication control system established by influenza virus in infected cells is directed at the preferential synthesis of the NP and NS_1 proteins early and at delaying the synthesis of the M_1 protein (306). The NP protein is synthesized early presumably because it is needed for the synthesis of template RNAs and vRNAs. The NS_1 protein is needed for other early functions (as discussed later). It is likely that the synthesis of the M_1 protein is delayed because this protein stops the transcription of vRNA into viral mRNA (402) and functions in the transport of vRNA-containing nucleocapsids from the nucleus to the cytoplasm (208).

Posttranscriptional Processing of Viral mRNAs

Most pre-mRNAs in higher eukaryotes contain introns that must be precisely removed to generate functional mRNAs (reviewed in 307). Several conserved sequence elements have been found in all introns: 5' splice site, 3' splice site, and the branch site. In mammalian systems, an additional requirement is a polypyrimidine tract, which lies between the branch site and 3' splice site. Influenza virus exploits the host nuclear machinery that splices cellular pre-mRNAs. As discussed previously, in influenza A virus–infected cells, two full-length viral mRNAs, the NS_1 and M_1 mRNAs (encoding the influenza A virus NS_1 protein [NS_{1A}] and the M_1 protein, respectively) are spliced by the cellular splicing machinery in the nucleus to form smaller viral mRNAs that encode two other proteins, NS_2 and M_2, respectively. Whereas the entirety of the population of cellular pre-mRNA molecules is usually spliced, only a portion of the influenza viral precursor NS_1 and M_1 mRNA molecules are spliced, and both the unspliced and spliced viral mRNAs are exported to the cytoplasm (163). Incomplete splicing and nuclear export of unspliced viral pre-mRNAs is not restricted to influenza virus; it also occurs in cells infected with retroviruses, both “simple” retroviruses (such as avian and murine leukemia viruses) and “complex” retroviruses (*Lentiviridae,* which include human immunodeficiency virus type 1) (see Chapter 59).

Regulated Splicing of Viral NS_1 and M_1 mRNAs

In influenza A virus–infected cells, splicing is regulated such that the steady-state amount of the spliced mRNAs is only about 10% of that of the unspliced mRNAs (177,180). It is likely that the extent of splicing of NS_1 (or M_1) mRNA is determined by competition between the splicing rate and the rate of nuclear export of NS_1 (or M_1) mRNA.

The rate of splicing of NS_1 mRNA in influenza virus–infected cells appears to be controlled solely by cis-acting sequences in NS_1 mRNA itself. This conclusion stems from the observation that the rate of splicing of NS_1 mRNA encoded in an adenovirus recombinant is not significantly different from the splicing rate of NS_1 mRNA in influenza virus–infected cells (4 hours after infection) (3).

In contrast to the rate of splicing, the rate of nuclear export of NS_1 mRNA is not intrinsic to the NS_1 mRNA sequence, but rather varies depending on whether the NS_1 mRNA is expressed through a DNA vector or through influenza virus machinery in infected cells. When NS_1 mRNA is expressed with an adenovirus vector (3), its nuclear export is totally blocked because NS_1 mRNA is committed to the splicing pathway (in accordance with the splicesome retention theory). In contrast, in influenza virus–infected cells, nuclear export of NS_1 mRNA is efficient, and the rate of splicing of NS_1 mRNA largely, if not totally, determines the extent of splicing. It is not known how the nuclear export of unspliced NS_1 mRNA is facilitated in influenza virus–infected cells. Viral proteins do not appear to be required for the facilitated nuclear export of unspliced NS_1 mRNA (4).

Control of the Alternative Splicing of M_1 mRNA by Both Viral and Cellular Proteins

The splicing of M_1 mRNA of influenza A virus is complicated by the use of alternative 5' splice sites (183,312) (Fig. 22; see Fig. 10). When the 5' splice site at position 11 (the first virus-coded nucleotide at the 5' end of the mRNA is assigned position number 1) is used, mRNA3 is produced. The mRNA3 5' splice site, CAG\GUAGAU, fits the consensus sequence for 5' splice sites (C/AAAG\GUA/GAGU) closely. By contrast, the 5' splice site for the M_2 mRNA (at position 51), AAC\GUAUGU, does not fit the consensus as well because there is a C rather than a G at the 3' end of the 5' exon. The common 3' splice site, G7G(A)4(U)3GCAG\G, deviates from the consensus (U/CnNC/UAG\G) in that it does not contain an immediately adjacent polypyrimidine tract. When the M_1 gene is expressed in uninfected cells using a transient transfection DNA vector, only the strong mRNA3 5' splice site and not the weaker M_2 5' splice site is used (4,182). This raised the question of how the M_2 mRNA 5' splice site and the mRNA3 5' splice site are used in influenza virus–infected cells.

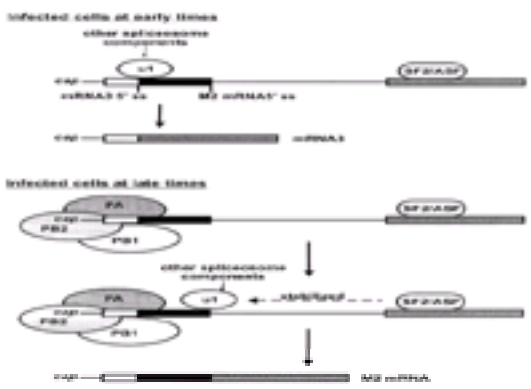

FIG. 22. Mechanism by which the viral polymerase complex and the cellular SF2/ASF splicing factor control the alternative splicing of influenza virus M_1 mRNA. See text for details of the mechanism. (Redrawn from ref. 310, with permission.)

It has been shown that the alternative splicing of influenza viral M_1 mRNA is controlled by both viral and cellular proteins (310,312) (see Fig. 22). The weaker M_2 5' splice site of M_1 mRNA is not used in infected cells at early times of infection because of the presence of the stronger mRNA3 5' splice site. The switch to the M_2 5' splice site occurs at later times in infected cells because the viral polymerase complex binds to a sequence near the 5' end of M_1 mRNA, thereby blocking the mRNA3 5' splice site (312). The polymerase complex binds to the sequence (AGCAAAAGCAGG), which is the complement of the first 12 nucleotides found at the 3' end of each of the virion RNA segments, and it is located immediately 3' to the 5' terminal 10 to 13 nucleotides that are “snatched” from host cell RNAs during viral mRNA synthesis. As a result of this binding, the polymerase protein complex acquires cap-binding activity, thereby enhancing the binding of the polymerase complex to the capped M_1 mRNA. As the cap-binding activity of the polymerase complex is also activated by the common 3'-terminal sequence during the initiation of viral mRNA synthesis (as discussed previously), it is likely that the 5'-viral mRNA sequence and the 3'-terminal sequence of vRNA bind to the same site on the PB1 subunit even though these two RNA sequences differ at 4 out of the 12 positions. Thus, the production of M_2 mRNA is delayed until sufficient amounts of the polymerase complex are synthesized, so that the time course of synthesis and assembly of functional polymerase complexes controls the time at which the M_2 5' splice site can be used in infected cells.

Although the binding of the viral polymerase to the 5' end of the M_1 mRNA causes the M_2 5' splice site to be the only available 5' splice site in the M_1 mRNA molecule, the weak M_2 5' splice site is poorly used unless the cellular SF2/ASF splicing factor is present (310). SF2/ASF is an SR protein, a member of a family of proteins containing a domain rich in serine and arginine and functioning in both alternative and constitutive splicing (83,162). The SF2/ASF protein binds specifically to a purine-rich sequence, called a *splicing enhancer sequence,* in the 3' exon of M_1 mRNA, and this binding, through a series of protein–protein interactions, results in the activation of the M_2 5' splice site (310) (see Fig. 22). Consequently, the control of the alternative splicing of influenza viral M_1 mRNA involves the viral polymerase complex, which determines the time at which the M_2 5' splice site can be activated in infected cells, and the cellular splicing factor SF2/ASF, which controls this activation. The latter function is totally dependent on the former function: SF2/ASF does not activate the M_2 5' splice site of M_1 mRNA when the mRNA3 5' splice site is unblocked. As a consequence of this alternative splicing mechanism, influenza virus has ceded the control of the level of expression of one of its gene products, the M_2 ion channel protein, to a cellular nuclear protein, the SF2/ASF splicing factor. Nonetheless, most of the M_1 mRNA molecules are not spliced, and these M_1 mRNA molecules are transported to the cytoplasm to direct the synthesis of the M_1 protein.

Viral mRNAs Are Not Cannabilized for Their 5' Caps

The viral polymerase exhibits selectivity by “snatching” caps from cellular pre-mRNAs, but not viral mRNAs (163). This selectivity is essential because if the 5' ends of viral mRNAs were also cleaved and used as primers, net synthesis of viral mRNAs would not occur. The mechanism for the selectivity emerged from studying the mechanism of alternative splicing of the viral M_1 mRNA, when it was found that the viral polymerase complex binds to the specific sequence 5' AGCAAAAGCAGG 3' found in all mRNAs that is complementary to nucleotides 1 to 12 of the 3' end of each vRNA segment. This polymerase binding also serves a common function: selective protection of the 5' ends of viral mRNAs, but not cellular pre-mRNAs, from endonucleolytic cleavage catalyzed by the polymerase complex (311). Thus, the three influenza virus P proteins carry out two disparate, opposing functions: the endonucleolytic cleavage of all capped mRNAs, and the selective protection of viral mRNAs, but not cellular pre-mRNAs, against this cleavage (311). The acquisition of these two very different functions stems from the fact that the complex of the three P proteins lacks any detectable activity in the absence of specific vRNA sequences (45,100,194,341) (Fig. 23; see Fig. 22). Influenza virus mRNAs lack the sequence complementary to the 5' end of vRNA; therefore, mRNAs can activate only cap-binding activity and not the endonuclease activity.

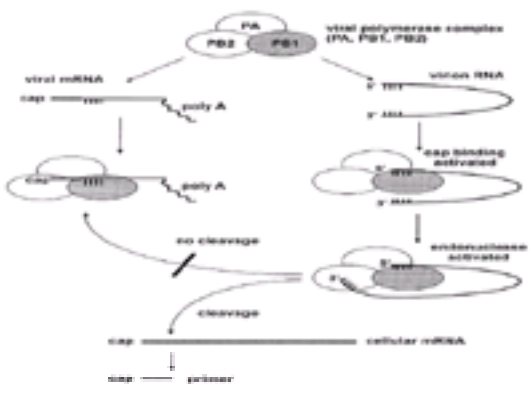

FIG. 23. The influenza viral P protein complex carries out two disparate, opposing functions. The viral P protein complex lacks any detectable activity in the absence of a specific RNA sequence. One specific RNA sequence to which it binds is the 5'-terminal AGUAGAAACAAG sequence present on all eight virion RNA segments. As a result of this binding, the viral P protein complex acquires cap-binding activity. Subsequent binding of the 3' end of the virion RNA results in the acquisition of cap-dependent endonuclease and transcriptase activities. The other specific sequence to which it binds is the AGCAAAAGCAGG sequence that is found both in viral template RNAs, where it is located at the 5' ends (not shown),

and in viral mRNAs, where it is located immediately 3' to the 5' sequence “snatched” from host cell–capped RNAs (shown). As a result of the latter binding, the 5' ends of viral mRNAs are protected against cleavage by the viral cap-dependent endonuclease. In contrast, the 5' ends of host cell mRNAs are not protected and are cleaved by the viral endonuclease. (Redrawn from ref. 311, with permission.)

Splicing in Cells Infected with Influenza B and C Viruses

Splicing also occurs in cells infected with influenza B and C viruses. As with influenza A virus, the NS_1 mRNA of influenza B virus is spliced to form a smaller NS_2 mRNA (25). Both the ORF and the intron of the NS_1 mRNA of influenza virus B/Lee/40 are larger than those of the NS_1 mRNA of influenza virus A/Udorn/72; the sizes of the NS_2 mRNAs of the two virus strains are similar. The 5' splice site, GAG\GUGGGU, of the NS_1 mRNA of influenza B virus fits the consensus sequence closely, whereas the 3' splice site, GAUCGGACAG\U, deviates from the consensus sequence in two ways: (a) absence of a pyrimidine tract immediately upstream of the 39-terminal AG of the intron; and (b) presence of a U rather than a G at the 5' end of the 3' exon. The extent of splicing of the influenza B virus NS_1 mRNA is regulated like that of the influenza A virus NS_1 mRNA; the steady-state level of the spliced influenza B virus NS_2 mRNA is only about 5% to 10% of that of the unspliced influenza B virus NS_1 mRNA (25). In contrast to the M_1 mRNA of influenza A virus, the M_1 mRNA of influenza B virus is not spliced.

Influenza C virus differs from both influenza A and B viruses in that its M_1 protein is not encoded by an unspliced mRNA that is colinear with the virion RNA, but rather is encoded by the mRNA that arises by splicing of the colinear RNA transcript (386). In cells infected with influenza C virus, only a small amount of a colinear M transcript has been detected. This transcript encodes p42, which is processed to p31 and CM2 proteins.

Translational Control Mechanisms

Two major translational controls operate in influenza A virus–infected cells: (a) suppression of the interferon (IFN)-induced block against all protein synthesis, thereby ensuring the efficient translation of virus-specific proteins in infected cells (141,163); and (b) shutoff of the translation of cellular mRNAs, resulting in the selective translation of viral mRNAs (163,176,188,316,317).

Unless a virus-specific defense mechanism were established, overall translation in influenza virus–infected cells would be inhibited because of the production of virus-specific RNA molecules that contain double-stranded regions. The potential for the formation of such double-stranded RNA (dsRNA) molecules stems from the presence of both positive-sense and negative-sense RNAs that are synthesized in the nucleus during infection (163). These viral dsRNA molecules would activate the cellular kinase PKR (141). This dsRNA-activated kinase is expressed constitutively in eukaryotic cells at low levels, and its synthesis is substantially induced by IFN (141,210). Because virus-specific dsRNA also induces IFN (reviewed in 141), increased levels of PKR would be expected to be present in influenza virus–infected cells, and it has been shown that PKR is initially activated during infection (141). Continued activation of PKR would lead to a global shutdown of protein synthesis because activated PKR phosphorylates the a-subunit of the initiation factor eIF-2 (141,210). This initiation factor forms the ternary complex (eIF-2) á g GTP á (met-tRNAi) that binds to the initiating 40S ribosomal subunit before mRNA is bound (135). Phosphorylation of the a-subunit of eIF-2 prevents the recycling of eIF-2–g GDP to form the functional form of eIF-2, eIF-2-GTP (reviewed in 296). Recycling has been shown to be catalyzed by the factor eIF-2B, which is trapped in an inactive complex with eIF-2-GDP when the a-subunit of eIF-2 is phosphorylated (reviewed in 296). Without this recycling of eIF-2 by eIF-2B, protein synthesis initiation is effectively blocked.

Influenza virus mounts a two-pronged attack against the action of PKR. The virus-specific NS_1 protein binds to, and hence sequesters, viral dsRNA molecules, thereby blocking the activation of PKR (102,202). In addition, a 58-kd cellular protein that inhibits PKR function is activated in influenza virus–infected cells (191,192). The experimental evidence indicates that this 58-kd cellular protein interacts directly with PKR, and that influenza virus activates the inhibitory activity of the 58-kd protein by dissociating it from its own natural inhibitor.

In contrast, the mechanism by which viral mRNAs are selectively translated over cellular mRNAs in infected cells is poorly understood. One of the key elements in the shutoff of host cell protein synthesis takes place in the nucleus of infected cells: newly synthesized cellular pre-mRNAs are degraded (143). This degradation is probably initiated by the cleavage of the 5' ends of cellular pre-mRNAs transcripts by the viral cap-dependent endonuclease (163). The resulting decapped RNAs would be more susceptible to degradation by cellular nucleases, as it has been shown that the 5' cap structure stabilizes RNAs against nucleolytic degradation both *in vivo* and in cell extracts (6). This degradation is probably significantly enhanced by the action of the viral NS_1 protein, which blocks the nuclear export of cellular mRNAs (as discussed later). As a consequence, the mRNAs in the cytoplasm of influenza virus–infected cells are composed of cellular mRNAs synthesized before infection (“old cellular mRNAs”) and viral mRNAs synthesized after infection (“new viral mRNAs”). Nonetheless, high levels of these old cellular mRNAs are found in the cytoplasm of infected cells (143). These cellular mRNAs are stable and also functional, as assayed by their efficient translation in reticulocyte extracts *in vitro* (143). Consequently, the shutoff of host cell protein synthesis, which is complete by about 3 hours after infection in several cell lines, probably does not result from the degradation or modification of cytoplasmic old cellular mRNAs. Based on the polysome distribution of several representative cellular mRNAs in uninfected and infected cells, it has been concluded that both the initiation and the elongation step in the translation of old cellular mRNAs are blocked in infected cells (142).

A molecular mechanism for a selective block against the elongation of cellular, but not viral, proteins has not been established. However, selectivity at initiation is probably due at least in part to the fact that influenza viral mRNAs are

efficient initiators of translation (79,142,143). Other possible mechanisms for the selective translational initiation of viral over cellular mRNAs include the following:

1. The inhibition of the activation and activity of PKR may be partial, rather than complete (142,163). Under such conditions, the translation of viral mRNAs would be favored over the translation of cellular mRNAs because influenza viral mRNAs are better initiators of translation (142,143).
2. The NS_1 protein may bind to the 5'-untranslated regions (5'-UTRs) of viral mRNAs, thereby causing, in a manner unknown, the selective translation of these mRNAs (61,74,251). However, no binding of the NS_1 protein to the 5'-UTR in full-length viral mRNAs has been detected (278). Nonetheless, the NS_1 protein may play a role in translation in infected cells because the NS_1 protein appears to be associated with the polysomes of virus-infected cells (50,78,166).
3. Cellular proteins function in the selective translation of influenza virus mRNAs in infected cells. It has been reported that: (a) the cellular RNA-binding protein GRSF-1 specifically binds to the 5'-UTRs of viral mRNAs (252); and (b) the human homologue of the Stauffen protein, which binds to dsRNA, interacts with the NS_1 protein that is associated with polysomes in infected cells (78).
4. One or more cellular protein initiation factors may be modified in influenza virus–infected cells (79), a strategy employed by some other viruses to ensure that their mRNAs are selectively translated (89).

Roles of the NS1 Protein

The nonstructural protein NS_1 of influenza A virus (NS_{1A} protein) was first identified in 1971 (188), but only recently have its multiple functions in the influenza virus life cycle become evident. The observation that provided the initial insight into these functions was that the NS_{1A} protein inhibits the nuclear export of poly(A)-containing mRNAs in transient transfection experiments (4,82,275). Subsequent experiments established that the NS_{1A} protein is a posttranscriptional regulator that has several activities.

Functional Domains of the NS_{1A} Protein

Two major functional domains of the 237–amino acid NS_{1A} protein have been identified. The first is a sequence near the N-terminus that constitutes the RNA-binding domain. The NS_{1A} protein binds with similar dissociation constants to three RNAs: poly(A), a specific stem-bulge in U6 snRNA, and dsRNA (202,278,279). This domain is also required for dimerization of the NS_{1A} protein, the state in which it exists both *in vivo* and *in vitro* (229).

Structural analysis of the RNA-binding domain of the NS_{1A} protein was facilitated by the demonstration that a fragment of the NS1 protein containing the first 73 N-terminal residues (NS_{1A} residues 1 to 73) possesses all the RNA-binding and dimerization activities of the full-length protein (276). Both nuclear magnetic resonance imaging and x-ray crystallography studies demonstrated that the dimeric NS_{1A} residues 1 to 73 RNA-binding domain in the absence of RNA adopts a novel six-helical chain fold (42,200) (Fig. 24). Mutagenesis studies demonstrated that any alanine replacement that causes disruption of the dimer also leads to the loss of RNA-binding activity, indicating that the dimer structure is essential for RNA-binding. Surprisingly, only one amino acid side chain is absolutely required for RNA-binding without significantly affecting dimerization, namely, the arginine side chain at position 38, which is in the second helix of each monomer (see Fig. 24). This result indicates that this arginine side chain probably interacts directly with the RNA target. This interaction is primarily electrostatic because replacement of this arginine with lysine had no effect on RNA binding. A second basic amino acid, the lysine at position 41, which is also in helix 2 (see Fig. 24), makes a strong contribution to the affinity of binding.

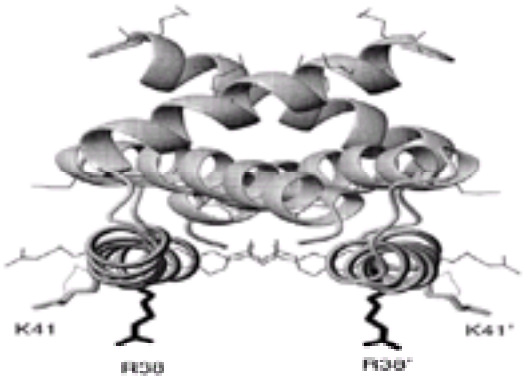

FIG. 24. The three-dimensional structure of the RNA-binding domain of the NS_{1A} protein. The four basic amino acids (R38, R38', K41, K41'), which are required for, or strongly enhance, RNA-binding are shown. All the other solvent-exposed basic amino acids are shown in stick diagram format. (Redrawn from data in refs. 42 and 357, with permission.)

It has been proposed that helix 2 and helix 2', which are antiparallel and next to each other in the dimer conformation, constitute the interaction face between the NS_{1A} RNA-binding domain and its RNA targets (42). This puts the NS_{1A} protein in the small group of proteins that bind to their specific RNA targets through one or more a-helices rather than by b-sheets. The dimeric six-helical NS_{1A} RNA-binding domain almost certainly does not bind in the major groove of a dsRNA target. Rather, at least one basic amino acid (arginine 38, arginine 38'), in each of the antiparallel helices 2 and 2' (see Fig. 24), probably contacts the negatively charged phosphate backbone of dsRNA. Consequently, the dimer structure serves to position arginine 38 on helix 2 so that it is a specific distance (16.5 Å) from the corresponding arginine

38' on helix 2'. This distance is close to that (17 Å) found between the antiparallel phosphodiester backbones surrounding the minor groove of dsRNA. Presumably, the NS_{1A} RNA-binding domain recognizes a comparable type of dsRNA conformation when it binds to all its specific RNA targets.

The second functional domain, which is located in the C-terminal half of the molecule, is not required for RNA binding but is required for the inhibition of the nuclear export of poly(A)-containing mRNAs (275). Although mutagenesis originally identified the amino acid sequence from 134 to 161 as the effector domain (275), it is likely that the effector domain extends from amino acid 74 (at the end of the RNA-binding domain) to amino acid 237, the carboxyl-terminal amino acid. This domain was presumed to be an effector domain that interacts with host nuclear proteins to inhibit nuclear RNA export (see later). On the basis of current information, it can be concluded that all naturally occurring influenza A viruses encode an NS_{1A} protein that contains an effector domain. At one time, it was thought that one naturally occurring influenza virus, A/Turkey/Oregon/71, encoded an NS_{1A} protein lacking the C-terminal sequence containing the effector domain (235). It has since been established, however, that naturally occurring A/Turkey/Oregon/71 virus encodes a full-length NS_{1A} protein, indicating that the previously characterized virus isolate was probably generated during multiple passages in the laboratory (331).

The NS_{1A} Protein Interferes with the Cellular Machinery that Produces the 3' Ends of Cellular mRNAs

The effector domain of the NS_{1A} protein interacts functionally with two essential proteins of the mammalian pre-mRNA 3' end processing machinery: the human 30-kd subunit of the cleavage and polyadenylation specificity factor (CPSF) (228); and poly(A)-binding protein II (PABII) (41). Many of the functions of these two cellular proteins have been established (Fig. 25). The CPSF factor binds to the AAUAAA poly(A) signal located about 15 to 30 nucleotides upstream of the cleavage site in pre-mRNAs and is required for both cleavage and polyadenylation of pre-mRNAs (350). The 30-kd subunit is one of the two subunits of CPSF that has been implicated in the specific binding to the AAUAAA polyadenylation signal (88,149). In contrast, the PABII protein functions after 3' cleavage of the cellular pre-mRNA, and is required for the processive elongation of poly(A) chains catalyzed by the cellular poly(A) polymerase (reviewed in 350). In the absence of PABII, poly(A) polymerase adds only short poly(A) tails to the 3' cleaved pre-mRNA.

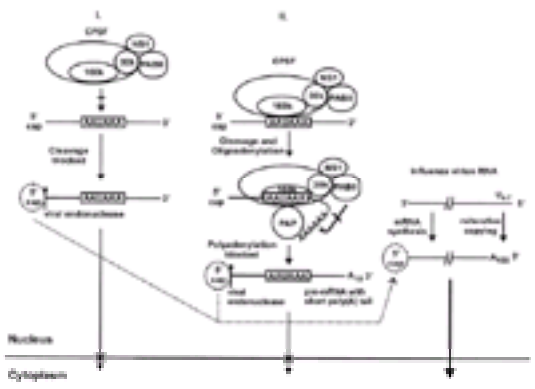

FIG. 25. Proposed two-pronged mechanism by which the NS_1 protein of influenza A virus inhibits the cellular 3' end processing system in infected cells. *Pathway I:* The binding of the NS_1 protein (and PABII) to the 30-kd subunit of cleavage and polyadenylation specificity factor (CPSF) blocks the binding of CPSF to the AAUAAA sequence of some cellular pre-mRNA molecules, thereby blocking 3' cleavage of these pre-mRNAs. The uncleaved pre-mRNAs remain in the nucleus. *Pathway II:* CPSF binds to the AAUAAA sequence of other cellular pre-mRNA molecules, despite the binding of the NS_1 protein and PABII to the 30-kd CPSF subunit. A short poly(A) sequence is then added to these cleaved pre-mRNAs by PAP in a CPSF-dependent reaction. Subsequent elongation of the short poly(A) sequence is blocked by the binding of the NS_1 protein to PABII, resulting in the nuclear accumulation of cleaved pre-mRNAs containing short poly(A) tails. In both pathways, an individual NS_{1A} protein forms a complex with both PABII and the 30-kd subunit of CPSF. In this complex, the two cellular 3'-processing proteins, 30-kd CPSF and PABII, also bind directly to each other. The presence of PABII in the 3'-processing complexes that are formed before 3' cleavage probably results from the binding of PABII to the NS_{1A} protein because PABII has not been found in 3' cleavage complexes formed *in vitro* in the absence of the NS_{1A} protein. Both species of the cellular pre-mRNAs that are sequestered in the nucleus are cleaved by the virion cap-dependent endonuclease to produce the primers required for viral mRNA synthesis. The 3'-terminal poly(A) sequence on viral mRNAs is produced by the viral transcriptase, which reiteratively copies a stretch of 4 to 7 U in the virion RNA templates. The poly(A)-containing viral mRNAs are exported from the nucleus. (Redrawn from refs. 41 and 228, with permission).

Binding of the influenza virus NS_{1A} protein through its effector domain to the 30-kd CPSF subunit dramatically inhibits CPSF function and hence inhibits the 3' end cleavage and polyadenylation of the cellular pre-mRNAs that are synthesized after infection (see Fig. 25, pathway I) (228). Indeed, influenza viruses that contain ts mutations in the NS_{1A} protein coding sequence inhibit 3' cleavage of cellular pre-mRNAs at the permissive, but not at the nonpermissive, temperature (313). Nonetheless, 3' cleavage of some cellular pre-mRNAs still occurs in virus-infected cells, followed by the addition of short poly(A) tails catalyzed by the cellular poly(A) polymerase (see Fig. 25, pathway II) (41). The subsequent processive elongation of these short poly(A) tails does not occur because the NS_{1A} protein inhibits the function of the cellular PABII protein (41).

Both the cellular pre-mRNAs that do not undergo cleavage and those that contain short (about 12 nucleotides) poly(A) tails accumulate in the nucleus of infected cell. The newly synthesized cellular pre-mRNAs that are trapped in the nucleus are almost completely degraded (144). This efficient nuclear degradation probably occurs because the viral

cap-dependent endonuclease removes the 5' terminal cap of the cellular pre-mRNAs and mRNAs (163), thereby facilitating degradation by cellular 5'-3' exoribonucleases in the nucleus. Consequently, little or no cellular mRNAs that are synthesized after infection should survive, and little or no proteins encoded by such cellular mRNAs should be synthesized in influenza A virus–infected cells.

In contrast to cellular pre-mRNAs, the nuclear export of viral mRNAs in infected cells should not be hindered by the NS_{1A} protein because the poly(A) tails of viral mRNAs are produced by the viral transcriptase and not by the cellular 3' end processing machinery (203,273,286).

The NS_{1A} Protein Inhibits the Splicing of Pre-mRNAs

By using transfection experiments, it was established that the NS_{1A} protein inhibits the splicing of cellular pre-mRNAs *in vivo* (82,201) and that purified recombinant NS_{1A} protein inhibits pre-mRNA splicing *in vitro,* catalyzed by nuclear extracts from uninfected cells (201,279). Splicing inhibition *in vitro* is mediated by the binding of the NS_{1A} protein to a specific stem-bulge in U6 snRNA (279), an snRNA that plays a central role in the splicing most of the cellular pre-mRNAs (307). In contrast, splicing inhibition *in vivo* requires the effector domain as well as the RNA-binding domain of the NS_{1A} protein (201).

However, it has not yet been conclusively established that the NS_{1A} protein inhibits the splicing of cellular pre-mRNAs in virus-infected cells. Any such inhibition is obscured by the rapid degradation of cellular pre-mRNAs that occurs in the nucleus of infected cells (143,163). The only evidence for the NS_{1A} protein-mediated inhibition of cellular pre-mRNA splicing in infected cells is that the NS_{1A} protein synthesized during virus infection is specifically associated with U6 snRNA molecules (202). Several important questions about the presumed inhibition of the splicing of cellular pre-mRNAs in virus-infected cells have not yet been addressed. Thus, it is not known how the NS_{1A} protein-mediated inhibition of pre-mRNA splicing would benefit virus gene expression and how the splicing of the viral M_1 and NS_1 mRNAs by the cellular splicing machinery could take place in the face of an overall inhibition of pre-mRNA splicing.

The NS_{1A} Protein Inhibits the Cellular Interferon Response

The several hundred cellular proteins that are induced by IFN-a/b constitute one of the initial host defenses against virus infection (62,327). Reverse genetics experiments have established that the NS_{1A} protein plays a crucial role in protecting influenza A virus against the cellular IFN response: influenza A viruses that lack the NS_{1A} gene replicate only when the cellular IFN response is defective (84).

One IFN-induced pathway involves PKR, and as discussed previously, the RNA-binding domain of the NS_{1A} protein, by binding to dsRNA molecules, inhibits the activation of PKR *in vitro* (101,102,202). Evidence that the NS_{1A} protein carries out this function in influenza virus–infected cells was obtained using mutant viruses that contain ts mutations in the NS_{1A} protein coding sequence (102).

IFN-induced pre-mRNAs are synthesized after infection and consequently should be subjected to the effector domain-mediated inhibition of nuclear export, followed by nuclear degradation (41,143,228,313). As a result, few or no IFN-induced mRNAs should survive, and few or no IFN-induced proteins should be synthesized in influenza A virus–infected cells. Consistent with such a role of the effector domain, laboratory-generated viruses that lack deletions of the effector domain fail to protect the virus against IFN (70). Only in Vero cells, which are defective in IFN production, do these mutant viruses grow as well as wild-type virus in tissue culture. The efficiency of replication of these mutant viruses in MDCK cells, which produce IFN, is inversely correlated with the length of the effector domain of the encoded NS_{1A} protein, and these viruses are attenuated in wild-type mice that produce IFN.

A Latent Nuclear Export Signal in the Effector Domain of the NS_{1A} Protein

An amino acid sequence in the effector domain of the NS_{1A} protein (amino acids 134 to 147) has the same spacing of hydrophobic residues (leucines) as other proven NES sequences (reviewed in 91). When this short NS_{1A} sequence is linked to a heterologous protein, it functions as an NES; that is, it transports the heterologous protein from the nucleus to the cytoplasm (198).

In uninfected cells that are transfected with the NS_{1A} gene, however, the NES of the NS_{1A} protein does not function. In contrast, in influenza virus–infected cells, the NES of a fraction of the wild-type NS_{1A} protein molecules is activated, and a substantial amount of the NS_{1A} protein is found in the cytoplasm. The mechanism by which the NES of the NS_{1A} protein is activated in infected cells has not yet been established. In addition, the functions of the NS_{1A} protein molecules that are exported to the cytoplasm of virus-infected cells have not been identified. Several possibilities have been suggested. For example, the binding of dsRNA by the NS_{1A} protein to inhibit the activation of the PKR kinase, a cytoplasmic enzyme, could occur in the cytoplasm (102,202). Furthermore, as discussed previously, NS_{1A} protein molecules in the cytoplasm may also play other roles in the translation of viral mRNAs.

Comparison of the NS_{1A} Protein with the Influenza B Virus NS_1 Protein (NS_{1B} Protein)

The NS_{1A} and NS_{1B} proteins share little sequence homology, and NS_{1B} proteins are usually larger than NS_{1A} proteins (26). For example, the NS_{1B} protein encoded by B/Lee/40 virus is 281 amino acids long, whereas the NS_{1A} protein encoded by A/Udorn/72 is only 237 amino acids long. However, the NS_{1B} protein binds to the same specific RNA targets as the NS_{1A} protein (356). In addition, as is the case with the NS_{1A} protein, an N-terminal fragment of the NS_{1B} protein

possesses all the RNA-binding and dimerization activities of the full-length protein and is largely a-helical (356). The NS_{1B} fragment is 93 amino acids long, and is thus about 20 amino acids longer than the functional N-terminal fragment of the NS_{1A} protein. A reasonable alignment between the two N-terminal fragments that perfectly aligns helix 2 and 2' can be made (357). Thus, influenza B viruses probably encodes an NS_{1B} proteins with a N-terminal, six-helical RNA-binding domain similar to that of the NS_{1A} protein.

In contrast to the RNA-binding and dimerization domain, the effector domain of the NS_{1A} protein is not conserved in the NS_{1B} protein (356). Thus, unlike the NS_{1A} protein, the NS_{1B} protein does not inhibit either the nuclear export of cellular mRNAs or the splicing of cellular pre-mRNAs (356). Consequently, these effector domain functions are not needed for the propagation of influenza B viruses in nature, and the function of the large C-terminal region of NS_{1B} proteins is not known. All naturally occurring influenza B viruses encode an NS_{1B} protein with such a large C-terminal region (235). This fundamental difference between NS_{1A} and NS_{1B} proteins probably contributes to the different biologic properties of influenza A and B viruses.

Viral Assembly and Release

Intracellular Transport of the Integral Membrane Proteins

The influenza virus integral membrane proteins HA, HEF, NA, M_2, and NB are synthesized on membrane-bound ribosomes and are translocated across the membrane of the ER in a signal recognition particle (SRP)-dependent manner (71,130). For HA, the N-terminal signal sequence is cleaved in the ER by signal peptidase: NA, M_2, and NB do not contain cleavable signal sequences. N-linked carbohydrate chains are transferred to HA, NA, NB, and CM2 (M_2 is not glycosylated) *en bloc* from a dolicyl lipid carrier, and trimming of terminal glucose residues from mannose-rich oligosaccharides occurs in the ER. The HA and NA have been used as prototypes from which much of what we know about the process of folding and oligomerization of an integral membrane protein has been learned. For HA, a large body of data indicates that there is a step-wise conformational maturation of the protein with independent folding of specific domains in the HA monomer, followed by trimerization and the completion of folding to the pH neutral form of HA (reviewed in 66). Once correctly folded and assembled, proteins are transported out of the ER to the Golgi apparatus. There, oligosaccharide chains may be further processed to the complex form. For influenza virus A/Aichi/68 HA, five carbohydrate chains are in the complex form, and two are in the high mannose form (359). HA and NA lack terminal sialic acid on their complex carbohydrate chains in influenza virus–infected cells, presumably because of the action of the viral NA (8,156). Palmitoylation of the three C-terminal proximal cysteine residues of HA (two in the cytoplasmic tail and one in the presumed TM domain) and palmitoylation of the M_2 protein cytoplasmic tail residue 50 (119,333,346) are thought to occur in the cis-Golgi complex. In HAs containing a furin cleavage site, cleavage activation occurs in the TGN (330).

In polarized epithelial cells, influenza viruses assemble and bud at the apical surface of cells (287,290), and it has been shown that HA (influenza A virus), HEF, NA (influenza A virus), M_2, and CM2, when expressed alone from cDNAs in polarized cells, are transported to the apical surface (128,140,291,398). Thus, apical transport is an intrinsic property of these proteins.

The last step in intracellular transport is the expression of the proteins at the plasma membrane. Although HA appears to be diffusely distributed over the surface, M_2, NA, and the influenza C virus HEF glycoprotein appear to cluster in patches (53,54,112,128,140,185,395,396). At the present time, the significance of this cell surface distribution is not known.

Role of M_2 Ion Channel Activity on Maturation of Hemagglutinin in the trans-Golgi Network

Amantadine has a second late effect during the replication of avian influenza virus A/chicken/Germany/34 (H7N1) fowl plague virus (FPV) Rostock. A premature conformational change occurs in its HA in the TGN during the transport of HA to the cell surface (95,96,332; (reviewed in 179). This form of HA is indistinguishable from the low-pH–induced form of HA. Several experiments indicate that the M_2 ion channel activity also functions in the TGN and associated transport vesicles, where it regulates intracompartmental pH and as a result keeps the pH above the threshold at which the FPV Rostock HA conformational change occurs (238,297,332,339; reviewed in 104,179). Consequently, in the presence of amantadine, the low-pH–induced conformational change occurs, and the resulting extrusion of the fusion peptide at the wrong time and in the wrong subcellular compartment results in the aggregation of HA molecules; hence, budding is greatly reduced (292). However, it is clear that the avian influenza virus FPV Rostock, even for an influenza virus with an HA that is cleaved intracellularly, is an exceptional case in requiring a functional M_2 protein ion channel activity in the TGN. Other viruses that also contain an H7 HA do not share such a requirement because their pH of transition to the low-pH–induced form is lower (96).

The Budding Process

Influenza viruses can be observed to bud from the plasma membrane of infected cells morphologically (reviewed in 51) (see Fig. 2). Electron microscopic observations suggest that the precursor to the envelope of a budding virion is a patch of cell membrane containing viral envelope proteins (reviewed in 51). Host cell membrane proteins appear to be excluded from virions. In addition, among virus-encoded proteins, there must be a positive selection or a rejection process. The ratio of M_2 to HA at the plasma membrane is about 1:4, whereas in the virus, it is 1:25 (185,395).

Interactions between the cytoplasmic tails of the viral integral membrane proteins and the internal proteins of the virion likely provide the necessary molecular information for formation of the budding particle. The 10- to 11-residue HA C-terminal cytoplasmic tail and the six residues constituting the predicted NA cytoplasmic tail are highly conserved among the HA and NA subtypes (47,233). Given the mutation rate of the influenza virus RNA genome, this sequence conservation suggests an important function for the HA and NA cytoplasmic tails. To elucidate such a function, reverse

genetics procedures were used to generate influenza viruses that lack either the cytoplasmic tail of HA (136,138) or the cytoplasmic tail of NA (86,217) or both cytoplasmic tails (137). Based on observation of properties of these mutant viruses, a model was proposed in which, for the normal budding of influenza virus, the requirements of the HA and NA cytoplasmic tail interactions with an internal component of the virion (most likely the M_1 protein) are so crucial for normal budding that these two cytoplasmic tail interactions are redundant (137). When both cytoplasmic tails are eliminated, the virions possessed greatly altered morphology (137).

The M_1 and M_2 proteins may also form crucial interactions during virus assembly and budding. It has been found that a monoclonal antibody (14C2) with specificity for the M_2 ectodomain restricts the size of plaque growth of a variety of influenza A virus strains (395). This M_2 antibody reduces the level of cell surface expression of M_2 and reduces the level of influenza virus particle formation in a single cycle of infection (129). Furthermore, this M_2 antibody also reduces the level of filamentous particle formation (285). When variant viruses resistant to the antibody were isolated, they were found to have compensating changes in the cytoplasmic tail of M_2 as well as in the N-terminal domain of the M_1 protein (394). These variant viruses resistant to the antibody retain the filamentous particle phenotype (285). Thus, the evidence suggests important roles for the putative M_1 and M_2 interactions in virus assembly.

The positive selection or exclusion process for inclusion of viral integral membrane proteins into virus particles may be affected by the organization of the lipids in the plasma membrane. It was long thought that the lipids of the plasma membrane functioned mainly as a solvent for membrane proteins (the fluid mosaic model) (315). However, more recently, this view has been refined to include lateral organization resulting from preferential packaging of sphingolipids and cholesterol into moving “platforms,” or “rafts,” in which specific membrane proteins become incorporated (29,314). Rafts can incorporate specific proteins, including many, but not all, glycophosphatidylinositol-anchored proteins and N-terminally myristoylated and palmitoylated cytoplasmic proteins (216; reviewed in 314). Rafts also incorporate some integral membrane proteins. HA and NA enter cholesterol-sphingomyelin–rich raft domains, and it is becoming increasing clear that the rafts are the site of assembly of budding virions at the plasma membrane (300,398). The M_1 protein also associates with these rafts, but this association is decreased in cells infected with viruses lacking both the HA and NA cytoplasmic tails (398). In contrast, the M_2 ion channel protein is largely excluded from rafts (398), thereby providing an explanation for the low amount of M_2 protein in virions as compared with HA (185,395). However, some M_2 is included into virions, and for the positive inclusion of M_2 into virions, it has been suggested that the extracellular domain of M_2 is required (250).

The mechanism by which the bud pinches off from the plasma membrane is not known. For retroviruses, there is a role for monoubiquitination of Gag in the budding process (255), and it is known that monoubiquitination of some receptor protein promotes their internalization and down-regulation (115). Thus, for retroviruses, budding involves cellular machinery, and this is also likely to be the case for influenza virus. To release fully formed influenza virions from the cell surface, the action of the viral NA is thought to be needed because in the absence of NA activity, sialic acid present on the carbohydrate chains of HA and cell surface molecules causes the virus to aggregate to itself and the cell surface through the HA sialic acid receptor–binding pocket (248).

Packaging a Segmented Genome

Production of an infectious influenza virus particle requires incorporation of at least one copy of the eight genomic segments. Nevertheless, random packaging cannot be distinguished from a selective mechanism for inclusion of a full complement of genomic RNAs. A random packaging mechanism in which any eight RNA segments of the genome are incorporated into virions would generate a maximum of 1 infectious particle for every 400 assembled ($8!/8^8$), a number within the range of noninfectious to infectious particles found in virion preparations. If 12 segments can be randomly packaged, 10% of virions would contain one copy of each of the eight segments (77).

A selective mechanism for incorporation of one copy of each RNA segment exists for the three segments of dsRNA of phage f6. The positive strand of each segment is packaged before synthesis of the complementary negative strands within particles. The particle-to-plaque forming unit ratio is close to 1, indicating that essentially all particles contain a complete complement of genome segments. Such precise packaging appears to be due to the serial (sequential) dependence of packaging of the positive-stranded RNA segments with the smallest segment entering first, followed by the medium dsRNA segment and then the largest dsRNA segment (277).

For influenza virus, particles containing more than eight RNA segments (77) and less than eight RNA segments (208) have been obtained consistent with random packaging. However, the specific interference with packaging of wild-type RNA segment 1, but of no other genome segment, by an internally deleted defective RNA segment 1, argues strongly for a specific mechanism of packaging, at least for RNA segment 1 (69,237).

GENETICS OF INFLUENZA VIRUSES

RNA Segment Reassortment

Influenza type A, B, and C viruses can form reassortants *in vivo* between members of the homotypic virus type but not between types. The emergence of new pandemic strains of influenza A virus usually results from such a reassortment. The consequence of this reassortment is the appearance of a new virus subtype containing a novel HA or NA that is immunologically distinct from those of the previous circulating strain. In the 20th century, there was the appearance of “Spanish” influenza in 1918 and 1919, with an HA related to those of swine viruses or H1 subtype viruses. Viruses of this subtype circulated until 1957, when viruses of the H2N2 subtype (Asian strains) were isolated. The H2 subtype HA has little or no cross-reactivity with the H1 HA. In addition to containing an H2 HA, the Asian strains had a new NA (N2). For 11 years, the H2N2 strains of influenza virus spread and changed until the next pandemic in 1968 with the introduction of a new H3 subtype (Hong Kong strains). In 1976, an H1N1 virus reemerged, and in 1997 and 1998, in Hong Kong, a

virulent avian H5 virus crossed the species barrier from birds to humans (see Chapter 47). The extensive changes in the HA or NA that lead to new subtypes of human influenza viruses are known as *antigenic shifts* (reviewed in 152 and 366) (see Chapter 47). These drastic antigenic changes come about from the reassortment of previously circulating human viruses and influenza viruses of animal origin. There is a great deal of evidence for the reassortment of RNA segments between human and animal viruses *in vivo* (365) and among human viruses in nature (392). It has been established that the A/Hong Kong/68 H3N2 virus contains the NA and all genes encoding internal proteins, except that for PB1, from an Asian (H2N2) strain; the genes for HA and PB1 are thought to be derived from a strain that is closely related to Eurasian avian viruses and A/equine/Miami/63 (H3N8 viruses) (145).

Theoretically, it might be expected that *in vivo,* the eight RNA segments of influenza A virus could give rise to 256 possible reassortants. However, a random segregation does not occur because certain viral proteins apparently need their matched strain-specific cognate protein. Nowhere is this more clearly demonstrated than with the requirement of the A/chicken/Germany/34 (H7N1) fowl plague virus Rostock HA (encoded by RNA segment 4), which always cosegregates with its cognate M_2 protein (encoded by RNA segment 7) (95,96).

RNA Mutations

Minor changes in antigenic character of influenza viruses occur as a result of the accumulation of amino acid changes due to mutation; of principal importance are changes in HA and NA. This process is known as *antigenic drift* and occurs with influenza A, B, and C viruses (reviewed in 152 and 366) (see Chapter 47). However, influenza A viruses show a high number of substitutions accumulating in a sequential pattern with time, whereas influenza C viruses isolated decades apart show much lesser degrees of variability (reviewed in 322). The mutation rate for the H3 HA (HA_1 domain) has been estimated to be 6.7×10^{-3} substitutions per site per year. The observation that the nucleotide substitutions in HA were found predominantly in HA_1 suggests that many substitutions may be selected for by the immune pressure (322 and references therein).

Many studies have been performed to analyze the rate of mutation of RNA viruses in tissue culture. Recent calculations, which take into consideration many artifacts of earlier studies, indicate the influenza virus mutation rate at 1.5×10^{-5} mutations per nucleotide per infectious cycle (253). However, although the influenza virus mutation rate is high compared with mammalian DNA genomes (10^{-8} to 10^{-11} per incorporated nucleotide), the mutation rate of influenza A virus is not markedly different from that of other RNA viruses (reviewed in 322).

RNA Recombination

RNA recombination is the joining of two separate RNA chains by crossing-over or copy-choice mechanisms. Historically for influenza virus, the term *RNA recombination* has been used to mean RNA segment reassortment. Unlike the situation with the positive-stranded RNA viruses, the *Coronaviridae* and *Picornaviridae* (see Chapter 23 and Chapter 35), in which RNA recombination occurs at high frequency, until recently, recombination had not been measured for influenza viruses. However, natural examples of RNA recombination have been found when HAs were identified that contain at the HA_1/HA_2 cleavage site either an insertion of 54 nucleotides derived from host 28S ribosomal RNA (151,242) or 60 nucleotides derived from the NP gene (241). In addition, using molecular genetic techniques (see later), RNA recombination within an RNA segment has been shown to occur (12).

Molecularly Engineered Genetics (Reverse Genetics)

The study of viruses and their interactions with host cells and organisms has benefited greatly from the ability to engineer specific mutations into viral genomes, a technique known as *reverse genetics* (368). The negative-stranded RNA viruses present a difficult problem because the virion RNA is assembled with an active transcriptase complex for the genome to initiate replication.

The finding that it was possible to isolate an active polymerase complex from influenza virus RNP cores by using CsCl-glycerol gradient centrifugation (121) made it possible to transcribe *in vitro* short synthetic RNA molecules containing the influenza virus 5'- and 3'-terminal nucleotide sequences (254). It was then shown that an artificial gene construct containing the 5'- and 3'-terminal nucleotide sequences flanking a chloramphenicol acetyltransferase (CAT) reporter gene could be replicated *in vivo* in cells infected with a “helper” virus. The artificial CAT gene was replicated and packaged into virions, and it could be passaged several times in cells (204). These important experiments made it possible to introduce mutations into the influenza virus genome (75). This technology was later improved by finding that the efficiency of isolating a virus containing a foreign gene is enhanced if the transcription of the synthetic RNA is performed in the presence of the influenza virus RNA polymerase complex (76) (Fig. 26). The application of a strong selection pressure against the helper virus is needed to isolate progeny virus containing the plasmid DNA-derived RNA segment, and for this reason, the method has been used mainly for analysis of the surface proteins of the virus, because of strong antibody-dependent selection procedures. Several important discoveries pertaining to individual influenza virus proteins, as well as demonstrating the use of influenza virus to serve as a viral expression vector, have been obtained by application of this reverse genetics technology (7,12,85,195,197,222,245,249,261,280,326,401; reviewed in 231 and 256).

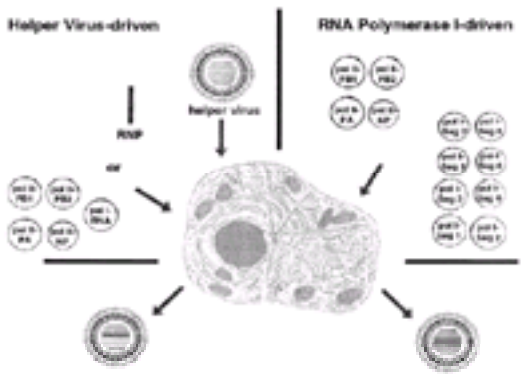

FIG. 26. Influenza virus reverse genetics. The initial replacement of individual RNA segments of influenza virus involved either the *in vitro* reconstitution of vRNPs or *in vivo* assembly of vRNPs after transfection of a cell with plasmids that use pol II promoters driving the expression of the PA, PB1, PB2, and NP proteins, and pol I promoters and terminators controlling viral genome synthesis. Influenza virus rescue entirely from plasmid DNA involves the transfection of plasmids encoding each of the eight RNA segments (under control of the pol I promoter and terminator), four of the proteins that make up the polymerase complex (under control of the pol II promoter) and is aided by, but does not require, the other five viral structural proteins. (Redrawn from ref. 257, with permission.)

Recently, two very similar systems have been established in which helper influenza virus is not needed, making it possible to manipulate every gene in the influenza virus genome (see Fig. 26) (80,232,264). In one system, the host cell is conscripted into making the vRNPs using specific properties of the cellular DNA-dependent RNA polymerase I (pol I) promoter and transcription terminator sequences. The essence of the system is that pol I transcription and termination signals permit the production of artificial influenza virus RNA segments with the same 5'- and 3'-termini as authentic vRNAs. Eight plasmids encoding each of the influenza virus genomic RNA segments under the control of the the pol I promoter and terminator are transfected into cells together with four plasmids encoding the polymerase complex proteins and NP cDNAs under control of an RNA polymerase type II (pol II) promoter (232). In the other system a pol I promoter and aribozyme are used (80,264).

MEANS OF CONTROLLING INFLUENZA VIRUS

Influenza virus causes widespread human disease. In a typical year, influenza virus afflicts 10% -20% of the U.S. population, causing up to 40,000 deaths. During pandemics, a much greater loss of life occurs. The 1918 pandemic was the worse of those pandemic: at least 20 million people died worldwide, many of whom were young adults in the prime of life (56). The reason for the high pathogenicity of this particular virus strain has not yet been ascertained (361). The potential for the reemergence of a highly pathogenic influenza virus strain is exemplified by the sudden appearance of a lethal virus in Hong Kong in 1997, which killed 6 of the 18 infected individuals (46). This virus was transmitted from birds to humans, contrary to the long-held dogma that such a transmission was not possible because the viral receptors on avian cells differ from those on human cells. The spread of infection was controlled because the entire live bird market population in Hong Kong was sacrificed and because the virus did not develop the ability to spread from humans to humans. Usually, influenza virus is readily transmitted from humans to humans by aerosol. For these and other reasons, influenza virus is now considered a dangerous threat as a reemerging viral disease (240).

A detailed description of the means of controlling future influenza virus epidemics is beyond the scope of this chapter. Many reviews of this subject have been published elsewhere (see 35 and 55). Here, we outline a few salient features of the currently employed means of control.

The primary means of controlling influenza virus epidemics at the present time is vaccination (55). The vaccine, which is made up of formaldehyde-fixed virus (killed) or surface glycoproteins (split), has to be reformulated each year because, as discussed previously in this chapter, the viral HA, the protein that elicits the primary neutralizing immune response, usually undergoes changes that render the previous year's vaccine ineffective against the new strain. Each year, the new vaccine takes about 6 months to produce, which is clearly too long to control a fast-moving epidemic. In addition, the 1997 Hong Kong virus demonstrated another difficulty, namely, that it could not be grown in embryonated eggs because it was so pathogenic that it killed the embryos before high levels of virus were produced (338).

In contrast to vaccines, antivirals can be rapidly employed to combat an epidemic, and the efficacy of antiviral therapy against influenza virus has been greatly enhanced because the virus can now be detected at an early time of infection using rapid diagnostic tests (55). Several approaches have been employed to identify compounds that specifically inhibit influenza virus replication. The antiviral drug amantadine (rimantadine) (Fig. 27) exemplifies a compound that was identified by a mass screening empirical approach. As discussed previously, amantadine only inhibits influenza A virus because its target site of action is the M_2 ion channel protein. These drugs have other limitations: they are effective against some, but not all, strains of influenza A viruses; virus mutants that are resistant to these drugs rapidly emerge; and these drugs cause undesirable side effects (e.g., anxiety, lightheadedness, seizures) (55,381).

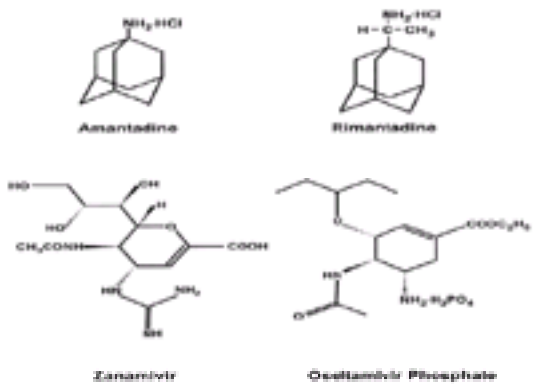

FIG. 27. Influenza virus antiviral compounds. The structures of amantadine, rimantadine (Flumadine), zamanivir (Relenza), and oseltamivir phosphate (Tamiflu) are shown.

Another approach, rational drug design, has been used to devise inhibitors of NA. As discussed previously, the sialic acid–binding site on NA was identified as a large pocket on the surface of each of the four subunits (49). The pocket is lined by amino acids that are invariant in all influenza A and B viruses that have been characterized. The crystal structure indicated that there should be an empty cavity in this pocket when sialic acid is in the pocket, which led to the prediction that substitution of the 4-hydroxyl group of sialic acid with an amino or guanidinyl group would increase the binding interaction of the resulting compound (349). X-ray crystallography confirmed this prediction. Two compounds (4-amino or 4-guanidino 2-deoxy-2,3-didehydro-D- *N*-acetylneuraminic acid) were synthesized, and they were found to be extremely effective inhibitors of NA of both influenza A and B viruses, and these compounds were orders of magnitude less active against an array of nonviral neuraminidases. These compounds, particularly the 4-guanidino derivative (Zanamivir or Relenza; see Fig. 27), inhibit influenza virus plaque formation and virus replication in ferrets (349). This drug has to be administered by nasal spray, and similar rational drug design resulted in another NA inhibitor that can be administered orally, GS4104 (Oseltamivir or Tamiflu) (336; see Fig. 27). Viruses resistant to these NA inhibitors have occurred infrequently *in vitro,* and many appear to map to HA (17,336). However, in the first year of their use (the 1999 to 2000 winter), these two new drugs were not as effective as had been hoped: they shortened the length of influenza virus symptoms by only about 1 day and did not decrease serious complications of influenza virus infections (e.g., pneumonia, hospitalization) (381). Research directed at developing more effective antiviral drugs against influenza virus is continuing.

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CHAPTER 47 Orthomyxoviruses

Peter F. Wright and Robert G. Webster

HISTORY

The highly contagious, acute respiratory illness known as influenza appears to have afflicted humans since ancient times. The sudden appearances of respiratory disease that persist for a few weeks and equally suddenly disappear are sufficiently characteristic to permit identification of a number of major epidemics in the distant past. One such epidemic was recorded by Hippocrates in 412 B.C., and numerous episodes were described in the Middle Ages. Historical data from 1500 until 1800 collected by Webster (518) and Hirsch (219) and reviewed by Potter (405) permit us to establish that features included (a) epidemics occurring relatively frequently but at irregular intervals; (b) epidemics varying in severity but usually causing mortality in the elderly; and (c) some epidemics, such as those in 1781 and 1830, appearing to spread across Russia from Asia. There are similarities with this historical picture and our current knowledge of the epidemiology of influenza—recent epidemics have appeared first in China and usually have had the highest mortality in the elderly.

Although influenza has killed untold millions throughout the centuries, the pandemic of 1918 to 1919 was particularly severe. Not only did Spanish influenza, as it was called, kill between 20 and 40 million people worldwide, it also altered the course of history (89). General Erich F.N. Von Ludenddorff, Chief of Staff of the Imperial German Army, blamed the failure of the Marne Offensive not on the influx of fresh American troops, but rather on the diminished strength of the German Army, which he attributed at least in part to influenza. All the armies in Europe were hit hard by this outbreak; in fact, 80% of the U.S. Army's war deaths were due to influenza.

The severity of the pandemic of 1918 to 1919 greatly accelerated the search for the causative agent of influenza. In the

late 1920s, Richard E. Shope (456) showed that swine influenza could be transmitted with filtered mucus, suggesting that the causative agent was a virus. In 1933, a virus was isolated from humans by Wilson Smith, Sir Christopher Andrewes, and Sir Patrick Laidlaw of the National Institute for Medical Research in London, England (466).

Recent retrospective seroepidemiologic analysis, also known as seroarcheology, suggests that the influenza epidemic in humans in 1889 and 1890 was caused by a virus antigenically similar to more contemporary Asian strains (H2N2) (Fig. 1) (345). The epidemic of 1900 may have been caused by a virus with a hemagglutinin similar to the H3N2 pandemic virus of 1968, A/Hong Kong-like strain, but with a neuraminidase like that of the A/Equine/Miami/1/63 strain. An influenza virus (H1N1) similar to those in swine is believed to have caused the catastrophic pandemic in 1918 to 1919 (89). The seroepidemiologic information is supported by sequence analysis of influenza RNA from lung tissue of humans who died at the peak of the pandemic (415,488).

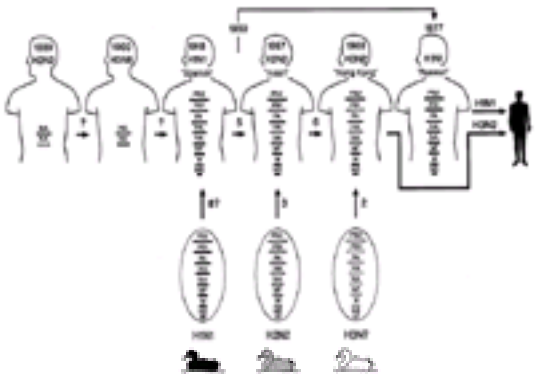


FIG. 1. Postulated evolution of the influenza A viruses currently circulating in humans. Seroarcheology suggests that H2N2 and H3N8 influenza viruses circulated in humans in 1889 and 1900, respectively. Phylogenetic evidence suggests that an influenza virus possessing eight gene segments from avian influenza reservoirs was transmitted to humans and pigs before 1918 and replaced the 1900 strain. This virus was probably carried from North America to Europe by American troops and caused the catastrophic Spanish influenza pandemic of 1918. In 1957 the Asian pandemic virus acquired three genes (PB1, HA, and NA) from the avian influenza gene pool in wild ducks by genetic reassortment and kept five other genes from the circulating human strain (257). After the Asian strain appeared, the H1N1 strains disappeared from humans. In 1968 the Hong Kong pandemic virus acquired two genes (PB1 and HA) from the duck reservoir by reassortment and kept six genes from the virus circulating in humans. After the appearance of the Hong Kong strain, the H2N2 Asian strains were no longer detectable in humans. In 1977 the Russian H1N1 influenza virus that had circulated in humans in 1950 reappeared and spread in children and young adults. This virus probably escaped from a laboratory and has continued to cocirculate with the H3N2 influenza viruses in the human population.

Since 1933, major antigenic shifts have occurred: in 1957 when the H2N2 subtype (Asian influenza) replaced the H1N1 subtype; in 1968 when the Hong Kong (H3N2) virus appeared; and in 1977 when the H1N1 virus reappeared. In 1997 the direct transmission of avian H5N1 influenza viruses to humans (see section on Evolutionary Pathways), with death of six of the 18 infected humans, provided evidence for the role of avian influenza viruses in the emergence of human strains (98,555).

NOMENCLATURE

Influenza viruses belong to the *Orthomyxoviridae* family of RNA viruses and is constituted of four genera: Influenza A virus, Influenza B virus, Influenza C virus, and Thogovirus. Influenza A viruses are subdivided into subtypes, and the current nomenclature system (534) includes the host of origin, geographic location of first isolation, strain number, and year of isolation (Table 1). The antigenic description of the hemagglutinin (HA) and neuraminidase (NA) is given parenthetically [e.g., A/Swine/Iowa/15/30 (H1N1)]. By convention, the host of origin of human strains is not included [e.g., A/Puerto Rico/8/34 (H1N1)]. There are 15 HA and nine NA subtypes; the subtypes differ by 30% or more in amino acid sequence homology (429). No new subtypes have been isolated since 1983, suggesting that there are a finite number of distinct influenza A viruses circulating in aquatic birds.

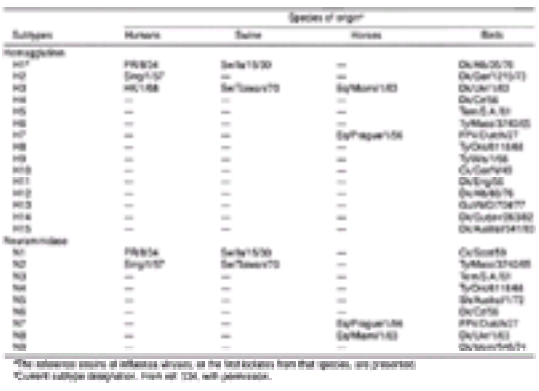


TABLE 1. *Hemagglutinin and neuramindase subtypes of influenza A viruses isolated from humans, lower mammals, and birds*

PROPAGATION

Influenza viruses can be grown in embryonated hens' eggs or in a number of primary tissue-culture systems (for technical details, see 389). Influenza virus was first grown in embryonated eggs (54). Cultivation of influenza viruses in eggs is still the system of choice for vaccine production and for the generation of large quantities of virus used in laboratory studies. Currently, tissue-culture systems [primary monkey kidney and Madin–Darby canine kidney (MDCK) cells] are generally used for the primary isolation of influenza viruses from humans (493). Many, but not all, strains of human influenza A viruses, and most avian influenza viruses, can be isolated directly in the allantoic cavity of embryonated eggs. Some human influenza A and B viruses must first be isolated in the amniotic cavity and subsequently adapted to growth in the allantoic cavity of the chicken embryo. Virus replication in these systems is detected by the ability of an aliquot of culture fluid to agglutinate erythrocytes, suggesting the presence of progeny. On initial passage in eggs, some influenza viruses agglutinate guinea-pig erythrocytes to higher titers than chicken erythrocytes, but on continued passage, the viruses usually agglutinate the latter to higher titers.

Influenza viruses will replicate, cause cytopathology, and produce plaques in a number of primary cell cultures including monkey kidney, calf kidney, hamster kidney, chicken kidney, and primary human respiratory epithelium. However, few cell systems other than primary kidney cells are suitable for plaque formation by influenza viruses unless trypsin is present to activate the HA molecule.

Exceptional viruses produce plaques in chicken embryo fibroblasts without trypsin. Alternative tissue-culture systems used for studies on influenza virus include primary organ cultures (502) and the allantois-on-shell system (133); African green monkey kidney (Vero) cells will support the replication of many influenza A and B viruses (169).

MORPHOLOGY AND NATURE OF THE GENOME

The structure of influenza viruses of types A and B is similar, whereas influenza C reveals a different pattern of surface projections. When examined by electron microscopy, negatively stained preparations of viruses derived from infected eggs or tissue-culture fluids reveal irregularly shaped spherical particles approximately 120 nm in diameter (Fig. 2). In contrast, most influenza virus isolates from humans and other species, after a single passage in culture, show a much greater shape variation, including the presence of greatly elongated forms (70,71). Morphologic examination of primary influenza virus isolates from avian species before passage in culture systems revealed that some strains are predominantly spherical, whereas others are extremely heterogeneous in size with long, filamentous, and bizarrely shaped particles (521). The morphologic characteristics of influenza viruses are a genetic trait (268), but spherical morphology appears to be dominant in passage in chicken embryos or tissue-culture systems. The M gene seems to be a major determinant of this morphologic difference, although the HA and NP genes also may contribute (462).

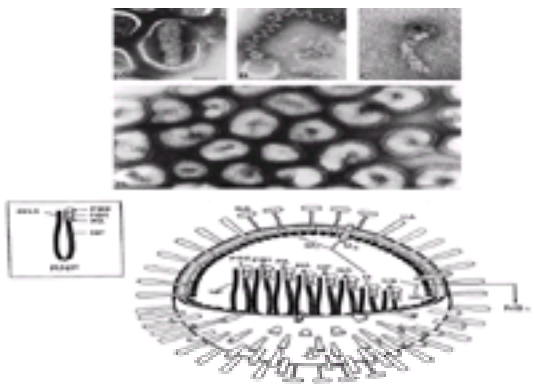


FIG. 2. Structure of influenza virus. Electron micrographs **(top)** and diagram **(bottom)** of influenza virus. **A–C:** The structure of the internal components; **(D)** the external view. A substantial fraction (up to 50%) of influenza virions contain large helical internal components **(A, B)**, which may contain individual ribonucleoprotein (RNP) segments **(C)** linked together. The individual RNPs each contain a binding site for the viral polymerase, as seen by the immunogold labeling of the end of the RNP segment **(C)**. The external view of the virions **(D)** illustrates the pleomorphic appearance and the surface spikes. Bar in all figures equals 50 nm. Courtesy of G. Murti, St. Jude Children's Research Hospital, Memphis, Tennessee. The diagram illustrates the main structural features of the virion. The surface of the particle contains three kinds of spike proteins: the hemagglutinin (HA), neuraminidase (NA), and matrix (M2) protein embedded in a lipid bilayer derived from the host cell and covers the matrix (M1) protein that surrounds the viral core. The ribonucleoprotein complex making up the core consists of at least one of each of the eight single-stranded RNA segments associated with the nucleoprotein (NP) and the three polymerase proteins (PB2, PB1, PA). RNA segments have base pairing between their 3' and 5' ends forming a panhandle. Their organization and the role of NS2 in the virion remain unresolved.

The most striking feature of influenza A and B virions is a layer of spikes projecting radially outward over the surface (357). These surface spikes on influenza A viruses are of two distinct types, corresponding to the HA and NA. The M2 surface component of the virus is present in small numbers and is not found in influenza B strains. Influenza C viruses possess only one type of surface spike, with both HA and esterase activity. On influenza A and B viruses, the HA spike seen by electron microscopy appears rod-shaped, and the NA spike is mushroom-shaped. These differences are seldom, if ever, apparent on intact particles but are revealed after isolation of the proteins after detergent disruption of the virion envelope (549). The HA and NA glycoproteins are attached by short sequences of hydrophobic amino acids to a lipid envelope derived from the plasma membrane of the host cell. Within the lipid envelope lies the matrix protein (M1), which is believed to be structural in function and plays a role in ribonucleoprotein (RNP) nuclear export. Inside the matrix shell are eight single-stranded RNA molecules of negative sense (i.e., the virion RNA is complementary to the messenger

RNA) associated with the nucleocapsid protein (NP) and three large proteins (PB1, PB2, and PA) responsible for RNA replication and transcription. The two virus-coded nonstructural proteins (NS1 and NS2) are found in infected cells.

The organization of the eight single-stranded RNA segments within the virion is still poorly understood. Biochemical and biologic studies support the idea that each RNA segment exists as a distinct ribonucleoprotein complex (82,388), whereas electron microscopic studies have shown that the internal component released from a disrupted influenza virion is a single large helix (8). Genomic RNAs of influenza virus are held in a circular conformation in virions and in infected cells in a terminal panhandle form that might play an important role in replication (229). Up to 50% of virions partially disrupted with Triton X-100 revealed large helical structures (see Fig. 2) (356). These helices also were detectable in spontaneously lysed virions and in thin sections of fixed particles. It is possible that the helices represent the native organization of RNP and that multiple RNPs arise from the degradation of helices. Whether particles with helices are infectious remains to be determined, as does the question as to whether there is a mechanism by which the eight individual RNA segments are packaged or whether this is a random process.

Influenza C virus has only seven RNA segments and contains only one surface glycoprotein (410). Tick-borne viruses (Dhori and Thogoto) are structurally similar to orthomyxoviruses and have six or seven RNA segments. Although the single envelope protein of Dhori virus does not share significant sequence homology with orthomyxoviruses, segments 5 and 6, which presumably code for the nucleoprotein and matrix proteins, respectively, share conserved amino acid sequences with influenza A, B, and C viruses (296,401). These viruses have not yet been given a taxonomic classification. Further information on the nature of the genomes in *Orthomyxoviridae* can be found in the accompanying chapter (see Chapter 46).

EPIDEMIOLOGY AND PATHOGENESIS IN NONHUMAN LOWER ANIMALS AND BIRDS

Influenza A viruses infect swine, horses, seals, and a large variety of birds as well as humans (112). Influenza B viruses infect humans and have been isolated from seals (382). Influenza C virus infects humans, dogs, and swine. Phylogenetic studies of influenza A viruses have revealed species-specific lineages of viral genes and have demonstrated that the prevalence of interspecies transmission depends on the animal species. It also has been revealed that aquatic birds are the source of all influenza viruses in other species, as illustrated in Fig. 3.

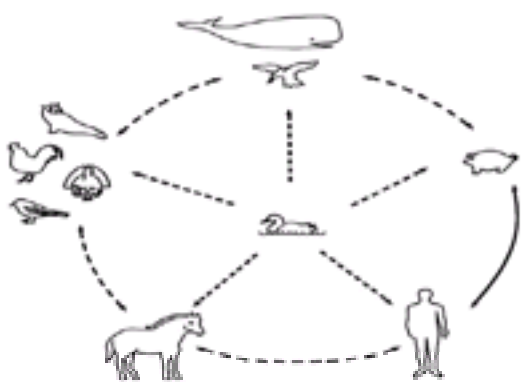


FIG. 3. The reservoir of influenza A viruses. The working hypothesis is that wild aquatic birds are the primordial reservoir of all influenza viruses for avian and mammalian species. Transmission of influenza has been demonstrated between pigs and humans (*solid lines*). There is extensive evidence for transmission between wild ducks and other species, and the five different host groups are based on phylogenetic analysis of the nucleoproteins of a large number of different influenza viruses.

Naturally Occurring Infections

Influenza in Birds

Representatives of each of the known subtypes of influenza A viruses have been isolated from aquatic birds (217). These viruses have been isolated worldwide from both domestic and wild species. The largest number of viruses have been isolated from feral water birds including ducks, geese, terns, shearwaters, and gulls, as well as from a wide range of domestic avian species such as turkeys, chickens, quail, pheasants, geese, and ducks, and less frequently from passerine birds such as starlings (365) and budgerigars (112). The disease signs associated with influenza A virus infections in avian species vary considerably with the strain of virus and species of bird. Infection with most strains of influenza virus are completely asymptomatic. However, a few strains produce systemic infection accompanied by central nervous system (CNS) involvement, with death occurring within 1 week. The viruses that belong in this last category are members of the H5 and H7 subtypes such as A/FPV/Dutch/27 (H7N7) and A/Tern/South Africa/1/61 (H5N3). The most serious problems associated with other influenza A subtypes (H1 through H15) are in turkeys, in which they cause chronic respiratory illnesses. For example, economic losses in a 2-year period in Minnesota exceeded $5 million. Infection in turkeys is thought to be caused by virus strains that are periodically introduced from migrating ducks. Influenza occurs much less frequently in domestic chickens, perhaps because they are housed indoors. However, in 1997, an H5N1 influenza virus gained access to chicken farms in Hong Kong, caused high mortality, and spread to poultry markets and to humans (see later).

In ducks, the majority of avian strains of influenza virus replicate in the lungs and in cells lining the intestinal tract (528). The viruses gain access by passage through the digestive tract of the duck, despite the low pH of the gizzard, and are shed in high concentration in the feces. Influenza A viruses of each of the major subtypes that infect humans (H1N1, H2N2, H3N2) also will replicate in ducks; however, after intranasal administration, these viruses replicate only in the

upper respiratory tract to a limited extent and not in the intestinal tract. Some human strains will replicate to a limited extent in the cloaca if administered rectally.

Studies on wild ducks in Canada from 1975 to 1999 have shown that (a) a high percentage (up to 20%) of juvenile birds are infected with influenza virus when the birds congregate before migration; (b) none of the birds show any symptoms of infection; and (c) many subtypes of influenza virus are enzootic (218). Influenza A viruses have been isolated from water samples collected from lakes in Canada at the time when wild ducks were marshalling before migration. Fecal samples collected from the shores of these lakes also revealed influenza A viruses. The high concentration of duck influenza viruses in fecal material and the isolation of influenza A viruses from lake water offer a logical mechanism for the transmission of avian influenza viruses among feral ducks and to domestic avian and mammalian species. The available information supports the continuous circulation of influenza in migrating waterfowl, with a lower incidence of virus in birds in their overwintering sites in the subtropics.

The avirulent nature of avian influenza infection in ducks may be the result of virus adaptation to this host over many centuries, creating a reservoir that ensures perpetuation of the virus. This speculation strongly suggests that ducks occupy a unique and very important position in the natural history of influenza viruses. Influenza viruses of avian origin have been implicated in outbreaks of influenza in mammals such as seals (523), whales (213), pigs (446), and horses (347), as well as in domestic poultry, especially turkeys.

Influenza in Swine

Swine are susceptible to infection with several different variants of the H1N1 and H3N2 subtypes of influenza A viruses. These include the H1N1 viruses of classic swine influenza, the H1N1 viruses antigenically similar to viruses isolated from avian sources, as well as the H1N1 viruses similar to strains from humans. The H3N2 variants of human influenza viruses also replicate in swine, and reassortants between human swine and avian H1N1 and H3N2 viruses have been detected in pigs (448). Other subtypes isolated from swine include H1N7 and H9N2 (45,393a) but have not established stable lineages.

Influenza in swine was first observed in the United States during the catastrophic 1918 to 1919 human influenza pandemic. The disease signs in pigs, as in humans, are characterized by nasal discharge, coughing, fever, labored breathing, and conjunctivitis (456). The isolation of swine influenza virus [A/swine/Iowa/15/30 (H1N1)] by Shope and retrospective serologic studies on humans indicated that the swine virus was antigenically similar to the type A influenza virus responsible for the human pandemic (345). Analysis of human lung samples from the 1918 Spanish influenza pandemic period by Taubenberger's group (488) established the HA sequence of three 1918 viruses and confirmed the relationship to classic swine influenza virus (415). Since then, swine influenza virus has remained in the swine population, circulating year-round, and responsible for one of the most prevalent respiratory diseases in pigs in North America.

In 1976, swine influenza virus (H1N1) was isolated from military recruits at Fort Dix, one of whom died of pneumonia (164). Antigenically and genetically indistinguishable isolates were subsequently obtained from a man and a pig on the same farm in Wisconsin (214). These studies confirmed the earlier serologic and virus isolation studies that implicated swine viruses in human disease (443). Serologic studies on slaughterhouse workers indicated that transmission of swine influenza viruses to humans occurs quite frequently (up to 20% of workers in 1977 had antibodies to swine influenza virus), but in the recent past, none of these incidents has resulted in an epidemic of disease in humans. However, swine virus occasionally is transmitted to humans and causes respiratory illness (91) and occasionally is lethal (432).

Influenza A viruses of the H3N2 subtype were first isolated from swine in Taiwan by Kundin (278) during an outbreak in humans. Since then, human H3N2 variants have been detected in pigs, usually in serologic studies, for the disease signs are generally mild. In Italy the A/Port Chalmers/1/73 (H3N2) virus established a stable lineage in pigs (64,65), and variants and reassortants containing avian H1N1-like genomes continue to circulate (62). In 1998 two new H3N2 reassortant influenza viruses emerged in American pigs (557). One reassortant [A/swine/Texas/4199-298 (H3N2)] contained the HA, NA, and PB1 genes from 1995 human H3N2 influenza viruses, the NP, M, and NS gene segments from classic swine influenza virus, and the PB2 and PA genes from avian sources. The second reassortant contained the HA, NA, and PB1 gene segments from human H3N2 virus and the remaining genes from classic swine influenza virus. Both reassortants caused respiratory disease and abortion in pigs; serologic surveillance indicates that the first of these [e.g., A/Swine/Texas/4199-298 (H3N2)] has become widespread in pigs in the U.S.

In 1979 an avian H1N1 influenza virus was transmitted to pigs in Europe and established a stable lineage that continues to circulate in pigs throughout Europe (448). H1N2 reassortants containing HA genes related to human H1N1 strains have been detected in pigs in Europe and Japan (44,481), each with different internal genes. Thus reassortant influenza viruses between H1N1 and H3N2 influenza viruses from pigs, humans, and avians are increasingly being detected, and some of these reassortants have been transmitted to and caused typical influenza in children (72). These observations lend support to the hypothesis that pigs may serve as an intermediate host facilitating interspecies transmission and serve as a “mixing vessel” for generation of reassortant viruses (448). The possession of receptors in pigs for both avian (*N*-acetylneuraminic acid, a 2,3 galactose) and human (*N*-acetylneuraminic acid, a 2,6 galactose) influenza viruses (232) again supports this contention. The direct transfer of H5N1 avian influenza viruses to humans in Hong Kong (98) indicates that pigs are not always involved in interspecies transmission but may be involved in adaptation that permits human-to-human transfer. Although the role of pigs in the emergence of human influenza virus is not fully resolved, the available evidence indicates that continued surveillance is important for both human and swine influenza preparedness.

Influenza in Horses

Although influenza virus was not isolated from horses until 1956, there are records indicating that influenza virus probably infected horses centuries ago (49). Two different subtypes of influenza A viruses infect and cause disease in horses, A/Equine/Prague/1/56 (H7N7) and A/Equine/Miami/1/63 (H3N8) (347). The former is commonly known as

equine-1, and the latter, as equine-2. Both viruses produce similar disease signs in horses, but equine-2 infections are usually more severe. The signs of disease include a dry hacking cough, fever, loss of appetite, muscular soreness, and tracheobronchitis. Secondary bacterial pneumonia almost always accompanies equine influenza. The recommendation to horse owners is that all equines recovering from influenza must be rested for 3 weeks after infection (49), or permanent disability can occur. A high incidence of inflammation of the heart muscle (interstitial myocarditis) has been recorded in horses during and after A/Equine/Miami/1/63 (H3N8) (equine-2) infections (155). International transport of horses to competitive events and breeding occurs globally, and the replication of viruses without disease signs in vaccinated horses now plays a part in international virus transmission (347).

Formalin-inactivated vaccines are available, but these vaccines in horses induce immunity that lasts only 3 months (50). The available evidence indicates that the short-lived immunity is a property of the immune response of horses rather than low vaccine potency or genetic drift (346). Antigenic drift is not extensive and thus is probably not the major factor in short-lived immunity to influenza viruses in horses (25,347). Since 1987, equine-2 influenza viruses have evolved into two lineages; isolates from Europe, Mongolia, and China belong to the “Eurasian family,” and those from the U.S. and Argentina to the “American family” (92).

Equine-1 influenza viruses have not been isolated from most countries since 1980, and the possibility has been considered that they are extinct or nearly so (519). However, unconfirmed reports of equine-1 from India and Egypt (231,460) raise the possibility that isolated pockets may still exist. Additionally, antibodies to equine-1 influenza virus have been detected sporadically in horses, either from vaccination or from subclinical infections. In 1989 a new H3N8 influenza virus appeared in horses in Northern China; it derived all of its gene segments from an avian influenza virus (183). This virus initially caused severe respiratory disease, with 20% mortality, but it is unknown whether this variant has established a stable lineage in horses. It is noteworthy that avian influenza viruses are replication competent in equine tracheas without producing disease signs (347), but to date only the H3N8 and H7N7 subtypes have been established in horses.

Influenza in Seals, Whales, and Mink

In 1979 to 1980, approximately 20% of the harbor seal (*Phoca vitulina*) population of the northeast coast of the U.S. died of a severe respiratory infection with consolidation of the lungs typical of primary viral pneumonia (153). Influenza virus particles were found in high concentrations in the lungs and brains of the dead seals. Antigenic and genetic analysis (523) established that this virus derived all its genes from avian influenza viruses and was associated with severe disease in mammals. Biologically, the virus behaved more like a mammalian strain of influenza, replicating to high titers in ferrets, cats, and pigs. In humans, the replication of A/Seal/Massachusetts/1/80 (H7N7) influenza virus is confined to the eyes and causes conjunctivitis. Infected persons recovered without complications, but antibodies to the virus were not detectable in hemagglutination inhibition tests (522), which has since been shown to reflect the inadequacy of this test in the detection of antibodies to avian influenza viruses in mammals (252). In squirrel monkeys, the seal influenza virus replicates in the lungs and nasopharynx after intratracheal administration and in the conjunctiva after administration to the eye (350). In this study, one monkey died of pneumonia, and the seal influenza virus was recovered from the spleen, liver, muscles, and lung, indicating that the virus has the capability for systemic spread in primates. Influenza viruses have subsequently been isolated from seals found dead on the New England coast of the U.S., including H4N5 in 1983, H4N6 in 1991, and H3N2 in 1992 (215).

Influenza A viruses (H13N2 and H13N9) have been isolated from the lungs and hilar nodes of one stranded pilot whale. The relationship between influenza virus infection and stranding remains unknown. Genetic analysis of the H13N9 whale virus has shown that the virus was introduced from birds (213). Influenza A viruses (H1N3) also have been isolated from the lungs and a liver of whales (Balaenopteridae) in the South Pacific (303).

Influenza viruses have been isolated from mink, which together with ferrets, belong to the genus *Mustela* and are naturally susceptible to influenza viruses. These viruses, which were of avian origin (H10N4), caused systemic infection and disease in the mink and spread to contacts (274). Collectively, these findings demonstrate that interspecies transmission of influenza A viruses occurs relatively frequently, mainly from aquatic birds to mammalian species. The epidemics tend to be self-limiting, and the newly introduced viruses are not maintained in animal species such as seals, whales, and mink.

Experimental Infections

Influenza in Mice

Mice are not naturally infected with influenza viruses but can be experimentally infected with both influenza A or B viruses. Newly isolated human influenza A viruses cause an inapparent infection of the upper and lower respiratory tract, where they may replicate to high titer depending on the strain. When large inocula (i.e., titer greater than 1,000 HA units) of non–mouse-adapted influenza viruses are administered, mice develop a toxic pneumonitis even in the absence of significant virus replication (17). Mice are an ideal animal model to study pathogenesis and the immune response to influenza viruses, and the availability of mice with targeted gene disruptions (103) is providing new insights into the molecular basis of virus–host interactions. Thus mice with a targeted defect in the STAT1 gene that are unable to respond to type 1 or type 2 interferons (IFNs) are 100 times more sensitive to lethal infections with A/WSN/33 (H1N1) (151). The clearance of influenza virus by $CD4^+$ T cells is inefficient in the absence of B cells (495). It is becoming apparent that whereas antibody responses are critical for protection from influenza, the various components of the immune response act together to provide optimal protection (419). Mutations in the HA, NP, M, or NS gene segments each can contribute to pneumovirulence of mouse-adapted viruses (43). Histopathologic findings in infected murine pulmonary tissues are similar to the pathologic changes during human infection described elsewhere in this chapter (210). Neonatal mice are more susceptible to lethal pneumonia than are adult animals (81).

The ability of influenza virus to replicate in the CNS of mice has been studied in detail. In adult mice, the intracerebral

administration of non–mouse-adapted human virus initiates an abortive infection of ependymal cells, and a noninflammatory hydrocephalus can result from destruction of ependymal cells (238). In neonatal mice, several cycles of replication can occur with nonneurotropic strains (145). The avian H5N1 influenza viruses that are transmitted to humans also are neurotropic in mice (149). The neurotropic A/WSN/33 (H1N1) virus was isolated by serial passage of the WS strain of H1N1 virus in the brains of mice (477). This virus undergoes multiple cycles of replication in the brains of adult and neonatal animals and causes fatal encephalitis (145). The loss of a glycosylation site at residue 130 of the N1 neuraminidase is required for the neurovirulence phenotype (293). It has been proposed that the binding of plasminogen by the neuraminidase of A/WSN/33 (H1N1) leads to plasmin cleavage of the HA and high pathogenicity (168).

Most strains of mice are susceptible to infection with influenza A viruses. However, the A2G strain of mice is resistant to the neurotropic, hepatotropic, and pneumotropic influenza virus variants (192). The resistant A2G mice carry a dominant allele that specifies an influenza virus–specific increase in sensitivity to IFN (192). The NS1 gene of influenza virus can modulate the induction of IFN and affect the pathogenicity of the virus (151).

Influenza in Ferrets

Human influenza A virus was first isolated in ferrets. They develop a febrile rhinitis after inoculation with throat washings obtained from a symptomatic person (466). Ferrets have proven to be a very permissive host for all influenza A and B viruses. Pathology in the ferret is largely confined to the nasal mucosa, in which the influenza antigen–containing ciliated epithelial cells desquamate and are replaced by antigen-negative cuboidal epithelial cells (465). Infection of the lower respiratory tract can occasionally be demonstrated, and the pathologic changes of bronchitis and pneumonia that occur are similar to those seen in mice and humans (465).

Ferrets have been used extensively to study various aspects of influenza virus biology, largely because they are the only small animal that regularly develops febrile illness when infected with naturally occurring influenza A viruses. Ferrets are used to produce postinfection antisera for the study of antigenic shift in the World Health Organization centers. These animals have been useful in studying the development of immunity to influenza virus infection (16,129,404). The level of attenuation of various strains of virus has been assessed in ferrets (465) and in ferret tracheal organ explant cultures (341).

Influenza in Primates

Influenza A virus can infect many species of Old and New World primates including chimpanzees; gibbons; baboons; and owl, squirrel, cebus, rhesus, African green, patas, cynomolgus, and cercopithecus monkeys (3255, 354). Gibbons, baboons, and chimpanzees can be naturally infected with epidemic human influenza A viruses; the gibbon and baboon develop illness (237). Illness has been observed in squirrel, cebus, cynomolgus, and rhesus monkeys experimentally infected with influenza A virus, but the development of illness requires intratracheal inoculation of virus (55,173). Three human influenza A viruses known to be virulent in humans produce illness in squirrel monkeys, making it possible to evaluate the genetic determinants of virulence of influenza A viruses for primates in this species (351).

EVOLUTIONARY PATHWAYS OF INFLUENZA VIRUSES

Studies on the ecology of influenza viruses have led to the hypothesis that all mammalian influenza viruses are derived from avian influenza reservoirs (520). Support for this theory comes from phylogenetic analyses of nucleic acid sequences of influenza A viruses from a variety of hosts, geographic regions, and virus subtypes.

A surprising discovery from phylogenetic analyses of amino acid changes (Fig. 4) was that avian influenza viruses, unlike mammalian strains, show low evolutionary rates (166). In fact, influenza viruses in wild aquatic birds appear to be in evolutionary stasis, with no evidence of net evolution over the past 60 years. Nucleotide changes have continued to occur at a similar rate in avian and mammalian influenza viruses, but these changes no longer result in amino acid changes in the avian viruses, whereas all eight mammalian influenza gene segments continue to accumulate changes in amino acids. The high level of genetic conservation suggests that avian influenza viruses in their natural reservoirs are approaching or have reached an adaptive optimum, wherein nucleotide changes provide no selective advantage. It also means that the source of genes for pandemic influenza viruses exists phenotypically unchanged in the aquatic bird reservoir.

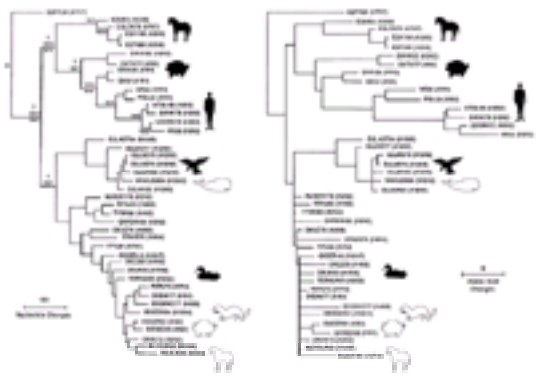


FIG. 4. Phylogenetic trees for 41 influenza A virus NP genes rooted to influenza B virus NP (B/Lee/40). **Left:** Nucleotide tree. Horizontal distance is proportional to the minimal number of nucleotide differences to join nodes and NP sequences. *Vertical lines*, Spacing branches and labels. Animal symbols (*black*) denote the five host-specific lineages. Animal symbols (*white*) denote viruses in that lineage that have been transmitted to other hosts. **Right:** Amino acid tree. A table of abbreviations for virus isolates shown in these trees may be found in Gorman et al. (166)

Phylogenetic analyses of the NP gene show that avian influenza viruses have evolved into five host-specific lineages (see Fig. 4): an ancient equine lineage, which has not been isolated in more than 20 years; a recent equine lineage; a lineage in gulls; one in swine; and one in humans. The human and classic swine viruses have a genetic sister-group relationship, which shows that they evolved from a common origin. It appears that the ancestor of the human and classic swine virus was an intact avian virus that, like the influenza virus currently circulating in pigs in Europe, derived all of its eight genes from avian sources (520). Studies of the NP and other genes in avian species reveal separate sublineages of influenza in Eurasia and the Americas. Studies on the 1918 influenza genome by Taubenberger's group (415) suggested that the precursor of the 1918 pandemic may have circulated in mammals before being transmitted to humans or alternatively may have been derived from a contemporary avian strain.

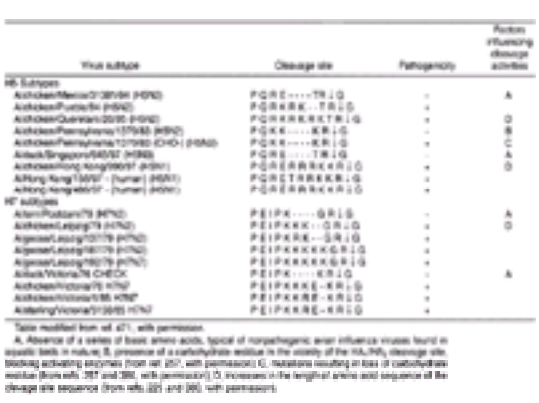
Influenza A viruses in humans usually evolve along a single-branch lineage, leading to the hypothesis that these viruses evolve by clonal reconstitution after widespread extinction (52). However, detailed analysis shows that some human influenza sublineages can cocirculate for a limited period (86,93), which causes added difficulties in the selection of vaccine strains. In contrast, the B and C viruses exhibit multiple-lineage evolution, which indicates the presence of cocirculating strains within the human population (551). The pattern of epidemiology and evolution of the B and C viruses suggests that they are at or approaching an evolutionary equilibrium with their human hosts, whereas the A viruses of humans are not and are prevented from it by perturbations arising from reassortment of human virus genomes with those from avian viruses.

Overall, the most important implication of phylogenetic studies is that the ancestral viruses that caused Spanish influenza in 1918, as well as the viruses that provided gene segments for the Asian/1957 and Hong Kong/1968 pandemics, are still circulating in wild birds, with few or no mutational changes.

MOLECULAR DETERMINANTS OF HOST RANGE AND PATHOGENESIS

The available evidence indicates that host range and pathogenesis are polygenic traits. Phylogenetic analysis of NP gene segments has established that there are five host-specific lineages and suggests the putative role of NP as one determinant of host range (148). Thus when the NP gene of FPV was replaced by the same gene segment of a human strain [A/Hong Kong/1/68 (H3N2)], the reassortants failed to replicate in chickens or chicken cells but replicated in MDCK cells (449). Similarly, if a human influenza virus NP gene was replaced by an avian counterpart, the reassortants were attenuated in monkeys (490). It has been proposed that the NP is a major determinant of host specificity (447) and that host-specific phosphorylation patterns of NP can affect the replication of influenza viruses (272). The other internal genes of influenza A viruses (PB1, PB2, PA, M1, M2, NS1, and NS2) also may play roles in host discrimination. The involvement of polymerase gene products as determinants of host range/pathogenicity has been demonstrated in a number of studies (9,26,449,450 and 451), and NS gene products have been implicated (151,498).

The HA plays a central role in the pathogenicity of avian influenza viruses (see review, 471). Infection with certain avian influenza viruses possessing the H5 or H7 HA is associated with high mortality in starlings and domestic poultry (365). The HA of these viruses is readily cleaved in tissue culture and does not require an exogenous protease for plaque formation. The virulent H5 and H7 viruses differ from the HAs of other influenza A subtypes in that their HAs possess multiple basic amino acids at the carboxyl terminus of HA_1 (Table 2). This permits cellular proteases that recognize multiple basic amino acids to cleave the HA and to render the virus infectious and capable of spread *in vitro* and *in vivo.* The number of basic amino acids may vary from four to six residues, and cleavability is influenced by the presence of an adjacent oligosaccharide side chain on the HA molecule (see Table 2). The proposed sequence requirement for HA cleavage by endogenous proteases, when carbohydrate is nearby, is X-X-R-X-R/K-R (X, nonbasic residues); otherwise, it is R/K-X-R/K-R (225,380,508). The cleavage enzyme for the HA with multiple basic amino acids appears to be located in the trans-Golgi apparatus, to be calcium dependent, and to have an acidic pH optimum (512).



Virus subtype	Cleavage site	Pathogenicity	cleavage activities
H5 Subtypes			
A/chicken/Mexico/31369/94 (H5N2)	PQRE----TR↓G	-	A
A/chicken/Pueblo/94 (H5N2)	PQRKRK..TR↓G	+	
A/chicken/Queretaro/2000 (H5N2)	PQHKRKKKTR↓G	+	D
A/chicken/Pennsylvania/1370/83 (H5N2)	PQKK----KR↓G	-	B
A/chicken/Pennsylvania/1210/83 (CHO-) (H5N2)	PQKR----KR↓G	+	C
A/duck/Singapore/3/97 (H5N3)	PQRE----TR↓G	-	A
A/chicken/Hong Kong/220/97 (H5N1)	PQRERRRKKR↓G	+	D
A/Hong Kong/156/97 - (human) (H5N1)	PQRETRRKKR↓G	+	
A/Hong Kong/483/97 - (human) (H5N1)	PQRERRRKKR↓G	+	
H7 subtypes			
A/tern/Pt/atom/79 (H7N3)	PEIPK----GR↓G	-	A
A/chicken/Leipzig/79 (H7N7)	PEIPKRK--GR↓G	+	D
A/gannet/Leipzig/1/2179 (H7N2)	PEIPKRK---GR↓G	+	
A/gannet/Leipzig/1/80179 (H7N2)	PEIPKKKKKGR↓G	+	
A/gannet/Leipzig/1/82179 (H7N7)	PEIPKKKKKGR↓G	+	
A/duck/Victoria/76 CHECK	PEIPK----KR↓G	-	A
A/chicken/Victoria/76 H7N2	PEIPKRKE-KR↓G	+	
A/chicken/Victoria/1/85 H7N7	PEIPKRRE-KR↓G	+	
A/starling/Victoria/1/2685 H7N7	PEIPKRRE-KR↓G	+	

Table modified from ref. 471, with permission.

A, Absence of a series of basic amino acids, typical of nonpathogenic avian influenza viruses found in aquatic birds in nature; B, presence of a carbohydrate residue in the vicinity of the HA_1-HA_2 cleavage site, blocking activating enzymes (from ref. 257, with permission); C, mutations resulting in loss of carbohydrate residue (from refs. 257 and 380, with permission); D, increases in the length of amino acid sequence at the cleavage site sequence (from refs. 225 and 380, with permission).

TABLE 2. *The cleavage site on the HA is a determinant of pathogenicity*

Two groups of proteases appear responsible for HA cleavage *in vivo.* One includes enzymes able to cleave avirulent as well as virulent HAs, such as plasmin, blood-clotting factor X-like protease, tryptase Clara, and bacterial proteases (273). Of these, tryptase Clara is of particular interest in that it is a product of specialized respiratory epithelial cells and has been isolated from rat lungs and shown to activate avirulent HA cleavage (266) The second group comprises proteases that cleave only virulent-type HAs with multiple basic residues at the cleavage site and include the subtilisin-related endoprotease furin and PC6 (225, 474). Based on the fact that a large number of pathogenic viruses of both H5 and H7 subtypes contain direct repeats that give rise to basic residue insertions of various lengths, and RNA secondary structure predictions, a mechanism for the generation of cleavage site insertion mutants, was proposed (394). It was postulated

that during RNA synthesis from the positive-sense template, the polymerase encounters a region of stable secondary structure at the coding sequence for the cleavage site, which can cause it to stutter.

The receptor specificity of influenza virus HAs depends on the host species from which they evolved. Human H3 viruses bind almost exclusively to sialyloligosaccharides terminated by SAa2,6 galactose, whereas avian and equine H3 viruses preferentially bind SAa2,3 glactose (427). This difference in specificity is tightly associated with the presence, in the structure of the HA receptor-binding site, of Leu-226 in human H3 viruses instead of Gln-226 in avian or equine viruses. H1 viruses isolated from humans after 1977, swine H1 viruses, and human H2 viruses are also preferentially SAa2,6 galactose specific.

The amino acids identified as constituting the heart of the HA receptor-binding site [Tyr-98, Trp-153, His-183, Glu-190, Leu-194, and Gln (Leu)-226] (529) are essentially invariant features of the HA of influenza A viruses. Immediately adjacent, Ser-227 is highly characteristic of duck viruses of all subtypes as well as of human/swine H3 viruses, whereas Ala/Gly-227 is present in the HA of some other mammalian viruses. Gly-228 covaries with Gln-226; as it is conserved in all duck and mammalian viruses except in human/swine H3 HA, which have Leu-226-Ser-227-Ser-228. Substitution of Gln-226-Ser-227-Gly-228 into a human H3 virus permitted virus replication in ducks, whereas substitution of Gln-226-Ser-227-Ser-228 did not (358).

The propagation of influenza viruses in different host cells including chicken embryos or MDCK cells can result in selection of variants with different receptor-binding specificities (146,423). Thus influenza virus grown in chicken embryos selects variants that preferentially bind to SAa2,3 galactose, which can affect the efficiency of vaccines (10,255). Optimally, human vaccines should be grown in cell substrates that favor SAa2,6 galactose–containing influenza viruses, but this would mean changes in vaccine preparation, which conventionally uses the chicken embryo.

There is evidence for a role of influenza virus NA in host-range discrimination and pathogenicity. Recent studies (168) have demonstrated that the A/WSN/33 (H1N1) neuraminidase has the ability to bind plasminogen and that this activity is dependent on the absence of carbohydrate and the presence of a lysine residue at the C-terminus of the NA (293). By concentrating the plasmin precursor at the surface of viral or infected-cell membranes, HA is readily available for cleavage after the generation of activated plasmin. The relevance of this form of cleavage activation of the HA to explain the pathogenicity of human influenza viruses is an interesting possibility, but it remains to be demonstrated whether this mechanism is generally applicable.

Thus pathogenicity and host range are very complex phenomena that can involve a multitude of interrelated determinants, including the properties of individual viral components, the interaction of such components with one another and with elements of the host cell, and immunologic and environmental factors.

INFLUENZA VIRUS GENETICS

Mutational Frequency

The following is a brief description of the genetics of the influenza A viruses, and the reader is referred to other reviews that cover the subject in greater depth (268,445,482).

The mutation frequency for influenza A virus HA and NA genes for any single nucleotide appears to be about $10^{-5.5}$ per replication (527). This rate is similar to that observed for the genes coding for the surface glycoproteins of a paramyxovirus and a rhabdovirus (402). This indicates that the rapid evolution of influenza virus strains in nature cannot be explained by a mutation rate higher than that of viruses that do not undergo rapid antigenic drift. The mutation rate of influenza A virus is nonetheless higher than that observed for bacterial DNA, suggesting that the RNA polymerases of influenza and other animal RNA viruses are less faithful than are DNA polymerases and are not able to correct errors by a proofreading mechanism. Thus although influenza A viruses do not differ in mutation rate from other RNA viruses, mutation occurs often enough to provide an ample source for the selection of naturally occurring variants.

Genetic Reassortment

Exchange of genetic markers between two influenza A viruses was first observed in 1949 during experiments in which an exchange of the HA serotype and neurotropism occurred between two viruses differing in these traits during mixed infection in mouse brain (56). Even before the segmented nature of the influenza A viruses became clearly defined by biochemical techniques, the nature of the genetic interaction between influenza A viruses suggested that influenza virus genes segregated independently (57). It was shown in 1951 that influenza viruses could undergo multiplicity reactivation (206); an influenza A virus whose infectivity had been inactivated by ultraviolet light produced infectious virus when a cell adsorbed two or more irradiated viruses. The first evidence that the gene exchange occurs with high frequency was obtained in 1952 when it was found that reassortant virus composed 27% of the progeny from a cross between two different influenza A viruses (58).

High frequency of reassortment is characteristic of influenza A, B, and C viruses, and the frequency of reassortment at any given locus can be as high as that expected for independently segregating genes [i.e., 50% (491)]. However, exchange of genes between influenza type A, B, or C viruses has not been observed (482). Thus the process of genetic interaction between influenza viruses differs from classic recombination in which breakage, exchange of DNA, and ligation occur. Instead, the exchange of RNA segments between two viruses (Fig. 5) occurs by the process of genetic reassortment in a manner analogous to the exchange of parental chromosomes during meiosis.

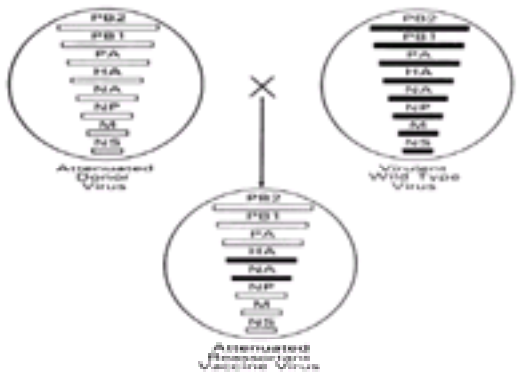


FIG. 5. Production of live attenuated viruses by genetic reassortment between two influenza A viruses by transfer of genes from an attenuated donor virus to a virulent wild-type virus. The attenuated reassortant live vaccine virus contains the genes that code for the hemagglutinin and neuraminidase from the virulent wild-type virus and the genes that confer attenuation from the attenuated donor virus.

With the development of conditional lethal temperature-sensitive (ts) mutants of influenza A virus, the process of gene exchange between influenza A viruses was studied in greater depth. Eight different reassortment groups are defined using ts mutants (307). If there is random reassortment of RNA segments, then linkage between segments should not occur, and this is indeed the case. It has been calculated that if the process were indeed random and the virus packaged any eight segments, then only one in 416 virus particles would receive a full complement of eight different genes (361). However, careful determination of the particle-to-infectivity ratios of four different preparations of influenza A or B virus indicated that one in 10 particles were infectious (107). Hirst (220,221) found that cells yielding reassortant viruses exceeded the expected number of cells doubly infected (calculated from multiplicity of infection, based on plaque formation) by a factor of 30 to 60 and concluded that non–plaque-forming particles are the principal source of viruses that exchange genes during mixed infection. Thus non–plaque-forming, presumably noninfectious virus can enter cells and participate in genetic interactions leading to replication of a full complement of influenza virus genes. Hirst (220) further suggested that because of significant variation in size of individual virions, the amount of RNA per particle could vary and that it could be unevenly distributed such that not every virion received a full complement of eight RNA segments; some particles might contain more than one genome equivalent. The infectivity-to-particle ratio of one to 10 could thus be explained by the packaging of more than eight segments per virion in a substantial number of virions. Alternatively, an unidentified mechanism could exist for precise selection of the full complement of eight segments. The resolution of this question remains an important goal in influenza virus genetic research.

Recombination

Classic recombination in which an influenza RNA segment contains genetic material from two different parental viruses is rare among influenza viruses (428). The gene coding for the NP protein of an influenza A virus was shown to have been produced by intracistronic recombination between the two parental NP genes (428). The viral RNA segment was a mosaic structure with sequences derived from segments 1 and 3. This RNA could have been produced by jumping of the polymerase from one segment to another, rather than by classic recombination (130). An influenza A virus HA gene was shown to contain an insertion of 54 nucleotides, which correspond to a region of host cell 28S ribosomal RNA (265). This occurred presumably by a mechanism of polymerase jumping, and the insertion, which encoded eight amino acids adjacent to the HA_1–HA_2 cleavage site, increased the pathogenicity of the virus for chicken embryos (265).

Complementation

When a cell is infected with two viruses that have defective functions at different loci, complementation can occur between the viruses; the function defective in one virus is provided by the coinfecting virus, and vice versa. After this occurs, infectious reassortant viruses that receive a full complement of eight nondefective genes are replicated. The first step in multiplicity reactivation and cross-reactivation is complementation, which is then followed by reassortment. Complementation between influenza A viruses has been most extensively evaluated between ts mutants under conditions in which mixed infection is carried out at the restrictive temperature (i.e., a temperature at which the ts viruses are unable to replicate). The ts mutants are arranged in complementation groups defined by the ability of two ts viruses to produce infectious virus during coinfection at restrictive temperature. The number of complementation groups so defined exceeds the number of RNA segments of influenza virus, and this finding suggests that intrasegmental complementation has occurred (203,311). Intrasegmental complementation occurs with each of the RNA segments except those coding for the M and HA proteins. Four complementation groups have been identified for RNA segment 8 (NS), which codes for two proteins (455). Thus for the NS ts mutants, it is possible that both intra- and intercistronic complementation occur. Three complementation groups exist for the NP gene, and this represents intracistronic complementation, because the NP gene is monocistronic. It is likely that intracistronic complementation occurs by the formation of a multimeric protein consisting of two defective subunits that are able to correct the defect present in each other. The resulting multimeric protein can function effectively at restrictive temperature and thus has the wild-type phenotype.

Heterozygosis

Heterozygotes are viruses that contain RNA derived from two parents at one or more loci. The suggestion offered previously that some influenza A viruses transiently contain more than eight RNA segments suggests that heterozygote formation might be a common feature of influenza A viruses. Methodologic difficulties make it almost impossible to assess the frequency of transient heterozygotes that form after mixed infection. However, a stable, partial heterozygote has been observed (451). It was suggested that the presence of a recombinant NP gene was functionally related to

heterozygosis (428).

Phenotypic Mixing and Transcapsidation

Infection of cells with two antigenically distinct influenza A viruses gives rise to progeny virus particles that contain HA from both parents (222,223). Such particles are inhibited in hemagglutination-inhibition (HAI) and neutralization assays by antiserum directed against either parent, and are referred to as phenotypically mixed particles. Phenotypically mixed particles can represent up to 84% of all virus progeny (220). Phenotypic mixing occurs between two different influenza A viruses (222), between influenza A and B viruses (167), and between influenza A and heterologous viruses such as Newcastle disease virus and vesicular stomatitis virus (VSV) (170).

Transcapsidation is a process by which the genome of one virus is carried in the coat of another virus. In dual infection of cells with influenza A virus and VSV, virus that contains the VSV genome in an influenza A virus coat composes about 1% of the progeny (433). Trancapsidation occurs between influenza A and B viruses such that 20% of progeny virus displaying only the HA of type B virus possess an HA gene derived from the type A virus (492). The influenza A HA can encapsidate a retroviral genome and extend the host range of the retrovirus (108).

Dominance

Certain influenza strains, including cold-adapted live, attenuated vaccine candidates, have been shown to interfere with the growth of other strains on coinfection (532). This dominance appears to be mediated by the M protein (533). This observation has potential implications for the use of live, attenuated vaccines in the face of an influenza outbreak.

Suppressor Reassortants and Suppressor Mutants

The product of one RNA segment can alter (i.e., suppress) the phenotype specified by a different RNA segment (158,452). In one instance, the ts phenotype specified by the PB2 gene was suppressed by the development of a mutation in the PA gene (494); this is referred to as extragenic suppressor mutation. In extragenic suppressor mutations that modify the ts phenotype of FPV mutants with a ts lesion in the PB2 or PB1 gene, the locus of the suppressor mutation is not known (452). The genetic mechanisms of reversion, extragenic suppressor mutation, and intragenic suppressor mutation were each able to correct a ts mutation in the HA gene (343).

Reverse Genetics

A major advance in the ability to manipulate the genome of influenza viruses occurred when Palese et al. (118,302) established the reverse genetic system, which permits the generation of influenza viruses containing genes derived from cloned cDNA. A further major advance occurred when Kawaoka et al. (366) demonstrated the generation of influenza A viruses entirely from cloned cDNAs with high efficiency. These advances permit complete manipulation of all genes of influenza viruses, and are considered in detail in the accompanying Chapter 46.

EPIDEMIOLOGY IN HUMANS

The influenza viruses are unique among the respiratory tract viruses in that they undergo significant antigenic variation. Both of the surface antigens of the influenza A viruses undergo two types of antigenic variation: antigenic drift and antigenic shift. Antigenic drift involves minor antigenic changes in the HA and NA, whereas shift involves major antigenic changes in these molecules resulting from replacement of the gene segment.

Nature of Antigenic Drift

Antigenic Composition of HA

HA is the major surface antigen of influenza viruses and with the neuraminadases defines the subtype-specific serotype of the virus. The HA from H3 human influenza viruses has been crystallized, and the three-dimensional structure has been determined (see Chapter 46). Five antigenic domains (A to E) have been defined on the HA_1 by comparative sequence analysis (Fig. 6) (413,535). Site A is centered around a protruding loop of residues 140 to 146. Residues 138, 139, 147, and 148 are conserved, thus preserving the structural foundation of the site. Site B, at the extreme membrane distal end of the molecule, is centered on a loop of residues 155 to 160, as well as the a-helix of residues 188 to 198 situated at the edge of the receptor-binding site. Site C is situated at the base of the globular domain in the antiparallel strands of HA_1. Site D is situated near the trimeric interface of the globular domains of HA_1 on the top of the molecule. Site E is near the bottom of the globular distal domain between sites C and A. Antigenic mapping with mouse monoclonal antibodies and sequence analysis of escape mutants confirm the number and location of the antigenic sites in H3 and H1 influenza viruses (156,527). Crystallographic studies of escape mutants have confirmed that antibodies bind to those regions of the molecule where amino acid substitutions permitting escape from neutralization have been found (276).

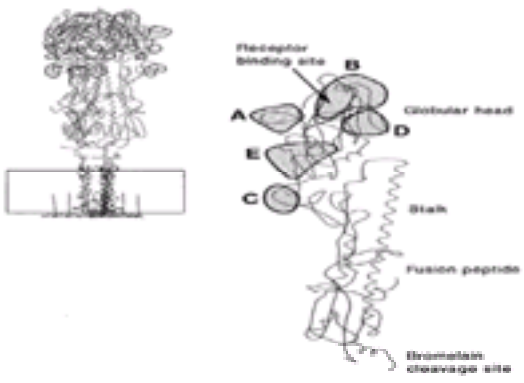


FIG. 6. The hemagglutinin of H3 influenza A virus, showing the location of the five antigenic areas on the globular head of the monomeric and trimeric molecules (535,539). In the HA spike, site *D* is buried and may not be involved in antibody binding. The fusion peptide is also buried in the intact molecule and is exposed only after a conformational change at low pH. The trans-membrane domain of the molecule (**box**) is a hypothetical model illustrating an a-helix, cytoplasmic tails, and palmitic acids.

Drift of HA

After the appearance of a new subtype, antigenic differences between isolates can be detected within a few years by using ferret antisera for analysis (Table 3). Analysis with monoclonal antibodies indicates that minor antigenic heterogeneity is detectable among different influenza virus isolates at any time (383,461,473,524). Antigenic drift occurs by accumulation of a series of point mutations resulting in amino acid substitutions in antigenic sites A to E at the membrane distal region of the HA. These substitutions prevent binding of antibodies induced by the previous infection, and the virus can infect the host. In influenza A viruses of humans and other mammals, antibodies play a role in selection of mutants, whereas in influenza A viruses in avian species and in influenza B and C viruses, antibody selection does not appear to play an important role in emergence of drift variants (4).

	Reference ferret antisera									
Strain designation	HK/68 1.	ENG/72 2.	VIC/75 3.	TEX/77 4.	BANG/79 5.	PHL/83 6.	MDS/86 7.	SHN/87 8.	BEI/89 9.	BEI/9 10.
1. A/Hong Kong/1/68	2560	1280	40	5	5	5	10	10	5	5
2. A/England/42/72	320	1280	80	20	5	5	5	5	5	5
3. A/Victoria/3/75	5	80	320	40	10	20	10	5	5	5
4. A/Texas/1/77	5	80	160	1280	160	320	320	10	10	5
5. A/Bangkok/1/79	5	5	80	640	640	640	640	20	10	5
6. A/Philippines/82/83	5	5	10	40	40	320	160	10	10	5
7. A/Mississippi/1/85	5	5	40	160	80	640	640	40	20	20
8. A/Shanghai/11/87	5	5	5	5	5	5	80	320	160	20
9. A/Beijing/353/89	5	5	5	5	5	5	5	160	320	40
10. A/Beijing/32/92	5	5	5	5	5	5	10	20	80	640
11. A/Johannesburg/33/94	5	5	5	5	5	5	5	10	20	160
12. A/Nanchang/933/95	5	5	5	5	5	5	5	5	10	20
13. A/Sydney/05/97	5	5	5	5	5	5	5	5	5	5

Tables courtesy of A. Klimov and N.J. Cox, Centers for Disease Control and Prevention, Atlanta, GA.

TABLE 3. *Antigenic drift of H3N2 viruses between 1968 and 1997 as demonstrated by their cross-reactivity in hemagglutination-inhibition tests*

Antigenic drift can be mimicked in the laboratory by growth of influenza viruses in the presence of monoclonal antibodies to a single antigenic site (e.g., to site A). However, it is not possible to select antigenic variants with mixtures of monoclonal antibodies to different antigenic sites (e.g., to sites A, B, C). The frequency of selection of variants by monoclonal antibodies to a single site *in vitro* is 10^5 (527,553); with a mixture of monoclonal antibodies to two different antigenic sites, the frequency would be 10^{10}. How then does selection of drift variants occur in humans who have polyclonal responses and a mixture of antibodies to each antigenic site? Analyses of postinfection human sera indicate a limited antibody repertoire (516), and studies on animal sera demonstrate that some are essentially monoclonal (101). The reactivity of human sera with monoclonal antibody escape mutants (362) suggests that the selection of variants may be sequential, involving several different humans.

Antigenic Composition of NA

The NA is the second most abundant spike protein on influenza virus, and with the HA, determines serotype specificity. The NA spikes are clustered in patches on the virion surface and are important in virus release from infected cells (357) (for additional information on structure and function see Chapter 46). The majority of the nine subtypes of influenza type A and influenza B NA have been crystallized (489), and the three-dimensional structures have been elucidated and are similar. The antigenic structure of the NA has been determined by comparison of amino acid sequences of naturally occurring variants and by analysis with monoclonal antibodies. At least two antigenic sites on the upper surface of the influenza NA encircle the sialic acid–binding activity and there is probably another on the bottom of the head (Fig. 7) (5). The detailed structure of one of these epitopes on N9 NA was established by crystallization of the Fab fragment of monoclonal antibody (NC41) and comprises five peptide loops located at the rim of the enzyme active site (501). A second epitope has been characterized with monoclonal antibodies on N8 NA and is located at the interface of two adjacent monomers in the tetrameric NA, involving at least two peptide loops on each monomer (residues 150 and 199 on one subunit and at 367, 399, 400 on the other subunit) (437). Antibodies to this epitope react with the NA only after the tetrameric molecule is formed.

FIG. 7. The neuraminidase of influenza A virus, showing the location of the antigenic sites. Schematic diagram of the four identical subunits of N8 neuraminidase viewed from above. The enzyme active center is shown (*asterisk*) surrounded by antigenic sites (*B*). Amino acid substitutions found in different escape mutants are plotted to illustrate that some antibodies combine with individual monomers, whereas others bridge two monomers. The best characterized epitope is NC-41 (*D*), comprising five peptide loops on each monomer adjacent to the enzyme active site (501). Another epitope located at the interface of adjacent monomers (*A, C*) involves at least two peptide loops in each monomer (437). Note that some peptide loops are involved in both epitopes (e.g., loop containing residue 367). N8 diagram courtesy of G. Taylor, University of St. Andrews, Scotland.

The interaction of antibodies with NA has been elucidated for monoclonal antibodies NC10 and NC41 in combination with N9 NA (308). The epitopes recognized by these monoclonal antibodies overlap. The surface areas on NA buried by interaction with the NC10 and NC41 antibodies are 716 $Å^2$ and 899 $Å^2$, respectively, and the buried surface areas contributed by the residues common to both epitopes are 594 $Å^2$ (83% of total) and 686 $Å^2$ (77% of total), respectively. The NC10 binding site is somewhat farther from the NA active site, consistent with observations that NC41 antibody can inhibit enzyme activity on trisaccharide substrates, but NC10 has inhibitory activity only against macromolecular substrates. Thus two different antibodies can combine with portions of the same epitope and show different biologic activities.

Other epitopes on the NA are at distant sites from the enzyme active center, o not include antibodies that select escape mutants, and remain to be fully characterized. One of these appears to be on the bottom of the head; it is difficult to imagine how antibody could gain access to this site on the virion unless the NA head is presented sideways on the virion.

Drift in NA

Antigenic drift also occurs in the NA of influenza viruses (90,390) and has been correlated with differences in amino acid sequences (80). The mechanism of antigenic drift in the NA of human influenza viruses is believed to be the same as for the HA. The process can be mimicked with monoclonal antibodies *in vitro,* and the frequency of selection of variants is approximately 10^5 (525).

Nature of Antigenic Shift

Since the first human influenza virus was isolated in 1933, antigenic shifts in type A influenza viruses have occurred in 1957, when the H2N2 subtype (Asian influenza) replaced the H1N1 subtype; in 1968, when the Hong Kong (H3N2) virus appeared, replacing H2N2; and in 1977, when the H1N1 virus reappeared (see Fig. 1). Each of these major antigenic shifts has several characteristics in common: (a) their appearance was sudden; (b) they first occurred in China; (c) they were antigenically distinct from the influenza viruses then circulating in humans; and (d) they were confined to the H1, H2, and H3 subtypes.

On the basis of phylogenetic evidence, a likely explanation for the appearance of new pandemic strains in humans is that they are derived from avian influenza viruses, either after reassortment with the currently circulating human strain or by direct transfer. There is ample evidence for genetic reassortment between human and animal influenza A viruses *in vivo* (520), and genetic reassortment also has been detected among human viruses (87). Genetic and biochemical studies have concluded that the 1957 and 1968 strains arose by genetic reassortment. As described earlier, the 1957 Asian H2N2 strain obtained its HA, NA, and PB1 genes from an avian virus and the remaining five genes from the preceding human H1N1 strain (257,450).

A new pandemic strain that emerged in Hong Kong in 1968 contained the H3 HA and PB1 genes from an avian donor (126,257) and the N2 NA and five other genes from the H2N2 strain circulating in early 1968. The HA of the human Hong Kong/68 strain differed by only seven amino acids from hypothetical Asian avian H3 strains (25), providing the strongest evidence yet that the Hong Kong virus originated by reassortment between an avian influenza virus present in Asia and the circulating human strain.

The second way that new pandemic viruses could occur in the human population would be if an avian or mammalian strain were transmitted to humans. The transmission of H5N1 and H9N2 influenza viruses to humans in Hong Kong establishes that this can occur (see later section, Transmission of Avian Influenza Viruses to Humans). Phylogenetic evidence favors this mechanism for the appearance of the Spanish influenza virus in 1918. The periodic transmission of swine influenza viruses to humans that presumably occurred at Fort Dix and the isolation of virtually identical viruses from pigs and humans (432) leave no doubt that such transmissions occur in nature and may be more frequent than originally thought. Most of these transmissions are dead-end transmissions, in that the viruses have little or no capacity to be transmitted secondarily to human contacts and initiate an epidemic. However, the disastrous pandemic virus of

1918 to 1919 did possess this property.

A third explanation for the origin of pandemic viruses is that virus that caused an epidemic many years previously remained hidden and unchanged in some place since that time. The appearance of Russian influenza (H1N1) provided support for this concept. This virus appeared in Anshan, northern China, in May of 1977 and subsequently spread to the rest of the world; it is identical in all genes to the virus that caused a human influenza epidemic in 1950 (360). Where was this virus for 27 years? The possible explanations include preservation in a frozen state, preservation in an animal reservoir, or retention in an integrated, as yet undetected form in the genetic material of a human or lower animal. The animal-reservoir option is unlikely, for the accumulation of mutations would have continued. There is no evidence for integration of influenza genetic material into the host genome, leaving the most likely explanation that in 1977, the H1N1 virus was reintroduced to humans from a frozen source.

From this discussion, it is apparent that there are multiple ways in which pandemic influenza viruses can arise, and each has probably played a role in the evolution of the influenza viruses currently circulating in humans. The primordial source of all influenza virus genes is from the aquatic bird reservoir, and many mutations and reassortments occur during adaptation to the human host.

Transmission of Avian Influenza Viruses to Humans

Although serologic studies (457,555) and the isolation of H7N7 influenza viruses from the eyes of humans with conjunctivitis (279,522) support the notion that avian influenza viruses could be transmitted to humans, it was the transmission of H5N1 influenza viruses to humans in 1997 that confirmed the direct transmission of avian influenza to humans apparently without an intermediate host. The avian H5N1 influenza virus that was transmitted to poultry and humans in 1997 caused high mortality in both species, killing 70% to 100% of chickens and six of 18 humans (73,98,478,479,555) and is unusual in having a large proportion of amino acid changes in all gene products except in the surface genes (558). It was therefore proposed that this H5N1 influenza virus might be a reassortant possessing the HA from A/goose/Guandang/1/96 (H5N1) (550) and the other gene segments from H6N1 or H9N2 viruses (Fig. 8) (177,224).

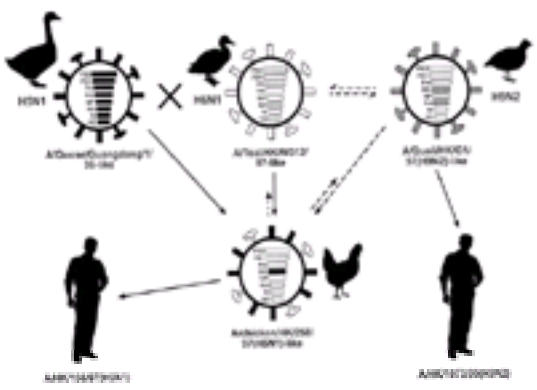


FIG. 8. The emergence of avian H5N1 and H9N2 influenza viruses that were transmitted to humans in Hong Kong. The avian H5N1 and H9N2 influenza viruses that were transmitted to humans in 1997 and 1999 contained the same six genes composing their replicative complex. The source of these six genes is from either a duck virus [A/teal/HK/W312/97(H6N1)-like] or a quail virus [A/quail/HK/G1/97(H9N2)-like] (177,224). The donor of the hemagglutinin in the A/HK/156/97(H5N1)-like viruses that killed six of 18 infected persons was probably a goose virus [A/goose/Guangdong/1/96/(H5N1)] (550). The H9N2 virus that was transmitted to two children in Hong Kong was identical in all gene segments to A/quail/HK/G1/97 (H9N2).

Surveillance studies in the live poultry markets in Hong Kong in December of 1997 revealed the presence of nearly identical highly pathogenic H5N1 virus in 20% of birds and in the majority of live poultry markets tested. In addition, H9N2 influenza virus was present in approximately 5% of birds. Two antigenically and genetically distinguishable variants of H5N1 were circulating among avians and humans (29,458,478). There was no correlation between lethality in humans and one or other of the variants. Both H5N1 variants were highly pathogenic in chickens, causing generalized systemic infection and, like the viruses from the index case, possessed a polybasic amino acid insertion at the cleavage site of the HA. They also contained a shortened stalk of the NA. It has been proposed that NA stalk deletions are a characteristic of pathogenic H5 and H7 viruses (312).

The majority of human and chicken H5N1 influenza viruses were highly pathogenic in mice, requiring no adaptation and spread after intranasal infection to the brain. A subgroup of the human and avian H5N1 viruses were nonpathogenic in mice (149,253,458). The slaughter of approximately 1.6 million poultry in Hong Kong on December 29 and 30, 1997, stopped the spread of H5N1 influenza viruses to humans. The failure of H5N1 to be transmitted from human to human, and the slaughter of poultry a matter of days before the human A/Sydney/05/95 (H3N2) strain began circulating in humans in Hong Kong probably prevented the generation of reassortants.

The H9N2 detected in the live poultry market in 1997 were genetically heterogenous with three serologically and genetically distinguishable groups (177). These H9N2 viruses were by definition not pathogenic in chickens in laboratory studies (they did not possess polybasic amino acids at the cleavage site of the HA and did not kill six of eight chickens after intravenous injection), but in the field, they caused respiratory problems, a decrease in egg production, and one isolate killed up to 80% of chickens. In 1998 through 1999, H9N2 influenza viruses was transmitted to humans and pigs. The initial report of five human cases in southern China (184) was confirmed by the isolation of H9N2 viruses from two children in March 1999 in Hong Kong (297). The children had typical influenza and recovered; the isolates were genetically similar to H9N2 isolates from quail [A/quail/Hong Kong/G1/97 (H9N2)] (see Fig. 8). The viruses isolated from

apparently healthy pigs during surveillance in the slaughterhouse in Hong Kong belonged to the H9N2 virus from ducks [A/duck/Hong Kong/Y280/97 (N9N2)]. Further characterization of the H9N2 viruses revealed that the human isolates from Hong Kong and the quail isolates (A/quail/Hong Kong/G1/97) shared the replicative gene complex (PB2, PB1, PA, NP, M, NS) with the H5N1-like viruses from chickens and humans in Hong Kong in 1997 (177), whereas the pig isolates contained the genome similar to A/duck/Hong Kong/Y280/98 (N9N2). Thus avian influenza viruses can be transmitted directly to humans and cause disease, but additional mutations and/or reassortant events are probably required to permit efficient human-to-human spread.

Maintenance of Influenza A Virus in Human Populations

There is no evidence of persistent or latent infection with human influenza viruses. The virus is maintained in humans by direct person-to-person spread during acute infection. Isolated communities become infected by the introduction of virus by an infected individual, often resulting in an explosive localized epidemic. Subsequent epidemics in such isolated communities are initiated by reintroduction of virus (46). Influenza virus activity can be detected in a large city during each month of the year, although each subsequent epidemic is likely from exogenous introduction of virus, as typified by the circulation of a limited number of strains globally each year (137,243,271,374,395). The minimal population size necessary to sustain influenza virus transmission during nonepidemic periods is unknown. On a global scale, influenza viruses are isolated from humans during most months of the year somewhere in the world (374). In the U.S., influenza virus epidemics generally occur during January through April, with occasional epidemics in December or May (374). One biologic property of the virus that could contribute to this seasonal distribution is the ability of influenza A viruses to survive in aerosol under the condition of low relative humidity that exists indoors during the winter months (204,439).

Transmission of Influenza Between Humans

Epidemiologic observations indicate that influenza A virus is most effectively spread from person to person by the aerosol route (319,339). It is probable that most influenza A virus infections are transmitted by droplets formed during coughing or sneezing. A high proportion of particles generated during a sneeze or cough are less than 2 μm in diameter; particles of this size are preferentially deposited in the lower airways of the lung (275). Infection of volunteers can best be achieved by aerosol administration of virus (6,109). The human infectious dose 50 (HID_{50}) of influenza A wild-type virus administered intranasally by drops is 127 to 320 tissue culture infectious dose 50s ($TCID_{50}$) (84,85), and that of virus delivered by an aerosol is 0.6 to 3 $TCID_{50}$ (6).

Preschool and school-age children are major vectors in transmission of influenza A viruses in the community (136,189,335,336). After introduction of the Asian influenza A virus (H2N2) in 1957 and the Hong Kong virus (H3N2) in 1968, epidemics occurred soon after school opened in September and October (242,374). Clearly, crowding that occurs in schools favors the rapid spread of virus by aerosol transmission of virus (69). The incubation period for influenza viruses is about 3 days for influenza A virus and 4 days for influenza B viruses (139).

The virus, host, and environmental factors that limit the size of an influenza virus epidemic have not been clearly delineated. One indicator that epidemics might occur the next winter is isolation of a virus with an altered hemagglutinin in the late spring during a miniepidemic (herald wave) (338). Such herald waves can precede both influenza A and B epidemics.

The immunologic status of the population as reflected in seroprevalence of antibody influences the size of an epidemic and which age is affected. This was seen in 1957, when a pandemic was caused by an H2N2 virus to which the vast majority of the population was fully susceptible (i.e., they had not had previous experience with influenza A viruses bearing a related HA or NA) (Fig. 9). During 1968, the first year of spread of the Hong Kong (H3N2) virus, excess mortality was about one half that caused by the H2N2 virus during its first year of prevalence. This dampening of the first H3N2 epidemic may have been a consequence of partial immunity induced by prior infection with H2N2 viruses that possessed a related NA antigen. Furthermore, the size of epidemics occurring during interpandemic periods are always smaller than those occurring during the introduction of a new virus subtype, although their cumulative mortality may exceed that of the rarer pandemics.

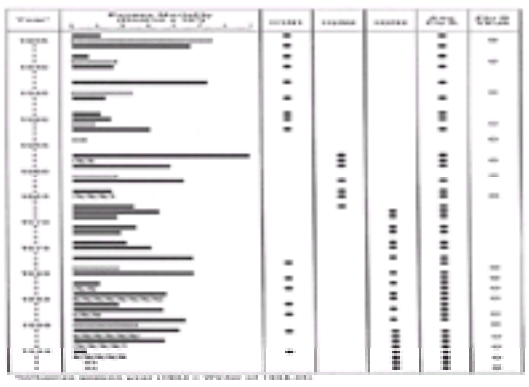


FIG. 9. Excess mortality caused by influenza A and B viruses (1934–1998). *1935*, Winter of 1934 through 1935. The excess mortality figures that are depicted use two sets of data with slightly different methods for their calculation (374,459). ■, Excess mortality predominantly caused by influenza A virus; ■, excess mortality caused by influenza B virus; ▨, excess mortality occurring during a year of mixed circulation of influenza A and B viruses.

There is no regular periodicity in the occurrence of epidemics (see Fig. 9), indicating that the appearance of an epidemic in any given year represents a subtle interplay between intrinsic properties of the virus, the extent of antigenic drift of the

virus, and the waning immunity in the population (93). During 9 of the 22 years of circulation of H1N1 virus from 1934 to 1956, there were epidemics associated with excess mortality. During years when H2N2 or H3N2 viruses were the sole influenza A viruses circulating, excess mortality occurred in 64% and 80% of years, respectively.

The temporal curves of individual influenza virus epidemics are similar. Seeding of a community with virus is followed by a relatively sharp, single peak of virus activity, as indicated by school and industrial absenteeism and, slightly later, excess mortality (374). Exceptions to this pattern were observed in 1918 and 1957, in which introduction of a pandemic virus was followed by two waves of epidemic activity within 1 year (242,374,514).

Morbidity

Influenza viruses have a major impact on morbidity, with increases in hospitalizations and visits to health care providers. With large databases, the morbidity has recently been defined in children (367), in women of all ages with high-risk conditions (368), and in pregnant women (369). In the elderly, who remain the group at highest risk, the impact of influenza has been shown to extend beyond influenza and pneumonia illnesses to all acute and chronic respiratory conditions and to congestive heart failure (371). Hospitalization due to influenza A virus infection averaged over 8 years (1970 to 1978, which did not include a pandemic year) were 1 per 2,900 for the 1- to 44-year-old age group, 1 per 1,100 for the 45- to 64-year-old age group, and 1 per 270 for those older than 65 years (18,20). High rates of hospitalization also are observed in children younger than 5 years, with the highest rates in children being in those younger than 1 year (396). The observation that 14% to 16% of children seeking medical care for febrile respiratory illness are infected by influenza virus indicates influenza's contribution to outpatient morbidity (163,548).

The major factor that diminishes morbidity after exposure to influenza A virus is the level of preexisting immunity from past exposure, through vaccination or natural infection, to viruses bearing related surface glycoproteins. The more severe illness seen in naive individuals explains the high morbidity seen in infants and young children undergoing first infection (396) and the high morbidity seen in all age groups after the introduction of new pandemic viruses such as in 1918 (H1N1), 1957 (H2N2), and 1968 (H3N2) (242).

Another factor that appears to contribute independently to morbidity is age at the time of infection. Regardless of the level of antibody to the HA, infections in young adults are more often asymptomatic than are those in children (102,136,160,335). In the elderly, influenza poses special risks of debilitating illness (19). It is important to emphasize that infection rates and attack rates reflect the delicate interplay between immunity in the population and the relative virulence of the virus and can thus vary greatly after introduction of an influenza virus into a population (69,136,160,243,418). Immunity to influenza viruses is incomplete, and morbidity can occur after reinfection with the same or with a drift strain (96,97,136,141,142,467). However, the morbidity after reinfection is usually less than that after first infection (96,97,160,467). Significant morbidity and mortality with influenza A viruses can occur during nosocomial spread of infection (152,186).

The economic impact of influenza A virus infection is considerable (256). The estimated total cost (direct and indirect) of each of three different epidemics varied from $1 to 3 billion depending on the size of the epidemic (256).

Mortality

Influenza virus is uniquely important among respiratory viruses as a cause of excess mortality. During the period from 1934 to 1990, influenza B virus was associated with excess mortality in 16 (26%) of 63 years examined, whereas excess mortality associated with influenza A virus was seen in 38 (60%) of 63 years (see Fig. 9). A recent article has created a severity index for judging the impact of influenza epidemics (459). The authors found that epidemics associated with the circulation of influenza A H3N2 viruses have higher mortality than do those seen with H1N1 or B strains. Their estimate of the total mortality attributable to influenza in the U.S. for the 20-year period from 1972 to 1992 was 426,000 deaths, but in some years, influenza B infections can be a serious problem (e.g., 1990).

Although morbidity with influenza A virus is concentrated in people younger than 20 years, the highest rates of excess mortality occur in persons older than 65 years (22,94,117,469). However, excess mortality is seen in all ages (94). During the 1918 epidemic, the excess mortality rate was extremely high in young adults, as well as in those older than 65 years, but this pattern has not been seen since (374). Various underlying medical conditions place an individual at high risk for serious consequences of influenza virus infections. These conditions include cardiovascular disease, pulmonary disease, metabolic disease, neoplastic disease, and pregnancy (20,21,171,369). The highest risk for pneumonia is seen in those older than 65 years who have both a cardiovascular and pulmonary condition (22). Mortality due to influenza is not confined to the bedridden, because up to 50% of all deaths occurred in people who were chronically ill but medically stable and functioning independently (19). The average yearly rate of death due to influenza A virus infection is 1 per 2,200 adults 65 years or older (7). For a pandemic year (1957 to 1958) in which the excess mortality totaled 70,000 persons, 1 in 300 of adults older than 65 years died of influenza infection. Although excess mortality is highest during the first year of circulation of a new subtype, cumulative interpandemic mortality significantly exceeds that caused during the first year (374,459).

Strain Differences in Virulence

Although there has been remarkable similarity in the clinical manifestation of influenza A viruses belonging to different subtypes (95,109,476), some differences between virus strains are suggested by their epidemic behavior (271,514,547). In a prospective long-term study of urban children, H3N2 viruses were more frequently associated with croup than were H2N2 strains (271). The H1N1 virus that caused the 1918 epidemic was associated with very high mortality in young adults, an age group that was not at high risk for mortality in subsequent H1N1, H2N2, or H3N2 epidemics (512). Host, viral, and/or environmental factors that contributed to this unusual pattern have not been identified. Furthermore, encephalitis lethargica is epidemiologically associated only with the H1N1 viruses in 1918 and years immediately thereafter (412). In comparison to H3N2 viruses, H1N1 viruses, when reintroduced in 1977, caused relatively mild illness

in most but not all investigational outbreaks in susceptible children and young adults (141,159,400,547).

PATHOGENESIS IN HUMANS

Pattern of Virus Shedding

Influenza virus replicates throughout the respiratory tract, with virus being recoverable from the upper and lower tracts of people naturally or experimentally infected with virus. The pattern of virus replication in six adult volunteers administered an influenza A/HK/68-like H3N2 virus, in relation to the onset of clinical symptoms, IFN response, and serum and nasal wash antibody responses, is presented in Fig. 10) (420). Virus replication peaks at about 48 hours after inoculation and declines somewhat slowly thereafter, with little shedding after days 6 to 8. Peak virus titers in symptomatic volunteers range from $10^{3.0}$ to $10^{7.0}$ $TCID_{50}$/mL of nasopharyngeal wash (349), with a positive correlation between the amount of virus shedding and the severity of the clinical response. Individuals who shed less than $10^{3.0}$ $TCID_{50}$/mL are either asymptomatic or have only minor upper respiratory tract symptoms. Even after infectious virus can no longer be recovered, viral antigen can be detected for several days in cells and secretions of infected individuals (33,113,487). In children naturally infected with influenza viruses, virus is most frequently recovered 1 to 2 days after the onset of symptoms and can be found for up to 13 days thereafter (143). The peak titer is achieved on the first day of illness and averages $10^{4.0}$ $TCID_{50}$/mL of nasal wash (188).

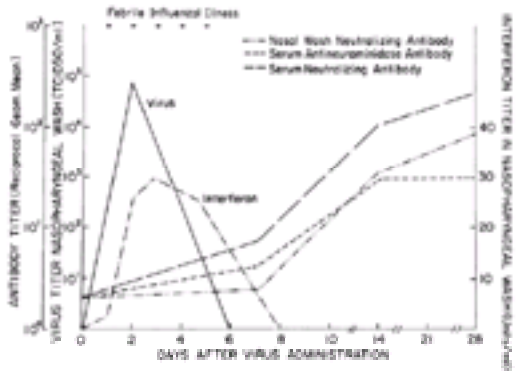


FIG. 10. Six seronegative volunteers received $10^{4.0}$ $TCID_{50}$ of wild-type A/Bethesda/1015/68 virus intranasally on day 0. From ref. 149, with permission.

Pathology

Influenza A virus induces pathologic changes throughout the respiratory tract, but the most significant pathology is present in the lower respiratory tract (207,208,209 and 210,513). During bronchoscopy of persons with uncomplicated influenza infections, acute diffuse inflammation of the larynx, trachea, and bronchi were observed with mucosal inflammation and edema. Light-microscopic studies of infected cells revealed that the columnar ciliated cells become vacuolated, edematous, and lose cilia before being desquamated. Within 1 day after the onset of symptoms, desquamation of the ciliated and mucus-producing epithelial cells down to a one-cell-thick basal layer is observed; in other areas, the thickened and hyalinized basement membrane is exposed. Submucosal edema and hyperemia occur with an infiltration by neutrophils and mononuclear cells (310). Viral antigen is present predominantly in epithelial cells and mononuclear cells, but is infrequently found in the basal cell layer (344). In a more severe primary viral pneumonia, there is an interstitial pneumonitis with marked hyperemia and broadening of the alveolar walls, with a predominantly mononuclear leukocyte infiltration and capillary dilation and thrombosis. The alveolar walls become denuded of epithelium; intraalveolar edema and exudate are present; hyaline membranes cover alveolar walls; and intraalveolar hemorrhage occurs. Influenza virus–specific antigen is present in type 1 and 2 alveolar epithelial cells, as well as in intraalveolar macrophages (344). Necrotizing changes may occur with rupture of walls of alveoli and bronchioles. Influenza virus has been obtained from lungs at autopsy in titers of $10^{2.0}$ to $10^{5.7}$ 50% egg infectious doses per gram of tissue (300).

At a cellular level, influenza virus shuts off cell protein synthesis and induces apoptosis as an additional means of cell destruction (216). From the third to fifth day after onset of illness, mitoses appear in the basal cell layer, and regeneration of the epithelium begins. During this time, reparative and destructive processes may be present simultaneously. Complete resolution of the epithelial necrosis probably takes up to a month, because pulmonary-function abnormalities persist beyond the period of symptomatic recovery from the acute phase (190).

Immune Response to Infection

Innate Immune Response

Human influenza viruses replicate exclusively in superficial cells of the respiratory tract. They are released from the apical surface of the cell, which may limit more systemic spread but facilitate accumulation of virus in the lumen of the respiratory tract for transmission to the next susceptible host (37). The apical release of virus raises not yet explored questions of how antigen recognition and modulation of infection by antibody may occur. The extremely short incubation period between infection and clinical illness implies that innate immunity or preformed cognate recognition components are important.

The respiratory tract has a series of protective mechanisms against influenza infection, including the mucin layer, ciliary

action, and protease inhibitors that may prevent effective cell entry and virus uncoating. Once the epithelial cell is infected, proinflammatory cytokines, notably interleukin-6 (IL-6) and IFN-a, are induced and released from these cells. These cytokines reach their peak by day 2 after experimental infection (199). These two cytokines correspond in their release with the highest clinical symptom score, mucus production, fever, and viral load. Other cytokines either appeared later [IL-8 and tumor necrosis factor-a (TNF-a), or did not increase IL-b, IL-2, or TGF-b in the respiratory secretions or serum. The importance of IFN in regulation of influenza replication is suggested by the observation that the NS-1 protein is an IFN antagonist. Deletion of NS-1 leads to increased induction of IFN and a virus that is highly attenuated, except in a mouse with an interruption in the IFN-induction pathway (151). It is generally believed that IFN contributes to recovery from infection, and the early cytokine pattern directs the immune response toward a Th1 pattern (150).

A transient increase in natural killer cell activity occurs after influenza infection (121,292).

Cognate Immune Response

Humoral Immunity

In 80% of naturally acquired human infections, a serum antibody response can be documented (136). After primary infection, HA-specific antibodies of IgG, IgA, and IgM isotypes are detected, whereas in the secondary response, IgG and IgA antibodies predominate (47,349). B cells producing influenza virus–specific IgG, IgA, and IgM antibodies are present in the peripheral blood of normal individuals and of individuals undergoing infection with an influenza A virus (59,552). Serum antibody plays a role in resistance to or recovery from influenza illness in humans. A protective effect of transplacentally acquired antibody has been inferred from the observation that a significant direct correlation exists between age at the time of symptomatic influenza A virus infection in infants and level of maternally transmitted antibody measured in cord serum (408).

During infection of humans with influenza A virus, antibodies to the HA, NA, NP, and M proteins are produced (88,403). Antibodies to the HA and NA glycoproteins are associated with resistance to infection and/or illness in humans and in laboratory animals, whereas antibodies to the internal M1 and NP proteins are not (12). Passively transferred monoclonal antibody to the M2 protein is protective in mice, and M2 antibodies develop in humans infected with influenza A viruses (36,499). A universal influenza A vaccine has been proposed based on the extracellular domain of the M2 protein (364). Studies of vaccinia recombinant viruses expressing each of 10 gene products of the influenza A virus clearly identified the HA and NA glycoproteins as primary protective antigens, and the primary mediators of the resistance to virus challenge induced by these glycoproteins were antibodies (123). Because of the importance of antibodies to the surface glycoproteins in resistance to infection, only the antibody response to these proteins is discussed further. The reader is referred to four reviews that have considered immunity to influenza virus infection in detail (12,85,349,403).

The level of serum antibody to the HA and NA correlates with resistance to illness and with restriction of the influenza virus replication in the respiratory tract of humans (76,352). This has been shown after experimental infection with influenza virus (76) or after natural infection (85). HA antibodies can prevent infection by neutralizing the infectivity of the virus (509), whereas NA antibodies mediate their antiviral effect primarily after infection has been initiated by restricting spread of virus within the respiratory tract of the host (453).

It was previously thought that $CD8^+$ T cells were solely responsible for clearance of virus during primary infection or during reinfection. However, it has been demonstrated that passively transferred antibodies can clear an influenza A virus infection in the absence of B or T cells in the host (442).

Although immunity mediated by infection can be long lived, reinfection with antigenetically closely related influenza A viruses occurs, indicating that immunity against this highly infectious virus induced by a single infection can be incomplete (141,142,467). Two mechanisms have been identified that can contribute to this incomplete immunity. First, there is a gradual diminution in the total amount of serum (IgG), local IgA antibodies, or antibody-secreting cells within the first year after the first infection (239,240,545). Second, after infection with a new influenza virus subtype, antibodies are generated that react with only a limited number of antigenic sites on the HA glycoprotein, whereas after several infections, antibodies are generated that have a broad range of specificities (516). Consistent with this is the greater degree of resistance to infection with antigenic drift strains in humans previously infected twice with prototype virus than in those with only one previous infection (97).

Cellular Immunity

The role of cellular immunity in clearance of influenza virus has been well defined in the murine model but less well in humans (251). There are two subsets of effector lymphocytes in humans. The first subset consists of cells that mediate cytoxicity that is restricted by class I histocompatibility antigens and has the $CD8^+$ phenotype. These cytotoxic lymphocytes (CTLs) appear in the blood of infected or vaccinated individuals on days 6 to 14 and disappear by day 21 (122). Most studies have examined memory CTLs, which are detected *in vitro* by stimulation of peripheral blood lymphocytes with antigen. These CTLs exhibit a cross-reactive pattern of virus specificity (i.e., they lyse target cells infected with influenza A viruses belonging to any subtype, but not target cells infected with influenza B virus) (553). However, a more specific subpopulation of CTLs lyses only cells infected with homologous virus. Human class I–restricted CTLs recognize either glycoprotein HA or nonglycoprotein antigens (M, NP, PB2), which can account for their subtype-specific and cross-reactive specificities, respectively (134,553). Depending on human leukocyte antigen (HLA) haplotype, individuals can differ considerably in the antigens that their CTLs can recognize (321). The memory CTLs characterized after infection or vaccination of humans have the cross-reactive pattern of cytotoxicity (120,122). Passively transferred class I–restricted CTLs mediate recovery from influenza pneumonia in mice (553). The prechallenge level of memory CTLs did not correlate with susceptibility to infection or illness after experimental administration of wild-type virus, but it correlated with accelerated clearance of virus from the respiratory tract of humans (321).

The other class of CTLs is restricted by histocompatibility class II antigens and has the $CD4^+$ phenotype (248,472) and

thus belong to the T-helper class of T cells. The $CD4^+$ T cells have two functional properties. First, they are helper T cells, providing help both to B cells for antibody production and to class I–restricted CTLs for their proliferation (281,282,553). It is interesting to note that the helper T cells specific for M or NP antigen can provide help to B cells secreting HA antibody (282,283,441), and in this manner, can augment the antibody response to protective antigens. Second, the $CD4^+$ T cells can have cytolytic activity, with a range of antigen specificities similar to those of class I–restricted CTLs (553). Little further is known of the role of these T cells in immunity in humans or of the kinetics of their responses after infection or vaccination.

$CD4^+$ and $CD8^+$ T cells both can contribute to immunity to influenza A virus infection. The important role of $CD4^+$ T cells in immunity to influenza virus infection has been defined from studies in $CD8^+$ T cell–deficient animals, which can clear influenza A virus by a $CD4^+$ T cell–dependent mechanism (28,116,295). Passively transferred influenza virus specific $CD4^+$ T-cell clones are not able by themselves to clear an ongoing influenza virus infection, but require functional B cells to accomplish this clearance (442). Thus the primary antiviral activity of the $CD4^+$ T cell is to provide help to B cells to produce antiviral antibodies. Because $CD4^+$ T cells recognize many epitopes on influenza viral proteins, a large number of T cells are available to provide such help (66).

$CD8^+$ T cells also are able to mediate clearance of influenza A virus infection in $CD4^+$ T cell–deficient animals (115,295). The $CD8^+$ T cells not only clear infectious virus from experimentally infected animals, but also clear influenza virus RNA from lung tissue. Thus the immune system is functionally redundant in its ability to clear influenza A virus infections. Animals deficient in both $CD4^+$ and $CD8^+$ T cells succumb to infection, indicating that nonspecific mediators of immunity are not sufficient in the absence of T cells to restrict influenza A virus infection significantly (116,295). Although passively transferred $CD8^+$ T cells that have been activated *in vitro* with influenza virus antigen can modify infection with influenza A virus, it remains problematic to induce protective $CD8^+$ T cells by immunization (287,475). The mechanisms by which influenza A virus $CD8^+$ T-cell clones clear influenza A virus infections, be it by cytolysis or antiviral cytokine secretion, remains unknown. The number of $CD8^+$ T-cell epitopes on viral proteins is much more restricted than the number of B-cell or $CD4^+$ T-cell epitopes (30). Cytolysis of influenza virus–infected cells also can be mediated by antibody plus complement, and antibody-armed lymphocytes (195,409,434).

Lymphproliferation is another measurement of a cellular response to influenza. Lymphocytes responsive to influenza antigens have been isolated from the blood and lower respiratory tract secretions of influenza virus–infected humans, but only the former have been studied extensively (244,553). Lymphocyte blastogenic responses to influenza antigens in humans increase after influenza A virus infection, beginning between the third and sixth day after infection (104), and return to baseline levels by day 28. Influenza antigen-specific cutaneous reactivity or *in vitro* blastogenesis in humans correlates weakly with susceptibility to infection with influenza A viruses (234).

Influenza A virus infection can depress skin test reactivity to common antigens used to assess cutaneous delayed hypersensitivity (38,246,414). In addition, depression of the peripheral blood lymphocyte blastogenic responses to mitogenic and antigenic stimulation (104,246,292) is seen during the acute stage of infection and early convalescence. Despite this transient depression of delayed hypersensitivity and blastogenic responses to noninfluenzal antigens or mitogens, influenza virus–specific cellular responses develop (106,122). The mechanisms underlying the depression in the function of lymphocytes in influenza indicated earlier have not been defined, but influenza A virus can abortively infect human lymphocytes *in vitro* (48) and possibly *in vivo* (537).

Mucosal Antibody Response

Francis (140) was the first to detect neutralizing activity to influenza virus in nasal secretions, and subsequently, resistance to experimental influenza A virus infection in the mouse was found to be mediated primarily by antibody present in bronchial secretions (128). The neutralizing antibody in nasal secretions of humans is primarily of the immunoglobulin A (IgA) isotype (11) and is locally produced (431). Antibody to both the HA and NA can be detected in local secretions, but the former is easier to detect and thus has been studied more frequently (85). Furthermore, IgA antibodies, which are the primary mediators of resistance to influenza A virus infection in the upper respiratory tract, can act intracellularly to inhibit replication of virus and likely actively participate in clearance of infectious virus from the epithelial cells of the respiratory tract (314,315,416,417).

During primary viral infection, IgA, IgG, and IgM hemagglutinin-specific enzyme-linked immunosorbent assay (ELISA) antibodies appear in nasal washes, with IgA and IgM antibodies detected more frequently than IgG antibody (349,353). Most of the HA-specific IgA and IgM antibodies are actively secreted locally, whereas local secretion could be demonstrated only infrequently for IgG antibody. Individuals with a local IgA response also had a serum IgA response. Local IgA antibody stimulated by natural infection is detectable for 3 to 5 months after infection, and there is local IgA memory for influenza antigen (349,545). After secondary infection, local antibody is also primarily of the IgA isotype. Again, those individuals with a local IgA response also have a serum IgA response, and the magnitude of the serum IgA HA antibody response correlates with that of the local response. Nasal wash IgG appears to be predominantly a transudate from serum, with a serum-to-nasal wash concentration gradient of 350:1 (353).

The presence of local IgA antibody induced by infection with an attenuated virus correlated with resistance of volunteers to infection and illness after challenge with a virulent wild-type virus (239). Challenge of children with an attenuated live vaccine showed similar findings (40). Resistance to experimental wild-type infection also correlated with the level of local HA antibody present at the time of virus administration, with IgG and IgA each contributing to resistance (76).

Effect of Infection on Bacterial Flora and Phagocytic Cell Function

Influenza A virus infection does not generally significantly alter the normal bacterial flora (39,102); however, several exceptions have been noted. In patients with chronic bronchitis, influenza A virus infection was followed by an increased incidence of colonization with *Streptococcus pneumoniae* (463). During an outbreak of meningococcal and influenza A

virus infections, influenza virus infection was found to be significantly associated with infection or carriage of *Neisseria meningitidis* (554). Furthermore, pharyngeal cells from experimentally infected volunteers demonstrated an increased adherence for pathogenic bacteria during acute disease (124).

Infection of human polymorphonuclear (PMN) and mononuclear (MN) leukocytes with influenza A virus *in vitro* depresses the ability of these cells to respond to a chemotactic stimulus (1,63). During influenza A virus infection in humans, PMN and MN leukocytes obtained from peripheral blood also exhibit depressed chemotaxis *in vitro* (1). Phagocytosis has generally not been affected by infection of PMN or MN leukocytes *in vitro,* but infection does decrease bactericidal capacity of the phagocytic cells (1). Similar findings have been reported in experimental animals, although not by all investigators (377,438). Influenza A virus infection of macrophages *in vitro* can inhibit their ability to help or augment the proliferative response of lymphocytes to mitogens (422). Perhaps these virus-related alterations in phagocytic cell function act in concert with the destruction of the ciliated epithelium to set the stage for the secondary bacterial infections that can occur during influenza virus infections of humans.

CLINICAL FEATURES IN HUMANS

Adults

Infection with influenza A viruses results in a spectrum of clinical responses ranging from an asymptomatic infection to a primary viral pneumonia that rapidly progresses to a fatal outcome. Reviews of the signs and symptoms of illnesses due to different strains of influenza A virus have been published (102,109,476). The typical uncomplicated influenza syndrome is a tracheobronchitis with the additional involvement of small airways (513). The incubation period can be as short as 24 hours or up to 4 or 5 days, depending in part on the dose of virus and the immune status of the host.

The onset of illness is usually abrupt, with the occurrence of headache, chills, and dry cough, which is rapidly followed by high fever, significant myalgias, malaise, and anorexia. Substernal tightness and soreness can accompany the cough. The most prominent sign of infection is fever that often peaks within 24 hours to as high as 41°C, but more commonly is in the 38°C to 40°C range. The fever usually begins to decline on the second or third day of illness and is usually gone by the sixth day; the median duration is 3 days (109). The elderly can have high fever, lassitude, and confusion without respiratory signs.

Other than fever, the physical findings in influenza are usually minimal, although patients can appear quite toxic. Nasal obstruction, rhinorrhea, and sneezing often occur, and pharyngeal inflammation without exudate is common. Conjunctival inflammation and excessive tearing may occur. Influenza A virus usually does not cause purulent conjunctivitis, but has been recovered from the conjunctiva of a person with conjunctivitis who was infected with a virus of seal origin (522). Viral antigen is detectable in conjunctival cells and secretions (487). Small cervical nodes can be felt in a minority of cases. Chest radiograph and auscultatory findings are usually normal, although occasionally patchy rales and rhonchi are heard.

As the fever is declining, the respiratory signs and symptoms such as rhinorrhea and coughing may become more intense. The cough frequently changes from a dry, hacking nature to one that is productive of small amounts of sputum that are usually mucoid but can be purulent. After the fever and upper respiratory tract symptoms resolve, cough and weakness can persist for 1 to 2 additional weeks. Illness is more frequent and more severe in cigarette smokers (249). It is important to emphasize that influenza virus can also give rise to asymptomatic infection, as well as to lesser illnesses such as rhinitis and/or pharyngitis.

Tracheobronchial clearance of radiolabeled particles is depressed during acute influenza virus infections but returns to normal levels about 1 month later (61). Clearly, the loss of the mucociliary blanket is a factor in the predisposition to secondary bacterial pneumonia seen after influenza infection. Although air flow in large airways is usually unaltered in uncomplicated influenza, there is a transient increase in bronchial reactivity to chemical and particulate stimuli during natural infection (503). Small peripheral airways are often affected in uncomplicated influenza A virus infections (190). This small-airway abnormality can persist after symptomatic illness has subsided. Alterations in pulmonary gas exchange, with a depression of the diffusing capacity of carbon monoxide and an increase in the alveolar–arterial oxygen tension gradient, also are seen (226). Although significant abnormalities in large and small airways can be demonstrated during acute infection and early convalescence, uncomplicated influenza appears to cause little permanent damage in the lung, even in patients with chronic obstructive lung disease (288,464).

Children

The clinical manifestations of influenza in children are similar to those seen in adults, but there are some distinct differences between these two age groups.

First, children have higher fever, and febrile convulsions can occur (163,407,546,548). At least 14% of fevers associated with respiratory tract symptoms that result in a visit to a pediatrician are caused by influenza viruses (163,548).

Otitis media, croup, pneumonia, and myositis are more frequent in children (205,271). Influenza A and B virus infections increase the risk of developing otitis media as well as the risk of recurrence of middle ear disease in children (205). In approximately 12% of naturally infected seronegative children, otitis media develops (548). From 24% to 33% of children hospitalized with influenza A virus infection have otitis media (384). Control measures that are effective in preventing influenza virus infection or illness decrease the incidence of otitis media in children. Although less well documented, bacterial sinusitis may follow acute influenza.

Studies in children in an urban setting have shown that influenza A viruses are important causes of laryngeotracheobronchitis (croup) (271). In the H2N2 era, influenza A virus infection was associated with 8% of all croup requiring hospitalization, and in the H3N2 era, with 24% of all such croup. Croup occurred predominantly in children

younger than 1 year. Influenza-associated croup can be especially severe and occasionally requires intubation (42,51).

It is important to emphasize that influenza A and B viruses are a significant cause of serious lower respiratory tract disease in children (42,162,163,271,298,363,381). In a prospective study of 121 susceptible (seronegative) young children seen in a clinic during an H3N2 virus outbreak in 1974, five of 60 infected infants had clinical and radiographic evidence of pneumonia (546). In addition, influenza A virus infection has been shown to exacerbate asthma (277,330,399).

In children, myositis is usually localized to the gastrocnemius and soleus muscles and is characterized by pain on walking. It has its onset as respiratory illness wanes; the muscles are tender and swollen; serum levels of muscle enzymes are elevated; the course is usually benign and reversible; and light-microscopic changes of muscle necrosis and an inflammatory cell infiltrate occur (100,325,329). Children (especially those younger than 3 years) have a higher incidence of gastrointestinal manifestations such as vomiting and abdominal pain (102). Infection of neonates can present as unexplained fever (322) and can be life-threatening (24,322).

Differential Diagnosis

The respiratory tract manifestations of influenza virus infections are not specific and can be seen during infection with other viral respiratory tract pathogens, including respiratory syncytial virus, parainfluenza viruses, coronaviruses, adenoviruses, rhinoviruses, and *Mycoplasma pneumoniae* (271). Because the severe forms of viral pneumonia are extremely rare with other respiratory viruses in adults, it is reasonable to assume that during an epidemic of influenza A virus in the community, diffuse interstitial viral pneumonia in an adult is likely to be due to influenza virus. The recent documentation that another wintertime virus, respiratory syncytial virus, can cause equally severe illness in the elderly makes even this assumption tenuous (125). The protean manifestations of influenza A virus demand that influenza virus be considered in the differential diagnosis of fever in neonates and of myositis, febrile convulsions, and encephalopathies occurring in the general population during influenza epidemics. The diagnosis can be established only by recovery of virus, by demonstration of the presence of viral antigen or viral genetic material in clinical specimens, or by detection of an increase in specific antibody titer in serum or nasal secretions (74,85a,440,515).

Lower Respiratory Tract Complications

Three distinct syndromes of severe pneumonia can follow influenza infection in children or adults. Complications are most common in the elderly (301).

Primary Viral Pneumonia

Primary influenza virus pneumonia was first described in 1959 (300,310) in fatal cases caused by the 1957 Asian influenza A virus (H2N2), although retrospectively, it had been observed during previous influenza A epidemics (165,344). Primary viral pneumonia occurs predominantly in individuals at high risk for the complications of influenza virus infection (i.e., the elderly or patients with cardiopulmonary disease), but 25% of the cases are in normal individuals and an additional 13% in pregnant women. The typical case of primary viral pneumonia develops abruptly after the onset of influenza illness. It progresses within 6 to 24 hours to a severe pneumonia with rapid respiration rate (30 to 60 respirations per minute), tachycardia (greater than 120 beats/min), cyanosis (in 80% of patients), high fever (average of 39°C), and hypotension. The illness may rapidly progress to hypoxemia and death in 1 to 4 days; the presence of frothy hemoptysis, tachypnea, and cyanosis portends a fatal outcome. Auscultatory findings include bilateral crepitant inspiratory rales. Examination by radiograph reveals mottled densities in two or more lobes and diffuse symmetrical interstitial infiltrates or areas of consolidation; cavitation or pleural effusion suggests bacterial superinfection. Electrocardiographic abnormalities occur, but are not those consistent with myocarditis. Pathologic findings in the trachea and bronchi are similar to those previously described during acute uncomplicated disease, but bronchiolitis also is present. Significantly, pathologic findings are present in the alveoli (210,310). The clinical, laboratory, and physiologic findings in primary viral pneumonia are not specific. The erythrocyte sedimentation rate is usually normal; a leukocytosis with band forms is seen in a little fewer than half the cases; and the partial pressure of arterial oxygen is depressed, often markedly.

In nonfatal cases, initial improvement occurs from 5 to 16 days after onset of pneumonia, with resolution of radiographic changes taking up to 4 months (541). Survivors can develop diffuse interstitial fibrosis with a decrease in diffusion capacity of carbon dioxide and a decrease in arterial oxygen tension (285,398,541). Influenza B virus can cause severe disease but has not been reported to be associated with fatal primary viral pneumonia in normal individuals (131,500).

Combined Viral–Bacterial Pneumonia

A combined viral–bacterial pneumonia is approximately 3 times more common han primary viral pneumonia. The bacteria most commonly involved are *Streptococcus pneumoniae, Staphylococcus aureus,* and *Hemophilus influenzae,* although other microorganisms can play a role (35,154,300,310,397,454). Clinically, this syndrome may be indistinguishable from primary viral pneumonia, except that the symptoms of pneumonia appear somewhat later after onset of influenza symptoms, and chest radiographs taken early in the course of pneumonia are more apt to reveal the presence of pleural effusion. Virus has been recovered from the lungs (in a titer of $10^{2.0}$ $TCID_{50}$/g) and from pleural fluid (310,313). The erythrocyte sedimentation rate is elevated more frequently than in primary viral pneumonia. Because there is no effective distinction between the two syndromes, the diagnosis depends solely on the demonstration of bacteria in the sputum, in fluid obtained at bronchoscopy, or in the pleural fluid. The case fatality rate for combined viral–bacterial pneumonia is about 10% to 12% (35,300,397). However, coinfection with influenza and *S. aureus* has a higher fatality rate of up to 42% (425).

There are strains of *S. aureus* that secrete proteases capable of activating the infectivity of influenza virus by proteolytic cleavage of the HA. These strains play a synergistic role in experimental pneumonia in mice (485,486). Such

protease-secreting bacterial strains could well be an added pathogenic factor in combined viral–bacterial pneumonias in humans.

Influenza Infection Followed by a Bacterial Pneumonia

In this syndrome, an individual has a typical influenzal illness and is beginning to improve, but then develops shaking chills, pleuritic chest pain, and an increase in cough productive of bloody or purulent sputum (300). Cyanosis and a marked increase in respiratory rate are less common than with a primary viral pneumonia. Examination by radiograph reveals local areas of lung consolidation but not diffuse pneumonia. Often influenza A virus is not recovered from the upper or lower respiratory tract, leukocytosis is common, and the erythrocyte sedimentation rate is elevated. This is generally a milder form of pneumonia, although a case fatality rate of about 7% has been described (300).

Extrapulmonary Manifestations of Influenza

Viremia

Viremia is unusual in influenza virus infection (267,332). Virus has been isolated from the blood in low quantity (present only in undiluted blood specimens) from a patient in the fourth day of influenza illness (359). In experimental or natural infection, virus has been recovered from the blood during the 1- to 3-day incubation period but not thereafter (264,470). Virus was present in barely detectable amounts in the blood of two patients with fatal primary viral pneumonia on the sixth day after the onset of illness and was recovered at necropsy from the liver and spleen of one patient (289). In addition, the presence of viremia has been inferred because virus was present in low titer in extrapulmonary tissues such as heart, liver, spleen, kidney, adrenal glands, and meninges obtained from patients dying of pneumonia (245,421).

Myositis and Cardiac Involvement

In adults, a diffuse form of the myositis occurs with generalized pain, tenderness, and weakness of muscles, increased serum levels of muscle enzymes, myoglobinemia, and myoglobinuria (331). Fatal acute renal failure can follow this form of the disease. The pathogenesis of the myositis remains uncertain. Muscle cell necrosis with or without inflammation can occur, and although influenza virus has been recovered from muscle, the relation between the virus and the myositis remains unclear (147,263,331).

Attempts at virus recovery from the heart of patients with myocarditis associated with fatal pulmonary infection have largely been unsuccessful (132,300,310,381). Virus was recovered from the heart of a patient with fatal pneumonia, but postmortem examination revealed arteritis, not myocarditis (381). Clinical findings and cardiac-function studies in patients with severe pulmonary infection suggest that myocardial dysfunction is not a direct result of influenza A virus infection (53,300). In uncomplicated influenza A virus infection, electrocardiographic abnormalities have been observed (506). Virus has been recovered from pericardial fluid in two cases (211,542), and influenza A and B virus–associated myopericarditis has been described (3).

Reye Syndrome

Reye syndrome, which is a neurologic and metabolic disease of children and adolescents, is characterized by a rapidly progressive noninflammatory encephalopathy and fatty infiltration of the viscera, especially the liver, resulting in severe hepatic dysfunction, as indicated by elevated serum transaminase and ammonia levels. This syndrome has followed respiratory viruses, varicella virus, and gastrointestinal infections (230). Histologically there is electron-microscopic evidence of mitochondrial swelling (391). Influenza B in particular but influenza A H1N1 and H3N2 viruses as well have been identified as antecedent infections (230). The administration of therapeutic doses of salicylates appears to be a risk factor in the development of Reye syndrome (23). The incidence of Reye syndrome after influenza infection was estimated to be between 0.37 and 0.88 cases per 100,000 children younger than 18 years. The incidence was likely to have been underestimated because milder forms of Reye syndrome have been described (294). The onset of the CNS and hepatic symptoms usually begins as respiratory tract symptoms wane. The case fatality rate varies and has been estimated to be between 22% and 42% (230). The disease associated with influenza B virus occurs in children aged 0 to 18 years (median age, 11 years) and is more frequently seen in a rural than in an urban environment (230). The etiology and pathogenesis of this syndrome are unknown. Virus has been isolated from affected tissue in low titer in several instances (376,392), and the syndrome has been observed in three siblings of different HLA types (540). A syndrome similar to, but milder than, Reye syndrome was observed in India during the 1957 influenza A (H2N2) virus epidemic (247). Noninflammatory encephalitis and hepatic dysfunction occurred in association with that influenza epidemic. There has been a dramatic decrease in Reye syndrome cases in the U.S. in both varicella-associated and non–varicella-associated cases. This reduction is thought to be associated with reduced use of salicylates, which are contraindicated for use in influenza infection (23).

Central Nervous System Involvement

A wide spectrum of CNS involvement has been observed during influenza A and B virus infections in humans (31,99,110,111,201,316,317,381,407), ranging from irritability, drowsiness, boisterousness, and confusion to the more serious manifestations of psychosis, delirium, and coma. Physical signs of altered reflexes can be elicited. The pathogenesis of these CNS symptoms is unclear. Nonspecific metabolic effects such as hypoxia resulting from severe pulmonary infection contribute to the CNS signs and symptoms.

Two specific CNS syndromes have been suggested to accompany influenza infection: influenzal encephalopathy and postinfluenzal encephalitis. Encephalopathy occurs at the height of influenza illness and may progress to death (99,135,228). The cerebrospinal fluid (CSF) is usually normal, the brain at autopsy shows severe congestion, and histologic changes are minimal. Lungs of such patients show changes typical of influenza and can yield virus in high titer. The postencephalitic syndrome is extremely rare and occurs from 2 to 3 weeks after recovery from influenza. The CSF

findings indicate inflammatory changes, and recovery occurs in the majority of cases. The association of the postencephalitic syndrome with influenza virus infection is less certain because virus is not usually recovered, and initial serum specimens usually contain antibodies (135). Influenza A virus has only rarely been recovered from the brain or CSF (135,384,407), and attempts to isolate it from the CNS of individuals dying of primary viral pneumonia have been unsuccessful (300,310).

It has been suggested that the syndrome of encephalitis lethargica followed by postinfluenzal encephalitic Parkinson's disease was associated with the influenza epidemics of 1918 and the epidemics following shortly thereafter (412). The epidemics of encephalitis lethargica followed 1 year after the epidemics of influenza, and these were followed in turn by postencephalitic Parkinson's disease about one decade later (412).

Febrile convulsions leading to hospitalization occur in children with and without underlying CNS abnormalities. An increase in the incidence of the Guillain–Barré syndrome (GBS) has not been seen after influenza A or B virus epidemics.

Toxic Shock Syndrome

Toxic shock syndrome has been seen in association with influenza virus infection in humans and is believed to be the consequence of the bacterial exotoxin (TSST-1) secreted by colonizing *S. aureus* strains (83,305).

Infection During Pregnancy and Fetal Risk

Pregnant women in the second or third trimester have an increased risk of developing fatal influenza disease (300,310,369,444,468). The increased mortality is generally seen during the years after introduction of a new pandemic strain, as in 1918 and 1957 (171,444,543), although using a large data set, the impact can be appreciated in the interpandemic period (369). Virus is usually not recovered from the fetus (310). An increase in congenital anomalies and hematologic malignancies has been reported after influenza virus infection in pregnancy (13,172,185,411). However, no consistent association between specific defects or malignancies and influenza epidemics has emerged, and the virus itself has not been implicated as a cause of congenital defects (250,306,334).

Influenza Infections in Immunosuppressed Patients

Influenza viruses can infect and cause severe disease in immunocompromised individuals (299,426,531), but more often the illness that occurs resembles that seen in fully immunocompetent persons. Prolonged shedding of virus occurs in immunosuppressed persons (426,531).

Influenza B and C Virus Infections

Influenza B virus can cause the same spectrum of disease as that seen after influenza A virus infection, and severe illness can occur (15,271,544). The frequency of serious influenza B virus infections requiring hospitalization is about four-fold less than that of influenza A virus (271). Influenza B virus illness occurs in epidemic form and predominantly involves adolescents and school-age children, but adults and the elderly also can be involved (15,75,161,191,363,375,418). Myositis and gastrointestinal symptoms are more common manifestations of influenza B than of A virus infection (75,127,262,284).

Influenza C virus causes sporadic upper respiratory tract illness and is rarely associated with severe lower respiratory tract disease (140,337). By early adulthood, most individuals (96%) have antibody to influenza C virus (378). Administration of influenza C virus to volunteers induced mild coryza with some systemic symptoms (241).

PREVENTION AND CONTROL

Antivirals

Amantadine and Rimantadine

Amantadine hydrochloride and rimantadine, an analogue of amantadine, are currently licensed for prophylactic and therapeutic use against influenza A virus in humans in the U.S. Both have a tricyclic structure with an amine side group and have antiviral properties against all subtypes of influenza A virus but not against influenza B or C viruses (504). The concentration of amantadine and rimantadine that inhibits virus plaque formation ranges from 0.2 to 0.4 µg/mL for sensitive influenza A viruses and is greater than 100 µg/mL for influenza B viruses. The former level of drug is found in the blood and nasal secretions after oral administration.

The antiviral activity of amantadine and rimantadine is exerted after adsorption of virus to cells but before primary transcription (196). Resistant strains of influenza A virus can be isolated *in vitro* and *in vivo.* In experimental studies, amantadine-resistant mutants can spread to susceptible contacts and cause disease, including fatalities, indicating that acquisition of amantadine resistance is not associated with attenuation (483,526). Amantadine and rimantadine inhibit virus replication by blocking the acid-activated ion channel formed by the virion-associated M2 protein. The primary antiviral action of these compounds results from blocking the flow of H^+ ions from the acidified endosome into the interior of the virion, a process necessary for release of the transcriptionally active ribonucleoprotein complex for transport to the nucleus. In addition, a second effect of these compounds is to block maturation of the HA during transport from the endoplasmic reticulum to the plasma membrane (196).

At the recommended adult dose of amantadine or rimantadine (i.e., 200 mg orally), side effects are unusual. At dosages of 300 to 400 mg per day, significant symptoms referable to the CNS such as depression, difficulty concentrating,

nervousness, sleep disturbances, and gastrointestinal complaints occur. These effects are more frequently observed in recipients of amantadine than of rimantadine and are more evident in the elderly, in whom a reduced dose of 100 mg orally is recommended. Amantadine is cleared by the kidneys, and the dose must be adjusted with renal impairment, whereas rimantadine is metabolized by the liver. Compared with amantadine, rimantadine has the desirable properties of (a) fewer side effects, (b) one dose can be administered daily rather than two, and (c) clearance of drug is not affected by the level of renal function.

Amantadine and rimantadine are useful for prophylaxis against H1N1, H2N2, and H3N2 influenza A virus infections in adults and children. During an epidemic involving both influenza A H1N1 and H3N2 viruses, amantadine and rimantadine protected against influenza-like illness (78% and 65%, respectively), documented influenza illness (91% and 85%), and influenza infection (74% and 66%) (105). Lower efficacy rates against documented illness (70%) and infection (39%) were observed for amantadine prophylaxis during an H1N1 virus epidemic.

Amantadine or rimantadine also is useful in the therapy of illness caused by influenza H1N1, H2N2, and H3N2 viruses. Symptomatic improvement, including accelerated clearance of local symptoms and fever, occurs about 1 day earlier in treated patients, and peripheral airway dysfunction also resolves more quickly. Accelerated clearance of symptoms is seen in children infected with an H3N2 virus treated with rimantadine compared with control children receiving an antipyretic (187). Virus was cleared more rapidly in such treated individuals than in controls. Development of serum and nasal wash influenza antibodies was not inhibited by amantadine treatment.

Recommendations for prophylactic and therapeutic uses of amantadine include prophylactic use in individuals at high risk who have not been vaccinated, as well as hospital personnel who could spread infection in the hospital. Therapeutic use includes individuals with uncomplicated influenza who have a high temperature of less than 48 hours' duration. It would be helpful to know that amantadine is useful in treating primary viral pneumonia, but efficacy in this clinical setting remains to be established. Updated recommendations for use of these compounds by the Advisory Committee on Immunization Practices are published yearly (67).

Neuraminidase Inhibitors

The first NA inhibitors included DANA (2-deoxy-2,3-dihydro-*N*-acetylneuraminic acid; Neu5Ac2en) and its *N*-trifluoroacetyl analog FANA, which were effective *in vitro* but did not inhibit replication of influenza viruses in animals (324,387). After solution of the structure of the NA (79,505), DANA served as the lead compound in the rational design of drugs targeting the NA. Replacement of the 4-hydroxyl group on DANA with a guanidino group filled the unoccupied pocket in the NA active site with an inhibition constant (K_i) of 2×10^{-10} mol/L (510). The compound zanamivir or [GG-167] is a specific inhibitor of the NA of all influenza A and B viruses.

Zanamivir must be administered intranasally or inhaled for optimal effect. An additional NA inhibitor can be taken orally. This was achieved through the discovery and use of a hydrophobic pocket in the enzyme-active center that could accommodate lipophilic groups necessary to improve the inhibitor's oral biovailability (270). The oral compound oseltamivir or GS 4104 (Fig. 11) is converted to oseltamivir carboxylate or GS 4071 in the body. The resulting molecule retains the carboxylate bond of sialic acid but also makes use of a hydrophobic group. This group induces the binding cleft to form a hydrophobic pocket, which holds the drug in place through hydrophobic interactions. The carbocyclic inhibitor GS 4071 is as potent as zanamivir against the NA of both influenza A and B viruses when tested in NA inhibition assays, and it demonstrated antiviral efficacy in mice and humans when administered orally (198,326). A third compound targeting the NA and designated RWJ-270201 also is under development; preliminary reports indicate that it is effective on all subtypes of influenza A and B, is as potent as both of the NA inhibitors given earlier, and is administered orally (181).

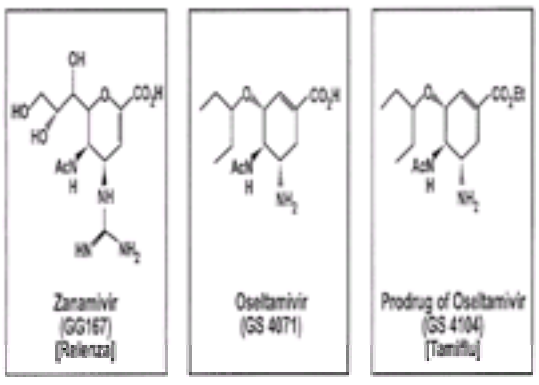


FIG. 11. Neuraminidase inhibitors. Zanamivir and oseltamivir were designed based on the structure of the enzyme active site of the neuraminidase. Zanamivir is inhaled or administered intranasally, whereas the prodrug of oseltamivir is administered orally. The prodrug oseltamivir phosphate (GS 4104) is converted predominantly by hepatic esterases to oseltamivir carboxylate (GS 4071).

Both zanamivir and oseltamivir are well tolerated in lower animal models and in humans (for reviews, see 179,182) and are efficacious for prophylaxis and treatment of influenza in humans. Both zanamivir and oseltamivir have been approved for use in humans. To determine the dose-related prophylactic activity of zanamivir in humans, it was administered intranasally 2 or 6 times daily for 5 days beginning 4 hours before inoculation of the virus. For all zanamivir recipients, prophylaxis was 96% effective against viral shedding, 82% effective against infection, and 95% effective in preventing febrile illness ($p < .001$), reduced peak viral titers by 2.0 log $TCID_{50}$/mL ($p < .001$), and reduced the median duration of

viral shedding by 3 days ($p < .001$) and the frequency of febrile illness by 85% ($p < .01$).

The efficacy of zanamivir against acute influenza was shown in double-blind placebo-controlled field trials. For all infected patients, the median time to alleviation of all major influenza symptoms (i.e., headache, myalgia, cough, and sore throat) was 4 days compared with 5 days for the placebo group (200). When treatment was started within 30 hours after infection, the treated group recovered 2 days earlier, and if treatment was started less than 30 hours after infection, the alleviation of symptoms was up to 4 days. Thus early diagnosis and treatment are necessary. Similar efficacy has recently been shown for oseltamivir (197). Recommendations for the use of each compound have recently been published by the Centers for Disease Control and Prevention (CDC) (68). Oseltamvir is currently recommended for those as young as 1 year.

Resistant variants to the NA inhibitors have been selected under drug pressure *in vitro* but only with difficulty and at a much lower frequency than amantadine (78,178,181,318). The emergence of resistance is first detected by appearance of mutations around the receptor-binding site of the HA, which compensates for reduced NA activity by lowering the affinity of binding to receptors. The second step in emergence of resistant mutants is selection of variants with mutations in the inhibitor binding site of the NA. The most common substitution with zanamivir occurs at residue 119, where Glu was typically replaced by Gly and less frequently by Asp or Ala. Less frequently substitutions occur at residues 292. Glu 119 is conserved among all influenza viruses and is one of the “framework” residues, which do not directly participate in substrate catalysis but stabilize the enzyme structure. The interaction of Glu at residue 119 and 227 with the 4-guanidinium moiety of zanamivir provides the high potency and selectivity of the inhibitor, and changes at residue 119 weaken the interaction between the mutated NA and the drug (77).

Characterization of these mutants reveals that the functional integrity of their NA activity is impaired (77,181,318). They have reduced NA activity, are heat labile, and have a narrower range of pH optima. Thus zanamivir-resistant mutants that contain structural changes in their NA and HA may not succeed in nature.

There is no evidence of selection of zanamivir-resistant mutants during clinical trials. Typical zanamivir treatment in humans (5 days) is likely to be too brief for development of resistance. However, prolonged treatment and immunocompromised status may create conditions conducive to the selection of drug-resistant mutants. A zanamivir-resistant mutant that emerged after treatment of an immunocompromised child was reported (180). The HA of the influenza B virus present in the patient's endotracheal tube aspirate after 1 week of treatment with zanamivir had reduced affinity for the *N*-acetylneuraminic acid 2,6-galactose-receptor determinant present on human tracheal epithelium. After 2 weeks of treatment, the isolated mutant had acquired an additional substitution in the NA-active site. Future large-scale studies are needed to determine whether resistant mutants can be detected after *in vivo* use in humans. Now that zanamivir and oseltamivir have been approved for use in humans, it is important to monitor for the emergence of resistance to these inhibitors under field conditions.

Other Antivirals Against Influenza

Ribavirin

Ribavirin, which is a synthetic nucleoside analogue that has antiviral activity against influenza A and B viruses in tissue culture and mice, currently does not have a defined role in the prevention of or therapy for influenza infections in humans (504). The antiviral activity is exerted after adsorption, penetration, and uncoating have taken place and can be reversed by adding an excess of guanosine or xanthosine to the tissue-culture medium. The *in vitro* inhibitory activity of ribavirin for influenza A and B viruses is 3 to 6 µg/mL, which is considerably higher than the peak levels achieved in serum after a single dose of 400 mg. Orally administered ribavirin has an inconsistent prophylactic or therapeutic effect, whereas aerosol administration has a detectable therapeutic effect.

Interferon

Interferon and IFN inducers induce resistance to influenza infection in mice (60,157,484). In humans the administration of exogenous IFN or an IFN inducer before experimental or natural challenge with a virulent influenza virus fails to reduce the level of illness or the quantity of virus shed (212,327). Studies with recombinant IFN-a2 indicate slight protection against illness and virus shedding (496).

Vaccines

Inactivated Vaccines

Vaccine Formulation

Inactivated influenza A and B virus vaccines are licensed for parenteral administration in humans. The strains to be included each year for the annual vaccination program and recommendations for use are made by the Public Health Service (PHS) (67,235,269) for an extensive discussion of these vaccines). Seed viruses for the A and B components of the vaccine are naturally occurring virus strains that replicate to high titers in the allantoic cavity of embryonated chicken eggs, the substrate used for vaccine production. Alternatively, the influenza A components may be reassortant viruses that contain internal genes from the A/PR/8/34 (H1N1) virus, which confer a high level of growth in eggs plus genes coding for surface antigens of the current influenza A virus (424).

Virus present in allantoic fluid is purified and concentrated by zonal centrifugation or column chromatography and inactivated with formalin or b-propiolactone. These purification procedures have greatly reduced the incidence of local and systemic reactions. The quantity of immunoreactive HA in each dose is standardized to contain the amount recommended by the Advisory Committee on Immunization Practices (67), which is usually 15 µg per component for adults and older children or 7.5 µg for children younger than 3 years. The quantity of NA is not standardized because this

glycoprotein is quite labile during the process of purification and storage (260,261). Each 0.5-mL dose of vaccine contains approximately 10 billion virus particles, and one egg yields one to three doses of vaccine. Vaccine also contains variable but small quantities of endotoxin, egg-derived protein, free formaldehyde, and preservative, which do not appear to contribute to the reactogenicity of the vaccines for humans. The route of immunization is intramuscular.

The vaccines currently in use are designated whole-virus (WV) vaccine or subvirion (SV) (split or purified surface antigen are alternative terms) virus vaccine. The WV vaccine contains intact, inactivated virus, whereas the SV vaccine contains purified virus disrupted with detergents that solubilize the lipid-containing viral envelope, followed by chemical inactivation of residual virus. Experimental vaccines have been prepared and evaluated in humans that contain either baculovirus-expressed HA alone (280) or purified NA alone (236). An experimental inactivated WV vaccine containing an NA antigen immunologically related to the epidemic virus and an unrelated HA appears to be less effective than conventional vaccine (379). Only inactivated vaccines containing both relevant surface antigens will be considered further.

It has recently been observed that human influenza A and B viruses isolated in the allantoic cavity of embryonated chicken eggs can have altered antigenicity and possess mutations in the HA that promote growth in eggs (370,536). Studies that have compared the efficacy of viruses egg-passaged or cell-culture grown indicate that the egg-passaged viruses are protective against cell-culture viruses grown in the predominant virus isolated from humans (194,254,432). In humans, immunization with vaccines containing an HA egg made from grown virus induces less neutralizing antibody to virus possessing the nonadapted HA than to the homologous virus (370). At present, egg-grown viruses are used in the inactivated virus vaccine.

Vaccine Reactogenicity

Reactogenicity of vaccines has been evaluated in four large-scale, placebo-controlled trials in adults and children (67). The vaccines contained a monovalent influenza A H1N1 virus or a mixture of H1N1, H3N2, and B viruses. In each instance, vaccine was standardized with respect to quantity of HA. Satisfactory serologic responses were achieved in children and adults with doses of vaccine that caused minimal clinical reactions. Reactions generally occurred within the first 24 hours and lasted for 1 to 2 days; these included systemic manifestations such as fever, malaise, myalgia, and headache, as well as local manifestations such as pain, erythema, induration, and tenderness at the site of inoculation.

In adults, the frequency of systemic reactions to monovalent or trivalent WV or SV vaccines containing from 6 to 65 µg of HA was not different from that of recipients of placebo. Only when vaccine containing 118 µg of HA was used were systemic reactions significantly greater than those in placebo recipients. Reactions were observed most frequently in vaccinees lacking detectable preexisting serum antibody. Local reactions occurred in 3% to 5% of adults and rarely caused significant morbidity. The reactogenicity of influenza B virus vaccine is similar to that of influenza A virus.

In children from ages 6 months to 18 years, a difference in reactogenicity of WV or SV vaccine was apparent. WV vaccinees exhibited significantly more systemic reactions than did SV vaccinees. Febrile reactions with occasional febrile convulsions occur in young children younger than 2 years after administration of WV vaccine containing either monovalent H3N2 or B antigen. These observations form the basis for recommending that only SV vaccine be used in children younger than 12 years. WV vaccines cause an increase in systemic reactions at doses as low as 4 µg of HA, whereas SV vaccines were acceptable at dosages as high as 40 to 60 µg. Reactions in children are less frequent after the second or booster inoculation of WV vaccine.

Local or systemic allergic reactions to vaccine components occur rarely. It is believed that the majority of these reactions are due to residual egg protein (67). High-risk patients who have egg allergies may be immunized by using a specific protocol (355).

An increase in the number of reported cases of the GBS occurred after the National Influenza Immunization Program in 1976 to 1977 (430,436). About 1 in 100,000 individuals who received the swine influenza virus vaccine developed GBS, with a relative risk in vaccinees about four- to eight-fold greater than that in unvaccinated subjects. Influenza virus vaccine produced after 1977 had not been associated with an increased risk of GBS, until a recent report implicated a low but definable excess risk (one to two cases per million vaccinees) with the 1992 to 1993 and 1993 to 1994 vaccines (286). The reason for the difference between 1977 and subsequent years is not known.

Vaccine Immunogenicity

Prior antigenic experience with an influenza A virus within the same subtype is an important determinant of the serum antibody response to SV and WV vaccines. An example occurred in 1977 when those younger than 24 years in 1977 represented a unprimed population to H1N1 viruses, which had last circulated in 1957. In contrast, most persons 25 years or older probably had one or more infections with H1N1 viruses and thus represented a primed population. A dose of either SV and WV vaccine containing 20 to 30 µg of HA induced an acceptable level of antibody in primed adults. In contrast, 2 to 3 times as much vaccine was required to induce a similar antibody response in unprimed individuals (i.e., 60 µg or more of HA). In general, increasing the dose of vaccine increases the level of antibody achieved, but there is a rather shallow dose–response curve with WV and SV vaccines in primed and unprimed individuals (342,385). Responses to individual antigens in trivalent vaccines are as great as responses to the same components administered separately. The neuraminidase-inhibiting antibody response to WV and subunit vaccines in primed and unprimed individuals followed the same patterns as the HAI antibody response (261). The serum antibody response of elderly subjects to inactivated virus vaccines is variable but is generally lower than that of younger subjects (386). The antibody response of children older than 6 months was similar to that of unprimed adults but with two doses of SV vaccine needed to achieve a satisfactory level of antibody on primary immunization of children up to age 8 years. In infants younger than 6 months, inactivated vaccines are less immunogenic than they are in older infants and children (174). The serum antibody response of the young seronegative vaccinee can be quite strain specific, which is an important property to bear in mind

when antigenically distinct viruses of the same type or subtype are cocirculating (291).

The level of antibody induced in unprimed or primed individuals given two doses of SV vaccine decreased 75% over an 8-month period. The rate of decline of antibody after SV vaccine administration is faster than that observed after infection in young children.

Parenteral administration of vaccine leads to an increase in nasal wash antibody in 25% to 50% of primed vaccinees (340). A correlation between an increase in serum and an increase in nasal wash antibody has been observed, and the nasal wash response is dose dependent (340,530).

Antibody responses in patients with renal failure or with cancer are less than those in control individuals (34,175,507). Vaccine responses in high-risk patients with pulmonary or heart disease not receiving immunosuppressive drugs do not differ from those of controls. Immunization of immunocompromised individuals can induce antibody responses dependent, in part, on the level of immunocompetence (67,119,333).

Carefully controlled studies comparing the antibody response after intradermal and parenteral administration of vaccine containing comparable amounts of HA antigen indicated that intradermal administration offers no advantage over parenteral administration (373). A systematic comparison of intranasal versus parenteral administration of vaccine in primed and unprimed individuals using a vaccine of known HA content has not been performed, but intranasal administration of inactivated vaccine induces little systemic response (41). The oral route of immunization with inactivated virus vaccine induces secretory IgA antibody but little serum antibody (511). The efficacy of oral immunization has not been compared with that of parenterally administered inactivated vaccine.

Vaccine Efficacy

Parenteral vaccination with influenza A and B viruses consistently induces resistance to illness and, to a lesser extent, infection with influenza A and B viruses (14,114,128,138,233,323,328). The resistance induced is manifested by a reduction in the frequency and severity of the illness, including otitis media in children (202). Efficacy ranges from 60% to 80%, with the higher value seen after challenge with homologous virus and the lower value after challenge with virus that has undergone antigenic drift.

Resistance to experimental or natural challenge correlates with the level of serum HAI antibody induced to the infecting strain. In those situations in which vaccine efficacy was low or not detectable, surface antigens of the epidemic virus differed significantly from those of the vaccine virus, or vaccines were of low potency and thus did not stimulate sufficient antibody to the epidemic virus. A polyvalent influenza A virus vaccine containing antigens not related to the subtype present in the epidemic virus did not induce resistance to natural challenge (342). Vaccine is effective in the elderly who are at high risk for morbidity and mortality, but the level of efficacy is often less than that seen in young adults (21,176,393,435). Vaccination protects the vaccinee, but unvaccinated individuals also can benefit if the extent of vaccination is sufficient to dampen the epidemic spread of virus in the community (335,517). The duration of immunity after vaccination has not been evaluated systematically, but the relatively rapid decline in antibody levels suggests that protective immunity is of short duration. At any rate, antigenic changes in the influenza virus predicate annual immunization with current vaccines.

Inactivated vaccines boost HLA-restricted cytotoxic T cells in adults (406), but their ability to stimulate CTLs in unprimed individuals has not been evaluated. In contrast to virus infection, inactivated vaccines stimulate a poor CTL response in unprimed mice. However, WV vaccine stimulates T cells that mediate delayed-type hypersensitivity (T_d-cells) (2). In cell-transfer studies in mice, T_d-cells have been implicated as an important mediator of cellular infiltration in the lungs; these cells also increase mortality during influenza infection of mice (290). It is important to emphasize that potentiation of influenza illness in human vaccine recipients in any way analogous to that occurring after measles or respiratory syncytial virus vaccination has not been observed. Inactivated influenza virus vaccines have a consistent record of efficacy and safety.

Although influenza vaccine is currently recommended for annual vaccination of high-risk groups, most of the studies that have demonstrated efficacy have involved populations that received one or two doses of vaccine shortly before experimental or natural challenge. In one study in a boys' boarding school, the value of annual administration of vaccine was assessed over a 7-year period from 1970 to 1976, during which time three outbreaks occurred that involved different H3N2 drift viruses (227). The protective effect of inactivated influenza A vaccines was limited to boys after they received their first vaccination. As a consequence of the ineffectiveness of revaccination, the cumulative attack rate in the three epidemics was similar in vaccinated and unvaccinated boys. This raised a question concerning the current recommendation for annual vaccination with inactivated vaccine. However, subsequent studies have confirmed that annual vaccination with inactivated virus vaccines is indeed beneficial (114).

Vaccine Recommendations

Annual vaccination is recommended for persons regardless of age who have chronic conditions, such as (a) heart disease, (b) bronchopulmonary disease, (c) renal disease, (d) diabetes or other metabolic disorders, (e) children receiving long-term aspirin therapy, and (f) women in the second or third trimester of pregnancy (67). In addition, individuals older than 65 years should be vaccinated annually. Health care providers should be vaccinated as well as others in contact with high-risk groups.

Live Virus Vaccines

Live influenza virus vaccines are currently not a licensed product, but experimental live vaccines have been developed and evaluated for their usefulness in the immunoprophylaxis of influenza illness in adults (372) and in children (27). There are two major reasons for continued interest in the use of live vaccines in humans. First, the current inactivated

vaccines are recommended primarily for those at high risk of influenza virus infection and yet do not, in those groups, provide complete protection. In contrast, live vaccines appear to have the potential to broaden the indications for influenza vaccine and hence to have an impact on the overall morbidity and perhaps spread of the disease. Second, local immunity is increasingly recognized to play a major role in resistance to most respiratory pathogens (40). Respiratory tract infection with a live attenuated vaccine has been found to be the most efficient method of stimulating such immunity. Because infection of the respiratory tract stimulates systemic as well as local immunity, and theoretically is a good way of stimulating cell-mediated immunity, all components of the host's immune response are brought into play.

As previously indicated, influenza A viruses (and to a lesser extent, influenza B viruses) are unique among viruses that infect the respiratory tract in that they undergo significant antigenic variation on virtually a yearly basis. The strategy for developing a live virus vaccine must take this property into account. It is not feasible to attenuate each new variant of influenza virus that appears in nature by multiple passages in a heterologous host(s), a method that has been successfully used to attenuate viruses that do not show antigenic variation. Therefore, a strategy is needed in which attenuation can be achieved in a single step by transfer of attenuating genes from an attenuated donor virus to each new epidemic or pandemic virulent virus (348).

Because resistance to influenza A virus is mediated by the development of an immune response to the HA and NA glycoproteins, the genes coding for these surface antigens must come from the epidemic virus, whereas the attenuating genes are derived from the attenuated parent. This scheme is possible because of the segmented nature of the influenza genome and is illustrated in Fig. 5, in which the HA and NA genes are derived from the virulent wild-type virus, and the other genes are derived from the attenuated donor virus. In this approach, it is necessary that the genes that confer attenuation not code for the HA and NA glycoproteins, as these genes must be derived from the new epidemic or pandemic virus. In the figure, the attenuated reassortant vaccine virus was intentionally shown to receive each of the six other internal genes from the attenuated donor virus because such reassortants would be expected to exhibit the same level of attenuation each time a new vaccine virus is produced. Many donor viruses have been evaluated for their ability to attenuate new epidemic influenza A viruses reproducibly, including significant work in Russia with the A/Leningrad strains (259). However, only the background for the A/Ann Arbor/6/60 (H2N2) cold-adapted (ca) donor virus developed by John Maassab is presented, because this virus shows considerable promise as a vaccine candidate (27,114,348).

The influenza A/AA/6/60 (H2N2) ca donor virus was developed by passage of the wild-type virus at progressively lower temperatures in primary chick kidney cell cultures until a mutant was identified that replicated efficiently at 25°C, a temperature restrictive for the replication of wild-type virus (304). The donor virus and reassortants derived from it have three phenotypes: (a) the temperature-sensitive (ts) phenotype (i.e., restricted replication at temperatures of 38°C to 39°C, temperatures permissive for the replication of wild-type virus); (b) the ca phenotype (i.e., efficient replication at 25°C); and (c) the attenuation (att) phenotype (i.e., restricted replication in the upper and lower respiratory tract of ferrets). New live, attenuated reassortant virus vaccines are generated by mating the ca donor virus with a virulent wild-type virus and then selecting reassortant progeny at 25°C (restrictive for replication of wild-type virus), in the presence of an H2N2 antiserum, which inhibits replication of the viruses bearing the surface antigens of the attenuated A/AA/6/60 (H2N2) ca donor virus. A large series of H1N1 and H3N2 reassortants has been evaluated in humans and found to be satisfactorily (a) infectious, (b) attenuated for seronegative children and immunologically primed adults, (c) immunogenic, and (d) genetically stable. The viruses are poorly transmissible. The attenuation manifested by ca reassortants was accompanied by markedly reduced replication of virus in the upper respiratory tract of susceptible adults compared with that of volunteers infected with wild-type virus. Similar studies in other age groups revealed that the level of replication of ca reassortant viruses is (a) greatest in seronegative infants or children because viral growth is not limited by immunity induced by prior infection with influenza viruses; (b) intermediate in children and young adults who have had prior experience with related influenza A viruses; and (c) lowest in the elderly who have been infected many times with related influenza A viruses. Despite the efficient replication of ca reassortant viruses in young, fully susceptible, seronegative pediatric subjects, the vaccinees experience few symptoms, and those that occur are confined to the upper respiratory tract. The immunogenicity of the ca reassortants parallels their level of replication. Thus the acquisition of the six transferable genes of the ca donor virus by new wild-type viruses has reproducibly attenuated these viruses for susceptible adults and children.

The genetic basis of the ts, ca, and att phenotypes of ca reassortant viruses was determined. The PB1 and PB2 genes independently specify the ts phenotype, and the PA gene specifies the ca phenotype. Each of the three polymerase genes specifies the att phenotype (480), and only seven amino acid differences between the ca and wild-type viruses that could contribute to attenuation are present in these genes.

The ca reassortant vaccines induce resistance to experimental or natural challenge with wild-type influenza A viruses in adult and pediatric subjects who have had some prior experience with influenza A viruses (114,348,372). In addition, they are highly immunogenic in seronegative subjects, and this suggests that the vaccine would be efficacious in this population (239). Recent very impressive efficacy, 93% overall, against culture-confirmed influenza A/H3N2 and B was shown in a pediatric population aged 15 to 71 months after either one or two doses of trivalent ca reassortant vaccine given as a nasal spray (27). In the elderly, coadministration of live ca virus vaccine and inactivated virus vaccine may be more efficacious than inactivated virus vaccine alone against natural infection with influenza A virus (497).

Because the populations to be immunized have such disparate experience with influenza A viruses, it is not surprising that different vaccines (i.e., live vs. inactivated virus vaccine) or different vaccine schedules would be needed for each population. The suggested use and schedule of annual immunization with ca reassortant and inactivated virus vaccines and the reasons for the choice of vaccine in the pediatric, young adult, and elderly adult populations are (a) seronegative pediatric population older than 6 months may preferentially get live vaccine because this type of vaccine has been shown to be more immunogenic in this population; (b) previously primed children and adults younger than 65 years may get either vaccine because efficacies of live and inactivated vaccines are comparable for this population, although a live virus vaccine may be better accepted because of an easier route of delivery; and (c) elderly (older than 65 years) may get live plus inactivated virus vaccine because this combination is more efficacious in this population (348,497). Additional studies are needed to support this suggested use, and licensure of the live vaccine must be achieved over the

next few years.

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CHAPTER 48 *Bunyaviridae:* The Viruses and Their Replication

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CLASSIFICATION

The family *Bunyaviridae* was established in 1975 to encompass a large group of arthropod-borne viruses sharing morphologic, morphogenic, and antigenic properties (55). The family currently contains four genera of animal-infecting viruses (*Bunyavirus, Hantavirus, Nairovirus,* and *Phlebovirus* genera) and one genus of plant-infecting viruses (*Tospovirus* genus). Most viruses in the family were isolated from or are transmitted by arthropods, primarily mosquitoes, ticks, sand flies, or thrips. Hantaviruses are exceptions; these viruses are rodent borne and are transmitted in aerosolized rodent excreta.

Viruses in the family *Bunyaviridae* were classified historically based on their antigenic relationships. Serogroups were identified that contain viruses related by their reactivity in any serologic test. Bunyaviruses, phleboviruses, and nairoviruses were further divided into complexes of closely related members of a serogroup. Genetic characterization of newly discovered viruses has largely circumvented serologic classification methods, particularly for the hantaviruses, which are very difficult to isolate and propagate in cell cultures.

Efforts to classify viruses as species resulted in a revision in the catalogue of viruses in the family *Bunyaviridae.* The most recent report from the International Committee on the Taxonomy of Viruses lists 47 species (and four tentative species) in the *Bunyavirus* genus, 22 species in the *Hantavirus* genus, seven species in the *Nairovirus* genus, nine species in the *Phlebovirus* genus (and 16 ungrouped viruses), and eight species in the *Tospovirus* genus (and five tentative species) (169). In addition, seven groups (19 viruses) and 21 ungrouped viruses are probable members of the family, but have not been characterized molecularly or antigenically. Because classification criteria vary among the genera, as well as among virus families, it is certain that the taxonomy of the family *Bunyaviridae* will continue to evolve.

VIRION STRUCTURE

Morphology

Virions are spherical, 80 to 120 nm in diameter, and display surface glycoprotein projections of 5 to 10 nm, which are embedded in a lipid bilayered envelope approximately 5- to 7-nm thick (Fig. 1A). Electron microscopy and biochemical studies of the bunyavirus La Crosse virus suggest that there are 270 to 1,400 glycoprotein spikes per virion (76,137). The spikes are generally thought to consist of heterodimers of the viral glycoproteins, G1 and G2; however, one study suggested that homodimers make up the minimal surface unit of the phlebovirus, Uukuniemi virus (176). Studies have not been conducted to exclude the presence of homodimers for other viruses in the family. The glycoproteins interact to form surface morphologic units that vary among viruses in different genera. Viruses in the *Phlebovirus* genus (e.g., Rift Valley fever, Punta Toro, sandfly fever Sicilian, and Uukuniemi viruses) have round, closely packed morphologic units, approximately 10 to 11 nm in diameter, with central cavities approximately 5 nm in diameter (49,120,194). Negative staining of glutaraldehyde-fixed particles, as well as freeze-etching techniques, demonstrated that the surface units of Uukuniemi virus are penton-hexon clusters arranged in a T=12, P=3 icosahedral surface lattice with hexon–hexon distances estimated at 12.5 to 16 nm for stained viral particles and 17 nm for freeze-etched samples (213).

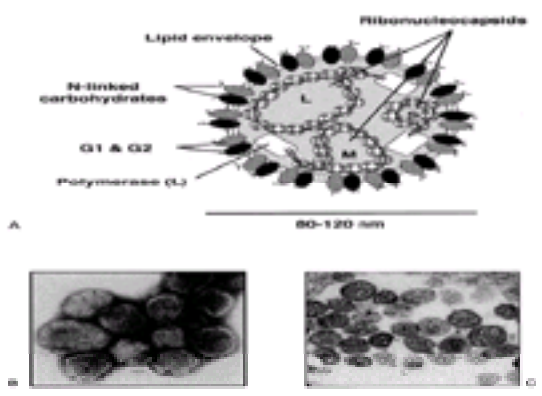

FIG. 1. Morphology of *Bunyaviridae* virions. **A:** Schematic cross section of virus. The three RNA genome segments (S, M, and L) are complexed with nucleocapsid protein to form ribonucleocapsid structures. The nucleocapsids and RNA-dependent RNA polymerase are packaged within a lipid envelope that contains the viral glycoproteins, G1 and G2. Note that there is no matrix protein. **B:** Electron micrograph of glutaraldehyde-fixed, negatively stained Hantaan virions (*Hantavirus* genus). The morphologic units on the surface form a grid-like pattern. As described in the text, viruses in other genera have varying characteristic surface structures. **C:** Thin-section electron micrograph of Puumala virus (*Hantavirus* genus). The interior of the virions has a filamentous or coiled-bead appearance, presumably due to the presence of the ribonucleocapsids. Micrographs courtesy of T. Geisbert, K. Kuhl, and J.E. White, U.S. Army Medical Research Institute of Infectious Diseases, Frederick, MD.

The surface structure of viruses in the *Hantavirus* genus (e.g., Hantaan virus) also are distinctly ordered and have a unique square grid-like appearance (see Fig. 1B) (124,221). In contrast, virions in the *Bunyavirus* genus (e.g., Anhembi, La Crosse, and Bunyamwera viruses) have surfaces covered with knob-like morphologic units with no detectable order (120,204). Similarly, no obvious order was found for the small surface structures with central cavities observed on viruses in the *Nairovirus* genus (e.g., Crimean Congo hemorrhagic fever, Qalyub, and Dugbe viruses) (17,120).

The appearance of viruses in the *Tospovirus* genus (e.g., tomato spotted wilt and impatiens necrotic spot viruses) has been likened to that of the nairoviruses (128). Other than the presence of glycoprotein projections, which are observed as a fringe on negatively stained virions, distinctive surface structure has not been noted for these viruses (98).

The interior of virions, as observed by thin-section electron microscopy, has a filamentous or coiled bead appearance, presumably due to the presence of the ribonucleocapsids (see Fig. 1C) (41,63,79).

Biochemical and Biophysical Properties

The composition and structure of virions has been inferred from biochemical and morphologic studies. An overall chemical content of 2% RNA, 58% protein, 33% lipid, and 7% carbohydrate was estimated for Uukuniemi virus (139). Sedimentation coefficients of virions range from 400 to 500 S, and their buoyant densities in sucrose are 1.16 to 1.18 g/cc, and in CsCl, 1.20 to 1.21 g/cc. Treatment with lipid solvents or nonionic detergents removes the viral envelope and results in loss of infectivity for arthropods and mammals (121,128,139). For plants, the envelope is not required for infectivity, as demonstrated in studies with tomato spotted wilt virus, for which repeated mechanical passage among plants resulted in a defective virus that was unable to produce enveloped particles but was able to infect and replicate in plant cells (170). These studies provide indirect evidence that the L polymerase protein remains associated with ribonucleocapsids and is active despite the absence of intact virions.

GENOME STRUCTURE AND ORGANIZATION

Viral Genome

Virions contain three single-stranded RNA genome segments designated large (L), medium (M), and small (S). All three gene segments of a virus have the same complementary nucleotides at their 3' and 5' termini. The terminal nucleotide sequences are highly conserved among viruses within a genus, but differ from those of viruses in other genera (Table 1). Base-pairing of the terminal nucleotides is predicted to form stable panhandle structures and noncovalently closed circular RNAs. Direct support for base-pairing comes from electron microscopy of RNA extracted from virions, in which three sizes of circular RNAs were evident (77).

Genus/Virus	Consensus S, M, L terminal nucleotides	Gene sizes (accession no.) S	M	L
Bunyavirus				
Bunyamwera	3′ UCAUCACAUG– 5′ AGUAGUGUGC–	961 (X00053)	4458 (M11852)	6875 (X14383)
Hantavirus				
Hantaan	3′ AUCAUCACUG– 5′ UAGUAGUAGC–	1696 (M14626)	3616 (M14627)	6530 (D10921)
Nairovirus				
Dugbe	3′ AGAGUUUCU– 5′ UCUCAAAGA–	1712 (M20159)	4888 (M94133)	12255 (U15018)
Phlebovirus				
Rift Valley fever	3′ UGUGUUUC– 5′ ACACAAAG–	1690 (X53771)	3885 (M11957)	6404 (X56464)
Tospovirus				
Tomato spotted wilt	3′ UCUCGUUA– 5′ AGAGCAAU–	2916 (D10540)	4821 (S48091)	8897 (D12068)

TABLE 1. *Terminal nucleotide sequences of the S, M, and L genome segments of representative members of the family Bunyaviridae*

The RNA segments are complexed with N to form individual L, M, and S nucleocapsids, which appear to be helical (138). Nucleocapsids released by nonionic detergent treatment of virions often also appear as circular structures in electron micrographs, suggesting that the complementary RNAs can base-pair even when complexed with protein in an estimated ratio of 4% RNA:96% protein (137,157,165). This hypothesis is supported by data showing cross-linking of the ends of nucleocapsid-enclosed RNAs by treatment with psoralens, which are photoreactive, nucleic acid, cross-linking agents

(165).

At least one each of the L, M, and S ribonucleocapsids must be contained in a virion for infectivity; however, equal numbers of nucleocapsids may not always be packaged in mature virions, as suggested by various reports of equimolar or nonequimolar ratios of L, M, and S RNAs (16,81,207). Unequal complements of the ribonucleocapsids may contribute to the size differences of virions observed by electron microscopy (204).

In addition to ribonucleocapsids containing virion sense RNA (vRNA), certain viruses in the *Phlebovirus* and *Tospovirus* genera encapsidate small amounts of complementary sense RNA (cRNA) of their ambisense genes (i.e., S, but not M for Uukuniemi virus, and both M and S for tomato spotted wilt virus) (102,191). For the bunyavirus, La Crosse virus, S segment cRNA was detected in virions synthesized in insect cells, but not in mammalian cells (165). The significance of this finding, if any, is not known.

Coding Strategies of Viral Genes

Both similarities and noteworthy differences in the coding strategies of viruses in the family *Bunyaviridae* are known. Viruses in each genus encode all structural proteins (N, G1, G2, and L) in virus cRNA. Nonstructural proteins are encoded in the M segment cRNA of phleboviruses and bunyaviruses and in the S segment cRNA of bunyaviruses. In addition, phleboviruses encode a nonstructural protein in their S segment vRNA, and tospoviruses encode nonstructural proteins in both their M and S segment vRNAs. Therefore bunyaviruses, hantaviruses, and nairoviruses use strictly negative-sense coding strategies, but phleboviruses and tospoviruses use ambisense coding strategies (Fig. 3).

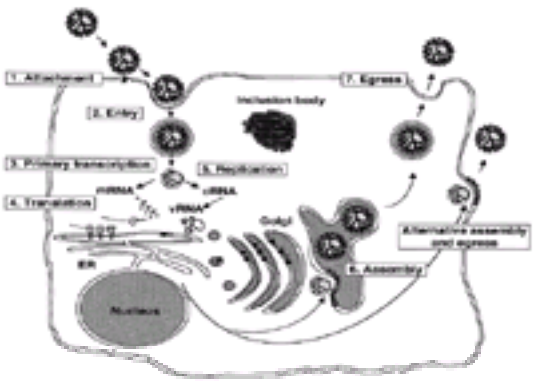

FIG. 3. Replication cycle of viruses in the family *Bunyaviridae.* Steps in the replication cycle are numbered as follows: *1*, attachment of virions to cell-surface receptors; *2*, entry via receptor-mediated endocytosis followed by membrane fusion, allowing viral ribonucleocapsids and RNA-dependent RNA polymerase access to the cytoplasm; *3*, primary transcription; *4*, translation of viral proteins; *5*, replication of vRNA via a cRNA intermediate; *6*, assembly of virions at the Golgi or plasma membrane; *7*, egress by budding into the Golgi followed by exocytosis, or budding through the plasma membrane. For several members of the family, inclusion bodies are found in the cytoplasm. *ER*, endoplasmic reticulum; *black and white boxes*, receptor; *open circles*, N; *shaded circles*, G1 and G2

S Segment Strategies

Viruses in the *Bunyavirus* genus have smaller S segments than those of viruses in other genera (Fig. 2A). Two polypeptides, N and NSs, are encoded in overlapping reading frames in cRNA (15). The presence of NSs in virus-infected cells was demonstrated for several members of the genus (46). Only one S segment mRNA species can be found in bunyavirus-infected cells (26); therefore N and NSs likely are generated by ribosomal recognition of start codons in both reading frames.

FIG. 2. Genomic structure and coding strategies of viruses in the family *Bunyaviridae.* All members of this family have a genome that consists of three single-stranded RNA segments (S, M, and L) that have either a negative-sense or ambisense coding strategy. Numbers of nucleotides for the L, M, and S segments listed below the boxes, which represent relative sizes of the gene segments and show regions encoding the viral proteins. **A:** S-segment coding strategies. Hantaviruses and nairoviruses use negative-sense coding to express a single protein, N from their virion complementary-sense RNA (cRNA). Bunyaviruses also use negative-sense coding but express two proteins, N and NSs, from overlapping open reading frames (ORFs) of cRNA. Phleboviruses and tospoviruses use ambisense coding to express two proteins, N and NSs, from separate, subgenomic mRNAs. Lines, N coding regions; solid, NSs coding regions. **B:** M segment coding strategies. Bunyaviruses, hantaviruses, nairoviruses, and phleboviruses all use negative-sense coding to express two surface glycoproteins, G1 and G2, from a single ORF of cRNA. Bunyaviruses and

phleboviruses encode an additional nonstructural protein, NSm, in the same ORF. M segment products are processed from a precursor polyprotein by cotranslational cleavage. Tospoviruses generate two subgenomic messages and use ambisense coding to express G1 and G2, from an ORF in cRNA, and NSm, from an ORF in vRNA. *Dots*, G1 and G2; *cross-hatch*, NSm coding regions; *solid*, G1 precursor region of nairoviruses. **C:** L segment. All viruses in the family use negative-sense coding to express the L protein, which is the RNA-dependent RNA polymerase, from an ORF in cRNA. Vertical dashes, L coding region.

Hantaviruses and nairoviruses encode larger nucleocapsid proteins than do viruses in other genera, but are not known to encode NSs proteins (see Fig. 2A) (117,187,217). Although some hantaviruses (e.g., Sin Nombre, Puumala, and Prospect Hill viruses) have an overlapping reading frame with N that has a coding potential of approximately 6 kd, NSs proteins have not been detected in hantavirus-infected cells (141,195,197). Only one S segment mRNA, similar in size to the coding region for N, was identified in hantavirus- or nairovirus-infected cells, indicating that transcription termination occurs shortly after the translation stop codon (81,186,216). Certain hantaviruses (e.g., Sin Nombre virus) have long 3' noncoding regions (>700 nt) containing numerous repeated sequences. These repeats may result from polymerase slippage on the vRNA template (195).

The ambisense coding strategy of the S segments of phleboviruses and tospoviruses produces N from a subgenomic mRNA that is complementary to vRNA, and NSs from a subgenomic mRNA of the same polarity as vRNA (see Fig. 2A) (37,82). Evidence that the mRNA for NSs is copied from cRNA (after genome replication) comes from time-course studies. For the phlebovirus Uukuniemi virus, N was detected at 4 to 6 hours after infection, whereas NSs did not appear until 8 hours after infection (192,205). Likewise, the mRNA for NSs of the tospovirus, tomato spotted wilt virus, was detected in infected plant cells 15 hours later than for N (196). In addition, studies with the phlebovirus, Punta Toro virus, demonstrated that protein synthesis inhibitors arrest production of NSs mRNA, but not N mRNA (84). These results suggest that protein synthesis must occur before the NSs mRNA can be made. The requirement for protein synthesis is probably related to the need for an adequate supply of newly formed N to encapsidate nascent cRNA, which in turn is then available as a transcription template for NSs mRNA (see transcription section later).

M Segment Strategies

The M segment encodes the two envelope glycoproteins, G1 and G2, in a single open reading frame (ORF) of cRNA. Some viruses encode NSm proteins, and others do not. Except for the tospoviruses, which use an ambisense strategy to generate NSm from a subgenomic mRNA, a single mRNA, nearly equivalent in size to the cRNA ORF, has been detected in virus-infected cells (30,86,118,186). The G1 and G2 gene order varies between genera and even among viruses of a single genus; however, these discrepancies are due to nomenclature anomalies rather than to functional differences. That is, the G1 and G2 designations refer to the electrophoretic migration of the envelope proteins, with G1 being the more slowly migrating band on an sodium dodecylsulfate (SDS)-polyacrylamide gel.

Bunyaviruses have a cRNA M segment gene order of 5'-G2-NSm-G1-3' (see Fig. 2B) (53). The synthesis of NSm in bunyavirus-infected cells was confirmed by immune-precipitation with antisera to viruses or to synthetic peptides representing amino acids predicted from the NSm coding region (53,56,134).

Hantaviruses have a M segment cRNA gene order of 5'-G1-G2-3' (188). Immune-precipitation with antisera to peptides representing predicted G1 or G2 amino acids showed that the carboxyl terminus of G1 extends to within 17 amino acids of the signal sequence for G2 and that the carboxyl terminus of G2 extends to the end of the ORF (188). Consequently, with the possible exception of a very short intergenic region, no coding information for a NSm polypeptide is available.

A gene order of 5'-G2-G1-3' was determined for the nairovirus, Dugbe virus, by expression of portions of the genes with recombinant baculoviruses and by amino-terminal sequence analysis of authentic G1 (116). These studies indicated that the amino terminus of G1 was about 50 amino acids downstream of the nearest predicted signal sequence. Consequently, it was postulated that G1 arises by proteolytic processing of a precursor protein (116). Further studies are needed to confirm this finding and to determine if additional nonstructural proteins are encoded in the nairovirus M segment.

The M segment gene order and the coding strategy varies among viruses in the *Phlebovirus* genus. The tick-borne phleboviruses (e.g., Uukuniemi virus) encode only G1 and G2 proteins (178) in cRNA, whereas the mosquito- and sandfly-vectored phleboviruses also have coding information for a NSm protein. For Rift Valley fever virus, the cRNA gene order is 5'-NSm-G2-G1-3' (32). The gene order for Punta Toro virus is 5'-NSm-G1-G2-3', but the reversed order has no significance, as indicated at the beginning of this section. The NSm protein of Rift Valley fever virus, but not that of Punta Toro virus, could be detected in virus-infected cells (83,93).

Like the S segments, the M segments of tospoviruses use an ambisense coding strategy. The cRNA has a gene order of 5'-G2-G1-3', and the vRNA encodes NSm (108). There are separate, subgenomic messages for the G1–G2 precursor and for NSm (101,108). NSm is readily detected in infected plants and is the only M segment nonstructural protein in the *Bunyaviridae* family to have a defined role (i.e., it is a movement protein, as discussed later).

L Segment Strategies

A conventional negative-sense coding strategy was found for all L segments of viruses in the family *Bunyaviridae.* For each L segment described thus far, there are fewer than 200 nucleotides of total noncoding information, and there is no evidence for additional coding regions in either the cRNA or vRNA (36,47,130,131,183,210).

STAGES OF REPLICATION

The principal stages of the replication process for viruses in the *Bunyaviridae* are illustrated in Fig. 3 and are summarized.

1. Attachment, mediated by an interaction of viral proteins and host receptors.
2. Entry and uncoating, by endocytosis of virions and fusion of viral membranes with endosomal membranes.
3. Primary transcription of viral-complementary mRNA species from genome templates using host cell–derived primers, and the virion-associated polymerase.
4. Translation of primary L and S segment mRNAs by free ribosomes, translation of M segment mRNAs by membrane-bound ribosomes, and primary glycosylation of nascent envelope proteins.
5. Synthesis and encapsidation of viral-complementary RNA to serve as templates for genomic RNA or, in some cases, for subgenomic mRNA.
6. Genome replication.
7. Secondary transcription of mRNA from newly synthesized genomes and of ambisense mRNAs from cRNA.
8. Continued translation and RNA replication.
9. Morphogenesis, including accumulation of G1 and G2 in the Golgi, terminal glycosylation, and acquisition of modified host membranes, generally by budding into the Golgi cisternae.
10. Fusion of cytoplasmic vesicles with the plasma membrane and release of mature virions.

Attachment and Entry

Viral Attachment Proteins and Cellular Receptors

The mechanisms by which members of the family *Bunyaviridae* gain access to the host cell's cytoplasm appear similar to those reported for many other enveloped viruses. The first step involves an interaction between cell-surface receptors and viral attachment proteins, G1 and/or G2. The functions of G1 and G2 during attachment and entry may vary among the genera. For the bunyavirus, LaCrosse virus, it was suggested that G1 might be used for attachment to mammalian cells, whereas G2 might be used for attachment to mosquito cells (113). However, studies involving proteolytic digestion of virions or incubation of virions with glycoprotein-specific antibodies implicated G1 as the attachment protein for mammalian cells, mosquito cells, and mosquitoes (67,71,75,96,148,201). For viruses in other genera, little is known concerning the use of G1 or G2 for attachment. The presence of neutralizing and hemagglutination-inhibiting sites on both the G1 and G2 proteins of the phleboviruses, Rift Valley fever (94), and Punta Toro (159) viruses, and on those of the hantavirus Hantaan virus (9,35), suggest that both proteins may be involved in attachment, either directly or because of conformational requirements.

Host-cell receptors have not been identified for most viruses in the family; however, b $_3$ integrins, which are surface proteins found on endothelial cells and platelets, were found to facilitate cellular entry of some pathogenic hantaviruses (59,60). Evidence for this comes from studies in which cells were rendered resistant to hantavirus infection by pretreating them with vitronectin, the physiologic ligand of b $_3$ integrins, or with b$_3$ integrin–specific antibodies. Consistent with these findings, stably transformed CHO cells expressing b$_3$ integrins supported hantavirus infections, whereas normal CHO cells did not. A nonpathogenic hantavirus, Prospect Hill virus, was found to use b $_1$ integrins rather than b$_3$ integrins, suggesting a role for viral receptors in determining virulence (60). Whether integrins are involved in virion attachment, or a postattachment entry event, has not been determined.

Entry of the Viral Genome into the Host Cytoplasm

Shortly after attachment, viruses in the *Phlebovirus* and *Nairovirus* genera were observed in phagocytic vacuoles (49,181). This finding suggests a mode of viral entry similar to that first described for alphaviruses in which the virus is endocytosed in coated vesicles (119). Acidification of the endosomes is thought to promote a conformational change in G1 and/or G2 that facilitates fusion of the viral and cell membranes, allowing the viral genome and polymerase access to the cytoplasm. Consistent with this, treating cells with ammonium chloride to prevent acidification of endosomes inhibited infection by the bunyavirus, California encephalitis virus, or the phlebovirus, Uukuniemi virus (74,176). Additional indirect support for membrane fusion as a mode of entry came from experiments demonstrating the ability of viruses in the family to mediate syncytia formation at low pH (10,64,65 and 66,86).

Whether one or both of the envelope proteins are necessary for viral entry has not been defined for most members of the family. For bunyaviruses, several lines of evidence indicate that G1 is involved not only in viral attachment to cells, but also has a role in membrane fusion. Protease sensitivity assays, detergent partitioning experiments, and antibody-binding studies, suggest that G1 undergoes a conformational change at pH conditions in which fusion is observed (65,74,147). In addition, a mutant of La Crosse virus, generated by passage in the presence of a monoclonal antibody to G1, displayed a reduced capacity to undergo pH-dependent conformational changes at low pH, and to cause cell-to-cell fusion (65). G1 alone, however, when expressed from recombinant vaccinia viruses, could not cause cell–cell fusion, suggesting that an association of the two glycoproteins is probably needed for membrane fusion (23,86).

Primary Transcription

Transcription Initiation

After uncoating of viral genomes, primary transcription of negative-sense vRNA to mRNA is initiated by interaction of the virion-associated polymerase (L) and the three viral RNA templates (18,167). Only ribonucleocapsids, not free RNA, can serve as transcription templates, as demonstrated in studies with expressed phlebovirus and bunyavirus L and N proteins. In these studies, transcription depended on the presence of both proteins and was unaffected by the presence

of other viral proteins (43,112).

Like influenza viruses, viruses in the family *Bunyaviridae* prime mRNA synthesis with capped oligonucleotides that are scavenged from host mRNAs. Unlike influenza viruses, which take primers from newly synthesized mRNAs in the host cell's nucleus (85), members of the *Bunyaviridae* family use primers cleaved from cytoplasmic host-cell mRNAs. A result of this mode of mRNA transcription is the presence of 5' terminal extensions of approximately 10 to 20 heterogeneous nucleotides that are not found in vRNA (14). Studies using anti-cap antibodies to immunoselect mRNAs (73) provided direct proof for the presence of caps on the scavenged primers and substantiated earlier, indirect findings that showed stimulation of La Crosse virus transcription by methylated cap analogs such as m ^{7}GpppAm, or naturally capped RNAs (144). Further evidence for capped extensions of mRNAs was provided by a study with the tospovirus, tomato spotted wilt virus, in which plants were coinfected with the tospovirus and with a positive-strand RNA virus (alfalfa mosaic virus). The tospovirus acquired 5' mRNA extensions with nucleotide sequences that matched those of the other virus. Presumably, these extensions retained the cap structures normally found on the positive-strand RNA viral messages (42). Cleavage of the capped primers is accomplished by endonucleolytic activity associated with virions (144). The endonuclease activity was demonstrated to localize to the L protein of Bunyamwera virus in studies in which vaccinia virus–expressed L was shown to transcribe mRNAs with heterogeneous extensions analogous to those found on authentic viral mRNAs (90).

For several viruses in the family, there appears to be a nucleotide or nucleotide motif preference for endonuclease cleavage. That is, although heterogeneous in sequence, the 5'-terminal extensions often have a preponderance of specific mono-, di-, or trinucleotides at the –1 to –3 positions with respect to the 5' terminus of their mRNAs. These preferences vary among the genera, and sometimes among viruses within a genus. For example, in one study, almost all cDNA clones of the S mRNA of the bunyavirus, Germiston virus, displayed U or G at the –1 position (19). In contrast, the bunyavirus, snowshoe hare virus, was found most commonly to have A as the 3'-terminal nucleotide of the S mRNA extensions (52). For phleboviruses, C was detected most often at the –1 position of the M mRNA of Rift Valley fever virus and also at the –1 position of the N and NSs mRNAs of Uukuniemi virus (30,193). For the hantaviruses, Hantaan and Sin Nombre viruses, the preferred –1 nucleotide was G (38,81), and for the nairovirus, Dugbe virus, C was most common (91). Examination of terminal nucleotides of 20 clones of N mRNAs of tomato spotted wilt virus revealed no base preference at the endonucleolytic cleavage site (209); however, a preference for A was observed in studies in which oligonucleotides were acquired from messages of another plant virus (42). A selective preference for an entire primer sequence was observed with La Crosse virus in persistently infected mosquitoes. In both embryonating and dormant eggs, specific, but different, 5'-extensions were observed on the majority of mRNAs (39). It was not determined whether the paucity of heterogeneous sequences related to poor availability of diverse host mRNAs, or to a preference by La Crosse virus for certain mRNAs as a source of primers.

A preferred primer sequence, or a favored nucleotide at the site of cleavage, might imply that a restricted or specific subset of host mRNAs is used for generating transcription primers, perhaps because of a need for limited base pairing with the viral genome. In one study, the 3'-terminal nucleotides of the scavenged host primers often were similar to the 5'-terminal viral nucleotides (91). It was proposed that after transcription of two or three nucleotides of the nascent mRNA, the viral polymerase might slip backward on the template before further elongation, resulting in a partial reiteration of the 5'-terminal sequence. This mechanism would explain findings with the bunyavirus, Germiston virus, which had an insertion of U or GU between the primer and viral sequences (211). Likewise, the mechanism could explain the finding that Bunyamwera virus mRNAs, which were synthesized by vaccinia virus–expressed L protein, had insertions of GU or AGU (90). Polymerase slippage also was suggested to be a possible reason for the AG insertions observed in a few mRNAs of the tospovirus, tomato spotted wilt virus (42).

An extension of this concept, termed “prime and realign,” was proposed for mRNA transcription of hantaviruses (58). According to this model, priming by host oligonucleotides with a terminal G residue would initiate transcription by aligning at the third nucleotide of the viral RNA template (C residue). After synthesis of a few oligonucleotides, the nascent RNA could realign by slipping backward two nucleotides on the repeated terminal sequences (AUCAUCAUC) (see Table 1), such that the G becomes the first nucleotide of the nontemplated 5' extensions. The frequent deletion of one or two of the triplet repeats in hantaviral mRNA supports this sort of slippage mechanism and suggests that sometimes the initial priming might start at the C residue of the third triplet in the conserved sequence rather than at the C of the second triplet (58). A prime-and-realign model also was postulated for transcription of hantaviral vRNA and cRNA, except that transcription would initiate with pppG alignment at the third nucleotide (C residue) of the template RNA. After synthesis of several nucleotides, polymerase slipping would realign the nascent RNA such that the initial priming G residue would overhang the template. It was further theorized that nucleolytic activity of the L protein might remove the overhanging G, leaving a monophosphorylated U residue at the nascent 5' end. The presence of the monophosphorylated U on Hantaan virus RNA was experimentally demonstrated (58).

Indirect evidence suggesting that a prime-and-realign method of initiation is used by phleboviruses was obtained by using a reconstituted transcription system to study the polymerase recognition sequence at the 3' termini of the ambisense S segment RNA of Rift Valley fever virus. In those studies, mutational analysis of the terminal nucleotides revealed that the first 13 nucleotides are required for polymerase recognition, but that one of the two terminal dinucleotides (UGUG) could be removed without deleterious effects on transcription (161). These data also suggested that realignment is not a prerequisite for transcription initiation.

Host Factors

Beyond the need for host-derived primers for initiating mRNA synthesis, little is known about the role that host factors might play in viral replication. Although transcriptional host factors have not been identified, they cannot presently be excluded. Whereas inhibitors of host-cell protein synthesis, such as cycloheximide and puromycin, have been found to have no effect on primary transcription in other negative-strand RNA virus families (e.g., *Orthomyxoviridae, Rhabdoviridae,* and *Paramyxoviridae*), conflicting data exist on their effects on viruses in the *Bunyaviridae* family. Early reports indicated that the bunyaviruses, Bunyamwera and Akabane viruses, were sensitive to cycloheximide (1) and that either no or greatly reduced amounts of S mRNA could be detected in La Crosse bunyavirus–infected cell cultures treated with cycloheximide or puromycin (145,162). In addition, La Crosse virion–associated polymerase produced only

incomplete transcripts *in vitro* unless rabbit reticulocyte lysates were added to provide a coupled transcription–translation system. In the coupled system, drugs that inhibit protein synthesis also inhibited full-length mRNA synthesis and resulted in the reappearance of the incomplete transcripts (13,163). This led to a hypothesis that translation of the nascent bunyavirus S mRNA is required to prevent premature termination of primary transcription products and that the nascent chain may be interacting with its template to cause premature termination. Such a translation requirement was postulated to be similar to that observed in certain bacterial systems, whereby ribosomes prevent RNA–RNA interaction and thus premature termination (163).

For the bunyavirus, Germiston virus, although S segment mRNA was inhibited in cell culture by either anisomycin or cycloheximide, full-length S transcripts could be obtained in an *in vitro* transcription system without added translational capabilities (61). Further studies, however, revealed that these full-length transcripts were obtained only under conditions of increased temperature, and that premature termination products were obtained under more usual transcription conditions. Adding of reticulocyte lysate improved transcription significantly and restored the ability for full-length transcription to occur (211).

The mechanism of the inhibition of transcription by proteinase inhibitors was more clearly defined for Germiston virus, in that adding the translation inhibitors cycloheximide, puromycin, or anisomycin, but not edeine, inhibited transcription. Because edeine inhibits translation by preventing the 40S and 60S ribosomal subunits from complexing, but still allows the 40S subunit to scan the transcript, the need for ongoing protein synthesis was postulated to be at the level of ribosome scanning, and to be independent of the need for actual translation (211). These data are consistent with an earlier hypothesis that scanning ribosomes prevent premature termination by preventing base-pairing between the template RNA and the transcript, which would cause the polymerase to halt and terminate prematurely. This theory is based on the finding that if inosine was substituted for guanosine in the *in vitro* transcription reaction, thus destabilizing base pairing, less premature termination was observed (13).

Results conflicting with these findings were obtained with the bunyavirus, snowshoe hare virus, in that strand-specific cDNA probes were able to detect full-length S segment mRNA (but not vRNA) readily in the presence of puromycin or cycloheximide (52). These results suggest that primary transcription depends neither on ongoing host protein synthesis nor on ribosomal scanning. A similar finding was obtained with La Crosse virus transcription in mosquito cells, in that translation inhibitors had no effect on transcription, suggesting that host-cell factors may influence the ability of the viral polymerase to function in the presence of such inhibitors (166). At present, it is unclear whether the variations reported in transcriptional requirements for viruses in this genus are due to differences in their transcriptional properties or to differences in the sensitivities of methods used.

Transcription Termination

Differences between vRNA and mRNA are found, not only at their 5'-termini but also at their 3'-termini. For gene segments with simple negative-sense coding, mRNAs are truncated at the 3'-termini by approximately 100 nucleotides as compared with cRNA (19,20,30,52,145,146,158,164). Potential transcription-termination sites have been proposed for the S segments of the bunyaviruses La Crosse (145) and snowshoe hare viruses (51,52) at or near the genomic sequence, 3'-G/CUUUUU, which is similar to other negative-strand RNA viral transcription termination–polyadenylation signals (72,174). U-rich regions also were observed at putative transcription-termination sites for the M and S segments of the bunyavirus Germiston virus (3'-AUGUUUUUGUU and 3'-GGGGUUUGUU, respectively) (19) and the M mRNA of hantaviruses (81). Despite these findings, there is little evidence to suggest that the mRNAs of viruses in the family *Bunyaviridae* are polyadenylated (146,158,205). Moreover, transcription-termination sites without the homopolymeric U5 or U6 tract have been proposed for numerous gene segments of viruses in the family. For example, a C-rich motif was proposed as the site of termination for the S mRNA of Sin Nombre virus (CCCACCC) (81), and purine-rich regions have been suggested as termination sites for the S and M segment mRNAs of snowshoe hare virus (5'-GGUGGGGGGUGGGG and 5'-GGUGGGGGGUGGGG, respectively), (51) and the M segments of the phleboviruses Rift Valley fever and Punta Toro viruses (5'-UGGGGUGGUGGGGU and 5'-GGUGAGAGUGUAGAAAG, respectively) (30). If these sequences are involved in transcription termination, the mechanism by which they are recognized will require further study.

Interestingly, the polymerase protein of Bunyamwera virus, expressed with a recombinant vaccinia virus, was able to transcribe virion-derived nucleocapsids, and some of the S transcripts terminated at the authentic transcription-termination site, but most did not (90). These results suggest that an additional factor(s), of either viral or cellular origin, may be required for consistent transcription termination. Likewise, in studies with the bunyavirus, Germiston virus, transcription termination was abolished if the drug edeine was included in reaction mixtures. Two suggested mechanisms to explain these findings were proposed. One possibility is that transcription termination involves an interaction between the transcriptase and a newly synthesized viral protein, such as NSs. This could not be a familial characteristic, however, because hantaviruses and nairoviruses do not encode NSs proteins. A second possibility is that the abnormal presence of scanning 40S ribosomes might mask the transcription-termination site (90,211).

The mechanism of transcription termination of the ambisense genes of phleboviruses and tospoviruses may differ from negative-sense genes, and is likely to involve RNA secondary structure. The transcription-termination sites for both the N and NSs mRNAs of Punta Toro virus were mapped by hybridizing a series of synthetic oligonucleotides corresponding to vRNA or cRNA to the messages. The results indicated that the 3' termini of both mRNAs are within 40 nucleotides of one another (50). Computer analysis of the intergenic region and the sequences encoding the 3' termini of the messages revealed a long, inverted complementary sequence that could potentially form a hairpin structure.

Similar stable hairpin structures are predicted to occur in the intergenic regions of the S and M segments of tospoviruses (37,104,108). For the S segment of the phlebovirus Uukuniemi virus, although there is a noncoding region of 70 nucleotides between the N and NSs genes, hybridization studies demonstrated that the 3' ends of the subgenomic messages overlap one another by about 100 nucleotides. Thus the 3' end of the NSs mRNA extends into the coding region of N, and the 3' end of the N mRNA terminates just before the coding sequences for N. A short palindromic sequence in the intergenic region (including the 3' ends of each mRNA) was predicted to allow formation of an A/U-rich

hairpin structure (193). Similar (but shorter), energetically favored structures have been identified in intergenic regions of the S segments of the arenaviruses, Pichinde and lymphocytic choriomeningitis viruses, which also use ambisense coding (175). For the *Bunyaviridae,* such structures could affect transcription termination only if they can form while the genome is complexed with N, and there is no direct evidence that this occurs.

In addition to secondary structure, particular sequences or motifs also may play a role in transcription termination of ambisense mRNAs. Comparing the S-segment sequences of several phleboviruses revealed the presence of G-rich regions and similar sequence motifs in the intergenic regions of Rift Valley fever, Toscana and sandfly fever Sicilian viruses, but not for Punta Toro or Uukuniemi viruses (62). Currently, there is no conclusive evidence that either of these mechanisms (i.e., secondary structure or gene sequence) is important for transcription termination with viruses in this family.

Genome Replication

For negative-strand viruses, the change from primary transcription to replication requires a switch from mRNA synthesis to synthesis of full-length cRNA templates and then vRNA. For viruses in the family *Bunyaviridae,* the polymerase protein, acting either alone or in concert with undefined viral or cellular factors, must first function as a cap-dependent endonuclease to generate a primer for transcription of a nonencapsidated, subgenomic mRNA. At some point, the polymerase must switch to a process of independently initiating transcription at the precise 3' end of the template to produce a full-length transcript. The processes involved in making that switch from primary transcription to genome replication have not been defined completely for any member of the family. Presumably, some viral or host factor is required to signal a suppression of the transcription-termination signal responsible for generation of truncated mRNA. There is no question that genome replication and subsequent secondary transcription are prevented by translational inhibitors such as cycloheximide. These results indicate that continuous protein synthesis is required for replication of the genome. Although not proven, it is likely that synthesis of N is required for genome replication, as described for other negative-strand RNA viruses such as the rhabdovirus, vesicular stomatitis virus, and the paramyxovirus, Sendai virus (see Chapter 38 and Chapter 41). For these viruses, encapsidation by N seems to serve as an antitermination signal, thus allowing full-length genome synthesis. It was suggested that the vesicular stomatitis virus NS protein also is involved and acts to control the availability of N (78). A similar mechanism appears plausible for viruses in the family *Bunyaviridae,* whereby N would function to regulate replication and might be tempered by the presence of nonstructural proteins, where they exist. A model for genome transcription and replication based on information presented earlier, and modified slightly from that proposed by Simons (190), is presented in Fig. 4.

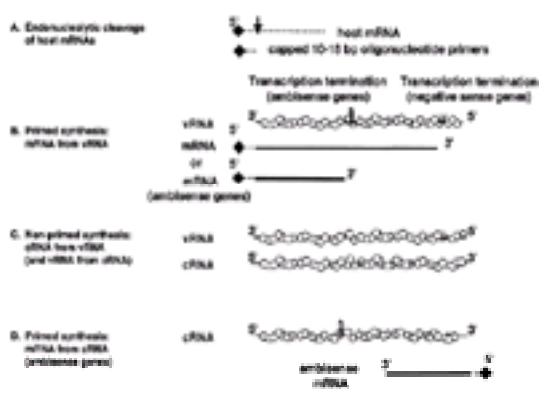

FIG. 4. Transcription and replication of RNA (*Bunyaviridae* family viruses). **A:** Transcription of viral mRNA is preceded by endonucleolytic cleavage of host mRNA caps and adjacent oligonucleotides (*solid diamond*) by viral polymerase (cap snatching). **B:** The capped oligonucleotides prime transcription of viral mRNA from the virion-encapsidated RNAs (vRNAs). Negative-sense genes are copied to yield mRNAs nearly as long as the vRNA template. Ambisense genes (S segments of phleboviruses and tospoviruses, and M segments of tospoviruses) are copied to yield mRNAs complementary to the 3' portion of the vRNA template. A hairpin structure (*hairpin shape*) is believed to be involved in transcription termination of ambisense genes. An undefined transcription termination signal (*V*) is found near the 5' end of the vRNA template for negative-sense gene segments. **C:** Replication involves primer-independent synthesis of complementary RNA (cRNA) from vRNA templates followed by primer-independent synthesis of progeny vRNA from cRNA templates. **D:** For viruses that use ambisense coding strategies to express nonstructural proteins (NSs of phleboviruses and tospoviruses and NSm of tospoviruses), the cRNA also serves as template for mRNA synthesis. As in primary transcription, capped, host-derived oligonucleotides prime synthesis of the ambisense mRNAs, and transcription termination occurs at a predicted hairpin structure.

Translation and Processing of Viral Proteins

Viral polypeptides are synthesized shortly after infection, with S and L mRNAs translated on free ribosomes, and M mRNAs translated on membrane-bound ribosomes. Expression products vary among the genera, and even within a genus.

S Segment Products: N and NSs

The N proteins of viruses in the family range in size from approximately 19 kd for bunyaviruses to 54 kd for hantaviruses and nairoviruses (see Fig. 2A). Posttranslational modifications to N have not been described. Few studies have characterized interactions of N and viral RNA. Biochemical analysis of RNA binding by N of Hantaan virus suggested that specific recognition of vRNA by N occurs in the terminal, noncoding regions of vRNA (189). For Hantaan and Puumala viruses, it was suggested that the carboxyl-terminal portion of N is needed for nucleic acid binding (68). In contrast, both

amino-terminal and carboxyl-terminal portions of N of the tospovirus, tomato spotted wilt virus, were postulated to bind to RNA (172).

In addition to N, viruses in the *Bunyavirus, Phlebovirus*, and *Tospovirus* genera produce NSs proteins. Sizes of NSs range from 10 kd for bunyaviruses to more than 50 kd for tospoviruses (see Fig. 2A). The NSs protein of the phlebovirus Rift Valley fever virus is phosphorylated and accumulates in the nuclei of infected cells, where it forms fibrillar structures (200). The major phosphorylation sites of the Rift Valley fever virus NSs were mapped to serine residues located near the carboxyl terminus of the protein (100). Expression studies revealed that fibrils could form in the absence of other viral proteins; however, if the carboxyl-terminal region of NSs was removed, NSs was still transported to nuclei, but did not coalesce into fibrils (224). Similar fibrillar structures of NSs were observed for some strains of tomato spotted wilt virus, but the structures appeared only in the cytoplasm. For other strains of tomato spotted wilt virus, NSs was found to be distributed throughout the cytoplasm but did not form discernible structures (103). The NSs protein of the phlebovirus Uukuniemi virus was also found to be distributed throughout the cytoplasm. This protein was not phosphorylated but did associate specifically with the 40S ribosomal subunit (192). The significance of this association, if any, was not determined.

The characteristics and functions of NSs are not well defined, and there is no evidence that NSs proteins serve the same function for viruses in different genera. Comparing the deduced amino acid sequences of NSs for five different phleboviruses revealed homologies of only 17% to 30% (62). However, comparison of the NSs gene sequences for a number of strains of a single phlebovirus, Rift Valley fever virus, showed that certain areas were highly conserved (182). These data suggest that there may be a strong evolutionary pressure to maintain distinct portions of the NSs for individual viruses, but that the remainder of the protein can diverge without affecting the function of NSs.

For phleboviruses and tospoviruses, it is unlikely that NSs is involved in early stages of replication because their ambisense mRNAs are produced only after vRNA has been copied to cRNA; therefore, NSs appears later in infection than the structural proteins. Consequently, it is likely that NSs is involved in late stages of replication, in morphogenesis, or in the spread of newly formed viruses. Consistent with this, a mutant of the phlebovirus Rift Valley fever virus, with an in-frame deletion of about 70% of the coding information for NSs, was found to replicate in Vero and mosquito cells, but not in human lung diploid cells. In addition, the deleted virus was avirulent in mice and hamsters, as compared with wild-type virus (132). These data suggest a role for the phlebovirus NSs in host range and perhaps in virus dissemination. Similarly, disruption of the coding sequences of NSs in a genetically engineered Bunyamwera virus resulted in a mutant virus that produced smaller plaques, displayed reduced shutoff of host-cell protein synthesis, and showed poorer cell-to-cell spread as compared with wild-type virus (22). The mechanism resulting in these effects remains to be determined.

M Segment Products: G1, G2, and NSm

The viral envelope glycoproteins, G1 and G2, are translated from a single mRNA complementary to vRNA. The polyprotein precursor of G1 and G2 is not seen in infected cells, and has been observed only by *in vitro* translation of RNA transcripts in the absence of microsomal membranes (178,202,205). For most viruses, both G1 and G2 are preceded by signal sequences; therefore, cleavage of the polyprotein precursor is likely mediated by host signalase (54). The nairovirus, Dugbe virus, differs in that there is no signal sequence immediately preceding G1 (the second polypeptide encoded in the ORF); therefore, this protein may be processed from a precursor (116). All M segment polyproteins display variable numbers of predicted transmembrane regions, and a hydrophobic sequence at the carboxyl terminus, indicative of a membrane anchor region. Thus the M segment translation products of viruses in the family *Bunyaviridae* are typical class 1 membrane proteins, with the amino terminus exposed on the surface of the virion and the carboxyl terminus anchored in the membrane.

M segment gene products have a cysteine content of approximately 4% to 7% (32,83,101,108,110,116,188). For related viruses, the positions of the cysteine residues in the polyprotein are highly conserved, suggesting that extensive disulfide-bridge formation may occur and that the positions may be crucial for determining correct polypeptide folding. The secondary structure of the proteins also is involved in immunogenicity, as indicated by the finding that neutralizing or protective epitopes are often nonlinear (11,215).

The nonstructural protein, NSm, produced by phleboviruses and bunyaviruses, is translated from the same mRNA as G1 and G2, whereas that of tospoviruses comes from an ambisense mRNA. There is no known functional equivalence of NSm proteins of viruses in different genera. Hantaviruses and nairoviruses produce no known M segment products except for G1 and G2 (116,188). However, because nairovirus-infected cells display several viral polypeptides of unknown origin (25,116,219), it is possible that additional precursor–product relationships may still be uncovered. In contrast, some viruses in the *Phlebovirus* and *Bunyavirus* genera produce NSm from the same M segment mRNA as G1 and G2. The NSm protein of bunyaviruses is encoded between the G1 and G2 proteins (53,134). Its hydropathy profile suggests that it is a membrane-bound protein, and expression studies demonstrated that it localizes to the Golgi along with G1 and G2 (134). Although a function for this protein has not been identified, it was suggested that it might be involved in facilitating virion assembly in the Golgi (134).

For the phlebovirus, Rift Valley fever virus, a NSm of approximately 14 kd is cleaved from the amino terminus of the polyprotein translation product. Sequence studies revealed five in-frame translation-initiation codons upstream of the amino-terminus of G2. Mutational analysis of those codons indicated that the first four can be used efficiently to translate G2 and G1. NSm was found to originate from the second ATG, and a 78-kd polypeptide, representing uncleaved NSm and G2, was translated from the first ATG (92,203). Pulse-chase experiments revealed no precursor–product relationship between the 78- and 14-kd proteins; thus it appears that use of the first and second ATGs, respectively, is what dictates generation of these proteins (31). Both the 14-kd NSm and the 78-kd polypeptide are found in abundance in Rift Valley fever virus–infected cells, (32,92,202), suggesting that they might play a role in replication or morphogenesis.

An even larger NSm (~30 kd) is cleaved from the amino terminus of the M segment polyprotein of the phlebovirus, Punta

Toro virus. *In vitro* expression studies indicated that both envelope glycoproteins could be produced in the absence of the NSm coding region (123), but other studies to evaluate the use of the 13 potential translation-initiation codons present in the NSm coding information have not been reported. No homology between the NSm proteins of Punta Toro and Rift Valley fever viruses was apparent (83).

In contrast to Punta Toro and Rift Valley fever phleboviruses, the tick-borne phlebovirus, Uukuniemi virus, does not produce an NSm. The first (and only) initiation codon preceding sequences of the envelope glycoproteins is located 17 amino acids upstream of the amino terminus of G1 (178); hence the G1 protein of Uukuniemi virus appears to be analogous to the 78-kd NSm–G2 fusion product produced by Rift Valley fever virus (although there is no obvious sequence homology of these predicted products). Until a function can be assigned to NSm, it is impossible to determine whether Uukuniemi virus replicates in the absence of such a function or accomplishes whatever function is required without removal of a portion of the amino terminus of G1.

Unlike all other viruses in the family, the NSm protein of tospoviruses is translated from an ambisense mRNA (101). It is also the only NSm protein in the family to have an assigned function. By subcellular fractionation of infected plants, or in thin-section immunoelectron microscopy studies, the NSm of tomato spotted wilt virus was found to be present in cell wall–containing fractions or associated with aggregates of nucleocapsids and with the plasmodesmata (101). Expression of the NSm protein in plant cells or insect cells revealed that the protein first appeared near the cell surface and later as tubular structures protruding from the cell surface. In infected leaf tissues, the tubules were observed only in the plasmodesmata (198). These data were interpreted to indicate that the tospovirus NSm protein is involved in cell-to-cell movement of the nonenveloped ribonucleocapsid structures across the cell walls in infected plants.

All G1 and G2 proteins examined to date possess asparagine-linked oligosaccharides. Two broad classes of asparagine-linked oligosaccharides, complex or high-mannose (simple), generally are found on mature glycoproteins (reviewed in 111). Often both types are attached to the same polypeptide chain. As described for the hemagglutinin protein of influenza virus, as well as other glycoproteins (reviewed in 99,105), for oligosaccharides to evolve from the high-mannose type to the complex type, they are normally transported through the Golgi, where mannose residues are trimmed and terminal residues added. Examination of the oligosaccharides attached to the G1 and G2 proteins of Uukuniemi virus in infected cells revealed that G2 has mostly high-mannose glycans, whereas G1 contains both complex and a novel intermediate-type oligosaccharide (152). Similar results were obtained with the bunyaviruses Inkoo and La Crosse viruses (115,152), whereas the glycoproteins of Hantaan virus were found to be mostly of the high-mannose type (8,180,185). These findings indicate that the proteins are incompletely processed through the Golgi. This is likely related to retention of G1 and G2 in the Golgi, where assembly and release occur.

Between four and nine potential glycosylation sites were identified in the deduced amino acid sequences of the G1 and G2 proteins of viruses in the family (32,53,70,83,101,108,110,178,188). The number of sites actually used in mature virion proteins has not been defined for all viruses; however, G2 of the phlebovirus Rift Valley fever virus was demonstrated to be glycosylated at its single available site and at least three of four possible G1 sites (92). Examination of glycosylated tryptic oligopeptides of the envelope glycoproteins of several bunyaviruses suggested that all three potential glycosylation sites on G2 are used and at least one of two sites on G1 (214). The single glycosylation site available in the G2 protein of Hantaan virus is used, and this site is conserved among numerous other hantaviruses (7,185). Potential N-linked sites also were identified in the NSm proteins of phleboviruses and tospoviruses, and at least for Rift Valley fever virus, are used to some extent (31,92). The NSm of Bunyamwera was found not to be glycosylated (110).

L Segment Product: L Polymerase Protein

The L proteins of viruses in the family *Bunyaviridae* range in size from about 237 kd for phleboviruses to 459 kd for nairoviruses (see Fig. 2C). This huge difference in protein sizes might reflect varying functional activities of the enzymes, but as yet, there are no data to support this. There are no known processing or posttranslational modifications to the L proteins (208). Comparing the deduced amino acid sequence of the L proteins of viruses in the family *Bunyaviridae* revealed several conserved motifs commonly associated with RNA polymerases (69,131,160,173). In addition, two novel regions, found at the amino terminus of the L protein of the phlebovirus Rift Valley fever virus, were shown to be conserved only in the polymerases of viruses in the *Bunyaviridae* and *Arenaviridae* families (131).

The function of the L protein as the viral polymerase was confirmed by using L protein expressed from vaccinia virus recombinants to transcribe authentic bunyavirus ribonucleocapsid templates (89). Endonuclease activity also was demonstrated, *in vitro,* with expressed Bunyamwera virus L protein, providing evidence that the L protein is responsible for generating the capped primers needed for transcription (90)

Transport of Viral Proteins, Assembly, and Release

One of the earliest notable features found to distinguish members of the family *Bunyaviridae* from other negative-strand RNA viruses was that virions are formed intracellularly by a budding process at smooth-surface vesicles in the Golgi (Fig. 5A) (16,114,133,212). The plant-infecting members of the family, the tospoviruses, also appear to assemble in the Golgi; however, it was suggested that instead of budding, there is a coalescence of Golgi membranes around the ribonucleocapsids (95). Although most members of the family examined have been found to mature in the Golgi, budding at membranes other than those associated with the Golgi also has been reported for some viruses. The hantaviruses, Sin Nombre and Black Creek Canal viruses, were shown to bud preferentially from the plasma membrane of infected cells (63,168). Similarly, the phlebovirus, Rift Valley fever virus, was found to bud from the plasma membrane as well as in the Golgi of primary rat hepatocytes (3). Because Rift Valley fever virus is a hepatotropic virus and can cause liver necrosis and death in animals, these results raise questions of possible differences in morphogenesis in target versus nontarget cell types that might affect pathogenesis and/or immune defense. The reason(s) for maturation of viruses in the family *Bunyaviridae* in the Golgi complex as opposed to the more usual mode of viral morphogenesis (budding at the plasma membrane) are not known completely; however, important clues have been obtained by studying the viral proteins'

transport to and retention in the Golgi.

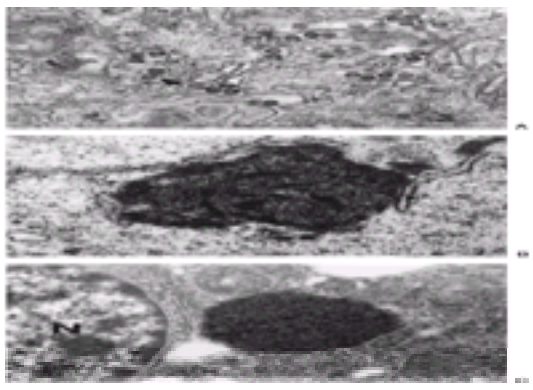

FIG. 5. Thin-section micrographs showing virions and cytoplasmic inclusion bodies found in cells infected with viruses in the family *Bunyaviridae.* **A:** Rift Valley fever virus (*Phlebovirus* genus) in the Golgi cisternae of primary rat hepatocytes (3). *Arrow*, A particle budding through Golgi membranes. Magnification ×54,000. **B:** Inclusions found in the brains of rats infected with Seoul virus (*Hantavirus* genus). Magnification ×53,000. **C:** Immunoelectron micrograph of Seoul virus inclusion bodies reacted with antibodies to the nucleocapsid protein. *N*, Nucleus. Magnification ×65,000. Micrographs courtesy of T. Geisbert, U.S. Army Medical Research Institute of Infectious Diseases, Frederick, MD.

Golgi Targeting and Retention

Expression of M gene segments of representative phleboviruses, hantaviruses, and bunyaviruses demonstrated that G1 and G2 are targeted to the Golgi in the absence of other viral components (29,123,134,151,180,218). When expressed individually, the first glycoprotein of the M segment ORF (i.e., encoded in the 5' portion of the M segment) was able to exit the endoplasmic reticulum (ER), but the second glycoprotein remained in the ER (23,107,122,125,150,177). These data indicate that the signal for Golgi transport resides in the first protein encoded in the M segment ORF and that dimerization of G1 and G2 in the ER is necessary for efficient transport of the downstream product.

The Golgi complex actually consists of several subcompartments, including the cis-, medial, and trans-Golgi (reviewed in 126). The exact location in the Golgi where the viral glycoproteins accumulate has not been conclusively determined. By using the fungal antibacterial reagent, brefeldin A, which inhibits transport of proteins out of the ER and causes a redistribution of the Golgi component to the ER, the glycoproteins of the phlebovirus Punta Toro virus were found to be localized in the cis/medial Golgi membranes (27). Similar redistribution of G1 and G2 from the Golgi to the ER were observed after brefeldin A treatment of cells infected with vaccinia virus recombinants expressing the M segment of Bunyamwera virus (134). Immunohistochemical and electron-microscopy studies of Uukuniemi virus demonstrated that budding may begin as early as pre-Golgi intermediate compartment and that virus budding continues in the Golgi stack (88). Similar studies are needed to determine if localization is the same for viruses in other genera.

The mechanism by which G1 and G2 are arrested in the Golgi is not completely known. To map the Golgi targeting and retention signal(s) of phleboviruses, a series of deleted and chimeric genes were constructed and their expression products examined for Golgi targeting and retention. The cytoplasmic tail of the first glycoprotein encoded in the M cRNA was found to contain the targeting signal (4,5,122). For Uukuniemi virus, the minimal transport signal was a 30-amino-acid peptide within the cytoplasmic tail (5). Similar expression studies with M2 segment constructs of Hantaan virus also demonstrated that the cytoplasmic tail of the upstream glycoprotein was needed for exit of the proteins from the ER. Although G2 did not have a targeting signal, results suggested that it might contribute to retention in the Golgi (6).

The NSm proteins are not known to play a role in transport or Golgi retention. For representative phleboviruses and bunyaviruses, expression of G1 and G2 in the absence of NSm had no effect on their transport to the Golgi (23,107,123,134,218). However, when the entire M segments were expressed, NSm colocalized to the Golgi with G1 and G2, suggesting an interaction of these proteins before their exit from the ER.

Kinetics of Transport

Examination of the types and amounts of oligosaccharides attached to viral proteins has been used to assess transit of the proteins through the Golgi compartments. For example, shortly after primary glycosylation of nascent proteins at the ER, oligosaccharides are susceptible to cleavage by endoglycosidase H (endo-H), an enzyme that cleaves only high-mannose residues. Later, after removal of glucose residues at the rough ER, migration of the glycoproteins to the smooth ER and Golgi, trimming of residues, and attachment of peripheral sugars, the oligosaccharides are no longer susceptible to endo-H cleavage. This acquired resistance to endo-H therefore generally indicates that the proteins have been processed through the Golgi. The time required to convert between endo-H susceptibility and resistance correlates with the time needed for protein transport from the ER to the Golgi.

For the phlebovirus, Punta Toro virus, heterodimerization occurred between newly synthesized G1 and G2 within 3 minutes after protein synthesis, and the dimers were found to be linked by disulfide bonds. The dimeric G1/G2 proteins were observed both during transport and after accumulation in the Golgi complex (27). For another phlebovirus, Uukuniemi virus, it was found that the transport of G1 and G2 from their site of synthesis on the rough ER through the Golgi occurred at an estimated 2 to 3 times slower rate than that of most viral membrane glycoproteins destined to be transported to the plasma membrane (57). That is, endo-H resistance was achieved at 45 and 90 to 150 minutes for G1 and G2 of Uukuniemi virus (106), compared with 15 to 20 minutes for the hemagglutinin protein of influenza virus or the G protein of vesicular stomatitis virus (33,40,199). The finding that Uukuniemi virus G1 and G2 have different transport

kinetics (i.e., G1 is incorporated into virions 20 minutes faster than G2) suggests that the dimers may arise from different precursor proteins, possibly because faster-folding G1 can not dimerize with slower-folding G2 until G2 has reached its correct conformation (223). In this same study, the G1 and G2 proteins of Uukuniemi virus were found to exit the ER quickly, but did not enter the Golgi for 15 to 20 minutes. The investigators interpreted these findings as indicating that the G1 and G2 proteins may dimerize in an intermediate compartment between the ER and Golgi (223).

Assembly and Release

Unlike most other negative-strand RNA viruses, members of the *Bunyaviridae* do not have a matrix (M) protein to link the integral viral envelope proteins and their nucleocapsids and to act as the nucleating step for assembly. The absence of M protein suggests a direct interaction between the ribonucleocapsids, which accumulate on the cytoplasmic side of vesicular membranes, and viral envelope proteins, which are displayed on the luminal side. Electron microscopy of cells infected with the phleboviruses, Punta Toro or Karimbad viruses, revealed that ribonucleocapsids and spike structures (i.e., viral envelope glycoproteins) were present only in regions of Golgi membranes where budding appeared to be occurring, and not on adjacent areas of the same membrane, suggesting a transmembranal interaction of N with G1 or G2 (194).

The signal directing the ribonucleocapsids to the budding compartment is not known. Excess ribonucleocapsids of hantaviruses (see Fig. 5B and Fig. 5C) tospoviruses, and nairoviruses were found to accumulate in large cytoplasmic inclusions, suggesting that only ribonucleocapsids that interact with the envelope proteins are transported to the Golgi (17,63,80,97,129,206). It is likely that the transmembrane domains of G1 or G2 that are exposed on the cytoplasmic face of the membrane are involved in this interaction. Candidate transmembrane regions have been predicted from hydropathic characteristics of derived amino acid sequences representing the envelope proteins of all members of the *Bunyaviridae* examined to date. Direct examination of the phlebovirus, Karimabad virus, by enzymatic digestion of exposed proteins embedded in intracellular membranes, demonstrated that approximately 12% of G1 and/or G2 was exposed on the cytoplasmic face of membranes in infected cells and was accessible to digestion. A large protease-resistant fragment was identified, which was presumably sequestered in the membrane in a manner that rendered it safe from enzymatic digestion (194). These enzyme-resistant fragments may therefore represent transmembrane regions of proteins, which could provide the interaction between ribonucleocapsids and the cellular membranes required for envelopment. For Uukuniemi virus, the cytoplasmic tail of G1, which is at least 70 residues long, may be a logical candidate for interaction with the nucleocapsids (156,178). Similar cytoplasmic tails also are predicted for other members of the family.

After budding into the Golgi cisternae, virions apparently are transported to the cell surface within vesicles analogous to those in the secretory pathway. The release of virus from infected cells presumably occurs when the virus-containing vesicles fuse with the cellular plasma membrane (i.e., by normal exocytosis). Numerous viruses in the family have been observed late in infection within vesicles or in the process of exocytosis by electron microscopy (41,181,194). In polarized cells, the phleboviruses, Rift Valley fever and Punta Toro viruses, were found to be released primarily from the basolateral surface, whereas the hantavirus, Black Creek Canal virus, was released from the apical surface (3,28,168). Such polarized release of viruses might be important for disseminating virus during natural infection to produce a systemic disease (28).

The assembly and release of tospoviruses may differ from those processes of other viruses in the family. A model was proposed in which Golgi membranes with integral viral envelope proteins wrap around ribonucleocapsids. These particles may then fuse with each other or with ER membranes to release single enveloped particles into the cisternae (95). Mature virions accumulated within the ER cisternae likely remain there until ingested by thrips. Release of tospoviruses from insect cells probably occurs via secretory exocytosis similar to that of animal-infecting members of the family (222).

EFFECTS OF VIRAL REPLICATION ON HOST CELLS

The cytopathic effects observed in cultured cells infected with members of the family *Bunyaviridae* vary widely, depending on both the virus and the type of host cell studied. Viruses in all genera except the *Hantavirus* genus are capable of alternately replicating in vertebrates (or plants for tospoviruses) and arthropods and generally are cytolytic for their vertebrate/plant hosts but cause little or no cytopathogenicity in their invertebrate hosts (12,87,114,222). Some viruses display a very narrow host range, especially for arthropod vectors. Although the reason for this has not been defined completely, studies on La Crosse variant and revertant viruses suggested that the specificity was related to G1, probably at the level of viral attachment to susceptible cells (201). In natural infections of mammals, viruses are often targeted to a particular organ or cell type. For example, bunyaviruses such as La Crosse virus appear to be neurotropic (142); the phlebovirus, Rift Valley fever virus, is primarily hepatotropic (2,3,44,153,154); and the hantavirus, Hantaan virus, persists in rodent lungs (109). It will be interesting to determine whether this targeting is due solely to host-cell receptors or to other factors such as differences in effects on host-cell metabolism in targeted cell types versus the unnatural situation in cultured vertebrate cell lines.

Effects on Host-Cell Metabolism

In vertebrate cells, bunyaviruses and some phleboviruses were found to cause a reduction in host-cell protein synthesis, which became more prominent as the infection progressed. For example, by 5 hours after infection, Bunyamwera virus–infected cells showed reduced levels of host protein synthesis, and by 7 hours, there was almost no synthesis (149). Similar results were obtained in La Crosse virus–infected cells (115). Rift Valley fever virus–infected cells displayed reduced host protein synthesis, which gradually became more pronounced from 4 to 20 hours after infection (140). In contrast, a reduction in host protein synthesis did not occur, even late in infection, with another phlebovirus, Uukuniemi virus, or with the nairovirus Dugbe virus (25,155,205), both of which are transmitted by ticks rather than mosquitoes. Host protein synthesis was slightly inhibited, however, in a *Xenopus laevis* (frog) cell line infected with the tick-borne nairovirus, Clo Mor, or the phlebovirus, St. Abb's head (220). Hantaviruses not only cause no detectable

reduction in host macromolecular synthesis (45,184), but they also routinely establish persistent, noncytolytic infections in susceptible mammalian host cells, a finding consistent with their nonpathogenic persistence in their natural rodent hosts (109).

The mechanism of host protein shutoff has not been determined; however, recent studies with Bunyamwera virus derived from cDNA indicated that mutations introduced into the NSs coding region drastically reduced the shutoff of host protein synthesis (21,22). Whether this is a direct consequence of eliminating NSs or a secondary effect remains to be determined. It is noteworthy that some of the viruses that do not shut off host protein synthesis do not produce an NSs protein (e.g., hantaviruses and nairoviruses) and/or do not produce NSm proteins (e.g., Uukuniemi and hantaviruses).

The arthropod-borne members of the family, like most other arboviruses, cause little detectable cytopathology in mosquito cell cultures, and viral persistence is readily established (24,48,135,136,179). Unlike cultured vertebrate cells, mosquito cells infected with the bunyavirus, Marituba virus, displayed no reduction in host macromolecular synthesis; thus viral infection apparently does not drastically interfere with normal cellular processes (24). One suggested reason for this is that, in arthropod cells, excess viral proteins do not accumulate in the cells but rather are more efficiently processed into mature virions (135). Another possibility is that viral transcriptase may be less active in arthropod cells than in mammalian cells and that the endonuclease activity of the polymerase (which is used to acquire transcriptional primers) is detrimental to host-cell messages. A reduced level of activity of the viral transcriptase would, therefore, produce less damage to host-cell messages and consequently to protein synthesis (179).

Persistence, both in insect and mammalian cells, can be mediated by defective interfering (DI) viruses. Conventional DI particles, which displayed deletions only in L, were described for Bunyamwera virus. The defect in L was found to be a single internal deletion, amounting to 72% to 77% of the L RNA segment (143). Defective L RNA segments and resultant DI particles also were reported for the bunyavirus, Germiston virus (34), and for the tospovirus, tomato spotted wilt virus (170,171). The tospovirus deletions, which were generated by repeated high-multiplicity passage, amounted to 60% to 80% of the L segment (171). Interestingly, the L deletions identified both in the tomato spotted wilt and Bunyamwera DI particles were in-frame, thus allowing translation of truncated L polypeptides (143,171). The significance of this finding is not known, but conservation of the terminal sequences and the observed encapsidation of these shortened L segments suggest that they may retain all signals necessary for replication, transcription, and translation, and might be able to interfere at several steps in the replication process.

Persistent infections of viruses in the family have also been described that do not involve typical DI particles. For Bunyamwera virus, temperature sensitive and plaque morphology mutants were recovered from persistent infections of mosquito cells (48,135). These carrier cultures were found to be resistant to superinfection with homologous virus. In addition, virus released from the cultures inhibited infection of normal mosquito cells by standard virus (48). No evidence for deleted RNAs was obtained; thus classic DI particles were apparently not involved (48,179). For the hantavirus, Seoul virus, persistence in mammalian cell cultures was found to be related to short deletions in the conserved terminal regions of the gene segments, particularly the 5' end of the L segment (127). It will be interesting to determine if similar deletions occur in rodents persistently infected with hantaviruses.

CONCLUSION

The family *Bunyaviridae,* a large and widely diverse group of viruses, is divided into five genera based on molecular properties and serologic relationships among its members. Viruses in different genera display common morphologic, biochemical, and genetic attributes, yet have unique replicative properties. All viruses have three-segmented, single-stranded RNA genomes and encode their N, G1/G2, and L proteins in the S, M, and L genome segments, respectively. Viruses in this family generally display an unusual Golgi-associated morphogenesis and usually acquire their envelopes by budding into intracytoplasmic vacuoles. Recent advances in the molecular biology of viruses, including the development of *in vitro* replication systems and infectious clones, should allow rapid progress in furthering our understanding of these viruses. Among the unresolved questions to be answered are the following: Can the polymerase carry out all of the replication functions by itself, or are cellular factors involved? What dictates the switch in polymerase function from primer-dependent mRNA synthesis to primer-independent cRNA synthesis? What are the functions of the nonstructural proteins encoded in the S and M segments of some viruses? What are the protein–protein and protein–nucleic acid interactions? How do virions assemble to include a full complement of the genome segments? What factors permit viral persistence? It is likely that the answers to these questions will differ for the many diverse viruses in this family.

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CHAPTER 49 Bunyaviruses

Stuart T. Nichol

Bunyamwera virus was originally isolated from *Aedes* species mosquitoes in Uganda during a yellow fever study in 1943 (253). This seemingly insignificant event would open the door to the discovery and characterization of a large new family of viruses, the *Bunyaviridae.* After the isolation of Bunyamwera virus, several additional arboviruses were discovered that clearly did not fit in the classic arbovirus group A and B antigenic groups (which later formed the *Alphavirus* and *Flavivirus* genera of the *Togaviridae,* respectively) and were assigned to what became known as group C (39,59). During the next decade, further virus discovery and detailed serologic characterization led to refinement of the virus groupings and formation of closely related groups, including the Bunyamwera, California, group C, and Simbu serogroups. These serologic studies, together with biochemical and morphologic analyses (194), led to the concept of a Bunyamwera "supergroup" consisting of these groups of closely related viruses that could be linked by weak but repeatable serologic cross-reactions. Eventually, the family *Bunyaviridae* was defined, which now contains more than 350 viruses with worldwide distribution and which includes several important human, livestock, and plant pathogens.

Electron microscopic thin-section studies have shown these viruses to have similar morphologies, forming generally spherical particles 80 to 120 nm in diameter (Fig. 1), although oval or more elongated forms can also been seen, particularly with the hantaviruses. The viruses share a common genetic organization of three predominantly negative-stranded RNA segments (S, M, and L). Based on antigenic, genetic, and ecological relatedness, they are organized into five genera: *Bunyavirus, Hantavirus, Phlebovirus, Nairovirus,* and *Tospovirus.* A listing of the most notable members of each genus is shown in Table 1. Most of these viruses, with the exception of the hantaviruses, are primarily maintained in nature by replication in arthropod hosts with alternating amplification cycles in vertebrate species. The arthropod vector varies with different viruses, and although mosquitoes are most frequently used, phlebotomine sandflies, culicoid flies, ticks, and thrips are also common vectors. Transovarial and venereal transmission have been demonstrated for several of these viruses in their arthropod hosts. These viruses are generally noncytopathic in arthropod cells but can cause reduction in host macromolecular synthesis and cytopathic effect (CPE) in susceptible vertebrate cells. In contrast, hantaviruses are found in specific rodent reservoir species in nature and are generally noncytopathic in susceptible vertebrate cells. The geographic range and natural history features of the vertebrate or arthropod hosts play an important role in determining the regional or global virus distribution and disease epidemiology.

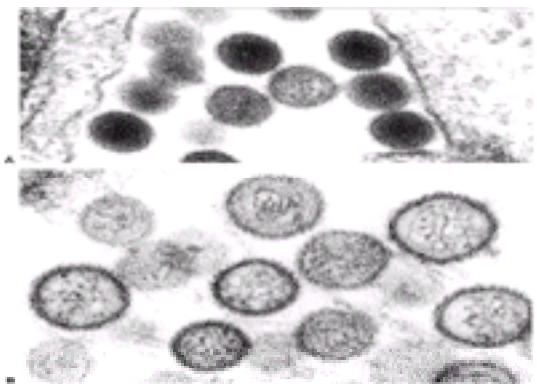

FIG. 1. Electron micrograph thin-section images of viruses of the family *Bunyaviridae.* **A:** Rift valley fever virus (genus *Phlebovirus*). (Courtesy of Cynthia Goldsmith and Tom Ksiazek.) **B:** Sin Nombre virus (genus *Hantavirus*) (135,000x magnification). (Courtesy of Cynthia Goldsmith and Luanne Elliott.).

TABLE 1. Most notable family *Bunyaviridae* virus members

BUNYAVIRUS GENUS

The *Bunyavirus* genus now contains more than 150 viruses that are found throughout the world with the exception of Australia. Almost all of these viruses are transmitted by mosquitoes and have amplification cycles in a variety of vertebrate hosts.

History

After the original isolation of bunyamwera virus from *Aedes* species mosquitoes in Uganda in the 1940s, the virus was found to be distributed throughout much of sub-Saharan Africa and associated with cases of febrile illness (100,143,253,278). Over several decades, many additional distinct bunyamwera serogroup viruses were detected in mosquitoes in the Americas, several of which were associated with disease in humans or livestock. For example, Cache Valley virus, which was isolated from mosquitoes trapped in Utah in 1956, is now known to be distributed throughout much of the United States and possibly elsewhere in the Americas (33,117).

The California serogroup viruses are found throughout a broad range of ecological zones ranging from the tropics (e.g., Guaroa and Lumbo viruses) to temperate and arctic zones (e.g., Inkoo and snowshoe hare viruses). Most of these viruses are vectored by mosquitoes of the *Aedes* genus. The type member, California encephalitis virus, was isolated in California in 1941 during now classic studies involving processing of large numbers of mosquito pools for virus isolation attempts by inoculations into suckling mouse brains. The virus was shown to be associated with three cases of encephalitis in California (108). Another member of the serogroup, La Crosse virus, was isolated from the brain of a child who died of encephalitis in La Crosse, Wisconsin in 1960 (281). It was found to be antigenically related to, but distinct from, California encephalitis virus and has been shown to be a significant cause of arboviral encephalitis in the United States. Snowshoe hare virus was originally isolated from a snowshoe hare captured in Montana in 1959 and is now known to be distributed throughout Alaska, Canada, and the northern United States (104). This virus is considered a variety of La Crosse virus and has been shown to be infrequently associated with febrile central nervous system (CNS) disease in children and adults. Jamestown Canyon virus was isolated from a pool of *Culiseta inornata* mosquitoes captured in Colorado in 1961 (104). This virus (including the closely related Jerry Slough variety) is now thought to be second only to La Crosse virus as a cause of arboviral encephalitis in the United States.

A large number of group C viruses were isolated during the 1950s and early 1960s, predominantly in South and Central America (248). Many have been shown to be associated with acute febrile illness in humans. Several Simbu serogroup viruses were also isolated at this time from throughout the world, including Oropouche virus in Trinidad in 1955 and Akabane virus in Japan 1959 (5,128). Akabane virus is an important cause of fetal malformations in cattle in Australia, Japan, Korea, and Israel and is also present in Africa (128). Oropouche virus has caused several major epidemics of febrile illness in Brazil (219).

Infectious Agents

The classic method for isolation of bunyaviruses is either intracranial inoculation of suckling mice or infection of susceptible cell lines such as Vero cells. Thin-section electron micrographs show that the viruses of the *Bunyavirus*

genus have a typical overall morphology with predominantly spherical or ovoid particles of about 68 to 110 nm in diameter with surface fringe projections of about 7 nm. However, negative-staining studies show *Bunyavirus* genus viruses to have a distinctive surface structure consisting of small knoblike morphologic units but no distinct organization pattern (173) (Fig. 2).

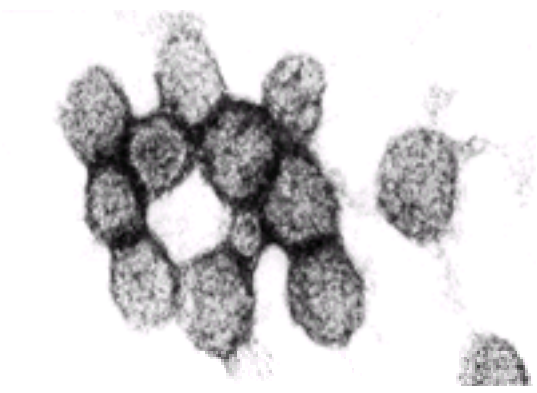

FIG. 2. Electron micrograph negative-stain image of La Crosse virus (genus *Bunyavirus*) (135,000× magnification). (Courtesy of Mary Lane Martin and Helen L. Regnery.)

Pathogenesis and Pathology

Human infections with California encephalitis, La Crosse, or Jamestown Canyon viruses are initiated by bite of a virus-infected mosquito. The course of the infection has been extensively modeled in mice (129,133,209). Similar to the human infection, the outcome of the infection in mice is dependent on the age of the animal and strain of virus used, with subcutaneous challenge of newborn mice most closely mimicking the natural human infection. Virus initially spreads from the site of inoculation into the skeletal muscle, which is the major site of replication. The virus spreads to the plasma, presumably through the lymphatic channels, delivering the virus to skeletal and cardiac muscle where further rounds of replication occur. The resulting plasma viremia spreads the virus (possibly through vascular endothelial cells) to the CNS, where the virus replicates in neurons and glial cells causing considerable neuronal necrosis. Death occurs 3 or 4 days after infection.

The lesions observed in the brains of fatal La Crosse encephalitis cases differ from those in infected suckling mice. In humans, and to a large extent in adult mice infected intracranially, one sees cerebral edema, perivascular cuffing, glial nodules, mild leptomeningitis, and occasional areas of focal necrosis which are typical of acute severe viral encephalitis. Lesions are mostly in the cerebral cortex, with some in the brain stem (134).

Infection of natural vertebrate amplifying hosts of these viruses, such as adult chipmunks (La Crosse virus) or snowshoe hares (snowshoe hare virus), results in inapparent infections and viremias sufficient to infect mosquitoes (242). *Aedes triseriatus* mosquitoes are the principal vector of La Crosse virus, and their experimental infection with California encephalitis serogroup viruses has been studied in detail (18,274,280,295). Virus ingested through a viremic blood meal infects the epithelial layer surrounding the mosquito midgut. Replication of the virus results in release of virus across the basal lamina into the hemocele, allowing transport of the virus to tissues throughout the body. Virus infection of mosquitoes other than the principal vector species usually results in poor penetration of the virus across this midgut barrier. Experimental infections using reassortant viruses have shown that virus M genomic segment (encoding the glycoproteins and NSm protein) is an important component in the correct match between virus and host mosquito, which allows efficient transit across the midgut barrier (18). Once across the barrier, virus replication occurs in a wide variety of tissues, including the ovaries and salivary glands (274). Release of virus from the salivary glands allows virus transmission from the female mosquito to the vertebrate host during feeding. Mosquitoes begin to be infectious about 1 or 2 weeks after ingestion of virus, an interval referred to as the *extrinsic incubation period.*

The infection of the ovaries is thought to be crucial to virus maintenance in mosquito populations in that this results in transovarial transmission of the virus from the female to the offspring, allowing the virus to overwinter in infected eggs (295). Male mosquitoes play no role in the vertical transovarial transmission of virus nor in the horizontal transmission through amplifying vertebrate hosts because they do not take a blood meal. However, virus is detected in the gonads of transovarially infected males and can result in venereal transmission of the virus horizontally within the mosquito population (280). This combination of transmission mechanisms allows efficient maintenance of the La Crosse virus in *A. triseriatus* populations. Similar processes are thought to function in host mosquito infections with other California serogroup viruses (285), and the work done with these viruses and their arthropod hosts often serves as a model for understanding virus maintenance and transmission mechanisms in other family *Bunyaviridae* viruses.

The Simbu serogroup virus, Akabane virus, is an important cause of disease in livestock, causing epizootics of abortions and congenital defects in cattle, sheep, and goats (110). Experimental infections of pregnant cattle or ewes with Akabane have been shown to produce viremia, followed by virus replication in the placenta and fetal tissues, and resulting in congenital defects such as microencephaly and hydrocephalus (110,153,208). Similarly, experimental infection of pregnant hamsters results in death of the fetus (4).

Epidemiology and Ecology

California Serogroup Viruses

La Crosse virus is the most significant of the California encephalitis serogroup viruses in terms of causing human disease

in the United States. The primary vector is the forest-dwelling, tree-hole breeding mosquito, *A. triseriatus.* This vector is found throughout the northern Midwest and northeastern United States. La Crosse virus is maintained in these mosquitoes by transovarial transmission, which allows overwintering of the virus in mosquito eggs (297). During the summer months, squirrels, chipmunks, foxes, and woodchucks become viremic following La Crosse virus infection and are important amplifying hosts (279,312). Most La Crosse encephalitis cases occur in the summer and early fall months when risk of bite from infected female mosquitoes is highest. Most cases are in the Mississippi and Ohio river basins, with more than 90% of cases occurring in Wisconsin, Minnesota, Iowa, Indiana, Ohio, and Illinois (31,135). During the 1980s, several cases were also reported in West Virginia and Georgia (249). Cases have now been reported from 28 states, and an incidence in endemic areas is about 20 to 30 per 100,000, exceeding that of bacterial meningitis (175). Cases are usually seen in residents in proximity to hardwood forests or in people entering forested areas for recreation (111). The breeding of *A. triseriatus* in discarded tires and other artificial containers has brought the vector into more urban settings (182).

Jamestown Canyon virus (including the closely related Jerry Slough variety) is also an important cause of arboviral encephalitis in the United States. The virus is vectored by *C. inornata* mosquitoes and several *Aedes* species mosquitoes and is broadly distributed across much of North America. Vertical transmission of the virus has been demonstrated in several *Aedes* species mosquitoes (109). White-tailed deer are the most likely vertebrate amplifying host. Unlike La Crosse virus, which mainly causes encephalitis in children, Jamestown Canyon virus appears to cause disease predominantly in adults. Together, the California encephalitis serogroup viruses are responsible for about 60 to 130 reported cases of encephalitis per year, although there is likely considerable underreporting of the disease.

Bunyamwera Serogroup Viruses

Bunyamwera virus is present throughout much of sub-Saharan Africa and appears to be an important cause of acute febrile illness in humans. The virus has been isolated from humans in Uganda, Nigeria, and South Africa (100,143,278). More recently, a reassortant of Bunyamwera virus has been isolated from hemorrhagic fever patients in Somalia and Kenya (S.T. Nichol and colleagues, unpublished observations). In addition, human antibodies reactive with Bunyamwera virus have been detected in most of sub-Saharan Africa, with high prevalence (up to 82%) recorded in some locations (253). Isolation of the virus from several *Aedes* species mosquitoes has implicated them as the primary vector (143,144,253). Antibodies reactive with Bunyamwera virus have been detected in domestic animals, nonhuman primates, rodents, and birds, and viremias capable of supporting mosquito transmission have been recorded in experimentally infected rodents, bats, and primates (253,301). However, the role of a potential vertebrate amplifying host is currently unclear.

Several additional Bunyamwera serogroup viruses are present in the Americas and are infrequently reported to cause acute febrile illness in humans. Cache Valley virus is found throughout the United States, Canada, and Mexico and has most frequently been isolated from *C. inornata* mosquitoes. The virus often infects sheep and possibly all ruminants, and infections have been associated with embryonic and fetal death, stillbirths, and multiple congenital malformations in sheep (80,174). More recently, the virus was the cause of a fatal encephalitis in a human (241). It remains unclear whether Cache Valley and serologically closely related viruses may play a role in syndromes of congenital malformations and embryonic losses in humans in North America (34).

Simbu Serogroup Viruses

Simbu serogroup viruses are global in distribution and are principally vectored by biting midges of the genus *Culicoides.* Since the original isolation of Oropouche virus from a febrile patient in Trinidad in the 1950s, the medical importance of the virus, particularly in the Amazon basin regions of northern Brazil and Peru, has become increasingly evident (219). The virus has been shown to be associated with several large epidemics of febrile disease, with attack rates as high as 83% (219,229). Most of these outbreaks were in relatively urban areas, leading to the suggestion that there is likely separate urban and sylvatic cycles (71,227). High antibody prevalence in populations in endemic areas suggest that human infection can be quite common in such areas (15,229,296).

The principal urban vector in Brazil appears to be the tiny biting midge, *Culicoides paraensis* (216,227). Isolates have also been made from mosquitoes such as *Culex pipiens quinquefasciatus,* but its vector competence is poor (71,116). The *C. paraensis* midges breed in rotting vegetative matter, and seasonal populations can become high in agricultural areas where buildup of debris, such as banana tree stalks or cacao husks, may occur (218). These midges feed predominantly in the early evening hours and are quite anthropophilic. Infected humans develop viremias sufficiently high to transmit virus to uninfected midges and appear capable of serving as the vertebrate amplifying host during urban epidemics (218). Serologic data also suggest that primates, sloths, or even birds may be potential hosts in the sylvatic cycle. Recent phylogenetic studies indicate that distinct Oropouche virus lineages are present in different geographic areas, with lineage I viruses including the prototype strain from Trinidad and most of the Brazilian strains, with lineage II containing six Peruvian strains isolated between 1992 and 1998 and two strains from western Brazil isolated in 1991, and with lineage III containing viruses isolated in Panama during 1989 (234).

Akabane virus is widely distributed throughout Australasia (Australia, Japan, Korea, Taiwan), the Middle East (occasionally extending as far north as Turkey), and sub-Saharan Africa (2,64,183,271,299). The virus is an important cause of disease in livestock with periodic outbreaks of abortions, stillbirths, and congenital malformations being recorded in cattle, sheep, and goats in Australia, Japan, Korea, Israel, and Turkey. It is currently unclear why no disease outbreaks have been observed in Africa, despite the widespread presence of the virus (2).

The virus is vectored by midges of the genus *Culicoides,* and although *C. brevitarsis* is the primary vector in Australia (195,260), different species are important in Asia and the Middle East (3,131,151). These midges have short flight ranges, but wind dispersal is thought to play a role in movement of infected *Culicoides* over substantial distances, occasionally resulting in disease epizootics in areas where the disease is not enzootic (20,24,195,239,240).

Clinical Features

The California serogroup viruses, La Crosse, Jamestown Canyon, and the prototype California encephalitis virus, all cause a similar encephalitic disease in humans (230). Indeed, because of serologic cross-reactivity, many of the early disease descriptions of California encephalitis cases were likely due to Jamestown Canyon or La Crosse viruses rather than California encephalitis virus, which is now known to be less common (36,70,259). The major difference between the viruses is in age dependence of the spectrum of clinical disease observed, in that La Crosse virus infections tend to be more severe in children, whereas Jamestown Canyon virus infection is more severe in adults. The spectrum of disease ranges from inapparent or mild febrile disease through to fatal encephalitis (57,175,281).

The incubation period is about 3 to 7 days. Most affected patients report sudden onset of fever, followed by stiff neck, lethargy, headache, and nausea and vomiting, and symptoms usually end within 7 days. About half of patients exhibit seizures, and up to 30% develop coma and exhibit a longer disease course. About 65% of patients have meningitis on presentation, with both mononuclear and polymorphonuclear cells present in cerebrospinal fluid. Despite mild neurologic symptoms often being present at the time of patient discharge, surprisingly little residua is found. The most important sequela is epilepsy, which is observed in about 10% to 15% of children, almost always patients who had seizures during the acute phase of illness. In addition, about 2% of patients have persistent paresis (56,57,103,107,175).

Several thousand cases of acute febrile illness can occur during Oropouche virus epidemics observed throughout the Amazon basin regions of Brazil and Peru. Humans acquire the infection by bite from infected midges. The incubation period is about 4 to 8 days, followed by sudden onset of fever, with arthralgia, myalgia, severe headache, chills, photophobia, and prostration. Occasionally, rash, meningitis, or meningismus is seen (217). Most of the symptoms resolve within 3 to 5 days, although the myalgia may last 1 or 2 weeks. Viremias are detectable in most patients 2 to 3 days after onset of illness. Rather unusually, about half of patients exhibit recurrence of some disease symptoms 1 to 10 days after initial recovery (291). Oropouche virus is also suspected to be infectious by aerosol based on reported laboratory infections (219).

Diagnosis

A wide variety of serologic assays have been used in the diagnosis of infections with viruses of the genus *Bunyavirus,* including complement fixation (CF), hemagglutination inhibition (HI), neutralization (NT), immunoglobulin M (IgM) capture enzyme-linked immunosorbent assay (ELISA), and reverse transcriptase–polymerase chain reaction (RT-PCR) (32,46,73,105,150,241). Diagnosis of California serogroup virus infections relies on serologic methods because virus is generally absent from blood or secretions during the phase of CNS disease. The HI test is considerably more sensitive for these viruses than CF, but NT assay or RT-PCR is needed for confirmation and subtype identification. The IgM capture ELISA works well for diagnosis of most infections by viruses of the genus *Bunyavirus* but has not been widely applied to date (73,127,241). Virus isolation attempts complement serologic and genetic assays, and isolation is best achieved by intracranial inoculation of suckling mice or by infection of susceptible cells, such as Vero cells.

Prevention and Control

Prevention and control are difficult for the mosquito-borne California and bunyamwera serogroup viruses because of the widespread presence of the mosquito vector hosts and relatively sporadic nature of the associated human or livestock diseases. Broad application of insecticides as carried out in earlier decades is no longer ecologically acceptable. Relative to human disease, use of fine mesh netting and personal protectants containing diethyltoluamide (DEET) are highly recommended in areas where people are at high risk of being bitten by virus-infected mosquitoes.

Similar vector control challenges exist for the Simbu serogroup viruses vectored by *Culicoides* species midges. With Oropouche virus disease in Brazil and Peru, avoiding the buildup of rotting organic debris, such as banana tree stalks and cacao husks, in agricultural areas may help curtail population levels of *C. paraensis* and reduce the risk of seasonal epidemics (218). Avoiding exposure (treated netting, DEET repellents) to these midges during their early evening feeding hours is also recommended. Similar protective measures are recommended for Akabane virus. In addition, a successful formalin-inactivated Akabane virus vaccine has been developed in Japan using virus-infected hamster lung cell cultures (152), and a similar product is planned in Australia. With knowledge of the distribution and seasonality of Akabane disease outbreaks, vaccination of at-risk animals before pregnancy is feasible.

PHLEBOVIRUS GENUS

The viruses of the genus *Phlebovirus* are present throughout the world, with the exception of Australia, and are more diverse in terms of arthropod vector than those of the other arthropod-borne genera. Most virus members are associated with phlebotomine sandflies, hence the genus name *Phlebovirus* (see Table 1). However, there are prominent exceptions, such Rift Valley fever (RVF) virus, a medically and agriculturally important virus in Africa, which is primarily associated with *Aedes* species mosquitoes. In addition, Uukuniemi virus, which has been used in a number of laboratory studies, is associated with the tick *Ixodes ricinus.*

History

RVF virus was first isolated in 1930 by Daubney and colleagues from an infected newborn lamb, as part of investigation of a large epizootic of disease causing abortion and high mortality in sheep (66). Large RVF epizootics in various areas of sub-Saharan Africa have been noted since that time, and clinically compatible outbreaks have been retrospectively identified as far back as 1912 (78). It was several decades later before serologic and molecular similarities to phlebotomine fever viruses were noted. Sandfly fever Sicilian and Naples viruses were first isolated from American troops with febrile illness in the Palermo region of Sicily, Italy in 1943 and Naples, Italy in 1944, respectively, by inoculation of acute-phase sera into human volunteers and passage in newborn mice (231,232). Outbreaks of compatible

human illness in the Mediterranean region date back to the time of the Napoleonic Wars, and association of the disease with sandflies was suggested as early as 1905 (112).

Infectious Agents

The classic general method for isolation of phleboviruses is either intracranial inoculation of suckling mice or infection of a susceptible cell line, such as Vero, LLC-MK2, or BHK-21 cells. Sandfly fever Sicilian and Naples viruses usually cause CPE in susceptible cells, but often blind intracranial passage is necessary before a lethal infection of the suckling mice is achieved (232,233). RVF viruses are generally cytopathic, grow well in a wide range of tissue culture cells, and result in fatal infection of mice after intracranial challenge. Thin-section electron micrographs show viruses of the *Phlebovirus* genus to have a typical overall morphology with predominantly spherical or ovoid particles of about 90 to 100 nm in diameter with surface fringe projections of about 6 to 7.5 nm. These viruses are distinct when viewed by negative staining, with sharply defined surface structures of small round morphologic units about 9.5 nm in diameter with a visible central hole (173) (Fig. 3).

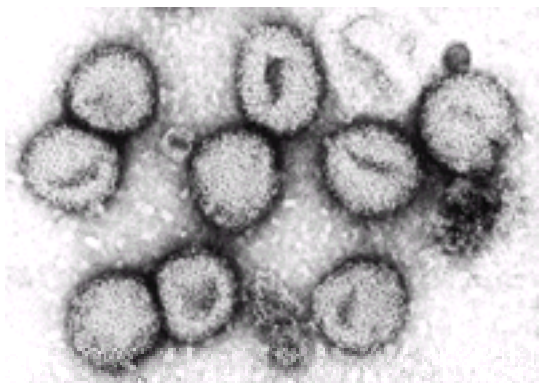

FIG. 3. Electron micrograph negative-stain image of Uukuniemi virus (genus *Phlebovirus*) (135,000× magnification). (Courtesy of Mary Lane Martin and Helen L. Regnery.)

Pathogenesis and Pathology

Several excellent reviews of RVF pathogenesis and disease exist (66,75,270). RVF virus infections of livestock are often recognized by the onset of abortion “storms,” which sweep through livestock producing areas, with simultaneous acute febrile disease in humans. Most human infections with RVF virus result in a relatively mild febrile illness. The incubation period is about 2 to 6 days and is followed by abrupt onset of fever, chills, and general malaise. Susceptibility of livestock to RVF infection varies considerably, depending on a variety of factors, including livestock species and age and strain of virus. Based on data from experimental infections and analogy with other arbovirus infections, the general pattern of infection likely follows the course that, after inoculation of virus by mosquito bite, virus is transported by lymphatic drainage to the regional lymph node where local replication takes place. Virus spills over into the circulation, causing the primary viremia and spread of the virus to the major organs. Replication in the lymph nodes, spleen, liver, adrenals, and lung and kidney tissues results in high viremias; in severe cases, hepatic necrosis is prominent, and necrotic foci can be observed in the brains of cases exhibiting the less frequent encephalitic form of the disease (6,187,286). RVF virus replication in cells is highly cytotoxic, suggesting that most cellular destruction in acute illness is likely due to direct virus killing of host cells.

Pathologic features of the disease in livestock varies considerably. Leukopenia is frequently seen during the first 3 or 4 days of infection when fever and viremia are usually at their highest. Altered serum enzyme levels (e.g., aspartate aminotransferase and sorbitol dehydrogenases) indicative of hepatocyte destruction are often seen during the acute phase. Leukocytosis often occurs in the early phase of recovery. Thrombocytopenia and fibrin thrombi in several organs suggest that disseminated intravascular coagulopathy (DIC) may be a feature of severe disease in livestock, as is seen in hemorrhagic infections in humans (62,268).

Infection of pregnant animals frequently results in abortion of the fetus. Abortions early in the course of infection are probably the result of the high fever associated with the acute phase of illness; later abortions are more commonly the result of direct infection of the fetus with resulting hepatic necrosis (270). No clear correlation between RVF infection and abortion has been seen in human infections (179).

Epidemiology and Ecology

The geographic distribution of sandfly fever Sicilian and Naples viruses closely follows that of their sandfly host, *Phlebotomus papatasii* (102,238,273). This distribution extends from the Mediterranean basin throughout the Middle East and Arabian peninsula, north up into areas around the Caucus mountains, and as far east as Pakistan and India. Sandflies are most numerous in the warmer months, are found at ground level, and feed in the early evenings. Unfortunately, their small size allows them to pass through untreated mosquito netting.

A related phlebovirus, Toscana virus, is hosted by *P. perniciocus* and is found in central Italy, Cyprus, Portugal, and Spain. Relatively high (20% to 25%) antibody prevalence rates suggest that human infections with Toscana virus are widespread and frequent in endemic areas (23,81). In addition, many acute lymphocytic meningitis and meningoencephalitis cases, particularly those in children, occurring in the summer months are attributable to Toscana infection (21,22,81,180,202). These viruses replicate in their sandfly hosts, and virus transstadial and transovarial transmission have been demonstrated (16,58,275,277). Sexual transmission of Toscana virus among sandflies has also

been shown (276). The relatively low efficiency of transovarial transmission demonstrated in experimental infections suggests that vertebrate amplifying hosts are required to maintain these viruses in endemic areas. However, such wildlife hosts have not yet been identified. Data showing that viremic humans can infect *P. papatasii*, together with attack rates as high as 75%, and records of urban outbreaks of sandfly fever suggest that humans can on occasion serve as the amplifying vertebrate host (106,232).

The ecology and epidemiology of RVF is complex and poorly understood. The geographic distribution of RVF covers much of Africa, from Senegal to Madagascar and from Egypt to South Africa, with most reported epizootics in livestock being reported in East and Southern Africa (270). Beginning in fall 2000, the presence of RVF in the Arabian peninsula was confirmed for the first time, with large epizootics identified in Saudia Arabia and Yeman (T.G. Ksiazek and colleagues, unpublished observations). Mosquitoes have long been known to play an important role in RVF epizootics, with epizootics frequently occurring at times of unusually high precipitation (167). More recent data have indicated that floodwater *Aedes* species mosquitoes of the subgenera *Aedimorphus* and *Neomelaniconion* are likely the principal vectors. The ecology of RVF has been best studied in Kenya, Zimbabwe, and South Africa. In these regions, damboes or vleis, which are shallow depressions up to several hundred meters across near streams and fed by ground water, are thought to play a central role (264). These damboes flood at times of unusually heavy rainfall, which triggers a population explosion in floodwater *Aedes* species mosquitoes.

Isolation of RVF virus from unfed male and female *Aedes mcintoshi* mosquitoes hatched at a dambo in the endemic area of Kenya during an interepidemic period demonstrated the maintenance of the virus between epidemics and the transovarial transmission of the virus in such mosquitoes (166). *A. mcintoshi* is thought to be the principal maintenance vector in Kenya and Zimbabwe (270). The biology of these mosquitoes is such that they are among the earliest mosquitoes to hatch from eggs after flooding. If the eggs contain virus (by transovarial transmission), infected mosquitoes hatch and feed on nearby livestock, potentially initiating a local epizootic. Viremic livestock act as amplifying hosts, transmitting the virus to other floodwater *Aedes* species mosquitoes as well as to other species of mosquitoes, including culicines and anophelines, promoting further amplification and spread of the virus. When such a cycle is initiated at damboes throughout a livestock-producing area, it can give rise to a regional, relatively synchronous eruption of RVF activity with associated abortion storms such as those observed in livestock at various intervals in East and Southern Africa (212,264). Although such a scenario is an attractive explanation of the dynamics of RVF epizootics, more data are necessary to confirm it.

Hemorrhagic fever cases in humans are usually seen 1 or 2 weeks after the appearance of abortions and disease in livestock. Cases are usually among farmers and others living in close proximity to livestock. Humans likely acquire infection through a bite from infected mosquitoes when vector densities are very high. However, contact transmission appears to be most important with RVF. People involved in the birth or abortion of livestock, butchering of animals, or abattoir workers are at high risk of infection during epizootics (1,44,306). RVF virus is also highly infectious by aerosol, having resulted in many infections of laboratory personnel working with the virus (91,255). The relative contribution of infectious aerosols or fomites to transmission is unclear. Although less than 1% of human infections result in severe disease (hemorrhagic fever, encephalitis, retinitis), this can add up to a substantial number of cases given the large scale of some epizootics. For instance, an estimated 27,500 RVF infections occurred during the recent 1997 and 1998 RVF outbreak in the Garissa district of Kenya (308), and about 200,000 human cases with 600 deaths were estimated to have occurred during the RVF outbreak in Egypt in 1977 and 1978 (154,177).

In contrast to the long history of periodic RVF epizootics in East and Southern Africa, the relatively recent large RVF outbreaks in Egypt in 1977 and 1978 and in West Africa in Mauritania and Senegal in 1987 are quite different situations (317). In Egypt, retrospective studies showed that RVF had not been enzootic before 1977 (178). Virus activity disappeared after 1981, only to be reintroduced in 1993 (10). These data suggest that although mosquitoes capable of acting as epizootic vectors (e.g., *Culex* species) exist in Egypt, mosquitoes capable of RVF transovarial transmission are probably lacking. It is thought that RVF was most likely introduced into Egypt from enzootic areas in Sudan (177,212).

The 1977 outbreak of RVF near the Senegal river was the first confirmed outbreak in West Africa. This is thought to have been precipitated by dams built on the river, which caused flooding and increased mosquito populations. Unlike in Egypt, antibody surveys in West Africa suggested that RVF was present earlier than 1977, and activity continues in various areas of the region. Similar to the situation in East and Southern Africa, flood water *Aedes* species mosquitoes (of the subgenus *Aedimorphus* in West Africa, as opposed to the subgenus *Neomelaniconion* in East Africa) are likely the enzootic vector (89,317), and continuing epizootics may be linked to unusual increases in precipitation (282).

Clinical Features

Human infection with sandfly fever virus results in an acute febrile illness with rapid onset. The incubation period lasts 2 to 6 days, followed by fever and general malaise, often accompanied by headache, photophobia, and back and joint pain (17,232). The disease is self-limiting, lasting 2 to 4 days, before rapid and complete recovery.

Experimental RVF infection of new-born lambs and kids suggest that the incubation period is usually 24 to 36 hours, but it can be as short as 12 hours (75,76 and 77). Fever, which is often biphasic, is accompanied by listlessness, lack of appetite, abdominal pain, increased respiration rate, and lack of movement. Mortality rates of greater than 90% can be seen in animals less than 1 or 2 weeks of age, with animals rarely surviving more than 24 to 36 hours after onset of illness. Older lambs and kids and adult sheep and goats are often less susceptible to RVF infection, with disease ranging from peracute to inapparent infection. Most older animals develop a febrile acute illness similar to the disease seen in 1- to 2-week-old animals, but with morality rates ranging from 5% to 60%, and a longer incubation period, ranging from 1 to 6 days. High and prolonged viremias, widespread tissue damage, vasculitis, and hepatic necrosis are seen in the more severe cases. Abortions in pregnant animals are frequently observed, with rates varying from 40% to 100%.

Human infections rarely lead to severe illness, with most infections resulting in a self-limiting influenza-like illness. About 0.5% of infections, however, lead to severe hemorrhagic fever. In such cases, it is likely that the reduction of the

antithrombotic function of the endothelial cells initiates intravascular coagulation and extensive necrosis of hepatocytes and other infected cells. The release of procoagulants into the circulation, together with the extensive liver damage that severely impairs synthesis of coagulation factors and removal of circulating activated coagulation factors are likely important factors resulting in DIC in the hemorrhagic fever cases (210,214).

In less than 0.5% of human RVF infections, retinal vasculitis or encephalitis can be observed 1 to 4 weeks after recovery from the acute illness. Encephalitis begins with headache, meningismus, and confusion. Often, fever reappears, and symptoms of severe cases include hallucinations, stupor, coma, and death. Focal necrosis is seen in the brain, which likely involves direct cell destruction by the virus. In addition, the onset of encephalitis after viremia has ceased and after RVF-specific antibodies are readily detectable suggests there may be an immunopathologic component to these late complications (213). Indeed, polymorphonuclear and mononuclear cell infiltration associated with necrotic lesions implicates delayed hypersensitivity or cytotoxic T cells (213).

Diagnosis

Sandfly fever should be suspected when patients present with a classic “3-day fever” in areas where phlebotomine sandflies are numerous (81,231,232 and 233). Similarly, Toscana infection should be suspected in patients from rural or semirural areas where phlebotomine sandflies are present, who are suffering from acute CNS disease during the summer months (23,202). Diagnostic confirmation using acute or early convalescent-phase samples is readily achieved by virus isolation by intracranial inoculation of suckling mouse or susceptible tissue culture cells or by IgM capture ELISA.

Differential clinical diagnosis of RVF varies depending on the region in question. The disease should be suspected in RVF endemic regions, following abnormally high precipitation, increased rates of abortion in livestock, and appearance of acute influenza-like illness in individuals in close contact with potentially infected livestock (e.g., farmers, veterinarians, abattoir workers). Virologic diagnosis is usually quite simple given the high viremias present throughout the acute phase of illness and the ease of growth of the virus when inoculated intracranially into suckling mice, or in a wide variety of susceptible tissue culture cells (270). Serologic testing is also straightforward, particularly if paired sera (one taken acutely and the other 1 or 2 weeks later) are available. IgM and IgG ELISA tests using inactivated RVF virus–infected cell lysates or slurries have proved particularly useful in outbreak investigations (189,308).

Prevention and Control

The sandfly hosts of sandfly fever Naples and Sicilian and Toscana viruses have short flight range, and locomotion is often by hopping, making localized use of insecticide sprays particularly effective near residences. The use of repellants and treated bed nets and screens is also recommended in such areas.

Immunization of livestock remains the most effective way to prevent RVF epizootics and human cases. Several vaccines exist. Live attenuated viruses based on the mouse neuroadapted Smithburn RVF strain are used in Kenya and South Africa (37,254). Formalin-inactivated wild-type viruses have also been used in Egypt and South Africa (82). The modified live Smithburn vaccine has good efficacy in sheep (but not cattle), with a single dose inducing lasting immunity in sheep 6 to 7 days after vaccination (61). However, it appears to be only partially attenuated, causing abortions or teratology in some pregnant animals (11). The formalin-inactivated vaccines are safer but are expensive and induce only short-lived immunity in sheep and cattle. The main livestock vaccination problem is the long periods between epizootics and their irregular appearance, which makes it difficult to convince livestock owners to vaccinate on a regular basis. Recent advances in the use of satellite imagery and surface ocean temperatures to predict regions at higher than usual risk of RVF activity may allow the timely vaccination of animals before potential epizootics (167).

In an African setting, humans are probably best protected by vaccination of livestock to prevent amplification of virus, thus limiting human exposure and epizootic potential. Formalin-inactivated RVF vaccines have been developed for immunization of laboratory and field workers at risk of exposure (176,223) but are unlikely to be used on a larger scale, given their limited commercial potential. New-generation live attenuated RVF vaccine strains (MP-12 and clone 13) are also currently under consideration (188,193,235). RVF virus is highly infectious by aerosol, as shown by numerous infections of laboratory personnel (91,255). Workers at risk of exposure wear protective clothing to avoid contact with potentially infectious materials and aerosols. The antiviral drug ribavirin may be of some benefit in the treatment of sandfly fevers and RVF, although human efficacy data are still lacking (63,211,298).

NAIROVIRUS GENUS

The nairoviruses are almost exclusively tick-borne viruses, although a few isolations have been made from culicoides flies and mosquitoes (see Table 1). Several serogroups exist, but the most important ones are the Crimean-Congo hemorrhagic fever group (CCHF), which includes CCHF and Hazara viruses, and the Nairobi sheep disease (NSD) group, which includes NSD and Dugbe viruses (see Table 1).

History

The *Nairovirus* genus was named after NSD virus, which was originally isolated in Nairobi, Kenya in 1910 by inoculation of sheep with the blood of sheep with acute gastroenteritis (185). The virus is now known to be present throughout various parts of Africa, and possibly India. CCHF was first recognized in the Crimean peninsula in the mid-1940s, when a large outbreak of severe hemorrhagic fever among agricultural workers was identified. The outbreak included more than 200 cases and a case fatality of about 10% (54). Similar disease cases were later reported throughout the European and central Asian republics of the former Soviet Union, Romania, and Bulgaria. However, the virus was first isolated from a patient with a 1-day fever in Kisangani, Democratic Republic of Congo, in 1956 (250,307). It was some years before the connection was established, but subsequent serologic studies and virus isolations from Asia and Europe revealed that the viruses from the different outbreaks and geographic regions were essentially the same virus, which then became

named CCHF virus (40,55).

Infectious Agents

The classic method for isolation of CCHF viruses has been by intracranial or intraperitoneal (i.p.) inoculation of patient acute-phase blood samples or ground tick pools into newborn mice. Isolation in tissue culture cells is generally less sensitive but provides a faster result in terms of diagnosis (244). Susceptible tissue culture cells include LLC-MK2, Vero, and BHK-21 cells, and SW-13 cells are particularly useful for plaque assay (298). Maximal virus yields (up to 10^7 to 10^8 plaque-forming units per milliliter) are obtained 4 to 7 days after inoculation. The virus frequently produces little CPE and often results in a noncytopathic persistent infection of the cells. Initial isolations of NSD virus were by subcutaneous or intravenous inoculations into sheep. More routinely, similar methods to those used for CCHF have been used, namely intracranial or i.p. inoculation of newborn mice. NSD virus will also grow in a wide variety of tissue culture cells with production of large cytoplasmic inclusions. Thin-section electron micrographs show viruses of the *Nairovirus* genus to have an overall morphology typical of other viruses of the family *Bunyaviridae,* forming predominantly spherical particles about 90 nm in diameter with an indistinct surface fringe of about 7 nm. These viruses, however, are quite distinct when viewed by negative staining, possessing very small morphologic surface units with no central hole (unlike the phleboviruses) (173).

The genome of these viruses consists of three negative-stranded RNA segments, S, M and L, encoding the N nucleocapsid, G1 and G2 glycoproteins, and L polymerase, respectively (171,172; M.D. Parker and colleagues), with the N, G1, and G2 representing the major antigens. Animals susceptible to experimental infection with CCHF virus include newborn mice, newborn white rats and cotton rats, guinea pigs, rabbits, rhesus monkeys, sheep, donkeys, and calves. Mice, sheep, and goats can be experimentally infected with NSD virus.

Pathogenesis and Pathology

Vertebrate Hosts

CCHF and NSD viruses are maintained in nature predominantly in their respective tick hosts. Although the viruses can persist in the ticks through the various life stages (transstadial transmission) and can be passed to the offspring (transovarial transmission), vertebrates are needed to provide blood meals for the ticks, and they can become infected and develop viremias capable of supporting virus transmission to uninfected ticks (69,98,165,168,246). In this manner, vertebrates may play an important amplifying role in the virus natural cycle. In the case of CCHF, a variety of livestock (sheep, goats, cattle, ostriches), large wild herbivores, hares, and hedgehogs may become infected, resulting in an inapparent or subclinical disease (118,245,247,267). Sheep and goats are implicated as being important vertebrate hosts of NSD virus and infection often results in severe disease (67). These animals are presumably subcutaneously infected when bitten by infected ticks. In experimental CCHF virus infections, such as may occur in sheep and ostriches, a viremia develops 1 to 4 and 3 to 9 days after infection, respectively, with maximal titers of about 10^3 to 10^4 mouse intracerebral median lethal dose (LD^{50}) per milliliter (99,266). Virus can be detected in the visceral organs, such as the spleen, liver, and kidneys, up to 5 to 10 days after infection, and CCHF-specific antibody responses are detectable about 7 to 10 days after infection.

In experimental NSD virus infections of sheep, goats, and suckling mice, virus replicates to high titers in the lung, liver, spleen, and other organs of the reticuloendothelial system (192). Vascular endothelium appears to be the primary cell target. Virus replication in the endothelial cells results in edema and necrosis of the capillary walls of the mucosal surfaces of the intestine, gallbladder, and female genital tract, leading to congestion, hemorrhage, and catarrhal inflammation.

Human Infections

In contrast to the inapparent infection characteristic of CCHF infection of other vertebrate hosts, human infections often result in severe hemorrhagic fevers. On introduction of the virus, there is likely local replication followed by blood- and lymph-borne spread of the virus to the major organs, including liver, where high levels of replication take place (30,265,267). Congestion, edema, and focal hemorrhage and necrosis are seen in most organs. DIC is evident early, with thrombocytopenia, elevated prothrombin ratio, and activated partial thromboplastin time present during the first few days of illness, and likely plays a central role in disease progression. Little is known regarding host cell receptor use or factors influencing virus virulence. Both IgM and IgG antibodies are usually detectable about 7 days after onset of illness and are detectable in all survivors by day 9 (243). Antibody responses are rarely detectable in fatal cases.

NSD virus has been isolated from sick patients in Uganda, and the identical or closely related Ganjam virus from febrile patients in India, but their importance in terms of human disease is unclear.

Epidemiology and Ecology

The epidemiology of CCHF reflects the complex ecology and geographic distribution of the ixodid tick hosts of the virus, particularly those of the genus *Hyalomma* (118,267,269). Although CCHF has been isolated from about 30 different tick species, evidence is lacking for many of these as to whether they truly represent vectors or merely reflect the isolation of virus from engorged ticks that have fed on a viremic vertebrate host. Only some ticks of three genera, *Hyalomma, Dermacentor,* and *Rhipicephalus,* have actually been shown to be capable of transstadial transmission of CCHF after feeding on a viremic host, that is, passing the virus through the various stages of the life cycle, from larva to nymph to adult. Transovarial transmission of CCHF has also been shown to occur with some of the tick species in these genera. Moreover, the overall global pattern of geographic distribution of CCHF cases corresponds most closely with the distribution of *Hyalomma* ticks, suggesting their principal role as vector.

There is evidence that CCHF virus is present throughout much of sub-Saharan Africa (from Egypt to South Africa, and

from Senegal to Madagascar), Bulgaria, former Yugoslavia, northern Greece, European Russia, the Arabian peninsula, Iraq, Pakistan, the former Soviet Union (particularly the former Central Asian republics), and Xinjiang province of Northwest China.

Different tick species or subspecies are likely the principal vector in different regions. For instance, *Hyaloma marginatum marginatum* is thought to be an important vector for the virus in Bulgaria, Greece, and European Russia. This is a two-host tick; the larva and nymphs develop on birds or small mammals such as hares and hedgehogs, but the adults feed on large vertebrates such as livestock or occasionally humans. Amplifying viremias occur in the hares and hedgehogs and also in livestock such as sheep and goats, likely making them an important component of the virus natural life cycle. Although some species of ground-feeding birds are susceptible to CCHF infection, several are not, but these may play an important role in providing virus-infected immature ticks with a blood meal and in their dispersal over local and large distances (118,315,316). In contrast, *H. marginatum rufipes* and *H. marginatum turanicum* are two of the more important vectors in West and Southern Africa (35,269); *H. anatolicum anatolicum* in the former Central Asian Soviet Republics and *H. asiaticum* in China (310). However, other ticks may play significant roles. Each tick species has different host preferences, which are reflected in the local epidemiology of the disease.

Most human infections are acquired by bite from infected ticks, contact with infected ticks during their removal from animals or human body, or contact with blood or tissues of infected livestock. As one would expect, most cases are among humans living or working in close contact with livestock (sheep, goats, cattle, or ostriches) in endemic areas, including shepherds, ranchers, and abattoir workers. Nosocomial outbreaks have also been reported in several regions, with infection of medical personnel after treatment of or surgery on a patient with unsuspected CCHF.

The distribution of NSD virus mirrors the distribution of the principal tick host, *Rhipicephalus appendiculatus.* The virus is present throughout much of Africa; from as far north as Ethiopia and Somalia, extending through much of East and Central Africa, to as far south as the Mozambique coastal plain (68,79). The distribution likely extends through parts of the Middle East and into India, based on the detection of the identical or closely related Ganjam virus from *Haemaphysalis intermedia* ticks (65).

Clinical Features

The incubation period for CCHF infections is commonly 3 to 7 days but varies depending on type of exposure (265). Symptoms usually appear abruptly and can include severe headache, dizziness, nausea, fever, neck and abdominal pain, and chills. Overall myalgia and malaise occurs, and hepatomegaly may be seen. Hemorrhage frequently appears by 3 to 6 days after onset of illness, with a petechial rash observable on the trunk and limbs. Patients are often apathetic or obtunded by this stage, with delirium or coma occurring in the more severe cases. Blood may be seen in the urine, and bleeding from the nose, gums, vagina, and other mucosal surfaces or needle puncture sites may occur about 5 days after onset. The case fatality is about 30%, with most deaths occurring 5 to 14 days after onset of illness (269).

Diagnosis

The clinical differential diagnosis of CCHF varies in the different regions where the disease is known to occur and can be difficult in the early stages. In general, the disease can be suspected in patients presenting with a rapid onset of severe influenza-like symptoms and known exposure to tick bite or contact with blood or tissues of potentially infected livestock or humans. The early stages can be confused with sandfly fevers in regions where the diseases overlap; and leptospirosis, Omsk hemorrhagic fever, other viral hemorrhagic fevers (yellow fever, hemorrhagic fever with renal syndrome [HFRS], Ebola), tick typhus, and mycotoxicoses can appear similar in the more advanced hemorrhagic phase of the illness. Clinical values can be helpful in diagnosis, with leukopenia and thrombocytopenia both frequently being present early in the disease (265,289).

Laboratory diagnosis of CCHF is ideally done by virus isolation or antigen-detection ELISA from patient acute-phase sera (28,139,228,304). Virus and antigen are usually detectable up to 1 to 2 weeks after onset of illness. For CCHF isolation, intracranial or i.p. inoculation of patient acute-phase blood samples into newborn mice is probably most sensitive, although quicker results can be obtained by inoculation into susceptible tissue culture cells, such as LLC-MK2, Vero, and BHK-21 cells, followed by fluorescent antibody (FA) staining (244). Fatal cases rarely show significant antibody responses; however, in those destined to survive, IgM and IgG antibodies are frequently detectable after about 1 week of illness. RT-PCR analysis has proved a useful complement to serodiagnosis (29,228).

Prevention and Control

The best means of prevention is to minimize exposure to the virus. Treatment of clothing with pyrethroid preparations that repel and even kill ticks is highly recommended for those likely to come in contact with CCHF-infected ticks, such as slaughterhouse workers, sheep shearers, veterinarians, and others involved with livestock. Also, habits that avoid virus-contaminated blood or tissues contacting the skin should be practiced, including wearing of gloves and avoiding crushing ticks with fingers. Medical personnel should practice standard barrier-nursing techniques during care of suspected CCHF patients. Formalin-inactivated vaccines have been prepared from virus-infected suckling mouse brains and used in Bulgaria and other parts of Eastern Europe and the former Soviet Union, but their efficacy is unclear (284,292).

Treatment of patients is by means of supportive and replacement therapy with blood products. Administration of immune plasma has been tried on numerous occasions, but its usefulness remains unclear (163,290). Based on the susceptibility of the virus in experimental tissue culture and mouse infections to the antiviral drug ribavirin (283,298), several CCHF cases have been treated with ribavirin, with encouraging results (88).

HANTAVIRUS GENUS

History

The discovery of hantaviruses traces back to 1951 to 1953 when United Nations troops were deployed during the border conflict between North and South Korea. More than 3,000 cases of an acute febrile illness were seen among the troops, about one third of which exhibited hemorrhagic manifestations, and an overall mortality of 5% to 10% was seen (74,158,252). The disease was initially termed *Korean hemorrhagic fever* but is now referred to as HFRS. A typical case would exhibit influenza-like symptoms for about 1 week, followed quickly by a hypotensive phase marked by thrombocytopenia, often with petechial hemorrhage, and shock in about 10% to 15% of patients. Fatalities were primarily due to renal failure. A similar disease (epidemic hemorrhagic fever) had been recognized many years earlier in northeastern China (157) and had also been described in eastern former Soviet Union (41). Despite considerable efforts, it took about 25 years until the field mouse, *Apodemus agrarius,* was identified as the rodent reservoir, and the virus was eventually isolated. At that time, all identified viruses of the family *Bunyaviridae* were known to be arthropod-borne viruses. Thus, it was a big surprise when this rodent-borne virus was shown to share characteristics of viruses of the family *Bunyaviridae.* The virus was named *Hantaan virus* after the Hantaan river close to the location of some of the initial Korean cases and became the prototype of the *Hantavirus* genus (157). During the course of the early studies in Korea, it became clear that HFRS cases were also occurring in urban areas. Years of investigation finally demonstrated that the urban cases in Korea, China, and Japan were associated with infection with Seoul virus, which is hosted by the rats *Rattus norvegicus* and *R. rattus* (156,157,256,263). The common rat is now global in its distribution, and study of rats from a number of port cities throughout the world showed that Seoul virus–infected rats are present throughout the world (156).

For more than 50 years, a similar, but generally milder, disease termed *nephropathia epidemica* was described in various parts of Scandinavia (203). After the isolation of Hantaan virus, the virus was shown to react with sera from convalescent-phase patients with nephropathia epidemica (203). This led to the discovery of Puumala virus as the cause of this form of HFRS and the bank vole, *Clethrionomys glareolus,* as the virus host. Additional hantaviruses were identified as researchers realized that many different murid rodents carried their own specific hantaviruses (Table 2). Notable discoveries included the finding of Thottapalayam virus in India in tree shrews (*Suncus murinus*), the only insectivore implicated as a hantavirus host. Prospect Hill virus was the first hantavirus shown to be associated with a rodent species indigenous to the Americas, namely the meadow vole, *Microtus pennsylvanicus.* However, Prospect Hill virus is not know to be associated with human disease. Later, Dobrava virus was shown to be the cause of severe HFRS (similar to that seen with Hantaan virus) in the Balkan region and was found to be associated with *Apodemus flavicollis* (14).

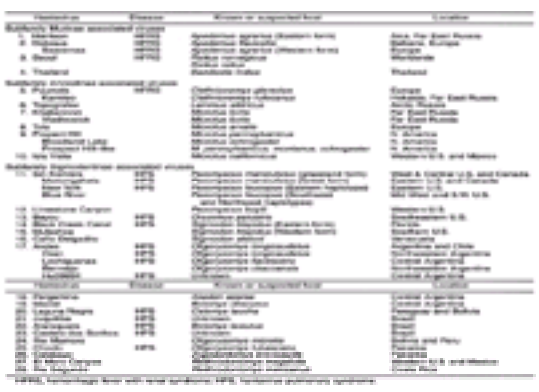

TABLE 2. *Hantaviruses and their their murid hosts*

The most recent major event in the history of hantaviruses was the discovery of hantavirus pulmonary syndrome (HPS) in the southwestern United States in 1993 (201). A cluster of cases identified in the Four Corners region (meeting point of New Mexico, Arizona, Colorado and Utah) presented with an influenza-like illness with fever, headache, muscle aches, and chills; disease rapidly progressed to a more severe respiratory disease with bilateral pulmonary infiltrates, respiratory failure, shock, and death occurring about 2 to 10 days after onset of illness in almost 50% of the cases (138). Within a couple of weeks of the initial outbreak, a newly identified virus, Sin Nombre virus, was shown to be the cause of HPS, and rodent reservoir was shown to be the common deer mouse, *Peromyscus maniculatus* (52,201). Within the next several years, HPS was shown to occur throughout the Americas from Canada to Patagonia and was found to be caused by least 10 different hantaviruses, each associated with different rodent species (see Table 2). During the study of the hantaviruses associated with HPS and HFRS, many additional hantaviruses not know to be associated with human illness were discovered in a wide variety of rodent hosts (see Table 2).

Infectious Agents

Virtually all of the hantaviruses have been difficult to isolate and propagate in tissue culture cells. Vero cells (monkey kidney), particularly the E6 clone, are thought to be most susceptible and are the usual cell of choice for work with hantaviruses. The viruses generally grow slowly, taking 5 to 14 days to produce maximal yields of progeny virus. Growth of these viruses usually results in little, if any, CPE. Electron microscopic studies of thin sections show the hantavirus particles to have a morphology fairly typical of members of the *Bunyaviridae* family, forming predominantly spherical or ovoid particles 80 to 120 nm in diameter. Elongated particles averaging about 170 nm in length have also been observed, which is atypical of other *Bunyaviridae* family viruses (173). Viruses of the Hantavirus genus are the most distinct of any of the genera when viewed by negative staining, which reveals surface structures in an unusual square gridlike pattern with each morphologic unit being about 8 nm in diameter (Fig. 4).

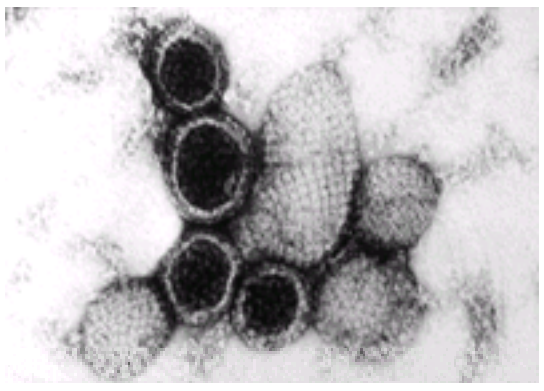

FIG. 4. Electron micrograph negative-stain image of Hantaan virus (genus *Hantavirus*) (135,000× magnification). (Courtesy of Mary Lane Martin and Helen L. Regnery.)

Three major virus antigens are present in infected cells: the virus nucleocapsid (N) protein and glycoproteins GP1 and GP2 (see Chapter 48 for more detailed description). The immunofluorescence staining of infected cells using anti-N antibody shows characteristic punctate staining throughout the cytoplasm. Glycoprotein-specific antibodies tend to show a perinuclear staining pattern typical of proteins accumulating in the Golgi compartment (258). Typical of viruses in the family *Bunyaviridae,* hantavirus glycoproteins are thought to accumulate in the Golgi as a first step in the maturation and assembly of mature virions in the Golgi complex. No accurate disease model exists for HFRS or HPS; however, hantaviruses have been shown to grow in a number of experimentally infected animals, including laboratory mice, rabbits, and nonhuman primates.

Pathogenesis and Pathology

Rodents

The hallmark of a hantavirus infection of a natural rodent reservoir species is the absence of overt disease symptoms and the establishment of a prolonged persistent infection that may last several months or years (19,92,160,161,200,311). Virus is horizontally transmitted among rodents, and infection is thought to be primarily by scratching and biting, although infectious aerosols may also play a role. These conclusions are based on studies examining the prevalence of hantavirus-specific antibodies in rodent host populations, which frequently show a greater number of scars of seropositive individuals relative to seronegative rodents, and often highest seropositive rates are seen in older males (96). Results from experimental infections of host rodents indicate that initial local virus replication leads to viremia, which peaks about 2 weeks after infection and results in dissemination of the virus throughout body (92,125,160,161,200,311) (Fig. 5). Hantaviruses appear to target primarily endothelial cells, with highest concentrations of virus antigen being observed in the lungs and kidneys (199). Hantaviruses are largely noncytopathic, with no virus-induced cell death observable in infected animals. Virus is shed in urine and feces and probably in the saliva too, based on the observation of considerable virus replication in the salivary glands. The reduction and eventual clearance of the viremia correlates with the induction of hantavirus-specific antibodies, which first become detectable about 14 days after infection, peak about 50 days after infection, and remain detectable for the life of the rodent. Despite the presence of serum neutralizing antibodies, the virus persists and is shed for several months after infection, although peak shedding occurs about 2 to 10 weeks after infection (126,161) (see Fig. 5). No overt disease symptoms are observed in naturally or experimentally infected rodent hosts.

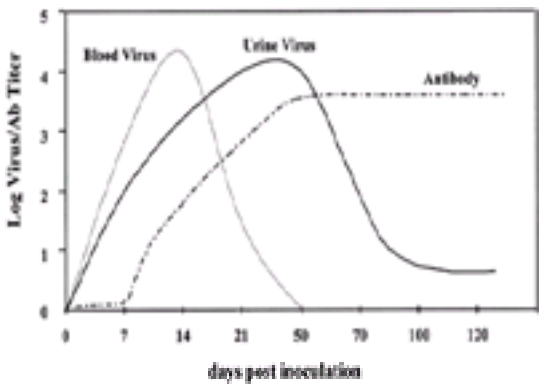

FIG. 5. Dynamics of a typical hantavirus infection of its primary reservoir rodent host. (Modified from ref. 200, with permission.)

Humans

In stark contrast to the asymptomatic hantavirus infection of primary rodent reservoir species, human infections frequently result in disease, with HFRS or HPS resulting depending on the hantavirus type. Hantaviruses are transmitted from the infected rodent reservoir to humans by inhalation of infectious aerosols produced from the rodent excreta. The early events in the human infection are poorly understood. Virus entry into cells is thought to be mediated by cell surface integrins, with pathogenic hantaviruses, such as Sin Nombre, New York, and Hantaan, using b $_3$-integrins, whereas so-called nonpathogenic hantaviruses, such as Prospect Hill and Tula viruses, use b $_1$-integrins (93,94). In the absence of

data regarding human infection with nonpathogenic hantaviruses, it is difficult to verify whether cell receptor usage does indeed correlate with virulence of the viruses in humans. Absence of reported disease with such viruses may merely reflect less human contact with these viruses owing to elements of the rodent host ecology. For instance, deer mice (*Peromyscus maniculatus*), which host Sin Nombre virus, are described as the most abundant small mammal in North America (38). They are generalists, being found throughout a wide variety of ecological zones, and are frequently found in peridomestic settings, bringing them into close and repeated contact with humans living in rural areas. These features may provide the best explanation of why most HPS cases in North America are associated with Sin Nombre virus infection. Studies of hantavirus infection of polarized cells, such as those done with Black Creek Canal virus, show that hantavirus entry into the host cell is predominantly through the apical cell surface (224). Such studies also show that most release of virus is occurring at the apical surface, in contrast to most other family *Bunyaviridae* viruses studied.

The incubation period before onset of illness lasts about 7 to 21 days. As in the rodent infections, no CPE is evident in hantavirus-infected human cells. Hantavirus-specific antibodies and T cells are detectable at the time of onset of disease symptoms, consistent with immune-modulated changes, rather than virus-induced cell death being responsible for the disease symptoms (85,148). Virus RNA is usually detectable in patient blood during the early stages of disease. At the time of death, virus antigens are detectable in endothelial cell layers throughout the body, but predominantly in lung endothelial cells in the case of HPS, or kidney endothelial cells in the case of HFRS (124,313). Acute tubulointerstitial nephritis can be seen in kidney biopsies from HFRS patients. Mild to massive proteinuria, hematuria, and a rise in the serum creatinine level are typical. The exact mechanism of kidney failure in HFRS patients is unclear. Mild to moderate interstitial infiltration of lymphocytes, plasma cells, monocyte-macrophages, and polymorphonuclear leukocytes (mainly eosinophilic granulocytes and neutrophils) can be seen in kidney biopsy specimens from Puumala virus–associated HFRS patients (272). Also, an increased expression of the cytokines tumor necrosis factor-a, transforming growth factor-b, and platelet-derived growth factor has been observed at the same sites, mainly in the peritubular area of the distal nephron. Expression of endothelial adhesion molecules CD54 (intercellular adhesion molecule-1), CD106 (vascular cell adhesion molecule-1), VCAM, and CD31 (platelet endothelial cell adhesion molecule-1) PECAM can also been seen at the same locations. Similarly, high numbers of cytokine-producing cells can be seen in the lungs (but not the kidneys and livers) of patients with fatal HPS, suggesting that local cytokine production may play an important role in pulmonary edema and high case-fatality (about 50%) seen in HPS, although the exact alteration in pulmonary endothelial cells leading to capillary leakage is still unclear (186). Although hantaviruses infect a number of laboratory animals, including mice, hamsters, and nonhuman primates, no good model of the human disease has been described. Severity of illness in humans appears to correlate with HLA type, at least in the case of Puumala virus–associated HFRS (196,197). Severe disease was shown to be associated with the HLA-B8 DRB1*0301 haplotype, whereas the HLA-B27 allele was underrepresented in hospitalized cases and those that possessed the allele had benign overall clinical course and mild renal involvement. Severity of HPS disease has also been suggested to be linked to HLA-B*35 (F.T. Koster, unpublished data).

Epidemiology and Ecology

The geographic distribution of hantaviruses and epidemiologic patterns observed for HFRS and HPS reflects the distribution and natural history features of the hantavirus-infected rodent hosts of these viruses (Fig. 6; see Table 2). This is because hantavirus infection of the rodent primary reservoir species produces a persistent infection with prolonged shedding of the virus, whereas infection of humans results in an acute infection with relatively quick clearance of virus and recovery in survivors. Epidemiologic data accumulated since the 1950s for a variety of hantaviruses, including HPS-associated hantaviruses, suggest that human-to-human virus transmission does not occur, and virtually all human infections are acquired by exposure to infected rodent excreta, with the one exception of an Andes virus–associated HPS outbreak in Patagonia, Argentina (see later).

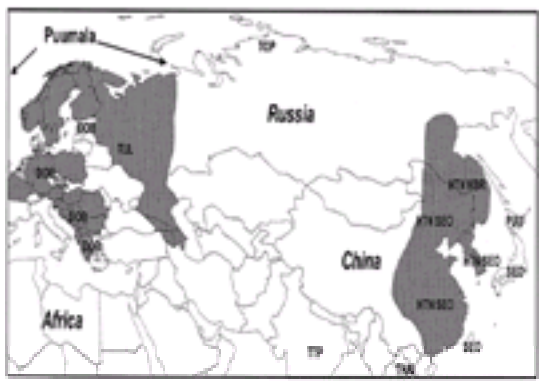

FIG. 6. Approximate distribution of hantaviruses and hemorrhagic fever with renal syndrome (HFRS) in the Old World. HTN, Hantaan; SEO, Seoul; PUU, Puumala; THAI, Thailand; TTP, Thottapalayam; KBR, Khabarovsk; TOP, Topografov; DOB, Dobrava; VLD, Vladivostok.

Hantaan virus is found in various parts of eastern China and Korea and Far Eastern Russia and is present in *Apodemus agrarius mantchuricus,* a mouse that is common in agricultural fields (51). Adults in rural areas (e.g., farmers, forest workers, or troops stationed in the field) are the most at risk for infection, and HFRS cases are seen to peak in the fall. This likely reflects a mixture of factors, including increased abundance of virus-infected mice in the fall, coinciding with increased human activity in the fields associated with harvest of crops, and movement of rodents into the houses as winter approaches (155). Most cases are 20 to 50 years in age, and cases in children younger than 10 years of age are uncommon (49,157). A greater number of male than female cases are seen, consistent with predominant exposure being in the fields rather than in the homes. Hantaan-related HFRS is probably most important in China, where about 100,000 cases are reported each year. On the order of 300 to 900 HFRS cases are estimated to occur in Korea each year, and

about the same number of cases are thought to occur in Far Eastern Russia on an annual basis.

Seoul virus is associated with the domestic rats *Rattus norvegicus* and *R. rattus* (159,263) and has a worldwide distribution owing to the relatively recent global spread of rats to port cities and beyond through international shipping (156). Seoul is the only hantavirus known to cause disease in urban areas because the other hantaviruses are associated with rodents that are predominantly rural in their distribution. Surprisingly, Seoul virus–related HFRS cases appear to be rare outside of China and Korea and institutions housing laboratory rat colonies, the exact reason for which is unclear. Seoul virus–related HFRS cases in general tend to be more moderate than those associated with Hantaan virus, although mortality rates still range to about 1% to 5%. Although most cases are in cities, Seoul virus–related HFRS cases are also seen in some rural areas in China. The seasonality of Seoul virus–related outbreaks are different to Hantaan-related HFRS, with most cases occurring in spring and early summer as opposed to fall and early winter (50). The annual number of cases is unknown; figures for Seoul virus– and Hantaan-related HFRS are often not separated. However, Chinese reports suggest that Seoul virus–related HFRS in urban areas of several provinces has been on the increase since its discovery in 1981 (47,256). A genetically related virus, Thailand virus, is found in *Bandicota indica* rodents in Thailand but is not known to cause human disease.

Dobrava virus has more recently been shown to be the cause of severe HFRS (similar to that seen with Hantaan virus) in the Balkans and perhaps elsewhere in southern Europe (9,13,206). The primary rodent reservoir is *Apodemus flavicollis,* which is widely abundant in this region. However, a distinct lineage of Dobrava virus can be found in *Apodemus agrarius agrarius* (a subspecies distinct from that which carries Hantaan virus in Asia), which is of greater abundance in more northern areas (12,51,198). Although *A. flavicollis* do not frequent houses and other man-made structures, they will often enter campsites where food may be present. Most cases of Dobrava-related HFRS occur in rural areas, involving men with occupations such as shepherds, woodcutters, or military personnel who work or sleep in open areas where these rodents may be abundant. Unlike Hantaan-associated HFRS, most Dobrava-associated HFRS cases occur in the late spring and summer months when activity in the rural areas is greatest. Epidemics of disease are seen at times when rodent populations are high and have been observed in recent years when military conflict in parts of the Balkan region have resulted in substantial numbers of troops or civilians living in open country (170). Average years see perhaps a couple of hundred HFRS cases occurring in the region, although not all are associated with Dobrava virus infection (see later discussion of Puumala virus). Seroprevalence rates can be high in some rural areas; for example, antibody positivity rates of up to 14% were seen in mountainous areas of northern and western Greece (8).

Nephropathia epidemica, generally the mildest form of HFRS associated with Puumala virus, is found throughout Scandinavia and Europe west of the Ural mountains. The virus is hosted by the bank vole, *Clethrionomys glareolus.* Puumala virus–associated HFRS is the most common form in Europe. In Sweden and Finland, the number of cases in rural residents usually peaks in November and January, although cases in urban residents appear to peak in August, which likely correlates with vacationing in summer cabins in rural areas. A male-to-female case ratio of about 2:1 is seen, and the disease is rare in children (27). Local epidemics usually reflect increased local bank vole densities, which tend to cycle with a 3- to 4-year periodicity. About 30% of Puumala virus infections result in reported disease, based on data from areas of Finland with high surveillance for disease (27). Also, antibody prevalence rates of up to 20% can be seen in known endemic areas in northern Sweden (120). In recent years, there has been an increased awareness of Puumala virus–associated HFRS in other areas of Europe. For instance, a large outbreak (60 cases) was recorded in southern Belgium in 1993, which mirrored an increase in the local bank vole population (60). Hantavirus disease in Belgium, northeastern France, and Germany mainly occurs in fall and spring, and extent of activity reflects the alterations in the size and structure of bank vole communities (215). Large Puumala virus–associated HFRS outbreaks are periodically recorded in several areas of European Russia, particularly Udmurtia and Bashkortostan. A genetically distinct Puumala-like virus has also been identified in *Clethrionomys rufocanus* voles in Far Eastern Russia and on Hokkaido Island, Japan but is not known to be associated with human disease (136). Several additional hantaviruses are known to be associated with other subfamily Arvicolinae rodents but are not known to cause human disease. Topografov virus is present in lemmings in Arctic regions of Russia, and at least five different hantaviruses are associated with various Old World and New World rodents of the *Microtus* genus (see Table 2).

Since the discovery of Sin Nombre virus and HPS in 1993, there has been an enormous expansion in the number of hantaviruses known to be associated with the New World subfamily Sigmodontinae rodents (see Table 2). In terms of the United States and Canada, by far the most important virus is Sin Nombre virus because it is responsible for most of the HPS cases identified in North America (184). Sin Nombre virus is present in deer mice (*P. maniculatus,* grasslands form) throughout western and central United States and Canada (184). The initial 1993 cluster of HPS cases occurred in the Four Corners region, and most subsequent cases have been identified in the southwestern United States. However, HPS cases have now been identified in 30 states and four provinces of Canada, and Sin Nombre virus–infected deer mice have been detected throughout most of its range, which stretches from the Yukon to Mexico and from California to the Appalachian mountains in the eastern United States and Canada (181,184). As of spring 2001, about 280 cases of HPS cases have been identified across the United States, with most cases occurring in the western region. Thirty-five cases have been confirmed in western Canada in the provinces of British Columbia, Alberta, Manitoba, and Saskatchewan. Survey of more than 9,000 sera from the U.S. general population found that about 0.2% of individuals had hantavirus-specific antibodies, indicating that hantavirus infection of humans in the United States is relatively rare (T.G. Ksiazek, personal communication). Even survey of 2,500 individuals in risk groups, such as those with occupations that would bring them in close contact with rural rodents, found hantavirus-specific antibodies in only about 0.5% of those sampled (L.R. Armstrong and associates, unpublished observations) (294,314), suggesting that infection of humans with Sin Nombre virus is rare. It appears that clusters of cases are generally associated with local increases in infected deer mice populations. The initial 1993 outbreak, which led to the discovery of the disease, was associated with unusually high deer mice populations that had risen to about 10 times normal levels owing to a prolonged El Niño effect, which produced conditions favorable for deer mice reproduction and survival in the Four Corners region (207). The peak of HPS cases occurs in late spring or early summer, and about equal ratios of males and females are affected (138). Clinical disease in children is rare. Retrospective studies suggest the disease had gone unrecognized since at least 1959 (90).

Several other hantaviruses are capable of causing HPS in the United States. The forest form of deer mice (*P.*

maniculatus nubiterrae), which are found predominantly throughout the Appalachian mountain range extending from Georgia to eastern Canada, harbor a genetically distinct Sin Nombre–like virus referred to as *Monongahela virus* (184,257). This virus has been associated with two or three HPS cases in the eastern United States. The white-footed mouse, *P. leucopus* (eastern haplotype), hosts another Sin Nombre–like virus, named *New York virus.* This virus has been responsible for at least two HPS cases in the northeastern United States (115,121). A different hantavirus, Blue River virus, is present in *P. leucopus* (western haplotypes) in the central and western U.S., but this virus is not known to be associated with human disease (191). Mitochondrial DNA (mtDNA) analysis of the different rodent hosts shows that each of these rodent hosts are genetically distinct (191) (S.P. Morzunov and S.T. Nichol, unpublished data). This is in keeping with the general pattern for hantaviruses, whereby each specific hantavirus is hosted by a single different species or subspecies of rodent, and each specific rodent species or subspecies serves as the primary reservoir for only a single specific hantavirus (200,220).

Other sigmodontine genera harbor HPS-associated hantaviruses in the U.S. Black Creek Canal virus was associated with a single HPS case in southern Florida and is hosted by the cotton rat, *Sigmodon hispidus* (eastern form) (225). The genetically distinct western form of *S. hispidus,* hosts a genetically distinct hantavirus, Muleshoe virus (226). Bayou virus was discovered in 1994 associated with a fatal HPS case in Louisiana (190). Subsequently, the virus was shown to be hosted by the rice rat *Oligoryzomys palustris,* to be present throughout the range of the rodent (147), and to be associated with a nonfatal HPS case in Texas (113). Although the HPS associated with Black Creek Canal and Bayou viruses is still predominantly a pulmonary disease, there does appear to be more renal involvement than seen in classic Sin Nombre virus–associated HPS (113,137,140).

The confirmation of an HPS case in Brazil in late 1993 marked the beginning of a wave of discovery of HPS cases and associated hantaviruses throughout South and Central America (see Table 2). Over 300 HPS cases have been identified in various parts of Argentina (website, http://www.healthig.com/hantavirus/hanta14.html), 90 in Chile (website, http://disap.minsal/epidmiologia/hantas/informedia.html), 40 in Paraguay, 26 in Brazil, 15 in Uruguay, and 12 in Panama. Notable outbreaks included the identification of 17 cases in the western Chaco region of Paraguay in 1995 to 1996 (132,305) and 20 cases in Patagonia, Argentina in 1996 (300). This unusual HPS outbreak in Patagonia was shown to be caused by a distinct hantavirus, Andes virus, hosted by *Oligoryzomys longicaudatus* (169). Similar to all the other studied hantavirus diseases, transmission of HPS-associated hantaviruses appears to be overwhelmingly from infected rodent to humans by inhalation of infectious aerosols. However, epidemiologic investigation of the Andes virus–associated HPS outbreak in Patagonia clearly demonstrated human-to-human transmission between a patient and her physician, and circumstantial evidence suggested several additional human-to-human virus transmissions during this particular outbreak (205,300). This does not appear to be a common property of Andes virus because no health care workers became infected in the course of handling Andes virus–associated HPS cases in a subsequent large HPS outbreak in Chile (45).

The finding of high prevalence of hantavirus-specific antibodies in the general population in some areas of South and Central America differs considerably from the epidemiologic picture seen in North America, where seropositivity rates of 0.2% of less are seen. For example, high rates of hantavirus-specific antibodies have been seen on surveillance of general populations in regions of Paraguay (40%), Salta province in Argentina (17%), and Los Santos province in Panama (13%) (87,293,305). These data suggest more frequent exposure of people to hantaviruses in these areas and more inapparent or subclinical infections than seen with North American hantavirus infections.

Phylogenetic analysis of hantaviruses and their rodent reservoirs have indicated that their evolutionary trees overlap with only a few exceptions (Fig. 7), suggesting that these viruses have coevolved with their rodent hosts over 20 to 30 million years (200,201,220). These data show that although HPS and many hantaviruses are only recently discovered, they are actually ancient.

FIG. 7. Hantavirus phylogenetic trees. Maximum parsimony phylogenetic trees based on the nucleotide sequences of the hantavirus entire S or M genome segments. Bootstrap values in excess of 50% are shown adjacent to internal nodes. HTN, Hantaan; DOB, Dobrava; SEO, Seoul; THAI, Thailand; PUU, Puumala; TOP, Topografov; KBR, Khabarovsk; TUL, Tula; PH, Prospect Hill; ISLA, Isla Vista; MULE, Muleshoe; BCC, Black Creek Canal; Bay, Bayou; LEC, Lechiguanas; ORN, Oran; RIOM, Rio Mamore; LN, Laguna Negra; SN, Sin Nombre; NY, New York; MON, Monongahela; BR, Blue River; ELMC, El Moro Canyon; RIOS, Rio Segundo. All sequences are available in GenBank, except those for Thottapalayam virus, which was kindly provided by Connie Schmaljohn. (Modified from ref. 200, with permission).

Clinical Features

After infection with Hantaan or Dobrava virus, there is a typical 2- to 3-week incubation phase followed by the sudden onset of influenza-like symptoms. The febrile phase lasts 3 to 5 days with high fever, headache, malaise, chills, and prostration. Flushing of the face and neck and conjunctival and pharyngeal injection (indicating capillary dilation) are

frequently observed. Increased capillary permeability is likely responsible for the retroperitoneal edema and lower back pain often described. The febrile phase is followed by a hypotensive phase lasting several hours to days characterized by marked thrombocytopenia and often petechial hemorrhage. Many patients exhibit low-grade DIC, and about 10% to 15% of patients show some degree of shock around this time. Usually, shock improves in 12 to 48 hours, and blood pressure returns to normal or the patient becomes hypertensive. Oliguria may occur, and historically, renal failure contributes to about half of the deaths (74,251,252). Recovering patients then go through a phase of diuresis, which may last several months. Obviously, not all patients go through all the stages described, with some of the more severe cases rapidly progressing through to death in a few days, whereas other, milder cases skip some phases of the disease or exhibit very mild symptoms and are not recognizable as HFRS. However, this tends to be the most severe form of HFRS, with a 5% to 15% mortality rate.

Seoul virus–associated HFRS tends to be milder, with a mortality rate of 1% or 2%. In general, the disease course resembles that observed with milder Hantaan and Dobrava virus–associated cases, although more prominent liver involvement often occurs (141). Puumala virus–associated HFRS (often referred to as nephropathia epidemica), is the mildest form of the disease, with a mortality rate of less than 1%. The phases of the illness are generally less pronounced, and symptoms are milder, with hypotension rather than shock, petechiae instead of more frank hemorrhage, and relatively mild renal involvement. There is some suggestion that cases in central Europe and Russia are more severe than those seen in Scandinavia. In contrast to HPS (see later), pulmonary edema is not a common on presentation of HFRS cases and, when seen, is not an initial symptom but is usually the result of overhydration of the patient.

Since the discovery of HPS in 1993, a considerable amount of data have accumulated on the clinical presentation of the disease, particularly for Sin Nombre virus–associated HPS, which is the most common form seen in North America (72,138). Similar to HFRS, in HPS, there is also a 2- to 3-week incubation period before rapid onset of an influenza-like illness. The febrile phase lasts about 4 days and includes fever, myalgia, malaise, and headache; gastrointestinal symptoms are also often observed at this early stage. Patients often present for medical attention with the onset of pulmonary edema, dyspnea, and hypoxemia. These features, together with the hemoconcentration that is frequently observed, are presumably linked to the hantavirus infection of the microvascular endothelial cells surrounding the lungs, which leads to increased permeability and fluid leakage into the lungs. Bilateral pulmonary infiltrates are often visible on chest radiographs. Deterioration of patient condition is often rapid, with death occurring in almost half of cases within 1 to 3 days of hospital admission. Clinical laboratory tests frequently reveal thrombocytopenia and prolonged partial thromboplastin time. Hypotension and shock can be prominent at this stage. Recovery of surviving patients is often remarkably quick, with rapid resolution of the lung lesion and shock within 3 to 6 days. Very similar clinical pictures are seen with HPS cases associated with other viruses found in rodents of the subfamily Sigmodontinae. HPS cases have also been associated with Sin Nombre virus–related Monongahela and New York viruses; Bayou virus; Black Creek Canal virus; Andes and related viruses Oran, Lechiguanas, and Hu39694; and Laguna Negra, Juquitiba, Araraquara, and Castelo dos Sonhos viruses. The clinical picture seen in HPS cases associated with Black Creek Canal and Bayou viruses differs slightly in that more renal involvement and elevated serum creatine phosphokinase are observed. Renal failure also appeared to be common with Oran virus–associated HPS in northern Argentina (164). Flushing, petechiae, and frank hemorrhage were seen in some HPS patients infected with Andes virus in Argentina and Chile, respectively (7).

Diagnosis

Clinical differential diagnosis of HFRS and HPS cases can be difficult because of the nonspecific early symptoms in both types of cases. In areas of China, Korea, Far Eastern Russia, Europe, and Scandinavia, where HFRS is known to occur, a febrile patient presenting with thrombocytopenia and renal symptoms should be questioned about potential rodent exposure and tested for evidence of hantavirus infection. The early signs of severe HFRS can be easily confused with those of leptospirosis, typhus, pyelonephritis, poststreptococcal glomerulonephritis, an acute abdominal emergency, or other hemorrhagic fevers. Mild HFRS can be confused with hepatitis A, mild leptospirosis, streptococcal pharyngitis, influenza, or nonsteroid antiinflammatory toxicity.

In the Americas, the early stages of HPS are extremely difficult to recognize, being easily confused with influenza. However, fever and myalgia and a history of potential rural rodent exposure, together with the appearance of shortness of breath and findings of thrombocytopenia, leukocytosis with left shift (higher ratio of immature-to-mature neutrophils), and atypical lymphocytes, would be strongly suggestive of HPS (204).

In keeping with the perception that both HFRS and HPS are immune-modulated diseases, virus antibodies are invariably detectable in patient sera near the time of onset of illness. Laboratory analysis of the presence of virus-specific antibodies has historically included indirect fluorescent antibody (IFA) testing. This has good sensitivity and hantavirus cross-reactivity, and use of a representative virus from each of the main rodent subfamilies harboring hantaviruses (i.e., Murinae, Arvicolinae, and Sigmodontinae) identifies infections caused by virtually all hantaviruses. However, false-positive results are not uncommon owing to rheumatoid factor problems and overall nonspecific “stickiness” of some patient sera. Some of these difficulties can be controlled for by the inclusion of appropriate negative antigens to detect and remove the effects of problem sera. Although of some utility for acute diagnosis, the inherent nonspecific background and subjectivity problems make the IFA test unsuitable for serologic survey studies. Acute diagnosis is best achieved by IgM capture ELISA using hantavirus-infected cell slurry as antigen and incorporating an uninfected cell slurry control (148). Recombinant nucleocapsid proteins with appropriate negative-control protein fractions have also proved useful as antigens in this assay format (83,86,101). Such assays have been shown to be highly sensitivity, rapid, and inexpensive. Considerable cross-reactivity among related hantaviruses is seen, such that inclusion of a limited number of representative virus antigens (e.g., Sin Nombre, Puumala, and Hantaan viruses) allows detection of virtually all acute hantavirus infections. The disadvantage of the cross-reactivity of the assay is the inability to identify precisely the virus based on a positive result. Western blot assays using recombinant nucleocapsid proteins or glycoproteins have also been effectively used for acute diagnosis, particularly with New World hantaviruses, and can provide some additional insights into virus identity (114,130).

Virus isolation attempts from acute clinical specimens are almost always negative. The exact reason for difficulty of

isolation of hantaviruses is unknown but is likely related to the presence of a strong immune response at the time of onset of illness, including serum neutralizing antibodies. Demonstration of the lack of amino acid substitutions during the isolation of Sin Nombre virus from infected lung material would suggest that virus adaptation to growth in tissue culture is not a major element in the difficulty of isolation of these viruses (53). Given the frequent difficulty in obtaining infectious virus, RT-PCR assay has been used effectively to complement immunodiagnostic assays and allow detailed genetic comparison of the hantaviruses (169,201,206,237). Given the potential for false-positive results because of cross-contamination, RT-PCR is not recommended as the sole means of hantavirus diagnosis.

Prevention and Control

The primary means of prevention is the reduction of rodent exposure. Effective measures include rodent-proofing of homes, correct storage of food, airing of seasonally closed cabins, and disinfection and removal of trapped rodents and rodent droppings (42,43,95). The lack of nosocomial transmission or person-to-person transmission reported for virtually all hantavirus outbreaks suggests that spread to medical personnel or contacts is usually not a concern. The one exception was the limited person-to-person spread seen during an Andes virus–associated HPS outbreak in Patagonia, Argentina in 1996 (205,300).

Several different inactivated virus vaccines for Hantaan and Seoul viruses have been used in Asia (reviewed in 119). These include virus-infected rodent brain–derived antigens inactivated with formalin or b-propiolactone (162,309). Although such products would not be suitable for use outside the region, good efficacy has been reported (119). Several monovalent and bivalent Hantaan and Seoul tissue culture–derived inactivated vaccines have also been produced in China (119), some of which may have high efficacy (48). In addition, a number of recombinant DNA approaches to vaccine development have been investigated (recombinant baculovirus, vaccinia, or Sindbis virus produced hantavirus proteins, naked DNA, and so forth), some of which show promise in experimental systems (119).

The sensitivity of Hantaan virus to the inhibitory effects of the antiviral drug ribavirin on virus replication in tissue culture or in animal models (123) has promoted clinical trials both with HFRS patients and HPS patients, with positive results reported in the case of HFRS (122).

TOSPOVIRUS GENUS

The tospoviruses are plant viruses that are transmitted propagatively by thrips (303). At least eight different species of thrips are reported to transmit these viruses, five in the genus *Frankliniella* and three in the genus *Thrips.* Several of these viruses are now recognized as being of significant agricultural importance (222). An excellent recent review of the tospoviruses is available on line (http://www.spg.wau.nl/viro/research/tospo.html).

The history of the tospoviruses goes back to 1915, with the recognition of spotted wilt of tomatoes in Australia (25), and then the subsequent isolation in 1930 of tomato spotted wilt virus (TSWV) (236), from which the genus gained its name (146). By the late 1940s, TSWV infections were greatly reduced in the United States and Western Europe owing to pesticide use controlling the onion thrips, *Thrips tabaci,* which was likely the primary vector at that time. The geographic spread during the 1960s and 1970s of the Western flower thrips, *Frankliniella occidentalis,* another efficient vector of TSWV, led to a rapid expansion of the virus (26,149,288). The virus is now known to be global in distribution, and is found throughout agricultural areas in warmer climate zones, and is prevalent in greenhouse cultivations in more temperate areas. In contrast to the other genus members, TSWV has an unusually wide host range, with more than 925 plant species, including 82 botanical families, reported to be susceptible to infection. These include several important crops, such as peanuts, peppers, tobacco, potatoes, peas, tomatoes, celery, lettuce, and ornamental flowers, with crop losses amounting to more than $1 billion dollars (97). In addition to TSWV, there are 12 other recognized tospovirus species (84).

In infected plant cells, several types of aggregates of virus proteins are observed, and mature virus particles accumulate in the endoplasmic reticulum (142). Although the spread of virus from plant to plant is by ingestion of the cell contents by thrips, acquisition of a virus envelope membrane containing the virus glycoproteins is still thought to be important for virus transmission by thrips. However, once inside the plant, complete virus maturation may be less critical because movement from cell to cell likely involves virus nucleocapsid movement through plasmodesmata cytoplasmic channels (146,262). In addition, to the structural proteins, nucleocapsid (N), glycoproteins (G1 and G2), and RNA polymerase (L), the tripartite genome of these viruses encode two nonstructural proteins, one from the S genome segment, NSs, and one from the M segment, NSm (145). The NSs protein accumulates in the salivary gland of infected thrips, suggesting a role in virus transmission (303). The NSm protein associates with virus nucleocapsids and localizes to plasmodesmata, where it forms tubular structures thought to be involved in virus cell-to-cell movement (146,262).

In contrast to the other arthropod-borne members of the *Bunyaviridae,* adults of the vector thrips do not acquire the virus, only larvae becoming infected when feeding on virus-infected plants (287). TSWV has been shown to be transstadially transmitted through molting, pupation, and emergence to the adult stage, and both larvae and adults can transmit the virus to plants (302). Unlike many of the other arthropod-borne bunyaviruses, there is no evidence of transovarial transmission for any of the tospoviruses. This presumably reflects the widespread and year-round replication of thrips in most climates, and the enormous number of susceptible plants, making vertical transmission of the virus in the vector thrips of lesser importance.

Control of tospovirus infections in a field setting is difficult owing to problems in eliminating thrips and the fact that many weed species surrounding cultivated fields serve as virus reservoirs. Several breeding programs have attempted to increase natural host resistence of plants to tospovirus infection, but with little success to date (261). Attempts to create genetically engineered host plant resistance are showing promising results (221,222).

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CHAPTER 50 *Arenaviridae:* The Viruses and Their Replication

Michael J. Buchmeier, Michael D. Bowen, and Clarence J. Peters

HISTORY

Arenaviruses cause chronic infections of rodents indigenous to Europe, Africa, the Americas, and perhaps other continents. These asymptomatically infected animals move freely in their natural habitat and may invade human habitation; when man comes in contact with excreted viruses, disease may result. Arenavirus infections of humans are common and in some cases severe. The mechanisms that have evolved to ensure chronic infection and intergenerational survival of the virus in rodents are complex, and their elucidation over the last 60 years has led to a deeper understanding of immunology and immunopathology.

The first arenavirus isolated was lymphocytic choriomeningitis virus (LCMV), discovered in 1933 during the study of samples from a St. Louis encephalitis epidemic (14). It was soon found to be a cause of aseptic meningitis (255) and to be identical to an agent that chronically infected mouse colonies (294). This launched a series of studies that showed LCMV to be a common human pathogen and a fascinating virus with many lessons for viral immunologists (50,51,260).

By the 1960s, several other viruses had been discovered and found to share common morphology (229,230), serology (262), biochemical features (159), and a natural history that depended on chronic infection of rodent hosts, leading to the recognition of the *Arenaviridae* family after the sandy (Latin, *arenosus*) appearance of the ribosomes often seen in thin sections of virions (261). Some of these viruses caused severe hemorrhagic fever (Table 1), whereas others were apparently not pathogenic for or rarely infected humans (Table 2). After the isolation of Tacaribe virus in 1956 (87), new arenaviruses continued to be discovered in the Americas on the average of one every 3 years, including three lethal new viruses, Guanarito (266), Sabia in 1990 (188), and Whitewater Arroyo in 1996 (see Fig. 2) (54,104).

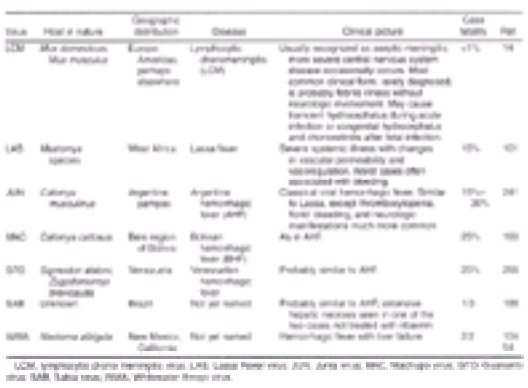

TABLE 1. *Arenaviruses that are known to be human pathogens*

Virus	Host in nature	Geographic distribution	Comments	Year isolated	Ref.
TAC	Unknown	Trinidad	Originally isolated from Artibeus bats in 1958–59; not found in subsequent attempts; bats may be reservoir	1958	57
ANA	*Oryzomys gaeldi*, *Neacomys guianae*	Brazil	Apparently has two rodent reservoirs	1964	250
PAR	*Oryzomys buccinatus*?	Paraguay	Reservoir not well established	1965	309
TAM	*Sigmodon hispidus*	United States	Antibodies detected in a few humans without disease	1963, 1965	56
PIC	*Oryzomys albigularis*	Colombia	Several asymptomatic laboratory infections	1967	292
LAT	*Calomys callosus*	Bolivia	Thought to be nonpathogenic for man	1965	311
FLE	*Oryzomys* spp?	Brazil	Two laboratory infections with disease	1975	251
OLI	*Bolomys obscurus*	Argentina	Found during field studies of Junin virus; still under evaluation	1969	210
IPPY	*Arvicanthus* spp.	Central African Republic	Proved to be an arenavirus in 1985; see Mopeia (MOP)	1970	84
MOP	*Mastomys natalensis*	Mozambique, Zimbabwe	One of several non-Lassa arenaviruses from *Mastomys* species in Africa	1977	321, 133
MOB	*Praomys*	Central African Republic	See Mopeia (MOP)	1983	110

TAC, Tacaribe virus; ANA, Amapari virus; PAR, Pirital virus; TAM, Tamiami virus; PIC, Pichinde virus; LAT, Latino virus; FLE, Flexal virus; OLI, Oliveros virus; IPPY, Ippy; MOP, Mopeia virus; MOB, Mobala virus.

TABLE 2. *Arenaviruses not known to be significant human pathogens*

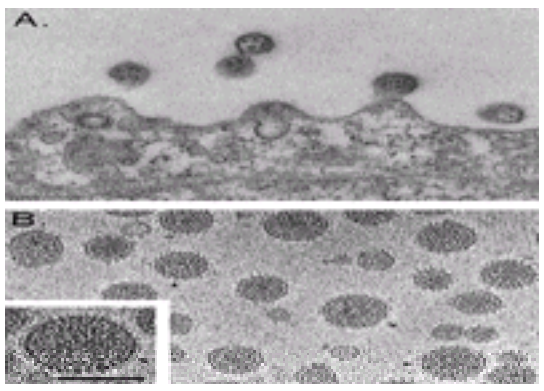

FIG. 2. Electron microscopy of lymphocytic choriomeningitis (LCM) virus. **A:** Thin section showing virions budding from infected BHK-21 cells. Typical 110-nm particles containing numerous electron-dense 20-nm ribosomes that were the basis for the family name. **B:** Cryoelectron microscopy of unstained, purified LCM virions at –4 microns defocus to illustrate surface topography. Glycoprotein spikes are evident (*arrows*) studding the viral envelope. Bar indicates 100 nm. (From J. W. Burns, R. Milligan, and M. J. Buchmeier, unpublished data.)

THE INFECTIOUS AGENT

Virion Structure

All arenaviruses are enveloped and have a bi-segmented RNA with a unique ambisense genomic organization (reviewed in ref. 283). Virions exhibit a pleomorphic morphology when examined by cryoelectron microscopy, ranging in size from 62 to more than 200 nM in diameter (Fig. 2). The surface of the virion envelope is studded with glycoprotein projections that consist of tetrameric complexes of the viral glycoproteins GP1 and GP2 (53). Internal nucleocapsids of another arenavirus, Tacaribe virus, have been shown to consist of two size classes of coiled circular filaments (238). Young and Howard (322a) investigated the structure of these RNAs and demonstrated that each segment is capable of forming twisted circular fibers of 20 nM, similar to the electron-dense particles observed within virons.

FIG. 1. Location of American arenaviruses with their natural host and approximate date of isolation. (Courtesy of Dr. S. Nichol, Centers for Disease Control.)

Genomic Organization

The genome of arenaviruses consists of two single-stranded RNA segments designated S (small) and L (large). In virions, the molar ratio of S to L RNAs is roughly 2:1. The complete S RNA sequence has been determined for eight arenaviruses and ranges from 3,366 to 3,535 nucleotides. The complete L RNA sequence has been determined for Tacaribe, LCM, and Lassa viruses and ranges from 7,102 to 7,279 nucleotides. The 3' terminal sequences of the S and L RNA segments are identical at 17 of the last 19 nucleotides (for S it is 5'...GCCUAGGAUCCACUGUGCG $_{OH}$3', and for L it is 5'...GCCUAGGAUCCUCGGUGCG$_{OH}$3'), and they are conserved among the arenaviruses (18,40,85,268,269). The 5'

terminus of each segment contains a tri- or diphosphate group and lacks a cap structure. The 5'-terminal 19 or 20 nucleotides at the beginning of each genomic RNA are imperfectly complementary with each corresponding 3' end. The 3' and 5' termini are thought to base-pair and form panhandle structures, which would account for the circular appearance of nucleocapsids when viewed by electron microscopy. An additional G residue, which would not participate in base-pairing with a corresponding 3'-terminal base, has been detected on the 5' end of some S genomic RNAs (40,116). In these cases, the panhandle structures would not contain flush ends, for there would be a single G overhang on the 5' side.

The S RNA segment contains two genes that encode three final gene products—the nucleoprotein (NP or N) and the envelope glycoproteins GP1 and GP2 (also termed GP-1 and GP-2, or G1 and G2). GP1 and GP2 are first expressed as a precursor protein, GPC (or GP-C), which is cleaved posttranslationally (49). The L RNA segment contains two genes that encode two gene products, the viral polymerase (L protein) and the Z protein, a small protein of undetermined function (142,268,270). On both segments, the genes are arranged in an ambisense orientation (Fig. 3) (141,270). The NP and polymerase genes reside at the 3' end of the S and L RNA segments, respectively, and are encoded in the conventional negative sense—that is, they are expressed through transcription of genome-complementary mRNAs. The genes located at the 5' end of the S and L RNA segments, GPC and Z, respectively, are encoded in mRNA sense but there is no evidence that they are translated directly from genomic RNA. These genes are expressed instead through transcription of genomic-sense mRNAs from antigenomes, full-length complementary copies of genomic RNAs that function as replicative intermediates. An unusual feature of arenavirus genomic RNAs is found in the intergenic regions that separate the two genes on each segment. The intergenic regions, which range from 59 to 217 nucleotides in length, are predicted to form one to three energetically stable, G-C–rich, stem-loop (hairpin) structures in both the genomic and antigenomic sense (Fig. 3) (19,141,316). The potential function of these stem-loop structures and the mechanisms of gene expression will be described in greater detail in the section, Viral Entry, Transcription, and Replication.

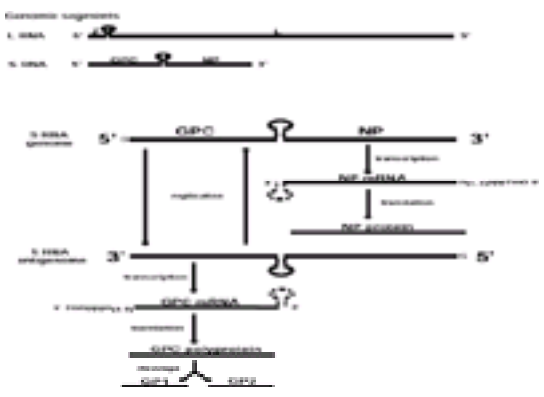

FIG. 3. Schematic diagram showing coding strategy for the L and S RNAs of the arenaviruses. Stem-loop structures indicate noncoding regions of each genomic segment. The lower portion of the diagram summarizes the transcription and replication events for the S genomic segment. As a consequence of this arrangement, the GPC and Z open reading frames can be transcribed only after initiation of replication of viral genomic RNA. Nontemplated nucleotides and a “cap” are shown at the 5' ends of N and GPC mRNAs. (Modified from ref. 283, with permission.)

Arenavirus Proteins

The NP is the major structural protein of arenavirions, with a molecular size of 60 to 68 kd. In Pichinde virus, a New World arenavirus isolated in Columbia, the NP represents 70% of the labeled virion proteins, and 1,530 molecules of NP are present in each virion. The NP is the major protein component of nucleocapsids and associates with virion RNA in the form of beadlike structures. NP can become phosphorylated in the later stages of acute infection, and this form increases in abundance during persistent infections *in vitro* (45,283). In infected cells, full-length NP is localized exclusively in the cell cytoplasm, but a 28-kd degradation fragment of NP accumulates in the nucleus of Pichinde-infected cells.

The GPC is a 70- to 80-kd polyprotein that is posttranslationally cleaved. A 58-amino-acid signal peptide is cleaved from the amino terminus before GPC is transported from the endoplasmic reticulum. Cleavage of GP1 and GP2 occurs in the secretory pathway between the medial and trans-Golgi network and requires prior glycosylation. GP1 derives from the amino-terminal portion of the GPC, and cleavage from GP2 occurs at or near a dibasic (arginine–arginine) motif that is conserved in all arenaviruses except Tacaribe virus. Equimolar amounts of the two glycoproteins are found in virions. GP1 (MW 40 to 46 kd) is the peripheral membrane glycoprotein with four to seven potential N-linked glycosylation sites (40,116). Because GP1 runs as a heterogeneous species on sodium dodecyl sulfate–polyacrylamide gel electrophoresis, it is speculated that not all potential glycosylation sites are used at the same time. GP1 molecules assemble into homotetrameric complexes that are held together by disulfide bonds. GP2 (MW, 35 kd) is the integral membrane glycoprotein, containing one to four potential N-linked glycosylation sites and a membrane-spanning domain. The carboxyl terminus of GP2 contains highly charged + or – residues and is thought to interact with NP in the cytosol, as evidenced by cross-linking studies. GP2 molecules, which are predicted to assume a coiled-coil conformation because of the presence of heptad-repeat sequences in the amino-terminal half of the molecule, assemble into homotetramers and form the stalk portion of the glycoprotein spike. GP2 homotetramers bind through ionic interactions with GP1 homotetramers, which comprise the globular head of glycoprotein spikes. GP2 is the viral fusion protein.

The arenavirus L protein has a reported molecular size of 180 to 250 kd (117,191). Motifs that are common to all RNA-dependent RNA polymerases and the polymerases of segmented negative-strand RNA viruses, as well as two regions found only in the polymerases of arenaviruses and bunyaviruses, are conserved in arenavirus L proteins. The L

protein is presumably a component of nucleocapsids, as polymerase activity has been associated with these structures.

The smallest protein encoded by the arenaviral genome is the Z protein (also called p11), which has a molecular size of 11 kd. The Z protein contains a RING-finger motif and binds zinc. It has been suggested that the Z protein is a structural protein and a component of nucleocapsids. Z protein has been detected in the cytoplasm and nucleus of LCMV-infected cells. Immunodepletion of Z protein in Tacaribe virus–infected cell extracts strongly inhibits synthesis of genomic RNA and NP mRNA, but a requirement of Z protein for transcription was seen only during the early stages of infection.

Viral Entry, Transcription, and Replication

The GP1 portion of the glycoprotein spike is responsible for initial binding to cellular receptor proteins, and antibodies directed to epitopes on GP1 can neutralize viral infectivity. GP1 binds to a 120- to 140-kd cellular glycoprotein identified as alpha-dystroglycan (57). Other arenaviruses (i.e., Lassa, Mobala, Oliveros) also bind to this glycoprotein, but Guanarito virus does not, suggesting that more than one cellular protein serves as a receptor for arenaviruses.

After attachment to cellular receptors, arenavirions are taken into the cell in large, smooth-walled endocytic vesicles that do not appear to be clathrin coated. Viral nucleocapsids are delivered into the cell cytoplasm by pH-dependent fusion of virion and endosomal membranes in response to acidification of the endosome (38,83). At acidic pH, GP1 dissociates from GP2, thus exposing the putative GP2 fusion peptide (83).

Once nucleocapsids are delivered into the cytoplasm, replication is initiated by transcription of subgenomic, genome-complementary NP and L mRNAs by the viral L protein, an RNA-dependent RNA polymerase (see Fig. 3). The mRNAs of arenaviruses are capped and contain typically four to five additional heterogeneous nucleotides at the 5' end, resembling the mRNAs of influenza viruses and bunyaviruses except that there are fewer extra bases in the mRNAs of arenaviruses. Thus it has been inferred that the arenaviral L protein uses capped primers “snatched” from cellular mRNAs by a methylated cap-dependent endonuclease domain of the polymerase, to prime transcription in a manner analogous to that described for the influenza virus polymerase complex, but this has yet to be demonstrated directly. Treatment of arenavirus-infected cells with the transcriptional inhibitor actinomycin D will reduce or inhibit viral replication in a dosage-dependent manner, presumably because the drug reduces the pool of cellular mRNAs available for “cap-snatching” by the L protein. The 3' ends of arenavirus mRNAs are not polyadenylated, and the precise structure of the 3' ends of mRNAs is somewhat unresolved. The intergenic region of the Tacaribe virus S RNA is predicted to fold into two stem-loop structures in both genomic (GPC gene) and antigenomic (NP gene) orientation. S1 nuclease mapping of NP and GPC mRNA species mapped the 3' ends of mRNAs to three or four bases located on the distal side of the stem, near the base, in the hairpin closest to each respective gene, and the ends of the mRNAs are not overlapping. The intergenic region of the Tacaribe virus L RNA is predicted to fold into a single stem-loop structure. In this case, the 3' ends of the L and Z protein mRNAs mapped to four or six bases located on the distal side of the hairpin stem. The 3' end of the L mRNA contains the complement of the 3' end of the Z mRNA, and vice versa. Determination of the 3' ends of LCMV NP and GPC mRNAs using an intramolecular circularization technique found that the 3' ends of the GPC mRNA corresponded to those described for Tacaribe virus. The NP mRNAs, however, terminated at many sites in the stem structure, on both the proximal and the distal sides of the hairpin (209). Stem-loop structures in the intergenic region of arenaviruses are thought to play a role in transcriptional termination, stabilizing the 3' end of mRNAs.

NP mRNAs can be detected as early as 2 hours after infection, and NP protein can be detected after 6 to 12 hours, depending on the multiplicity of infection. The L protein can be detected at 12 to 24 hours after infection, and its concentration increases dramatically during acute infection. NP mRNA and NP protein accumulate earlier than GPC mRNA and the GP1 and GP2 glycoproteins. NP protein accumulation either precedes replication or the two processes are contemporary. These data illustrate how the ambisense coding arrangement of the arenavirus genome temporally separates initial expression of NP and L proteins from expression of the glycoproteins and Z protein. In the presence of the protein synthesis inhibitor pactamycin, NP mRNA accumulates but replication and GPC mRNA transcription do not occur, thus demonstrating the need for *de novo* protein synthesis for RNA replication to take place.

To obtain expression of the GPC and Z proteins, a round of replication must take place in which S and L antigenomes, full-length complements to the genomic RNAs, are produced. Unlike the mRNAs, antigenomes and genomes do not contain cap structures or multiple, heterogeneous nontemplated bases at their 5' ends. The bulk of the current data suggests that genomes and antigenomes contain a single nontemplated G at their 5' terminus, although there is some evidence that this might not be a feature in LCMV genomes and antigenomes. A model has been developed to explain how the extra nontemplated G is generated. This model contends that genomic and antigenomic replication is initiated with a dinucleotide primer (pppGpC) that first base-pairs to positions 2 and 3 of the template RNA. After initiation of replication, the nascent RNA strand slips “backward” two bases to produce a single G overhang. Replication then continues until the full-length genomic or antigenomic RNA is made. The process also differs from transcription, in that the viral L protein does not terminate polymerization in the intergenic region of the template strand but rather “reads through” to produce full-length genomic and antigenomic RNAs. The transcription-to-replication switch mechanism is currently uncharacterized, but the intracellular level of Z protein may regulate this process.

After S and L antigenomes have been synthesized, they serve as templates for transcription of GPC and Z mRNAs, and as replication templates for new genomic RNAs. GPC mRNAs can be detected as early as 2 hours after infection, and the GPC polyprotein can be immunoprecipitated from infected cells by 12 hours after infection at high multiplicity of infection. The GPC is cleaved 75 to 90 minutes after translation, after the conversion of high-mannose oligosaccharide precursors to complex carbohydrates (glucosamine, fucose, galactose) and transport to the medial Golgi (52). Little is known about the kinetics of Z protein synthesis.

The assembly process of arenavirus particles is poorly characterized. Newly synthesized genomic RNAs, NP protein, and L protein are presumably assembled into nucleocapsids. For a yet-to-be-determined purpose, arenaviruses package host-cell ribosomes into virion particles. In arenavirus-infected cells, the intracytoplasmic distribution of ribosomes is altered, and inclusion bodies can be found that consist of ribosomes embedded in a matrix of virus-specific proteins.

Aggregates of electron-dense granules are found free in the cytoplasm of infected cells, as well as in juxtaposition to the plasma membrane, where patches of spikelike projections, presumably glycoproteins, are present. NP can be cross-linked to the carboxyl terminus of GP2, suggesting that interaction of these two proteins is essential for the assembly of virions at the cell membrane. Following association of electron-dense structures with the plasma membrane, budding takes place by “pinching off” of the lipid bilayer and subsequent separation of the virion and plasma membranes.

Persistent Infection and Defective Interfering Particles

Arenaviruses are known to produce persistent infections in their rodent host as well as *in vitro*. The two systems differ, however, in the fact that persistence *in vitro* is caused solely by alterations in viral gene expression and replication, whereas *in vivo* persistence is likely to be a combination of molecular biologic mechanisms and a failure of the host immune response to clear infection. The molecular mechanisms of arenaviral persistence have been investigated both in cell culture and in infected rodents. *In vitro*, most arenavirus infections progress over time from the acute phase to a persistent phase characterized by a reduction in the amount of infectious virus produced. During the acute phase of arenavirus infection *in vitro*, there is active viral replication and virus production, and usually little if any cytopathic effect (CPE). In persistent infection, cells remain infected for life, with alterations in transcription, replication, and expression of viral proteins, though it is possible that some cells may spontaneously clear arenavirus infections. There is an increase in the number of S genomic RNAs relative to the number of L genomic RNAs in cells persistently infected with LCMV (103). In both cultured cells and mice persistently infected with LCMV, there is a down-regulation of viral glycoprotein expression. In persistently infected L cells, expression of GPC, GP1, and GP2 is reduced, but a truncated GPC protein can be detected in abundance along with increased levels of NP (44). In persistent infections, full-length genomic segments and mRNAs can be detected, along with populations of heterogeneously sized, truncated RNAs that accumulate over time. The truncated RNAs detected in persistently infected cells *in vitro* tend to be less heterogeneous in size than those detected *in vivo*. Defective-interfering (DI) RNAs of arenaviruses are poorly characterized. DI RNAs from the brain tissue of mice persistently infected with LCMV have short deletions (3 to 41 bases) at the termini of both the S and L RNA genomes and antigenomes. These DI RNAs are thought to be competent for replication but not transcription and therefore are able to compete with genomes and antigenomes for viral proteins (210).

DI particles, which are able to interfere with the infectivity of standard virus, are produced during both acute and persistent arenavirus infections. Infection of L cells with LCMV at a high multiplicity of infection results in lower titers of standard virus and a higher ratio of DI to standard virus compared to an infection at a low multiplicity (183). When LCMV stocks were passed at a high multiplicity of infection in cell culture, the titer of infectious virus and DIs alternately rises and falls synchronously with each passage. This cycling effect is presumably caused by increased production of DIs during high-titer infection, which interferes with standard virus production. DIs are smaller than infectious virions (mean diameter 55 to 56 nm) and apparently lack one or both genomic RNAs. In DIs, GP1 and GP2 can be absent or nonglycosylated, and the GPC and NP often display alterations in molecular size (114,183,200). Treatment of cultured cells with DIs prior to, or up to 8 hours after, infection with standard LCMV dramatically inhibits replication of the standard virus (183,243). Concurrent intracranial injection of DIs with standard LCMV inhibits cerebellar disease in a suckling rat model. The mechanism of DI interference and its potential role in the development and maintenance of persistent infections has yet to be determined.

A DNA form of the LCMV S RNA has been detected in the spleen tissues of persistently infected mice and in persistently infected murine cell lines (174). Production of the DNA form of the LCMV S RNA was inhibited by the reverse transcriptase inhibitor zidovudine, suggesting that endogenous reverse transcriptases are responsible for this activity. The exact relevance of genomic reverse transcription to the biology of arenaviruses, and its potential roles in the maintenance of persistent infection and the immune response, remain to be elucidated.

Arenavirus Taxonomy and Relationships

The family *Arenaviridae* contains a single genus, *Arenavirus.* The 18 arenavirus species currently recognized in the genus are listed in Table 1 and Table 2 (66). Two antigenic groups within the genus, the Old World group and the New World (Tacaribe complex) group, were established using serologic assays. Epitopes shared by Old World and New World arenaviruses have been detected on NP and GP2 using monoclonal antibodies. Close antigenic relationships exist among Amapari, Junin, Latino, Machupo, and Tacaribe viruses, and among Flexal, Pichinde, Parana, and Tamiami viruses (139,271). Within the Old World arenaviruses, Ippy, Lassa, Mobala, and Mopeia share certain antigenic determinants that distinguish the African arenaviruses from LCMV virus (119,264,288,320,321).

Analysis of genetic sequence data has provided the most complete picture of arenavirus relationships. Phylogenetic analyses of the NP and/or GPC sequences separate the arenaviruses into two distinct clades corresponding to the Old World and New World groups and identified by serologic analyses (Fig. 4), and see Table 1 and Table 2). Two studies have reported clustering of Pichinde or Pichinde and Oliveros viruses with the Old World arenaviruses when glycoprotein sequences were analyzed. This observed relationship is an artifact of the method used to root the trees in phylogenetic analyses (midpoint rooting or rooting using a very distantly related outgroup). The greater sequence divergence within the New World arenaviruses compared to the Old World arenaviruses, especially in the more variable GP1 and GP2 genes, places the midpoint of inferred phylogenetic trees within the New World group, and this fact must be considered in studies of arenavirus phylogeny. Clustering of Pichinde and Oliveros within the Old World group is not observed in phylogenetic trees inferred from the more conserved NP sequences. The New World arenaviruses comprise three evolutionary lineages, designated A, B, and C (Fig. 4). Lineage A contains Flexal, Parana, Pichinde, Pirital, Tamiami, and Whitewater Arroyo viruses. Lineage B contains the four agents of arenaviral hemorrhagic fevers in the New World—Guanarito, Junin, Machupo, and Sabia, as well as Amapari and Tacaribe viruses. Latino and Oliveros viruses comprise lineage C. The observation that the four agents of South American arenaviral hemorrhagic fevers (Guanarito, Junin, Machupo, and Sabia) occupy a single lineage suggests that the highly pathogenic phenotype of these viruses may be a result of radiation from a common pathogenic ancestor. Monophyly of Junin and Machupo viruses within lineage B, both of which have rodent hosts of the genus *Calomys* (see Fig. 3 and Table 1 and Table 2), and monophyly of Flexal, Parana, and Pichinde within lineage A, three viruses with rodent hosts of the genus *Oryzomys*, provides evidence that arenaviruses are coevolving with their rodent hosts. Within the Old World arenaviruses, LCMV is most closely related to

the New World arenaviruses. Ippy virus occupies an intermediate position, whereas Mobala, Mopeia, and Lassa viruses make up two terminal groups. Considerable sequence diversity, up to 25% nucleotide and 13.5% amino acid divergence, is observed among Lassa virus strains in the NP gene (partial gene sequence), and some divergence was evident among Mopeia virus strains and among strains of LCMV. Analysis of genetic sequence data has clarified arenavirus relationships and provided insights into arenavirus evolution.

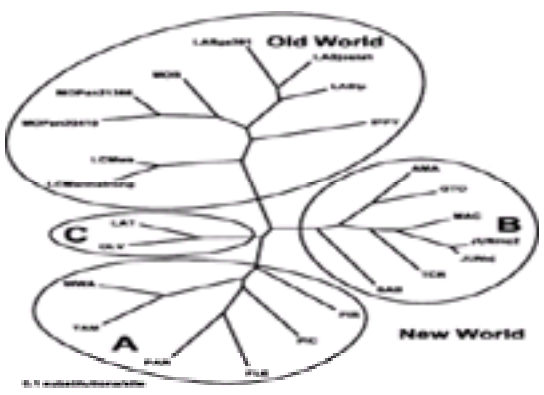

FIG. 4. Phylogenetic tree showing relationships among arenaviruses based on similarity in N gene sequences. Partial NP gene sequences were aligned using the PileUp program of the Wisconsin Package version 9.1-UNIX (Genetics Computer Group) followed by manual adjustment. The phylogenetic tree was estimated by maximum likelihood analysis performed by using PAUP version 4.0b1 [PAUP, Phylogenetic analysis using parsimony (and other methods). Sinauer Associates, Sunderland, MA]. Branch lengths are indicated by the scale bar. The Old World arenavirus group and three lineages (A, B, and C) within the New World arenavirus group are shown.

Genetics of Arenaviruses

Genetic reassortment, the association of heterologous S and L genomic RNAs within progeny virions following concurrent infection with parental virus strains, has been accomplished in the laboratory between wild-type and temperature-sensitive mutants of Pichinde virus. LCMV reassortants have been recovered after dual infections with wild-type parental strains. A Lassa-Mopeia reassortant virus that contains the L RNA of Mopeia and the S RNA of Lassa has been generated and was found to have lost the ability to kill suckling mice, a property of the Mopeia parent strain.

Diploidy, the property of having multiple genomic “alleles” packaged in the same virion, has been demonstrated for LCMV (259). Using temperature-sensitive mutants of Armstrong and WE strains of LCMV, wild-type reassortants were obtained that were diploid for the S RNA. They contained the L RNA of strain WE, and S RNAs of both strains.

Infectivity Assays and Serology

Arenaviruses are most often quantitated *in vitro* by plaque or quantal assay on Vero or L cells. CPE in cell monolayers is not typical of arenavirus-cell interactions. Plaque formation may require long incubation periods of up to 7 days for members of the Tacaribe group, but more commonly 4 to 5 days is sufficient for plaque development. Plaques may vary widely in size and appearance. For example, it has been reported that isolates of LCMV obtained from the brains of persistently infected mice are clear in appearance, whereas those from the spleens are turbid (6). Plaques obtained from a high multiplicity of inoculum or persistent infections in cell culture, both of which lead to high concentrations of DI virus, may be turbid or even exhibit a bull's eye appearance, presumably the result of alternating cycles of interfering virus production as the plaque expands (314).

In vivo LD_{50} assays for some must be used extensively to characterize arenavirus infections. Viruses that establish persistent infection in newborn mice, such as LCMV, are assayed by intracranial inoculation of 0.02 to 0.03 mL of virus dilution into adult (4- to 6-week-old) mice. Any of several inbred mouse strains may be used, but most commonly BALB/c, SWR/J, or outbred Swiss are employed. This assay is based on the fact that LCMV infection of the choroid plexus and meninges results in an acute, lethal T-cell-mediated response to viral antigens expressed in these tissues. This assay is more sensitive for LCMV strains than cell culture assays by a factor of two- to tenfold (180). As LCMV causes a persistent infection in neonatal mice, inoculation of neonates will result in a virus carrier state that might be interpreted as a negative result. Viruses in the Tacaribe complex of arenaviruses are usually lethal in newborn or suckling mice but may cause only a transient acute infection in adults. Infectivity assays for these viruses are performed in suckling mice or hamsters.

All arenaviruses are maintained in nature by persistent infection of specific species of rodent hosts, and congenital neonatal infection of the natural host usually results in lifelong infection. Persistently infected hosts may show no overt symptoms of infection, but they harbor high titers of virus and excrete infectious virus freely in their urine. Some of these characteristics may be seen in laboratory rodents. For example, hamsters infected with Pichinde virus and guinea pigs infected with LCMV may excrete virus in titers up to 10^5 plaque-forming units (pfu) per milliliter of urine. Many episodes of LCMV infection of laboratory and hospital personnel have been traced to exposure to mouse or hamster urine, contaminated cage dust, or litter, often in the absence of documented physical contact with the rodents (29,33,130,138).

A number of assays have been used to detect antibody responses to arenavirus infection. From the initial discovery of the agent in 1934 until the late 1970s, the complement fixation (CF) assay was most common. The CF assay predominantly detects nucleoprotein antigen, hence it is broadly specific (59). The CF test has largely been supplanted by indirect fluorescent antibody (IFA) and enzyme-linked immunosorbent assays (ELISA). Here again the dominant antigen is the NP. Monoclonal and polyclonal antisera against NP react across virus lines (47). Indirect

immunofluorescence assays utilizing virus-infected cells as substrate are widely used, particularly for analysis of field isolates and for work with the more dangerous pathogens (249). Antibody-positive sera usually produce granular cytoplasmic staining characteristic of intracellular nucleocapsid aggregates in arenavirus-infected cells. An identical pattern is seen when monoclonal antibodies to NP are utilized to stain infected cells (47). Antibodies to glycoprotein antigens produce a diffuse immunofluorescence staining in infected cells and also stain viral antigen on the surfaces of intact cells. The ELISA using gamma radiation–inactivated virus antigens provides greater sensitivity, more objectivity, and the capacity to accurately gauge the IgM antibody response, and it is supplanting the IFA in diagnostic and research applications. Neutralization assays are useful tools to establish relationships among closely related viruses and may be formatted as serum dilution (PRD_{50}) assays or as virus dilution log neutralization index (LNI) assays. PRD_{50} assays utilize a standardized, constant virus dilution containing 50 to 200 pfu mixed with an equal volume of diluted antiserum, incubated, and then plaqued (310). The LNI assay measures reduction of plaques from a high-titer virus pool mixed with a complement source and a single dilution (often undilute) of antibody source. Results are expressed as a $\log_{10}$ reduction of titer [$\log_{10} V/V_0$] where V_0 is the beginning titer and V is the titer after neutralization (13,147,181). This assay format also forms the basis of the neutralization kinetics assay, which, if properly applied, yields a neutralization coefficient, or K value, characteristic of a given antibody–virus interaction (139).

EXPERIMENTAL INFECTIONS OF ANIMALS

Arenaviruses readily infect mammals, and the outcome may be death, chronic infection, or immunizing acute infection. Rodents, particularly mice, guinea pigs, and hamsters, are excellent laboratory hosts for isolation; the outcome depends on virus strain, route of infection, and host genotype (244). Nonhuman primates are susceptible to many arenaviruses and may suffer serious and fatal disease with several of the human pathogens. There is little reliable information on infection of dogs, cats, livestock, or nonmammalian vertebrates, but these species are not epidemiologically implicated in transmission or as disease problems. LCMV has been reported to grow in mosquitoes, and several arenavirus isolates have been taken from arthropods, but there is no indication that these are relevant to the biology of the viruses in nature.

Pathology and Pathogenesis

Pathogenesis of arenavirus diseases is believed to involve initial replication at the site of infection in nonreservoir hosts, usually following aerosol deposition in the lung. The hilar lymph nodes, lung, and, later, other parenchymal organs are important sites of virus growth (73,168,286). Pneumonic foci are usually not present, although interstitial infiltrates and edema may occur during the course of infection. In infections by any route, the macrophage is usually identified as an early and prominent cell involved. As infection spreads, additional cell types reach equal prominence. Many epithelial structures are readily identified as containing antigen and nucleic acids. Widespread infection of the marginal zone and necrosis of lymphoid follicles of the spleen and lymph nodes are common, with the potential to blunt an effective curative immune response, and, at least in the adult LCMV-infected mouse, this is associated with cytotoxic-T-lymphocytic (CTL) destruction of critical antigen-presenting lymphoid cells (120,218,233,304). In spite of the extensive involvement of different cell types throughout the body, the pathologic changes are relatively subtle, with comparatively little frank necrosis.

The immune response to arenaviruses may be protective or deleterious or both. Cellular responses (delayed-type hypersensitivity and CTL) are readily measured and are believed to be major mechanisms in recovery and immunopathology. Antibody, although less prominently involved than in some infections, is clearly important in protection and in immune complex disease. The relative balances between protection and pathology, and between cellular and humoral responses, are determined by the virus strain and host; examples will follow.

Mouse and Other Rodent Models with LCMV

Role of T Cells in Recovery and Pathogenesis of Choriomeningitis in Mice.

Murine infection with LCMV is illustrative of the balance between immunologic protection and damage. The presence of fully functional, differentiated T cells is necessary to efficiently control the infection, but the T-cell response is also responsible for disease induction. The outcome of infection of the mouse with a given LCMV strain depends on the age and immunocompetence of the host, the route of inoculation, and the genetic background of the mouse.

Within a few days after intracranial (i.c.) inoculation of an adult mouse, LCMV replicates to high titers (>10^7 pfu/gm) in the choroid plexus and meninges, and viral antigen subsequently becomes the focus of an acute mononuclear cell infiltration driven by antigen-specific T cells (Fig. 5). During the acute infection, viral replication is limited largely to the leptomeninges and ependyma of the brain and does not penetrate into the parenchyma or involve neurons. Acutely infected mice usually succumb between 6 and 9 days after infection as a result of this T-cell-mediated acute inflammatory response; however, if the T-cell response is compromised by immunosuppression or by depletion of specific T-cell subsets, disease is prevented but viral clearance is delayed or blocked and a persistent infection ensues.

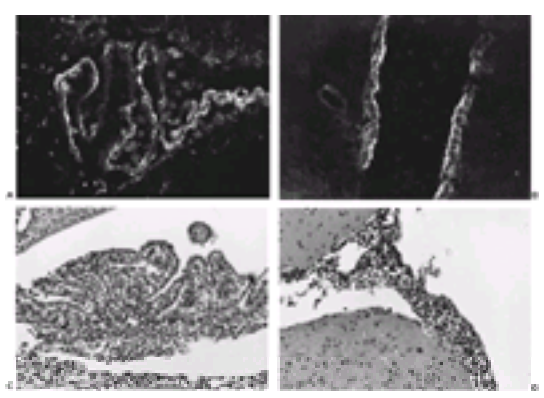

FIG. 5. Brain of a BALB/c mouse inoculated intracranially with LCMV Armstrong strain, 6 days previously. Abundant viral expression is seen in the choroid plexus (**A**) and ependymal cells lining the third ventricle (**B**) when stained by monoclonal antibody 2-11.10 that detects GP1 followed by fluorescein-labeled sheep anti-mouse IgG. Mononuclear cell infiltration is prominent in the choroid plexus (**C**) and the meninges (**D**).

The characteristic immunopathologic response following acute LCMV infection requires antigen-specific induction of CD8+ T lymphocytes that recognize LCMV antigens in the context of major histocompatibility complex (MHC) class I and are active *in vitro* in CTL assays on MHC class I–matched virus-infected target cells (325). The effector CTL can be cloned *in vitro* and used to reproduce the disease by direct central nervous system (CNS) inoculation or to mediate virus clearance from organs of acutely infected mice (55). The CD8+ cytotoxic T-cell response in LCMV infection has provided one of the most important experimental models available to immunologists for studying the nature of the signals required between cytotoxic T cells and targets to effect the lytic response (125,324). CTLs attack the infected cells and initiate dysfunction of the blood–brain barrier without any obvious morphologic correlates (9,50,306). Abnormal brain electrical activity leads to the onset of a convulsive disorder, loss of brain activity, and cardiovascular death (63). If anticonvulsants are administered, the onset of the seizure disorder is delayed by 1 to 2 days, and early morphologic signs of cell damage are seen (302). Most other arenaviruses inoculated intracranially into mice also induce this type of immunopathologic encephalitis, depending on the age of the mouse, the rapidity of growth of the virus, and the vigor of the immune response (231). Human CNS disease from LCMV is also thought to proceed by the same mechanisms as occurs in the intracranially inoculated adult mouse.

Role of Antibody in Recovery and Protection

In the immunologically naive animal, antibody plays an insignificant role in the resolution of acute LCMV infection. Evidence derived from studies of B-cell-depleted mice demonstrates no clear difference from intact controls in the development of acute choriomeningitis (61). Moreover, in studies of beta-2-microglobulin knockout mice, which lack functional CD8+ T cells, virus is not efficiently cleared (182,226). However, in certain circumstances, the importance of antibody in resistance to arenavirus infection should not be underestimated, because passive immunotherapy with neutralizing monoclonal antibodies up to 2 days after infection protects mice against lethal LCMV choriomeningitis (317). The effects of antibody were to lower virus titers in tissues including the brain and to attenuate the CD8+ T-cell response. In addition, efficient protection of offspring against chronic viremia could be passed from immune mothers by nursing, and intact IgG2a antibody was required (20). Mice protected in this manner were fully resistant to a second challenge infection given after the passive antibody had been eliminated, suggesting that the passive immunotherapy did not prevent transient infection or interfere with the memory T-cell response. Subsequent experiments have shown that antibody acts in a similar manner to protect neonatal mice against development of a persistent infection by the immunosuppressive, macrophage-tropic clone 13 ARM strain of LCMV as well (6); high doses of virus overcome antibody protection, suggesting that one effect of preexisting antibody is to restrict the replication of input virus as opposed to clearing virus-infected cells. This interpretation is further supported by the failure of passive immunotherapy in beta-2-microglobulin knockout mice, which lack functional CD8+ T lymphocytes. Following infection in these mice, virus titers are initially low or undetectable, but they rebound as the antibody is cleared (22).

In vitro neutralization of the Old World arenaviruses by antibody is usually inefficient, and significant amounts of neutralizing antibody do not appear until weeks after recovery. Even then, a constant serum dilution format (or an LNI test) with serum that has not been heated to destroy complement is needed to show neutralization (181,245). In spite of the unusual kinetics of appearance of such antibodies and their relative inefficiency, large amounts (e.g., 9 mL/kg) of convalescent serum selected for its LNI is protective or curative in both monkeys and guinea pigs infected with Lassa virus. With modern methods of preparation of therapeutic monoclonal antibodies and in the context of vaccines, these observations are significant.

With the notable exceptions of Pichinde and Latino viruses (292), Tacaribe complex arenaviruses are usually more readily neutralized, and passive antibody therapy has been used successfully in both monkeys and guinea pigs (91). When Junin virus therapy was modeled in guinea pigs, passive antibody spared the animals from disease, but, as observed in human Argentine hemorrhagic fever (AHF), there was a late neurologic syndrome associated with brain virus entry (166,169). In the guinea pig, antibody-dependent cellular cytotoxicity was thought to be important in protection because of the characteristics of *in vitro* cytotoxicity and because of the failure of $F(ab')_2$ to protect, despite being fully capable of mediating *in vitro* virus neutralization (164).

Development of the Carrier State in Mice

Inoculation of mice with LCMV, either by transplacental passage of virus from the pregnant mother to the fetus or by neonatal inoculation of virus, results in a lifelong persistent infection. The crucial difference that results in persistent infection in the neonate rather than acute, transient infection in the adult mouse is that the neonate encounters the virus prior to maturation of the cellular immune system; the neonate is unable to mount an effective antiviral CD8+ CTL response necessary to clear the infection. Persistent unresponsiveness to the virus is caused in part by the continuous presence of LCMV in the thymic environment, resulting in failure to ecognize the virus as foreign (324). Such persistently infected mice carry high levels of virus in their blood and tissues and shed virus in their urine for life (22,50,96,178,294). A consequence of persistent infection is the development of immune complex disease (48). Such mice may make a vigorous antibody response to LCMV antigens that is insufficient to clear virus, but this antibody, complexed with circulating viral antigen, lodges in the renal glomeruli and arterial walls, leading to chronic glomerulonephritis and arteritis (50). A genetic linkage was established between severity of disease, level of circulating immune complexes by C1q precipitation assay, and degree of humoral immune response to viral antigen. SWR/J animals show immune

responsiveness, immune complexes, and severe disease, whereas BALB/c are low responders with mild disease (237).

A carrier state can also be established in adult mice by immunosuppression prior to infection or by infection with macrophage-tropic immunosuppressive strains such as the clone 13 isolate. Clone 13 was derived from the spleen of a mouse persistently infected with LCMV Armstrong strain, and it has the capacity to induce a persistent infection in the adult immunocompetent mouse following intravenous inoculation. Clone 13 does not induce a CTL response in the acutely infected mouse and exhibits marked tropism for macrophage-lineage cells *in vivo* (37,202,203,273). Molecular genetic analysis of the clone 13 variant has mapped the imunosuppressive property to a single amino acid substitution (Glu to Phe) at amino acid 260 in the GP1 glycoprotein (203,267).

Macrophage tropism is not restricted to LCMV. The splenic macrophage is a primary target of infection for virulent Pichinde virus infection in MHA strain hamsters (227). Aggressive destruction of the reticuloendothelial system is a common feature of highly virulent arenavirus infections.

Termination of the LCMV Carrier State in Mice

Adoptive transfer of antigen-primed splenic T cells or cytotoxic T-cell clones is sufficient to terminate the LCMV carrier state (5,10,234). Both T-cell-subset depletion with monospecific antibody and analysis of the phenotypes of T-cell clones used in adoptive transfer experiments establish the CD8+ lymphocyte as the critical cell for virus elimination. This is further supported by the requirement for gamma interferon and for perforin, the T-lymphocyte component responsible for perforating membranes of target cells, in virus clearance.

Clearance is rapid after CTL are adoptively transferred, and initially there may be necrotic lesions and lymphoid infiltrates, presumably reflecting the cytolytic activity that is readily demonstrated *in vitro*. Different organs follow different kinetics of virus clearance; virus is cleared from the liver within 30 days, but neurons contain viral antigen for 90 days or more, and clearance of virus and antigen from the kidneys requires more than 200 days. Mechanisms of virus clearance from neurons are not well understood. As neurons lack MHC class I antigen, they are refractory to CTL lysis (161). Potential alternative clearance mechanisms include the action of cytokines and nitric oxide on persistently infected cells.

Virus-Induced Loss of Cellular Function

Although arenavirus infection is not overtly cytopathic in most tissues, evidence is mounting that the LCMV infection results in compromised function of specialized cells of the neuroendocrine system. Concentrations of the enzymes choline acetyl transferase and acetylcholine esterase are lowered in LCMV-infected neuroblastoma cells in culture (235). LCMV infection of the anterior pituitary of mice results in diminished synthesis of growth hormone without discernible cell death, but with a concomitant clinical syndrome consisting of retarded growth and hypoglycemia (236). Impairment of specialized cellular functions has been referred to as disruption of “luxury functions”—that is, functions not required for survival at the cellular level but that contribute to maintaining homeostasis of the organism. Similar observations have shown that LCMV can impair function of the insulin-producing beta cells of the pancreas and thyroid cells (173). Selective disruption of growth hormone release in a rat pituitary cell by virus infection is caused by interference with the growth hormone transactivator, GHF-1 (75). Treatment of infected cells with the antiviral agent ribavirin cures infection and restores normal growth hormone messenger-RNA levels.

Mice persistently infected with LCMV have extensive neuronal infection demonstrated by immunohistochemistry, but they have no evident lesions. These animals exhibit behavioral abnormalities, including decreased exploratory behavior when confronted with a new environment, deficits in Y-maze discrimination, and hypoactivity in locomotor testing (115,136). Low-dose scopolamine produces locomotor hyperactivity in the virus-infected group, suggesting a disruption of cholinergic function.

LCMV-Induced Ocular Disease

LCMV causes a destructive retinopathy following intracerebral inoculation into the newborn rat (224). Disorganization of the outer nuclear receptor layer is followed by loss of cells in the inner nuclear and ganglion cell layers, and finally the total disorganization and destruction of the retina. Infection is evident first in the optic nerve and inner nuclear layer, then in the pigment epithelium, and finally in all layers of the retina. Congenital retinitis occurs in offspring of LCMV-infected females and is accompanied by inflammation, macrophage infiltration, and eventual retinal detachment. In both neonatal and congenital infection, retinitis is diminished or prevented by immunosuppression, implying an immunopathologic response underlying the disease process. In the congenital model, retinitis persists in the absence of detectable viral antigen, suggesting that the retinitis can be viewed as a vertically transmitted autoimmune disease (80). Chorioretinitis and psychomotor retardation have also been reported in children with congenital LCMV infections (2,27,274).

LCMV-Induced Cerebellar Disease in Rats.

LCMV infection of 1- to 7-day-old rats interferes with normal cerebellar development (221,223). Intracerebral inoculation of LCMV (E350 strain) produces nonfatal but severe and permanent cerebellar hypoplasia and ataxia. Virus persisted for up to 4 months after infection. Evidence of an immunopathologic mechanism of disease was provided by demonstrating that antilymphocyte serum prevented cerebellar necrosis and resulted in higher brain virus titers than in control rats (222).

Ultrastructural studies of the infected rats revealed viral particles in the interstitial spaces of the apparently normal neural parenchyma at 5 days, followed by extravasation and infiltration of activated macrophages and concomitant cellular necrosis 1 to 3 weeks after infection. Several weeks after infection, an infiltration of plasma cells is accompanied by a gradual removal of viral antigen from the tissue (81). The late appearance of plasma cells in the brain tissue and concurrent disappearance of viral structures suggest that local antibody production is linked to viral clearance from

neurons.

Passive transfer of rat monoclonal antibodies to LCMV can block the cerebellar disease (22). Antibody given to the offspring as late as 48 hr after virus infection inhibits cerebellar hypoplasia. Offspring nursing on mothers who had previously received protective antibodies were also protected. Antibody substantially lowers viral titers in the brain and prevents infection of the cerebellar granular and Purkinje neurons. Cerebellar disease in the rat can also be prevented by injection of high concentrations of DI LCMV particles (102), perhaps due to homologous interference as there is no evidence of interferon activation.

Infection of the 4-day-old rat affects other sites as well. Persistent hippocampal abnormalities, consistent with a dysinhibition of gamma-aminobutyric acid (GABA)-ergic pathways in the dentate gyrus for long intervals, are observed after recovery from neonatal infection (242).

Hamster Infection

Syrian hamsters are susceptible to infection with Pichinde and LCMV. Strains of hamsters vary in susceptibility to Pichinde virus infection, with adults of the MHA strain being highly susceptible and those of the LVG strain being resistant. Peak serum virus titers in MHA animals are greater than 10^8 at 8 days after infection, and less than 10^3 in LVG animals. Cyclophosphamide immunosuppression of LVG hamsters converts them to an MHA-like susceptibility, suggesting that the differential observed is caused by a lack of effective immune response in the MHA strain. Major destructive effects of the infection in MHA animals are in liver and spleen. Primary target cells are macrophages in the marginal zone of the splenic white pulp, and Kupffer cells in the liver. Lesion sites lack mononuclear inflammatory cells, suggesting a destructive effect on the reticuloendothelium (227). Studies by Gee et al. (109) suggest that the targeting of Pichinde virus in the MHA hamster may be attributable to differences in the natural killer cell response to Pichinde virus infection.

The MHA hamster is also susceptible and the LVG hamster resistant to the WE strain of LCMV (113). Hamsters resistant to WE are susceptible to acute disease after immunosuppression. Immunosuppression does not spare susceptible hamsters, in contrast to the effects on T-cell-mediated immunopathology seen in mice intracranially inoculated with LCMV. The Armstrong strain of LCMV is not pathogenic for any hamster strain, even with sublethal irradiation or cyclophosphamide treatment (112). None of the surviving animals develop any evidence of chronic infection; viremia is cleared by 35 days and organs were free of antigen by immunofluorescence.

The disease induced in MHA hamsters by WE is characterized by a prolonged viremia, severe wasting and weight loss, and death 2 to 3 weeks after inoculation (110). There are relatively few histologically evident lesions; both hamster strains have scattered perivascular and other lymphoid infiltrates as well as lymphoid hyperplasia of spleen and lymph nodes. Fatal disease is characterized by segmental inflammatory ileal hyperplasia. Most infected cells are macrophages, and the disease, including the intestinal lesions, may be largely lymphokine mediated. Hamster strains that were genetically resistant had slightly lower viremias on day 3 and a marked decrease in viremia around day 7 to 14, concomitant with the onset of the humoral immune response measured by IFA. IFA titers were similar in both surviving and dying hamster strains. Neutralizing antibodies became detectable in all hamsters around day 21 and in surviving strains reached an LNI of 3.0 by day 35. The major distinguishing feature of the hamster strains destined to survive WE infections is the presence of foot-pad delayed-type hypersensitivity responses beginning on day 5 and reaching a maximum on day 7 (111).

Infection of outbred hamsters has also been studied with LCMV isolated from an infected transplantable tumor. The disease resembles that described in virus-contaminated hamster colonies that led to human epidemics. One third of 3-week-old hamsters died of an intestinal illness during the first month after inoculation; the remainder appeared healthy and required 1 to 3 months to clear viremia, but some were viruric for more than 6 months. Newborn hamsters fell into two groups: About one half appeared healthy but had prolonged viremia and high-titered viruria for 4 to 6 months. The other half were obviously diseased with poor weight gain, ruffled fur, blepharitis, and hunched posture. The diseased animals had much higher viremias and prolonged viruria. CF antibody responses were noted in both groups but were higher in the healthy animals. The wasted animals had histologic evidence of vasculitis and developed immune complex glomerulonephritis with renal failure. Congenital infection resulted in decreased litter size and a similar wasting disease, but it was possible to breed three generations of congenitally infected hamsters.

LCMV infection of the mouse has been the preeminent model for viral pathogenesis over the past 40 or more years. By manipulating the host age and genetic background as well as the strain of virus, the infection can be used to study mechanisms of virus clearance, virus persistence, and autoimmune disease in the mouse. Indeed, many of the fundamental concepts of modern cellular immunology have been formulated on the basis of investigation of the LCMV model. Useful models of viral pathogenesis are evident in studies of other species as well. Notably, LCMV infection of the rat may provide the most accurate paradigm for the neuropathologic consequences of congenital and neonatal infection in man, and infection of guinea pigs and monkeys has provided insight into the pathobiology of arenavirus hemorrhagic fevers.

Viral Persistence in Natural Rodent Hosts

Relatively few arenavirus experiments have been performed in the natural rodent host except with LCMV. Indeed, most studies with LCMV, however enlightening for basic immunobiology, have been designed to elucidate specific principles and apply to laboratory-selected virus strains and specific inbred mouse strains. Available data from other virus–host combinations (Table 3) show a complex pattern of infection.

Virus	Age of host at infection and subsequent viremia[a]		
	Fetal	Neonatal	Adult
LCMV, Lassa	Lifelong	Lifelong	Transient
Junin, Machupo	Fetal death	Lifelong	50% lifelong 50% transient
Guanarito	Unknown	Lifelong	>2/3 lifelong
Latino, Tamiami	Unknown	Transient	Transient

[a]Regardless of viremia, prolonged urinary virus excretion may occur and may be epidemiologically significant.
LCMV, lymphocytic choriomeningitis virus.

TABLE 3. *Duration of viremia after infection of rodent reservoirs with arenaviruses*

Lassa virus, like LCMV, induces chronic viremic infection in neonatal *Mastomys* and transient, immunizing infection in adults (228). This type of infection, which is efficiently transmitted to the fetus, would favor a maintenance cycle in which congenital, vertical transmission is the major feature. The agents of Bolivian and Argentine hemorrhagic fever can also induce chronic viremic infection in adult *Calomys* hosts, but the chronically viremic females are sterile (265,301,312). Finally, Tamiami and Latino have not induced persistent viremia in laboratory studies, although the possibility of mismatch between virus isolate and colonized host genotype might be responsible. It is also possible that maintenance strategies of these viruses result mainly in chronic viruria, with respiratory or salivary shedding (Fig. 6). For example, in spite of clearance of Tamiami virus from the blood of *Sigmodon hispidus*, and the decline in viral infectivity in organ homogenates, abundant viral antigen persisted in tissues for over a year in one study, and viruria was found for at least 28 days in another (151). The impact of these differences in host pathogenesis on the survival of the virus in wild rodent populations is clearly considerable (see Epidemiology section later) and the evolutionary significance of the different host adaptations is unknown.

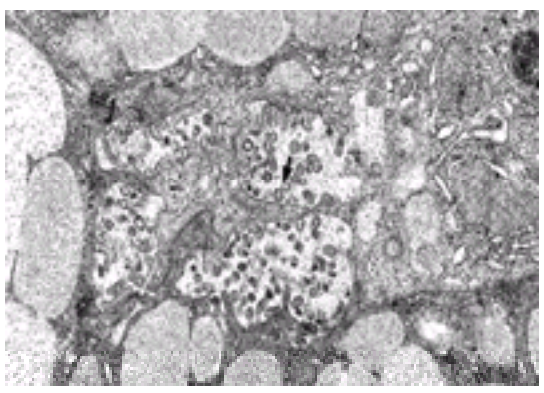

FIG. 6. Accumulation of Machupo virus (*arrow*) budding from acinar cells of the salivary gland of a virus-infected *Calomys callosus*; abundant virions in the lumen readily contaminate saliva (×22,000). (Courtesy of F. A. Murphy.)

The determinants of persistent viremia in the mature host are unknown, although a host genetic component has been identified. For example, selective breeding experiments in *Calomys callosus* identified a polygenic inheritance of the persistent viremic response following adult inoculation of Machupo virus (312). Viral genes are also important. The passage of either Machupo or Junin viruses to attenuate them for man has resulted in loss of the ability to induce chronic viremia in, and the appearance of pathogenicity for, their natural *Calomys* host.

There is a remarkable rodent species specificity seen among arenaviruses in nature. There is usually only a single major reservoir host. Amapari virus appeared to be an exception, with multiyear field observations in Brazil clearly suggesting chronic viremia and participation in maintenance for both *Oryzomys capito* and *Neacomys guianae* (251). However, closer examination of the viruses revealed that the *Orzomys* virus was indeed a different arenavirus, Cupixi, (M. D. Bowen et al., unpublished data). Similarly, initial field studies of Guanarito virus in Venezuela suggested that *Sigmodon alstoni* was the major reservoir (289), but laboratory infections of adult *Zygodontymys brevicauda* produced chronic viremia (107). This prompted a reexamination of the field situation; Guanarito virus was found to be a parasite of *Z. brevicauda* (106) and it was confirmed to be the cause of Venezuelan hemorrhagic fever (308). Viral antibodies and isolates from *Sigmodon alstoni* were related to an independent arenavirus species, Pirital virus (105).

Nonreservoir rodents may on occasion develop chronic infection and viruria, such as is seen after Machupo virus infection of hamsters or Lassa virus infection of inbred mice, but field data strongly support the concept of virus–natural host specificity (64). In the case of LCMV infection of the golden Syrian hamster, it does seem that under colony conditions virus infection can be maintained in the breeding population through viruria and result in dissemination of infected animals to the public (133).

The impact of chronic viral infection on the rodent is an unresolved issue. Most studies of LCMV have suggested that naturally occurring chronic viremic infection does not affect most aspects of the wild mouse's reproductive fitness, although some decrement in litter size may occur in carrier colonies and some transient effects have been found when virus is first introduced (179,276),. Neonatally infected reservoir hosts of Machupo and Junin viruses have somewhat retarded growth and in the case of Junin show an increased mortality; females reach adulthood with markedly decreased fertility. Pathologic lesions clearly attributable to the chronic arenavirus infection have been insignificant or found only in

studies of laboratory-adapted strains of LCMV inoculated into selected mouse strains (296).

Animal Models of Arenavirus Hemorrhagic Fevers

The strong evidence for immunopathogenesis of LCMV neurologic disease after i.c. inoculation of mice is not reflected in the arenavirus hemorrhagic fevers. Unfortunately, there are no realistic murine models that can be exploited, and most experimental studies rely on hamsters, guinea pigs, or nonhuman primates (245). Histologic evidence of tissue damage is minor, although viral antigens are often found in many apparently normal macrophages and parenchymal cells. The extensive T-cell infiltrates seen in murine LCMV are not present, and immunosuppression leads to fatal disease rather than prevention of disease (112,167).

Pichinde virus can be adapted to infect strain 13 guinea pigs with a virologic and pathologic picture that resembles that of human Lassa fever. Because Pichinde virus does not cause disease in man, this model has been exploited for data not easily obtainable from the more pathogenic viruses. After infection, the animal appears normal for approximately 7 days, and then fever and weight loss are seen. During this period, viremia is just beginning and most infected cells are macrophages. With time, macrophage infection becomes more abundant and epithelial cells of several organs begin to show infection. Interestingly, although arenaviruses infect endothelial cells readily in cell culture (11), there is relatively little involvement of endothelial cells *in vivo*, suggesting that indirect effects may be responsible for the increased permeability seen in patients and to some extent in this model. Few dramatic histologic lesions occur; there may be focal necrosis in liver and adrenal glands, mild interstitial pneumonitis in the lung, marginal zone necrosis in the splenic white pulp, and an unusual intestinal lesion with villus blunting. Immunohistochemistry shows that the degree of infection in the liver and adrenal is usually far more extensive than the mild areas of inflammation and necrosis visualized histologically. Furthermore, the intestinal lesion is not associated with infection of the epithelial cells themselves, but rather with extensive infection of the abundant underlying mucosal macrophages (68). Thus, the intestinal involvement may be similar to that seen in other conditions in which lymphokine secretion is thought to play an important pathogenic role; both tissue necrosis factor (TNF) and interleukin (IL)-6 are produced by macrophages from infected guinea pigs (16). The combination of TNF secretion and the intestinal lesion presumably accounts for the wasting, which reaches 25% of body weight (145,245). Viral antigen is extensively present in the perifollicular areas of the spleen and is presumed to be related to the failure of these animals to make a protective immune response either directly through infection or indirectly through an immunopathologic mechanism (323).

In contrast to the relatively modest pathologic lesions at the light microscopic level, there are extensive physiologic changes in the cardiovascular system, which are thought to lead to the death of the animal. There is a progressive decrease in cardiac index from 2 to 1 L/min/m^2 (an indication of the amount of blood pumped by the heart each minute, normalized to the body surface area), which is compensated for by a rise in the peripheral resistance to maintain the blood pressure until the last stages of infection around day 14 to 16 (253). The cause of this fall in cardiac index is not viral infection of the heart; extensive immunohistochemistry failed to detect viral antigens in cardiac muscle using the same techniques that readily found antigen in other organs. The cause seems to be related to the secretion of soluble mediators that decrease heart function, such as TNF-a (16), leukotrienes (189), platelet-activating factor (254), or endorphins (123). Abnormalities are also seen in catecholamines and serotonin (124). These changes, taken with the evidence of lymphokine secretion (16), suggest that the shock state here, and presumably in human arenavirus disease, has its origins in similar alterations of metabolism as seen in the septic shock syndrome (36). The pattern of mediators must be different, however, because the cardiac output is consistently low in Pichinde-infected animals but high in early septic shock. Interestingly, the cardiac output is low in lethal disease caused by hantaviruses, such as the newly described hantavirus pulmonary syndrome (88).

Significant pulmonary functional compromise is present as well. There is increased airway resistance, particularly late in the disease and in younger animals (122,253,272). This is associated with mild pulmonary edema, but it is primarily attributable to accumulation of lymphocytes near infected macrophages, or to bronchial epithelium in or near small bronchi. This would be an important element in the final collapse of the animals.

ARENAVIRUS INFECTIONS OF HUMANS

LCMV infections in humans are rarely fatal. Two fatalities resulting from overwhelming systemic disease resembled hemorrhagic fever cases (see later). A single well-studied encephalitis case showed mononuclear cell infiltrates in the meninges and around vessels and glial nodules in the deeper structures (307). Fluorescent antibodies detected viral antigen in meninges and in cortical neurons.

The gross and light microscopic lesions found at postmortem in arenaviral hemorrhagic fever patients rarely account for their demise (305). Petechial hemorrhages of the skin and internal organs, cutaneous ecchymoses, and evidence of mucosal bleeding are common. Serous effusions, interstitial pneumonitis and pulmonary edema, and evidence of secondary bacterial infection are sometimes seen. Microscopic evidence of disseminated intravascular coagulation or vasculitis is uncommonly found and is not a regular feature of these diseases. Adequate studies of vascular endothelium are not available.

Focal necrosis may be present in several organs including liver, kidney, and adrenal cortex; fluorescent antigen tracing or virus titration generally correspond to these lesions (197,304), as they do in experimental animal studies (68). These lesions usually contain a few mononuclear phagocytes as the only inflammatory cell contribution. Lymphoid depletion or necrosis in the marginal zone of the splenic periarteriolar lymphoid sheath are also present (304). Secondary changes associated with shock are usually found and may be responsible for the frequent presence of some degree of myocardial damage or myocarditis, but virus has not been detected in the limited cardiac tissue studied (245,304).

Hemorrhages are common in the South American hemorrhagic fevers, in keeping with the low platelet counts. Thrombocytopenia is probably caused, at least in part, by maturation arrest of the megakaryocytes attributable to the high levels of interferon present in these patients (184). In Lassa fever, thrombocytopenia is less marked, but liver

involvement is more extensive. Although death does not appear to be caused by hepatic failure, the liver is an important site of virus replication and the levels of aspartate transaminase (AST) in serum are proportional to the risk of dying (157,205).

Clinical Features

Arenavirus diseases begin with a gradual onset of fever and myalgia, followed by systemic and hematopoietic cell abnormalities. In the viral hemorrhagic fevers (VHF), a period of viremia lasting 1 to 3 weeks is accompanied by intense systemic symptoms; the occurrence of an effective immune response is concordant with clinical recovery and cessation of viremia. In LCMV infection, rare fatal cases have occurred with a pattern resembling that of VHF (279), but usually LCMV infection results in a febrile disease with accompanying viremia, often followed by CNS disease. During the CNS disease, there is no viremia, but virus is still detectable in cerebrospinal fluid (CSF), and the pathogenesis is thought to be immunopathologic.

Lymphocytic Choriomeningitis Virus Infections

The initial phase of LCMV infections is usually characterized by fever, myalgia, and other constitutional symptoms such as prostration, headache, photophobia, nausea, or vomiting, with occasional involvement of the testis or salivary gland (3,30,33,34,78,89,178,185,298). Leucopenia, thrombocytopenia, and sometimes mildly elevated AST or lactate dehydrogenase are expected during this period. Cough and chest pain may occur, and sore throat or mild abnormalities of the chest radiograph are occasionally found; indeed, LCMV cases have been serologically detected among patients examined for atypical pneumonia (216). Rash, arthralgia, and arthritis also are reported but are not common. Rarely, myocarditis has been attributed to LCMV infection (79,290).

Although LCMV is usually thought of as a viral meningitis, deeper neurologic involvement is manifest in a minority of cases, perhaps 10% or fewer in naturally observed outbreaks that provide some possibility of case-ascertainment (78,133,298). If a febrile prodrome is present, meningeal signs usually begin about 10 days later, often after a brief hiatus in symptoms and fever (Fig. 7). Encephalitis has been diagnosed in 5% to 34% of hospitalized patients with documented LCMV (4,97,211). Full recovery is usual, although occasional deaths do occur.

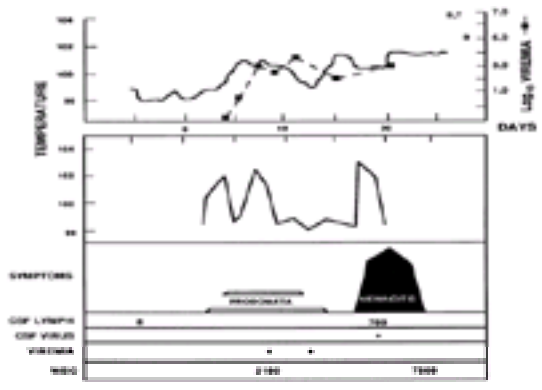

FIG. 7. Probable pathogenesis of human LCM. At the *bottom*, results of inoculation of a normal human volunteer with LCMV are shown (95). These and other human data (34,298) show an initial period of 1 to 2 weeks' viremia associated with fever, leucopenia, and systemic symptoms. This is followed by the onset of the cellular immune response with meningeal inflammation leading to mild transient CNS disease. Virus was isolated from the CSF. In the *top panel*, the course of infection in a patient with Hodgkin's disease, immunosuppressed by disease and chemotherapy, is shown. (Adapted from ref. 134, with permission.) LCMV was inoculated in hopes of favorably influencing the underlying disease. Viremia continued as long as measured; no IFA, CF, or neutralizing antibodies developed; and there was no overt CNS disease. At the time of death, possibly secondary to bacterial pneumonia, there were high titers of virus present in brain (*B*), spleen (*S*), and tumor tissue (*T*).

The clinical laboratory abnormalities at the time of CNS involvement are usually confined to the CSF, which shows an increase in cells into the hundreds or low thousands, predominantly mononuclear in type. Protein is usually modestly elevated (50 to 150 mg%) and sugar may be low in a fourth of the patients, sometimes reaching values below 20 mg/dL (4,33).

Rare neurologic presentations attributed to LCMV include ascending myelitis, transverse myelitis, and bulbar paralysis (4). The course of acute LCMV has also been complicated by hydrocephalus. One case was thought to have posterior fossa arachnoiditis and was treated with ventriculomastoidostomy (291). Another was transient (133), and a third required the placement of a ventricular reservoir (177). The latter case had evidence (by computed tomography) of periventricular edema and lateral ventricular enlargement, suggesting that the major lesion was ependymal inflammation. Both ependymal and meningeal inflammation are prominent lesions in experimental infections and in fatal LCMV encephalitis (307). Nerve deafness has been associated with LCMV infection in two cases (60,129,132). The occurrence of eighth nerve involvement in another Old World arenavirus disease, Lassa fever, suggests that these observations may represent a true causal relationship.

Lassa Fever

Infection with Lassa virus leads to the gradual onset of fever and malaise after an incubation period of about 10 days (range, 5 to 21 days). As the process develops, there is an increase in fever and myalgia, with severe prostration. Gastrointestinal manifestations such as abdominal pain, nausea and vomiting, diarrhea, or constipation are common.

Sore throat occurs in two thirds of cases and is usually accompanied by objective inflammatory or exudative pharyngitis. Retrosternal pain and cough are frequent, and pleural effusions may develop. Bleeding manifestations are seen in less than a third of patients but signal an unfavorable prognosis. Signs of increased vascular permeability such as facial edema or pleural effusions are present in a minority of patients and also suggest a poor prognosis. Mortality in hospitality patients is 15-20%.

A careful case-control study comparing Lassa fever to other febrile diseases seen in a west African hospital found several features significantly associated with Lassa fever, including bleeding, edema, exudative pharyngitis, conjunctivitis, and pharyngitis, but the positive predictive values were only 0.61 to 0.74 (204). The same study also found vomiting, sore throat, tachypnea, or bleeding to predict a 2.5-fold or higher increase of risk of death. In spite of the relative nonspecificity of the clinical findings, more than three fourths of patients thought to have severe Lassa fever are confirmed virologically. Lassa fever is a major pediatric problem as well (279,280). Disease is more difficult to diagnose clinically. Occasional cases of infants developing anasarca have been described.

The course of fatal Lassa fever is relentless, with progression of the signs and symptoms culminating in the onset of shock and death. In survivors, symptoms and viremia persist until, 2 to 3 weeks after onset, there is defervescence accompanied by the disappearance of virus from the blood. Pericarditis may occur in early convalescence, particularly in male patients. A case of polyserositis and recurrent pericarditis with constriction has been reported, which suggests that such complications should be sought more carefully (131).

Neurologic disease is not usually a dominant clinical manifestation in Lassa fever, but aseptic meningitis, encephalitis, global encephalopathy with seizures, and more subtle neurologic problems are well described (71,281,282). Cerebellar ataxia in convalescence is an uncommon but interesting occurrence. In convalescence, deafness is common; this is an important feature of Lassa fever, as it impacts on the community, and it provides an important diagnostic clue (100). Late in the course of disease or early in convalescence, unilateral or bilateral hearing loss was noted in 29% of prospectively studied patients (72). No treatment is available and the effects may be transitory or often permanent. The auditory patterns and clinical course resemble idiopathic nerve deafness (187).

The clinical laboratory provides few clues to the diagnosis. The leucocyte count can be low, normal, or modestly elevated. Platelet counts are generally normal but may be modestly decreased (98). Albuminuria is common. AST is usually at least mildly elevated and the degree of elevation, which parallels the viremia, is a useful predictor of mortality (157). Patients with AST values in the hundreds or thousands are at considerable risk of dying, even with ribavirin treatment. Chest radiography may show infiltrates, pleural effusions, or, more commonly, no abnormalities. Electrocardiographic (ECG) findings are often nonspecifically abnormal (70).

Argentine Hemorrhagic Fever

AHF begins insidiously with fever and malaise. Headache, myalgia, epigastric pain, and anorexia are common. Within 3 to 4 days, these symptoms give way to more severe prostration, nausea and vomiting, dizziness, and early indicators of vascular damage. The earliest signs are conjunctival injection, flushing over the head and sometimes upper torso, petechiae, and mild hypotension. Tremor of the tongue and hands is common and may resemble cerebellar disease. In the more severe cases, mucous membrane hemorrhages and ecchymoses at needle puncture points develop; shock supervenes and, with the hemorrhages, is an indicator of a poor prognosis. In some cases, there are extensive neurologic signs, including coma and convulsions, and these cases also have a high mortality (263). Usually around day 10 to 13, patients begin to improve as the appearance of IFA and neutralizing antibodies signals the onset of the immune response (74). Overall case fatality is 30% without therapy. Convalescence often lasts several weeks with fatigue, dizziness, and hair loss.

Clinical laboratory findings are quite helpful in the diagnosis of AHF. Leucopenia, thrombocytopenia, and proteinuria are present in virtually all patients with the disease and may be early manifestations. AST and alanine transaminase are usually normal or slightly elevated. The ECG may be nonspecifically abnormal, and chest radiography is usually normal in the absence of secondary infections.

Bolivian and Venezuelan Hemorrhagic Fevers

Bolivian hemorrhagic fever (BHF) resembles AHF clinically, just as the virus is closely related phylogenetically to Junin virus (154,287). Mortality is about 25% to 35%. Early information from the San Joaquin epidemic suggested that most Machupo virus infections resulted in disease sufficiently severe to be remembered as BHF (192), but a recent survey in the endemic region of the Beni has found ELISA IgG antibodies in persons with no previous history of BHF (C. J. Peters and T. G. Ksiazek, unpublished observations).

Venezuelan hemorrhagic fever also has a clinical spectrum similar to that of AHF, with thrombocytopenia, bleeding, and neurologic involvement being prominent (76,266,297). Sore throat and pharyngitis were noted with some frequency among initial cases, and deafness has occurred in convalescence. Although reported cases have had a somewhat higher mortality (34%), it seems likely that with increased case-finding there may be milder cases detected. Normal inhabitants of the endemic area have antibody prevalence rates of 0.1% to 3%, and household contacts, 10.5%.

Sabia Virus Infection

Sabia virus was isolated from a fatal case of hemorrhagic fever presenting in Sao Paulo, Brazil, in 1990 (188). Because of the extensive liver necrosis associated with the clinical presentation, yellow fever was a leading diagnosis, but it was excluded virologically. Later, the virus was shown to be an arenavirus, and a laboratory worker performing the studies developed severe disease resembling AHF (299). This virus once again infected a virologist in 1994, resulting in a febrile, leucopenic, thrombocytopenic syndrome that was promptly treated with intravenous ribavirin. There was an apparent clinical and virologic response. The reservoir remains unknown; it is presumed to be a rodent found around the

small community of Sabia outside Sao Paulo, where the only known natural infection occurred. Without additional information, it must be assumed that this virus has a high morbidity and mortality as well as the aerosol infectivity expected from an arenavirus.

Whitewater Arroyo Virus

Whitewater Arroyo (WWA) virus was first described as a zoonotic persistent infection in woodrats (*Neotoma albigula*) in New Mexico (104). This and related reservoirs are widely distributed throughout the southwestern United States. The virus is most closely related to Tamiami virus, an arenavirus originally found in cotton rats (*Sigmodon hispidus*) in South Florida (56). Little evidence for human infection or disease with either of these North American arenaviruses was found until the period from June 1999 to May 2000. Two fatal cases with a clinical picture suggesting acute hemorrhagic fever were shown to have preliminary evidence of acute WWA virus infection (54). Confirmation of these findings and a search for additional cases are underway.

Other Arenavirus Infections

Most arenaviruses are infectious for humans based on surveys of potentially exposed laboratory workers and residents of endemic areas. In most cases, actual infections have not been observed for their clinical effects, so information about the consequences is relatively limited. The newly described Argentine arenavirus, Oliveros, provides an example (213). This virus circulates in the same agricultural zones as Junin virus and LCMV, but *Bolomys obscurus* is its natural reservoir. Even though these rodents comprise about 4% of the captures during longitudinal studies of small mammals in agricultural areas, and one fourth are infected, no antibodies were detected in 200 normal residents of the area or in 100 patients suspected of having AHF but found to be seronegative to Junin virus. Nevertheless, it remains an open question whether this virus might not be pathogenic for humans.

Laboratory workers provide another source of information concerning arenavirus pathogenicity, because these viruses are quite infectious as small-particle aerosols. Pichinde virus has caused a number of laboratory infections without any overt disease (46). Flexal virus has resulted in two symptomatic laboratory infections and should be regarded as potentially dangerous. Tacaribe virus has also resulted in a single example of febrile disease with mild CNS symptoms (J. Casals, personal communication, 1980).

Little is known about the pathogenicity of Old World arenaviruses other than LCMV and Lassa virus (65,118). They are usually assumed to be less pathogenic for humans than Lassa virus, based on their low virulence for monkeys and guinea pigs (245,303). They are studied at biosafety level (BSL)-3, but with additional precautions to avoid aerosols of the substantial amounts of virus shed from infected animals.

Maternal and Fetal Infections

Several arenaviruses are known to infect the fetus of the natural host when pregnant rodents are viremic. In some cases, this results in no damage to the developing embryo, but in other situations there may be fetal death (see Table 3). Thus it is not unexpected that maternal infections of nonreservoir rodent species may also lead to fetal infections. The extent of fetal invasion in infected humans is surprisingly high as well, and the impact on the fetus is often severe (Fig. 8). LCMV infection of the fetus has been associated with both fetal death at early stages of development and fatal neonatal infection (175). Sheinbergas and his colleagues found an association between maternal infection with LCMV and neonatal hydrocephalus with chorioretinitis in Lithuania (275); isolated cases have also been also reported from Germany and France (62).

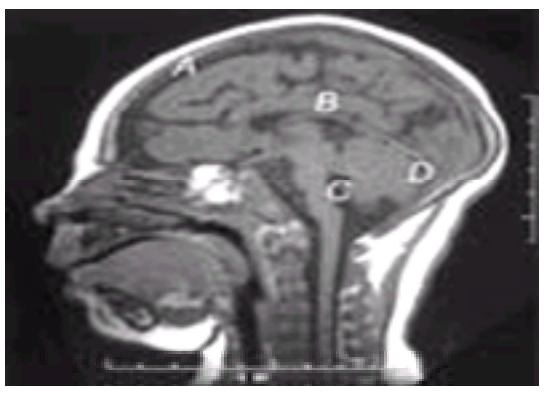

FIG. 8. T1-weighted magnetic resonance imaging (MRI) scan of the brain of a 10-month-old child with congenital LCMV infection. The child has severe developmental delay, spastic quadriparesis, and medically intractable epilepsy. The MRI scan reveals microcephaly, a lissencephalic frontal lobe (*A*), and hypoplasia of the corpus callosum (*B*), the brainstem (*C*), and the cerebellum (*D*). (Courtesy of D. Bonthius, University of Iowa.)

It is becoming increasingly apparent that LCMV is an important cause of fetal abnormalities in the United States. One report described the unusual occurrence of this syndrome in twins (27). Seven additional cases have been reported, primarily from the midwestern and southwestern states (28,32,318). The emerging picture from recent observations in the United States and Europe is of a congenital infection that follows symptomatic acute febrile disease or asymptomatic infection of the mother in mid-to-late pregnancy. The fetus is severely affected with combinations of psychomotor retardation, microcephaly, hydrocephaly, visual loss, and seizures, and it usually has evidence of aqueductal stenosis, periventricular calcifications, and chorioretinitis. The infants are not known to be chronically infected but may have a somewhat unusual antibody pattern (275). Any child with CNS or ocular disease who does not have clear-cut evidence of

intrauterine infection with one of the TORCH pathogens (toxoplasmosis, rubella, cytomegalovirus, herpes) should be evaluated for LCMV infection.

Lassa virus also causes unusually high fetal mortality. Gravid women have been recognized to have an increased risk of death from Lassa fever, and prospective studies have shown that this is particularly pronounced—30%—in the third trimester, compared to a 13% mortality in nonpregnant women (252). Fetal loss was 87%; all infants infected in the last trimester died *in utero* or during the neonatal period. Viremia, which is correlated with the risk of dying in Lassa fever patients, was higher in pregnant than in nonpregnant women. High concentrations of virus were found in fetal tissue, and in placenta as well (304). The biologic basis for these findings is unknown, but it seems likely that, once infected, the immature fetus is unable to mount an effective T-cell response to control the virus infection; maternal T cells would not be able to attack the placental infection because of the lack of MHC class I or II antigen expression on placental cells (140). Thus, the fetus and its supporting tissues would be a source of high-level virus production.

Pregnant women in the last trimester are also at an increased risk of dying from AHF, and the outlook for the fetus is grave (43). Anecdotal data for BHF also suggest that Machupo virus is abortogenic (86,154).

Differential Diagnosis

Lymphocytic Choriomeningitis

Lymphocytic choriomeningitis is probably greatly underdiagnosed because it is not sufficiently appreciated and because of the cumbersome methods of laboratory diagnosis formerly available (149). It is important to establish the diagnosis to prevent treatment for other conditions that can be confused with LCMV, and to signal the need to eradicate the virus-carrying rodents from the place of infection, particularly with the threat of maternal–fetal transmission. The diagnosis is often suggested by a history of pet rodent contact, but only during the hamster-borne epidemics of the 1970s was this commonly the actual source of LCMV. Most human LCMV infections are acquired from wild mice, and mouse exposure may not be noted in the typical clinical history. Useful clues to LCMV as a cause of aseptic meningitis include fall or winter season, a history of a distinct prodromal febrile illness, CSF cell counts that are unusually high compared to those of many other causes of aseptic meningitis, and hypoglycorrachia. Other clinical syndromes that should lead to a suspicion of LCMV infection would include nonbacterial orchitis, nonbacterial parotitis not thought to be caused by mumps virus, sudden-onset deafness, or fever, thrombocytopenia, and leucopenia.

Lassa Fever

Lassa fever should be suspected in virtually any febrile patient returning from endemic areas of Africa, particularly after a rural sojourn in Nigeria, Sierra Leone, Liberia, Guinea, or another west African country. Within a few days of onset, classical Lassa fever should progress to prostration and the other symptoms previously described, often including pharyngitis, retrosternal pain, gastrointestinal complaints, and proteinuria. However, mild or atypical cases can be diagnosed or excluded only with appropriate laboratory tests. Differential diagnoses should include malaria, shigellosis, leptospirosis, rickettsial disease, relapsing fever, and typhoid; if doubt exists, these entities should be treated empirically.

South American Hemorrhagic Fevers

Any patient from known endemic areas in South America should be under suspicion if fever, dizziness, and myalgia are accompanied by leucopenia, thrombocytopenia, and proteinuria. The relatively frequent discovery of “new” arenaviruses in the Americas implies that suggestive findings in patients with rural exposure or rodent contact anywhere in the Americas should lead to the consideration of arenavirus disease. Influenza, hantavirus pulmonary syndrome, and other febrile, myalgic diseases, as well as the more severe conditions noted for Lassa fever, enter into the differential diagnosis.

Laboratory Diagnosis

Lymphocytic Choriomeningitis

During the acute febrile phase, virus isolation from blood is the best approach. Viremia may also be detectable during the meningitic phase, but then the CSF may be more productive (34,95). Most clinical isolates of LCMV will be lethal for intracranially inoculated mice or intraperitoneally inoculated guinea pigs, but some strains will require back-challenge with a known virulent LCMV strain, or antibody measurement to demonstrate the presence of LCMV in the original inoculum; mice can also be injected with endotoxin to provoke lethality (137). Isolation in cell culture (e.g., Vero cells) is less expensive, less hazardous, and less likely to suffer cross-contamination, but it is probably slightly less sensitive than animal inoculation. Polymerase chain reaction (PCR) tests applied to CSF have also shown promise (239). Antibody is often present in serum and/or CSF during acute cases, and ELISA and IFA for IgM have been widely used to detect it (77,97,181,186). Neutralizing antibodies appear late after onset and are technically demanding to demonstrate, lessening their diagnostic importance (4,13,181). CF tests are relatively insensitive and antibodies disappear quickly; high CF titers are thought to indicate recent infection. The optimal test for serosurveys needs to be established by careful comparison of the major candidates, ELISA, IFA, and neutralization.

Lassa Fever

Lassa virus is easily isolated from blood or serum during the febrile phase of the disease up to 14 or more days after onset, even after the appearance of IFA antibody. Virus can also be detected in necropsy tissues (102,157). Vero cell cultures examined by FA allow a diagnosis in 5 to 7 days or sooner. Lassa virus antigen can be detected by ELISA capture in serum within 4 hours of beginning testing, and as it becomes negative, IgM antibodies appear (31). Antigen detection by ELISA is robust and reliable in rapidly fatal cases, even if the specimens are not handled properly for virus isolation (31). Reverse transcriptase (RT)-PCR is also a sensitive test for virus RNA, being positive in the blood of 23 of

29 patients at admission and of 29 of 29 patients by the third day of hospitalization (82,293).

Antibody can be detected by CF, IFA, or ELISA. IFA using Lassa-infected Vero cells as substrate is widely used (319). Interpretation is subjective, and discrepancies between laboratories are common. IFA IgG “seroreversion” has been reported and thought to represent loss of antibody by previously seropositive individuals (206). Lassa IgG and IgM can also be detected by ELISA (31,146,232). ELISA IgM titers appear earlier and persist longer than IFA IgM titers. IgG ELISA antibody persists for long periods, whereas IFA antibody appears to wane below detectable limits within several years.

South American Hemorrhagic Fevers

Like Lassa virus, Junin, Machupo, Guanarito, and Sabia viruses are infectious by aerosol and the human and rodent specimens should be processed with appropriate precautions in BSL-4 laboratories. Virus can be isolated from blood or serum during the acute febrile phase until 10 to 12 days after onset by inoculation of newborn hamsters or mice (41). Inoculation of cell cultures (Vero, BHK-21) is safer and more easily performed under the required BSL-4 conditions. Co-cultivation of peripheral blood mononuclear cells with Vero cells seems to be more sensitive than suckling mouse or Vero cell inoculation for the isolation of Junin virus from AHF patients (7). Virus can also be isolated from necropsy tissues, except from the brain. In rodent studies, virus is readily isolated from blood, urine, throat swabs, and autopsy tissues (156,162). Antigen-capture ELISA allows detection of viral antigen in blood, serum, or tissue homogenates. These tests could be used for acute diagnosis of patients suspected of having Junin, Machupo, Sabia, or Guanarito, and they are often the first available diagnostic result in rapidly fatal cases when patients die before the appearance of antibody. Antigen detection is also possible in rodent urine, blood, and throat swabs during ecologic surveys (213).

Serologic diagnosis of AHF and BHF is usually made by CF (193,315) and IFA (201,249,321). The lack of specificity and/or sensitivity of these tests is often a major problem. The plaque neutralization test can distinguish between antibodies to Machupo and Junin viruses and is used for evaluation of convalescent plasma units intended for therapeutic use in AHF (93). The ELISA test is the most useful and practical for rapid detection of IgG and IgM antibodies in a clinical setting and seroepidemiologic surveys (108,143). The antibodies detected by ELISA persist more than 30 years in some cases. Guanarito and Sabia IgG and IgM antibodies can also be detected by IFA and/or ELISA.

Treatment

Medical Management

Supportive therapy is important in the management of patients with arenaviral hemorrhagic fevers (246,248). Avoidance of travel and general trauma, gentle sedation and pain relief with conservative doses of opiates, the usual precautions for patients with bleeding diatheses (such as avoiding intramuscular injections and acetylsalicylic acid), and careful maintenance of hydration are indicated. Bleeding should be managed by platelet transfusions and factor replacement as indicated by clinical judgment and laboratory studies.

Management of shock is difficult. Modest increases in hematocrit indicate a generalized vascular permeability problem, but not of the magnitude seen in diseases such hantavirus pulmonary syndrome (88); nevertheless, vigorous infusion of crystalloid carries a high risk of pulmonary edema. Cautious administration of fluids and early use of pressors is indicated. Because of the low cardiac output seen in the Pichinde guinea pig model of arenaviral hemorrhagic fever, and based on clinical experience with the human disease, careful monitoring is important and Swan-Ganz catheterization is desirable.

Containment

Patients with arenaviral hemorrhagic fevers are thought to pose a minimal risk of contagion in the early stages of disease. As the process continues, more extensive viral infection of target organs and increasing blood virus titers may increase the opportunities for dissemination. Nosocomial outbreaks and infection of multiple contacts have occurred, but they are distinctly uncommon and the index patient has generally been severely ill at the time of the incident (58,172,219,244). The most dangerous exposure is parenteral and must be avoided through staff training. Thus, patients with these diseases should be treated in mask, gown, and glove isolation. Protection to care givers and other patients should be enhanced by the addition of respiratory protection against small-particle aerosols (23,99,246). Close personal contacts should be monitored for fever for a period of 3 weeks. The patient may continue to excrete virus in urine or semen for weeks after recovery, so body fluids should be monitored for infectivity before the patient is released; meanwhile, a program of counseling emphasizing addition of disinfectant to toilets before use and protection of sexual partners should be followed. Special precautions are indicated when blood and other body fluids are handled in the clinical laboratory (92).

Antiviral Drugs

Although several compounds have shown *in vitro* efficacy, only the guanosine analog ribavirin has had practical application. Although the mechanism of action is unknown, all arenaviruses tested have been inhibited in cell culture by similar concentrations of ribavirin, and the drug is effective in all arenaviral hemorrhagic fever models in which it has been studied (144,145,150,163,190,207,245,280,284). Ribavirin-treated experimental infections with Machupo or Junin viruses have sometimes led to a late neurologic disease, but activity against the visceral form of the disease has been a uniform finding. The drug is efficacious in Lassa fever and is the therapeutic agent of choice in that disease. Ribavirin has been of use principally in Lassa fever patients with a poor prognosis, usually being reserved for those with an AST value above 150. Preliminary results with ribavirin have been positive in AHF (94). Anecdotal data on early treatment of human Machupo and Sabia infections have been promising (26,171).

Ribavirin has been considered for the prophylaxis of persons exposed to arenaviral hemorrhagic fevers. In experimental

studies of Pichinde- or Lassa-infected guinea pigs, prophylaxis for periods such as 7 to 10 days only delayed the onset of acute disease by a similar period of time. Howver, drug administration for 14 days prevented disease (190,280,284). In the case of Junin virus, prophylaxis of guinea pigs for 14 to 21 days prevented acute disease, but it did not prevent Junin virus entering the CNS and causing fatal encephalitis (163). Thus, high-risk exposures to arenaviruses are probably best managed by expectant treatment at the first sign of fever, rather than dealing with the uncertainties of prophylaxis and its small but significant risk.

Passive Antibody

Therapy of Junin virus infections with convalescent plasma has been carefully studied in humans and experimental animals and is quite efficacious, with reduction of mortality from 15% to 30%, to 1% to 2% if initiated within the first 8 days of illness (93,196). The administration of convalescent plasma (usually two to three units, depending on the neutralizing titer) is the treatment of choice. About 10% of these patients will return 3 to 6 weeks later with neurologic signs such as fever, headache, cerebellar tremor, and cranial nerve palsies. This late neurologic syndrome is usually self-limited. Serum antibody titers peak later and at a higher level, and serum-to-CSF antibody ratios are increased in comparison with those in patients without this syndrome, suggesting the prior invasion of the CNS by virus. Late eradication of virus by T cells may be a factor in its pathogenesis. The syndrome resembles other experimental arenavirus neurologic diseases seen after passive antibody therapy (91,165). The *in vitro* neutralization characteristics and limited studies in animal models suggest that passive antibody therapy would probably be useful in Machupo and Guanarito virus infections (91).

Lassa virus infections are more difficult prospects for antibody therapy, because the volumes of plasma needed based on animal studies are large; experimental studies of IgG for intravenous administration indicate that this could be a useful means of treatment only if selected, highly active preparations were available (147,150,245). The future of antibody therapy in any of these diseases lies in development of standardized monoclonal antibody preparations of proven efficacy (264,271). Experimental studies of passive protection by monoclonal antibodies have been successful in the LCMV model (20,21,317).

Central Nervous System Disease

Ribavirin penetrates poorly into the CSF acutely; no established antiviral therapy exists for arenavirus infection in the CNS. LCMV may cause acute hydrocephalus from ependymal infection and inflammation, leading to a need for surgical shunting (177). Steroids have not been evaluated in this condition. The late neurologic syndrome seen after immune plasma treatment of AHF is generally self-limited and should be treated expectantly (93).

Epidemiology

The epidemiology of arenavirus diseases depends on the patterns of infection in the reservoir hosts and on the factors that bring man in contact with the rodents. Only in the case of LCMV infection of the colonized Syrian hamster has a rodent host besides the natural reservoir been shown to be responsible for human infection and, in spite of occasional virus isolations, no arthropod or other intermediate has been implicated. Tacaribe virus was isolated from 11 of 398 fruit-eating bats in Trinidad. The isolations occurred over a 3-year period, but experimental infection of bats and later attempts to isolate the virus failed, so the actual reservoir remains in doubt (87).

Interhuman transmission is uncommon except with Lassa virus. Nosocomial spread thought to be through aerosols has been a rare but devastating occurrence with Lassa (219,220), Machupo (247), and Junin viruses (195). Recent examples of person-to-person transmission of Lassa (P. E. Rollin, personal communication, 2000) and Bolivian (T. G. Ksiazek, personal communication, 2000) hemorrhagic fevers emphasize the occasional tendency of these diseases to spread among contacts, sometimes in a manner suggesting aerosol transmission. All three viruses can be transmitted to sexual partners by convalescent men. Although the mechanism of transmission is not precisely known, Lassa virus has been isolated from semen 6 weeks after acute disease.

All arenaviruses form stable, infectious aerosols and have been important causes of laboratory infections and deaths. Human pathogens must be handled with care and the VHF viruses (Lassa, Junin, Machupo, and Guanarito) are manipulated under BSL-4 containment.

PATTERNS

In Rodents

Very few longitudinal field studies have succeeded in integrating laboratory observations on pathogenesis and virus excretion of arenaviruses into a coherent description of how viruses are maintained and spread within rodent populations (64). It is thought that most LCMV and Lassa virus infections of reservoir hosts are congenitally transmitted, maintaining a tight linkage between virus and host, with little opportunity for interruption in the chain of infection. Indeed, there is evidence that carrier LCMV dams may transovarially infect their offspring (217). Horizontal spread from infectious nasal secretions, saliva, or urine may occur by aerosols or contact with infected materials in the environment, although the most efficient mechanism for infection is thought to be bite (278,295). Oral mucosa, conjunctiva, or intact skin are not efficient portals of experimental LCMV infection. Horizontal infection of weanling mice would produce only a transient immunizing infection; contact of newborn mice with infected mice from outside the nest is unlikely in the wild, and mothers excreting virus from a previous immunizing infection would protect their offspring via maternal antibody (20,294). However, if there is some marginal loss of reproductive fitness in chronically viremic animals, as suggested for LCMV, there must be a method to generate a compensatory number of carriers. *Mus* habits are such that this could most readily be achieved by an infected male mating with an uninfected female, leading to a transplacentally infected litter of carrier mice (277). The large number of infected wild mice in Europe and the Americas make the possibility of infection of mouse

colonies a real concern (225,244,281).

In the case of Junin virus, the cycle is even more complicated. Neonatally infected carrier females are virtually sterile as adults, as is the situation with Machupo virus, minimizing their role in producing chronically viremic offspring (300,301,311,312). Carrier males may simultaneously inseminate and infect virus-negative females, leading to production of congenitally infected offspring of both sexes. It has also been suggested that adult-infected Junin carrier females may be fertile (300). In spite of the complexity of this situation, which has been hypothesized to lead to cyclic, fluctuating interactions between infection and rodent population density (159), the field data from a 2-year study in the endemic area of Argentina suggested that during that period considerable virus transmission among rodents was through aggressive encounters between adult males. This was facilitated by the concentration of the Junin virus reservoir species, *Calomys musculinus*, along fence lines and roadsides leading to a focally highly populated area (213,215).

What determines the distribution of arenaviruses within the range of the host rodent species? There are partial answers for LCMV and Junin viruses. The genus *Mus* arose in Asia and radiated to form several species, most notably for this discussion *M. domesticus* in western Europe and the Mediterranean basin, and *M. musculus* in central Europe and east to China (39). Both these species have been shown to carry LCMV over much of their range, and introduction of *M. domesticus* to the Americas in post-Colombian times has also brought the virus to the New World. There are reports of LCMV antibodies in Australian *M. domesticus*, and there is an isolate from Japanese mice in a port setting. Thus, LCMV has established a tight linkage with two closely related species and appears to be found wherever they occur. The virus is not evenly distributed within that range. For example, murine LCMV infection in Germany is focal and variable in different regions and correlates with the presence of human antibodies (1,35). Focality can be extreme because of the structured social and breeding system of mice; variations in infection prevalence can be seen within neighborhoods and even between nearby homes in the urban setting.

Junin virus was first discovered in 1959 as a consequence of an epidemic of the very characteristic disease AHF. Epidemics of several hundred cases have occurred each year since the initial recognition of AHF. The geographic distribution of the virus can be inferred from the distribution of cases, which has progressively extended from the initial focus around the town of Junin to an area in excess of 120,000 km^2 with a population of 3.5 million (194,199). This progressive extension has not been explained, nor has it reached the limits of the populations of its reservoir rodent, *Calomys musculinus.* Studies of virus distribution within and outside the endemic area have shown that there is a direct correlation with the prevalence of rodent infection (21 of 549, or 3.8% Junin antigen-positive inside and nil outside) (214,313). However, virus is not completely absent outside the endemic area, as occasional isolates have been made from the abundant *C. musculinus* populations in other regions of the Argentine pampas. Thus, it is unknown whether the high prevalence of infected rodents was a consequence of a change in the virus, a change in the rodent host, or some interaction between the two leading to a spreading disease problem.

The pattern seen with most other American arenaviruses in their hosts has been one of focality. The viruses are discovered and seem to be prevalent in a particular area (Table 1 and Table 2) and have not been found elsewhere, although systematic attempts to map them have been infrequent. The distribution of the African arenaviruses and the taxonomy of their rodent reservoirs is not well enough known to comment on the exact distribution of the viruses or changes in their activity.

In Humans

Lymphocytic Choriomeningitis

LCMV infection is widespread wherever *Mus domesticus* or *M. musculus* occur and are infected. In the United States, observations in the 1930s placed human antibody prevalence at 11% (12). More recently, prevalence in inner-city Baltimore was shown to be 4.7% and in Birmingham 4.3 to 5.1% (285). In Germany, rates of 9.1% were found in rural residents of the north and 1.2% in the south, where the prevalence of murine infection was also shown to be less common (1). In Argentina, LCMV cases occur in the same settings as AHF, and the LCMV seroprevalence in male residents of the pampas was 2.2% with regional variations; some localities had rates as high as 4% to 10% (8).

Reported rates of LCMV disease are difficult to obtain because of the lack of widely available diagnostic testing and of interest on the part of clinicians. From 1941 to 1958, LCMV accounted for 9.2% of CNS disease of suspected viral etiology studied in a referral hospital. The annual incidence was relatively constant throughout the period and showed a maximum in the colder months. The fall-to-winter increase is believed to result from *Mus* entering residences for shelter and food. Disease occurs in adults and children of both sexes. Because urban antibody prevalence rates are similar today, the small number of reported cases presumably represents diagnostic apathy. In an attempt to examine this situation, LCMV infection was sought in patients admitted with CNS disease to a major hospital serving a southern U.S. city and the suburban and rural area around it (239). Surprisingly, no evidence of acute LCMV infection was found, and indeed the prevalence of past infection was only 0.3% in persons under 30 years of age. It is unknown if the change in risk is a consequence of human behavior, mouse distribution, or prevalence of infection in mouse populations, but the older persons in the same area had a seroprevalence of 5.4%.

LCMV-infected colonies of hamsters can sustain virus transmission without overt evidence of infection (240,244). Several outbreaks of LCMV infection in humans have been linked to exposure to persistently infected hamsters or tumor lines contaminated with LCMV (3,121,135). Several laboratory outbreaks were associated with virus-contaminated transplantable tumors (15,30,130). The largest number of infections resulted from distribution of pet hamsters in the United States (181 reported cases from Dec 1973 to April 1974) and Germany (47 cases identified in 1968 to 1971) (3). Infection resulted not only after close contact with the animals, but also in persons present in the same room, implicating airborne virus in transmission in at least some cases. Chronically infected hamsters were identified in home and laboratory settings, and human disease rapidly decreased when infected animals were destroyed and suppliers of infected animals ceased distribution.

It is of interest that there are still no requirements for LCMV testing in hamster colonies, and that investigation of a 1989 outbreak in nu/nu mice undergoing tumor xenotransplants found a frozen tumor cell line that must have been infected in 1975 during a hamster-associated LCMV outbreak (90). Nude mice lack the capacity to clear LCMV infection, and if they become infected either by wild mouse contact or by LCMV-contaminated tumors, they will be persistently infected and pose a threat to those working in the area. Van der Zeijst et al. reported the presence of LCMV antigen in numerous hamster cell lines from several sources. Infectivity was shown to be transferable to uninfected cell lines by exposure to cellular extracts from the infected lines.

Lassa Fever

Human disease occurs maximally during the dry season, following the multimammate mouse reservoir breeding during the rainy season. The rodents enter village houses, and they are also plentiful in surrounding savanna and other disturbed habitats, resulting in extensive human infection in all age groups and both sexes. The recent wars in Liberia and Sierra Leone have increased transmission and resulted in an enhanced risk to expatriates operating in the area. Nigeria has continued to export occasional cases to Europe.

Argentine Hemorrhagic Fever

Junin virus infection has a regular fall increase in incidence, which results from the harvest activities of humans in agricultural fields at the same time that infected rodent populations are high. As the reservoir rodent, *Calomys musculinus*, has a predilection for linear habitats such as fence rows, these may be a particularly dangerous area for AHF transmission (213,215). In addition, there is aerosol transmission from mechanical harvesting and other activities. Hundreds and occasionally thousands of cases occur annually, with the highest attack rates in adult males (197). In addition, the endemic area has been progressively increasing until it is now 10 times its initial extent (194). The quantitative interactions among an increased geographic area of transmission, decreased human contact with the cultivars because of mechanization, and effects of different crops on disease transmission are unknown, but the result has been a largely unchanged annual incidence of disease. An interesting feature of AHF epidemiology has been the regular occurrence of disease in wives of farm workers. The incubation period suggests this is the result of transmission after hospital discharge (42).

Bolivian Hemorrhagic Fever

Calomys callosus, the reservoir of Machupo virus, is particularly suited to the cleared areas used for small plots of food crops in the Beni department of Bolivia. This leads to occasional cases of disease among rural residents, principally adult men, who live or visit these areas. These rodents may also enter small towns in the area, leading to more extensive epidemics affecting both sexes and all ages (154). The most impressive town-based epidemic occurred in San Joaquin in 1963–1964, when there were 637 cases and 113 deaths among the 3,000 residents (192). This epidemic led to the isolation of Machupo virus, the identification of the virus as a chronic rodent infection, and eventually the definition of *Arenaviridae* as a virus family (154,155 and 156,160,262). Trapping of rodents was shown to stop disease transmission in towns (193). Indeed, no cases of BHF were recognized after 1975 until 1993, when one case was noted, and three more occurred in 1994. These infections were acquired outside towns, but subsequently one of the cases transmitted the virus to six of eight family members, all of whom died (T. G. Ksiazek, personal communication, 1995).

Venezuelan Hemorrhagic Fever

VHF was recognized in 1989 and the virus isolated in 1990. The emergence of the disease in rural residents of the municipality of Guanarito in Portuguesa State, Venezuela, appeared to result from clearing of forest and changing land utilization to small farms. The highest risk is among adult males, and there is a harvest predominance, but some cases occur in rural residents of both sexes and all age groups (297). The initial epidemic produced 88 cases between September 1989 and December 1991, but only a handful of cases were recognized annually until an upsurge in 1997–1998 (297). Interestingly, field studies in the endemic area between the two peaks of transmission to humans showed a high prevalence of infection among the *Zygodontomys brevicauda* reservoir rodents, as well as confirming infection in surrounding areas that had never recognized disease (289). Unfortunately, no formal studies were possible during this time, but there were no obvious differences in rodent density or prevalence, weather conditions, or virus genotypes.

PREVENTION AND CONTROL

Rodent Control

Consistent, affordable, and sustainable rodent control has been successful only in a limited number of instances. If food sources are eliminated, peridomestic sites of harborage removed, and entry to homes denied by adequate construction, it is possible to maintain houses free of rodents. In some cases, this cannot be achieved with arenavirus reservoirs because of economic circumstances and the broad distribution of the rodent host; furthermore, in some situations, contact occurs outside the home.

In the case of LCMV, it is practical to eliminate the house mouse from homes and thus minimize exposure. Contact still occurs in barns and other situations, but the danger to humans is much less. There is a need for improved monitoring of colonies that produce rodents commercially, particularly mice and hamsters (135,244,281). The increasing use of rodents as pets or to feed pet reptiles has also led to “cottage” breeding of rodents, for which there is no likelihood of quality control. A recent incident exemplifies this problem: For alimentation of tamarinds, a zoo obtained baby mice from a local supplier, and the contaminated dietary supplement resulted in the death of several animals (225). LCMV is pathogenic for several nonhuman primate species (67,178,245), including Callitrichidae (marmosets and tamarinds), and has resulted in several outbreaks in zoos or laboratories when naturally infected wild mice entered the animal pens. In fact, the original

isolation of LCMV may well have arisen from such an incident (14).

Reservoirs of Lassa virus in west Africa appear to be *Mastomys erythroleucus* and *M. huberti*, which are numerous and widely distributed; the latter readily enters homes. Some decrease in human disease can be obtained through rodent control efforts, but ultimately a vaccine will be necessary.

C. musculinus, the major reservoir of Junin virus, is mainly a rodent of pampas and fields (215). Control of this rodent to prevent disease is impractical, and suggestions that certain crops may be associated with lesser risk have not been proven. Changing agricultural practices from the 1950s to encompass modern machinery and decrease human time spent in the fields has probably resulted in some decrease in the incidence of AHF, but the disease has continued to be a serious problem. Aerosol infection is thought to be a common way of acquiring Junin virus infection, especially for combine and tractor drivers. Lessening human–rodent contact should be attempted now that it is established that infected *C. musculinus* is found preferentially along fence lines in cultivated areas.

C. callosus, the reservoir of Machupo virus, prefers open areas and particularly adapts to cleared fields in which crops are grown. In the 1960s, the rodent invaded towns and the resulting epidemics of BHF were devastating but were controlled by eradicating *C. callosus* by trapping. The marked splenomegaly found in chronically infected rodents serves as a useful marker of field infection rates (153). Sporadic cases in farmers and residents of remote houses cannot be prevented solely by rodent control.

The major reservoir of Guanarito virus in Venezuela is *Z. brevicauda* (106). There is insufficient information on the epidemiology of the disease or rodent habits to suggest specific control measures.

Vaccines

The development of vaccines for arenaviruses carries several inherent problems. These viruses have been associated with chronic infections, neurologic syndromes, immunopathology, and fetal infection. Their immunology is poorly understood and the development of humoral immunity, particularly to the Old World arenaviruses, is late and low-titered (245). Inactivated vaccines have usually had little success (65), but the chances of reversion to virulence, or inadequate attenuation of such pathogenic viruses, make development of live vaccines a difficult approach as well. There is perhaps no group of viruses for which a vectored-vaccine strategy would be more appropriate than the arenaviruses, particularly the American arenaviruses, which have multiple distinct antigenic types, a defined but restricted geographic area of circulation, and apparently a similar pathogenesis.

Junin Virus

A classical live attenuated Junin vaccine referred to as Candidate #1 has been developed and field-tested in Argentina. The vaccine was compared in its virulence properties to an experimental strain called XJ Clone 3, which had been tested in at-risk laboratory workers and more than 600 volunteers and found to have excessive reactogenicity but apparent safety (198). Candidate #1 was found to be more attenuated than XJ Clone 3 for mice, new-born guinea pigs, and nonhuman primates, and to have low neurovirulence, no evidence of persistence, and no demonstrable phenotypic reversion (24,69,208). Seroconversion rates are high if highly sensitive neutralization tests are used, but titers are low compared to those seen in convalescence from natural infection. Strong evidence of cellular immunity as measured by lymphocyte stimulation assays is found, even in serologic nonresponders. The mechanism of virus attenuation is unknown. There are 39 nucleotide differences when the 1,433 nucleotides coding for GPC of Candidate #1 are compared to the sequence of an unrelated Junin isolate; there are a limited number of amino acid coding changes corresponding to the amino-terminal region of G1 (258). A number of phenotypic differences exist that favor attenuation, including sensitivity to complement inactivation in the absence of antibody and increased ease of neutralization (25,170). Interestingly, attenuated strains such as Junin XJ Clone 3 and Machupo SM80 become virulent for their natural *Calomys* hosts (152,176).

In 1988–1990, a double-blind, placebo-controlled field trial was carried out in 6,500 volunteers; the vaccine reduced morbidity from serologically confirmed AHF from 22 cases in controls to a single case in vaccine recipients ($p < .001$) (198). Since then, 175,000 doses have been administered to high-risk residents of the endemic areas and only occasional mild cases of AHF have been reported among the vaccinees. The total annual number of cases has fallen from several hundred to less than 200.

The vaccine has only minor reactogenicity in adults as measured in phase II and III studies, with no serious reactions observed in any recipient. Because of the need to extend coverage beyond the highest risk groups of adult male residents of rural areas with high incidence of AHF, studies have begun to include children and women. To date, no adverse reactions have been observed in 142 children as young as 4 years of age, and seroconversion rates have been high. In spite of counseling and pregnancy testing to avoid vaccination of pregnant women, 49 of 17,000 recipients have given birth within 9 months of vaccination; the occurrence of one case of anencephaly and one of meningomyelocoel in the offspring suggest that caution should be exercised in the use of Candidate #1 in women of child-bearing age, but there has been no direct implication of the vaccine as invasive or pathogenic for the fetus.

The success of the AHF vaccine highlights other hurdles to the solution of VHFs by human vaccines. There is little financial incentive and perhaps inadequate justification given the world health budgets to develop vaccines for most VHFs. Even in the case of the successful AHF vaccine, there is presently no means of producing the vaccine in Argentina and no prospect of vaccine production elsewhere. Finally, without the indicator human cases, a new surveillance system is needed for the presence of rodent infection to define the continuing risk from and spread of Junin virus.

In guinea pigs and nonhuman primates, Candidate #1 and XJ Clone 3 cross-protect against Machupo virus challenge. Although there is little cross-neutralization between Junin and Machupo viruses, Junin-vaccinated animals produce a

rapid neutralizing antibody response when challenged with virulent Machupo virus, suggesting cross-priming or perhaps cellular immunity as a basis for the cross-protection.

Lassa Virus

Of all the arenavirus diseases, Lassa fever has the greatest health impact, and prospects for its prevention through rodent control are least. Three approaches to vaccine development are often discussed: (a) Other Old World arenaviruses, including the Armstrong strain of LCMV, can provide protection against virulent Lassa virus challenge, and it has been suggested that because of their low virulence for nonhuman primates they might serve as human immunogens (148,303). Unfortunately, developing information to ensure the safety and efficacy of strains of these viruses in humans may be as difficult or more so than developing a vaccine from Lassa virus. (b) The development of genetically engineered killed immunogens that would elicit neutralizing antibodies, while supported by passive protection experiments in LCMV-infected mice and realistic Lassa fever animal models, suffers from a lack of understanding of the necessary epitopes for protection. (c) The use of Lassa genes in living virus vectors has had impressive initial success in the vaccinia system. (d) Naked DNA vaccination has been developed using the LCMV system (126,127 and 128,256,257,322), and this demonstrates the induction of long-lived immunity to infection. Most interesting is a recent report demonstrating successful immunization of neonatal mice with plasmid DNA expressing the LCMV nucleocapsid protein (NP) (127). Neonatally immunized mice exhibited a long-lived CD8+ T-cell response to epitopes in NP.

Vaccinia-vectored Lassa genes are protective in both guinea pigs and nonhuman primates (17). N-gene immunization protected guinea pigs but not monkeys, whereas immunization with vaccinia expressing the GPC gene prevented mortality in both experimental systems. Low levels of virus replication and mild clinical signs were detected in some vaccinated, virus-challenged animals. No neutralizing antibodies were detected, so the mechanism of protection may have been by cellular immunity. These studies establish the feasibility of Lassa fever vaccination by the appropriate presentation of GPC antigen and may eventually lead to protection for the extensive affected populations of west Africa.

CONCLUSION

Arenaviruses have provided important model systems for the study of host–virus interactions for more than six decades, and study of these models has illuminated a wide range of principles including persistent infection, virus-induced immunopathologic disease, cytotoxic T-cell recognition, and immunologic memory. Despite this wealth of knowledge, and even though arenavirus infections of humans remain a significant public health risk in much of the world, prospects for control of these diseases remains remote because of poor diagnostic protocols and sparse resource allocation to vaccine development and other control measures. In this sense, the arenaviruses may provide a useful model for many localized viral diseases where the knowledge but not the financial incentive for control or eradication exists.

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CHAPTER 51 Bornaviridae

Juan Carlos de la Torre

HISTORY AND CLASSIFICATION

Descriptions of horses with clinical signs likely to be Borna disease (BD) date back to the end of the 18th century, and by the late 1800s BD was recognized as a sporadically occurring encephalopathy that affected horses and sheep in central Europe (29). The disease name was derived from its continuous appearance between 1894 and 1896 around the city of Borna in Sauxony, Germany (29). Early studies demonstrated the infectious etiology of BD, and in 1926 its causative agent was identified as a virus, Borna disease virus (BDV). Since then, BDV has been found to cause central nervous system (CNS) disease in a broad range of vertebrate species, and it has become an important model for the study of persistent virus infection of the CNS (12). BDV has been molecularly characterized as a nonsegmented, negative-stranded (NNS) RNA virus (5,7). Based on its unique genetic and biologic features, BDV is the prototypic member of a new family, *Bornaviridae*, within the order *Mononegavirales* (8,32).

INFECTIOUS AGENT

Biologic Characteristics

Host Range

Horses and sheep have been regarded as the main natural hosts of BDV. In these species, BDV can cause BD, a fatal neurologic disease. Evidence indicates that the natural host range of BDV is wider than originally thought. Naturally occurring BDV infections have been documented in cattle and cats. In addition, sporadic cases of natural infection with BDV have been reported in other species including dogs, donkeys, mules, ostriches, alpacas, and pygmy hippopotami (15,28,29). Experimentally, BDV has a remarkably wide host range that includes species phylogenetically distant, from birds to rodents and nonhuman primates (15,28,29). Moreover, serologic data and recent molecular epidemiologic studies indicate that BDV can infect humans and might be associated with certain neuropsychiatric disorders (12,20).

The route of transmission is unclear. BDV infectivity and RNA have been detected in secretions and excretions of infected animals, suggesting that BDV could be transmitted through saliva, nasal and conjunctival secretions, urine, or feces (37). Infection may result from direct contact with these body fluids or by exposure to contaminated water and food (29). Different BDV isolates can exhibit significant diversity in phenotypic expression. However, despite its wide host range and phenotypic variability, molecular epidemiologic data have shown a remarkable sequence conservation, not only among BDV isolates within the same host species, but also among isolates from different animal species (12,15,28).

Cell and Tissue Tropism

BDV is noncytolytic and highly neurotropic. *In vivo* it replicates preferentially in neurons and astrocytes, but oligodendrocytes and ependymal cells can also be infected (29). In addition, cells derived from ectoderm become infected *in vivo* as a consequence of the centrifugal axonal spread of BDV to the periphery (29). The virus can also be present in peripheral blood mononuclear cells (PBMC) from naturally and experimentally infected animals (15,28,37). Besides brain-derived cells, other cell types can be infected in tissue culture by co-cultivation with infected brain cells or by inoculation with brain homogenates from infected animals (29).

Propagation and Assay in Cell Culture

BDV can be grown in a variety of neural and nonneural cell lines. None of these cells produce significant levels of extracellular cell-free virus, and cell-associated infectivity ranges from less than 0.01 to 0.1 infectious unit per cell. Hypertonic treatment of BDV-infected cells can release up to 50% of the total cell-associated infectivity (4). Homogenates from BDV-infected cells and tissues, as well as cell-free virus, can be quantified using an immunofocus assay.

Virion Morphology and Physical Characteristics

Electron microscopy studies of negatively stained cell-free BDV infectious particles have shown that virions are spherical with a diameter ranging from 70 to 130 nm (11,19,42). These particles contain an internal electron-dense core (50 to 60 nm) and a limiting outer membrane envelope, which appears to be covered with spikes approximately 7 nm long (19). Partially purified BDV infectious particles have a buoyant density in cesium chloride of 1.16 to 1.22 g/cm ^{3}, and in sucrose of 1.22 g/cm^3. Virus infectivity is rapidly lost by treatment at 56°C. Virions are relatively stable at 37°C, but after 24 hours incubation in the presence of serum, only minimal infectivity is recovered. Virions are inactivated below pH 5 and above pH 12, and by treatment with organic solvents, detergents, formaldehyde, and ultraviolet radiation.

Genome Organization and Encoded Proteins

BDV has a NNS RNA genome (about 8.9 kb in size, with a relative molecular mass (M_r) of about 3×10^6) (8,32). Extracistronic sequences are found at the 3' (leader) and 5' (trailer) ends of the BDV genome. As with other negative-strand RNA viruses, the ends of the BDV genome RNA exhibit partial inverted complementarity. Full-length plus-strand (antigenomic) RNAs are present in infected cells and in viral ribonucleoproteins.

There is no evidence for use of an ambisense coding strategy, and six major open reading frames (ORFs) are found in the BDV genome sequence (I to VI) (8,32) (Fig. 1). These ORFs code for polypeptides with predicted M_r of 40 kd (p40), 24 kd (p24), 10 kd (p10), 16 kd (p16), 56 kd (p56), and 180 kd (p180), respectively. Based on their positions in the viral genome and their abundance in infected cells and virion particles, together with their biochemical and sequence features, p40, p24, and p16 BDV polypeptides correspond to the viral nucleoprotein (N), the phosphoprotein (P) transcriptional activator, and matrix (M) proteins, respectively, found in other NNS RNA viruses. Two isoforms of the BDV N protein (p40 and p38) are found in BDV-infected cells. These two forms may be encoded by two different mRNA species (27), but differential usage of two in-frame initiation codons present in the BDV p40 gene could also contribute to the production of BDV p40/38. BDV p24 is an acidic polypeptide (predicted pI of 4.8) with a high Ser-Thr content (16%). Phosphorylation of serine residues is mediated by both protein kinase C and casein kinase II (33). An additional ORF, p10 or X, encodes a polypeptide of 10 kd present in BDV-infected cells (41). BDV p10 synthesis starts within the same mRNA transcription unit as p24, but 49 nucleotides upstream, and it overlaps, in a different frame, with the 71 N-terminal amino acids of p24. In contrast to other NNS RNA viruses, the putative BDV M protein is glycosylated, has a M_r of 18 kd (gp18), appears to be present in the virus envelope, and may participate in viral attachment to the cell (17).

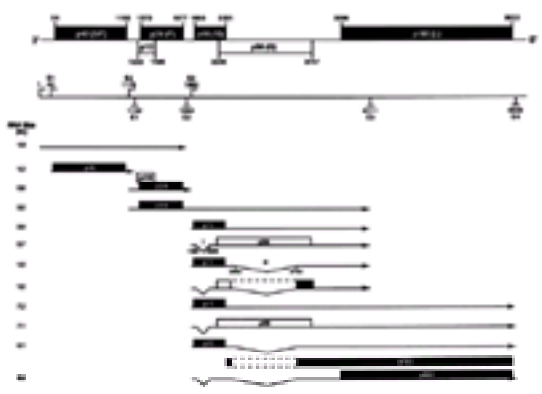

FIG. 1. Genomic organization and transcriptional map of BDV. BDV open reading frames are represented by *boxes* at the top. Different *shades* correspond to usage of different reading frames within the antigenomic polarity of the BDV genomic RNA. The location of transcription initiation and transcription termination sites are indicated by *S* and *E*, respectively. Positions of BDV introns I and II are indicated.

ORF p56 encodes the putative virus surface glycoprotein (G) and overlaps the C-terminus of ORF p16 in a different frame. The predicted protein is a 503-amino-acid polypeptide with a M_r of 56 kd. The p56 gene directs the synthesis of at least two glycosylated polypeptides of about 84 to 94 kd (GP-84/94) and 43 kd (GP-43), corresponding to the full length and C-terminus, respectively, of the p56 gene (10). GP-43 is produced via cleavage of GP-84/94 by the cellular protease furin. The cleaved N-terminal part of G has not been observed in BDV-infected cells. Whether this is because of its rapid degradation or the lack of suitable antibodies for its detection remains to be determined. GP-84 and GP-43 are sensitive to both endoglycosidases F and H. BDV ORF p180 is capable of encoding a polypeptide whose deduced amino acid sequence displays strong homology to other NNS RNA virus polymerases, members of the large (L) protein family (8,32).

CYCLE OF INFECTION

Virus Adsorption and Entry

There is evidence that a cellular receptor, of unknown identity, is required for BDV infection. Antibodies to both the virus G and M (gp18) proteins have neutralizing activity, suggesting that both are implicated in BDV adsorption and/or entry (11,17). Whether the carbohydrate modifications found in G and M (gp18) contribute to the adsorption process is unknown. Recent pseudotype studies have provided evidence that the N-terminal part (amino acids 1 to 244) of BDV p56 is sufficient for receptor recognition and virus entry. Lysosomotropic drugs and chemical treatments that cause depletion of cellular energy block BDV infection, indicating that BDV entry occurs by receptor-mediated endocytosis (10). Reduction in the pH of the endocytic compartment eventually triggers the fusion between viral and cell membranes. This fusion event releases the BDV ribonucleoprotein (RNP), which is then transported to the cell nucleus where viral

transcription and replication occur.

Transcription and Replication

BDV, unique among known NNS RNA animal viruses, has a nuclear site for genome transcription and replication (8,32). The nucleolus has been proposed as the site of BDV RNA synthesis (26). The genomic and antigenomic RNA molecules are neither capped nor polyadenylated. These RNAs exist as infectious RNP in the nucleus of infected cells (8).

Sequential and polar transcription results in decreasing molar quantities of BDV transcripts from the 3'- to the 5'-encoded cistrons. RNA species corresponding to the leader RNA have not been detected in BDV-infected cells. The viral mRNAs are polyadenylated and contain a 5' cap structure. Virus-specific mRNA synthesis is not inhibited by a-amanitin, and sequences at the 5' end of BDV mRNAs are homogeneous and genome-encoded. Thus, it is unlikely that transcription initiation of BDV mRNAs involves a cap-snatching mechanism similar to the one used by influenza viruses. Monocistronic viral mRNAs in BDV-infected cells are detected only for the N gene (see Fig. 1). The BDV genome contains three transcription initiation sites (S signals), and four transcription termination/polyadenylation sites (E signals) (8,32) (see Fig. 1). The S signals contain a semiconserved U-rich motif that is partially copied into the respective transcripts. A similar motif is not found within the S signals of previously described NNS RNA viruses. BDV E signals consist of six or seven U residues preceded by a single A residue, resembling the E signal motif found in other NNS RNA viruses. The BDV genome lacks the characteristic configuration of E signal–intergenic (IG) region–S signal found at the gene boundaries of other NNS RNA viruses. Instead, BDV transcription units and transcriptive signals frequently overlap.

Two of the BDV primary transcripts are posttranscriptionally processed by the cellular RNA splicing machinery (8,32). Two introns (I and II) have been identified in the BDV genome. BDV introns I and II span nucleotides 1932 to 2025, and 2410 to 3703, respectively, in the BDV antigenomic sequence. Splicing of intron I places the amino acid in position 13 of M, next to a stop codon, whereas splicing of intron II and I+II results in an mRNA containing a predicted ORF that corresponds to the first 58 amino acids of G fused to a new C-terminus of 20 amino acids. RNA species resulting from splicing of intron II and I+II are predicted to include an additional ORF that would encode a variant BDV L protein (p190) with 153 amino acids added to the N-terminus. Whether all these new predicted BDV polypeptides are synthesized in infected cells and tissues is unknown. RNA splicing can modulate the efficiency of termination-reinitiation of translation and leaky scanning mechanisms, thus contributing to the regulation of the expression of BDV gene products. A new BDV intron III, generated by alternative 3' splice site choice and comprising nucleotides 2,410 to 4,559 of the BDV genome, has been identified recently. Intron-III-spliced mRNAs were detected in cells from different types and species, and they have coding capability for two new viral proteins with predicted molecular masses of 8.4 and 165 (p165) kd. These findings underscore the proteomic complexity exhibited by BDV.

BDV-infected cells exhibit a heterogeneous pattern of viral antigen expression. BDV N, P, and p10 polypeptides are found in both the nucleus and the cytoplasm. N and P are the viral antigens expressed at highest levels. Only 1% to 10% of the infected cells express detectable levels of BDV G. Full-length BDV G (GP-84) appears to be restricted to the ER and nuclear envelope. The subcellular distribution of the BDV M has not been characterized. Both M and G are posttranslationally modified by glycosylation. M has been characterized as a biantennary complex type of glycoprotein, sensitive to endoglycosidase F, but not to endoglycosidase H or O-glycosidase (17). BDV G undergoes posttranslational cleavage by the cellular protease furin, with the resulting C-terminus (GP-43) reaching the cell surface (10,11). The newly exposed N-terminus of GP-43 is highly hydrophobic, and BDV-infected cells form extensive syncytia when subjected to low pH. These findings suggest that GP-43 may be involved in pH-dependent fusion after internalization of BDV by receptor-mediated endocytosis.

After infection, RNPs are transported into the nucleus for transcription and replication, and newly assembled RNPs are exported back from the nucleus to the cytoplasm. Thus, the nucleocytoplasmic transport of BDV macromolecules is an important factor in the life cycle of BDV. Co-localization studies suggest that in addition to NP and P, p10 is also part of the RNP complex (35). There is biochemical and genetic evidence for interactions between NP/P, P/P, and P/p10 (35). Nuclear localization signals (NLS) for NP and P have been characterized. The NP NLS has been mapped to the 13 N-terminal amino acid residues, with the motif 4KRR6 playing an essential role (18). Consequently, the p38 isoform of N lacks this NLS and accumulates in the cytoplasm. The biologic implications of the existence of two N isoforms with different subcellular targeting properties remain to be determined. P has two functionally independent proline-rich NLS located at its N- and C-terminal regions (36). The p10 sequence contains a characteristic leucine-rich nuclear export sequence (32). However, whether p10 has a nuclear export activity in BDV-infected cells remains to be determined.

Assembly and Release

Thin sections of BDV-infected cells show intracytoplasmic virus-like particles with morphologic characteristics similar to those described for partially purified cell-free BDV infectious particles (6). They have been observed below the plasma membrane of infected cells, and rarely in cytoplasmic vacuoles. These particles were not associated with cisternae of the endoplasmic reticulum, the Golgi complex, or other intracytoplasmic membranes. The assembly process and site of virus maturation have not been identified. Budding of BDV particles has been observed only at the cell surface of BDV-infected MDCK cells after treatment with *n*-butyrate (19). Whether this reflects the natural pathway for the exit of BDV remains to be determined.

PATHOGENESIS

Neuroinvasion and Propagation of BDV

The natural portal of entry for BDV has not been formally identified. However, field observations, together with results from experimental infections, suggest that a primary route of infection might be through the nose and the olfactory neuroepithelium (29). Experimental oral infection has been achieved in several animal species, suggesting that BDV

infection might also be initiated by the gastrointestinal route.

After initial replication in neurons located at the site of entry, BDV is transported intraaxonally in an anterograde or retrograde direction toward the CNS (29). Thus, following experimental intranasal inoculation of the rat, BDV initially replicates in the neuroreceptor cells of the olfactory epithelium. The virus then can gain access to the cells of the main olfactory bulb (MOB) by anterograde transport, subsequently reaching the MOB efferent targets. Further spread is then possible into numerous diencephalic and telencephalic areas, through polysynaptic neuronal connections. Likewise, experimental infection into the foot of a rat results in a retrograde transport of BDV in the sciatic nerve, and then from neuron to neuron into the CNS.

BDV spreads transsynaptically along neuronal chains and exhibits a preferential tropism for the limbic system (13). The hippocampus eventually harbors the highest viral load. Nevertheless, viral infection is not restricted to the limbic system and spreads throughout the CNS (13). Viral antigen accumulates in the nucleus, the perikaryon, and processes of infected neurons, whereas the nucleolus remains free of viral antigen. In the nuclei of infected neurons, aggregates of viral material form the Joest-Degen inclusion bodies characteristic of BDV infection (13). The inability to detect mature virus particles within the CNS during BDV propagation has led to the hypothesis that virus spreads in the form of RNP. BDV infection affects specific neurotransmitter pathways, and its distribution in the hippocampus coincides with that of some excitatory amino acid receptors, such as glutamate. This suggests that BDV might use these receptors for its transneuronal transfer (13). Glial cells are also permissive for BDV. Virus antigen and RNA appear rather early in astrocytes, followed by oligodendrocytes and ependymal cells, as well as Schwann cells in the peripheral nervous system. In the late stages of infection, BDV spreads centrifugally, probably by anterograde axonal transport, and viral markers can be detected in peripheral nerves of all tissues and organs, which eventually may become BDV positive. Neutralizing antibodies appear late during BDV infection and have been implicated in restricting virus replication to the nervous system, thus preventing generalized infection with BDV (40).

Neuropathogenesis of BD

BD is a nonpurulent polioencephalomyelitis caused by a T-cell-dependent immune mechanism. The rat is the most commonly used experimental model for the study of BDV pathogenesis (1,13,39). The age, immune status, and genetics of the rat, as well as viral factors and route of inoculation, can influence the course of infection. Adult rats infected intracerebrally or intranasally with BDV usually develop an immune-mediated biphasic behavioral disease. These rats display clinical signs and a histopathologic picture very similar to those found in horses with naturally acquired BD. Viral gene expression in limbic system structures, astrocytosis, and structural alterations of neurons within the hippocampus are the main histopathologic hallmarks of BDV infection (13).

The onset of clinical signs, including movement abnormalities and aggressive and hyperactive behavior, coincides with the appearance of an inflammatory reaction in the brain that reaches its maximum 30 to 40 days after infection. This extensive inflammatory reaction leads to neuronal destruction that may lead to hydrocephalus (1,13,39). Inflammatory cells form perivascular cuffs and also infiltrate the brain parenchyma. Both CD4+ and CD8+ T cells contribute to the immune-mediated pathology. CD8+ T cells are the effector cells, whereas CD4+ T cells play a helper role (1). Elimination, or functional blocking, of CD8+ T cells prevents both BDV-induced neurologic symptoms and histopathologic changes in the brain. In addition, lymphocytes isolated from rat brains during acute BD exhibit major histocompatibility complex (MHC) class I–restricted cytotoxic T-lymphocyte (CTL) activity *in vitro*. MHC class I antigens are expressed by astrocytes and neurons in BDV-infected rats, concomitantly with the peak of CD8+ T-cell infiltrate in the CNS and the onset of neuronal degeneration (1). The molecular mechanisms responsible for BDV-induced MHC class I expression by neurons are presently unknown. Inhibition of electric activity and exposure to specific cytokines can induce MHC class I expression in neurons (22). Virally infected neurons lacking MHC class I expression may avoid destruction by CTL. Antibodies do not seem to play any pathologic role in BD.

The initial aggressive hyperactive behavior is followed by apathy, somnolence, and depression. A significant number of chronically infected rats may develop obesity (28). This chronic phase is characterized by a steady decline in the inflammatory reaction, despite continuous high viral load in the CNS. A switch from a T_H1-like cellular immune response to a T_H2-like humoral immune response very likely contributes to this delayed immune tolerance (14).

Levels of interleukin (IL)-6, tumor necrosis factor (TNF)-a, IL-1a, and inducible nitric oxide synthase (iNOS) mRNAs are significantly increased in BD rat brains, and expression correlates with the degree of inflammation and severity of neurologic signs (1,39). Nitric oxide and these pro-inflammatory cytokines may contribute to BD neuropathogenesis or reflect nonspecific activation of microglia, thus being more a secondary than a primary pathogenic importance in BD.

BDV Chronic Infection in the Absence of CNS Inflammation

In contrast to the situation just depicted, BDV infection of the neonatal rat causes a life-long persistent infection that is characterized by the lack of a cellular immune response to BDV and the absence of clinical signs of BD. This infection has been designated a persistent, tolerant infection of the newborn (PTI-NB) rat (30). Brains of PTI-NB rats harbor a viral load comparable to that found in rats with acute BD, illustrating the noncytolytic replication of BDV. Although PTI-NB rats do not show clinical signs, they exhibit distinct deficiencies in emotional and cognitive functions, as well as physiologic and neurodevelopmental abnormalities resulting from damage to specific neuronal populations (30). BDV infection interferes with the development areas of the CNS that experience extensive postnatal maturation leading to cerebellar hypoplasia and progressive degeneration of the dentate gyrus of the hippocampus (12,16,30). Purkinje cells are the predominant, if not the only, cell type infected in the cerebellum. The central role played by Purkinje cells in supporting the multiplication, maturation, and migration of the granular cells could be altered by BDV infection. Astrocytes provide the physical track on which the granule cells migrate, as well as neurotropic support to surrounding cells. Hence, the intense astrocytosis observed in PTI-NB rats may also be involved in the cessation of granule cell migration. The hippocampus consistently harbors the highest viral load in PTI-NB rat brains, but there is little effect on the lifespan of these infected neurons, with the exception of the dentate gyrus (DG) where the neurons are progressively destroyed. The

majority (>85%) of DG granule cells are postnatally generated, and neurogenesis occurs in this area well into adulthood. The mechanisms underlying BDV-induced DG degeneration are presently unknown.

BDV and Brain Homeostasis

CNS alterations induced by BDV infection of immunocompetent adult rats can be attributed to the virus-induced cellular immune response. However, BDV-mediated impairment of brain activity can also occur without the hallmarks of inflammation and widespread cytolysis, because of the virus's ability to interfere with brain cell functions required to maintain CNS homeostasis.

Cytokines play an important role in CNS function, under both normal physiologic and pathologic conditions. Chronic up-regulation of proinflammatory cytokines IL-1a and b, IL-6, and TNF-a is observed in the hippocampus and cerebellum of PTI-NB rats (31). Brains of PTI-NB rats do not have immune cell infiltrates but exhibit marked astrocytosis. Astrocyte functions are essential to neurons; they provide a substrate for neuronal migration during brain development, and the astrocytic network participates in brain information processing in cooperation with neuronal networks. Astrocytes participate also in the elimination of neurotoxins and in the production of cytokines that can act as neuronal differentiation factors, and modulators of neuronal neurotransmitter gene expression. BDV-induced altered astrocyte function may affect complex animal behaviors and plasticity processes accompanying learning and memory, which are altered in PTI-NB rats.

PTI-NB rat brains have also increased expression levels of tissue factor (TF) (12). TF, a transmembrane receptor, is the primary initiator of the coagulation protease cascade that results in the generation of the protease thrombin. In the CNS, TF is produced by astrocytes. BDV-mediated TF up-regulation is caused by increased transcription of the TF gene and a stabilization of TF mRNA. There is increasing evidence that proteins of the coagulation and fibrinolysis systems may have functions in the CNS that are independent of blood clotting, such as regulating normal brain development and defending the brain against injury-related damage. Altered protease activities may contribute to neuronal damage, and thrombin has been implicated in several neurodegenerative diseases, including Alzheimer's disease. Interestingly, thrombin is predominantly expressed by dopaminergic neurons of the mesencephalon, suggesting a possible link between TF up-regulation and the dopaminergic abnormalities observed in BD rats (9,38).

BEHAVIORAL DISTURBANCES ASSOCIATED WITH BDV INFECTION

The acute phase of BD is characterized by movement disorders accompanied by excitability and hyperactivity. The later stages of the disease are frequently manifested by somnolence, depression, apathy, and anorexia. Mortality rates are as high as 80% to 100% in horses and 50% in sheep (29). Animals that survive remain chronically infected, and recurrent episodes with exacerbation of behavioral abnormalities may occur. Both host and viral factors influence significantly the behavioral abnormalities associated with BDV infection (29).

Pharmacologic studies indicate that behavioral disturbances associated with BD might be te result of BDV-induced alterations of the dopamine system. Dopaminergic activity is enhanced in the prefrontal cortex of BD rats, but dopamine receptor density is unchanged. Decreased numbers of D2 and D3 receptor binding sites are found in the nucleus accumbens of BD rats. This brain structure is a primary site of integration between limbic and motor information and participates in the control of motor, cognitive, emotional, and reward processes. Thus, abnormalities in the mesocorticolimbic dopaminergic network may constitute the neural substrate of hyperactivity in BD (38). Besides dopamine, alterations in other neurotransmitter systems have also been described in BD rats, including changes in mRNA levels of cholecystokinin, glutamic acid decarboxylase, and somatostatin (20). These pharmacologic abnormalities are likely to be a consequence of the inflammatory response. However, cortical cholinergic denervation has been observed in BDV-infected rats prior to the onset of encephalitis and in the absence of any significant cell destruction in the CNS (9).

In contrast to BDV-infected immunocompetent adult rats, PTI-NB rats develop a chronic infection with no overt signs of classic BD. Nevertheless, PTI-NB rats exhibit distinct cognitive, behavioral, and physiologic abnormalities (30). PTI-NB rats show impaired cognitive functions in different learning paradigms, including spatial discrimination and discriminated avoidance tasks. Results from open field and neophobia tests revealed emotional disturbances in PTI-NB rats. Moreover, these animals displayed hyperactivity when placed in a novel environment. Reduced body weight and length were also observed in PTI-NB rats, compared to age- and sex-matched uninfected controls. Impaired growth did not seem to be related to disturbances in growth hormone physiology. These differences in growth appeared only after weaning, suggesting that the feeding behavior might have been affected by perinatal BDV infection. In addition, PTI-NB rats exhibit an altered preference for salt.

BDV-infected tree shrews illustrate important aspects of the consequences of BDV persistent infection on the behavior of highly developed animals (29). Tree shrews (*Tupaia glis*) are phylogenetically at the root of the primate tree and exhibit a complex behavioral repertoire. Tree shrews inoculated with BDV develop a persistent infection associated with behavioral disorders related to limbic system functions. Interestingly, housing conditions have a strong influence on the behavioral manifestations of infection. Only a small number of animals kept in isolation show behavioral abnormalities, whereas all paired animals display profound sociosexual behavioral disturbances. These include a tendency to accept partners more quickly and a need for increased body contact, as well as a reversal in the role of the sex partners and an impaired breeding behavior of the females. Some animals kept in isolation also show neurologic symptoms. All these animals recover, but relapse of clinical symptoms occurs in some cases. It is unknown why clinical symptoms are observed only in some animals, although all have comparable viral load in the brain and similar titers of serum antibodies to BDV.

EPIDEMIOLOGY

Intranasal infection is very likely an important route of natural infection with BDV, allowing virus access to the limbic

system in the CNS by intraaxonal transport through the olfactory nerve (29). Animals may become infected by contact with saliva, excretions, or nasal secretions. Contact experiments have suggested that healthy carrier animals might represent a source of infection (29). BDV could also be transmitted by the oral/gastric route on exposure to contaminated food or water. Cases of BD are more frequent in some years than others and tend to occur in spring and early summer, suggesting that environmental factors can influence the circulation of BDV in the field (29). The extremely low levels of infectious virus shed by infected animals may explain the limited contagiousness of BDV.

Evidence indicates that the natural host range of BDV is wider than originally thought (15,28,29). Asymptomatic, naturally infected animals of different species have been documented in Europe, North America, Africa, and Asia, suggesting that the prevalence and geographic distribution of BDV may have been underestimated (15,28,29). It is an intriguing observation that clinical cases of BD appear to be restricted to central Europe. The natural reservoir of BDV has not been identified. BDV has not been isolated from insects, but ticks have been implicated in the transmission of an infectious encephalomyelitis similar to BD affecting ruminants and equidae in the Middle East. There is also some evidence for a possible role of rodents as a BDV reservoir (29).

Seroepidemiologic studies conducted in different laboratories over the last 10 years have consistently shown an increased BDV seroprevalence in neuropsychiatric patients (2,12,20). However, considerable variation in prevalence rates has been described, ranging from 1.6% to 30% in psychiatric patients and 0% to 3.5% in controls. Increased BDV seroprevalence has also been reported in immunosuppressed patients infected with human immunodeficiency virus (HIV) from Europe and Thailand, individuals with parasitic infections in Africa, and patients with neurologic disorders (2,12,15). However, the limited information about the composition of the subject groups analyzed, including geographic area, heterogeneity of diagnoses, and clinical status of patients may have significantly influenced these results. In addition, differences in the experimental procedures used to detect antibodies to BDV in human sera may also have affected the results.

Detection by reverse transcriptase polymerase chain reaction (RT-PCR) of BDV RNA sequences in tissues, secretions, and blood from naturally BDV-infected animals has been documented (15,28). Sequence analysis of amplified RT-PCR products is providing valuable information for the understanding of the epidemiology of BDV. The importance of these new molecular diagnostic tools is further stressed by the finding of a significant number of patients with asymptomatic BDV infection, who have low to nondetectable serum antibodies to BDV. Moreover, using RT-PCR procedures, several laboratories have detected BDV RNA in human blood (12,15,28). BDV RNA prevalences of 10% to 37% have been documented in the PBMCs of Japanese neuropsychiatric in-patients, whereas healthy blood donors had a prevalence of 0% to 4.6%. Similar findings have been described in studies done with German neuropsychiatric patients. A high BDV prevalence, RNA in both blood and serum antibodies, has also been documented in Japanese patients diagnosed with chronic fatigue syndrome. These findings remain controversial, because not all laboratories have found evidence of BDV RNA in human blood. As with naturally infected animals, a lack of correlation between detection of BDV RNA in blood and serum antibodies to BDV has been reported in humans. Sequence analysis revealed a high degree of conservation of both inter- and intrapatient BDV sequences, as well as a close genetic relationship between human and animal-derived BDV sequences (12,15,28). This finding is consistent with the remarkable genetic stability of BDV in animals. However, the recently identified subtype No/98 of BDV (23) differs at the nucleotide level from other known BDV strains by more than 15%. The identification of this new BDV field isolate that escapes detection by currently used diagnostic assays has important implications for the epidemiology of BDV in animals and humans. BDV has been isolated from PBMC (three cases) (3) and granulocytes (one case) (25) of neuropsychiatric patients. However, there are some concerns about possible contamination with laboratory sources of BDV, stressing the need for further studies to confirm these findings. BDV antigen and RNA have also been detected in human autopsy brain samples (12,15,28). These findings demonstrate that BDV can persist in the human CNS.

The source of BDV and routes for human infection are unknown. Circumstantial evidence suggests the possibility of a zoonosis (29). The close genetic relationship between BDV sequences derived from humans and animal strains provides support for this hypothesis. In this regard, it is of interest that domestic cats are susceptible to BDV, and a high BDV prevalence has been reported in domestic cats with “staggering” disease or with undefined neurologic symptoms, as well as in a significant number of asymptomatic cats (12,15,28). The detection of BDV RNA in PBMC and granulocytes suggests a possibility of blood transmission. As with other neurotropic viruses, a small number of BDV-infected cells present in a blood sample could reach the brain and initiate a persistent infection within the CNS. Thorough and comprehensive investigations will be required to accurately determine the prevalence and significance of BDV infection in humans.

PREVENTION AND TREATMENT

Live-virus vaccines have been used in the past to attempt protection against naturally occurring BD in horses and sheep. This practice was discontinued because of vaccine-related transmission of infectious BDV and other viruses. Experimentally, passive immunization with a BDV-specific CD4+ T-cell line (1) as well as inoculation of rats with a high dose of an attenuated BDV (24) have been shown to provide protection against BD.

Amantadine has been reported to effectively cure cells persistently infected with BDV. However, this finding has not been reproduced by other groups (33). Ribavirin inhibits BDV replication in MDCK cells persistently infected with BDV (21). Whether this finding can be extended to other cell types and to infected animals remains to be established.

PERSPECTIVES

BDV is the prototypic member of a new family of RNA animal viruses. Therefore, the investigation of BDV may uncover new aspects of the biology of these infectious agents. These investigations will greatly benefit from the establishment of a reverse genetic system, similar to those already developed for several NNS RNA viruses. The use of the newly established mouse model of BDV infection will be instrumental for detailed investigations of the mechanisms underlying the immune-mediated neuropathogenesis of BDV. The elucidation of the molecular mechanisms underlying BDV-induced

cerebellar hypoplasia and dentate gyrus neuronal degeneration will contribute to our understanding of neurodevelopmental disorders resulting from congenital and perinatal viral infections. There is compelling evidence that BDV can infect humans. However, the true prevalence of BDV infection in humans and its possible association with certain neuropsychiatric disorders remain to be determined. The development of standardized sensitive and reliable serologic and nucleic acid diagnostic tests will be necessary before this pressing question about BDV can be satisfactorily answered. These diagnostic tests will also permit rigorous examination of the prevalence of BDV in domestic and wild animals, and they will help to assess most of the implications for potential transmission to humans.

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CHAPTER 52 Reoviruses and Their Replication

Max L. Nibert and Leslie A. Schiff

Viruses with double-stranded RNA (dsRNA) genomes illustrate the potency of evolution at exploiting credible strategies for genome organization and replication. The dsRNA viruses include several human pathogens, most notably the human rotaviruses and Colorado tick fever virus, and numerous pathogens of other organisms including mammals, birds, fish, insects, and plants. In addition, studies of the dsRNA viruses have contributed a number of fundamental concepts to the fields of virology and molecular biology. To name some examples, we draw from work on the nonfusogenic mammalian orthoreoviruses (reoviruses), which are the primary focus of this chapter. Reoviruses provided one of the first systems for *in vitro* studies of RNA synthesis (52,426,445) and evidence for the 5' cap structure on eukaryotic mRNAs, its mechanism of synthesis, and its importance in mRNA translation (165). Early evidence for the consensus sequence for translation initiation in eukaryotic mRNAs was also obtained from work on reoviruses (249). The use of reassortant genetics to identify allelic differences in reovirus genome segments that segregate with phenotypic differences between reovirus strains (416), demonstrated the value of genetic approaches to virology at a time when such approaches were not yet developed for many other animal viruses. Moreover, several of these early genetic studies with reoviruses concerned the basis of viral pathogenesis and yielded some of the first evidence for the role of animal virus receptor-binding proteins in tropism and/or virulence (494). Studies with reoviruses also provided early evidence for the role of M cells in viral penetration of the mucosal barrier during entry into an animal host (512).

CLASSIFICATION

dsRNA Viruses

Viruses with dsRNA genomes are currently grouped into six families: *Reoviridae*, *Birnaviridae*, *Totiviridae*, *Partitiviridae*, *Hypoviridae*, and *Cystoviridae* (Table 1). Viruses in only two of these families, *Reoviridae* and *Birnaviridae*, are known to infect vertebrates, and those in only one, *Reoviridae*, are known to infect mammals. The six families are distinguished by their genome organizations, protein coding strategies, virion structures, and other differences, in addition to host range. They are sufficiently different in their RNA polymerase sequences, for example, to have earned speculation that at least some arose from different plus-strand RNA virus ancestors (241). Certain structural features are nonetheless shared across family lines and beg explanations in evolutionary terms. For example, the outer capsid layer of *Reoviridae*, the single capsid layer of *Birnaviridae*, and the outer capsid layer of *Cystoviridae* are all arranged with T=13 (laevo) symmetry (63,71,310), which is not seen elsewhere in the virus world. Similarly, the inner capsid layer of *Reoviridae*, the single capsid layer of *Totiviridae*, and the inner capsid layer of *Cystoviridae* are arranged with a form of T=1 symmetry in which the icosahedral asymmetric unit is defined by an asymmetric dimer of the major capsid protein (71,79,378), also not in other viruses.

Family	No. of Genera	No. of Genome segments	Hosts
Reoviridae	9	10–12	
Turreted[a]			
Orthoreovirus		10	Mammals, birds, reptiles
Aquareovirus		11	Fish, molluscs
Cypovirus		10	Insects
Fijivirus		10	Plants, insects[b]
Oryzavirus		10	Plants, insects[b]
Nonturreted[a]			
Rotavirus		11	Mammals, birds
Orbivirus		10	Mammals, birds, arthropods[b]
Coltivirus		12	Mammals, arthropods[b]
Phytoreovirus		12	Plants, insects[b]
Birnaviridae	3	2	Birds, fish, insects
Totiviridae	3	1–4	Fungi, protozoa
Partitiviridae	4	2–4	Fungi, plants
Hypoviridae	1	1	Fungi
Cystoviridae	1	3	Bacteria

[a]Proposed division based on presence or absence of turret-like protein projecting around fivefold axes from innermost capsid layer (206).
[b]Serve as vectors for transmission to other hosts.

TABLE 1. *dsRNA viruses*

The *Reoviridae* Family

Of the six dsRNA virus families, *Reoviridae* is the largest and most diverse in terms of the host range of its members (see Table 1). The genomes of these viruses comprise 10, 11, or 12 segments of dsRNA, each encoding one to three proteins (usually one) on only one of the complementary strands. Their mature virions have characteristic sizes (60 to 85 nm, excluding the extended fiber proteins that project from the surfaces of some members), no lipid envelope, and proteins arranged in concentric layers that generally reflect icosahedral symmetry. A distinguishing feature of their replication cycles, also found in dsRNA viruses in other families, is synthesis of viral mRNAs by virally encoded enzymes within the icosahedral particles.

Despite these similarities, viruses in the nine recognized genera of this family (see Table 1) exhibit substantial genetic, biochemical, structural, and biologic differences. Regions of significant sequence similarity across genus lines are few and of limited size, and thus evolutionary connections among the genera remain a matter of speculation (but see later for a recent exception concerning the genera *Orthoreovirus* and *Aquareovirus*). Ongoing exchanges of genetic material between genera, such as genome segment reassortment, appear unlikely given the extent of differences. Viruses in a subset of the genera (see Table 1) have a turret-like protein projecting outwardly from the innermost capsid layer and surrounding the fivefold axes of their particles, which suggests a division of the family into turreted and nonturreted groups (206).

The *Orthoreovirus* Genus

The *Orthoreovirus* genus includes the prototype members of the *Reoviridae* family, the nonfusogenic mammalian orthoreoviruses, which are the primary focus of this and the next chapter. Fusogenic orthoreovirus isolates from mammals, birds, and reptiles are discussed as well, but in a more limited fashion. Designation of the latter isolates as fusogenic reflects their capacity to cause infected cells to fuse together into large multinucleated syncytia. This is an unusual property for nonenveloped viruses, which lack a classical fusion glycoprotein. The mechanism and functional roles of syncytia formation in the life cycles of these viruses is the subject of active investigation (139,307,340) but seems to involve a fusion-associated small transmembrane (FAST) protein encoded by each (438).

Viral isolates were first assigned to the *Orthoreovirus* genus based on similar traits, including a genome comprising 10 segments of dsRNA, the size and morphology of particles by electron microscopy, the number and arrangement of structural proteins within particles, and serologic reactivities. More recently, nucleotide sequences derived from the viral RNAs have confirmed the appropriateness of grouping these isolates, both nonfusogenic and fusogenic, within the same genus. The capacity of related isolates of these viruses to reassort their genome segments upon co-infection of cells, giving rise to infectious reassortant progeny viruses, plays an important role in their evolution (87,189,235). It also suggests a practical approach for designating species of virus within the genus: If particular isolates can reassort genome segments to produce infectious reassortant progeny, then they can be said to belong to the same species (134). Reassortment among nonfusogenic mammalian isolates and also among avian isolates has been demonstrated (373,409), but reassortment between these two groups has not. No reassortment has been reported for the fusogenic mammalian or reptilian isolates.

The name reovirus involves an acronym for respiratory and enteric orphan (397), reflecting that the initial isolates came from human respiratory and enteric tracts but were not associated with any serious human disease. With the isolation and characterization of other dsRNA viruses and the adoption of a standard virus classification scheme, this acronym was retained for the encompassing family of dsRNA viruses, *Reoviridae*, all members of which could then be called reoviruses. As a consequence, the prefix ortho- was added to the name of the initial isolates (orthoreoviruses) and their genus (*Orthoreovirus*) to distinguish them from other members of the family. Despite these formal changes in nomenclature, the orthoreoviruses are still commonly referred to as reoviruses.

Nonfusogenic Mammalian Orthoreoviruses

The nonfusogenic mammalian orthoreoviruses are ubiquitous agents (349) that infect and can be isolated from a wide variety of mammalian species including humans (390,459). Some recent studies have described new isolates from humans, pigs, dogs, and cats (162,189,222,240,316). There is little evidence for restrictions to the range of mammalian species in which a given isolate can replicate. Nonfusogenic mammalian orthoreoviruses can also be isolated from a variety of fresh- and saltwater sources (297,331), consistent with their pervasiveness and predominantly enteric route of infection. Although association with serious human disease is rare, sporadic cases suggest this can occur (222). In addition, although still debated (461), some data suggest that these viruses are a common cause of extrahepatic biliary atresia and other types of cholestatic liver disease in human infants (122,474). A recent survey of human sera collected

in Germany showed that 54% and 15% contained reovirus-specific IgG and IgA antibodies, respectively, indicating frequent human infections (412). The next chapter (Chapter 53) contains a current review of these viruses as agents of disease and tools for studies of viral pathogenesis. Some basic properties of the nonfusogenic mammalian orthoreoviruses are listed in Table 2.

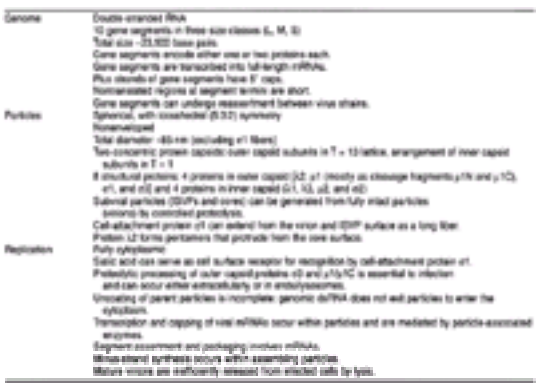

TABLE 2. *Properties of Nonfusogenic Mammalian Reoviruses*

Three distinct serotypes of nonfusogenic mammalian orthoreoviruses are identified by neutralization and hemagglutination-inhibition tests (390,397,459). An isolate from a healthy child is the prototype for reovirus type 1 (type 1 Lang); an isolate from a child with diarrhea is the prototype for reovirus type 2 (type 2 Jones); and isolates from a child with diarrhea (type 3 Dearing) and a child with an upper respiratory illness (type 3 Abney) are prototypes for reovirus type 3 (375,391,397). Although these prototypes are studied most extensively, other isolates from all three serotypes have been studied as well (209). Further discussions of the basis and significance of serotype, and its relationship to the evolution of these viruses, are found later. According to nucleotide sequences, the nonfusogenic mammalian isolates constitute one distinct phylogenetic group (species) within the *Orthoreovirus* genus (134), consistent with their capacity for genome segment reassortment.

Avian Orthoreoviruses

The avian orthoreoviruses remain less well characterized at the molecular level than the nonfusogenic mammalian isolates. They can be isolated from both domestic and wild birds, in which they can cause a variety of diseases including tenosynovitis (arthritis), a gastrointestinal maladsorption syndrome, and runting. The avian orthoreoviruses are divided into five or more serotypes by neutralization tests (501). Most avian orthoreoviruses promote the formation of multinucleated syncytia within infected cultures and are thus said to be fusogenic. Unlike the nonfusogenic mammalian orthoreoviruses, they lack the capacity to agglutinate red blood cells (340) and thus may lack the capacity to bind the cellular carbohydrate receptors that have been implicated in hemagglutination by the nonfusogenic mammalian orthoreoviruses (see later). Some isolates can be adapted for growth in mammalian cells (340,501) or will grow in mammalian cells under certain conditions (285). The particle forms and proteins of mammalian and avian orthoreoviruses have several features and properties in common (133,291,292). Recent sequence determinations for the small (S) genome segments of several avian orthoreovirus isolates showed that they constitute at least one other distinct phylogenetic group (species) within the *Orthoreovirus* genus (134), consistent with their capacity for genome segment reassortment.

Other Orthoreoviruses

Nelson Bay virus (NBV), an orthoreovirus isolate from an Australian flying fox, exhibits characteristics intermediate between the more classical mammalian and avian isolates. In particular, although it was isolated from a mammal and replicates in mammalian cell culture, it induces syncytia formation in those cultures and is thus fusogenic like the avian orthoreoviruses (171,508). A recent orthoreovirus isolate from a baboon research colony in Texas (designated baboon reovirus, or BRV) (262) exhibits properties similar to those of NBV, including replication and syncytia formation in mammalian cell culture, but it is distinguishable in certain respects (138). Nucleotide sequences determined for the S genome segments of these viruses indicate that the fusogenic viruses NBV and BRV represent two additional phylogenetic groups within the *Orthoreovirus* genus, distinct from both the nonfusogenic mammalian virus group and the fusogenic avian virus group (134). Fusogenic virus isolates from snakes have also been assigned to this genus (256,481), but identification of their relationships to the four defined phylogenetic groups awaits nucleotide sequence determinations. Recent evidence from structure determinations (338) and nucleotide sequences (149) suggests that most or all of the fusogenic viruses with 11 genome segments currently grouped in the genus *Aquareovirus* share significant homology with orthoreoviruses and may warrant grouping within the same genus.

The remainder of this chapter is devoted to a description of the nonfusogenic mammalian orthoreoviruses, which are henceforth called reoviruses for simplicity. When mentioned for comparison, other viruses are explicitly identified.

THE GENOME

Physical Characteristics

Double-Stranded

An observation that the viral inclusions in reovirus-infected cells stain orthochromatically green with acridine orange provided the first evidence that the genomic RNA is double stranded (188). Physicochemical properties and nuclease

sensitivities of the virion RNA, including resistance to the single-strand-specific nuclease S1, are consistent with its double-stranded nature (39,187,334,420). Moreover, S1 resistance suggests that the plus and minus strands of the genomic dsRNA are fully collinear and complementary (334). The base composition of the genome indicates both G/C and A/U ratios of one, again suggesting that the genome contains two complementary strands (39,187). X-ray diffraction studies of the isolated genomic dsRNA indicate that it adopts a classical A-form duplex structure, constituting a right-handed double helix with about 10 base pairs per turn, a 30-Å pitch (3-Å translation per base pair), and nucleotides oriented at 75° to 80° to the long axis (15). Other physical characteristics of the dsRNA genome as it is found within virus particles are discussed later.

Segments and Nomenclature

The dsRNA genome of reoviruses is routinely isolated from virus particles in the form of discrete segments. Early evidence from electron microscopy, sedimentation, and chromatography showed that these segments are divided among large (L), medium (M), and small (S) size classes (36,141,185,477,488). Separation on polyacrylamide gel electrophoresis provided the first evidence for 10 discrete segments: three large (L1, L2, L3), three medium (M1, M2, M3), and four small (S1, S2, S3, S4) (428,489). Both electron microscopy and quantitation of 3' hydroxyl groups demonstrated that the 10 isolated genome segments are linear and have two free ends (141,185,312,477). In addition to the different sizes, early evidence that each of the 10 segments is unique included that neither denatured segments from the different size classes nor plus-strand RNAs transcribed from them can cross-hybridize (39,488,489). Many subsequent studies, including sequence determinations and *in vitro* protein translations, have confirmed that reoviruses contain 10 distinct genome segments.

Because they are commonly isolated as discrete units, the 10 genome segments are thought to take this form within reovirus particles. Nevertheless, reports of longer-than-segment-length dsRNA molecules released from reovirus particles under certain conditions (141,185,190,233) might indicate that the genome segments are linked end to end within particles, either noncovalently or through a covalent interaction that is usually broken when the particles are disrupted. Relevance of the form of the genome segments within particles to plus-strand RNA synthesis is discussed later.

As mentioned earlier, the 10 genome segments are routinely distinguishable on polyacrylamide gels, and purified virions contain equimolar quantities of the 10 species (428). Homologous segments from different isolates commonly exhibit differences in their electrophoretic mobilities (370), despite having the same or very similar sequence lengths. The numbering scheme standardly used for the genome segments within each size class reflects the relative migrations in Tris-acetate polyacrylamide gels of the segments from isolate type 3 Dearing (419). Once the segments' protein-coding capacities were determined (302,332), this numbering scheme became meaningfully applicable to all isolates. For example, the genome segment that encodes the core turret protein l2 is always designated to be L2, because that is the case for type 3 Dearing using the standard gel systems. It is important to recognize, however, that with changes in gel system, the order of genome segments within each size class can change; for example, the medium segments of type 3 Dearing migrate in the order M1-M2-M3 in Tris-acetate gels, but M2-M1-M3 in Tris-glycine gels (373). In addition, homologous genome segments from different isolates can show different orders of migration; for example, the small segments of type 3 Dearing migrate in the order S1-S2-S3-S4, but those of type 1 Lang migrate in the order S1-S2-S4-S3 (373). Last, different genome segments from the same size class can co-migrate in certain cases, giving the appearance of nine or fewer segments.

The fact that reoviruses have segmented genomes has important biological and experimental consequences because of the phenomenon of genome segment reassortment. Differences in the electrophoretic migration of genome segments from two parental viruses used for co-infection provide a simple means for determining the parental origin of each genome segment in a reassortant virus (332,373,419). Differences in electrophoretic migration of the viral proteins and restriction fragment length polymorphisms defined using DNA copies of the genome segments have also been used in confirming the genotypes of reassortant viruses (346).

Modifications Including the 5' Cap

Each reovirus genome segment has an identical blocking group at the 5' end of its plus strand: a dimethylated cap 1 structure like that formed on cellular mRNA precursors by cell nuclear enzymes (91,166,313) (Fig. 1). In the case of the reovirus plus-strand RNAs, however, the cap is formed by viral enzymes within viral particles (165,168). The cap is important for efficient protein translation from the free plus-strand RNAs (61,333) because of recognition by cellular translation initiation factor eIF-4E, and it enhances the stability of the free plus-strand RNAs by protecting them from 5' exonucleases (164). In the reovirus life cycle, the cap may also be important for packaging into viral particles and/or minus-strand synthesis, because plus-strand RNAs either lacking a 5' cap or containing a 5' cap 2 structure (with a third methyl group added by a cell cytoplasmic enzyme) are found in large amounts in reovirus-infected cells but are not found in viral particles (121,166,313).

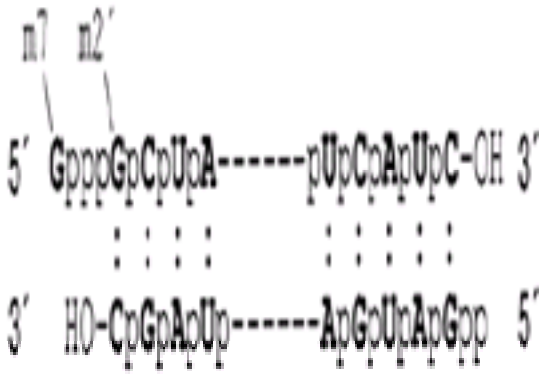

FIG. 1. Conserved features of reovirus dsRNA genome segment termini. Both plus (*top*) and minus (*bottom*) strands are shown. Nucleosides are shown in *bold. Dashes* in strand interiors indicate nucleotides that vary in number and sequence from segment to segment. *Colons* represent base-pairing.

The 5' end of each genomic minus strand is thought to be an unblocked diphosphate (25,91) (see Fig. 1); however, the resistance of this terminus to labeling with polynucleotide kinase provides some evidence for blockage by an unknown structure (313). The presence of a diphosphate at the minus-strand 5' end, and not a triphosphate as would be initially present following minus-strand synthesis from nucleoside triphosphate precursors, probably reflects the activity of an RNA triphosphate phosphohydrolase within reovirus particles (25,91). As stated earlier, both 3' termini of each genomic dsRNA segment take the form of unblocked hydroxyl groups (312) (see Fig. 1). Other modifications to the genomic ribonucleotides, such as methylation or deamination, have not been demonstrated.

Sequence-Based Features

Sequence Determinations

Initial sequence determinations were of terminal regions of the reovirus genome segments and mRNAs (12,170). Subsequent full-length determinations were made directly from genome-derived RNA strands using reverse transcriptase as the sequencing enzyme (31,120), from one or more cloned DNA copies of the segments (26,77,189,213,235,335,414,503,505,506), or from DNA copies of the segments obtained by reverse transcriptase-polymerase chain reaction (RT-PCR) without subsequent cloning (198,303). A complete genome sequence has been determined for prototype isolate type 3 Dearing and encompasses a total of about 23,500 bp (506). Lengths of the individual genome segments from this isolate range from 3,916 bp for L2, to 1,196 bp for S4 (Fig. 2). Nearly complete genome sequences have also been determined for prototype isolates type 1 Lang and type 2 Jones. The lengths of individual genome segments of type 1 Lang and type 2 Jones are either identical or very similar (give or take four nucleotides) to those of type 3 Dearing, except for the S1 genome segment, which encodes the serotype-determining and receptor-binding protein s1 and has lengths of 1,463, 1,440, and 1,416 bp in type 1 Lang, type 2 Jones, and type 3 Dearing, respectively. Several genes from other reovirus isolates have been sequenced as well.

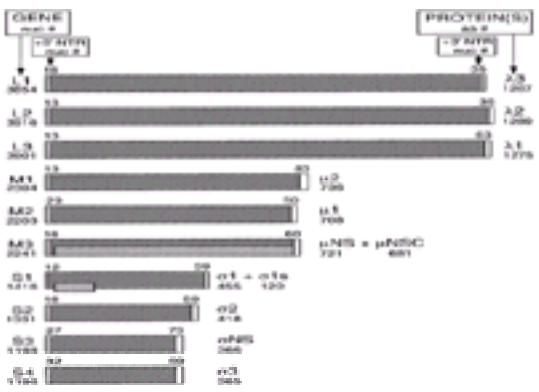

FIG. 2. Coding strategies of the 10 genome segments of reovirus type 3 Dearing. Segments are drawn approximately to scale and are oriented so that left-to-right corresponds to 5'-to-3' for the protein-coding plus strands. The segment names and lengths in nucleotides (*nuc* #) are listed at *left.* The portion of each segment encompassed by protein-coding sequences is *hatched.* Short nontranslated regions (*NTR*) at the ends of each segment remain unshaded, and the lengths of these regions in nucleotides (*nuc* #) are designated above. Names of encoded proteins and their lengths in amino acids (*aa* #) are listed at *right.* The S1 segment encodes two proteins as shown: The s1s protein (*offset, lighter hatching*) initiates at a second initiator codon in a different reading frame from s1 (*darker hatching*). A second protein, µNSC (*lighter hatching*) also arises from the M3 segment, but it is thought to initiate at a second initiator codon in the same reading frame as µNS (*darker hatching*).

Protein-Coding Strategies and Nomenclature

Proteins encoded by the 10 reovirus genome segments can be resolved by electrophoresis on different types of sodium dodecyl sulfate (SDS)-polyacrylamide gels (30,93,100,105,370,449,531). Twelve primary translation products are currently known, with eight genome segments coding for one protein each, and two coding for two each (see Fig. 2). Eight of the 12 proteins (l1, l2, l3, µ1, µ2, s1, s2, and s3) are structural—that is, present within mature reovirus particles—whereas four (µNS, µNSC, sNS, and s1s) are nonstructural and are thought to mediate functions during the intracellular steps in reovirus replication. Genetic analyses with reassortant viruses (332) and *in vitro* translation of isolated mRNAs (302) were used to determine which proteins are encoded by each of the gene segments.

The names of the reovirus proteins include Greek letter designations for proteins of large (l), medium (µ), and small (s) size classes. Each protein is encoded by a genome segment of the corresponding size class. However, the numbering scheme does not always correspond between proteins and their encoding genome segments. For example, although the second most slowly migrating genome segment L2 encodes the second most slowly migrating protein l2 in the standard gels, the third most slowly migrating genome segment L3 encodes the most slowly migrating protein l1 (see Fig. 2). There are several reasons for these discrepancies, including (a) that the numbers were first assigned to the proteins of reovirus type 3 Dearing according to their relative electrophoretic migrations in a standard phosphate-urea gel system (449)

before the protein-coding capacities of the genome segments were determined, and (b) that the two low-copy structural proteins l3 and µ2, as well as the four nonstructural proteins, were identified and named later than the others. In addition, the names of some proteins have been changed over time; for example, nonstructural protein µNS was originally called µ0 (531). Nevertheless, the protein names and their attribution to particular genome segments are now standardly defined as in Figure 2.

Each reovirus genome segment is transcribed end to end by enzymes within viral transcriptase particles to yield full-length, plus-strand copies that serve as mRNAs. Each mRNA includes one long open reading frame (ORF) that spans almost the whole length of the plus-strand sequence. In each case, this long ORF encodes a predicted protein that approximates the size of the larger protein attributed to that segment (see Fig. 2). In addition, the long ORF in each plus strand begins with an AUG that is protected from RNase degradation upon the binding of ribosomes (249). Reovirus translation is generally consistent with the scanning model for eukaryotic translation initiation. The AUG at the beginning of the long ORF in each plus strand is thus almost always the 5'-most AUG in the RNA sequence and is consistently located within an excellent to reasonable context for translation initiation according to the eukaryotic consensus (248). Some of the differences in translational efficiency among the reovirus plus-strand RNAs (169) may be attributable to differences in approximating this consensus, although other determinants are probably also important (see later). The S1 genome segment is unique in encoding a second protein product, s1s, in a separate but overlapping ORF from the larger product s1 (145,218,404). The initiation codon for s1s translation is the first out-of-frame AUG within the s1 ORF (29,78,328,335). The start codons for the s1 and s1s ORFs are separately protected from RNase degradation upon binding of ribosomes (246). The M3 gene segment also encodes a second protein, µNSC, but in this case translation appears to start from the second or third in-frame AUG within the same ORF as µNS (303,503). Important sequence determinants for translation from these downstream start codons in S1 and M3 remain to be fully defined (see later). Additional small ORFs are found in all reovirus genes, but evidence for their translation in cells is lacking. There is also no evidence that proteins are translated from the minus RNA strands of reovirus, consistent with the fact that free minus strands are not released into the cytoplasm as part of the replication cycle (410).

Terminal Nontranslated Regions

Nontranslated regions at the 5' and 3' ends of the reovirus plus strands (outside the boundaries of the long ORF in each) represent only a small part of the genome (see Fig. 2). The lengths of the 5' nontranslated regions range from 12 (S1 and L2 genome segments from certain isolates) to 32 (S4 genome segment) nucleotides. As shown for other eukaryotic mRNAs, such variations in length of the 5' nontranslated regions in the reovirus plus strands may affect their relative translational efficiencies (385). The 3' nontranslated regions range from 32 (L1 genome segment) to 80 (M1 genome segment from certain isolates) nucleotides. Among homologous genome segments from different isolates, lengths of the nontranslated sequences are often, but not always, conserved (135,303).

The plus strands of all genome segments analyzed to date contain the extreme 5' tetranucleotide 5'-GCUA and the extreme 3' pentanucleotide UCAUC-3' (12) (see Fig. 1). In addition, longer regions of sequences near the ends of the genome segments, extending through the nontranslated regions and into the protein-coding regions, are often less variable between isolates than more internal sequences (87,189,235). Sequences near the 5' and 3' ends of the reovirus plus strands also show a propensity to form base pairs in RNA secondary structure predictions, suggesting that these regions may interact in the free plus strands to form panhandle-like structures that may be important for RNA functions (87,274,526). Though a minimal portion of the genome, the nontranslated regions are likely to include sequences important for RNA packaging, recognition by the viral RNA polymerase for initiating plus- and minus-strand synthesis, and translational efficiency; however, the exact sequences that fulfill these functions have yet to be delimited for reoviruses.

PARTICLES

Virions and Partially Uncoated Derivatives

The mature reovirus virion contains all eight viral structural proteins (Fig. 3), but it is not known to contain any of the viral nonstructural proteins or any cellular proteins. The virion is the predominant form of particle released from infected cells in culture or animals or after artificial disruption of infected cell cultures in the laboratory. Virions can readily undergo partial uncoating to yield two distinct types of subvirion particles: infectious (or intermediate) subvirion particles (ISVPs) (51,223,425) and cores (298,426,449). These subvirion particles differ in protein content, in the conformation of certain proteins, and in other physicochemical and biological properties (Table 3, and see Fig. 3). The primary difference between ISVPs and virions is the lack of one outer capsid protein, s3, in ISVPs. The primary difference between cores and virions is the lack, in cores, of three outer capsid proteins: s3, s1, and µ1. ISVPs and cores can be routinely generated by treating virions with purified proteases *in vitro*. ISVPs and cores, or particles similar to these forms, also occur naturally in the course of infection and play specific roles in both early and later stages of reovirus replication (see later section). Some authors have discussed “subviral particles” (SVPs), in which case they are usually describing ISVP-like particles (16,84,440,443).

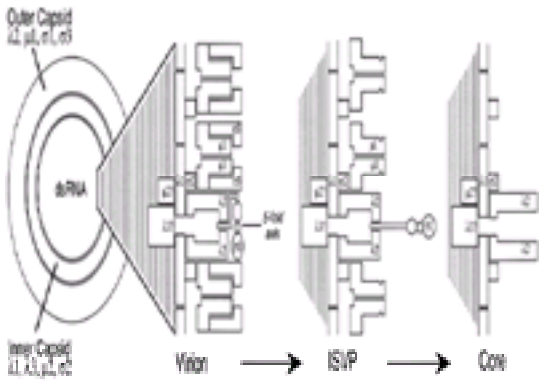

FIG. 3. Diagrams of reovirus structural elements. The two concentric capsids and centrally located dsRNA genome in reovirus virions are drawn in cross-section at *left.* Proteins in the outer capsid and inner capsid are designated. A wedge of the virion capsids, encompassing an axis of fivefold symmetry (labeled), is drawn in greater detail and shows approximate locations and interactions between reovirus proteins (labeled) as currently understood. Positions of open or potential channels through the outer capsid at axes of fivefold (P1) or sixfold (P2 and P3) symmetry are indicated (nomenclature from ref. 310). The position and interaction indicated for the l3 and µ2 proteins are hypothetical. The position of the nonextended s1 protein in virions is also hypothetical. Comparable wedges from a reovirus infectious subvirion particle (ISVP) and core are shown at *right* and reveal the loss and change in conformation of outer capsid proteins in these particle forms. The ISVP is distinguished by absence of the s3 protein, by an endoproteolytically cleaved µ1 protein (*notching*), and by a conformationally altered (extended) s1 protein. The core is distinguished by additional absence of the µ1 and s1 proteins and by a conformationally altered l2 protein. The conformational change in l2 and loss of s1 have opened a channel through the l2 “turret” that protrudes from the core surface.

Feature or property	Virion	ISVP	Core
Buoyant density in $CsCl_2$ (g/cm³)	1.36	1.39	1.43
Sedimentation value (S)	700	630	470
Molecular weight (MDa)			
From diffusion coefficient	100	ND	52
Estimated from components	127	103	49
Particles per mL at 1 OD_{568} (× 10^{12})	2.1	2.7	4.4
Diameter (nm)			
Negative-stain electron microscopy	73	64	51
Cryoelectron microscopy	85	80	60
Low-angle x-ray diffraction	83	ND	60
Outer capsid proteins			
σ3	Present	Absent (degraded)	Absent (degraded)
µ1	Present	Present as δ conformer (extended)	Absent (eluted)
µ1/δ1 C	Present	Present as δ1/δ1 + δ fragments	Absent (degraded)
λ2	Present	Present	Present as conformer
Inner capsid proteins	Present	Present	Present
10 dsRNA gene segments	Present	Present	Present
Oligonucleotides	Present	Variable	Absent
Infectivity after attachment to cell surface	High	High	Low
Interactions with membrane bilayers	No	Yes	No
Transcription-related activities			
Initiation and capping	Yes	Yes	Yes
Elongation	No	No	Yes

TABLE 3. *Characteristics of common reovirus particle forms*

A representative study in which all three of the preceding particle types were compared by negative-stain electron microscopy indicated diameters of 73 nm for virions, 64 nm for ISVPs, and 51 nm for cores (51). Diameters obtained from cryoelectron micrographs, in which shrinkage artifacts common to negative staining (200) are minimal, were 85, 80, and 60 nm for virions (excluding the s1 fiber), ISVPs (excluding the s1 fiber), and cores (excluding the l2 turrets), respectively (131,310). The latter values concur with those obtained by low-angle x-ray diffraction (199). Virions, ISVPs, and cores exhibit distinct morphologies in negative-stain electron micrographs (51,109,163,238,278,298,309,449,478). Virions appear roughly spheroidal, with smooth perimeters, except for flattened areas that are apparent in certain orientations. Few details of subunit arrangement are apparent in negatively stained virions. ISVPs appear even more spheroidal than virions; furthermore, regions of their perimeters often have a saw-toothed appearance suggesting structural subunits (µ1 protein). Long fibers (s1 protein) are sometimes seen extending from the surfaces of virions and ISVPs (163). Negatively stained cores are more distinctive in that they have prominent turrets (l2 protein) protruding from their surfaces.

Radial and Axial Distributions of Proteins Within Particles

Observations from negative-stain electron micrographs first defined reovirus virions as icosahedrally symmetrical objects comprising two radially concentric, separable protein shells (inner capsid and outer capsid) surrounding the centrally condensed RNA genome (109,278,298,478). The description of two radially differentiated protein layers within reovirus virions was supported by data from dynamic light scattering (200) and low-angle x-ray diffraction (199). The latter studies also showed the outer capsid to be substantially thicker than the inner capsid and to have a higher water content. Some of these early studies suggested axial differentiation of proteins within reovirus particles as well, in that the protruding turrets of l2 protein were seen surrounding the fivefold axes in cores (298,426,449,500) and, somewhat later, the projecting fibers of s1 protein were seen to extend from the fivefold axes in certain virions and ISVPs (22,161,163). Evidence for extrusion of the viral plus-strand transcripts from the fivefold axes of actively transcribing cores supported the notion of axial differentiation of proteins and their associated functions (27,516).

Components of Viral Particles

Early studies used negative-stain electron microscopy to describe the precise arrangements of proteins and their symmetry relationships within the reovirus capsids. These studies, although accurate in several details for the outer capsid (238,309), were largely superseded by ones using cryoelectron microscopy and three-dimensional image analysis (130,131,310). An analysis of the structure of cores at 3.6 Å resolution using x-ray crystallography has recently defined the inner capsid structure in much greater detail (378). Information about each structural protein, in addition to that immediately following, can be found in the Proteins section.

Core Components

T=1 Core Shell

The core shell (inner shell in virions) is formed by 120 copies of the l1 protein. The l1 subunits are arranged with T=1 symmetry, with a parallel dimer of l1 composing the asymmetric unit. Five of these asymmetric units, containing a total of 10 l1 subunits, surround each icosahedral fivefold axis in the core. Twelve of these decameric units of l1 then interact to form the complete core shell. This shell is flat and thin but approximately continuous, being permeated by small, radially

directed channels at only a few positions including the fivefold axes (378). Such channels are seemingly required to allow entrance of ribonucleoside triphosphates and exit of newly synthesized mRNAs during transcription by cores (see later). The two l1 subunits per asymmetric unit have similar, but nonidentical, structures, which allows them to pack tightly within the decamers and intact shell. Five copies of l1 from each decamer approach the fivefold axis and interact with each other around it. The other five copies of l1 from each decamer approach the five surrounding threefold axes and interact with the homologous copies of l1 from adjacent decamers in the shell. An N-terminal region of l1 was not fully visualized in core crystals, but the portions of it that were visualized are located internal to the main core shell, they assume an extended conformation, and appear attributable to the copies of l1 that approach the threefold axes (378). Thus, an N-terminal region (240 amino acids) of the copies of l1 that approach the fivefold axes remains to be placed in the core structure.

Internal Transcriptase Complexes

A proteinaceous structure internal to the l1 shell has been visualized to date only at lower resolution by cryoelectron microscopy and x-ray crystallography (130,378). This structure has a flower-like appearance and projects below the shell by 65 Å (central stalk) to 80 Å (surrounding petals) at each of the 12 fivefold axes. Biochemical evidence suggests that it, or other poorly visualized internal structures, are formed in whole or part by an N-terminal region of l1 and the two low copy number core proteins l3 (12 copies) and μ2 (12 to 24 copies) (130). Functions assigned to these proteins through other studies (see later) have led to the proposal that these complexes represent the viral transcriptase complexes, which transcribe the genomic dsRNA segments into mRNAs (see later). Additional work is needed to define the structures of these complexes in more detail.

External Turrets and Nodules

Sitting directly atop the l1 shell are two other core proteins: l2 and s2. A pentamer of l2 forms each of the 12 characteristic turret-like structures or “spikes” that project by more than 100 Å above the l1 shell on the core surface, surrounding the fivefold axes (a total of 60 l2 subunits per particle). The individual monomers of l2 within each pentamer are identical and exhibit an extended, multidomain structure (378). Each monomer spirals around the fivefold axis, enclosing a large solvent cavity within the center of the turret and giving the turret walls an interlocking structure. Three domains in each monomer, including the one l2 domain that contacts l1, represent enzymes involved in mRNA capping (see later). The catalytic pockets of these enzymatic domains face into the central cavity of each pentameric turret. A channel through the base of the turret connects the fivefold channel through the l1 shell (see earlier) with the turret cavity and is very likely important for the movement of nascent transcripts. The opening at the top of the l2 turret exhibits variable diameter in different visualizations of cores, as determined by different orientations of a series of outermost, apparently nonenzymatic, flaplike domains of l2 (131,281,310,378). A more closed conformation of these flaps may be important for retaining nascent transcripts inside the turret for capping (378), whereas a more open conformation may be important for release of the transcripts after capping is complete (27,378,516). Specific mechanisms for regulating these conformational changes remain unknown. In virions and ISVPs, however, the flaps uniformly adopt a more closed conformation (130,131,310). The closed conformation may be important for the capacity of l2 to bind the s1 fiber protein at the fivefold axes (see later).

Monomers of s2 form the 150 ovoid nodules that sit atop the l1 shell and decorate the core surface at the twofold axes and surrounding the threefold and fivefold axes (131,310,378). Each of the 60 copies surrounding the fivefold axes makes a minor contact with one l2 subunit. The 60 near-fivefold and 60 near-threefold copies of s2 have similar, but nonidentical, structures, allowing them to make distinct contacts with the underlying l1 molecules. The 30 twofold copies of s2 were only partially resolved at high resolution in the crystal structure of cores, because they can adopt either of two equivalent, twofold symmetrical orientations at each of these positions. Each twofold copy of s2 appears to make minor contacts with surrounding near-fivefold and near-threefold copies of the protein. Because each s2 copy appears to make contacts with l1 across subunit boundaries within the l1 shell, s2 is proposed to serve as a stabilizing clamp for that shell (378).

Although they are core proteins and thus classically identified as components of the inner capsid, proteins l2 and s2 occupy structural positions that are clearly distinct from those of the core shell protein l1. They sit atop the l1 shell, at radial positions between the core shell and the T=13 outer shell (see later). In fact, the extended conformation of l2 allows it to extend upward into the outer capsid lattice and to occupy positions within the outer lattice surrounding each fivefold axis (see later). Thus, it is possible to describe s2 and the bottom, l1-binding domain of l2 (see earlier) as forming an incomplete, intermediate protein layer in reovirus particles, which separates the T=1 core shell and T=13 outer shell and is probably important for interactions between them. By extending up into the outer shell, l2 may be especially important for holding the two shells together.

Outer Capsid

T=13 Outer Shell

The reovirus outer capsid layer has been analyzed using a number of different styles of electron microscopy. The results of these studies agree that the outer shell has subunits arranged in a skewed, T=13 (laevo) lattice (131,238,309,310). The lattice is formed primarily by 600 molecules of protein μ1. The stoichiometry of μ1 fails to account for 180 of the expected 780 (13 × 60) subunits in a classical T=13 structure. The remaining positions are occupied instead by 60 molecules of protein l2, which substitute for μ1 around each fivefold axis such that one molecule of l2 occupies three of the μ1 subunit positions. This lattice exhibits a very similar structure in virions and ISVPs, except that in virions the s3 protein is also present (see later).

The μ1 protein appears to be organized in trimeric complexes within virions and ISVPs (131,238,309). The trimers are most apparent at higher axial radii in ISVPs, where adjacent trimers make contact across local twofold axes (131). At lower radii, other intertrimer contacts are seen, giving μ1 a structure that interlocks across the outer capsid. Because the

μ1 network is substituted around each fivefold axis by l2, not all μ1 subunits are in identical positions. The copies of μ1 adjacent to l2 appear to contact that protein directly. Only some of the 600 μ1 subunits appear to be in proper position to contact the s2 nodules projecting from the surface of the core.

The mass of μ1 and s3 is arranged around large solvent channels that pass axially through the outer capsid (131,310). Two different types of channels are present because of substitution by l2 around the fivefold axes. The two channel types are designated P2 and P3 and range in diameter from 2.5 to 11 nm at different axial radii in ISVPs. Each P2 channel is partially blocked by a l2 subunit. The diameters of both P2 and P3 channels are decreased at higher radii in virions by the presence of s3 (see later). The purpose of these channels is not known, but they may be required for conformational changes in μ1 involved in membrane penetration (see later).

An observation that the μ1 and s3 proteins bind to each other in solution (83,260,437,471) is reflected in intimate contacts between these proteins within the virion outer capsid (131). Difference maps between images of virions and ISVPs permit densities to be attributed specifically to s3 and suggest that each s3 subunit is elongated in an axial direction. The s3 subunits project 34 Å above the outer extent of μ1 and look like “fingers” on the virion surface (131,310). Each copy of s3 appears to contact as many as three adjacent μ1 subunits and may in this way serve to stabilize the outer capsid structure. Six and four subunits of s3 are placed around the perimeters of the P3 and P2 channels, respectively, and appear to contact each other side to side to form complete and partial hexamers. Because of the presence of l2, not all s3 subunits are in identical positions. One of the s3 copies adjacent to each l2 subunit appears to contact that protein directly.

The arrangement of μ1 in virions and ISVPs exhibits only minor differences (131,219), despite the fact that μ1 has undergone an additional endoproteolytic cleavage in ISVPs. Thus, the major difference between these two particle types is the loss of 600 s3 subunits without a major perturbation of underlying structures in the outer capsid or core regions. This observation contributes to an interpretation that the primary contacts within the outer shell are made among subunits of μ1 and l2, while s3 effectively decorates this lattice. However, only μ1 in complex with s3 appears to be competent for assembly into the outer shell (83,437).

Extending Fibers

In negatively stained images of some virions and ISVPs, s1 is seen as a long fiber that projects out more than 40 nm from the particle surface (131,163). The spacing of these fibers suggests location at the fivefold axes, as does work with antibodies indicating that s1 and l2 are in close proximity within virions (202,260). Small portions from near the base of the s1 fiber are all that is standardly visualized by cryoelectron microscopy and three-dimensional reconstruction of virions and ISVPs (130,131,310), probably because of the flexible nature of these fibers. The placement of s1 atop l2 is further supported by a recent study demonstrating the absence of s1-attributed density in a cryoelectron microscopy reconstruction of cores that have been “recoated” *in vitro* with the μ1 and s3 proteins, but not s1 (83). The s1 fibers seen extending from particles in raw micrographs (131,163) have a head-and-tail morphology, with the head distal to the particle-bound end. The extreme base of the tail is proposed to interact with l2, at a binding site created by five l2 subunits. Because each s1 fiber most likely represents a homotrimer of that protein (see later), binding of s1 to l2 appears to involve a three-to-five symmetry mismatch. Some evidence suggests that s1 can also assume a less extended conformation in virions (131,163), but the nature of this alternative conformation remains undefined. Different conformations of s1 may be important for its roles in mediating binding to cell surface receptors (see later). The s1 protein appears to be lost from cores by elution following conformational changes in the outermost flaplike domains of l2 (131).

RNA Components

Centrally Condensed dsRNA

Low-angle x-ray diffraction, cryoelectron microscopy, and x-ray crystallography all indicate that the genomic dsRNA is found in reovirus particles within a central sphere, about 49 nm in diameter, and that it is well ordered so that adjacent helices are locally parallel and separated by distances of 25 to 27 Å (131,199,378). Regions of local disorder in the parallel packing scheme are expected to permit the RNA to pack into the available space. Given the measured interhelix distances and the size of the 10 genome segments, calculations indicate that the central cavity should provide just enough room to accommodate the total genome (341). Thus, large quantities of protein are unlikely to be bound to the packaged dsRNA, consistent with other observations. An additional conclusion is that random packaging of more than 10 segments per particle is an unlikely explanation for the high ratio of particles to plaque-forming units (pfu) in reoviruses. The arrangement of the genomic dsRNA has relevance to the mechanism of reovirus transcription (see later). The dispositions of the segment ends are unknown, but the end containing the 3' end of the minus strand is expected to be bound by the RNA polymerase to permit efficient onset of transcription upon entry of the particle into cells.

Oligonucleotides

About 25% of the RNA in purified virions is in the form of small, single-stranded oligonucleotides (37,427), which can be grouped into two major classes (34). Quantities can vary widely with conditions of virus growth, including temperature (253), as well as between virions obtained from the same purification (150). About 70% of the oligonucleotides (~2,000 copies per virion) terminate with a 5'GC(U)(A) and are two to nine residues long (35,38,348). The conserved 5' sequences suggest that these are the products of abortive transcription. They are often termed initiator oligonucleotides, and those packaged into virions are generated in the final stages of morphogenesis (38,151). Oligoadenylates constitute a second major class of oligonucleotides, which are two to 20 residues long and present in about 850 copies per virion (34,348,462). Like the initiator oligonucleotides, they appear to result from an alternative activity of the viral transcriptase and are also generated in the final stages of morphogenesis (532). Whereas virions contain both classes of oligonucleotides, cores lack them (73,151,223,425,449). Whether oligonucleotides are present in ISVPs is controversial (84,223,425). Their exact locations within virions remain unknown (199). Functions of the oligonucleotides are also

unknown, but there is evidence that they are not required for infectivity (73,151).

Molecular Weights

Molecular weights for virions and cores of reovirus type 3 Dearing were determined from diffusion coefficients to be 129.5 and 52.3 megadaltons (MDa), respectively (150). The total weight of the genomic RNA is calculated to be 15.1 MDa from sequences of the 10 genome segments of this isolate. Oligonucleotides are estimated to contribute another 5 MDa to the weight of virions (348). Thus, proteins are expected to contribute roughly 109 and 37 MDa (85% and 70% of totals) to the molecular weights of virions and cores, respectively. These weights correspond well with those obtained by calculation from sequence-predicted masses of the proteins and current estimates for their copy numbers in particles (see Table 3). Similar estimates can be made for ISVPs (see Table 3). For these calculations, it was assumed that the proteins in virions are full length, given the lack of evidence for loss of mass by protein cleavage, and good correlations between molecular weights calculated from sequences and those estimated from gels. The crystal structure for the reovirus core indicates that the assembled forms of l2 and s2 are indeed full length (except for lacking their initiator methionines), and that the assembled form of l1 extends to its sequence-predicted C terminus (the N terminus of l1 was not well visualized in the crystal structure) (378).

Purification of Particles and Their Infectivities

Murine L929 cells are commonly used for plaque assay and for growing virions for purification. For purification, virions are released from cells by sonication and then dissociated from other components by treatment with deoxycholate and extraction with freon (39,128,163,223,420,449) or a newer freon substitute (308). Final purification of virions is commonly achieved by centrifugation through a cesium chloride (CsCl) gradient. Values for sedimentation and buoyant density of reovirus particles are shown in Table 3 (51,150,163,449). Banded virions are dialyzed into buffer (often 150mM NaCl, 10mM $MgCl_2$, 10mM Tris, pH 7.5) for storage at 4°C, where they can remain stable and infectious for extended periods. An average yield from this procedure is 1×10^{13} virions (~about 1.8 mg) per 1×10^{8} cells. The equivalence 1 OD_{260} = 185 µg protein per mL = 2.1×10^{12} virions per mL is routinely used for estimating the concentration of purified virions (449).

Chymotrypsin is most commonly used to convert virions to ISVPs or cores *in vitro* (51,163,223,344,425,426,449,454,467). At fixed conditions (buffer, enzyme concentration, temperature, time of treatment), the concentration of virions included in the mixture determines whether ISVPs or cores are obtained as products (51,54,57,60,163,223). The particular conditions yielding one or the other particle form can vary between isolates (101,127,223). ISVPs or cores generated by this treatment can be purified by centrifugation through CsCl gradients. Purified ISVPs are commonly stored in the same buffer as virions, but a different buffer (1M NaCl, 100mM MgCl2, 20mM HEPES, pH 8.0) was found to maintain cores in a nonaggregated state (101). Comparisons of protein quantities in virions and subvirion particles suggest the following equivalences: 1 OD_{260} = 2.7×10^{12} ISVPs per mL and 1 OD_{260} = 4.4×10^{12} cores per mL (100).

When purified virions are tested for infectivity by plaque assay in L cells, a particle-to-pfu ratio of from 50:1 to 500:1 is commonly obtained (14,144,226,467). The basis for this high ratio is unknown; it could reflect either that defective particles are present in the preparations or that productive infection involves a complex process that only a statistically determined subset of infectious particles can routinely complete. In a few studies, a ratio approaching 1:1 between particles and infectious units has been reported; however, these studies included special circumstances such as protease activation of viral particles and measurement of infectious units by a fluorescent focus assay (454). In general, the efficiency of infection is mildly enhanced in preparations of ISVPs, and reduced by several orders of magnitude in preparations of cores. An exception to the enhanced infectivity of ISVPs is seen with isolates type 3 Dearing and type 3 clone 31, which have a s1 cell-attachment protein that is sensitive to protease cleavage during the generation of ISVPs and that exhibits an associated loss in infectivity (85,342).

Other Types of Icosahedral Reovirus Particles

Top Component Particles

“Empty” virions, which contain markedly reduced quantities of dsRNA gene segments and oligonucleotides, account for a large proportion of the particles purified from reovirus-infected cells (380,449). Their buoyant density is 1.29 g/cm^3, lower than that of full virions and consistent with their lack of RNA (150,163,255,449); thus, they migrate above full virions in CsCl gradients and are called top component particles. Both their protein content and morphology are very similar to those of full virions, except that negative stains can enter their central cavity (449,500). Protease treatments of empty virions can be used to generate empty ISVPs and empty cores (130,277,449,500). Empty particles have been used in studies that address the function of reovirus proteins (196,277,416) and have provided evidence for the inwardly protruding transcriptase complexes within reovirus particles (130). The derivation of empty virions is unknown; however, they are unlikely to be precursors of full virions during morphogenesis (226). Methods for increasing the relative yields of empty virions from infected cells have been described (255).

Particle Assembly Intermediates from Infected Cells

Isolation of particles from infected cells can provide insight into reovirus assembly and other aspects of replication. Gradient fractionation of assembly intermediates from infected cells tentatively identified distinct particle populations engaged in minus-strand RNA synthesis (replicase particles) and plus-strand RNA synthesis (transcriptase particles) (323). The latter particles were found to represent cores that are associated with variable amounts of the nonstructural protein µNS (323,532). Particles obtained from infections with temperature-sensitive (ts) or other assembly-restricted mutants are useful for identifying putative assembly intermediates that accumulate behind the restrictive block. Core-like particles that accumulate at nonpermissive temperature with ts group B and G mutants provide evidence for generation of the core as a central intermediate in virion morphogenesis (322). Similarly, particles containing core proteins l1, l3, and s2, but lacking other core components, have been shown to accumulate at nonpermissive temperature with one

L2-segment ts mutant and have been proposed to suggest a role for this particle type in core assembly (204).

Particles Assembled In Whole or In Part from Recombinant Proteins

Coexpression of subsets of recombinant reovirus core proteins in HeLa cells using vaccinia virus vectors has resulted in production of empty core-like particles containing the following proteins: l1 and s2; l1, l2, and s2; l1, l3, and s2; and l1, l2, l3, and s2 (513). The minimal protein complement for core-like particle assembly in this system thus appears to be l1 and s2. These findings provide evidence for packaging of l3 independently of l2. Additional assemblies of core protein µ2, outer capsid proteins, or viral RNA in this type of system have not yet been reported.

ISVPs and cores, generated from virions by *in vitro* protease treatment and then purified, can serve as platforms for *in vitro* assembly of the missing outer capsid proteins. This process, recently termed recoating (219), was first indicated by the binding to ISVPs of s3 from infected cells (16). A subsequent study using *in vitro* translated, recombinant s3 demonstrated its feasibility for more controlled studies (430). The recent study demonstrates that approximately full levels of baculovirus-expressed recombinant s3 can be bound to ISVPs *in vitro*, yielding recoated particles that are very similar to virions in a large number of structural and biological properties (219). The recoating approach has also been extended to cores, to which baculovirus-expressed recombinant µ1 and s3 proteins, or µ1, s3, and s1 proteins, can be bound (83,151). The utility of this general approach lies not only in its capacity to address the structure and assembly of the reovirus outer capsid but also in the fact that the resulting particles demonstrate infectivity properties attributable to the *in vitro* assembled proteins (see later). Another variation to this approach involves the binding of nonstructural protein µNS to cores (66), yielding particles that appear to mimic transcriptase particles isolated from infected cells (323).

PROTEINS

The eight species of viral proteins in mature reovirus virions (structural proteins) have particle-based functions that are critical to initiation of infection and synthesis of the capped viral mRNAs (Table 4). These same proteins may also play regulatory or other “nonstructural” roles within the infected cell. General characteristics and functions of these proteins are discussed later. The five core proteins (l1, l2, l3, µ2, and s2) are described first, followed by descriptions of the three additional proteins in the virion outer capsid (µ1, s1, and s3). Examination of proteins in reovirus-infected cells initially revealed two other virally encoded proteins that are not present in mature virions (nonstructural proteins) and that are now called µNS and sNS (531). A smaller form of µNS, called µNSC, is also found in infected cells (260). Nucleotide sequencing (29,78,246,328,335) plus *in vitro* translation of viral mRNAs and comparison with proteins from reovirus-infected cells (80,145,218,404) identified an additional nonstructural protein, s1s (227), encoded by the second ORF in the S1 genome segment. All four nonstructural proteins are discussed in detail later (see Table 4).

Encoding segment	Protein	Mass (kd)	Copy no. per virion	Location in virions	Presence in particle forms*	Function or property
L1	λ3	142	12	Inner capsid	V, I, C	RNA-dependent RNA polymerase
L2	λ2	145	60	Outer capsid, core spike	V, I, C	Guanylyltransferase, methyltransferases
L3	λ1	143	120	Inner capsid	V, I, C	Binds RNA, Zn metalloprotein, NTPase?, RNA helicase?, RNA triphosphatase?
M1	µ2	83	20	Inner capsid	V, I, C	Binds RNA, NTPase?
M2	µ1	76	600	Outer capsid	V, I	N-myristoylated, cleaved into fragments, role in penetration, role in transcriptase activation
M3	µNS	80	0	Nonstructural	—	Binds core, role in secondary transcription, probable role in RNA assortment or replication
	µNSC	75	0	Nonstructural	—	Unknown
S1	σ1	49	36	Outer capsid	V, I	Cell attachment protein, hemagglutinin, primary serotype determinant
	σ1s	14	0	Nonstructural	—	Dispensible in cell culture
S2	σ2	47	150	Inner capsid	V, I, C	Binds ds RNA
S3	σNS	41	0	Nonstructural	—	Binds ss RNA, probable role in RNA assortment or replication
S4	σ3	41	600	Outer capsid	V	Sensitive to protease degradation, binds ds RNA, zinc metalloprotein, effects on translation

*V, virion; I, ISVP; C, core.

TABLE 4. *Reovirus proteins*

Structural Proteins in the Core

l1

The l1 protein (142 kd, 1,275 amino acids) is the primary constituent of the core shell (296,500,513) and is present in 120 copies per particle (100,378). In recombinant form, it requires coexpression with core nodule protein s2 to assemble into icosahedral particles (513). Recombinant l1 was also shown to interact with core turret protein l2 and low-copy core protein l3 (460). In native particles, l1 has extensive contacts with s2 and l2 (378), and it is thought to interact with one or both of the low-copy-number core proteins, l3 and µ2, as well (130,378). An N-terminal region of l1 was proposed to project into the core interior based on its protease sensitivity in top component particles (130) and to be more hydrophilic and flexible than the rest of the protein based on sequence analyses (198). This N-terminal region was not fully visualized in core crystals, but the portions of it that were visualized are located internal to the main core shell and assume an extended conformation (378). The l1 sequence contains a conserved CCHH zinc-finger motif near the junction between this N-terminal region and the rest of the protein (26,198), and l1 was shown to bind Zn^{2+} in a blotting assay (407) and in core crystals (378). The role of the zinc finger remains unknown.

Functions of the l1 protein are only partly understood. Recent reassortant analyses showed that the L3 genome segment encoding l1 contributes to determining differences in the NTPase activities of cores (354,355), consistent with the presence of nucleotide-binding motifs near the l1 N-terminus (26,44,354). Moreover, recent studies with partially purified recombinant l1 protein showed it to possess not only NTPase activity but also RNA triphosphate phosphohydrolase and helicase activities (44,45). These findings tentatively assign l1 to mediating the first step in mRNA capping by reovirus particles as well as to mediating RNA duplex unwinding during one or more step in reovirus plus- and/or minus-strand RNA synthesis. A conformational change in l1 during transcription by cores was previously noted (366). Consistent with

its RNA-directed enzymatic activities, the l1 protein was shown to have an RNA binding activity (both ssRNA and dsRNA, but ssRNA preferred) that is not specific for reovirus RNA and is mediated by sequences near the N-terminus of l1, not including the zinc finger (46,263,407). In another reassortant analysis, the L3 genome segment was shown to be a genetic determinant of isolate-dependent differences in viral yields in L cells (295).

s2

The s2 protein (47 kd, 418 amino acids) interacts with l1 to form the core shell (296,500,513). Initial estimates suggested 120 or 180 s2 copies per particle (100,449), but the recently determined core crystal structure showed that s2 forms 150 nodule-like features that reside fully external to l1 on the core surface (378). In addition to binding to l1, 60 copies of s2 in cores share a small region of interaction with l2 (378). Furthermore, it appears likely that s2, and not l1 as previously inferred (306), binds to the base of major outer capsid protein µ1 in virions and plays a role in anchoring it in the particle (378).

The functions of s2 remain poorly understood. The s2 protein shows weak affinity for dsRNA (120,407), but the significance of this finding appears uncertain given the external location of s2 in cores (378). In recombinant form, s2 must be coexpressed with core shell protein l1 for assembly of icosahedral particles to occur (513). A ts mutant whose lesion maps to the S2 genome segment encoding s2 (373) fails to assemble particles or to synthesize dsRNA at nonpermissive temperature, suggesting a role for s2 in one or more steps in replicase particle assembly, including RNA selection or replication (99,104,160,216,296). Selection and characterization of ts^+ revertants from this mutant showed the reversion events to be exclusively intragenic and to involve true reversion at s2 residue 383 in all instances (99). In reassortant analyses, the S2 genome segment encoding s2 was shown to be a genetic determinant of isolate-dependent differences in the induction of interferon a/b in cultured murine cardiac myocytes (436).

l2

Pentamers of the l2 protein (144 kd, 1,288 to 1,289 amino acids) form the turret-like projections that surround the fivefold axes of cores (367,500), providing a total of 60 l2 subunits per particle (100,131,367,378). There is evidence for l2 interactions with each of the other seven structural proteins: l1, l3, µ2, and s2 in cores and µ1, s3, and s1 in the outer capsid of virions (83,131,202,204,306,310,378,460,500). However, the evidence for l2 interactions with low-copy core proteins l3 and µ2 within particles remains weak or indirect, and these interactions now seem less likely given the recent core crystal structure showing that l2 sits fully atop the l1 shell (378). A l2 fragment lacking about 15 kd can represent more than 10% of the l2 molecules in certain preparations of virions (100,260), but the significance of this fragment is unknown. It may represent cleavage and loss of a C-terminal region of l2, as recently shown to occur *in vitro* after exposing cores to mild heat and protease (281).

The l2 protein plays important roles in particle assembly. Some ts mutants whose lesions map to the L2 genome segment encoding l2 (332) synthesize dsRNA and form core-like particles, but not whole virions, at nonpermissive temperature, suggesting a role for l2 in outer capsid assembly (160,322). A small C-terminal region of l2 appears to constitute the binding site for s1 after a larger C-terminal portion of l2 undergoes a structural rearrangement from its position in cores (83,131,281,378). Another ts mutant whose lesion maps to L2 was shown to accumulate core-like particles that are deficient in l2, the low-copy core protein µ2, and the genomic dsRNA segments at nonpermissive temperature (204). The characteristics of these particles suggest a role for l2 in replicase particle assembly (including RNA selection, replication, and packaging) as well as in outer capsid assembly (204). Also suggesting a role for l2 in RNA replication or packaging was a genetic study with reassortant viruses showing that the L2 genome segment determines an isolate-dependent difference in the generation of genome segment deletions and defective interfering (DI) stocks during serial passage of reoviruses at high multiplicity of infection (MOI) (67).

The l2 protein also mediates enzymatic activities in 5' capping of the reovirus mRNAs. A series of studies with native and recombinant l2 confirmed that it mediates the RNA guanylyltransferase activity in mRNA capping (93,152,279,280,286,424). Reovirus cores incubated with [a-^{32}P] GTP form covalent l2–GMP complexes, consistent with this activity (93,152,281,424). An early study localized the GMP linkage to lysine 226 in the N-terminal half of l2 (152), but a more recent study contested that finding and proposed lysine 190 as the linkage site (279). The latter study also showed that an N-terminal 42-kd region of recombinant l2 is sufficient for guanylyltransferase activity. The crystal structure of reovirus cores revealed that this region constitutes an independent domain that also provides the contacts to l1 and s2 in cores (378).

Evidence that l2 mediates one or both of the methyltransferase activities in mRNA capping, for which S-adenosyl-L-methionine (SAM) is the methyl donor (422), remains more indirect, as recombinant l2 has not yet been shown to mediate these activities (279,286). This lack of activity by recombinant l2 may reflect that it is monomeric whereas only the pentameric form of l2 in cores may be active at methyl transfer (279,286,378). Evidence for the methyltransferase activity of l2 includes that it is the only core protein labeled by 8-azido-SAM (414), that it includes a small central region of sequence with similarity to the SAM-binding motif of other viral and cellular methyltransferases (242), and that both l2 in cores and recombinant l2 bind to SAM in an ultraviolet (UV) cross-linking assay via a central region of sequence that overlaps the previously identified SAM-binding motif (242,280). Most recently, the core crystal structure revealed that a site in each of two separate domains formed by a large central region of l2 sequence can bind to S-adenosyl-L-homocysteine, the product of methyl transfer from SAM, suggesting that these two domains represent the two capping methyltransferases (378). Which domain represents the 7-N-methyl-transferase and which the 2'-O-methyltransferase remain undefined, but the domain formed by the more C-terminal sequences, and including the previously identified SAM-binding site (242,280), is proposed to be the 2'-O-methyltransferase (378). A small region of l2 sequence that approximates the A element of a GTP-binding motif (414) may constitute part of the RNA 5'-end binding site of one of the methyltransferases (the proposed 2'-O-methyltransferase) (378).

Conformational changes in an external portion of the l2 turret formed by C-terminal sequences of l2 are known to occur during generation of cores, resulting in an increase in the diameter of a channel through the top of the turret (130,131).

This conformational change may be important for allowing or regulating transcript capping and release from cores (27,378,516). It may also be important for allowing s1 loss during viral entry (83,131). The mechanism of this conformational change in l2 and its probable regulation by other viral proteins remain poorly characterized.

In other reassortant analyses, the L2 genome segment encoding l2 has been shown to be a genetic determinant of isolate-dependent differences in the crystallization of core particles (101), growth in enteric tissues of newborn mice after intragastric inoculation (49), transmission between newborn mice (237), growth and injury in different organs of mice with severe combined immunodeficiency disease (SCID) (194), induction of myocarditis in newborn mice (433), and induction of interferon a/b and sensitivity of viral growth to interferon a/b in cultured murine cardiac myocytes (436).

l3

l3 (142 kd, 1,267 amino acids) is a low-copy core protein (62,105,215), estimated to be present in only 12 copies per particle, or one copy per icosahedral fivefold axis (100). It has not been definitively localized within particles, but it was shown to be a component of the core shell that is not exposed to the core exterior (76). It is thought to be a constituent of the protein complexes that project into the interior of reovirus particles near the fivefold axes (130,378). Recombinant l3 was shown to interact with core shell protein l1 and core turret protein l2 (460). It is suspected to interact with those same two proteins, and perhaps also with the other low-copy core protein µ2, within particles (130,194,433).

The functions of l3 appear to relate to its role as a component of the reovirus RNA polymerase. A ts mutant whose lesion maps to the L1 genome segment encoding l3 (373) is severely reduced for dsRNA synthesis (104,216) and forms empty particles at nonpermissive temperature (160), suggesting a role for l3 in one or more steps in replicase particle assembly including RNA selection or replication. An early reassortant study indicated that the L1 genome segment determines an isolate-dependent difference in the pH optimum of core transcription, suggesting that l3 may be the viral transcriptase (127). A more recent reassortant study showed that L1 contributes to determining a difference in the amounts of transcripts produced by cores, consistent with the previous suggestion (519). A central portion of the deduced amino acid sequence of l3 includes sequence motifs common to the catalytic regions of RNA polymerases (69,241,243,326). Furthermore, recombinant, purified l3 demonstrates poly(C)-dependent poly(G) polymerase activity *in vitro* (460). These findings suggest that l3 represents the catalytic subunit of the reovirus RNA-dependent RNA polymerase, which is likely to mediate both plus- and minus-strand RNA synthesis within reovirus particles. The L1 genome segment encoding l3 was also shown to be a genetic determinant of isolate-dependent differences in growth in L cells and cultured mouse heart cells (295), growth in MDCK cells (383), induction of myocarditis in newborn mice (433), and growth and injury in different organs of SCID mice (194).

µ2

µ2 (83 to 84 kd, 736 amino acids) is another low-copy core protein, estimated to be present in about 20 copies per particle (100). It has not been definitively localized within particles but is thought to reside near the fivefold axes, either interior to (130,378) or within (204) the core shell and possibly contributing to the protein complexes that project into the interior of reovirus particles near the fivefold axes (130,378). It has not been demonstrated to interact with any other reovirus protein, but it was proposed to interact with the core turret protein l2 (204), the other low-copy core protein l3 (130,194,433), and perhaps with the core shell protein l1 as well (130,378).

The functions of µ2 are only partly understood. Temperature-sensitive mutants with a lesion in the M1 genome segment encoding µ2 fail to synthesize dsRNA at nonpermissive temperature, suggesting a role for µ2 in one or more steps in replicase particle assembly, including RNA selection or replication (99,503). Constitutive expression of µ2 in L cells allows complementation of one of these ts mutants (527). Recombinant µ2 protein was recently shown to have an RNA-binding activity (both ssRNA and dsRNA) that is not specific for reovirus RNA (65). Recent reassortant analyses showed that the M1 genome segment encoding µ2 determines a difference in the temperature optimum of core transcription (519) and contributes to determining differences in the amounts of transcripts produced by cores (519) and the NTPase activities of cores (355). Together, these findings suggest that µ2 represents a cofactor or second subunit of the reovirus RNA polymerase. The M1 genome segment was also shown to be a genetic determinant of isolate-dependent differences in plaque size and extent of cytopathic effect in L cells (318); growth in cultured murine cardiac myocytes and bovine aortic endothelial cells (294,295); growth in MDCK cells (383); induction of myocarditis in newborn mice (433,434), levels of viral RNA synthesis, induction of interferon a/b and sensitivity of viral growth to interferon a/b in cultured murine cardiac myocytes (432,436); and growth and injury in different organs of SCID mice (194). It was hypothesized that the role of µ2 protein as a determinant of viral cytopathology may explain its low level of translation *in vivo* (388), but a higher level of µ2 expression by reovirus type 1 Lang versus type 3 Dearing was recently demonstrated (527). Most recently, µ2 was suggested to play a role in determining the rate of viral inclusion formation in reovirus-infected cells (299).

More Structural Proteins in the Outer Capsid

µ1

The µ1 protein (76 kd, 708 amino acids), together with s3, forms the bulk of the virion outer capsid (449). It is present in virions in 600 copies, in the form of 1:1 complexes with s3 (100,220,260). It also interacts with core proteins l2 and s2 in virions (131,306,378). The µ1 protein is modified by addition of a myristoyl (C_{14} saturated fatty acyl) group to its N-terminus following loss of the initiator methionine (347) (Fig. 4). In virions, most µ1 appears to occur as a 72-kd fragment, µ1C, that is generated from µ1 by proteolytic cleavage (530). N-terminal sequencing localizes this cleavage between residues 42 and 43 in µ1 (220,364), indicating that µ1C is a C-terminal fragment of µ1 (see Fig. 4). The cleavage of µ1 to µ1C also yields a 4.2-kd myristoylated N-terminal fragment, µ1N, that is retained in particles in stoichiometric amounts (347) and is quite likely the same virion component identified as component viii in earlier studies (449). The capacity of µ1 to undergo this cleavage is sensitive to the residues surrounding the µ1N–µ1C junction, and these residues are also important for determining µ1 interactions with s3 (471). Based on sequence similarity to the

VP4–VP2 autolytic cleavage junction in the VP0 protein of enteroviruses, cleavage at the μ1N–μ1C junction is thought to be autolytic as well (347). A small amount of μ1 in virions (commonly £5%) appears to remain uncleaved, similar to the VP0 protein of enteroviruses. Genetic studies have implicated μ1 in determining isolate-dependent differences in the sensitivity of virions and ISVPs to inactivation with ethanol, phenol, and heat (129,208,497).

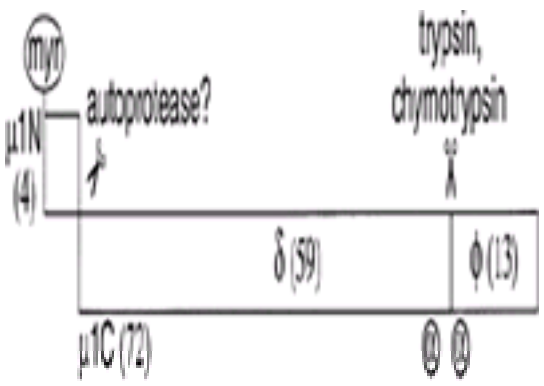

FIG. 4. Outer capsid protein μ1. Most (95%) of the μ1 protein (76 kd) in virions undergoes an assembly-related cleavage (*scissors*) into small amino-terminal and large carboxyl-terminal fragments designated μ1N (4 kd, as indicated) and μ1C (72 kd), respectively. An autoprotease activity appears to be involved in this cleavagen. The μ1N fragment (like uncleaved μ1) is modified by an amide-linked myristoyl group (*myr*), probably at its extreme amino terminus. When ISVPs are generated by the action of exogenous proteases, such as trypsin or chymotrypsin, on virion proteins, the μ1C fragment undergoes additional cleavage (*scissors*) nearer its carboxyl terminus. The products are large amino-terminal and small carboxyl-terminal fragments designated d (59 kd) and f (13 kd), respectively. The d–f cleavage junction is flanked by predicted long amphipathic a-helices (a) which are proposed to play a role in membrane interaction and penetration by reoviruses. The small amount of uncleaved μ1 protein in virions is also cleaved during generation of ISVPs to produce fragments μ1d [μ1N + d] and f.

In the cytoplasm of infected cells, 90% of free M2-encoded protein is μ1, whereas 95% of the protein complexed with s3 has been cleaved to μ1C (260). This finding suggests that the μ1N–μ1C cleavage is linked to formation of the μ1–s3 complex. This hypothesis is further supported by studies with ts mutant viruses and mutant s3 and μ1 molecules expressed in transfected cells (43,471). For example, μ1 expressed from an M2 genome segment that lacks the signal for myristoylation neither forms complexes with s3 nor undergoes cleavage to μ1C (471). Several lines of evidence suggest that when s3 and μ1 interact, both proteins undergo conformational changes that influence their functions in viral entry and assembly, respectively (430,437). μ1 interaction with s3 also inhibits the dsRNA binding activity of the latter and thereby interferes with its translational effects, suggesting a regulatory function for μ1 prior to its assembly onto particles (471,521). Complexes of μ1 and s3 are almost certainly the form of these proteins that is assembled onto cores in generating mature virions (83,260,430,437).

Several lines of evidence now indicate that a primary function of particle-bound μ1 is in penetration of the cellular membrane barrier during entry into cells at the onset of infection. A genetic difference in the capacity of ISVPs of reovirus isolates type 1 Lang and type 3 Abney to permeabilize membranes, causing ^{51}Cr release from L cells, was mapped with reassortants to the M2 genome segment encoding μ1 (277). Mutants of type 3 reoviruses Abney or Dearing, which were selected for ethanol resistance and (in the case of two of these mutants) shown by both reassortant analysis and M2 nucleotide sequencing to harbor resistance mutations in their μ1 proteins (208,497), show correlative changes in ISVP-mediated ^{51}Cr release and thermostability consistent with an effect of μ1-determined particle stability on membrane permeabilization (208). Certain μ1-specific monoclonal antibodies can block ISVP-mediated ^{51}Cr release from L cells at a postattachment step (207) and can also block virion-initiated infections of L cells at a step following both attachment and proteolytic processing (483). When assembled at nonpermissive temperature, virions of ts mutant A279 and also of reassortants containing the M2 genome segment of this mutant show a defect in entry into L cells characterized by accumulation of infecting particles within lysosomes (203). The strong hemolytic activity of ISVP-like particles derived from recoated core particles lacking s1, compared with the negligible hemolytic activity of cores, implicates μ1 in this form of membrane premeabilization as well (83). A role for the N-terminal myristoyl group on μ1/μ1N in membrane penetration was proposed (347) but remains unproven. Binding of alkyl sulfate detergents to virions, with an associated block to μ1 cleavage at the d–f junction by exogenous proteases (see later), was proposed to reflect an early, reversible step in membrane binding by μ1 during entry (82).

Another function of particle-bound μ1 appears to be in regulation of the particle-bound RNA transcriptase. An isolate-dependent difference in the conditions required for conversion of virions to cores, with associated activation of the transcriptase, was mapped with reassortants to the M2 genome segment encoding μ1 (127). Biochemical studies suggest an association between loss or rearrangement of μ1 and transcriptase activation (127,146), although the precise mechanism of this effect remains undefined. The structural changes in μ1 involved in transcriptase activation may be important for membrane penetration as well.

Despite marked sequence conservation among the M2 genome segments from different reovirus isolates (208,220,505), genetic analyses with reassortant viruses have associated M2 with a number of isolate-dependent differences in viral phenotypes, including the several described earlier. In addition, the reduced neurovirulence of reovirus type 3 clone 8 after intracerebral inoculation into newborn mice was shown to be determined by its M2 genome segment (210). Interference between reovirus isolates upon co-infection of L cells was shown to be genetically determined by the M2 genome segment and to involve steps in infection following uncoating (392,394). Polymorphisms in the M2 genome segment were also linked to isolate-dependent differences in reovirus-induced apoptosis and the inhibition of DNA

synthesis in cultured cells (383,475).

The µ1 and µ1C proteins undergo a distinct proteolytic cleavage near their shared C-terminus after exposure to proteases in vitro (51,220,223,344,425), within endocytic compartments of cultured cells early in infection (59,82,84,440,467), and within the intestinal lumen of newborn mice after peroral inoculation (28,50). This cleavage, which occurs after residue Tyr-581 with chymotrypsin and after residues Arg-584 with trypsin, generates a large (59 to 60 kd), acidic, N-terminal fragment named µ1d when derived from µ1, or d when derived from µ1C, as well as a small (12 to 13 kd), basic, C-terminal fragment named f (344) (see Fig. 4). The µ1d/d and f fragments remain bound to ISVPs in stoichiometric amounts (344). The C-terminal cleavage of µ1/µ1C was previously proposed to be required for the penetration of cell membranes during viral entry (344), but the normal infectivity and hemolytic activity of ISVP-like particles in which little or no cleavage at the d–f junction has occurred strongly suggest that this is not the case (82,83). Thus, at present, the biological significance of the d–f cleavage remains unknown. A small number of µ1/µ1C molecules remain uncleaved at the d–f junction in most or all preparations of ISVPs (100,344).

Several studies suggest that a fraction of µ1 and its fragments in virions undergo modifications in addition to myristoylation. It was reported that 2% to 4% of µ1C molecules in virions are O-glycosylated on serine or threonine residues (250). Evidence was also presented for the presence of one or more phosphoserine residues per molecule of µ1C in virions (251). It was further shown that several of the µ1C and component viii (µ1N) molecules in virions are both polyadenylated and ADP-ribosylated (75). Although cryo-electron microscopy reconstructions indicate that the µ1 protein in virions and ISVPs is centered in trimeric clusters (131), other data suggest that it is linked into disulfide-bonded dimers (211) through cysteine 679 in the f portion of µ1 (220). The role(s) of this disulfide bond in assembly or infection remains unknown.

s3

The s3 protein (41 kd, 365 amino acids), together with µ1, forms the bulk of the virion outer capsid (449), where it interacts with proteins l2 and µ1 and perhaps also with s1 (131,163,261,306,310,482). It is present in virions in 600 copies and in the form of 1:1 complexes with µ1 (100,220,260). Genetic studies with reassortant viruses implicated s3 in determining differences in the sensitivity of virions to inactivation with heat and SDS (129). In addition, by recoating ISVPs with recombinant s3 *in vitro*, the thermostability of those particles can be increased to approximately that of virions (219). Thus, s3 may play a role in stabilizing virions for survival in environments outside cells (345).

Balancing its stabilizing effects, the s3 protein must be removed from virions, by proteolysis in the natural setting, for infection of cells to proceed beyond the steps of receptor binding and endocytic uptake and trafficking (19,219,467,483,499). Within cells, s3 proteolysis is thought to occur within endocytic vacuoles, preceding penetration of viral particles into the cytoplasm, and to be predominantly mediated by acidic cysteine proteases of lysosomal origin (19,20,244,442,467). Genetic studies indicate that mutations in the S4 genome segment encoding s3 play a critical role in the establishment and/or maintenance of persistent reovirus infections, and that this effect at least partly involves mutations in s3 that enhance its sensitivity to proteolysis during entry into cells (6,19,499,511). Proteolytic removal of s3 from reovirus particles also occurs in the lumen of the small intestine during infection of newborn mice by the enteric route (28,50) and may be required for infection to proceed by that route in some cases (28), possibly by allowing viral particles to attach to M cells, through which they can be translocated across the epithelial barrier (10). Proteolysis of s3 is most likely mediated by alkaline proteases of pancreatic origin in the enteric system (28,50). A protease-sensitive region centrally located within the s3 sequence has been described (407), and additional C-terminal sequences have been identified to influence the rate at which s3 is cleaved by both acidic and alkaline proteases (219,499).

In addition to its functions before and during viral entry, s3 plays an important role in outer capsid assembly. A ts mutant whose lesion maps to the S4 genome segment encoding s3 accumulates dsRNA-containing core-like particles at the nonpermissive temperature (110,322,437). Biochemical studies with this mutant suggest that, at the nonpermissive temperature, s3 is misfolded such that it cannot interact with µ1 (283); thus it appears that s3 may be required to interact with and promote a conformational change in µ1 that enables these proteins to assemble onto core particles (437). The interaction between s3 and µ1 also causes a conformational change in s3 that renders it more susceptible to proteolysis (430), consistent with the requirement for s3 proteolysis during viral entry. Other evidence suggests that s3 may have a function early in particle assembly or RNA selection, replication, or packaging involving its association with the reovirus mRNAs soon after they are synthesized in infected cells and its continued association with these nucleoprotein complexes through the process of minus-strand synthesis and the consequent generation and selection of the 10 genomic dsRNA molecules (13). A role for s3 in RNA selection or packaging is also suggested by recent evidence that mutations in s3 are needed to permit mixing of genome segments from different reovirus isolates within certain reassortants (386).

Biochemical studies reveal that s3 binds dsRNA in a sequence-independent manner (211), and a dsRNA-binding activity has been localized to a C-terminal fragment of s3 (311,407). This C-terminal fragment contains two copies of a basic amino acid motif that has been proposed to contribute to dsRNA binding (115,282,311,485). The capacity of s3 to bind dsRNA is inhibited by association with µ1 and enhanced when certain N-terminal sequences of s3 are either removed by proteolysis or mutated (43,311,407,431,471,485,521). The positive effect of these N-terminal mutations in s3 is thought to occur through a reduction in µ1 binding to s3 and/or a reduction in steric hindrance to dsRNA binding provided by interactions between different s3 regions. Emerging evidence suggests that the dsRNA-binding activity of s3 is important for its function in translational control within infected cells (see later section).

The s3 protein occurs as a zinc metalloprotein in virions (407), and s3 binds Zn^{2+} in biochemical assays (283,407). A zinc-finger motif is found in an N-terminal portion of s3 (407). Current evidence indicates that this zinc finger includes a CCHC motif (283) similar to that found in retrovirus nucleocapsid proteins. The coordination of Zn^{2+} appears to play a structural role in s3, because mutations in the coordinating residues eliminate the capacity of s3 to bind to µ1 (431) and decrease its stability *in vitro* (431) and in cells (283). There is no evidence to support an association between the Zn^{2+}-

and dsRNA-binding activities of s3.

In most infected cells, s3 is localized to the cytoplasm and becomes concentrated in perinuclear regions where viral assembly takes place (408). However, in transfected cells and following infection with some reovirus isolates, s3 has been shown to localize to the nucleus (408,520). Nuclear localization of s3 is inhibited by mutations that destroy dsRNA binding and also by coexpression with µ1 (520). To date, no function has been ascribed to nuclear s3.

An early genetic study with reassortant viruses indicated that the S4 genome segment encoding s3 determines an isolate-dependent difference in inhibition of cellular RNA and protein synthesis (417). A recent study provided evidence that such differences in effects on translation of cellular mRNAs may be based in genetically determined differences in s3 localization within cells, with s3 proteins that are more broadly distributed in the cytoplasm providing greater sparing of cellular mRNA translation, possibly by blocking activation of the interferon-induced and dsRNA-activated protein kinase PKR (408). The differences in localization may be attributable to differences in s3–µ1 affinity between isolates (408). Other studies suggest roles for s3 in the regulation of viral transcription (16) and translation. The s3 protein was found to be enriched in the translation initiation factor fraction of eukaryotic cells expressing a cloned S4 genome segment (264) and was suggested to stimulate the translation of late (uncapped) viral mRNAs within infected cells (265,266). Several studies found that expression of s3 can stimulate expression of reporter genes (178,275,282,290,413,471,521). It was hypothesized that the effect of s3 on translation relates to its capacity to bind dsRNA and thereby sequester the activator of PKR, which phosphorylates eIF2a and inhibits translation initiation (214,275,463,521). This hypothesis is supported by the observation that s3 can substitute for the analogous dsRNA binding activity of vaccinia virus protein E3L (32).

s1

According to current estimates, the s1 protein (49 to 51 kd, 455 to 470 amino acids) is present in no more than 36 copies per virion outer capsid, or one s1 trimer per icosahedral vertex (100,465). In purified virions, some of the s1 trimers may be lost, yielding particles with fewer than one per vertex (257). A minimum of three s1 trimers per particle was found to be required for full infectivity (257). Earlier interpretations that s1 takes the form of a dimer or tetramer (22,29,30,161,343) appear to have been in error. The s1 protein interacts with l2 and perhaps also with s3 in the virion outer capsid (131,163,202,261,306,310,482). It may be capable of adopting two very different conformations, one in which it extends as an elongated fiber from the particle surface and another in which it is more folded or retracted (22,131,161,163,345).

Sequence analyses yielded a model in which residues in the N-terminal third of s1 adopt an a-helical coiled-coil structure (29,163,343). Two-dimensional reconstructions from electron micrographs of s1 fibers later revealed significant substructure within the tail region (161). Related work identified repeats of hydrophobic residues within a central portion of s1 sequences and suggested that different morphologic regions in the tail result from sequences assuming distinct supersecondary structures: alternating regions of a-helical coiled-coil and b-sheet motifs (161,343) (Fig. 5). The head appears to be formed by sequences in the C-terminal third of s1 and to have a more complex globular structure (29,136,161,163,343,473).

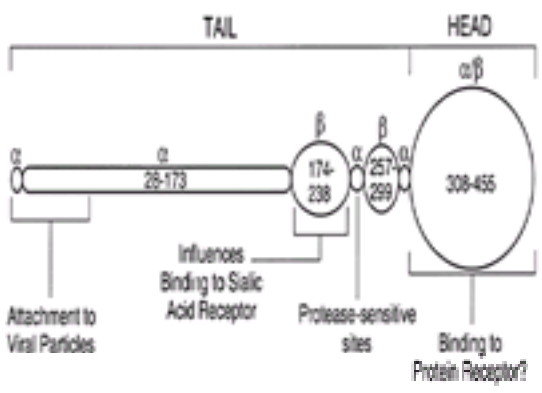

FIG. 5. Cell-attachment protein s1. Trimers of the reovirus s1 protein can be isolated from viral particles as extended fibers characterized by tail and head domains as shown. Distinct regions within the tail are proposed to be formed by sequences that assume a-helical coiled-coil and cross-b-sheet supersecondary structures as labeled [sequences assigned to the larger tail regions and head in s1 proteins from type 3 (*T3*) reovirus strains are indicated by position number]. Properties or activities associated with different regions of the type 3 s1 proteins are indicated.

Numerous studies have implicated s1 as the reovirus cell-attachment protein (14,22,124,144,261,293,363,470,490,491 and 492,517) and the protein against which serotype-specific neutralizing antibodies are directed (22,70,293,363,457,482,493,517). Sequencing of S1 genes from neutralization-resistant mutants identified two residues in the C-terminal third of type 3 Dearing s1 that influence neutralization by the monoclonal antibody G5 (31). Evidence that these mutants exhibit altered tropisms within the murine brain (234,455,456) led to a hypothesis that the C-terminal head region of s1 mediates receptor binding. Sequence similarities between type 3 Dearing s1 and an antiidiotypic monoclonal antibody directed against G5 identified other residues in the C-terminal third of s1 that may serve as receptor contacts (68,509). Other work based on the antiidiotypic antibody has identified candidate cellular receptors for this region of s1 (see later section). In contemporary work, a C-terminal proteolytic fragment of s1 and deletion mutants that retain a large C-terminal portion of s1 were found to retain L-cell binding activity (136,336,473,518). Deletion and site-directed mutagenesis within a C-terminal portion of s1 indicate that the extreme C-terminus and other conserved amino acids in this region contribute to the structure and function of the head domain (136,473).

The s1 protein also serves as the viral hemagglutinin (22,30,70,293,495,517). Hemagglutination by type 3 reovirus isolates involves binding of s1 to sialic acid residues on the erythrocyte surface (14,175,362), large amounts of which are

attached to the major erythrocyte surface protein glycophorin A (361). Strong evidence now indicates that the region of type 3 s1 that mediates glycophorin A binding and hemagglutination is distinct from the region that mediates L-cell binding. Early such evidence came from monoclonal antibodies that identify distinct s1 epitopes involved in neutralization and hemagglutination inhibition (70,457). Findings with deletion mutants (336) and proteolytic fragments (518) of type 3 Dearing s1 provided additional evidence that glycophorin A and L-cell binding are separable functions of this protein, most likely mediated by different protein regions. Analysis of deduced s1 sequences from 11 type 3 isolates that differ in capacity to agglutinate erythrocytes and bind glycophorin A subsequently provided evidence that a small central region in the s1 tail, characterized by b-sheet motifs, is involved in these properties (118). These findings were supported by studies on growth in murine erythroleukemia (MEL) cells, which indicate that growth in MEL cells is dependent on virus binding to sialylated receptors on the cell surface, that certain type 3 isolates defective for sialic acid binding (118) are also defective for growth in MEL cells, that these isolates can undergo selection for sialic acid binding and growth in MEL cells by serial passage in those cells, and that this selection involves genetic changes in the S1 genome segment resulting in amino acid changes in the central b-sheet region of the s1 tail that was previously implicated in sialic acid binding (see Fig. 5) (88,396). Recent work using chimeric and truncated recombinant s1 proteins concur with this localization (86). Binding to sialic acid appears not to be involved in hemagglutination or attachment to other cells by type 1 and type 2 reoviruses (118,360), but the alternative moieties that are bound by these isolates remain to be identified. The fact that neutralization and hemagglutination inhibition epitopes overlap in the type 1 Lang s1 protein suggests that the separation of protein and carbohydrate receptor binding functions indicated for type 3 s1 proteins may not hold true for type 1 s1 proteins (70,457). In addition, a more distal region of the s1 tail, closer to the head in primary sequence and putative structure, was recently implicated in hemagglutination by type 1 Lang s1 by using chimeric and truncated recombinant s1 proteins (86).

A flexible “neck” region has been described to separate the fibrous tail from the globular head of s1 (137). It has been recognized for some time that this region of isolated native or recombinant type 3 Dearing s1 protein is sensitive to proteolysis (22,136,137,465,518). Sequences that influence or impart this sensitivity have now been identified, as have the sites for proteolysis by intestinal serine proteases (85,137). The mechanism for protease sensitivity is proposed to involve disruption of a-helical coiled-coil structure within a limited region of the s1 tail (85). The sensitivity of type 3 Dearing s1 to proteolysis by intestinal proteases and the consequent reduction in infectivity provides a logical mechanistic explanation for the reduced capacity of reovirus type 3 Dearing to infect mice by the enteric route (85,342). In addition, because the protease-sensitive region is found between the s1 head and the sialic binding region in the tail (85,137), findings with ISVPs containing protease-cleaved type 3 Dearing s1 provide further evidence for the separation of the two receptor binding activities within two different regions of the protein (342).

The long a-helical coiled-coil tail region of s1 may allow for projection of the more distal receptor binding regions away from the particle for better accessibility for binding to those receptors. A recent study indicated that engagement of receptors on cells via the C-terminal regions of s1 leads to reversible conformational changes in more N-terminal tail regions of s1, as well as in other capsid proteins, suggesting that conformational changes in the s1 tail may be important for relaying information about receptor binding to other capsid proteins as part of the entry process (156). Consistent with this hypothesis is the finding that certain mutations in the long a-helical region of the s1 tail that are selected during persistent infections can reduce s1 oligomer stability and have effects on reovirus entry (511). Recent evidence suggests that s1 sequences near the base of the s1 tail (including residues 36 and 54) mediate a glycosyl hydrolase activity, which may be important for reovirus infections in the mammalian intestine by permitting hydrolysis of soluble or surface-bound mucin proteins and thereby better access to cell surface receptors (47).

Sequences near the extreme N-terminus of s1 are thought to form a virus-attachment region that anchors this protein to the l2 protein in viral particles (see Fig. 5). A small region of hydrophobic amino acids at the extreme N-terminus was first predicted to fulfill this function through a coupling of sequence analysis and electron microscopy (22,161,163,343). Subsequent work with C-terminally truncated recombinant s1 proteins demonstrated that residues between positions 3 and 34 (of reovirus type 3 Dearing s1) are required for virion anchoring (270,284).

A number of studies indicate that s1 folds into its native conformation via a multistep process that provides a useful model for folding of an oligomeric fiber protein (259). Monomers of s1 do not bind to cells and they exhibit extreme protease sensitivity, indicating the importance of oligomerization for s1 structure and function (268). Folding of s1 involves two distinct trimerization events. An N-terminal region of s1, which includes a portion of the predicted long a-helical coiled coil, possesses a capacity to undergo oligomerization independently of other sequences (23,136,269,271,473). N-terminal trimerization occurs co-translationally and does not require ATP or cellular chaperone proteins HSP70 and HSP90 (181,182,267,271). In contrast, trimerization of C-terminal s1 sequences is a posttranslational event that requires ATP and both of those chaperones (181,182,267,271). Additional maturation events in both N- and C-terminal regions are required to generate the final s1 structure (271).

A wide variety of phenotypic differences among reovirus isolates have been mapped to the S1 genome segment by reassortant analyses, and many of these have been proposed to relate to the function of s1 as the reovirus cell-attachment protein. Recent examples include studies that have implicated S1/s1 as a genetic determinant of phenotypic differences in inhibition of cellular DNA synthesis in L cells (416,475), induction of apoptosis in L cells (475,476) and MDCK cells (383), efficiency of infection of cultured murine cardiac myocytes (432), induction of biliary atresia in newborn mice (510), growth and injury in different organs of SCID mice (194), viral growth and neutrophil response in a rat model for reovirus-induced pneumonia (325), and the capacity of viral mutants selected during persistent infections to grow in the presence of ammonium chloride as well as in mutant cells that have been cured of persistent infection (499,511).

Nonstructural Proteins

sNS

The sNS protein (41 kd, 366 amino acids) is predicted to have a high a-helical content (381,504) but a low propensity to form a-helical coiled coils (303). It appears to exist as a small oligomer in free solution (179,183). A ts mutant whose

lesion maps to the S3 genome segment encoding sNS (373) is severely reduced in dsRNA synthesis at nonpermissive temperature, suggesting a role for sNS in replicase particle assembly including RNA selection or replication (104,216). sNS shows strong affinity for ssRNA, including, but not specific for, the reovirus mRNAs (179,180,183,184,211,382,458). It binds poorly to dsRNA (180,211,382). Binding to ssRNA is cooperative and can lead to displacement of short DNA oligonucleotides from RNA–DNA hybrids (180). A small region of sequence at the extreme N-terminus of sNS is important for ssRNA binding (179). Complexes containing sNS and ssRNA can be readily isolated from infected cells and resolved on columns or gradients (179,183,184,211,458). According to immunoprecipitation studies with protein-specific antibodies, sNS binds to the reovirus mRNAs shortly after they are synthesized in infected cells, and it remains bound in these complexes, along with other viral proteins, through the process of minus-strand synthesis and the consequent generation and selection of the 10 genomic dsRNA molecules (13). Thus, sNS appears likely to play one or more important roles in the selection, replication, and/or packaging of reovirus RNA for the assembly of progeny particles. A possible role in translation was also acknowledged (179). 15–19S complexes obtained from infected cells and containing sNS and ssRNA were reported to have poly(C)-dependent poly(G) polymerase activity (184); however, that activity was also demonstrated for purified reovirus l3 protein (460) and has not been demonstrated for purified sNS (179,382), suggesting that the activity in 15–19S complexes (184) was caused by contaminating l3.

µNS and µNSC

Although first thought to be a cleavage product of µNS (80 kd, 721 amino acids) (260), µNSC appears instead to be formed by alternative translation initiation at the second or third AUG in the M3 mRNA, in the same ORF as µNS (303,503). µNS and µNSC are thus likely to share the same sequences except for an extra 4 to 5 kd of sequence at the N-terminus of µNS. Possible functional differences between µNS and µNSC have not been addressed. A C-terminal portion of µNS/µNSC is predicted to have a high a-helical content and similarity with myosins (503), as well as a propensity to form a-helical coiled coils (303). It appears to take the form of a small oligomer in free solution (66). The µNS protein has been shown to associate with the cytoskeleton in infected cells, to which it may anchor other viral components during replication or assembly (319). According to immunoprecipitation studies with protein-specific antibodies, µNS and/or µNSC binds to reovirus mRNAs shortly after the RNAs are synthesized in infected cells and before the RNAs associate with other viral proteins (13). In addition, µNS and/or µNSC remains bound in these complexes, along with other viral proteins, through the process of minus-strand synthesis and the consequent generation and selection of the 10 genomic dsRNA molecules (13). Thus, µNS and/or µNSC appears likely to play one or more important role in the replication, selection, and/or packaging of reovirus RNA for the assembly of progeny particles. One reassortant study showed that the M3 genome segment encoding µNS/µNSC determines an isolate-dependent difference in the specific genome segments that undergo deletion during serial passage of viruses at high MOI, consistent with a role in RNA replication or packaging (67). Other studies found that µNS is a component of rapidly sedimenting particle assembly intermediates that can be isolated from reovirus-infected cells (323,322,532). Although disputed by subsequent work (446), one of these studies (323) provided evidence that µNS is a major component of the secondary transcriptase particles that produce most (95%) of the viral mRNAs within reovirus-infected cells (see later section). Recent evidence that recombinant µNS protein can bind to cores in large amounts *in vitro*, but does not inhibit their transcriptional and capping activities, is consistent with the original evidence of Morgan and Zweerink (323) and suggests that µNS may play a role in transcript production and outer capsid assembly within infected cells (66).

s1s

The s1s protein (14 to 15 kd, 119 to 125 amino acids) is highly variable between reovirus isolates, even within the same serotype (78,117,135). It is strongly basic, and an N-terminal cluster of basic residues is its most conserved feature. A role for this basic protein in binding to nucleic acid was proposed (218), and localization of s1s to both cytoplasm and nucleus was noted (40,384). As a second translation product derived from the S1 genome segment, s1s might be responsible for some of the differences in biologic properties that have been mapped to S1 and tentatively attributed to the other protein derived from this segment, the cell-attachment protein s1 (see earlier section). When expressed to high levels in uninfected mammalian cells, recombinant s1s does not affect cellular DNA synthesis or cause cytopathic effects; however, when expressed to high levels in cells that have also been infected with certain reovirus isolates, recombinant s1s potentiates the viral inhibition of cellular DNA synthesis and the cytopathic effects of infection as well (147). These findings suggest a role for s1s in reovirus effects on host cells. Recent evidence for a reovirus mutant having a stop codon early in the s1s ORF indicates that this protein is dispensable for reovirus growth in L and MDCK cells as well as for induction of apoptosis in L cells (384). Whether it is required for replication in certain cells or performs some other function related to growth and/or spread in host animals remains to be determined. The s1s protein has alternatively been called p14 (80), s1bNS (328), ss (404), and s1NS (40).

GENETICS

Mutants

Mutants Obtained by Selection, Screen, or Passage

Temperature-Sensitive Mutants

Reovirus ts mutants were classically defined by their reduced capacity to replicate at 39°C as opposed to 31°C (159). Recent studies indicate that 40°C is a more effective temperature for distinguishing certain ts mutants (102,203). Temperature-sensitive mutants from 7 of the 10 genetic groups (genome segments) of reovirus type 3 Dearing were first isolated after chemical mutagenesis with nitrous acid (groups B, D, and E), nitroso-guanidine (groups C, F, and G), or proflavin (group A) (159,212). Mutants from the other three groups were isolated from type 3 Dearing stocks subjected to serial passage at high MOI (group H) or from persistently infected cultures (groups I and J) (3,4,8). All groups but F were assigned to discrete genome segments by reassortant analyses (332,368,369,373). The prototype group F isolate was assigned to the M3 segment based on its capacity to complement ts mutants from the other groups (369). A recent study failed to confirm the original findings for the prototype isolates of groups F, H, and J (99), indicating that these isolates

cannot fulfill their initial promise of providing isolated ts lesions in the M3, M1, and S1 genome segments, respectively. The same and another recent study nonetheless described a new set of ts mutants (99,102), one of which has been shown to represent a new prototype isolate for ts group H (M1 segment) (99). To date, sequencing studies have identified nucleotide and amino acid changes in mutants from groups C (102,507), E (504), G (110), and H (99). Temperature-sensitive mutants in groups A, B, C, D, E, G, and H have been analyzed extensively with respect to their morphologic, biochemical, and biologic properties (discussed at other places in this chapter).

Suppressor Mutants

Reversion of ts mutants to the ts^+ phenotype might occur through a change in the same gene, either at the site of the original mutation (true reversion) or at a second site (intragenic suppression). Alternatively, mutation in a second gene might suppress the defect produced by the original mutation (extragenic suppression). A recent study found that one reovirus ts mutant (tsC447, with a lesion in the S2 genome segment encoding core nodule protein s2) undergoes true reversion exclusively (102); nevertheless, a number of revertants of other reovirus ts mutants result from extragenic suppression (371, 372, 374). A commonly described mechanism for extragenic suppression involves mutation in a gene product that interacts directly with the original ts product. Interactions between reovirus protein pairs µ1-s3, µ1-l1, and l2-s1 were inferred in this manner (221, 306).

Deletion Mutants and Defective Stocks

Some reovirus isolates accumulate deletion mutants during serial passage at high MOI (5, 67, 356, 358, 411). Genome segments L1 and L3 are most commonly deleted, but L2 and M1 deletions are also seen. A series of deleted M1 segments were analyzed and found to lack internal sequence regions (525). Based on the smallest deleted M1 segments, the minimum sizes for retained 5'- and 3'-terminal regions of the plus strand were 132 to 135 and 182 to 185 bases, respectively, suggesting that these regions contain the essential signals for RNA synthesis and packaging. One genetic study compared isolates type 1 Lang and type 3 Dearing, which differ in their capacity to accumulate deletion mutants (67). This study showed that the L2 genome segment determines whether or not deletions accumulate, and that the M3 genome segment determines in which segments the deletions occur. The L2 and M3 gene products l2 and µNS/µNSC may play a role in synthesis or packaging of reovirus RNA such that they influence the generation or amplification of deletion mutants.

Some reovirus stocks that have been serially passed at high MOI can be classified as DI stocks (3, 5, 6 and 7, 67, 119, 356, 358, 411). The traits of defective stocks include the presence of deletion mutants, the capacity to interfere with growth of wild-type (wt) virus (see later), and the capacity to establish persistent infections. The role of deletion mutants in the latter two properties is unclear, because defective stocks contain a variety of mutants, including a high frequency of tsG (S4 genome segment) mutants (3, 5), some of which are known to mediate interference (3, 81). Given that the nondeleted genome segments in defective stocks are normal with respect to transcription and reassortment, it was suggested that interference and facilitation of persistent infections are linked to specific mutations in nondeleted segments such as S4, rather than to the deleted ones (5, 6, 67).

Neutralization-Resistant Mutants

Neutralizing monoclonal antibodies directed against the cell-attachment protein s1 were used to select neutralization-resistant mutants of isolate type 3 Dearing, and changes in the mutants were localized to two residues in the C-terminal third of s1 (31, 456). The mutants were found to be altered in their pathogenic behaviors within newborn mice (234, 455, 456), possibly because their mutations fall in or near a receptor-binding region of s1.

Inactivation-Resistant Mutants

Physicochemical agents (e.g., heat or ethanol) can cause large reductions in reovirus infectivity, and differences between isolates in their sensitivities to these agents can be attributed to specific genome segments by reassortant analyses (128, 129). One such difference in ethanol sensitivity was genetically mapped to the M2 genome segment (129). Ethanol-resistant mutants were subsequently isolated in which relevant mutations were also localized to M2, within a 200-amino-acid central region of the encoded major outer capsid protein µ1 (208, 497). These mutants exhibit increased thermostability and a decreased capacity to permeabilize cells as detected by release of ^{51}Cr (208), a marker for membrane rupture during viral entry (56, 277). Thus, mutants selected with physicochemical agents may be used to study the structure and function of reovirus particles, as well as to define the mechanisms of inactivation by these agents.

Other Mutants

Other types of reovirus mutants obtained after passage in cells or animals, with or without a defined mechanism for selection, have been described. These include small-plaque mutants (7), cold-sensitive mutants (8), mutants selected during persistent infections in L cells and genetically mapped to the S1 and S4 genome segments (119, 499, 511), mutants selected for binding to sialic-acid-containing receptors by growth in MEL cells and genetically mapped to the S1 genome segment (88), and mutants with altered organ tropism and virulence obtained after passage in mice and genetically mapped to several genome segments including M1 and S1 (193, 194, 434, 435). A particularly fortuitous mutant is one of those selected for growth in MEL cells which, in addition to having a mutation in S1 that affects the sialic binding activity of s1, has a second mutation in S1 that introduces a stop codon near the beginning of the s1s-encoding ORF (88). This mutant was used to show that expression of s1s is dispensable for reovirus replication and induction of apoptosis in L and MDCK cells (384).

Genetically Engineered Mutants or Mutations

It has been demonstrated that reovirus mRNAs can be infectious after transfection into cells (386, 389), and this infectious RNA system has been used to construct a double ts mutant using virally produced RNAs obtained from two different

single ts mutants (387). Despite the clear promise of this approach for genetic engineering of reoviruses, it has not yet been modified to allow the efficient recovery of infectious particles containing genome segments derived from cloned and mutated DNA copies. As a result, genetic engineering with reoviruses has been largely restricted to studies in which isolated mutant gene products have been analyzed for activity *in vitro* or in transfected cells. In a recent study, recombinant µ2 protein constitutively expressed in L cells was shown to complement the growth of a tsH mutant (a lesion in the M1 genome segment encoding low-copy core protein µ2) at nonpermissive temperature (527). This offers a new genetic approach for identifying sequence determinants of µ2 function and promises to be applicable to other reovirus gene products as well. In addition, a series of recent reports have demonstrated that genetically engineered reovirus outer capsid proteins µ1, s1, and s3 can be bound to the surfaces of subvirion particles (ISVPs or cores), regenerating virion-like particles that are infectious (83,151,219). This offers another new genetic approach for identifying sequence determinants of µ1, s1, and s3 functions during the entry phase of productive infection.

Interactions Between Viruses

Reassortment

Reassortment of genome segments between reoviruses occurs not only in cultured cells but also in mice (496). Sequencing studies indicate that reassortment in nature is a major determinant of reovirus evolution (see later). Reassortment was first demonstrated in two-factor crosses (157,159). L cells were co-infected with two ts mutants at permissive temperature, and reassortants were detected by assaying for ts^+ progeny at nonpermissive temperature. If a cross between two ts mutants did not produce ts^+ reassortants, the mutants were considered to have lesions in the same genome segment. On the other hand, if a cross between two ts mutants resulted in ts^+ reassortants, the mutants were considered to have lesions in different genome segments. In this way, the available ts mutants were separated into five different genetic groups (157). The discovery of electrophoretic differences for protein µ1/µ1C in certain ts mutants provided an additional marker (µ+ versus µ–, to indicate wt versus faster mobility) that could be used in genetic crosses to evaluate reassortment (106). Three-factor crosses were performed using this mobility marker in combination with the ts phenotype and confirmed that reovirus genome segments behave as discrete genetic elements during reassortment (107). In subsequent experiments, ts mutants representing the five remaining genetic groups were identified, and separate co-infections involving one ts mutant and one wt isolate were used in reassortant analyses to assign each ts group to a particular genome segment (see earlier section). Reassortment of genome segments between wt isolates, irrespective of serotype, has been subsequently demonstrated in numerous studies.

If reassortment of genome segments from two co-infected isolates were a random process, then almost all of the progeny from the co-infection should be reassortants [1,022 of 1,024 (210) possible combinations of genome segments from the two parents]. In practice, however, a much lower frequency of reassortants has been seen among co-infection progeny [3% to 20% in one series of crosses (159)]. A variety of factors could contribute to reovirus reassortment's exhibiting such deviation from randomness, including that not all genome segments or their protein products from two isolates can be productively paired without accommodating mutations. Supportive data in this regard came from a statistical study of 83 reassortants derived from reoviruses type 1 Lang and type 3 Dearing. These reassortants showed significant deviations from randomness for the distributions of parental alleles in certain of the possible pairings of the 10 genome segments (346). Possibly reflecting the same or a similar phenomenon is evidence that reovirus reassortants commonly contain mutations in the S4 genome segment, suggesting that such mutations may be necessary to improve the fitness of certain reassortants for independent replication (228,229,386).

Complementation and Interference

Complementation refers to interactions of viral gene products in co-infected cells that result in enhanced yield of one or both parental viruses without a change in genotype. Interference refers to the capacity of certain viruses to decrease the yield of other viruses upon co- or superinfection. Although reassortants were readily identified after co-infections with reovirus ts mutants, enhanced yields of progeny at the nonpermissive temperature were often not observed (157,159). This lack of complementation was explained by the phenomenon of interference. Temperature-sensitive mutants from groups A, B, C, and G were found to interfere with the growth of wt virus, whereas those from groups D and E were not (81). Genetic crosses established that interference is a dominant trait. The degree of interference with the growth of wt virus can be substantial (50% or more), and it increases if the MOI of the interfering ts mutant is increased (81). Significant complementation occurs only if both parental ts mutants are noninterfering (81,216). Interference with growth of wt virus is also a property of defective high-passage stocks, which may reflect the frequent presence of interfering group G ts mutants (3,5).

The molecular basis of interference with reoviruses remains poorly defined in most cases. It was suggested that interfering ts mutants encode mutant proteins that become incorporated into progeny virions and reduce their infectivity (5). In support of this model, one study identified dominant-negative mutations in s1 that interfere with the capacity of wt s1 to assemble into functional oligomers (271). Mutant proteins from interfering ts mutants may act similarly to interfere with assembly and function of reovirus protein complexes. Recent studies on the mechanism of interference during co-infections with certain pairs of wt reoviruses revealed that interference occurs by 8 to 10 hours after infection, after viral entry and uncoating but before or during accumulation of newly synthesized dsRNA (392,394). These results are consistent with a model in which interference occurs at the stage of progeny particle assembly (394). Genetic evidence from one of these studies identifies the M2 genome segment (which encodes major outer capsid protein µ1) as the primary determinant of interference in this system (392).

Other Types of Interactions

There is no evidence for recombination between either homologous or heterologous reovirus genome segments. Reoviruses can undergo multiplicity reactivation after inactivation with genome-damaging agents like ultraviolet radiation (300), probably reflecting the capacity of viruses to reassort mRNAs produced from nondamaged genome segments. There is recent evidence for phenotypic mixing of proteins within progeny virions assembled during co-infections with

pairs of reovirus isolates that have distinguishable properties attributable to those proteins (393). A recent study found no evidence for super-infection exclusion during temporally asynchronous infections of cultured cells with pairs of reovirus ts mutants, which may have significance for the evolution of reoviruses in nature by allowing co-infection and reassortment even when different viruses infect cells at different times (236).

Naturally Occurring Variation

Exploitation for Genetic Studies

Reovirus isolates obtained from nature commonly exhibit qualitative or quantitative differences in phenotypes of biological interest. Substantial progress in attributing these phenotypic differences to individual genome segments, and thus presumably to specific sequence differences between the two parental alleles of these segments, has come from the generation and characterization of reassortant viruses derived from the two parents. Early studies used this approach to identify the S1 segment as the primary determinant of a difference between reoviruses type 1 Lang and type 3 Dearing in tropism and virulence for newborn mice after intracranial inoculation (492,494). Numerous subsequent studies, involving these and other isolates, have succeeded in attributing other phenotypic differences to single genome segments (127,210,237,354,417,434). In other cases, reassortant analysis has attributed the phenotypic difference to more than one genome segment at a time, indicating that genetic differences in two or more viral components are involved (49,194,355,433,499,519).

The availability of reovirus genome sequences has permitted another type of study that takes advantage of naturally occurring variation among isolates. In this approach, the protein sequences deduced from individual genome segments of different isolates are compared in an effort to link specific, variable sequences with a phenotypic difference that is known to be linked to these segments. In successful applications of this approach, a small variable region in the cell-attachment protein s1 was implicated in the failure of s1 from four type 3 isolates to bind sialylated proteins on the erythrocyte surface (118), and a single variable amino acid in s1 was implicated in the susceptibility of s1 from two other type 3 isolates to undergo cleavage by intestinal proteases (85). In each of these cases, additional studies have confirmed the reliability of the original conclusions (85,88). Other applications have been reported for studies of the l1 and s3 proteins (198,235).

Evolution of Reoviruses

Although useful for scoring reassortants, electrophoretic mobility differences between homologous genome segments and proteins of different reovirus isolates are not generally meaningful for classification (209,370). Nucleotide and deduced amino acid sequences provide the most reliable information for understanding reovirus evolution (241). Sequences are available for all 10 genome segments of the prototype isolates type 1 Lang, type 2 Jones, and type 3 Dearing. Except for the serotype-determining S1 segment, homologous segments from the prototypes are similar at the level of 70.6–97.8% and 80.0–99.3% identity in pairwise comparisons of nucleotide and deduced amino acid sequences, respectively. For every gene but S1, the type 2 Jones sequence is most distinct, which led to a suggestion that it diverged from a common ancestor prior to type 1 Lang and type Dearing (506).

The s1-encoding S1 genome segments of reoviruses exhibit the greatest extent of variability among the 10 segments. The variability includes differences in length, the need for multiple gaps to align deduced s1 sequences, and reduced sequence similarity (only 26% to 49% identity in s1 sequences in pair wise comparisons) (135,343). Among the three prototype isolates, the type 3 Dearing S1 sequence is most divergent. On the other hand, examination of S1 genes from a set of 11 type 3 isolates revealed no differences in length or need for gaps to optimize s1 alignments, and sequence divergence similar to that between prototypes for other genome segments was seen (86% to 99% identity in pair wise comparisons of deduced s1 protein sequences) (117). In sum, these findings suggest that three versions of the S1 genome segment, corresponding to the three serotypes, arose from progenitors at different times in the past and have diverged at a rate similar to that of other segments since those times.

Reassortment of genome segments between viruses is another important force in reovirus evolution. Variable rates of change in third codon bases seen for different genome segments from type 1 Lang and type 3 Dearing suggest that the segments in these two isolates were combined by reassortment after having had distinct evolutionary histories (506). A study of S2 genome segment sequences from 11 type 1 and type 3 isolates was the first to show convincingly that a segment other than S1 has diverged independently of the serotype of isolate from which it was obtained for sequencing (87). Subsequent studies have yielded similar findings for L2, S3, and S4 genome segment sequences from multiple type 1, type 2, and type 3 isolates and also showed topological differences in the phylogenetic trees drawn for these and S2 sequences obtained from the same isolates (87,189,235). These findings indicate that each of these genome segments has had an evolutionary history distinct from those of many or all of the other segments with which it is combined in the current isolates, consistent with ongoing reassortment of segments in nature. The same studies also showed that evolution of the genome segments is independent of host of origin. Despite this strong evidence for evolution at the level of individual genome segments, one study has shown evidence for the existence of coevolving sets of reovirus proteins (482) and another has shown evidence for nonrandom segregation of segments during generation of reassortants in the laboratory, indicating bias for or against certain RNA or protein pairings (346). The significance of these possible biases on the evolution of reoviruses in nature remains uncertain.

REPLICATION CYCLE

A diagram of the reovirus replication cycle is shown in Figure 6. Subsequent sections of this chapter discuss details of the depicted events, but a brief description follows here. The first step in reovirus infection is attachment of the virion (or ISVP) to receptor molecules on the cell surface. Following *attachment*, virion particles are taken up from the cell surface by receptor-mediated *endocytosis* and delivered into vacuoles resembling endosomes and lysosomes. Within these vacuoles, reovirus outer capsid proteins undergo specific proteolytic cleavages, which are acid dependent and represent an essential step in the infection process. In some situations, ISVPs are generated by *extracellular proteolysis*, and the

entry of such particles is acid independent and may not require endocytosis. The mechanism by which reoviruses interact with and penetrate the vacuolar or plasma membrane barrier is unknown, but it appears to depend on preceding *proteolysis*, as only the ISVP form exhibits *membrane interaction* in available assays. Coincident with or following *penetration*, a derivative of the ISVP (perhaps a core particle as shown in Fig. 6) becomes transcriptionally active and begins synthesis of the 10 capped viral mRNAs. These products of *primary transcription* are used for the translation of viral proteins by the cellular *protein synthesis* machinery. In addition, the early transcripts associate with newly made viral proteins to form progeny RNA *assortment* complexes. Within subsequently derived replicase particles, each of the 10 packaged plus strands serves as a template for *minus-strand synthesis.* Viral mRNAs are also transcribed by progeny subviral particles (*secondary transcription*), which have a unique protein composition. These "late" transcripts serve as the primary templates for viral protein synthesis later in infection. The final steps of virion *capsid assembly* are not well defined but may involve the addition of preformed complexes of outer capsid proteins to core-like particles. Mature virions undergo an inefficient process of *release* from infected cells following cell lysis.

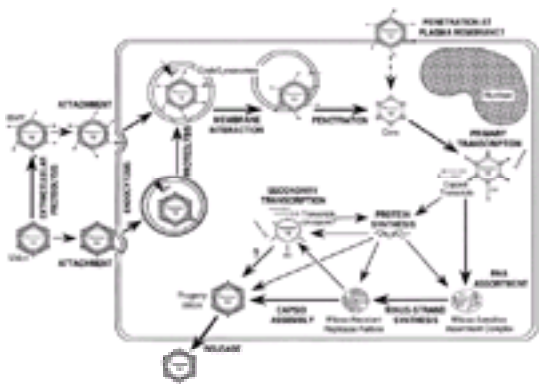

FIG. 6. Reovirus replication cycle. See text for detailed explanation. The primary steps in replication are labeled in *bold capital letters.* Virions, ISVPs, and cores, as well as other assembly-related particle forms are indicated. Capped plus-stranded RNAs—as part of the 10 dsRNA genome segments, as cytoplasmic transcripts, and as assortment elements—are indicated (*5'-terminal open circles* represent caps). Uptake from the cell surface is shown to involve receptor-mediated endocytosis, with viral particles sequentially associated with clathrin-coated pits, clathrin-coated vesicles, endosomes, and lysosomes (the last three compartments are acidic as shown). The roles played by individual reovirus proteins are not indicated here.

Early Steps in Replication: Entry into Cells and Production of Primary Transcripts

Attachment to Cell Surface Receptors

Numerous studies have investigated the attachment of reoviruses to erythrocytes and other cells, some of which are capable of undergoing productive infection. Most studies have utilized cells *in vitro* (see later), but some have investigated the binding of reoviruses to different cells within host animals. These studies generally indicate that defined receptor moieties, exposed on the cell surface, are responsible for binding reovirus particles via specific interactions with the viral hemagglutinin and cell-attachment protein s1. Attachment to surface receptors is considered a required first step in the productive infection of cells. Interactions of cell surface proteins with other reovirus proteins, contributing to attachment or subsequent steps in infection, remain possible but undefined.

Hemagglutination

Hemagglutination has been used as a convenient assay to study the attachment capabilities of reovirus particles. Isolates from all three serotypes agglutinate human type O erythrocytes, but only type 3 isolates agglutinate bovine red cells. This difference between strains type 1 Lang and type 3 Dearing was mapped to their S1 genes, implicating the encoded structural protein s1 as the reovirus hemagglutinin (495). Hemagglutination can also be mediated by purified s1, and biochemical and molecular genetic studies have suggested that a central region of sequences in type 3 s1 proteins is involved in this activity (see earlier section).

A decrease in hemagglutination is commonly noted after erythrocytes are treated with periodate or proteases, suggesting that one or more glycoproteins on the red cell surface serves as the receptor for virus binding prior to agglutination (14,421). There is strong evidence, including the sensitivity of hemagglutination to treatment of red cells with neuraminidase and to treatment of virions with sialoglycoproteins, that sialic (N-acetylneuraminic) acid residues are essential components of the erythrocyte receptor for type 3 reoviruses (14,88,175,361). Glycophorin A is the primary sialoglycoprotein found on human erythrocytes and is capable, in purified form, of binding to the s1 proteins of type 3 Dearing and other type 3 strains (118,336,361,482,518); thus, glycophorin A was specifically proposed to represent the erythrocyte receptor for type 3 reoviruses (361).

Hemagglutination by type 1 and type 2 reoviruses has been studied less thoroughly than that by type 3 isolates, but it does have some different characteristics. Unlike type 3 Dearing and some other type 3 strains, reovirus type 1 Lang is deficient in binding to sialoglycoproteins, including glycophorin A, as determined by its S1 gene (118,360). The erythrocyte receptor for type 1 and type 2 reoviruses remains undefined.

Attachment to Other Cells

Studies of reovirus attachment have been performed with a variety of cultured cells, utilizing different virus strains and techniques of analysis. Attachment to L cells by strain type 3 Dearing appears to be mediated by one or more

populations of saturable receptors. Estimates for the number of attachment sites per cell range between 1×10^4 and 5×10^5, and the receptors exhibit a dissociation constant for virus binding of 0.25 to 13 nM (9,14,144,173,406). Maximal attachment is generally achieved after 30 to 90 minutes regardless of temperature (14,144,173). Studies in other cell types or with strain type 1 Lang have provided similar estimates for the copy numbers and dissociation constants of receptors (9,144,173,288,406,479). Competition experiments have disputed whether type 3 Dearing and type 1 Lang bind to the same (261) or different (9,144,490) receptors on L cells; a recent study argues for one receptor being shared by these two strains, plus a second being restricted to type 3 Dearing (9). There is general agreement that attachment is mediated primarily or exclusively by the s1 protein.

The nature of the L-cell receptor(s) for reovirus type 3 Dearing is well studied but remains incompletely defined (reviewed in ref. 405). There is generally consistent evidence for involvement of a sialoglycoprotein based on the decreased virus-binding capacity of L cells treated with proteases, periodate, neuraminidase, or particular lectins (14,173,174,395). The capacity of type 3 Dearing virions or s1 protein molecules to bind L cells is similarly decreased in the presence of sialoglycoproteins (174,360,361,509), gangliosides (14), or free sugars that contain a-linked sialic acid residues (362). The latter observations have been used to argue that the L-cell receptor, like the erythrocyte receptor described earlier, includes sialic acid. One study suggested that the a-anomer of sialic acid is the minimal receptor determinant required for reovirus (type 3 Dearing) recognition (362). Correlation between the capacity of certain type 3 strains to bind glycophorin and their capacity to bind and productively infect MEL cells (88, 396) is consistent with a model in which binding to sialic acid is critically important for infection of at least some cells.

Other data suggest that one or more specific proteins, perhaps sialylated glycoproteins, can serve as cell surface receptors for type 3 Dearing. Initial data in this regard were acquired using an antiidiotype approach (reviewed in ref. 158). Antibodies were raised against the anti-s1 monoclonal antibody G5, which blocks the binding of type 3 Dearing to cells and thereby neutralizes its infectivity. These preparations of G5-specific antibodies, as well as similarly derived monoclonal antibodies, exhibited behaviors predicted for antiidiotypic antibodies: They were capable of blocking the binding of type 3 Dearing to cells, presumably by mimicking within their own structures a portion of the attachment site on s1 and thus binding to s1-specific receptors on the cell surface (68,339,509). A monoclonal antiidiotypic antibody was subsequently used to identify a 67-kd membrane glycoprotein, having notable structural similarity to the b-adrenergic receptor, as a probable receptor for reovirus type 3 Dearing (94). Subsequent studies have provided mixed support for this hypothesis (89,90,125,406), and it currently seems unlikely that this protein represents the sole or even primary receptor for reoviruses on many cells. Other reports describe receptors for reovirus type 3 Dearing that include the epidermal growth factor (EGF) receptor (469), a 54-kd protein on endothelial cells recognized by both type 3 Dearing and type 1 Lang (479), and multiple sialoglycoproteins on L and human erythroleukemia cells (89). Reports describing specific receptors for reovirus type 1 Lang are less frequent but include a 47-kd protein from mouse intestinal epithelial cells (490) and undefined moieties on Caco-2 cells (9) and mouse ependymal cells (339).

Whether sialic acid or another protein element(s) alone is sufficient to mediate reovirus attachment to cells resulting in productive infection remains in question. A two-step mechanism involving initial binding to sialic acid and subsequent interaction with a specific protein receptor has been suggested (362). Available data suggest that differences in receptors are likely to exist between cell types and between reovirus serotypes or strains. It may also be important to distinguish attachment that leads to productive infection from attachment that is nonproductive. The capacity of attachment to lead to virus uptake from the cell surface by endocytosis may be an important distinguishing feature in this case (89,395). The importance of multivalent attachment via two or more of the s1 oligomers present in viral particles remains undefined. Differences in the attachment process for virions and ISVPs are also unknown.

Endocytic Uptake and Proteolytic Activation

Thin-section electron micrographs of cells soon after infection reveal reovirus particles associated with clathrin-coated pits or vesicles at or near the plasma membrane, suggesting that uptake from the cell surface can occur by receptor-mediated endocytosis (56,59,395,467) (see Fig. 6). Particles are subsequently observed within vacuoles resembling endosomes and lysosomes (56,59,177,395,442,467). Treatment with colchicine and nocodazole does not inhibit endocytosis, but it does inhibit the movement of these virus-containing vacuoles toward perinuclear regions, suggesting that vacuole translocation occurs along microtubules (177). Only some of the proteins to which reovirus particles bind on the cell surface may be capable of signaling endocytic uptake (89,395).

Experiments with radiolabeled virions provide evidence for proteolysis of viral proteins within hydrolytic compartments of the endocytic pathway (late endosomes or lysosomes), starting 20 to 30 minutes after infection at 37°C (442,443,467). The proteolytic cleavages are notable in that they involve only certain outer capsid components. Specifically, degradation of protein s3 and conversion of protein µ1C to its stable cleavage product d are observed, generating particles very similar to ISVPs that result from *in vitro* proteolysis (59,84,344,440,467). The reproducibility of these limited cleavages suggested that the generation of ISVP-like particles by proteolysis within lysosomes may be a required step in reovirus infection.

More direct evidence for proteolytic activation as a required step in infection arose from an observation that infection is inhibited by the weak base ammonium chloride, which raises the pH within endosomes and lysosomes (72). Later experiments revealed that an early step in infections initiated by virions, but not ISVPs, is inhibited by this agent (50,119,287,467). In addition, the extent of inhibition was found to correlate with the extent to which cleavages of proteins s3 and µ1C in infecting virions are blocked. It was suggested that, by raising the pH within endosomes and lysosomes, ammonium chloride inhibits the activity of acidic proteases within those compartments and blocks the generation of ISVPs. ISVPs may resist the block to infection by ammonium chloride because they have undergone proteolysis *in vitro* and thus no longer contain an acid-dependent step in their replication cycle (467). Proteolysis appears to activate reovirus particles for the next steps in infection, membrane interaction and penetration (see later).

In certain settings, proteolytic activation of virions occurs outside cells, before attachment and endocytosis (see Fig. 6). When newborn mice are perorally inoculated with virions, proteolysis to generate ISVPs occurs within the lumen of the

small intestine and is probably mediated by pancreatic serine proteases (28,50). Extracellularly generated ISVPs are apparently required to initiate infection of mouse tissues by that route (10,28), but the basis for this requirement is unknown. ISVPs generated *in vitro* may have the capacity to bypass endocytic uptake and to penetrate into the cytoplasm directly through the plasma membrane (see later).

Interaction with and Penetration of Cell Membranes

As with other nonenveloped viruses, the mechanism by which reoviruses cross the membrane barrier during entry into cells (see Fig. 6) remains poorly defined. Three assays have demonstrated a direct interaction between reovirus particles and lipid bilayers. One involves the capacity of particles to effect the release of ^{51}Cr from preloaded L cells (56,277). Another revealed the capacity of particles to induce conductance through artificial planar bilayers (472). Finally, reovirus particles can mediate lysis of erythrocytes, resulting in the release of hemoglobin (82,83,219). Membrane interaction in all these assays is restricted to ISVPs: Virions and cores did not exhibit the activity. Moreover, the membrane interactions did not require acid pH. These findings are consistent with a model in which ISVPs are required intermediates in the process of reovirus entry into cells and in which the acid-dependent step in reovirus entry (the one inhibited by ammonium chloride) is proteolytic activation rather than membrane interaction (277,345,467,472).

Available data implicate the outer capsid protein µ1, which covers most of the surface of ISVPs, in mediating direct interactions with membranes. A strain difference in the capacity of ISVPs to mediate ^{51}Cr release from L cells was mapped to the M2 gene, which encodes µ1 (277). Changes in the ^{51}Cr release phenotype have been associated with certain µ1 mutations (208). Studies with recoated cores indicate that the µ1 protein is the only additional outer capsid protein that must be present in those particles for erythrocyte lysis to proceed (83). Features of the µ1 protein noted to be consistent with a role in membrane interaction include the modification of it and its amino-terminal fragment µ1N with a myristoyl group (347), and the presence of a predicted pair of long, amphipathic a-helices flanking the cleavage junction between the µ1d/d and f fragments of µ1/µ1C that are found in ISVPs (344). A conformational change in µ1 is expected to precede reovirus-membrane interaction according to one model (277,344,345). The relative importance to membrane interaction of s3 degradation and µ1/µ1C cleavage into fragments µ1d/d and f remains somewhat unclear (20); however, some data indicate that the µ1/µ1C cleavage into µ1d/d and f is dispensable (82,83).

Direct interaction of viral components with a cell membrane is expected to be one step in a process that gives reovirus particles access to the cytoplasm, where there are substrates for transcribing and capping the viral mRNAs and ribosomes for translating them (see Fig. 6). As nonenveloped viruses, reoviruses cannot use fusion as a general mechanism for this process, and little is known about the steps that follow membrane interaction and permit reovirus components to penetrate into the cytoplasm. Suggested possibilities are that membrane interaction results in local disruption of the bilayer such that virus enters the cytoplasm directly through a large breach, or that a more precise, pore-like structure is formed by viral proteins within the membrane, through which viral components enter the cytoplasm (56,59,344,345,472).

A current model (see Fig. 6) suggests that when ISVP-like particles are generated from virions by proteolytic activation within late endosomes or lysosomes, membrane interaction and penetration are most likely to occur within and from those vacuoles (56,59,345,467). When ISVPs are generated *in vitro*, however, they may be capable of penetrating directly through the plasma membrane, prior to endocytosis (see Fig. 6) (56,59,277), although other work suggests that ISVPs also undergo endocytic uptake and sequestration into vacuoles prior to penetration (467). Whether penetration through plasma membrane by extracellularly generated ISVPs occurs routinely during reovirus infections remains to be determined.

Plus-Strand RNA Synthesis (Transcription)

Notable features of reovirus transcription include the stability and productivity of the transcriptase and its encasement within icosahedral particles (24,52,127,230,272,426,445). A common system for *in vitro* transcription includes reovirus cores, ribonucleoside triphosphates, and an ATP-regenerating source. The products of transcription are single-stranded RNAs, which are released from the transcribing particle (24,27). Reovirus transcription is conservative and asymmetric (24,272,445). Ten distinct transcripts have been identified, which by hybridization and sequencing (247,301) represent full-length copies of the 10 genomic plus strands. Except for abortive initiation (see later), there is no evidence that any of the gene segments give rise to less-than-full-length transcripts as part of the viral replication strategy.

If appropriate substrates are provided, transcripts produced by purified reovirus particles *in vitro* can undergo capping and methylation at their 5' ends, indicating that the capping enzymes are also present within the particles (see later). Capping imparts increased stability to reovirus mRNAs (164) and increases the efficiency with which many mRNAs are translated within eukaryotic cells (424). Reovirus transcripts are not polyadenylated: their 3' ends are fully complementary to the 5' ends of the genomic minus strands that serve as templates for their synthesis.

Transcriptase Activation

Early studies showed that the virion-associated transcriptase can be activated by heat (52) or by treatment with proteases such as chymotrypsin (426). For protease treatments yielding cores as end products, transcriptase activation was correlated with loss of protein µ1 from particles (55,127,223). In addition, a strain difference in the treatment conditions needed to generate cores and activate transcription was mapped to the M2 gene, which encodes µ1 (127). A number of parameters (e.g., the concentration and nature of monovalent cations, divalent cations, proteases, and viral particles) were found to affect whether transcriptase activation occurs during protease treatments, in general correlating with whether cores were produced (51,54,55,60,127,223).

Although some studies suggest that ISVPs are transcriptionally active (16,84,425,440), others indicate that the transcriptase is latent in ISVPs but can be activated under certain conditions without additional proteolysis (54,55,58,146,403). One study correlated the switch-on of transcriptase activity in ISVPs with a change in the µ1d

fragment of μ1 (146). From these studies, a model was proposed in which virions undergo transcriptase activation via two steps (55,59,146,345). The first step requires proteolysis and produces ISVPs. The second step is protease independent, produces transcriptionally active particles, and may be linked to penetration during entry into cells.

Although virions cannot make full-length transcripts, they have been reported to synthesize short oligonucleotides when given appropriate substrates (151,515). This important finding suggests that the transcriptase is constitutively active in virions but capable of limited or no elongation. Capping enzymes are also active in virions (74,231,515); thus, transcriptase activation must primarily involve relief of a block to elongation. The nature of this block is unknown, but it may relate to movement of the dsRNA templates (151,226,424). Changes in the outer capsid are essential for activation (see earlier), implying that interactions between outer and inner capsid proteins may produce the elongation block. One study suggested that structural changes in the core occur at the onset of transcription (366), and it may be that these changes are required for elongation but are inhibited by the outer capsid proteins in virions and ISVPs. A conformational change in l2 (131) may be important for transcriptase activation in that it opens a fivefold channel through which the substrates or products of transcription may be required to pass.

Initiator oligonucleotides are the major transcription products produced *in vitro* by both virions and cores, even though cores can at low frequency elongate these into full-length transcripts (151,514,522). This and other findings indicate that the onset of elongation is the rate-limiting step in reovirus transcription (230,514,515,522). Three phases of transcriptase activity are thus suggested: efficient, repetitive initiation yielding large amounts of initiator oligonucleotides, an inefficient onset to the elongation process, and elongation yielding smaller amounts of full-length transcripts (515).

Primary Transcription Within Cells

Two phases of transcription by reoviruses are known to occur within infected cells, based on the derivation of the transcribing particle. Primary transcription is mediated by particles derived by partial uncoating of infecting particles (59,84,440,442,443). It results in production of capped transcripts that serve both as mRNAs for translation and as templates for minus-strand synthesis within progeny particles. Primary transcripts are detected by 2 hours after infection, reach a maximum at 6 to 8 hours, and decrease to undetectable levels by 12 hours (224,442,530). Secondary transcription is mediated by particles assembled from newly synthesized RNA and protein molecules and is the source of 95% of the transcripts produced during infection (216,523). Secondary transcripts have been reported to remain uncapped, but this has been disputed (see later section).

Primary transcription may be subject to regulation within cells, but some authors have disputed these findings as well (224,441,530). Transcripts from four “pre-early” genome segments (L1, M3, S3, and S4) have been reported to be preferentially synthesized during the first several hours of infection (357,451,489). Moreover, transcription from the six other genes was suggested to require new protein synthesis because it is blocked with cycloheximide (258,425,489). One hypothesis is that a cellular repressor prevents transcription of the six later genes and that a protein encoded by a pre-early gene interferes with its activity (258,357,523).

The nature of the primary transcriptase particle remains in question; however, because of their transcriptional activity *in vitro*, cores are assigned this role in one model (see Fig. 6). In one study, transcriptionally active particles derived from input particles were isolated from infected cells and were shown to have the characteristics of cores, perhaps indicating that additional uncoating occurs in association with or subsequent to penetration (59). Because proteinase K was used to separate the particles from cell debris in this study, however, degradation of outer capsid components may have occurred before the particles were characterized (523). Other studies suggest that the primary transcriptase particle is more similar to an ISVP, in that it still contains the μ1 protein (16,84,425,440,443).

The intracellular site of primary transcription also remains undetermined. Thin-section electron micrographs have been reported to show small numbers of core-like particles free within the cytoplasm of newly infected cells (49,59), suggesting that the primary transcriptase particle is introduced fully into the cytoplasm (see Fig. 6). In fact, most reovirus particles in such micrographs are located within endocytic vacuoles (59,442,467). Although these may be particles that have not yet succeeded in initiating infection, they might also represent the primary transcriptase particles. Thus, another model was proposed in which the primary transcriptase particle remains within a vacuole but inserts its l2 pentamers through the membrane so that mRNAs can be extruded into the cytoplasm (467).

Discrete Enzyme Activities in Reovirus Transcription and mRNA Capping

Each of the steps in reovirus transcription and mRNA capping (Fig. 7) (167) is mediated by an enzyme present in cores and encoded by a viral genome segment. The 5' cap of reovirus transcripts includes a 7-N-methyl guanosine linked by three phosphate groups to the 5'-terminal, template-encoded guanosine present in all reovirus mRNAs (153,165). The 5'-terminal, template-encoded guanosine is 2'-O-methylated (153,165). The next residue in all reovirus mRNAs is cytidine, which can also undergo 2'-O-methylation, but by a cellular cytoplasmic methyltransferase (121). Reovirus capping activities can be uncoupled from transcription and readily function when exogenous substrates, including preformed oligonucleotides and reovirus mRNAs in some cases, are incubated with viral particles (168,321,337).

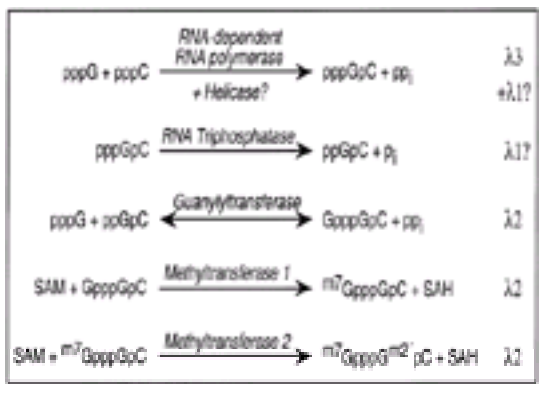

FIG. 7. Enzymatic activities in transcription and capping by reoviruses. Polymerization of nucleoside triphosphates (shown here for the first nucleotide pair) as specified by a genomic dsRNA template is mediated by the dsRNA-dependent RNA polymerase. The RNA triphosphatase generates a nucleosidyl diphosphate at the 5' end of the nascent mRNA and sets the stage for formation of the dimethylated 5' cap by sequential actions of the guanylyltransferase and methyltransferases. The role of an independent helicase in reovirus transcription is proposed but not proven. Individual reovirus proteins proven or suspected to mediate each activity are indicated at *right.* SAM, S-adenosyl methionine; SAH, S-adenosyl homocysteine.

RNA Polymerase

Early work failed to associate polymerase activity with any individual reovirus proteins after disruption of cores (226,230,500). The transcriptase (dsRNA-dependent RNA polymerase) was later suggested to be formed by portions of both core proteins l1 and l2 because of their apparent labeling by pyridoxal phosphate during transcription (320,321). Recent work indicates that the minor core protein l3 is likely to be the catalytic subunit of the transcriptase (69,127,243,326,460). A limited spectrum of activity [poly(C)-dependent poly(G) polymerase] has been reported for l3 expressed in isolation, which probably indicates that other core proteins are needed to form the complete transcriptase (460). The l3 protein is also likely to be a primary component of the ssRNA-dependent RNA polymerase that operates during minus-strand synthesis. Sequences in reovirus RNAs that are specifically recognized by the polymerase (e.g., initiation sites) have yet to be defined.

RNA Helicase

The double-stranded nature of the reovirus genome necessitates an RNA duplex unwinding activity for transcription to proceed. This activity might be inherent in the polymerase but also may be localized to another core protein and take the form of a classical RNA helicase (44,354,355,460). One report suggested that the antiviral drug ribavirin inhibits reovirus replication by interfering with helicase activity (376). Some recent evidence suggests that helicase activity may be associated with core shell protein l1 (44,354).

RNA Triphosphatase

An RNA triphosphatase (RNA triphosphate phosphohydrolase) initiates formation of the 5' cap on reovirus mRNAs by releasing inorganic phosphate from the originally triphosphorylated 5' end of the transcript, thereby yielding a 5' diphosphate group (see Fig. 7). An NTPase nucleoside triphosphatase has been found in reovirus virions and cores (53,231,354,355) and may represent this enzyme. Recent work with recombinant l1 protein indicates that it has such an activity, suggesting that it performs this function during 5' cap generation by reovirus particles (45).

RNA Guanylyltransferase

An N-terminal domain of protein l2 has been defined as the reovirus guanylyltransferase (280,378). Its enzymatic mechanism includes formation of a covalent l2–GMP intermediate, probably at lysine 190, using GTP as substrate (93,152,279,423). The GMP is subsequently transferred to the 5' diphosphate of reovirus mRNAs (see Fig. 7). The guanylyltransferase is promiscuous in its specificity for GMP-acceptors in that PP_i, GDP, GTP, and both ppG- and ppA-terminated oligonucleotides work as substrates (93,167). A GTP-dependent, pyrophosphate exchange activity associated with cores (484) represents guanylylation plus its reverse reaction. After methylation, transcript termini become resistant to pyrophosphate exchange (167); thus, reversibility of guanylylation may be required to ensure that proper 5'GpppG caps are formed on transcripts to act as substrates for methylation (see later).

RNA Methyltransferases

Final proof is lacking for the protein that mediates methyltransferase activities to modify the 5'-terminal guanosines of reovirus mRNAs (see Fig. 7), but a role for l2 is strongly indicated by the presence of two methylase-like domains identified in the crystal structure of core-bound l2 (378). SAM serves as methyl group donor for these reactions yielding S-adenosyl homocysteine (SAH) as a by-product (154,167). Indeed, SAH was found to bind both methylase-like domains of l2 in core crystals (378), and one of these regions was found to undergo cross-linking to radiolabeled SAM in other experiments (280). The 5'-terminal structure of reovirus mRNAs is specifically recognized by the methyltransferase(s), as GpppG and GpppGC can act as methyl acceptors but GppG, GppppG, and GpppGA cannot (424).

Organization and Mechanism of the Transcription Machinery

The sequential action of enzymes that synthesize reovirus mRNAs implies a precise structural organization within the

core. Some studies suggest that capping of nascent mRNAs occurs when they are only 2 to 15 nucleotides long (167,321), in which case the transcriptase and capping enzymes must be close together. Because preformed oligonucleotides and reovirus mRNAs can be capped by cores (168,321,337), the capping enzymes must be accessible to external solvent; however, antibodies have not been found to inhibit any transcription-related activity (424).

Transcription-related enzymes may be located in complexes near the fivefold axes of reovirus particles. Electron micrographs of transcribing cores suggest that mRNAs are released through the l2 spikes that protrude upward around the fivefold axes of cores (27,500). This idea is supported by reconstructed images from cryoelectron microscopy showing a channel through the l2 pentamer that is open in cores (131,310). The low copy number in which l3 is found in cores (about 12 copies) has also been used to argue that it is located near the fivefold axes.

Each of the 10 reovirus gene segments may represent an independent transcriptional unit. Both *in vitro* and in cells later in infection, transcripts from the 10 segments are produced at rates that are approximately proportional to the reciprocals of their lengths (24,225,445,489,530). Given that transcript elongation is the rate-limiting step in transcription (see later), this finding suggests a model for transcription in which each of the 10 genes is transcribed independently, and not as part of a linked array (225,226,424). A feature of this model is that it involves at least 10 transcriptases within the reovirus core (445). Electron micrographs of transcribing cores with multiple mRNAs being simultaneously extruded around the particle perimeter support this idea (27). Both moving transcriptase and moving template models for reovirus transcription have been proposed (226,424,515). Experiments in which transcription was inhibited by cross-linking genomic dsRNA are consistent with both these models (337,424); however, the moving template model is generally accepted. According to this model, a set of transcription-related enzymes is fixed near the base of each l2 spike within the core. It is proposed that the entire length of each dsRNA gene segment moves past the fixed transcriptase and that the nascent mRNA is directed past the capping enzymes and out the l2 spike (378,516). The liquid crystalline state of the packaged genomic dsRNA (131,199) may permit it the freedom of movement required by this model.

Later Steps in Replication: Amplification of Viral Products and Assembly of Progeny Virions

Translation of Viral mRNAs

Shortly after the onset of infection, there is a gradual increase in synthesis of reovirus proteins and a decrease in synthesis of cellular proteins (186,417). Newly synthesized viral proteins are detected by 2 hours after infection, and by 10 hours most of the proteins synthesized in the infected cell are viral in origin (530). The capacity of viral mRNA to associate with ribosomes may be a host-range determinant, as certain avian reoviruses can enter mammalian cells and transcribe their early mRNAs yet fail to initiate protein synthesis (42,285). The mechanism by which viral protein synthesis comes to predominate in infected cells is not well understood.

In addition to regulation of translation of viral versus cellular mRNAs, translation of the different reovirus transcripts is regulated. Reovirus mRNAs differ in translation frequency by as much as 30-fold (225). Because the different genome segments are transcribed at variable rates, inversely proportional to segment length, and because the transcripts appear to have similar stabilities, differences in the amounts of each viral mRNA provide one source of variability in the amounts of each protein that are synthesized (258,530). A second source of variability is in the translation frequency of the different mRNAs. Such variability has been found in both infected cells and *in vitro* translation systems (17,169,191,273,304,305,530,531). Nucleotide sequences of reovirus mRNAs at the –3 and +4 positions flanking the initiator AUG contribute to differing translation efficiencies *in vitro* (245); however, other nucleotide positions may influence the efficiency of translation of these mRNAs *in vivo* (41,329,385). Because the 5' nontranslated regions of the reovirus mRNAs are short (12 to 32 bases), this length may also contribute to translation rate; for example, the two reovirus proteins made in largest quantities—µ1 and s3—have the longest 5' nontranslated regions. For the S1 mRNA, from which two unrelated proteins are translated, it was proposed that translation rates are regulated at the level of polypeptide elongation (41,126,148). The observation that the M1 mRNA is translated much less efficiently *in vivo* than *in vitro* has suggested that its translation may be influenced by negative regulatory factors in cells (388). Reovirus mRNAs also may differ in capacity to compete for components of the translation machinery (64,285,377). Several studies suggest that levels of translation may be affected by the influence of different viral mRNAs on negative regulators of translation, such as the dsRNA-activated protein kinase (41,48,400).

Assortment and Packaging of the 10 Viral mRNAs

The mechanism ensuring that 10 unique dsRNA segments are assembled into each newly formed virion remains unknown. Pieces of evidence that the reovirus genes are assorted selectively are that a particle-to-pfu ratio as low as 1 has been reported (454), and that the central cavity of reovirus particles is not large enough to accommodate many more than the 10 unique segments (131,199,341). It is generally accepted that assortment of the 10 segments occurs at the level of the reovirus mRNAs, and that minus-strand synthesis to generate dsRNA occurs later, within a nascent viral particle (486,528,529) (see later and Fig. 6). It has been suggested that assortment and minus-strand synthesis may be more intimately linked than previously interpreted (13).

Assortment is likely to involve signals that identify an RNA as viral and not cellular, as well as signals that specify each of the 10 segments as unique. The relevance of either RNA–protein or RNA–RNA interactions to this process is unclear. All 10 reovirus gene segments contain short conserved regions at the 5' and 3' ends of their plus and minus strands, but it is not known if these sequences play any role in packaging. Sequences near the termini of some reovirus ssRNAs may interact in a panhandle-like structure, which may be important for packaging (87,274,526). Analysis of mutants containing internal deletions of their M1 genes identified sequences within 130 to 190 nucleotides of the 5' and 3' termini that contain the minimum required signals for packaging (525). The plus strand of each genomic segment contains a 5' cap identical to that on mRNAs made by primary transcriptase particles (166), so the cap might serve as a packaging signal as well.

One early study hypothesized that sNS plays a role in mRNA assortment and packaging because of its capacity to bind

ssRNA (211). Protein assemblies, containing primarily sNS and apparently capable of binding to selected regions of reovirus mRNAs, were subsequently isolated from infected cells, consistent with the idea that this protein plays a significant role in the earliest stages of particle assembly (183,184,458). In a more recent study, protein-specific antisera were used to precipitate protein–RNA complexes from infected cells in an attempt to identify components of the assorting particle (13). This work suggested that sNS, as well as μNS and s3, associate with individual reovirus mRNAs to form small nucleoprotein complexes soon after transcription, but that μNS or s3 (not sNS) binds to the mRNAs first. Recent evidence that sNS binds to ssRNA with positive cooperativity and demonstrates an ATP-independent, duplex-unwinding activity is consistent with a role for this protein in packaging and/or minus-strand synthesis (179,180).

Minus-Strand Synthesis: The Replicase Reaction

Each packaged plus strand serves as a template for synthesis of one minus-strand copy, which remains base-paired to the plus strand after synthesis and thereby regenerates the genomic dsRNA (1,398,399,410,528). Minus-strand synthesis proceeds from an initiation point at the extreme 3' end of each template, and once the minus strand is synthesized, it remains within the nascent progeny particle. The viral replicase catalyzes a single round of minus-strand synthesis. Initiation of minus-strand synthesis, but not elongation, appears to depend on continuing protein synthesis (489), but this effect remains poorly understood.

Although preparations of replicase particles are often heterogeneous and include particles with transcriptase activity (323,398,487,529), it seems likely that these two activities are associated with discrete types of particles (323). Particles with replicase activity have a buoyant density similar to that of virions, are morphologically indistinct, and contain the viral core proteins with reduced amounts of l2 and other outer capsid proteins relative to virions (323,529). The replicase activity associated with nascent particles is susceptible to RNase, but once minus-strand synthesis is complete, the particles become RNase-resistant (1). Recent work suggests that l3 acts as the ssRNA-dependent dsRNA polymerase of reoviruses (460), but the roles of other proteins in minus-strand synthesis remain undefined. At end of minus-strand synthesis, RNA polymerase is positioned to jump strands and begin plus-strand synthesis using the minus strand as template.

Secondary Transcription

Secondary transcription is defined as that mediated by newly assembled subviral particles within infected cells. These particles must be derived from those with replicase activity, and they are distinguishable by sedimentation behavior, by the presence of a full complement of dsRNA, by the presence of variable amounts of protein μNS, and by reduced amounts of l2 and the other outer capsid proteins (323,447,532). The appearance of “late” viral mRNAs represents transcription by progeny subviral particles (secondary transcriptase particles) (323,447,524,529). According to some studies, these secondary transcriptase particles contain latent capping enzymes and produce uncapped mRNAs (447,523,524). These uncapped viral mRNAs are proposed to be translated preferentially at later times in infection. Late viral transcripts begin to appear at 4 to 6 hours after infection, reach maximum amounts (greatly exceeding the quantity of primary transcripts) at 12 hours, and subsequently decrease (252,448,489,523). Whether they serve only for translation into proteins or whether they may also be packaged into progeny particles and serve as templates for minus-strand synthesis remains controversial (157,324,410,523). The recent evidence that recombinant μNS protein can bind to cores without inhibiting their transcription and capping activities, while at the same time blocking outer capsid assembly, is consistent with a model in which μNS serves to increase the production of capped transcripts within infected cells (66). It is not known if particles that engage in secondary transcription can go on to form mature virions (see Fig. 6).

Assembly of the Viral Capsids

A great deal remains to be understood about the protein and nucleoprotein complexes involved in assembling the inner and outer capsids in reovirus particles (see Fig. 6). The earliest reovirus particles may be ones that contain reovirus mRNAs primarily in complex with s3 and the nonstructural proteins μNS and sNS (13,183). The next particle along the assembly pathway is described to lack nonstructural proteins, to include both inner and outer capsid proteins, and to be RNase sensitive (1,323). These “replicase particles” contain an ssRNA-dependent dsRNA polymerase activity and appear to be engaged in minus-strand synthesis (323). With the completion of minus-strand synthesis, the replicase particles become RNase resistant, probably reflecting the occurrence of structural rearrangements within the particle (1,523). Additional copies of the outer capsid proteins may be added to form complete virion particles (323,532). A final step in morphogenesis is synthesis of the virion-associated oligonucleotides, which appears to occur as the viral transcriptase undergoes inhibition during outer capsid assembly (16,38,151,347,439,532).

Many of the reovirus structural proteins exhibit some degree of self-assembly, in the absence of viral RNA or a full complement of viral proteins. For example, cells coexpressing the major inner capsid proteins l1 and s2 form particles that are morphologically similar to core shells (513). When l2 and l3 are coexpressed with l1 and s2, they are incorporated into particles as well, the former creating spikes that project from the inner capsid surface as expected (513). Other findings suggest that l1 and s2 may self-assemble into oligomers (dimers or trimers) before or after interacting in larger assemblies (102,131). The l2 protein, however, does not form pentamers in the absence of other proteins (286). The exact order in which these proteins come together to form nascent infectious particles, and the roles of RNA and the minor core protein μ2 in this process, are unknown.

Assembly of the outer capsid is also poorly understood. The major outer capsid proteins s3 and μ1 bind together in solution to form hetero-oligomers (131,211,260,471,530). Whether the s3-associated cleavage of μ1 into fragments μ1N and μ1C (260,347,471) is required for assembly is unknown. The fact that a mutant with a ts lesion in S4 (which encodes s3) results in the accumulation of core-like particles as assembly intermediates (160,322) may suggest that preformed complexes of μ1 and s3 are required to bind to nascent particles. Similar observations with a ts mutant in L2 (which encodes l2) may suggest a role for l2 in initiating the binding of these complexes to particles (160,322,368). Recently developed systems for recoating reovirus cores with recombinant outer capsid proteins have provided additional evidence that preformed complexes of μ1 and s3 are required for assembly into particles and that the assembly of μ1 and

s3 can occur independently of s1 (83,151). A significant body of work has addressed the self-assembly of s1 oligomers. Sequences near the amino terminus of s1 are likely to mediate its interactions with l2 within virions (22,131,161,163,270,284,343).

Release from Cells

There is little or no evidence for a specific mechanism such as membrane budding or vesicular transport by which reovirus particles might be released from infected cells in the absence of cytolysis. Thus, release of newly assembled reovirus virions is thought to follow cell death and the associated breakdown of the plasma membrane. Recent evidence indicates that apoptosis is an important mechanism of reovirus-induced cell death in the case of type 3 reovirus isolates (114,383,475,476).

EFFECTS ON THE HOST CELL

Viral Factories and Interactions with the Cytoskeleton

Reovirus-infected cells develop cytoplasmic inclusions that are not bound by cellular membranes (11,188,379). Inclusions first appear as phase-dense granules scattered in the cytoplasm, but these move toward the nucleus and increase in size as infection progresses (160,379,415,453). Viral factories contain dsRNA (188,444), viral polypeptides (109,160,299,379,453), and both complete and incomplete particles (18,109,160,379), but they do not contain ribosomes (415). Particles are often found in crystalline arrays. The characteristics and temporal development of viral factories can vary with growth conditions and virus strain (160,299). The µ2 proteins of reoviruses type 1 Lang and type 3 Dearing appear to be the primary determinant of a difference in the rate of inclusion formation observed with these two strains (299). A mechanistic description of the viral factories as “organelles” for reovirus replication and assembly remains in its infancy.

Immunocytochemical studies reveal progressive disruption and reorganization of the vimentin (intermediate) filament network in reovirus-infected fibroblasts (415). Vimentin filaments are specifically incorporated into viral factories and may play a direct role in replication (109,415,418). The nonstructural protein µNS may associate with vimentin filaments to anchor structures involved in viral assembly to the network (319). Disruption or reorganization of intermediate filaments may cause changes in cell shape that typify the cytopathic effect of reovirus infection and may contribute to cell injury (415,418).

Immunocytochemical studies reveal little change in the distribution of microtubules and microfilaments within reovirus-infected cells (415). In addition, experiments with microtubule-disrupting agents suggest that intact microtubules are not required for productive reovirus infection in cultured cells (18,108,452). Microtubules extend directly through viral factories (18,108,109,160,415,453), nevertheless, and there is evidence to suggest that proteins s1 (18) and µNS (319) may each associate with them. Other studies implicate microtubules in the transport of reovirus particles within endocytic vacuoles early in infection (177).

Inhibition of Cellular DNA Synthesis and Induction of Apoptosis

One consequence of reovirus infection can be the inhibition of cellular DNA synthesis (142,186,416,475). Inhibition of DNA synthesis is detected 8 to 12 hours after infection of L cells with type 3 strains (142,186,416,475), occurs more rapidly as the MOI is increased (429), and has been suggested to involve a G1/S transition block (103,143,195,197). Inhibition of L-cell DNA synthesis also occurs with UV-inactivated virions, but not with cores or empty virions (196,255,416,429). In an early genetic study, the basis for a strain difference in inhibition by both live and UV-inactivated virions of T1L and T3D was mapped to the S1 genome segment (416), which encodes both the cell-attachment protein s1 and the nonstructural protein s1s. Genetic studies indicate that S1 is also the primary determinant of strain-specific differences in the induction of apoptosis (383,475,476). Like inhibition of cellular DNA synthesis, apoptosis can be induced by UV-inactivated, replication-incompetent virions and it can be blocked by antibodies directed against the cell-attachment protein s1 (476).

The observation that both strain-specific differences in DNA synthesis inhibition and apoptosis induction segregate with the S1 genome segment suggested that these two processes might occur through related pathways. Recent studies do not support this hypothesis. Studies using a s1s-deficient reovirus strain demonstrate that reovirus induces cell cycle arrest at the G2/M transition, that this effect requires the nonstructural protein s1s, and that it occurs in the absence of apoptosis (365). Current efforts are focused on understanding the cellular pathways that are involved in reovirus-induced apoptosis *in vitro* and *in vivo*. Evidence suggests that induction involves cellular proteases including calpains (114) and caspase 8 (92), is blocked by Bcl-2 overexpression (383), is dependent on NF-kB activation (98), and involves the TRAIL-receptor binding pathway (92). More work is needed to establish the relationships among the various molecules implicated to date in reovirus-induced apoptosis.

Given the fact that not all reovirus strains induce apoptosis to high levels, the importance of this process in reovirus replication and disease induction remains somewhat uncertain. Neither is it clear if any reovirus-encoded molecules function to block apoptosis. The results of several studies support the notion that reovirus-induced apoptosis does not negatively impact viral growth (383) and does not depend on efficient viral replication (114). However, virus-induced apoptosis may play a significant role in pathogenesis, as one recent study revealed a correlation between areas of viral growth in the central nervous system (CNS) and apoptosis within those tissues (359).

Effects on Cellular RNA and Protein Synthesis

Reovirus infection can also result in the inhibition of cellular RNA and/or protein synthesis (186,252,276,417,530). Viral replication is required for this effect; moreover, inhibition is MOI dependent, suggesting that increasing amounts of viral products have increasing effects (417). In one case, a strain difference in the inhibition of cellular RNA and protein

synthesis was mapped to the S4 genome segment, which encodes the s3 protein (417). This result was initially interpreted as evidence that s3 plays a direct role in the mechanism of translational inhibition, but more recent studies suggest that s3 functions to block an inhibitory signal in infected cells (408). Some cell lines support reovirus infection yet are resistant to inhibition of cellular protein synthesis (112,123,317).

The molecular mechanism(s) that result in protein synthesis inhibition in reovirus-infected cells have yet to be determined, but several lines of indirect evidence suggest that s3 may influence the level of translation by regulating the activation of the interferon-induced, dsRNA-activated protein kinase, PKR. Activation of the dsRNA-activated protein kinase results in phosphorylation of eIF-2a and inhibition of translation initiation. *In vitro* studies, and others in heterologous viral systems, indicate that s3 can down-regulate PKR by sequestering its dsRNA activator (32,214,275,521). The ability to interfere with PKR activation is consistent with the finding that expression of s3 in transfected cells can stimulate translation from a reporter gene (178,290,413,471,521).

As yet, we do not understand the molecular basis for reovirus strain-specific effects on cellular protein synthesis. The s3 molecules from strains with distinct phenotypes are highly homologous in regions proposed to mediate dsRNA binding (115,235). Several pieces of evidence are consistent with a model in which strain differences in s3–µ1 affinity determine the amount of free s3 available to bind dsRNA and block PKR activation (178,214,275,408,471,521). The finding that s3 is localized throughout the cytoplasm in cells in which cellular translation is not inhibited, but is localized to perinuclear viral factories in cells in which cellular translation is inhibited, provides support for a model in which s3 down-regulates the dsRNA-activated protein kinase locally, typically sparing viral but not cellular protein synthesis from the inhibitory activity of PKR (408).

There is some controversial evidence that late in reovirus infection there is a switch to cap-independent translation. There are reports that late reovirus transcripts may lack a 5' cap (446,524), and some studies suggest that reovirus infection induces a modification of the cell translation machinery such that uncapped mRNAs are preferentially translated over capped (cellular) mRNAs (447,448). In one study, extracts from reovirus-infected cells were shown not to support translation of capped mRNAs (450). It has been speculated that a cellular protein required for capped mRNA synthesis may be inactivated in these infected cells (523). To date, however, there is no evidence that either EIF-4G or EIF-4E, translation factors that are known to be modified or inactivated in other virus-infected cells, are inactivated in reovirus-infected cells (140,239). Other studies imply that infection does not result in modification of the existing translation machinery but instead induces a factor that stimulates translation of uncapped mRNAs (266); moreover, protein s3 can apparently function as such a factor *in vitro* (264,265). Reports in conflict with the preceding ones indicate that reovirus-infected cells can translate capped and uncapped mRNAs with equal efficiency (123,330), suggesting a hypothesis that the preferential translation of reovirus mRNAs in infected cells is mediated by mRNA competition for a limiting translation factor (64,285,377). The reasons for discrepancies between studies are unclear; they may reflect the complexity of mechanisms that contribute to regulation of translation in reovirus-infected cells.

Interferon Induction

Reoviruses can induce infected cells to express interferon (205,254,418,436). With ts mutants, interferon induction correlates with yields of infectious virus but not with amounts of viral dsRNA, ssRNA, or protein made in infected cells (254). Using myocarditic and nonmyocarditic reovirus strains, one genetic study mapped the viral determinants of interferon induction to the M1, S2, and L2 genome segments encoding core proteins µ2, s2, and l2, respectively (436). A subset of these segments determines myocarditic potential and mRNA levels in infected cardiac myocyte cultures (432). These results suggest that levels of viral mRNA may determine the extent of interferon induction.

It is generally accepted that the genomic dsRNA of reoviruses can serve as a potent inducer of interferon during infection (205,254,289). The reovirus replication cycle, throughout which the genome remains encased within a protein capsid, may have evolved in part to prevent the genomic dsRNA from inducing a florid interferon response. Regions of dsRNA formed within particular reovirus mRNAs (for example the S1 mRNA) (48) might also play a role in interferon induction. The induction of interferon b in certain cell types in response to reovirus infection was recently shown to depend on interferon regulatory factor 3 (353).

Treatment with UV has been used to study interferon induction by reoviruses (205,254). Interferon induction by viral particles is far more resistant to UV inactivation than is infectivity. UV-treated reoviruses induce interferon more rapidly than occurs during productive infection, suggesting that a different mechanism is involved. A range of UV doses was found to increase the potency of strain T3D as an interferon inducer; in addition, UV treatment turned one ts mutant (tsC447) into an extremely potent inducer, even at nonpermissive temperature. It was proposed that UV treatment destabilizes the viral core so that genomic dsRNA from input particles escapes into the cytoplasm (205).

Interferons may inhibit reovirus replication by a variety of mechanisms. In general, interferon a/b appears to block the translation of early reovirus mRNAs (502). As discussed earlier, interferon-induced expression of the eIF-2a kinase, PKR, may explain the inhibition of viral protein synthesis in some reovirus-infected cells (113,192,350,401); however, interferon g is a poor inducer of this kinase and decreases the yield of progeny virions without having a major effect on viral protein synthesis (176,402). In other studies, cells treated with interferon a/b were found to contain an 2',5'-oligo(A)-dependent endoribonuclease (RNase L) that cleaves reovirus mRNAs and may facilitate the antiviral state (21,314,315,351); however, whether an endoribonuclease contributes to inhibiting the translation of reovirus mRNAs within cells in which the dsRNA-activated protein kinase has been proposed to function remains controversial (314,315,352). Reovirus strains can differ in the extent to which their replication is inhibited by interferon (217). Several studies have demonstrated that reovirus can replicate efficiently in interferon-treated cells despite activation of antiviral effector enzymes (111,155).

Effects on Other Functions in Differentiated Cells

Some studies have identified reovirus effects on differentiated cells. In MDCK (polarized epithelial) cells, reoviruses

produced persistent infections characterized by failure to form tight junctions, reduced EGF receptor expression, and increased expression of the adhesion molecule VCAM (317). In 3T3 (primary fibroblast) cells, reoviruses also produced persistent infections, characterized in this case by decreased expression of EGF receptors and increased expression of insulin receptors (480). Thus, reovirus persistence in a variety of differentiated cells can significantly alter cellular functions, including some involved in cellular growth control.

A number of interesting early studies demonstrated that transformed cells are more susceptible to reovirus-induced cytolysis than are untransformed cells (132,201). Until recently, there were few clues to explain the molecular basis of these observations. Evidence now suggests the involvement of the EGF receptor-linked signal transduction pathway in the efficiency of reovirus infection in differentiated cells (464,466). More recent studies demonstrate that activation of the *ras* signalling pathway (an event downstream of EGF receptor activation) renders cells susceptible to reovirus infection, and it has been suggested that a component of the *ras* pathway might function to down-regulate PKR, thereby promoting translation of reovirus mRNAs and thus viral replication (463).

Other studies investigated how interactions between the reovirus cell-attachment protein and its cell surface receptor(s) affect functions in differentiated cells. In optic nerve glial cultures, ligand binding to the reovirus type 3 receptor stimulated expression of galactocerebroside and myelin basic protein (96,97). Ligand binding to the type 3 receptor in rats also caused changes in oligodendrocyte function resulting in altered myelin expression (95). In another study, interaction of reovirus with thyroid follicular epithelial cells caused induction of major histocompatibility complex (MHC) class II molecules (172). Because this occurred even in the absence of viral replication, reovirus attachment to cell surface receptors may be sufficient to affect the expression of cellular proteins, probably through receptor-linked signaling pathways.

Persistent Infections

Although commonly causing the death of cells in which they replicate (lytic), reoviruses can produce nonlytic, persistent infections in a variety of cultured cells (2,4,6,7 and 8,33,67,112,116,119,132,317,463,468,480,498). Continuous maintenance of persistently infected cultures for months or even years is possible. Reoviruses have not yet been reported to persist in host mammals in the natural world, but recent studies have shown evidence for delayed clearance or chronic infection in laboratory mice. One of these studies involved infection of normal mice with reoviruses selected during persistent infections in L cells and showed delayed clearance from the CNS (327). The other studies involved infection of SCID mice with wt reoviruses and showed selection of organ-specific variants during the course of the chronic infection (193,194). Mechanistic relationships between these chronic infections in animals and persistent infections in culture remain undefined. Persistent infections in cultured cells by reoviruses appear best described as carrier cultures (2,7,116,119,498) and have been proposed to involve two distinguishable phases, establishment and maintenance. Certain aspects of reovirus–cell interaction (e.g., proteolytic uncoating during viral entry) appear to influence both phases of persistent infection, however, suggesting that the two phases may not be as mechanistically separable as first proposed.

Establishment

Viral characteristics can influence the establishment of persistent reovirus infections. Establishment of persistently infected L cells can be facilitated with defective stocks obtained by serially passing viruses at high MOI (6,7,67,119). Defective stocks contain a variety of mutants (see earlier section) including some that favor the establishment of persistent infections (6). Wild-type isolates can differ in their capacities to establish persistent infections in L cells after undergoing serial passage at high MOI. In the case of reoviruses type 1 Lang and type 3 Dearing, this difference is determined by their L2 genome segments (67). A current model links the L2 gene product l2 to the generation or amplification of different mutants within defective stocks, including those that favor the establishment of persistent infection, but the mechanism of l2 activity in this regard remains unknown.

Cellular characteristics can also influence the establishment of persistent reovirus infections. Wild-type reoviruses can establish persistent infections in several differentiated cell lines (33,112,116,132,317,468,480,498). The basis for an apparent relationship between cell differentiation and establishment of persistent infection remains unclear, but the resistance of some cells to reovirus-induced inhibition of cellular macromolecular synthesis has been proposed to play a role (112,132). The resistance of some cells to reovirus-induced apoptosis, or other mechanisms of killing, may also be involved. Other evidence suggests that some differentiated cells may favor the establishment of persistent infection by restricting reovirus disassembly as part of the entry process (498). Consistent with the latter proposal is evidence that persistent infections in L cells can be established using wt reoviruses if the cells are treated with ammonium chloride, which interferes with viral entry, during the first few days of infection (72).

Maintenance

A variety of viral mutants accumulate in cultures during persistent reovirus infections. Some of these mutants may play no role in the maintenance of persistence but reflect that reoviruses undergo extensive genetic changes during long-term persistent culture (4,6,7 and 8). On the other hand, cell lines cured of persistent reovirus infection by passing with neutralizing antibodies (PI cells) are found to support reduced growth of wt reoviruses relative to viruses isolated from persistently infected cultures (PI viruses) (2,119,232). This finding argues that both viruses and cells undergo mutation during the maintenance of persistent infection, and the complementary nature of the effects of these mutations on viral growth suggests that a process of virus–cell coevolution is a characteristic feature of the maintenance phase (2,119,232). Current evidence suggests that at least an element of this coevolution is based on steps in reovirus entry, with PI cells evolving to resist one or more steps in viral entry, and PI viruses evolving to evade these cellular counter-measures (119).

Specific Mutations Involved in Persistence

Mutant S4 genome segments from defective stocks (3) appear to play a critical role in the establishment of persistent reovirus infections in L cells (6). Because persistent infection appears to require that cellular injury be attenuated, it was proposed (6) that these mutations in S4 might be required for attentuating the shut-off of host RNA and protein synthesis characteristic of most reovirus infections (417) or for enabling PI viruses to interfere with the growth of wt, lytic virus (5,81). Although the S4 gene product, s3, can influence the activation state of interferon-induced enzymes (214), interferon does not appear to play a protective role in persistent reovirus infection of at least one cell line (111).

More recent evidence suggests that mutations in the S4 genome segment are selected during the maintenance phase of persistent infection in L cells, as part of the virus–cell coevolution described earlier. Mutations in S4 determine the capacity of certain PI viruses to replicate in cells that have been cured of persistent infection (511). In addition, the S4 mutations in these and other PI viruses have been found to facilitate the proteolytic cleavage of the encoded outer capsid protein s3 (499). Interestingly, a recent study revealed that cured cells have a defect in the maturation of cathepsin L, a lysosomal cysteine protease that may be responsible for virion uncoating in L cells (20). These results provide support for the model in which viral mutations that increase the efficiency of disassembly enable reovirus to maintain a persistent infection in cells that have evolved to restrict this step. These findings lend support to the hypothesis that reovirus persistence in L cells involves a carrier culture in which continuing transmission of virus between cells by an extracellular route is required for maintenance (119).

Mutations in the S1 genome segment appear also to be selected during the maintenance phase of persistent infections in L cells and can determine the capacity of certain PI viruses to replicate in cells that have been cured of persistent infection, independently of any mutations in S4 (232,511). In these and certain other PI viruses, the mutations in S1 are linked to decreased stability of the s1 (receptor binding protein) oligomer and its decreased capacity to mediate sialic-acid-dependent hemagglutination (511). The decrease in s1 oligomer stability in these viruses has been proposed to play a determinative role in the maintenance of persistent infections (511), but further work is needed to clarify the relative roles of S1 and S4 mutations in this regard.

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CHAPTER 53 Mammalian Reoviruses

Kenneth L. Tyler

HISTORY

Although the name *reovirus* was not introduced until 1959 (153), the first isolation of what would later be classified as a reovirus (type 3) was made in 1951 (182,195). In the summer of 1953, a virus was isolated from a rectal swab taken from a healthy child (“Lang”) living in Cincinnati, Ohio (131). This virus subsequently became the prototypic reovirus serotype 1 strain (reovirus serotype 1 strain Lang, T1L) (153). Ramos-Alvarez and Sabin subsequently isolated two additional viruses from children hospitalized with summer diarrheal illnesses. One of these isolates was initially referred to as D5 because it was the fifth unclassifiable isolate from a child (“Jones”) with a diarrheal illness. This isolate subsequently became the prototype for reovirus serotype 2 (reovirus serotype 2 strain Jones, T2J) (153). Another stool isolate from a child (“Dearing”) subsequently became the prototype for reovirus serotype 3 (reovirus serotype 3 strain Dearing, T3D) (153). In 1957, Rosen and colleagues (140,144) recovered a virus from an anal swab taken from a baby (“Abney”) living in an institution in Washington, D.C., which became a second prototype strain for reovirus serotype 3 (T3 Abney).

In 1959, Sabin (153) proposed that the viruses discussed above be included in a new group of respiratory and enteric viruses, for which he coined the name reoviruses (**r**espiratory **e**nteric **o**rphan viruses). The new name was intended to emphasize the fact that these viruses were isolated from the respiratory and enteric tracts and that they were not associated with any known disease (hence, *orphan*). The distinguishing features of this new group included their size (which was larger than other known enteroviruses), the capacity to produce cytoplasmic inclusions in monkey kidney tissue culture, pathogenicity for newborn but not adult mice, and hemagglutination (HA) of human type O erythrocytes. The wisdom of establishing a separate group for the reoviruses was quickly confirmed when it was discovered that reoviruses contained double-stranded segmented genomic RNA (dsRNA; see Chapter 52 for references).

Investigations of the nucleotide and amino acid sequence diversity of many reoviruses has led to an improved understanding of the evolutionary interrelationships between viruses (26,42,43,64,89). Regardless of the gene analyzed, there is no correlation between the relatedness of viral strains and the geographic site, date, or host species from which they were originally isolated (26,42,64). Phylogenetic trees constructed based on variations in the nucleotide sequences of the *S1, S2, S3,* and *S4* genes differ substantially from each other as a result of reassortment of reovirus gene segments in nature. *S1* gene–based phylogenetic trees suggest there are at least five lineages for reovirus strains (42). Analysis of lineages based on the *S2, S3,* and *S4* genes show no relationship to viral serotype and show a variable number of potential clades or lineages (26,64,89) (Fig. 1).

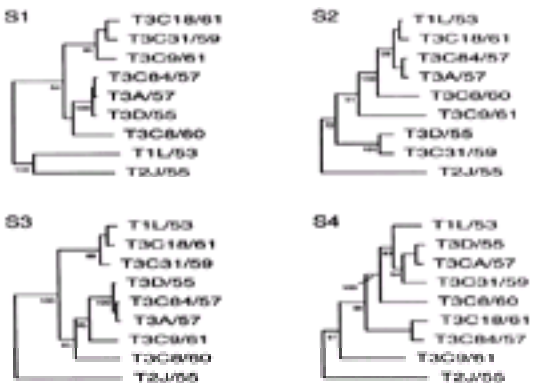

FIG. 1. Minimal-length phylogenetic trees for reovirus strains constructed from variation in the nucleotide sequence of the *S1, S2, S3,* and *S4* genes using the branch-and-bound algorithm of the parsimony program, PAUP. Trees are rooted at the midpoint of their longest branch. (Prepared by M. Goral and T. Dermody and reproduced from ref. 195, with permission.)

INFECTIOUS AGENT

The structure, genetics, and replication of reoviruses are described in detail in Chapter 52 (185). Reoviruses are nonenveloped viruses of about 850-Å diameter composed of two concentric icosahedrically symmetric protein capsids containing 10 discrete segments of genomic dsRNA. Each of the dsRNA segments encodes a single polypeptide, with the exception of the *S1* gene segment, which encodes two proteins derived from alternative reading frames. Most of the outer capsid shell is composed of complexes of two major proteins (µ1, s3). Trimers of a third protein, s1, are located at each of the virion's 12 vertices. The protein s1 is the viral receptor recognition protein (see later). The protein µ1 plays a crucial role in initiating membrane penetration and in facilitating access of virions and infectious subviral particles (ISVPs) to the cell cytoplasm during infection. The densely packed core capsid is composed primarily of four proteins (l1, s2, l3, µ2) and contains the segmented dsRNA genome. Another protein, l2, forms a spike extending from the core to the outer capsid.

During infection, reoviruses undergo partial uncoating, resulting in the production of ISVPs and cores (see Chapter 52 for review). ISVPs have lost s3 from the outer capsid, contain cleaved fragments of µ1/µ1C (designated µ1d, d, f), which remain virion associated and have the viral attachment protein s1 in an extended conformation. ISVPs are fully infectious and are generated at extracellular sites *in vivo* and within late endosomes or lysosomes of infected cells *in vivo* and *in vitro.* Subsequent conformational changes in µ1/µ1C facilitate interaction with membrane bilayers and allow subvirion particles to enter the cytoplasm where they become transcriptionally active.

Biologic Characteristics

Reoviruses grow in and produce cytopathic effects (CPE) in a wide variety of cultured cells. Although reovirus infection is usually cytocidal, under certain circumstances, noncytocidal persistent infection occurs (see Chapter 52 for a more detailed discussion of viral effects on host cells) (6,39). *In vivo,* chronic infections have been observed after infection of adult severe combined immunodeficiency (SCID) mice (62,65,66) and after inoculation of viruses derived from persistently infected *in vitro* cultures (PI viruses) into immunocompetent neonatal mice (113).

Mammalian cell lines derived from different species and tissues vary in the kinetics of viral multiplication, the time of appearance of infectious virus, and the rate of cell killing. CPE may correlate with the efficiency of virus-induced inhibition of host-cell protein synthesis and with the state of cellular differentiation (36). The M1 and S1 genes, encoding the µ2 core and s1 outer capsid proteins, respectively, are important determinants of reovirus strain-specific CPE in a variety of cell types (12).

The typical yield of virus is 1,000 plaque-forming units (pfu) per cell, with a particle-to-pfu ratio of 50:1 to 200:1 (6,81). Most viruses produced during infection remains cell associated, and physical disruption of cells (e.g., by several cycles of freezing and thawing) is required for complete recovery of virus during assay procedures. Studies of the one-step growth cycle in reovirus-infected L929 cells show an eclipse or lag phase lasting 6 to 10 hours followed by viral growth that peaks 15 to 18 hours after infection (81). ISVP-infected cells have viral yields similar to virus-infected cells, but the eclipse phase is shorter and maximal viral yield occurs earlier (81).

All three serotypes of reovirus agglutinate human red blood cells (HA) (41), but the capacity to agglutinate bovine red blood cells is restricted to certain T3 strains. Sialic acid residues present on glycophorin molecules are the reovirus receptor molecules on erythrocytes (see Cell and Tissue Tropism and Receptor Recognition for complete discussion and references). The s1 outer capsid protein is the viral hemagglutinin (199), and a discrete region (amino acids 198 to 204) of the s1 tail of T3 reovirus is crucial to HA (27,41) (Fig. 2).

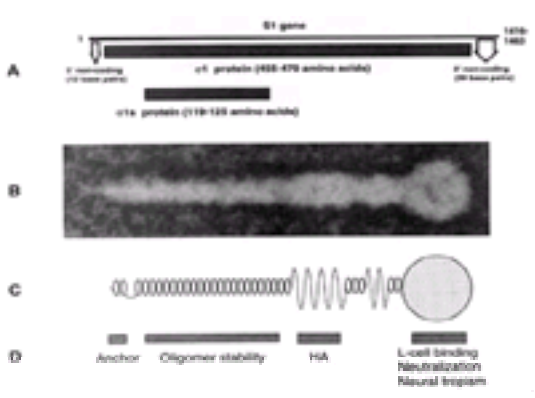

FIG. 2. Structure–function relationships of the T3 s1 protein. Model of s1 structure in which regions of proposed a-helical coiled-coil and b-sheet structure are depicted. Amino acid positions in the T3 s1 sequence are shown at *bottom.* Regions of sequence proposed to be important for hemagglutination (198,199,200,201,202,203 and 204), cell attachment (222–455), neutralization (340, 419), and a proposed receptor-recognition domain based on sequence similarity between s1 and antiidiotypic antibody (see text). (From ref. 195, with permission.)

Physical and Chemical Properties

Reoviruses are stable at room temperature and as aerosols. Reovirus T1L is more thermostable than T3D (72,182). Loss of infectivity is greatly accelerated at higher temperatures (81) and inhibited at 4°C. All the reovirus outer capsid proteins appear to play a role in virion thermostability. Loss of infectivity can result from heat-induced loss of the s1 viral attachment protein (47,48), and differences in thermostability between virions and ISVPs is determined by the s3 protein

(81). Mutations in the central region (amino acids 459 to 497) of the µ1 protein can enhance the thermostability of thermolabile T3D and T3A viruses.

Reoviruses are generally resistant to the effects of antibiotics commonly employed in tissue culture, including aminoglycosides and penicillin (141,169). Because they do not have an envelope, reoviruses are resistant to treatment with ether and other lipid solvents (168,182). They are also relatively resistant to a number of commonly used disinfectants, including formalin, Lysol, hydrogen peroxide, and phenol (47,48,168,182). Diluted Wescodyne (1/200) is of only limited efficacy in reducing reovirus infectivity (49). Most strains of reovirus T3 are inactivated by exposure to 95% ethanol (169). Ethanol-resistant mutants of T3D and T3A can be generated *in vitro* (72,200), making exclusive dependence on ethanol as a disinfectant problematic. Sodium hypochlorite (800 ppm chlorine) is extremely effective as a virucidal agent after brief exposure and should probably be considered the disinfectant of choice. Reoviruses are also sensitive to inactivation by ultraviolet light (106,133).

Viral outer capsid proteins appear to be particularly important in determining the susceptibility of reoviruses to inactivation by a variety of physical and chemical agents (see Chapter 52). For example, the *S4* gene, which encodes the s3 outer capsid protein, determines the relative susceptibility of reovirus strains to sodium dodecyl sulfate (SDS) and high temperature, whereas the *M2* gene, encoding the µ1 outer capsid protein determines sensitivity to phenol and ethanol (47,48). T3D mutants that are 100- to 1,000-fold less susceptible than wild-type virus to inactivation by ethanol contain point mutations in the *M2* gene, resulting in amino acid substitutions in the amino acids 425 to 459 region of µ1 (200).

The physical properties of reoviruses have practical implications for the storage of viral specimens and the disinfection of laboratory equipment and glassware. Reoviruses do not rapidly lose infectivity at room temperature, but optimal long-term storage should be at either –70°C or 4°C. Exposure of virus or specimens to ultraviolet light, which may be present in operating room suites or biosafety cabinets, should be avoided if isolation of reoviruses is desired. Disinfection of potentially contaminated materials can be done with either 95% ethanol, sodium hypochlorite solution (800 ppm chlorine), or preferably sequential application of the two solutions.

Propagation and Assay in Cell Culture

Mouse L929 fibroblasts (ATCC CCL 1) have been the most commonly employed cell line for experimental work, including viral growth, purification, and plaque assay, although Madin-Darby bovine kidney (MDBK), rhesus monkey kidney (LLC-MK2), and human embryonic intestinal (intestinal 407) cells are also sensitive lines for viral isolation (138). The standard assay for quantitating the amount of infectious virus present in infected tissues or culture stocks is the plaque assay, although assays of the 50% tissue culture infectious dose ($TCID_{50}$) are occasionally employed. The most commonly used plaque assay involves mouse L929 fibroblasts (57), although rhesus kidney cells can also be used (135). Plaque morphology varies with the virus strain and tissue culture system. In L929 cells, T1L and T3D generally form plaques 1 to 3 mm in diameter, whereas T2 plaques are significantly smaller (less than 0.5 mm) (109). The viral *S1* gene, encoding the s1 receptor-recognition protein, may be an important determinant of plaque size differences (109).

Host Range

Reoviruses are ubiquitous in their geographic distribution and host range. Virus is commonly found in river water, stagnant water, and untreated sewage (35). Reovirus concentrations in water supplies generally parallels the degree of contamination with *Escherichia coli,* suggesting that human feces may be a major source of virus (104,105).

Reoviruses have been isolated from humans, chimpanzees, Asian monkeys (*Macaca* species), African monkeys (*Cercopithecus* species), baboons, cattle, pigs, horses, cats, dogs, mice, birds, crustaceans, and even insects. Antibodies to reovirus have been found in an even larger group of animals, including dogs, cats, rabbits, rats, guinea pigs, horses, pigs, sheep, goats, bats, voles, camels, chickens and other birds, reptiles, fish, and 39 species of marsupials (182).

Naturally occurring reovirus infections have been described in wild mice and other small mammals, cattle and calves, sheep and lambs, pigs and piglets, horses, dogs, and cats (182). Serologic studies suggest that T3 strains can also infect laboratory rats and mice (91,99). The illnesses produced by reovirus infections in animals other than humans have involved primarily the respiratory, gastrointestinal, and nervous systems.

Perhaps the most interesting reovirus infections in animals are those in nonhuman primates. The close evolutionary relationship of these animals to humans raises the possibility that the reovirus-induced illnesses in these animals might also occur in humans. Cases of pneumonia and other respiratory illnesses have been found in both naturally and experimentally infected chimpanzees, macaques, marmosets, and *Cercopithecus* species (74,75,153,154,178). There are also isolated reports of other illnesses in primates, including cases of hepatitis and extrahepatic biliary atresia (T3) (145), meningitis (T2), and necrosis of ependymal and choroid plexus epithelial cells (T1) (153).

The isolation of reoviruses from humans and other primates, as well as cattle and calves and mice, suggests that biologic materials derived from these animals can potentially harbor reoviruses (40) and that these materials should be screened for reovirus before use in research studies or the derivation of biotechnology products.

Antigenic Composition and Determinants

Reoviruses are grouped into three serotypes, designated 1, 2, and 3, on the basis of neutralization (NT) and hemagglutination inhibition (HAI) tests (141). The s1 protein is the major serotype-specific antigen recognized in HAI and NT assays using type-specific polyclonal antisera (198,199). Serotyping has been facilitated by the availability of serotype-specific monoclonal antibodies (MAbs) specific for serotypes 1 (5C6) and 3 (9BG5).

Prototype viruses from the three serotypes are morphologically similar, but the cognate genes differ to varying degrees in their nucleotide sequences and in their migration patterns during polyacrylamide gel electrophoresis (see Chapter 52) (33). As would be predicted from the serologic studies, the greatest divergence between the prototypic T1L, T2J, and T3D strains occurs in the *S1* gene (24). At the amino acid level, the s1 proteins of T1L and T2J are more closely related (about 48% homology) than those of either strain to T3D (about 25% homology) (24,50). Despite this extensive divergence, there are at least five highly conserved domains of 22 to 34 amino acids (50). In contrast to s1, the other outer capsid proteins (l2, µ1, s3) of these strains have 90% or more, and in some cases 98% or more, homology (5,161,176,201). Similarly, high degrees of homology have been obtained from the available nucleotide sequence analysis of genes encoding viral core proteins (43,205).

Reovirus infection results in the production of both NT and HAI antibodies in the host. Studies with MAbs suggest that antibodies directed against the s1 and s3 outer capsid proteins, as well as antibody against the core spike protein l2, can neutralize reovirus infectivity. In general, the neutralizing l2 and s3 MAbs neutralize reovirus infectivity in a serotype-independent manner, although some MAbs show strain-specific patterns (69,84,94,183). By contrast, neutralizing s1 MAbs, as well as polyclonal monospecific s1 antisera, typically produce high-titer serotype-specific neutralization (21,69,84,94,166).

The NT epitope on the s1 protein of serotype 3 has been partially characterized through nucleotide sequence analysis of the *S1* gene of virus variants resistant to NT with s1 MAbs. Six mutants selected with two different MAbs contain single amino acid substitutions at either positions 340 or 419 of the s1 protein (10,11). These changes would result in nonconservative amino acid substitutions within the carboxyl-terminal globular head domain of s1 (58,119) (see Fig. 2). The importance of this region has been supported by studies of the binding of NT s1 MAbs to expressed truncated s1 proteins (115).

MAbs against the s1, s3, and µ1 proteins can all produce HAI (21,69,84,166,193). Analysis of the competition binding profiles of T3 s1 MAbs (21,166) suggest that the NT and HA epitopes on the T3 s1 protein are in distinct regions. These results are consistent with sequence data (11,199) and with the differential capacity of MAbs specific for other outer capsid proteins to inhibit s1-mediated HA and NT (193). MAbs specific for the s3 and µ1 outer capsid proteins may produce HAI through steric hindrance with the HA domains on s1 (193).

PATHOGENESIS AND PATHOLOGY

The capacity of reoviruses to produce disease depends on the outcome of a series of interactions between the virus and the host.

Entry Into the Host

Under natural circumstances, reoviruses are primarily respiratory and enteric viruses. A region of the s1 protein has sequence similarity with lysozyme (16). It has been speculated that this region of s1 might function as a glycosyl hydrolase (mucinase) and facilitate penetration of virions and ISVPs through the mucus layer overlying intestinal epithelial cells (16), although this remains unconfirmed. After oral inoculation into mice, reoviruses adhere to the apical surface of specialized intestinal microfold epithelial cells (M cells) overlying the dome of Peyer's patches (reviewed in 125) (Fig. 3). M cells are part of the gut-associated lymphoid tissue and are normally involved in the transepithelial transport of macromolecules from the intestinal lumen to the intercellular space and underlying Peyer's patches. Reoviruses can be sequentially visualized adherent to the luminal (apical) surface of M cells, inside intracellular vesicles, in the intercellular space between M cells and mucosal mononuclear cells inside Peyer's patch, and on the surface of mucosal mononuclear cells. A similar sequence of events occurs after reovirus entry through the respiratory tract in which viruses enter bronchial-associated lymphoid tissue through pulmonary M cells and then spread from the lungs to regional lymph nodes (111).

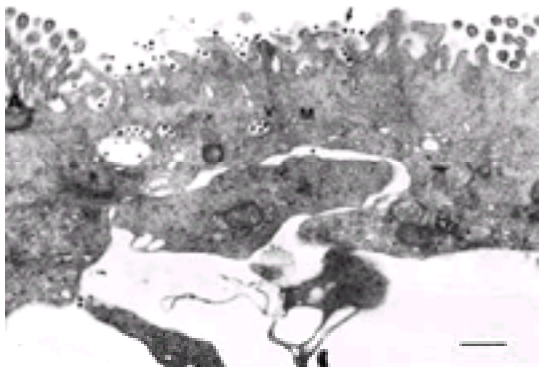

FIG. 3. Ileal epithelium overlying a Peyer's patch of an adult C $_3$H/Hej mouse. Thirty minutes after inoculation of an ileal loop with reovirus type 1, virions are found on the surface (*arrow*) and within vesicles (*V*) of an M cell (*M*). The apical surface of adjacent adsorptive cells (*A*) is virtually devoid of adherent virions (11,350× magnification). (From ref. 203, with permission.)

Binding to M cells, at least in the intestine, requires conversion of virions to ISVPs by endogenous intestinal serine proteinases (3). ISVPs are the predominant form of virus in intestinal tissues and in the intestinal lumen for the first 8 hours after peroral inoculation of virus and are the predominant form of virus that adheres to M cells and initiates infection (3,18). Inhibition of intraluminal proteolytic digestion, by peroral administration of serine protease inhibitors,

dramatically inhibits intestinal infection (3,9).

After oral inoculation of high doses of T1L, the epithelial cells of the ileum are infected, with relative sparing of the cells in the remainder of the small intestine and in the large intestine (reviewed in 125). Infection begins in epithelial cells adjacent to Peyer's patches in the crypts of Lieberkuhn. Infection of the crypt epithelial cells begins with viral binding to the basolateral membrane. This suggests that intestinal infection proceeds from the luminal surface through M cells to Peyer's patches and subsequently through the basolateral surface to epithelial cells. Attempts to demonstrate polarized infection of intestinal cells *in vitro* have yielded conflicting results, with studies indicating basolateral infection of isolated intestinal epithelial cells (197) but apical infection of cultured human colonic adenocarcinoma (Caco-2) cells (2). Reovirus T1L is also taken up by hepatic Kupffer's cells and subsequently excreted in the bile, raising the possibility that biliary excretion of virus may provide an additional mechanism of enteric infection (147,148).

Reovirus T1L and T3D show marked differences in pathogenicity after oral inoculation in suckling mice. T1L grows well in intestinal tissue after oral inoculation, whereas T3D does not (17,90,149,150). Both the *S1* gene (encoding the outer capsid protein s1) and the *L2* gene (encoding the core spike protein l2) determine the difference in capacity of T1L and T3D to survive in the gut, and the *L2* gene determines degree of viral shedding from the gastrointestinal tract (17,90). Recent studies suggest that the increased susceptibility of the s1 protein of some T3 reovirus strains to cleavage by intraluminal proteases may contribute to their reduced virulence after oral inoculation (25).

Spread of Virus

Reoviruses are able to spread from the gastrointestinal tract to extraintestinal organs and the central nervous system (CNS) after peroral inoculation (reviewed in 182,186, and 195). In newborn mice, T3C9 sequentially spreads from the intestinal tract into mononuclear cells of ileal Peyer's patches, through neurons of the myenteric plexus adjacent to Peyer's patches, and then ultimately through the vagus nerve to brain-stem neurons of the vagal dorsal motor nucleus (114). T1L follows a different pathway for extraintestinal dissemination. After transport across M cells, T1L can be sequentially detected in Peyer's patches, mesenteric lymph nodes, and, ultimately, the spleen, a pattern consistent with local lymphatic spread followed by bloodstream invasion (87).

Spread of reovirus to the CNS after peripheral inoculation into muscle has also been extensively investigated (180). After intramuscular inoculation, reovirus T3D spreads to the spinal cord through nerves (184). This spread, as well as the subsequent development of lethal encephalitis, can be prevented by surgical interruption of the nerve between the site of inoculation and the spinal cord as well as by antibody (see later). Studies using selective inhibitors of the fast and slow components of axonal transport indicate that the neural spread of T3 occurs by the microtubule-associated system of fast axonal transport (184). T3D appears capable of entering motor and sensory neurons as well as autonomic neurons (56,114). Viral antigen also appears to be transported both retrogradely and anterogradely through axons.

After intramuscular inoculation, the principal route of spread for T1L to the spinal cord is through the bloodstream. Viral antigen is detectable within the endothelium of blood vessels, although a limited degree of neural spread also occurs (56). Consistent with these results, T1L grows to significantly higher titer than T3D in cultured endothelial cells (103). Spread of T1L is not significantly inhibited by nerve section or by inhibitors of axonal transport, supporting the view that hematogenous rather than neural spread is the predominant pathway of dissemination to the CNS for T1L. Genetic studies using T1L × T3D reassortants indicate that the *S1* gene, which encodes the viral receptor-recognition protein s1, determines the pattern of spread of reoviruses from muscle to the CNS (184). These results are consistent with earlier studies suggesting that the s1 protein is also an important determinant of the extent of spread of virus from the gastrointestinal tract of mice after oral inoculation (87).

Cell and Tissue Tropism and Receptor Recognition

The s1 outer capsid protein serves as the receptor recognition protein (reviewed in 93)) (see Fig. 2). Topologically distinct receptor-binding domains exist in both the elongated fibrous “tail” domain of s1 and the distal globular head domain (93,118,158). A region of the s1 tail encompassing amino acids 198 to 204 is important for binding of T3 reoviruses to terminally sialylated oligosaccharides on erythrocytes, murine erythroleukemia, and L929 cells (27,31,41,152). Conversely, regions of the globular head of s1 contain receptor-binding sequences for neurons, neuroblastoma cells, murine thymoma cells, and L929 cells (93,118,158)). An antiidiotypic MAb (87.92.6) directed against a T3D s1-specific MAb (9BG5) contains an “internal image” of the binding domain of the T3D s1 protein (reviewed in 158). Amino acid sequence comparison of the antiidiotype MAb with s1 protein shows an area of homology that corresponds to amino acids 317 to 332 of s1. A synthetic peptide derived from this region of sequence homology can inhibit T3D binding to certain types of cultured cells, suggesting that this region of s1 may encompass a receptor-recognition domain. Studies of chronic reovirus infection in SCID mice indicate that organ-specific variants of reovirus are generated *in vivo* (65), but whether these variants include receptor-specific mutants or alterations in other proteins remains to be elucidated.

Perhaps the best characterized reovirus receptor is sialic acid. The reovirus receptor on erythrocytes are a-anomeric sialic acid residues on glycophorin molecules (128,129). Binding occurs to multiple sialoglycoproteins on other cells (30). Reovirus binding to sialic acid residues on erythrocytes and other target cells can be inhibited by treatment with neuraminidase or other sialidases and can be competitively blocked by sialylated oligosaccharides and brain gangliosides (4,60,61,126,129). However, reovirus binding to some cell lines, such as R1.1 thymoma cells, occurs in a sialic acid–independent manner (151), indicating that sialylated proteins cannot serve as the exclusive reovirus receptor.

P>A putative cell surface receptor for T3D has been isolated from murine neuroblastoma B104 and thymoma R1.1 cells using antiidiotypic antireceptor antibodies (reviewed in 158). The putative receptor molecule was a glycoprotein with a molecular mass of 67 kd. It was subsequently suggested that this receptor was structurally similar to the mammalian b-adrenergic receptor and that viral binding may occur on b_2-subtype receptors at a site distinct from that used by high-affinity ligands such as the antagonist iodocyanopindolol (ICYP) (97), although not all results are consistent with this

view (46). A substantial body of data appear inconsistent with the suggestion that a b-adrenergic receptor–like molecule serves as a receptor for T3 reoviruses. T3D clearly binds selectively and with high affinity to cells that lack detectable b-adrenergic receptor (159), does not trigger any of the pharmacologic activities associated with b-adrenergic receptor activation, and does not interfere with the binding or function of b-adrenergic receptor agonists (29,159). In addition, the binding of b-adrenergic receptor agonists and subsequent sequestration (down-regulation) of b-adrenergic receptors from the cell surface, does not reduce the capacity of reovirus to infect cells subsequently. Studies of the effects of competition between reovirions and b-adrenergic receptor antagonists have yielded conflicting results, with some studies indicating no effect (29,97) and others being consistent with a model in which reovirus binds to the antagonist domains of the b-adrenergic receptor (46).

Reovirus T3 can also bind to a heterodimeric cell surface receptor (designated p65/p95) composed of 65 kd and 95 kd subunits and expressed on the surface of some proliferating cells, include activated lymphocytes, neural embryonic precursor cells, and proliferating testicular cells (134,146,157). The p65 component of this receptor may be identical to the previously identified p67 receptor (see earlier). The heterodimeric p65/p95 receptor is associated with tyrosine kinase (TK) activity and may transduce a growth-inhibitory signal to proliferating cells (146,156).

Studies of the efficiency of reovirus infection in National Institutes of Health 3T3 cells suggest that receptor TKs may play a role in enhancing reovirus infection and cytopathicity. NR6 cells, 3T3 fibroblast derivatives lacking detectable epidermal growth factor (EGF) receptor, do not replicate T3D, fail to synthesize T3D proteins, and do not show detectable viral CPE (173). Transfection of these cells with the human EGF receptor gene, the *v-erbB* oncogene (172) or activated *Sos* or *Ras* (32,171), enables them to synthesize viral proteins, replicate virus, and become susceptible to viral CPE. The common denominator appears to be that efficient reovirus infection of 3T3 cells requires the presence of functional TK activity (172,173). NIH 3T3 cells transfected with a mutant gene encoding a kinase-dead EGF receptor are minimally cytopathic. The mechanism by which TK activity facilitates T3D infection has not been established. T3D can bind to the N-terminal extracellular domain of the EGF receptor, at a site distinct from the EGF ligand–inding site (174), but this does not appear to explain the effects of EGF and would not explain the results obtained with *v-erbB* or cells with activated *Sos* or *Ras*.

Perhaps the best synopsis of available data concerning reovirus receptor-recognition is as follows:

1. The s1 protein serves as the viral receptor-recognition protein, with distinct domains of the protein being involved in binding to different types of receptors on discrete populations of target cells.
2. One class of reovirus receptor, found on many cells including erythrocytes and L-cell fibroblasts, includes sialylated proteins. Binding to sialic acid residues is mediated by a domain in the s1 tail fiber (amino acids 198 to 204) (41).
3. There appear to be other, as yet incompletely characterized, reovirus receptors that are not sialylated proteins. Binding to these receptors may be mediated by regions of the s1 globular head (e.g, amino acids 317 to 332 or 340 and 419).

The importance of the s1 protein for tropism, particularly in the CNS, has been demonstrated *in vivo* as well as *in vitro.* After intracerebral inoculation into newborn mice, T3 reoviruses infect neurons and produce a lethal meningoencephalitis. T3 strains differ in their virulence and the peak titer they achieve in the brain but not in the topographic pattern of illness they produce (73), indicating that the general pattern of CNS tropism is serotype specific rather than strain specific. The pattern of injury produced by T1 reoviruses after intracerebral inoculation, as exemplified by the prototype T1L, is quite distinct from that produced by T3 reoviruses. The most striking difference is the failure of T1L to produce significant neuronal injury. Instead, T1L infects the ependymal cells lining the ventricles and produces ependymitis with associated hydrocephalus.

T1L × T3D reassortants demonstrate that the capacity of reoviruses to infect neurons (T3 pattern) or ependymal cells (T1 pattern) is determined by the viral *S1* gene, encoding the s1 receptor-recognition protein (reviewed in 180 and 195). Similar genetic studies have established the importance of the s1 protein in determining the capacity of reoviruses to infect discrete populations of cells in the pituitary and in the retina. The s1 MAb-resistant variants of T3D have attenuated neurovirulence and have impaired growth capacity in CNS tissue. After intracerebral inoculation, these viruses produce pathologic injury to only a subset of the regions damaged by wild-type T3D. These variants have single nonconservative amino acid substitutions within the globular head of the s1 protein (10,11) (amino acids 340 and 419) (see Fig. 2), suggesting that this region may play a critical role in CNS tropism and neuronal binding.

The importance of the s1 protein in neurotropism is also reflected in cultured neural cell lines. In primary explant cultures derived from fetal rat cortex, T3D, but not T1L, infects neurons. Both strains infect astrocytes. T1L, but not T3D, binds to the surface of isolated human and murine ciliated ependymal cells (175). The capacity of reoviruses to infect neural or ependymal cells is determined by the s1 protein (44). Infection of T3D in cultured neurons is inhibited by an antiidiotypic MAb containing a region of sequence homology to s1 (45), providing further evidence for the importance of s1 in neuronal cell binding.

Virus-Induced Cell Death

Reoviruses can induce apoptotic cell death in a variety of cultured cells *in vitro* (reviewed in 121). Apoptosis is characterized by specific changes in cell morphology, including nuclear chromatin condensation, margination, and fragmentation and shrinkage of cells with membrane blebbing (zeiosis). DNA isolated from apoptotic cells is cleaved at internucleosomal regions resulting in the appearance upon agarose gel electrophoresis of “ladders” of DNA fragments with sizes that are multiples of 180 base pairs. Reovirus strains differ in their capacity to induce apoptosis, with T3 strains typically inducing more apoptosis than T1 strains. Genetic studies using T1 × T3 reassortant viruses indicate that the *S1* and *M2* genes are key determinants of strain-specific differences in apoptosis (188,189). Apoptosis induction does not require viral replication and does not correlate with differences in the capacity of reovirus strains to replicate in infected cells (139,189). Apoptosis involves the activation of effector proteases, including calpains (38), and can be inhibited by

overexpression of *BcL-2* (139).

Reovirus-induced apoptosis also occurs *in vivo* (122) (Fig. 4). In the murine CNS, areas of virus-induced pathology, antigen istribution, and apoptotic cell death co-localize within the same brain regions. However, apoptotic cells may be either virus infected or merely in close proximity to infected cells, indicating that both direct and “bystander” mechanisms of apoptosis occur *in vivo.*

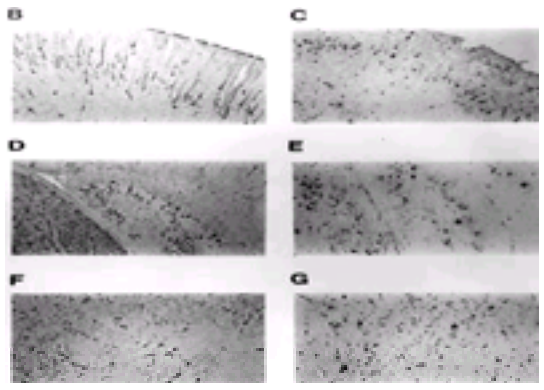

FIG. 4. Co-localization of reovirus T3D antigen detected by immunocytochemistry (*left panels*) and of apoptotic cells detected by TUNEL staining in the mouse brain after inoculation of T3D. Regions shown include cortex (*top row*), hippocampus with dentate gyrus (*center row*), and thalamus (*bottom row*). (From ref. 122, with permission.)

Reovirus-induced myocarditis is also associated with striking virus-induced destruction of cardiac myocytes in the absence of a significant inflammatory response (reviewed in 162). Cardiac myocyte injury is due to a direct cytopathogenic effect of virus and is not immune mediated. Genes encoding viral core proteins (164), notably M1 gene, are important determinants of the myocarditic potential of reoviruses. Cardiac injury *in vivo* correlates with cytopathicity in cardiac myocyte cultures *in vitro* (12) and in particular with the levels of viral RNA synthesis in infected cells (163). Myocarditic potential also inversely correlates with the amount of interferon-b (IFN-b) induced and the sensitivity of viral strains to its action (165).

Host Immune Response

Natural, as well as experimental, infections with reoviruses result in the induction of both humoral and cellular immune responses and have been used as models for the study of mucosal immunity in both the gut and respiratory tract (14,102,179,192,195). Type-specific and non–type-specific H_2-restricted cytotoxic T lymphocytes (CTLs) appear within the first week of intraperitoneal, peroral, or intranasal infection and can persist for at least several months (reviewed in 102,179,192, and 195). Reovirus-specific CTLs recognize epitopes on many reovirus proteins, including nonstructural proteins (70,71,98).

Peroral infection with reoviruses generates increases in virus-specific CD4+ and CD8+ intraepithelial lymphocyte populations. The cytokine profiles of reovirus-specific CD4+ helper T (T_H) cells indicate that T_H1 responses predominate over T_H2, with an associated increase in associated IFN-g (52). Similar results have been found in experimental reovirus serotype 2–induced murine diabetes (68). Virus-specific CD8+ CTLs generated within the intraepithelial lymphocyte population bear the a/b T-cell receptor (TCR) and are capable of major histocompatibility complex (MHC)-restricted lysis of virus-infected target cells (28). These CD8+ intraepithelial lymphocytes show disproportionate use of specific TCRs, including Vb-12, and -17, suggesting that infection results in a selective expansion of cells bearing TCR Vb (28).

The antibody response in mice depends on the route of viral inoculation and the H_2 haplotype of infected animals (100). Serum antibody titers are comparable after peroral and intradermal viral inoculation, and both routes of infection generate immunoglobulin G_{2a} (IgG2a) and IgG2b subclass antibodies (100). Antibodies generated in mice and rabbits recognize all the reovirus outer capsid proteins and the l core proteins (100). After peroral inoculation, mice develop an IgA antibody response in Peyer's patches as well as systemic IgG2a and IgG2b. Oral inoculation of neonatal mice with reovirus or microencapsulated virions induces IgA in the gastrointestinal tract (130), although this response is inhibited by transfer of maternal antireovirus antibodies through suckling (130).

In addition to inducing humoral and cellular immune responses, reovirus infection can also induce natural killer (NK) cell cytotoxicity (1,177) and the recruitment of neutrophils into infected tissues (112). Studies in SCID mice indicate that NK cell activity alone is insufficient to clear viral infection (177). In a model of reovirus-induced pneumonia, T3D induces a more prominent neutrophil response than T1L, and this difference is determined by the *S1* gene (112).

In an effort to understand better the roles of humoral and cellular immunity in reovirus pathogenesis, intestinal reovirus infection has been studied in mice deficient in all antigen-specific lymphocytes (SCID mice) (8,62,66,177) and in mice lacking CD8+ T cells (b_2-microglobulin–null mice) (8,101) or B cells (IgM transmembrane–null mice) (8). Although immunocompetent adult mice are not susceptible to reovirus infection, adult SCID mice orally inoculated with reovirus develop lethal infection (62,66). Passive transfer of reovirus-specific immune or normal lymphocytes from congenic mice protect SCID mice against infection (62). SCID mice fail to clear reovirus infection, indicating that antigen-specific lymphocytes are important for clearance of intestinal reovirus infection. Interestingly, the organ most prominently affected in reovirus-infected adult SCID mice is the liver (62,66). Viral virulence in SCID mice is associated with four reovirus gene segments (*S1, M1, L1, L2*) (66). Three of these genes (*S1, L1,* and *L2*) are significantly associated with viral titer in brain

and intestine, and three (*M1, L1, L2*) are associated with viral titer in liver or the presence of hepatic lesions (66) (Fig. 5).

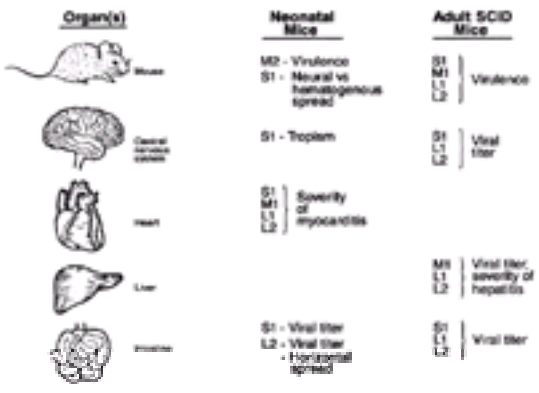

FIG. 5. Reovirus genes that determine virulence in different organs in immunocompetent neonatal and immunodeficient SCID mice. (From ref. 195, with permission.)

Mice lacking CD8 T cells clear infection normally (8,101), whereas mice lacking B cells and antibody have delayed clearance, indicating that both B cells and IgG play a role in intestinal clearance (8). Neonatal mice depleted of CD4+ T cells, CD8+ T cells, or both populations show enhanced pathology and higher viral titers in many organs (194).

The importance of immune cells in protection against reovirus infection has also been established in adoptive transfer studies. Transfer of reovirus-specific immune cells can protect neonatal mice against lethal infection with T3D and T3C9, and from neurologic disease produced by T1L (reviewed in 102,192). Protective cells can be derived from spleen after intraperitoneal immunization, or from Peyer's patches after peroral or intraduodenal immunization. Passive transfer experiments with reovirus-specific splenic lymphocytes indicate that both CD4+ and CD8+ immune cells are involved in protection and that removal of either population results in a significant decrement in the protective capacity of transferred cells. Intestinal lymphocytes mediating protection express CTL activity after secondary *in vitro* stimulation with reovirus-infected target cells (34). These cells are CD8+ and Thy-1+ and express TCRa/b (34).

Reovirus infection can result in up-regulation of class I and class II MHC expression in cultured cells *in vitro* and *in vivo.* For example, infection of human pancreatic b-cells or rat insulinoma RINm5F results in significantly increased class I, but not class II, MHC expression (22,23). Conversely, infection of rat FRTL-5 thyroid cells or murine M.5 thyroid epithelial cells does increase class II MHC expression (59,117).

Both polyclonal antisera and MAbs (neutralizing and nonneutralizing) directed against the T3 s1 protein can protect mice against lethal challenge with T3D and T3C9 as well as neurologic disease induced by T1L after peroral, intramuscular, or intracerebral viral challenge (reviewed in 102 and 192) (Fig. 6). *In vivo* protection can also be mediated by MAbs against each of the other outer capsid proteins, indicating that protective antibodies recognize multiple epitopes and that these are not confined to a single viral protein. *In vitro* properties of antibody, including isotype, complement-binding, capacity to neutralize infectivity or inhibit HA, do not directly correlate with their protective capacity *in vivo.* Antibodies that bind poorly are not protective, but among those that bind well, protection *in vivo* is not simply a function of relative binding avidity (183).

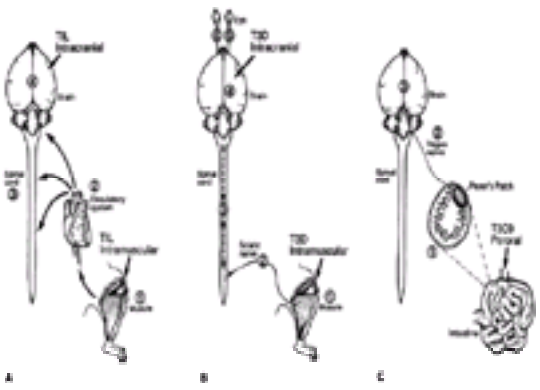

FIG. 6. Sites of antireovirus antibody action. For hematogenous spread after intramuscular or intracranial inoculation of T1L (**A**), antibody can inhibit primary replication in brain or muscle (*sites 1* and *4*) and the spread of virus through the bloodstream (*site 2*) to the spinal cord and brain (*sites 3* and *4*). For neural spread after intramuscular or intracranial inoculation of T3D (**B**), antibody can inhibit primary replication in muscle or brain (*sites 1* and *4*) and spread of virus through nerves to the spinal cord (*site 2*) and within the central nervous system (*sites 3* to *5*). For neural spread of virus after peroral inoculation of T3C9 (**C**), antibody can contribute to intestinal clearance of virus (*site 1*) and inhibit spread to (*site 2*) or growth within (*site 3*) the brain. (From ref. 195, with permission.)

Studies of the effects of protective MAbs on pathogenesis indicate that antibody-mediated protection may occur in the absence of a significant effect of antibody on primary replication of virus (183,191). However, antireovirus IgG can clearly play a role, independent of IgA, in the clearance of virus from mucosal primary sites of infection, such as the intestine (8). Protection against lethal T3D infection is associated with inhibition of neural spread of virus to the CNS and inhibition of growth and spread within the CNS (191). Protection against lethal peroral infection with T3C9 is associated with inhibition

of both viremic and neural spread of virus from intestinal tissue to the CNS and protection against T1L with inhibition of viremia (183,191) (see Fig. 6).

The effects of humoral and cellular immunity on reovirus infection appear complementary (194). Antibody is effective even when administered after CNS infection with reovirus has been established (190,196), whereas to be effective, passively transferred reovirus-specific immune cells must be given either before or within 24 to 28 hours of viral challenge. Antibody can protect against both peripheral and direct intracerebral challenge with virus, but immune cells are only protective against peripheral challenge with T3D, and fail to protect when virus is given intracerebrally.

In addition to humoral and cellular immune responses, cytokines and other mediators may play a role in modulating reovirus infection. Reoviruses can induce IFNs, tumor necrosis factor-a (TNF-a) and macrophage inflammatory protein-2 (MIP-2) (53,54,155). Induction of IFN depends on both the viral strain and the host cell and differs among mice of different strains (100). T3D, for example, is a better inducer of IFN in mouse L-cell fibroblasts than T1L. T3D also induces higher levels of chemokine mRNA expression for TNF-a and MIP-2 than T1L in pulmonary cells *in vitro* and within the lung after *in vivo* infection (53).

Reoviruses are susceptible to IFN-b, although strains differ strikingly in sensitivity, with T3D being much more sensitive than T1L (80,155). Differences in the levels of IFN-induced dsRNA-dependent protein kinase (PKR) may play a role in mediating effects of IFN-b on reovirus replication (155). The reovirus s3 protein inhibits the activation of PKR by preventing its interaction with dsRNA (13,15,204). Differences in IFN sensitivity of reovirus strains may depend in part on their s3 proteins (13,76). In cardiac myocyte cultures, reovirus induction of IFN-b is determined by viral core proteins and inversely correlates with the capacity of viruses to induce CPE *in vitro* and myocarditis *in vivo* (165). Depletion of IFN-b enhances the myocarditic potential of nonmyocarditic viral strains, suggesting a protective effect for IFN-b (165). These results contrast with studies of experimental reovirus serotype 2–induced murine diabetes, in which the severity of insulitis in mice correlates with increased expression of IFN-b and is ameliorated by administration of anti–IFN-b antibody (68), suggesting that IFN induction may play a pathogenetic rather than a protective role in this setting.

Under certain circumstances, reovirus infection may induce either autoimmunity or, conversely, a state of immunosuppression (67,124). Immunoregulatory abnormalities observed after reovirus infection may result, in part, from functional alterations in T-cell subpopulations (67,124). Infection of mice with T1L has been associated with the production of autoantibodies against a variety of host antigens, most prominently a variety of endocrine tissues and hormones (123). Suckling mice infected with T2J develop thymic atrophy with marked suppression of antibody production to both T-cell–dependent and T-cell–independent antigens. Transfer of splenocytes or thymocytes from normal uninfected mice restores the capacity of infected mice to produce antibody (124). Similar impairment of immune responses have been described after infection of suckling mice with T1L (124).

Pathology in Specific Organ Systems

A discussion of the pathology induced by reoviruses infection in different organ systems is beyond the scope of this chapter. Specific reviews detailing the effects of reovirus infection on the nervous system (180), heart (162), and gastrointestinal and hepatobiliary systems (125) are available.

CLINICAL FEATURES

Reoviruses remain as much orphan viruses today as when Sabin originally proposed their name. By early childhood, most individuals show serologic evidence of past reovirus infection. Despite this fact, it has been difficult to provide convincing evidence linking reoviruses to specific human diseases. Most reovirus infections must either be asymptomatic or blend imperceptibly with the minor respiratory and gastrointestinal illness of infancy and early childhood (77,82,95,167).

Some insight into the potential of reoviruses to produce human disease comes from studies of human volunteers. The most comprehensive of these studies involved intranasal inoculations of up to $10^{6.7}$ $TCID_{50}$ of reovirus into 27 adult (21 to 38 years old) male prisoners (142). Groups of nine volunteers were inoculated with either T1L, T2J, or T3D. After inoculation with T1L, virus was isolated from anal swab specimens of eight patients. The ninth patient had preexisting antireovirus antibodies, and virus was not isolated from his stool. Six of the eight (75%) initially seronegative patients seroconverted, with a significant increase in serotype-specific HAI antibody titers. Three of these individuals developed clinical illness, which began 24 to 48 hours after viral challenge and typically lasted for 4 to 7 days. The predominant symptoms included headache, pharyngitis, sneezing, rhinorrhea, cough, and malaise. A fourth individual had loose stools but was otherwise asymptomatic. In two other studies of human infection with T1L, a similarly high incidence of viral isolation and seroconversion was found. However, there was no clear association with clinical illness (78,79,86). None of the nine volunteers challenged with T2J developed clinical illness (142). T2J was isolated from throat or anal swab specimens in only two cases, although four individuals developed a serotype-specific HAI antibody response. Eight of the nine volunteers who received T3D had positive stool cultures (142). These eight individuals developed a serotype-specific rise in HAI antibody titer. One patient developed “mild rhinitis,” and the others remained symptom free.

In 1960, Rosen and colleagues (143) documented an outbreak of infection with T1 reoviruses among children in a welfare institution in Washington, DC. During a 9-week period, 22 of 73 children had at least one stool isolate of a T1 strain. Stool shedding lasted for at least 1 week in 54% of the children, and it lasted for at least 2 weeks in an additional 21%. In one case, virus shedding lasted for at least 5 weeks. All of the children with positive stool cultures developed a rise in serotype-specific HAI antibodies. In an attempt to define possible clinical correlates retrospectively, the record of patients with positive stool isolates were reviewed. During the time period of virus isolation, the children had rhinorrhea (81%), pharyngitis (56%), diarrhea (19%), and otitis media (19%). However, the frequency and nature of these complaints were not significantly different than those in the preisolation and postisolation periods.

There are similarities between the pathologic changes occurring in hepatobiliary disease produced in mice by reovirus

infection and those seen in human neonatal extrahepatic biliary atresia (EHBA) (reviewed in [187] and [202]). Serologic studies of infants with EHBA examining the prevalence of ant-reovirus antibodies or changes in antibody titer have yielded conflicting results, with some studies suggesting a possible relationship with reovirus infection ([7,63,110,137]) and others not ([19,20,51]). RT-PCR amplification of a region of the highly conserved L1 gene has been used to search for reovirus nucleic acid in fresh frozen liver and biliary tissues from infants with EHBA, choledochal cysts (CDC), and other hepatobiliary diseases ([187]). Reovirus RNA was detected in 78% (7 of 9) of tissue specimens from infants with CDC, in 55% (12 of 23) of those from infants with EHBA but in only 21% (7 of 33) of those with other liver diseases and in only 11% of autopsy controls without known hepatic or biliary disease. These differences were statistically significant and support an association between reovirus infection and both EHBA and CDC. Another study ([170]) using a different RT-PCR technique to amplify the *M3* gene on formalin-fixed tissue failed to detect reovirus RNA in 14 patients with EHBA or in 20 patients with neonatal hepatitis. The results may be in conflict owing to differences in RT-PCR methodology and the use of different tissue preparations (fresh frozen versus fixed).

Efforts have also been made to determine whether reovirus infection could be related to idiopathic cholestatic liver disease in adults, including primary biliary cirrhosis and primary sclerosing cholangitis ([107,108]), but no convincing data for an etiologic relationship exist.

Because one of the hallmarks of reovirus infection in rodents is CNS disease, it is perhaps not surprising that several case reports associating reoviruses with human CNS disease have appeared ([180]). One of the most convincing examples is a recently reported case of aseptic meningitis in a previously healthy 3-month-old infant ([83]). The child seroconverted, and a serotype 1 reovirus was isolated from cerebrospinal fluid (CSF) after inoculation onto green monkey kidney cells. There are similar but less well-documented cases of meningoencephalitis associated with both type 2 and type 3 reovirus ([92]). In some cases, virus was isolated only from stool and not CSF or neural tissue, and seroconversion was not documented ([116]), raising the possibility that reovirus infection was coincident rather than causal. In some cases with encephalitic as well as meningitic signs and symptoms, virus has been isolated from brain tissue ([85,92]).

It has been suggested that reoviruses may occasionally produce exanthemal illnesses in children ([96]). In one study, five children with maculopapular rashes and one with a vesicular rash were found to have seroconversion against T2. Two of these children also had virus isolated from throat or rectal swabs. In addition to rash, other symptoms included fever ([6]), malaise ([4]), anorexia ([4]), pharyngitis ([3]), cervical adenopathy ([2]), and diarrhea ([1]). The incidence of seroconversion and virus isolation was not significantly different than that in an asymptomatic age-matched population. Therefore, although it is clear that these children had reovirus infection, it remains in question whether it was responsible for the rash and other symptoms.

The list of other reported associations with reovirus infection ([182,195]) also includes keratoconjunctivitis and asymptomatic shedding of virus in urine.

In summary, most reovirus infections are asymptomatic or, at most, produce only minor upper respiratory or gastrointestinal symptoms that are indistinguishable from the upper respiratory infections and diarrheal illnesses that are regular features of infancy and early childhood. The potential association of T3 infection with extrahepatic biliary atresia must be considered possible rather than proven. Reoviruses may be an extremely rare cause of neurologic disease and exanthema, although there are a paucity of documented cases.

DIAGNOSIS

Diagnosis is based on isolation of virus from tissues or body fluids, detection of viral antigen in infected material, and serologic studies ([181]). Suitable specimens for virus isolation include stool, throat swabs, nasal washings, CSF, and urine. From a practical viewpoint, almost all isolates of virus have been from stool or, less commonly, respiratory secretions. For initial isolation from infected material, monkey kidney cells have generally been considered optimum. In addition to characteristic CPE, the presence of viral antigen can be confirmed using either immunofluorescence or immunocytochemistry ([181]). Immunocytochemical techniques can also be employed for the detection of reovirus antigens in tissue specimens ([181]).

An indirect immunofluorescence assay, using fixed infected cells as target antigen, has also been employed to detect antireovirus antibodies in human serum ([7,63,110]), although its sensitivity and specificity have never been established ([19]). Enzyme-linked immunosorbent assay (ELISA) techniques ([136,160]) have been widely used for detection of reovirus antibodies in serum and can be easily applied in most clinical laboratories. Results can be confirmed by plaque reduction and HAI assays, which are generally available only in research laboratories. The availability of nucleotide sequence information for a number of the reovirus genes provides the basis for the application of a variety of molecular techniques, including *in situ* and dot-blot hybridization and PCR for the detection of reovirus genomic and messenger RNA in infected tissues and cells. Reovirus RNA extracted from infected liver tissue has been detected by dot-blot hybridization using an *M2* gene–specific probe ([127]) and by RT-PCR using *L1, M2,* and *M3* gene–specific oligonucleotide primers ([127,170,187]).

Determination of the serotype of a viral isolate that has been identified as a reovirus has historically been based on the capacity of a panel of type-specific antisera to inhibit HA by the test isolate ([141]). Plaque-reduction NT assays can also be used to serotype unknown isolates, although they are generally not as reliable as HAI tests. The availability of type-specific neutralizing MAbs directed against the s1 protein of the prototypic T3D and T1L strains now allows serotyping based on plaque reduction assays or immunocytochemical reactions.

TREATMENT

Reoviruses are orphan viruses that have not been specifically shown to produce human diseases; hence, there has been limited investigation of antiviral therapy. Among the strategies that have been tested in experimental animals are the

administration of antiviral antibodies, virus-specific immune cells, and protease inhibitors (55). A number of vaccines have been developed for veterinary use. These typically contain either b-propiolactone or formalin-inactivated viruses and have been shown to induce high-titer antibody responses (178).

The activity of a number of agents, including ribivarin, acivicin, cicloxolone, and neplanocin A, has been tested in cell culture systems. Ribivarin has antiviral activity against reovirus *in vitro* (132) but has never been tested *in vivo.* The glutamine analog acivicin inhibits the growth of reovirus T3 in fibroblasts (88), although its mechanism of action has not been defined. Similarly, the monensin-like Golgi apparatus inhibitor cicloxolone sodium reduces reovirus particle yield and the proportion of infectious to noninfectious reovirus particles produced in infected cells (37). The *S*-adenosyl-L-homocysteine hydrolase inhibitor neoplanocin A and some of its derivatives have also been reported to have *in vitro* activity against reovirus (120).

EPIDEMIOLOGY

Epidemiologic surveys of the presence of antireovirus antibody in large populations of individuals in a variety of geographic and cultural settings have produced surprisingly uniform results. Most individuals have detectable antibodies against reoviruses of all three serotypes by the time they leave childhood (78,95,96,160). In an early study, the incidence of antireovirus antibodies rose from less than 25% in those younger than 1 year of age to more than 70% in those older than 3 years (96). A more recent ELISA study found that antibody prevalence increased steadily throughout life from about 35% of 6-month to 1-year old children, to 60% of 11- to 19-year-olds, to more than 85% in those older than 60 years of age (160).

Almost all reported or identified cases of reovirus infection are sporadic in nature, although one outbreak of serotype 1 infection in children has been described (143). Reovirus infections do not appear to have any striking seasonal predilection, although many of the original isolations of virus were from patients with summer diarrheal illnesses. The role, if any, played by animals as reservoirs for virus or as sources of human infection is unknown. Reoviruses of all three serotypes are also frequently isolated from water supplies and sewage effluents, presumably reflecting contamination of water with human and animal excreta. This could conceivably represent another source of virus for human infection.

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CHAPTER 54 Rotaviruses and Their Replication

Mary K. Estes

Rotaviruses were initially characterized as agents associated with gastroenteritis in animals. The history of the discovery of the rotaviruses exemplifies how studies of animal diseases can provide crucial insights into human viral disease (see Chapter 55). Numerous epidemiologic studies have shown that rotaviruses are the most significant cause of severe gastroenteritis in young children and animals (see Chapter 55). Rotaviruses cause diarrheal disease primarily in the young, but infection and disease in older children and adults can occur. Some outbreaks in adults are caused by non–group A rotaviruses. Rotaviruses have also been reported in association with syndromes other than diarrhea (e.g., encephalitis, exanthem subitum, otitis media, necrotizing enterocolitis, liver abscesses, intussusception). However, at this time, no convincing evidence proves rotaviruses cause any of these syndromes.

The clinical significance of the rotaviruses stimulated basic research on these agents. Studies since 1973 on the biochemistry, biology, and molecular and antigenic properties of the rotaviruses have resulted in a fairly detailed understanding of virus structure and the replication cycle. Discovery that the rotaviruses code for two glycoproteins that are targeted to the endoplasmic reticulum (ER) led to studies of these glycoproteins as general models of intracellular polypeptide processing. These observations have brought the rotaviruses into prominence as biologic tools for understanding ER functions as well as virus–host interactions in the gastrointestinal tract. Studies on the structure of rotavirus and rotavirus–antibody complexes using electron cryomicroscopy and three-dimensional image-processing techniques have highlighted the usefulness of these new methods to obtain significant biologic and structural information about large, complex viruses. Recognition that one of the six nonstructural proteins acts as a viral enterotoxin has widened our understanding of rotavirus pathogenesis. Recognition that five of the six nonstructural proteins bind nucleic acids has resulted in the realization that these proteins have unique properties, and understanding their properties is providing new fundamental information about RNA synthesis and expression. The clinical syndromes, epidemiology, techniques for viral cultivation, diagnosis of rotavirus infections, and vaccine status are discussed in Chapter 55.

VIRUS CLASSIFICATION

The rotaviruses compose a genus within the family *Reoviridae,* and rotaviruses share common morphologic and biochemical properties (Table 1). Salient features are that (a) mature virus particles are about 100 nm (1000 Å) in diameter and possess a triple-layered icosahedral protein capsid composed of an outer layer, an intermediate layer, and an inner core layer; (b) 60 spikes extend from the smooth surface of the outer shell; (c) particles contain an RNA-dependent RNA polymerase and other enzymes capable of producing capped RNA transcripts; (d) the virus genome contains 11 segments of double-stranded RNA (dsRNA); (e) the viruses are capable of genetic reassortment; (f) virus replication occurs in the cytoplasm of infected cells; (g) virus cultivation *in vitro* is facilitated by treatment with proteolytic enzymes, which enhances infectivity by cleavage of an outer capsid spike polypeptide; and (h) the viruses exhibit a unique morphogenic pathway (i.e., virus particles are formed by budding into the ER) and enveloped particles are evident transiently at this stage of morphogenesis. Mature particles are nonenveloped, and these virions are liberated from infected cells by cell lysis or by nonclassic vesicular transport in polarized epithelial cells.

Structure
65- to 75-nm Icosahedral particles
Triple-layered protein capsid
Nonenveloped (resistant to lipid solvents)
Capsid contains all enzymes for mRNA production
Genome
11 Segments of dsRNA
Purified RNA segments are not infectious
Each RNA segment codes for at least one protein
RNA segments from different viruses reassort at high frequency during dual infections of cells
Replication
Cultivation facilitated by proteases
Cytoplasmic replication
Inclusion body formation
Unique morphogenesis involves transient enveloped particles
Virus released by cell lysis or by nonclassic vesicular transport in polarized epithelial cells

TABLE 1. *General characteristics of rotaviruses*

Rotaviruses are classified serologically by a scheme that allows for the presence of multiple groups (serogroups) and for the existence of multiple serotypes within each group. A rotavirus group (or serogroup) includes viruses that share cross-reacting antigens detectable by a number of serologic tests, such as immunofluorescence, enzyme-linked immunosorbent assay (ELISA), and immunoelectron microscopy. Rotaviruses comprise seven distinct groups (A to G). Group A, B, and C rotaviruses are those currently found in both humans and animals, whereas viruses in groups D, E, F, and G have been found only in animals to date. Viruses within each group are capable of genetic reassortment, but reassortment does not occur among viruses in different groups (390). The group antigenic determinants, or common antigens, are found on most (if not all) of the structural proteins and probably on many of the nonstructural proteins as well. This has been documented by showing that monospecific antisera and some monoclonal antibodies (MAbs) specific for individual polypeptides cross-react with strains in addition to those to which they were made. However, cross-reactive epitopes on the inner capsid protein (VP6) are those usually detected by diagnostic ELISA.

Group A rotaviruses have clearly been established as causing significant diarrheal disease in the young. Group B rotaviruses have been associated with annual epidemics of severe diarrhea primarily in adults in China (338). Group C viruses have been sporadically reported in fecal specimens from children with diarrhea and in several outbreaks; their clinical significance remains unclear. Rapid diagnostic tests (ELISAs) and MAbs to detect non–group A rotaviruses have been established, and these are beginning to facilitate determining the clinical importance of these viruses (245,391). Very few non–group A rotavirus strains (a single group C porcine, group C human virus, and group B porcine virus) have been successfully cultivated (315,318,328,380). This inability to grow most non–group A viruses has hampered obtaining information on these viruses, although gene-coding assignments and sequence data have been obtained. Unless noted otherwise, this chapter focuses on information about the group A rotaviruses. Reviews on the non–group A rotaviruses and comparisons between the proteins of the group A and non–group A viruses have been published elsewhere (38,223,314).

Within each group, rotaviruses are classified into serotypes defined by reactivity of viruses in plaque reduction (or fluorescent foci reduction) neutralization assays using hyperimmune serum prepared in antibody-negative animals. Using such assays, 14 VP7 serotypes have been identified (Table 2; see Chapter 55), and strains of animal and human origin may fall within the same serotype (Table 3). Neutralization assays can measure reactivity of antibody with the two outer capsid neutralizing antigens (VP4 and VP7, see later). However, in most cases, the predominant reactivity measured is with the glycoprotein VP7. This may be because VP7 composes a greater percentage of the virion outer capsid (Table 4), or alternatively, with hyperimmunization, VP7 selectively induces highly specific antibodies. Identical classification of the same virus isolates using MAbs to VP7 unequivocally demonstrates that plaque-reduction neutralization assays with hyperimmune serum primarily measure reactivities with VP7 (29,82,353).

TABLE 2. *Group A rotavirus VP7 (G) serotypes*

TABLE 3. *Rotavirus VP4 genotypes [P] and serotypes (P)*

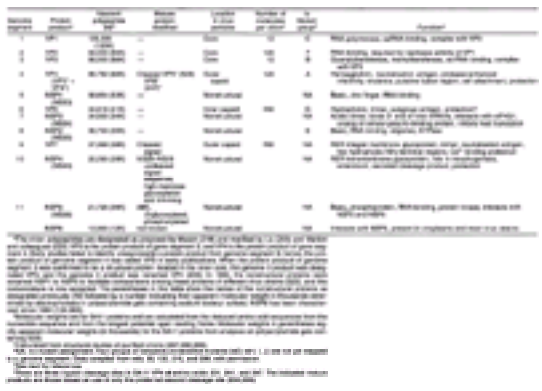

TABLE 4. *Rotavirus proteins*

In some cases, a rotavirus strain will not react clearly in reciprocal neutralization assays with hyperimmune antiserum. This is usually because the two viruses being compared possess distinct immunologic forms of VP4 (the second outer capsid protein), which is also a neutralization antigen (158). Because the genes that encode these separate neutralization antigens can segregate (reassort) independently, it is not surprising that some virus isolates can possess heterologous neutralization (VP4, VP7) antigens (158). Classification of rotaviruses is by a binary system (similar to that used for influenza viruses) in which distinct serotypes of VP4 and VP7 are recognized (141,160,304). However, a lack of readily available typing serum or MAbs to different VP4 types has hampered classification of VP4 (P) serotypes. Instead, properties of VP4 have been studied by sequence analysis, and current evidence suggests the existence of at least 20 different genotypes of VP4 (see Table 3). Genotypes of VP4 and VP7 are determined by sequence or hybridization analysis, whereas serotypes are determined by reactivity with polyclonal or monoclonal antisera (104). For VP7, a correlation between genotype and serotype has been established. Such a correlation is less clear for VP4, although sequence variation between amino acids 84 and 180 has been suggested to be useful to define P-type–specific epitopes (196).

Serotype designation thus reflects the expression of neutralization epitopes on both VP4 and VP7. Increasingly, epitope expression on these outer capsid proteins has been found to be dependent on the specific combinations of VP4 and VP7 in this complex capsid (57). Although the molecular basis of these interactions is not yet well understood, new structural information clearly documents the presence of specific interactions between VP4, VP6, and VP7 within this complex capsid. The serology of the epitopes in proteins that interact in the capsid is clearly complicated and likely will not be fully understood until high-resolution structural data on the capsid are available.

VIRIONS

Structure

The morphologic appearance of rotavirus particles is distinctive, and three types of particles can be observed by electron microscopy (Fig. 1 and Fig. 2). The complete particles resemble a wheel with short spokes and a well-defined smooth outer rim. The name *rotavirus* (from the Latin *rota,* meaning “wheel”) was suggested based on this morphology (119). The complete infectious particles historically were called double-shelled particles, but newer structural data show they are triple layered. Double-layered particles (previously called single-shelled particles) lacking the outer shell are described as *rough particles* because their periphery shows projecting trimeric subunits of the inner capsid. Single-layered core particles are seen infrequently; they usually lack genomic RNA and are aggregated.

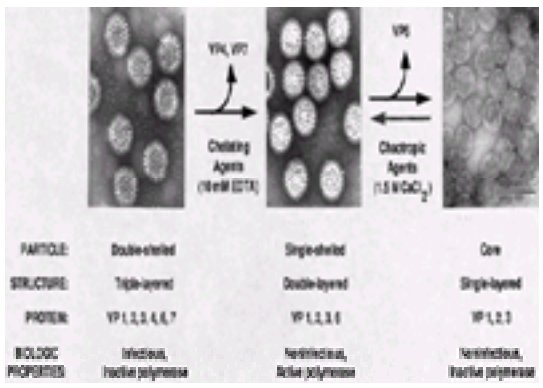

FIG. 1. Structural and biologic properties of rotavirus particles. Electron micrographs show typical triple-layered, double-layered, and single-layered core particles seen after staining with 1% ammonium molybdate. The double-layered and core particles can be produced by sequential degradation of infectious triple-layered particles as shown. The proteins and biologic properties of the particles are detailed in the text. Bar = 100 nM. (Modified from ref. 113, with permission.)

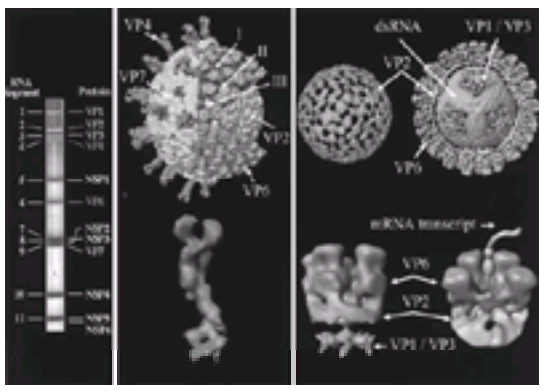

FIG. 2. Gene coding assignments and the three-dimensional structures of rotavirus particles. **Left:** A polyacrylamide gel shows the 11 segments of dsRNA that comprise the rotavirus genome (A:Si:SA11 strain in this case) and the proteins encoded by each of these genes. **Center:** A cutaway of the 23 Å three-dimensional structure of a complete rotavirus particle showing the outer, intermediate, and inner shells of the capsid structure. The proteins in the different shells and channels are indicated. The structure of a single spike, shown in the *bottom center panel,* illustrates the dimeric nature of the spike, which is most pronounced in the exterior portion. The globular shape of the interior portion may allow the VP4 spike to make multiple contacts with the VP6 trimers that surround the type II channels. **Right:** The three-dimensional structure of the single-layered particle at a threshold where the boundaries of the two molecules of VP2 that make the icosahedral asymmetric unit are shown. On the right, a cutaway from the 19 Å structure of a double-layered particle in which the VP6 and VP2 layers have been partially removed, shows the mass densities due to the genomic double-stranded RNA and the enzymatic complexes composed of VP1 and VP3. The *left portion of the bottom figure* illustrates a side view of the enzymatic complex found at the fivefold positions in the particles beneath the VP2 and VP6 layers; the *right portion* shows a proposed pathway for the nascent mRNA molecule exiting through a type I channel. These structures were determined by image processing of electron micrographs of particles embedded in vitreous ice (287,288,289,290 and 291). (Modified from ref. 79, with permission; also including an unpublished structure of the VP4 spike kindly prepared by B.V.V. Prasad.)

Three-dimensional reconstructions using cryoelectron microscopy and image processing techniques to study triple- and double-layered rotavirus particles embedded in vitreus ice have provided the most detailed description of particle structure (287,289,324,388) (see Fig. 2). These studies unequivocally establish that particles possess icosahedral symmetry with a T=13*l* (levo) icosahedral surface lattice for the two outer layers. A distinctive feature of the virus structure is the presence of 132 large channels spanning both shells and linking the outer surface with the inner core.

Three types of channels can be distinguished based on their position and size (see Fig. 2). Twelve type I channels run down the icosahedral fivefold axes, 60 type II channels are those on the six-coordinated positions surrounding the fivefold axes, and 60 type III channels are those on the six-coordinated positions around the icosahedral threefold axes. Type III channels are about 140 Å in depth and about 55 Å wide at the outer surface of the virus. On entering the particle, these channels constrict before widening to their maximum width, which is close to the surface of the inner shell. Similar features and dimensions are seen in the other two types of channels, except that type I channels have a narrower (about 40 Å) opening at the outer surface of the virus. These channels are involved in importing the metabolites required for RNA transcription and exporting the nascent RNA transcripts for subsequent viral replication processes. The channels at the fivefold axes are conduits for the export of messenger RNA (mRNA) that first interacts with the enzyme complexes composed of VP1 and VP3 that are present at the inner surface of the fivefold axes of the single-layered particle (198,199,291) (see Fig. 2). The single-layered particle possesses a T=1 symmetry and is composed of 120 molecules of VP2 arranged as 60 dimers that surround the genomic dsRNA that is highly ordered, with about 25% of the genome making up an internal dodecahedral structure (291) (see Fig. 2). This organization with two molecules in the icosahedral asymmetric unit has also been referred to as a T=2 structure (145). All segmented dsRNA viruses contain 120 molecules of a core protein (VP3 for bluetongue virus, lambda 1 for reoviruses, the single capsid protein for cytovirus) that surrounds an ordered genome (145,154,300,399). Although the inner shell protein shares similar features among these dsRNA viruses, rotaviruses and orbiviruses are distinguished by housing their enzymatic functions entirely *within* the inner shell, leading them to be called *nonturreted viruses,* and nascent mRNA transcripts are released through channels penetrating the two capsid layers at the icosahedral vertices (154,200). This capsid architecture contrasts with the structure of the turreted reoviruses, aquareovirus and cypovirus, where the polymerase enzyme is housed within the core, but the capping enzymes are incorporated as pentameric turret-like projections that extend through the inner capsid layer at each icosahedral vertex (154).

The VP2 layer is surrounded by 780 molecules of VP6 arranged in trimers and positioned at all the local and strict threefold axes of the T=13 icosahedral lattice. The addition of the VP6 layer to virus-like particles formed from VP2 imparts greater morphologic homogeneity and long-term stability to the particles (396), suggesting that VP6 may play a major role in providing structural integrity to the rotavirus capsid. The double-layered particle is about 705 Å in diameter, and the structure of the VP6 shell is very similar to the VP7 shell of bluetongue virus; both these proteins consist of two distinct domains: a distal eight-stranded b-barrel domain and a lower a-helical domain (292,301).

Studies of virus particles revealed structural features not seen previously (287). Sixty spikes extend from the smooth surface of the outer shell (see Fig. 2). These protein spikes are situated at an edge of the type II channels surrounding the fivefold icosahedral axes and are composed of the hemagglutinin (VP4). This was first demonstrated by showing that 2 Fab subunits of a monoclonal antibody that recognizes VP4 bind on the sides near the tips of the spikes (288). Higher-resolution reconstructions of native particles, of a reassortant lacking spikes, and of spikeless particles (formed by removing VP4 by treatment at high pH) confirmed that VP4 is the spike and showed that the spike is multidomained with a radial length of about 200 Å, with about 120 Å projecting from the surface of the virus (324,388). The head of the spike is bilobed and attached to a square-shaped body formed by two rods. The anchoring base displays a pseudo-sixfold symmetry, and this may represent a novel folding motif in which the single polypeptide of VP4 contributes

similar but nonequivalent domains to form the arms of the hexameric base (324,388).

In the VP4 spike, no twofold axis of symmetry is strikingly evident. The location of the spike does not correspond to any local or strict twofold axis of the icosahedron, and thus, the dimeric configuration is apparently asymmetric. This is consistent with the observation that the two Fab fragments of a VP4 MAb bind to the sides near the tip of the spike at different angles, indicating that the antigenic sites are not in structurally identical environments (288). It is unclear whether this asymmetry in the spike serves a specific function. The VP4 spike extends inward about 80 Å into the outer virion surface and interacts with two molecules of VP7, but inside, it appears that the large globular domain of VP4 interacts with all six of the VP6 molecules surrounding the type II channel. However, there may be more specific interactions between the VP4 dimer and two of the six VP6 molecules. The extensive VP4–VP7 and VP4–VP6 interactions imply that VP4 may participate in maintaining the precise geometric register between the inner and outer capsids as well as affect functional domains. The VP7 outer layer consists of trimers, or 780 molecules, of this protein. VP7 interacts with the tips of the VP6 trimers.

The existence of each shell and the ability of VP7 and VP4 to interact directly with VP6 have been demonstrated by the production of virus-like particles composed of VP2, VP2/6, VP2/4/6, VP2/6/7 by expression of the respective proteins (84,194) or coassembly of purified proteins of each layer onto preformed VP2 particles (62,63,398). These results show that the structural proteins have the intrinsic properties of self-assembly and suggest high-affinity interactions among the proteins.

Although VP4 appears to be a dimer, its elongated shape with multiple domains resembles the hemagglutinin of influenza virus, which is a trimer and has distinct rodlike and globular domains (see Chapter 46). The rodlike portion of the influenza hemagglutinin forms a triple-stranded coiled-coil of a-helices. VP4 is predicted to contain extended a-helical heptad repeats typical of coiled-coils (193,223), and higher-resolution analyses will be required to discern whether the rodlike domains of VP4 are folded as a-helices. A similar coiled-coiled region is also present in the homotrimeric reovirus 1 hemagglutinin, although this protein is much longer and structurally distinct (94) (see Chapter 52). Coiled-coils have been found to participate in both intracellular vesicle and viral membrane fusion activity (330) and are found in the fusion proteins of a variety of viruses, including retroviruses, coronaviruses, paramyxoviruses, filoviruses, and influenza viruses. Although rotavirus is not an enveloped virus, similarities exist between rotaviruses and viruses whose surface glycoproteins mediate fusion. For example, the spike proteins of all these viruses (a) are involved in binding to the cellular receptor, (b) are synthesized as precursors and require a proteolytic cleavage for their function, (c) contain a fusion peptide region that is part of a polar hydrophobic sequence, and (d) contain one or more heptad repeats. It remains to be determined whether the a helical heptad repeat or putative fusion domain in the rotavirus spike protein are important for virus entry or during viral morphogenesis in cells, a process that involves a budding of particles through the membrane of the ER (see later).

Physicochemical Properties

The different rotavirus particle types possess distinct biophysical and biologic properties (see Fig. 1). Infectivity depends on the presence of the outer capsid. Calcium is required for particle stability apparently by stabilizing the outer capsid glycoprotein VP7, and the crucial concentration of calcium needed to stabilize the outer capsid is dependent on the virus strain (124,311). Treatment of particles with calcium-chelating agents (e.g., EDTA and EGTA) removes the outer capsid and results in a loss of infectivity (40,110). Calcium has been detected in triple-layered but not double-layered particles, and this may represent binding to potential calcium binding sites present in the protein sequence of VP7 (16,92,323). Single-layered particles can be produced by disruption of double-layered particles with chaotropic agents such as sodium thiocyanate or high concentrations of calcium chloride (7,77).

Triple- and doubled-layered particles can be separated by centrifugation in gradients of cesium chloride (CsCl) or sucrose, where they possess distinct densities and sedimentation values. Triple-layered particles have a density of 1.36 g/cm^3 in CsCl and sediment at 520S to 530S in sucrose, whereas double-layered particles have a density of 1.38 g/cm ^{3} and sediment at 380S to 400S (346). Single-layered core particles have a density of 1.44 g/cm ^{3} in CsCl and a sedimentation coefficient of 280S (28). The three types of particles can also be separated in agarose gels (125).

Rotavirus infectivity and particle integrity are generally resistant to fluorocarbon extraction and exposure to ether, chloroform, or deoxycholate (110,117,346,378), reflecting the absence of an envelope on mature particles. Chloroform treatment reduces infectivity slightly and destroys hemagglutinating activity (31,110). Sodium dodecyl sulfate (0.1%) inactivates infectivity, but exposure to nonionic detergents can enhance infectivity, presumably by disrupting aggregates (372).

Rotavirus infectivity is relatively stable to inactivation, and it survives well on porous (paper, cotton cloth) and nonporous (aluminum, china, latex) environmental surfaces (1). Infectivity is stable within the pH range of 3.0 to 9.0 (110,215,259,377,378), and bovine and human samples have retained infectivity for months at 4°C or even 20°C, when stabilized by 1.5 mM $CaCl_2$ (329). The infectivity of bovine and simian rotaviruses is relatively stable even at 45° to 50°C, but hemagglutinating activity is lost rapidly at 45°C. Infectivity and hemagglutinating activity are destroyed by repeated freezing and thawing (23,31,110), and the hemagglutinating spikes are removed by treatment at high pH (11).

Virus infectivity can be inactivated by disinfectants such as phenols, formalin, chlorine, and b-propiolactone (320,367). Ethanol (95%), perhaps the most effective disinfectant, exerts its effect by removing the outer capsid (23,31,37,192,347).

In general, studies of the structure and inactivation of rotaviruses have yielded similar results whether the viruses studied were of animal (bovine or simian) or human origin. However, human rotavirus strains are distinguishable by being more difficult to cultivate, and their hemagglutinin, if present, is more difficult to detect (186). In addition, human strains seem to lose their outer capsid more easily than other strains. For example, purification of triple-layered human rotavirus particles is generally difficult in gradients of CsCl because of loss of the outer capsid; instead, metrizamide gradients may be required for purification of these viruses (352). Analysis of the determinants of density and stability by characterization

of reassortants has shown that a reassortant may have an intermediate density compared with two parental viruses, and physical interactions among the structural proteins appear to be responsible for heterogeneity in particle density (61). In addition, the stability of infectivity during fluorocarbon extraction, CsCl gradient purification, and subsequent storage at 4°C can be dependent on the type of genome segment 4 (VP4) in particles (61).

Genome Structure

The viral genome of 11 segments of dsRNA is contained within the virus core capsid. Deproteinized rotavirus dsRNAs are not infectious, reflecting the fact that virus particles contain their own RNA-dependent RNA polymerase to transcribe the individual RNA segments into active mRNAs. Hydrodynamic studies of the flexibility or “stiffness” of isolated rotavirus RNA segments in solution indicate that packaging of these RNA segments into the rotavirus capsid requires intimate protein–RNA interactions (177). The proteins directly responsible for segment packaging remain unknown; the structural proteins present in core particles (VP1, VP2, and VP3) are obvious candidates, but nonstructural proteins may also play a role. The genome RNA is highly ordered within the particle, with about 25% of the genome making up a dodecahedral structure, and VP2 interacts with the RNA. Several points of contact between the inwardly protruding portion of VP2 interact with the RNA surrounding each fivefold axis (195,291) (see Fig. 2).

The nucleotide sequence of all of the 11 rotavirus RNA segments for several rotavirus strains (SA11, human KU, bovine Rf) is now known. The prototype simian SA11 strain was the first genome completely sequenced. The sequences from different rotavirus strains show general features (Fig. 3) around the structure of each of the genome segments. Each positive-sense RNA segment starts with a 5'-guanidine followed by a set of conserved sequences that are part of the 5'-noncoding sequences. An open reading frame (ORF) coding for the protein product and ending with the stop codon follows, and then another set of noncoding sequences, which contains a subset of conserved terminal 3' sequences and which ends with two 3'-terminal cytidines, is found. Almost all mRNAs end with the consensus sequence 5'-UGUGACC-3', and these sequences contain important signals for gene expression and genome replication. The lengths of the 3'- and 5'-noncoding sequences vary for different genes, and no polyadenylation signal is found at the 3' end of the genes. All of the sequenced genes possess at least one long ORF after the first initiation codon. This is usually a strong initiation codon based on Kozak's rules (191). Although some of the genes possess additional in-phase (genes 7, 9, and 10) or out-of-phase (gene 11) ORFs, current evidence indicates that all the genes are monocistronic, except gene 11 (224). The last 4 nucleotides of the mRNAs can function as translation enhancers (66).

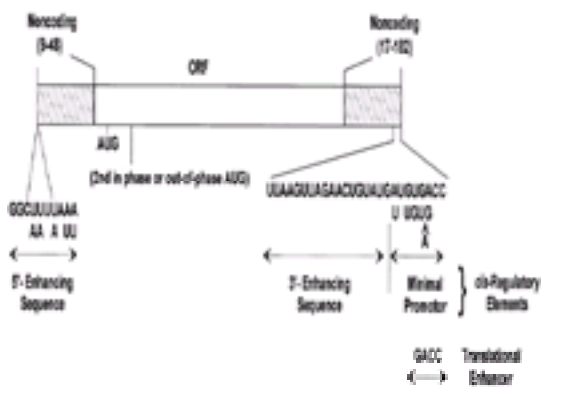

FIG. 3. Major features of rotavirus gene structure. Schematic shows the overall structure of rotavirus genes from the published sequences of genes 1 to 11. All eleven rotavirus genes lack a polyadenylation signal, they are A + U rich, and they contain conserved consensus sequences at their 5' and 3' ends. Variations in the conserved ends are also shown. The prototype SA11 genome segments range in size from 667 (segment 11) to 3302 (segment 1), with a total 18,556 base pairs. The *bottom arrows* show cis-regulatory elements of rotavirus mRNA required for replication of transcripts assayed in a cell-free replication system (268,381). The 5'- and 3'-noncoding regions at the termini of the mRNA are predicted to interact and stably base-pair to form a panhandle structure (265,268), and interactions between the 38-terminus with the nonstructural protein NSP3 may promote translation of the viral mRNA (368). The penultimate 5'-GACC-3' is a translation enhancer (66).

The rotavirus gene sequences are A + U rich (58% to 67%), and this bias against CGN and NCC codons is shared with many eukaryotic and viral genes. The dsRNA segments are base-paired end to end, and the positive-sense strand contains a 5' cap sequence $m^7GpppG^{(m)}GPy$ (165,227). Similar features of the RNA termini (capped structures and 5'- and 3'-conserved sequences) are found in the primary structures of the genome segments of other viruses (e.g., reovirus, cytoplasmic polyhedrosis virus, orbivirus) in the *Reoviridae* and in other virus families with segmented genomes (*Orthomyxoviridae, Arenaviridae,* and *Bunyaviridae*). One of the most intriguing aspects of rotaviruses and all segmented dsRNA viruses relates to how these viruses coordinately replicate and package the 11 viral mRNAs. The 11 mRNAs must share common cis-acting signals because they are all replicated by the same polymerase, and these signals are likely to be formed by secondary structures rather than by primary sequences. In addition, each mRNA must also contain a signal that is unique to it alone because the 11 mRNAs must be distinguished from one another during packaging. Generally, the conserved terminal sequences in genome segments contain cis-acting signals important for transcription, RNA translation, RNA transport, replication, assembly, or encapsidation of the viral genome segments. Some of the cis-acting signals for rotavirus RNA replication and translation have been identified (see Fig. 3), but assembly or encapsidation signals remain unknown (266).

Rotaviruses are the only known agents of mammals or birds that contain 11 segments of dsRNA. In most cases, the electrophoretic pattern of the genome of the group A viruses is composed of four high-molecular-weight dsRNA segments (1 to 4), two middle-sized segments (5 and 6), a distinctive triplet of segments (7 to 9), and two smaller segments (10 and 11). When this basic pattern is not seen, the rotavirus being analyzed may be either a group A avian virus, a non–group

A virus, a group A virus that contains rearrangements within individual genome segments (Fig. 4), or a new unique group A virus. Analysis of genomic electropherotypes is a relatively easy, rapid, and popular technique for virus detection and for molecular epidemiology studies to monitor virus outbreaks and transmission. However, because distinct RNA patterns can arise by different mechanisms (shift, drift, rearrangements), these profiles cannot be used as the sole criterion for classification of a virus strain (54,107).

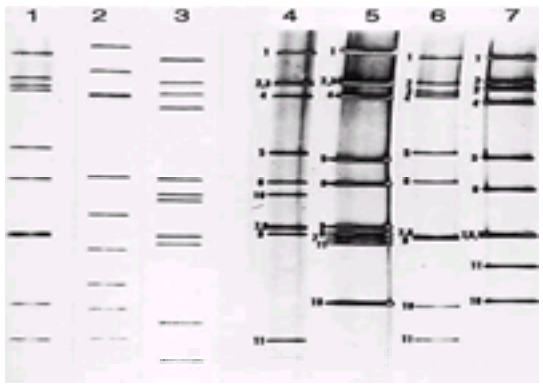

FIG. 4. Electropherogram of rotavirus RNA segments. The RNA segments were separated by electrophoresis in a 10% polyacrylamide gel and visualized by staining with silver nitrate. The RNA patterns of a group A rotavirus (SA11, *lane 1*), a group B rotavirus (adult diarrhea rotavirus isolate from China, *lane 2*), and a group C rotavirus (porcine pararotavirus, *lane 3*) are shown. The rearranged RNA patterns of three group A rabbit rotavirus strains (C11, *lane 4*; Ala, *lane 5*; and R2, *lane 7*) and their cognate segments compared with those of SA11 (*lane 6*) are also shown. The cognate genes were identified by hybridization with cDNAs for each SA11 RNA segment (348).

Nucleic acid hybridization combined with Northern blot is a second assay used to classify viruses based on the relatedness of genome segments (113). This method has been found to be useful to classify genetically related viruses into different genogroups (244) and to identify the origin of specific RNA segments in virus reassortants. This method has also been useful to characterize viruses possibly involved in cross-species transmission (242,243).

In viruses with genome rearrangements, typical RNA segments are missing or are decreased in concentration in an electrophoretic profile, and these are replaced by additional, more slowly (or rarely more rapidly) migrating bands of dsRNA (see Fig. 4). The slowly migrating bands represent concatemeric forms of dsRNA containing sequences specific for the missing RNA segments (reviewed in 89). The more rapidly migrating bands appear to represent deletions. Viruses with genome rearrangements of this type have been isolated most frequently from immunodeficient, chronically infected children (164,269), asymptomatically infected immunocompetent children (27,219), and animals [calves (279), pigs (26), or rabbits (348,358)]. They have also been obtained *in vitro* after serial passage of a tissue culture–adapted bovine rotavirus at high multiplicity of infection (163). Virus isolates with rearrangements in segments 5, 6, 8, 10, and 11 have been characterized, with the greatest number having rearrangements detected in segments 5 and 11. Viruses with a rearranged segment 11 have some selective advantage (better growth or stability) (221), so they are detected more easily, rather than occurring more frequently.

Viruses isolated and characterized that contain rearranged genome segments are generally not defective, and the rearranged segments can reassort and replace normal RNA segments structurally and functionally (6,30,140). Biophysical characterization of such particles has shown that up to 1,800 additional base pairs can be packaged in particles without causing detectable changes in particle diameter or apparent sedimentation values. However, the density of particles containing rearranged genomes may be increased, and the increase in density is directly proportional to the number of additionally packaged base pairs (228). These results indicate that rotaviruses have considerable capacity to package additional genomic RNA, although the upper limit is unknown. Whereas a total of 11 RNA segments are invariantly packaged, there appears to be much less constraint on the length of individual RNA segments assembled into the maturing virus particle. Because the amount of viral RNA present in virions can vary by as much as 1,800 nucleotides, a headfull mechanism for RNA packaging as seen for the dsRNA phage phi 6 (235) may not operate for rotaviruses.

In most cases, the profiles of virus-encoded proteins in cells infected with rotaviruses with rearranged genomes are similar to those seen in cells infected with standard rotavirus strains (6,30,220,230,279,321,348). This indicates that the rearrangement of the sequences in a segment apparently has left the normal reading frames and their expression unaltered. Sequence analyses of rearranged genome segments have confirmed this and revealed mechanisms by which the rearrangements arise.

In most cases, the rearrangements result from a head-to-tail duplication that occurs immediately downstream from the normal ORF, and hence the rearranged segment retains its capacity to express its normal protein product. For one rearranged virus, the protein profile was distinct from that seen with standard virus. In this case, the gene rearrangement was in genome segment 5, and no protein of normal or larger size was detected in infected cells (163). Further characterization of this rearranged segment showed that because of the introduction of a point mutation in the ORF, a truncated protein lacking the C-terminal half of the protein is made. Because this rearranged virus is nondefective, the C-terminal half of this protein is nonessential for rotavirus replication, at least in cell culture (162). Another mutant lacking the cysteine-rich zinc finger motif in the genome segment 5 protein, NSP1, is also viable (350). Genome rearrangements (concatemerization and deletion) are thought to be a third mechanism of evolution (in addition to antigenic shift and drift) of rotaviruses (89,361). Studies of rearranged segments are helpful to identify sequences critical for replication and RNA packaging (89).

CODING ASSIGNMENTS AND ROTAVIRUS PROTEINS

The coding assignments and many properties of the proteins encoded in each of the 11 genome segments are now well established (see Table 4 and Fig. 2), although new protein functions continue to be identified. Protein assignments have been determined by *in vitro* translation using mRNA or denatured dsRNA and by analyses of reassortant viruses. The information on SA11 was completed first, and SA11 became the prototype virus. Comparative studies of other rotavirus strains have shown that the absolute order of migration of cognate genes may differ among virus strains. Therefore, identification of cognate genes must be based on hybridization with gene-specific probes, on reassortant analysis, or on biochemical or immunologic identification of the protein synthesized in a cell-free translation system programmed with mRNA specific to the gene. The ability to obtain sequence information directly from dsRNA or single-stranded RNA (ssRNA) and the accumulating nucleic acid sequence databases make it possible to identify cognate genes based solely on sequence homology.

The rotavirus genome segments code for structural proteins found in virus particles and nonstructural proteins found in infected cells but not present in mature particles (Fig. 5; see Table 4 and Fig. 2). Six of the genome segments code for structural proteins found in virus particles. Another six proteins are nonstructural, although a few virus strains may lack NSP6. Early studies often presented conflicting conclusions concerning the numbers and locations of the rotavirus proteins. Many of these conflicts were resolved, as reviewed elsewhere (25,112,155,223,231), when it was recognized that posttranslational modifications (glycosylation, trimming of carbohydrate residues, and proteolytic cleavages) occur after polypeptide synthesis. In addition, strain variations (such as the presence of more than one glycosylation site on VP7 in some bovine, equine, porcine, and human rotavirus strains) provide explanations for other differences in polypeptide patterns.

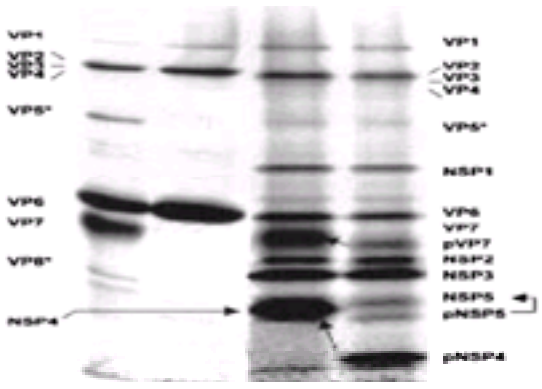

FIG. 5. Rotavirus polypeptides in purified virus particles and in infected cells. ^{35}S-methionine–labeled polypeptides in purified triple-layered virus (*lane 1*), double-layered virus (*lane 2*), or monkey kidney cells infected with SA11 and labeled for 1 hour, 6 hours after infection (*lanes 3 and 4*), were separated on a 12% polyacrylamide gel. The polypeptides were visualized by fluorography. The glycoprotein precursors to VP7 (pVP7) and to NSP4 (pNSP4) are seen only when N-linked glycosylation is blocked by treatment of the cells with the inhibitor tunicamycin (*lane 4*). The structural polypeptides are labeled on the *left* and the nonstructural polypeptides are labeled on the *right.* The two NSP5 bands (an unmodified and a modified form) are only seen clearly in cells treated with tunicamycin (*lane 4*). The VP2 and VP3 polypeptides were not resolved in this gel. To resolve VP2 and VP3, the polypeptides in purified virus must be separated on gels with a different cross-linking ratio than the standard used in the gels to analyze the infected-cell polypeptides (203). The triple-layered virus was grown in the presence of protease, so that uncleaved VP4 is not detected, but the VP4 cleavage products VP5* (60K) and VP8* (28K) are seen (*lane 1*). NSP6 was run off this gel and thus is not detected.

The nomenclature of the viral proteins (as originally proposed for SA11 proteins) designates structural proteins as viral protein (VP) followed by a number, with VP1 being the highest-molecular-weight protein, and proteins generated by cleavage of a larger precursor being indicated by an asterisk [VP4 is cleaved to produce VP5* and VP8* (103,108)]. Initial studies referred to the nonstructural proteins as NS followed by a number indicating the protein's molecular weight. This nomenclature has been replaced by NSP1 to NSP6 to facilitate comparisons among cognate nonstructural proteins of different molecular weights (223) (see Table 4). In fact, much of the literature before 1988 on “VP3” refers to the genome segment 4 product; before 1994, NS53, NS35 referred to NSP1, NSP2, and so forth (see Table 4). The new nomenclature is used throughout this chapter.

The Core Proteins

The proteins of core particles (VP1, VP2, VP3) function as structural proteins and each have affinity for ssRNA, but only VP1 shows sequence-specific recognition of viral RNA. Each of the core proteins plays a role in the processes of RNA transcription and replication. Viral transcription is easily studied *in vitro* by assays using double-layered particles (75), and replicase activity is assessed by studying negative-stranded RNA synthesis produced *in vitro* by single-layered particles that can function to replicate exogenously added mRNA (64,268). Several findings indicate that VP1 is the viral RNA polymerase and functions as both the viral transcriptase and replicase: (a) all VP1 sequences share the four common motifs conserved in the sequences of all RNA-dependent RNA polymerases (76,223); (b) VP1 binds nucleotides and cross-linking of the photoreactable nucleotide azido–adenosine triphosphate (ATP) to VP1 inhibits transcription (366); (c) VP1 specifically binds to the 3' end of viral mRNA (261); (d) temperature-sensitive (ts) mutants of VP1 do not synthesize ssRNA at the nonpermissive temperature (58); (e) VP1 is a component of baculovirus-expressed particles and replication intermediates that have replicase activity (264,396). Although VP1 is the candidate RNA polymerase, reconstitution experiments have shown that VP1 requires VP2 for replicase activity (264).

VP2 binds RNA nonspecifically (36,195) and interacts with the dsRNA lining the VP2 shell (291). However, ts mutants mapping to VP2 do not synthesize dsRNA (216). VP1 and VP2 are both required for replicase activity (264,396), and the essential role of VP2 in replication is linked to its ability to bind mRNA (264).

Of the core proteins, the least is known about VP3. Studies with virus-like particles have shown that VP3 can bind to the N-terminus of VP2, a region of the innershell protein to which VP1 and ssRNA also bind that is required for RNA replication (195,397). VP3 covalently binds a-^{32}p-guanosine triphosphate (GTP), indicating it is a guanylyltransferase (202,278), and VP3 is a methyltransferase (59), indicating that VP3 is a multifunctional capping enzyme. It is expected that one of the rotavirus core proteins should possess helicase activity needed for the transcription process, but to date no rotavirus core protein has been associated with this activity.

The Nonstructural Proteins

The rotavirus genome codes for six nonstructural proteins (NSP1, NSP2, NSP3, NSP4, NSP5, NSP6), which are variously involved in replication (NSP1 to 3, 5, 6) and morphogenesis (NSP4). All these nonstructural proteins, except NSP4, interact with nucleic acid. The effects and necessity of the nonstructural proteins for viral replication remain to be studied in more detail. However, the predictions or observations that most of the nonstructural proteins possess a basic charge [see Table 4], their demonstrated ability to bind RNA (36,161,178,222,280), their presence in subviral particles with replicase activity (260), and the dsRNA-negative phenotype of ts mutants that map to their genome segments (58,130) suggest these proteins function as part of replication complexes, as chaperones to transport RNAs or proteins to the sites of RNA replication, translation, assembly, or to gather the genome segments for packaging. A better understanding of the functions of the nonstructural proteins in the replication cycle is being obtained as these proteins are expressed and studied using recombinant DNA methods, often in conjunction with confocal microscopy.

NSP1 appears to be nonessential for virus replication, at least in cultured cells, and some studies have implicated this gene as a virulence factor in mice (41), but not in piglets or rabbits (39,70). NSP1 is the least conserved of the viral proteins, showing even more sequence variation than VP4 and VP7. It contains a relatively conserved zinc finger domain, but this domain is missing in some virus strains, showing it is not essential for virus replication and genome segment reassortment (257,364). NSP2 is an oligomeric NTPase that is localized to viroplasms, possesses helix destablizing activity, and may be involved in RNA encapsidation and virulence (53,354). NSP3 binds to the 3' end of viral mRNAs and represents a prototype of a new kind of sequence-specific RNA-binding protein that requires only four nucleotides for RNA recognition (281). NSP3 functions similarily to the cellular poly(A)-binding protein and binds eIF4G and inhibits cellular protein synthesis by this novel mechanism (Fig. 6) (276,277,280). NSP3 also enhances the translation of viral mRNA (368). NSP5 is an *O*-glycosylated phosphoprotein that self-assembles into dimers (132). NSP5 has autokinase activity and exists in several phosphorylated isomers in infected cells (4,32). When coexpressed in uninfected cells, NSP2 induces the hyperphosphorylation of NSP5 (3). The mechanism by which NSP5 hyperphosphorylation occurs are not known, but may involve a cascade of events initiated by the NTPase activity by which NSP2; NSP5 has been shown to interact with NSP2 (3,134,282,379). NSP6 is encoded in an alternative ORF on gene segment 11 of most rotaviruses and is known to interact with NSP5 and accumulate in viroplasms (224,363). NSP1 and NSP3 show a diffuse cytoplasmic staining in cells, and both fractionate with cellular cytoskeletal elements rather than concentrating in viroplasms (12,162,222). NSP2, NSP5, and NSP6 are associated with viroplasms and coexpression of NSP2 and NSP5 (probably NSP6 too, from the construct that made NSP5—although this was not evaluated) is sufficient for the formation of viroplasms (114). NSP2, NSP5, and NSP6 may function to recruit or move other viral proteins from their sites of synthesis into the viroplasms, or they facilitate RNA encapsidation or movement of nascent viral particles from the viroplasms to the ER membrane.

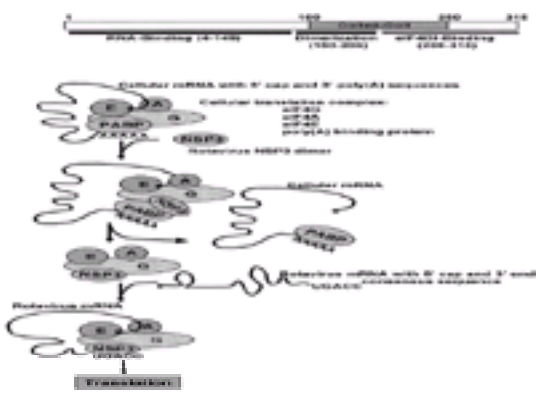

FIG. 6. Modulation of host cell translation initiation by rotavirus NSP3. **A:** Schematic of features of NSP3 defined by sequence and mutational analyses (276). **B:** Model of how NSP3 affects host cell translation. The rotavirus NSP3 functions similarly to the poly(A)-binding protein (PABP) and binds the same site on the cellular translation initiation factor eIF4G, thus evicting PABP bound to cellular mRNAs from the cellular translation initiation complex. The 3' sequence GACC on rotavirus mRNAs functions as a viral-specific translation enhancer by a process that involves recognition by NSP3. Rotavirus-specific mRNAs containing NSP3 are preferentially translated in virus-infected cells. (Modified from ref. 368, with permission).

NSP4 is the only nonstructural protein that does not bind to RNA. NSP4 has been studied extensively because it plays a role in viral morphogenesis and is important in virulence by functioning as an enterotoxin. NSP4 is a nonstructural glycoprotein that has multiple domains (Fig. 7). NSP4 is a 20K primary translation product, it is cotranslationally glycosylated to a 29K species, and oligosaccharide processing yields the mature 28K protein that is a transmembrane protein of the ER (101,171). The 175–amino acid polypeptide backbone of NSP4 consists of an uncleaved signal

sequence, three hydrophobic domains with two N-linked high mannose glycosylation sites being in the first hydrophobic domain, and a predicted amphipathic a-helix that overlaps a folded coiled-coil region. The H2 transmembrane domain traverses the ER bilayer, and the C terminus, which is hydrophilic, forms an extended cytoplasmic domain (35,51,102,171). The carbohydrate moieties remain sensitive to endoglycosidase H digestion, and oligosaccharide processing of the Man9GlcNAc carbohydrate added to NSP4 stops at Man8GlcNAc, with the mannose-9 species predominating (35,171), indicating that no further trimming occurs in the Golgi.

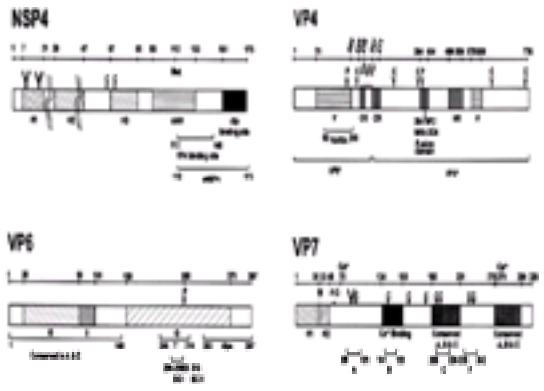

FIG. 7. Features of four rotavirus proteins: NSP4, VP4, VP6, and VP7. **Top left:** Schematic of structural properties of NSP4 and its proposed topography in the membrane of the endoplasmic reticulum. H1, H2, and H3 indicate the three hydrophobic domains and (Y) the glycosylation sites. Most of H2 and a small part of the upstream amino acids traverse the endoplasmic reticulum (ER) membrane ( ). Residues in the H1 domain are localized in the ER lumen, whereas amino acids from the end of the H2 domain to amino acid 175 are cytoplasmic. The amphipathic a-helical (*AMINO ACIDH*) domain overlaps a coiled-coil regin. The conserved cysteines (*C*), putative VP4, and double-layered particle (*dlp*)-binding sites are shown. This diagram is based on sequence data and biochemical analyses (51,102,171,274,357). **Top right:** Schematic of structural and antigenic properties of VP4 based on the analyses of nucleotide sequences of different virus strains and escape mutants selected with neutralizing monoclonal antibodies (MAbs). The two major cleavage products (VP8*) and (VP5*), the trypsin cleavage sites for SA11( ), predicted heptad repeat (*HR*), variable region (*V*), conserved region (*CR*), conserved cysteines (*C*) and prolines (*P*), hemagglutination (*HA*) and sialic acid (*SA*)-binding domain, one neutralization-positive (*NP*) NP2 (amino acid 394) MAb site, and the putative fusion domain are indicated. Other escape mutants selected with neutralization positive MAbs have been mapped to amino acids 150, 155, 180, 183, 187, 188, 190, and 194. The numbering shown here for VP4 is based on a protein of 776 amino acids as determined for SA114fM, rhesus rotavirus MMU18006, and bovine rotavirus 486. VP4 of human rotavirus strains are 775 amino acids, having lost an amino acid between residues 134 and 136. This diagram is based on sequence data and on analyses of neutralization escape mutants (213,351). **Bottom left:** Schematic of structural properties of VP6. The hydrophobic (*O*), hydrophilic (*I*), trimerization (*T*), double-layered particle assembly domain (*dlpa*), conserved proline (*P*), SGI MAb 255 and SGII MAb–binding sites, and conserved sequence domains among group A and C rotaviruses, are indicated. **Bottom right:** Schematic of structural and antigenic properties of VP7. The two hydrophobic domains H1 and H2 (amino acids 6–23 and amino acids 33–44), conserved sequence domains among group A, B, and C rotaviruses, putative Ca^{2+}-binding site, conserved cysteines (*C*), the signal cleavage site ( ), second in-frame methionine with a strong initiation codon (*M*), glycosylation site (*Y*), and neutralization epitopes A, B, C, and F are shown. The mature VP7 starts at Q51. This diagram is based on sequence data and on analyses of neutralization escape mutants (97,212,349).

NSP4 plays a distinct role in virus assembly. The C-terminal cytoplasmic domain (amino acids 161 to 175) functions in viral morphogenesis by acting as an intracellular receptor on the ER membrane (17,355,356). NSP4 binds newly made double-layered particles and mediates the budding of these particles into the ER lumen (see later). A receptor role for NSP4 is supported by the observation that double-layered particles bind to ER membranes containing only NSP4 (15,16,233). The amphipathic a-helix region, distinct from the receptor domain, is predicted to adopt an a-helical coiled-coil structure, and this region is thought to mediate oligomerization of the virus-binding domains into a homotetramer (357). Sequence analyses have identified at least four genetic groups of NSP4 (Table 5). The highest sequence diversity of NSP4 is located in the cytoplasmic domain. It is unclear whether this sequence diversity is important and driven by interactions with specific residues on VP6 or divergent regions on VP4 or VP7, or both.

Genogroup	Strain followed by species of origin	
	Human	Animal[a]
A	1076, RV5, S2, DS-1, A26, E210, E231, B37, KUN, CHMY 7, US1205, L26	BrUK, BoNCDV-Lincoln, BoBRV033, BrBS231, BoA5, BoA3, SiSA11-SEM, LaALA, LaC-171, LaBAP-2, LaRI-2, EqH-2, EqFI23, EqA3
B	Wa, M, ST3, RV3, RV4, AU32, M37, UK70, 1180E, A-75, F45, CH-32, CH-55, CH80, CH82TA, J-12, TH26, TR28, YO, MO, ITO	PrOSU, PoGottfried, PoYM, PoA34, PoA35L, POA138, PoA411, PoX8776, PoCR84-8, PoBEN-144
C	RU-1, 132/92	CaRS-15, CaCU-1, SuRRV, FoFRV-1, FeFRV64
D		MuEW, MuEC, MuEHP

[a]Abbreviations: Si, simian; La, lapine; Pr, porcine; Ca, canine; Mu, murine; Eq, equine; Fe, feline; Bo, bovine.
From refs. 19, 47, 52, 71, 86, 156, and 158, with permission.

TABLE 5. *Group A rotavirus NSP4 genogroups*

Glycosylation of NSP4 or of a cellular protein is required for removal of the transient envelope from budding particles.

This is known because if cells are infected with a rotavirus variant that possesses a nonglycosylated VP7 and the cells are treated with tunicamycin (that only prevents glycosylation of NSP4), all the particles observed in the ER remain enveloped (155,270,341). Glycosylation of NSP4 is not required for its binding activity to double-layered particles or for oligomerization, but it is required for interaction with calnexin (15,236,356). NSP4 also has a binding site for VP4 (17), and NSP4 may play a role in removing the transient envelope (359). Heterooligomers of NSP4, VP4, and VP7 have been detected in enveloped particles (210,284), and calcium has been shown to be important for oligomerization of these proteins in the ER (285) and for proper folding of VP7 epitopes and outer capsid assembly (92,322). The precise mechanism of how (a) the envelope on particles is removed, (b) the heterooligomeric complexes function in particle budding through the ER, and (c) the outer capsid is assembled onto the newly made double-layered particles remains poorly understood. Rotavirus particle transport, maturation, and assembly remain an interesting model to understand the transport of protein complexes across the ER membrane as well as envelope particle formation. Recently, a fraction of VP4 and VP7 has been detected at the cell surface of virus-infected cells (247), and a cleavage product of NSP4 has been detected in the media of cells before release of virus (400). These new data indicate that our understanding of rotavirus protein trafficking and particle formation and exiting from cells is incomplete.

NSP4 has other pleiotropic properties besides its intracellular role in morphogenesis. In 1996, NSP4 was shown to induce age-dependent diarrhea in mice that mimics disease caused by rotaviruses (19,156). This provides one possible explanation for how NSP4 might function as a virulence factor as determined by reassortant analyses (157). Further work showed that NSP4 functions as an enterotoxin and that the extracellular administration of NSP4 to the intestinal mucosa or to crypt cells from mice, and to human intestinal cell lines, can trigger a signal transduction pathway that leads to mobilization of intracellular calcium [Ca^{2+}]i and chloride secretion (19,91,237).

The complex structure of the intestinal architecture and potential cross-talk among the many distinct intestinal cell types highlights that many possible pathways may influence the response of host-differentiated enterocytes or secretory crypt epithelial cells to rotavirus replication and its enterotoxin (Fig. 8). The current model for the mechanism of action of intracellular and extracellular NSP4 indicates this protein may reduce glucose absorption in epithelial cells as well as induce chloride secretion (149,237). NSP4 can also affect the cytoskeleton in polarized epithelial cells (345), but how these pleiotropic properties of NSP4 influence the function of NSP4 in morphogenesis or pathogenesis remains to be clarified.

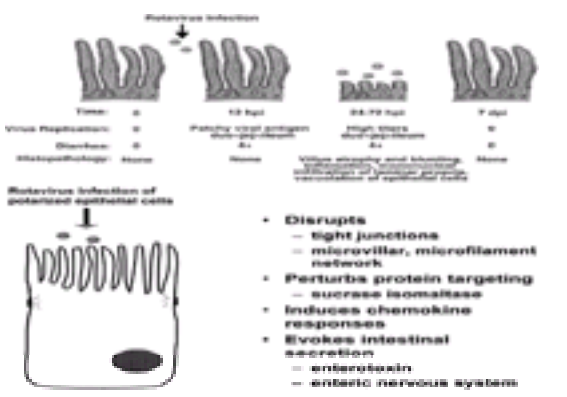

FIG. 8. Rotavirus pathogenesis. **Top:** Schematic of the histopathologic and clinical effects of rotavirus infection of intestinal cells. Rotaviruses-induced diarrheal disease occurs early after infection before significant histopathology of the infected mature enterocytes of the intestine. This diagram is based on studies in piglets infected with virulent porcine rotavirus (371). It is thought that the early, profuse, secretory diarrhea is caused by the action of the rotavirus enterotoxin, NSP4 (111) (see text). Histologic changes are seen at later time points when virus titers are high and loss of the differentiated enterocytes leads to malabsorption. The acute infection and disease normally resolves within 7 days in immunocompetent hosts. **Bottom:** Rotavirus infection of polarized epithelial cells triggers many effects that contribute to pathogenesis. Disruption of epithelial cell structure and function occurs slowly (18 to 24 hours after infection) and may lead to cell death and alterations of paracellular pathway of fluid movement (43,68,168,170,234,251,343). Rotavirus infection of epithelial cells activates epithelial cell signaling that can trigger cross-talk among the diverse types of cells that make up the villus architecture and activate the enteric nervous system, intestinal secretion, and immune responses (111,209,305).

Rotavirus, and NSP4 alone, induce age-dependent diarrhea and age-dependent chloride permeability changes in mice lacking the cystic fibrosis transductance resistance (CFTR) channel (10,237). These results indicate that NSP4 is a novel secretory agonist because other known secretogues that induce chloride changes by activating cyclic adenosine monophosphate, instead of by mobilizing [Ca^{2+}]i as a secondary mediator, fail to cause disease in CFTR knockout mice (237). The ability of NSP4 to elevate [Ca^{2+}]i has been exploited to identify novel calcium-binding proteins in cancer cells (386). Some virulent/avirulent pairs of rotaviruses contain mutations in NSP4, and these sequence changes correlate with altered protein function (182,401). However, not all attenuated viruses have been shown to contain mutations in NSP4 (10,52,373), a finding that is not unexpected because there are many mechanisms of virus attenuation, and other viral proteins (NSP1, NSP2, VP3, VP4, VP7) are virulence factors (44). Immunization with NSP4 can induce immunity that protects neonatal mice from disease induced by virus (19,20,395). The identification of NSP4 as the first viral enterotoxin highlights similarities between viruses and other microbes that cause diarrheal diseases and emphasizes a greater complexity of toxin action and gastrointestinal track–pathogen cross-talk than previously recognized.

The Major Structural Proteins

Three of the rotavirus structural proteins (VP4, VP6, VP7) have been studied extensively because of their unique biochemical, antigenic, and biologic roles in rotavirus replication and assembly (see Fig. 7). Known functions of these

proteins are described below. Antigenic properties and the role of these proteins in pathogenesis and immunity are considered in Chapter 55.

VP4, the protein product of genome segment 4, is a nonglycosylated outer capsid protein (13,218,226). Knowledge of the diverse properties of VP4 resulted in an increased awareness of the importance of this protein in the biology of the rotaviruses. VP4 is the hemagglutinin in many virus strains (173) and the cell attachment protein (84,308). Proteolytic cleavage of VP4 into VP5* (molecular weight about 60,000 [60K]) and VP8* (about 28K) results in an enhancement of viral infectivity (103,108). Cleavage of VP4 enhances penetration (but not binding) of virus into cells (73,122,175). Particles containing cleaved VP4 also possess lipophilic activity and may cause rapid release of ^{51}Cr from cells and of fluorescent dyes from liposomes or intestinal vesicles (309). VP4 is also associated with restriction of growth of certain rotavirus strains in tissue culture cells (142) and with protease-enhanced plaque formation (173). Finally, VP4 has been implicated as a virulence determinant in mice and piglets (253). VP4 induces neutralizing antibodies (158,252) and antibodies directed to VP4 neutralize virus *in vitro* (45,80,144,158,184,352) and passively protect mice against heterologous rotavirus challenge *in vivo* (256). Further studies have shown that VP4 effectively induces protective immunity in animals (214,255) and is immunogenic in children and animals (78,325,344).

Features of VP4 with potential biologic relevance have been found by analyzing nucleotide and predicted amino acid sequence data (see Fig. 7). Direct amino acid sequence analysis revealed the sites of proteolytic cleavage of VP4 of SA11 (205). Both VP4 and VP8* have blocked NH2-termini, and VP5* of SA11 is composed of two polypeptide species with slightly different amino acid sequences at their NH2-termini. Comparison of these data with the nucleotide sequence of VP4 identified two trypsin cleavage sites (arginine 241 and arginine 247), with position 247 being the preferred cleavage site (205). In some cases, a third cleavage may occur at arginine 231 (14). These arginines 241 and 247 are conserved in every rotavirus VP4 sequence analyzed, and cleavage at arginine 247 correlates with enhancement of infectivity (14,127,206,223).

Comparison of the properties of the VP4s of distinct sizes is useful to map conserved domains (223,263). In most animal rotavirus strains studied to date, VP4 contains 776 amino acids. A VP4 of animal (bovine B223) and human rotavirus strains containing 772 amino acids have been described (96,126,150). In most human rotavirus strains, the deduced VP4 contains 775 amino acids instead of 776 amino acids, with the difference being that the human strains have one less amino acid between residues 134 and 136 (136,176). VP8* of the human strains is, therefore, one amino acid shorter. In several human rotavirus strains, a third potential trypsin cleavage site of either lysine or arginine is found just upstream of the 3'-proximal arginine site (amino acid 246 in human strains, amino acid 247 in animal strains) (137,260). There is no direct evidence that this additional cleavage site in some human viruses correlates with virus virulence.

The mechanism of cleavage activation of infectivity is not clear from either sequence or biochemical analyses. It is unknown whether cleavage at both sites results in removal of the small intervening peptide and whether the minor and major cleavage sites shown for SA11 are really the same for other virus strains. It is assumed that cleavage of VP4 activates an early step of replication, which may be triggered by one or both of the regions generated by cleavage or by a possible conformational change in the VP4 cleavage products. The new NH2-terminus generated by cleavage in the rotavirus VP4 is not hydrophobic; however, it contains many polar amino acids (some of which may be charged at neutral pH), and cleaved VP4 possesses lipophilic activity (246,309). The mechanism of activation of rotavirus infectivity is not understood, but it appears to be different from that demonstrated for other viruses, such as the orthomyxoviruses and paramyxoviruses; in these viruses, cleavage activation of the hemagglutinin or fusion protein generates a highly conserved apolar amino-terminus, which is brought toward the membrane by a significant conformational change of the spike protein, and virus infectivity can be blocked by oligopeptides that mimic this sequence (see Chapter 40 and Chapter 45).

A region in VP4 (amino acids 384 to 401) has been identified that shares some homology with internal fusion sites in Semliki Forest and Sindbis virus proteins (213). This region in the alphavirus E1 protein has been implicated in cell fusion, leading to the hypothesis that these sequences might perform a similar function in VP4 (213). If this sequence is indeed a fusion domain, it remains unknown whether the fusion activity functions to mediate virus entry into cells, to mediate virus maturation during budding across the membrane of the ER (see below), or both. Conservation of this putative fusion region among rotaviruses lends credence to the suggestion that it has an important function in the replication cycle. Recent studies have shown that VP5* can permeabilize lipid vesicles (88).

The predicted amino acid sequence of VP4 indicates that the amino terminal 70% of the protein is rather hydrophobic (193,223). The predicted amino acid sequence of VP4 has a net negative charge at pH 7.0, and secondary structure predictions reveal that the amino-terminus before the trypsin cleavage site contains numerous random coils and turns, suggesting a globular structure. Analysis of the secondary structure of VP8* predicts the presence of 11 b-strands separated by loops and flanked by two small a-helices (166). Four cysteines (at amino acids 216, 318, 380, and 774) are conserved in the VP4 of all rotavirus strains sequenced (136,176,213), except those of the bovine rotavirus B223 (150). An additional cysteine is present at position 266 in the human M37 strain (136) and at amino acid 203 in SA11 4fM, bovine 486, and rhesus rotavirus. *In vitro* studies of the intrachain disulfide bonds in the VP5 and VP8 cleavage fragments of the rhesus and simian SA11 viruses have indicated that many animal rotaviruses contain two intrachain disulfide bonds: one in VP8 and one in VP5 (263). In contrast, human rotaviruses appear to contain only a single bond in the VP5 fragment (263). Sequences flanking the trypsin cleavage sites (amino acids 224 to 235 and 257 to 271) are relatively conserved in different virus strains and may be structurally important in keeping the trypsin cleavage site accessible.

The sequences of segment 4 from viruses in different serotypes have been compared to look for regions of divergence. Sequence variation is not evenly distributed across VP4 (see Fig. 7). The greatest variation is seen between amino acids 71 and 204 in VP8*, and sequence analyses between amino acids 84 and 180 have been found to correlate with VP4 P types (196,197). Because VP8* contains the locations of greatest sequence diversity, the binding sites of strain-specific neutralizing MAbs, and hemagglutination activity (118), although cross-reactive neutralizing MAbs bind to sites mapped to VP5* (213,351), it initially was tempting to speculate that VP8* is more externally exposed on the virus particle. A mechanism of immune selection could then explain the regions of sequence diversity in VP8*. Contrary to this

hypothesis, a cross-reactive neutralizing MAb that induces mutations at amino acid in VP5 was shown to bind to the sides near the tip of the spike (288). Thus, it appears that at least part of the VP8 domain may interact with VP6, and VP5 is more external. This is consistent with the observation that a neutralizing MAb that destabilizes the outer capsid and appears to affect VP4–VP7 interactions induces substitutions in neutralization escape mutants at amino acid 180 and amino acid 183, which flank a conserved proline (402). Analyses of mutants that have lost hemagglutination activity have suggested that amino acid residues 150, 155, 187, 188, 190, and 194 in VP8* are involved in binding to sialic acid (166,207,402). High-resolution structural information is needed to resolve the precise folding of VP4 and its interactions with VP6 and VP7.

VP6 is the major structural component of virions, and it plays a key role in virion structure because of its interactions with both the outer capsid proteins VP4 and VP7 and the core protein VP2. VP6 spontaneously forms trimers (105) and is extremely stable; these characteristics and the presence of conserved epitopes among many virus strains explain why this is the major antigen targeted in diagnostic assays. VP6 trimers can be disassociated and reassembled by changing pH (365). Removal of VP6 from double-layered particles results in a loss of polymerase activity, but it is unknown whether VP6 plays a structural or functional role in this process (28,317). VP6 is a hydrophobic protein that is highly antigenic and immunogenic (105,138,313), and VP6 may play a role in inducing protective immunity. Delivery of a nonneutralizing MAb to VP6 to severe combined immunodeficient mice can reduce chronic virus excretion (46), and 2/6–virus-like particles (VLPs) can induce protective immunity in mice (249), but not in rabbits (67) or piglets (393). Analyses of deletion mutants, virus variants, ts mutants, and chimeric proteins have begun to dissect the domains of VP6 (see Fig. 7). Thus, the trimerization domain is reported to be between amino acids 246 and 315, and a domain necessary for the formation of double-layered particles is located between amino acids 281 and 397 (2,365) (see Fig. 7). Residues 296 to 259 and 305 are important for determining the subgroup I epitope recognized by MAb 255/60 (327). Proline 308 has been implicated in trimer stability (326). The N-terminus is predicted to be an amphipathic a-helix and to be crucial to virus assembly, possibly by functioning in transporting VP6 to viroplasmic inclusions (217). Studies with MAbs to VP6 have suggested that amino acids 58 to 62 are not accessible in viral particles, and these could be located at the interface with VP2; other domains may be accessible through the outer shell of the virus (364).

VP7 is an outer capsid glycoprotein that is the second most abundant protein species in the virion. VP7 was initially thought to be the only antigen containing neutralization determinants (142,174), and this stimulated numerous studies of its structure, biosynthesis, and function. Biochemical analyses rapidly determined that VP7 is a glycoprotein that contains only N-linked high mannose oligosaccharides, which are processed by trimming (13,101,171). $Man_8GlcNAc_2$ and $Man_6GlcNAc_2$ oligosaccharide residues are found on intracellular VP7 and Man $_6GlcNAc_2$ (and to a lesser extent Man5GlcNAc2) is found on mature virus particles (171,172). VP7 is cotranslationally glycosylated as it is inserted into the membrane of the ER, and insertion is directed by a cleavable signal sequence found at the amino-terminus of the protein (34,102,171).

Comparisons of the deduced amino acid sequence of VP7 from a number of animal and human strains reveal interesting and potentially biologically relevant features about VP7 (see Fig. 7). The ORF of 326 amino acids begins with an initiation codon with a weak consensus sequence. A second, in-frame initiation codon with a strong consensus sequence is located 30 codons downstream. Each of these first two initiation codons precedes two regions of hydrophobic amino acids (H1 and H2), which can act as the signal sequence to direct VP7 to the ER, although the second codon is thought to be the major species used in cells. A third in-frame initiation codon is also present downstream from the second hydrophobic domain in some strains. One glycosylation site, at amino acid 69, is used in the SA11 VP7. Other virus strains contain up to three potential glycosylation sites; only two glycosylation sites are apparently used, but the locations of the used sites are unknown (189,190,319). The site of cleavage of the signal peptide in VP7 is glutamine 51, and this amino-terminal residue in VP7 from purified virus is blocked by pyroglutamic acid (337).

Studies to determine the signals specifying retention of VP7 in the ER have shown that VP7 does not contain the sequence KDEL found to confer retention for some other ER proteins (239). Two regions (one spanning amino acids 51 to 61, and the second between amino acids 61 and 111) that mediate retention of VP7 in the ER have been identified (283,286,337,382). Further sequence analyses identified a consensus peptide LPXTG [STGAE], where X indicates any other amino acid, within the ER retention domain of VP7 (223). This sequence is of interest because it is found in bacterial surface proteins and is proposed as being responsible for a posttranslational modification necessary for proper anchoring of proteins to the bacterial plasma membrane. The LPITGS sequence is highly conserved and found in rotavirus VP7 between amino acids 57 and 63 (223), and mutation analyses have shown the ITG are essential residues for ER retention (211). Although these residues are critical for retention, the method by which VP7 remains in the ER is unresolved. After its insertion into membranes, VP7 is resistant to digestion with proteolytic enzymes, suggesting it is not a membrane-spanning protein (101,171). VP7 remains membrane associated after high salt treatment and release of microsomal contents at alkaline pH (171). These findings suggest VP7 is an integral membrane protein with a luminal orientation. Further studies are required to determine why VP7 is retained in the ER, and whether posttranslational modification or conformational properties are responsible for membrane association. Structural reconstructions of virion VP7 have shown it is trimeric and VP7 forms oligomers with other proteins (VP4 and NSP4) in infected cells (210). These oligomers, and protein rearrangements and interactions with calcium, appear to be important in the assembly of VP7 into the outer capsid (92,284,285,323).

The normal site of VP7 synthesis and processing is exclusively in the ER, and rotaviruses are not transported to the Golgi apparatus. However, this pathway of protein targeting can be altered. Deletion of the two regions shown to mediate VP7 retention in the ER results in the processing of VP7 through the Golgi and into the extracellular medium. When transported through the Golgi, VP7 is modified to a form that contains complex carbohydrates (9,286). These results could be of practical significance for the production of soluble forms of VP7 for vaccines. Although these studies have only followed the fate of an individual protein, it would also be interesting to determine whether infectious virus could be redirected through such alternative pathways. Unfortunately, a method to alter a specific rotavirus gene and recover it in infectious virus is not yet available.

Mature VP7 lacks all amino acids proximal to Gln 51 but many conserved amino acids (18 of 35) are present before the second hydrophobic domain. In addition, none of the 11 amino acids in the second hydrophobic domain is highly

conserved, raising the question of what causes this extensive sequence variation. VP7 is the dominant target for cytotoxic T-lymphocyte (CTL) activity in mice (120,254), and the second hydrophobic domain has been shown to be the CTL target within VP7 (120). Four of six other regions showing sequence variation in VP7 are major neutralization sites in virions (97) (see Chapter 55).

THE ROTAVIRUS REPLICATION CYCLE

Overview of the Replication Cycle

Figure 9 shows a schematic of the rotavirus replication cycle. Many of the details of this cycle are from studies of rotaviruses infecting monkey kidney cells in cell monolayers. New insights about virus replication are coming from studies in polarized intestinal epithelial cells. Other information has come from assays to probe the specific steps in the replication cycle based on the expression and interaction of individual proteins and RNAs in *in vitro* systems, and conclusions from these studies are slowly being confirmed in the context of virus replication systems using confocal microscopy.

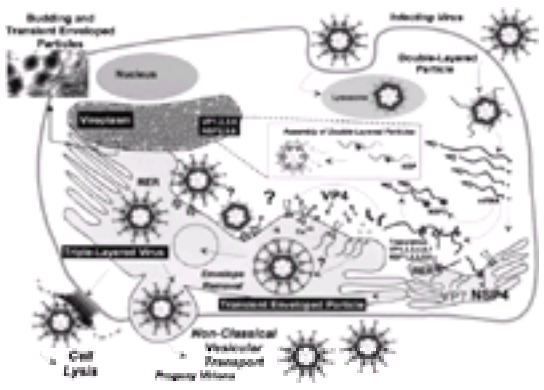

FIG. 9. Major features of the rotavirus replication cycle: VP4 , VP7 , NSP4 representations. For details, see section on the replication cycle. The unique rotavirus assembly process involves NSP4, which serves as an intracellular receptor for particle budding into the endoplasmic reticulum. The insert shows a thin section illustrating enveloped rotavirus particles budding into the endoplasmic reticulum. The viral glycoproteins do not traffic to the Golgi. In polarized epithelial cells, particles are released by a nonclassic vesicular transport mechanism (170).

Although the natural cell tropism for rotaviruses is the differentiated enterocyte in the small intestine, rotavirus replication has been studied primarily in continuous cell cultures derived from monkey kidneys. In these cells, the replication cycle is fairly rapid, with maximum yields of virus being found after 10 to 12 hours at 37°C or 18 hours at 33°C when cells are infected with high multiplicities (10 to 20 PFU/cell) (72,225,294). Recent studies examining rotavirus replication in differentiated human intestinal cell lines (CaCo-2 cells) grown on permeable filter membranes have reported that the virus replication cycle is slower with maximal yields of virus being detected on the apical surface of cells 20 to 24 hours after infection (169,170). Significantly, electron microscopic studies of virus replication in polarized intestinal cells indicate that the replication process in these differentiated polarized cells has some distinct differences compared with the virus replication cycle in nonpolarized cell cultures. These emerging differences are described later.

The general features of rotavirus replication (based on studies in cultures of monkey kidney cells) are as follows:

1. Cultivation of most virus strains requires the addition of exogenous proteases to the culture medium. This ensures activation of viral infectivity by cleaving the outer capsid protein VP4.
2. Replication is totally cytoplasmic.
3. Cells do not contain enzymes to replicate dsRNA; hence, the virus must supply the necessary enzymes.
4. Transcripts function both to produce proteins and as a template for production of a negative strand. Once the complementary negative strand is synthesized, it remains associated with the positive strand.
5. The dsRNA segments are formed within nascent subviral particles, and free dsRNA or free negative-stranded ssRNA is never found in infected cells.
6. Subviral particles form in association with viroplasms, and these particles mature by budding through the membrane of the ER. In this process, particles acquire their outer capsid proteins.
7. Cell lysis releases particles from infected cells grown on monolayers.

In polarized intestinal cells, virus entry may occur almost exclusively through the apical membrane or through both the apical and basolateral membranes, depending on the virus strain (see later). In addition, virus replication in polarized enterocytes alters differentiated enterocyte cell function by perturbing cellular protein trafficking, the cytoskeleton, and tight junctions (43,168,251). Finally, virus is released from polarized enterocytes by a novel vesicular transport that does not result in extensive cytopathic effects (170).

Stages of the Replication Cycle

Adsorption, Penetration, and Uncoating

The initial stages of virus replication have been examined by biochemical and morphologic (electron microscopic) procedures, and the molecular details of the early event of rotavirus infection are complex. Only triple-layered particles containing VP4 attach to cells when monitored by electron microscopy (168,272) or by cell binding (84) or infectivity

assays (40). Virus attachment is by VP4 (84,308) or its cleavage product VP5 (394). Binding to cells does not require cleaved VP4 (73,122) or glycosylated VP7 (271). Rotavirus entry into cells appears to be a multistep process (232) that likely requires both VP4 and VP7.

The identity of the cellular receptor for all rotaviruses is not known, but a study of the binding of radiolabeled SA11 to MA104 cells found about 13,000 receptor units per cell. Initial binding is sodium dependent, pH insensitive between 5.5 and 8.0, and dependent on sialic acid residues in the membrane; virus binds but is not internalized at 4°C (121,180,272,383), and probably involves more than one molecular species on the cell (335). A glycoprotein of 300K to 340K on murine enterocytes that binds to rhesus rotavirus and to VP4 has been reported, but whether this is a receptor remains unknown because this crude enterocyte extract was not purified or tested for its ability to compete directly for rotavirus binding to host enterocytes (22).

Demonstration that neuraminidase treatment of red blood cells reduces virus binding first indicated a role of sialic acid in virus attachment (23). Sialic acid–containing compounds, such as fetuin and mucin, also inhibit virus binding to cells (180,392), adding rotaviruses to an increasing number of viruses (such as reoviruses and influenza) that require sialic acid for binding to cells. However, these studies did not determine whether virus binds directly to sialic acid or whether sialic acid maintains the configuration of the binding site without directly interacting with the virus.

Additional evidence indicates that the type of glycoconjugate (i.e., ganglioside, neutral glycolipid, glycoprotein) that actually functions as an *in vivo* receptor for a given rotavirus strain depends on the rotavirus strain or type of host cell being studied. For example, the use of mouse-derived host cells with a heterologous simian rotavirus may not be the most advantageous combination to identify an *in vivo*–relevant simian rotavirus receptor. Initially, it was thought that most animal rotaviruses recognize sialic acid as an active receptor epitope (121), whereas the infectivity of tissue culture cells by human rotaviruses is resistant to neuraminidase (121,392). This apparent dichotomy of binding activities of different rotavirus strains appeared to have been resolved by the demonstration that binding to sialic acid is not an essential step for animal rotavirus entry into cells (231); thus, neuraminidase-resistant mutants can be selected that retain full infectivity for cells, indicating that animal rotavirus particles contain at least two binding sites for cells. In addition, human rotavirus strains initiate infection of cultured cells by a sialic acid–independent mechanism (121,180). Recent studies have found that most rotaviruses initiate infection by a sialic acid–independent mechanism and that this represents a change in paradigm (69). Perhaps more important, sialic acid–dependent viruses initiate infection of polarized intestinal epithelial cells efficiently only by the apical membrane, whereas sialic acid–independent viruses can efficiently infect polarized cells by either the apical or basolateral surfaces (68). These results indicate that sialic acid–dependent and –independent rotaviruses likely use different receptors, although studies in nonpolarized cells have suggested that they may share a second receptor (232). The consequences of these differences in modes of cell entry are not fully understood at this time, but it is likely that further studies on rotavirus entry into cells will reveal other virus–host interactions that affect the outcome of infection and pathogenesis.

A cell surface monoganglioside functions as an *in vivo* receptor for the OSU porcine rotavirus strain, and sialic acid is required for OSU virus–binding activity (306). Taken together, all the studies on rotavirus binding and entry suggest that unusual mechanisms may be involved. These include binding two different sites on the cell membrane, possibly with two different proteins on the virus, and direct membrane penetration of bound viruses rather than conventional receptor-mediated endocytosis. It is possible that rotavirus binding occurs initially by interaction with a sialic acid–containing receptor followed by a separate interaction with a second, nonsialylated receptor. Conformational changes in viral binding proteins induced by initial binding events may also mediate some of the early events. Other studies have suggested that a_1b_1-, a_xb_2-, a_vb_3-, and 4_4b_1-integrins are involved in rotavirus cell entry, probably functioning as postbinding coreceptors (81,148,153).

After binding occurs, virus is internalized. The enhancement of rotavirus infectivity by proteolysis is reportedly due to facilitating the penetration step (73). Internalization does not take place at 0° to 4°C, indicating that this step requires active cellular processes (180,272). All virus is internalized by 60 to 90 minutes after binding (180). The mechanism of internalization (penetration) into cells is controversial.

Both morphologic and biochemical approaches have been used to investigate the mode of entry of rotaviruses into cells. Early electron microscopy studies suggested that virus entry (SA11 strain) occurs by endocytosis (272,293) and that incoming particles are rapidly transported to lysosomes. Clear documentation of uptake of trypsin-treated virus particles into coated pits, coated vesicles, and secondary lysosomes by electron microscopy confirms that rotavirus particles (porcine OSU strain) enter cells by receptor-mediated endocytosis and suggests that uncoating might occur by the effect of lysosomal enzymes (208). Use of the calcium ionophore A23187 to increase the intracellular Ca^{2+} concentration during the early stages of replication can block uncoating (208). These results supported early hypotheses that low Ca^{2+} concentrations in the intracellular microenvironment may be responsible for uncoating. This idea was originally proposed because it was known that removal of the outer capsid of particles and activation of the endogenous polymerase could be accomplished by calcium chelation (77,155).

Studies of the uptake of a human rotavirus whose infectivity is independent of sialic acid binding and absolutely dependent on trypsin cleavage suggest that the mode of rotavirus entry into cells differs depending on whether virus particles have been pretreated with trypsin (339). Infectious virus pretreated with trypsin apparently enters cells by direct penetration of particles through the cell membrane into the cell cytoplasm. In contrast, non–trypsin-treated particles were taken up by phagocytosis, and such virions were sequestered into lysosomes 20 minutes after virus attachment to the cell membrane. Mere phagocytosis of particles into lysosomes was thought to be unrelated to rotavirus replication (339). Direct release of nucleic acid through spaces between the capsomeres and cell membrane pores after the attachment of trypsin-activated virus particles onto cells has also been proposed; this mechanism is considered to be analogous to injection of nucleic acid into bacteria by phages (340). The inability to detect virus shells lacking nucleic acid attached to cells makes this observation difficult to interpret.

The concept that trypsin-treated and non–trypsin-treated virus enter cells by different mechanisms is supported by a

study of the kinetics of entry of the sialic acid–dependent rhesus rotavirus into MA104 (175). Trypsin-activated rhesus rotavirus is internalized with a half-time of 3 to 5 minutes, whereas nonactivated virus disappears from the cell surface with a half-life of 30 to 50 minutes. Only trypsin-activated virus results in productive infection. Trypsin-treated virus can mediate the rapid release of ^{51}Cr from infected cells and this activity does not occur at 4°C. The specificity of the permeability alterations was examined by showing that double-layered particles do not mediate them and that neutralizing anti-VP4 MAbs (but not nonneutralizing antibodies to VP6 or VP7) inhibit the ^{51}Cr release. Unfortunately, permeability results with non–trypsin-treated virus were not reported. However, these data suggest that virus entry occurs by direct penetration of the cell membrane, which results in permeabilization of the cell membrane (175).

Other viruses that initiate infection by mechanisms involving receptor-mediated endocytosis often depend on the acidification of endosomes for partial uncoating or entry into the cell. The importance of acidification of endosomes for the initiation of infection of rotaviruses has been studied by several groups (123,175,179,208). In all cases, lysosomotropic agents (ammonium chloride, chloroquine, methylamine, amantadine) have little inhibitory effect on virus replication as measured by viral RNA synthesis, polypeptide synthesis, virus yields, or shutoff of host protein synthesis. Energy inhibitors (sodium azide and dinitrophenol) have a minimal effect on rotavirus infection, and this has been taken to suggest that rotaviruses do not use endocytosis to enter cells (175). Other endocytosis inhibitors, such as dansylcadaverine and cytochalasin D, or the vacuolar proton–ATPase inhibitor bafilomycin A1, also do not block rotavirus entry. These results indicate that neither endocytosis nor an intraendosomal acidic pH or a proton gradient are required for rotavirus entry into cells.

It seems clear that the passage of rotaviruses from endocytic vesicles to the cytoplasm does not occur by a pH-dependent fusion mechanism, but this does not rule out the possibility that rotaviruses are still taken up by endocytosis. Direct demonstration of virus fusion with membranes or hemolysis is lacking, and without this, the data suggesting direct penetration can have other interpretations. Protease cleavage of VP4 is important for rapid entry into cells, and particles containing cleaved VP4 possess lipophilic activity and can affect release of fluorescent dyes from liposomes and isolated membrane vesicles. Rotavirus entry into cells can also be monitored by coentry of toxins, such as a-sarcin, into cells (85,201) and by a cell–cell fusion from without assay (115). Most observations are consistent with the hypothesis that virus enters cells after direct interactions with a receptor on the plasma membrane (246,309,310).

The outer capsid proteins of rotavirus that are solubilized from virus particles are able to permeabilize cellular membranes (310), and it has been proposed that the outer capsid proteins are solubilized within an endocytic vesicle because of low Ca^{2+} concentrations. The decrease in calcium concentrations within the endosomal vesicle might trigger conformational changes in the capsid, capsid solubilization, and vesicle lysis (310). In this Ca^{2+}-dependent endocytosis model, acidification of the endosome would not be needed for the infectious process. It is also possible that more than one mechanism, including endocytosis and direct entry, is operative for rotaviruses, as has been proposed for poliovirus and reoviruses (33,90). Further studies are needed to determine whether the common endocytosis-mediated entry pathway exists for rotaviruses as originally thought.

A single report that the protein product of genome segment 10 influences virus adsorption to cells further complicates the question of the mechanism of virus attachment to cells (312). This study of a series of reassortants suggests that the nonstructural protein NSP4 may be indirectly involved in adsorption by influencing the arrangement of outer shell proteins assembled in infectious particles. This observation remains to be confirmed but is of interest based on the new information that NSP4 can affect calcium mobilization in cells and the proposal that rotaviruses may enter cells by a Ca^{2+}-dependent endocytic mechanism.

Transcription and Replication

The synthesis of viral transcripts is mediated by an endogenous viral RNA-dependent RNA polymerase (transcriptase), which has a number of enzymatic activities. The transcriptase is a component of the virion, and this enzyme complex of VP1 and VP3 has been localized to a flower-shaped structure attached to the top of VP2 at all the fivefold axes. Properties of the transcriptase have been inferred by studying the characteristics of products from *in vitro* transcription reactions and by electron microscopy analysis of transcribing particles (75,198,218,226,317). Rotavirus particles contain the enzymatic activities needed for synthesis of capped messenger RNA, including transcriptase, nucleotide phosphohydrolase, guanyltransferase, and methylases. These activities have either been demonstrated biochemically or they are inferred are because rotavirus transcripts made *in vitro* in the presence of *S*-adenosyl methionine possess a methylated 5'-terminal cap structure, $m^7GpppGm$ (165), and transcription is inhibited by pyrophosphate (334). Particles also contain a poly(A) polymerase activity whose precise function remains unknown; it has been postulated to be responsible for the synthesis of oligo(A) molecules (135).

The virus-associated transcriptase is latent in triple-layered particles and can be activated *in vitro* by treatment with a chelating agent or by heat shock treatment (77,334). Such treatments result in removal of the outer capsid proteins with conversion of triple-layered particles to double-layered particles (77). In infected cells, triple-layered particles have been shown to be uncoated to double-layered particles, and it is thought that transcription in cells occurs from such particles (74). Consistent with this idea, cells are susceptible to infection by liposome-mediated transfection of double-layered particles, indicating that simple delivery of these particles into the cell cytoplasm permits transcription to proceed (21). Transcription is asymmetric, and all transcripts are full-length positive strands made off the dsRNA negative strand (227). The exact site of transcription within the cytoplasm has not been precisely localized, although it is thought to occur in viroplasms.

The dynamic transcription process is beginning to be understood. Rotavirus transcription occurs within structurally intact double-layered particles and requires a hydrolyzable form of ATP, and studies with analogs that inhibit transcription suggest that ATP is required in reactions other than polymerization (334). ATP may be used for initiation or elongation of RNA molecules. Structural studies of transcribing particles indicate that RNA synthesis occurs within the core of the virion, near the fivefold axes, because this is the location of the transcriptional complexes composed of VP1 and VP3. Newly transcribed RNA exits the core through the type I channels in the VP2 layer, and multiple mRNA transcripts can be

released simultaneously from an actively transcribing particle (198) (see Fig. 2). Each genome segment is thought to be transcribed by a specific polymerase complex, and the resulting transcript exits through the channel system at the axis adjacent to its site of synthesis. This mechanism of transcription offers an explanation of why no dsRNA virus contains more than 12 genome segments.

The synthesis of positive- and negative-stranded RNA has been studied in SA11-infected cells (336), in a cell-free system using extracts from infected cells (260), and in an electrophoretic system that allows separation of the positive and negative strands of rotavirus RNAs in acid urea agarose gels (267). Analysis of the kinetics of RNA synthesis in infected cells showed that positive- and negative-stranded RNAs are initially detected 3 hours after infection (336). After 3 hours, the level of transcription increases until 9 to 12 hours, at which time the levels of positive-stranded RNAs are maximum. The ratio of positive- to negative-stranded RNA synthesis changes during infection, and the maximal level of negative-stranded RNA synthesis occurs several hours before the peak of positive-stranded RNA synthesis.

The delay in obtaining maximal positive-stranded RNA synthesis has been hypothesized to be due to a requirement for the accumulation of stoichiometric amounts of a protein (e.g., VP6) necessary for the assembly of transcriptase particles. Both newly synthesized and preexisting positive-stranded RNA can act as templates for negative-stranded RNA synthesis throughout infection (260). The observation that the level of RNA replication does not increase continually in conjunction with the increasing levels of positive-stranded RNA suggests that RNA replication is regulated by factors other than simply the level of positive-stranded RNAs in the infected cell. Based on nuclease sensitivity in this system, about 20% of the RNA made *in vitro* is double-stranded, and 80% is single-stranded. The synthesis of dsRNA *in vitro* has been determined to be an asymmetric process in which a nuclease-sensitive positive-stranded RNA acts as template for the synthesis of negative-stranded RNA.

It is assumed that rotavirus RNA replication, like that of reovirus, takes place in a conservative fashion; that is, both parental dsRNA strands remain within partially uncoated particles. After its synthesis, dsRNA remains associated with subviral particles, suggesting that free dsRNA is not found in cells. Characterization of the subviral particles (complexes separable by sedimentation through sucrose gradients and by equilibrium centrifugation in CsCl gradients), in which dsRNA synthesis occurs both in infected cells and in the cell-free system, indicates that these replicase complexes consist of core proteins, VP1 and VP2, small amounts of the protein VP6, large amounts of the nonstructural protein NSP3, and lesser amounts of NSP1 and NSP2 (152). These types of results indicate that some of the NSPs (NSP2, NSP3, NSP5) might be involved in the RNA replication process based on (a) the presence of these NSPs in replication complexes isolated from infected cells (125,262), (b) the nucleic acid–binding properties of some of the NSPs (42,162,178,222,280,379), (c) localization of some NSPs to viroplasms (12,273), and (d) the RNA negative phenotype of ts mutants mapped to the genes that encode these NSPs (58,131)).

A model was hypothesized in which rotavirus double-layered particles were assembled by the sequential addition of VP2 and VP6 to a precore replication intermediate consisting of VP1, VP3, NSP2, NSP3, and NSP5 (125). Because of the inability to separate particles completely with transcriptase and replicase activities with these methods and problems of contamination of some fractions with proteins from neighboring fractions, it has remained unclear whether the components characterized in this system are those necessary for RNA replication or simply present in these complexes for other reasons. A foreign reporter molecule was also engineered into a rotavirus genome segment and tested for expression in cells co-infected with a helper virus (139). This system identified cis-acting nucleotides at the 3' end of the genome necessary for gene expression, but the lack of direct evidence of synthesis of negative strands did not permit determination of whether these signals were necessary for RNA replication. Unfortunately, the efficiency of these systems was too low to ensure clear evaluation of the roles of cis- and trans-acting sequences and proteins required for replication and packaging. Instead, it seems likely that detection of an increased reporter signal was due to enhanced translation in rotavirus-infected cells, possibly owing to the presence of the 3'-translation enhancer on the expressed reporter gene (66).

The role of individual proteins and specific protein complexes in RNA replication and viral morphogenesis will probably not be solved until they are studied *in vitro* with pure species of native rotavirus proteins and viral RNAs. Progress toward this goal came with the development of a template-dependent replication system that uses only viral core proteins and exogenously added purified mRNAs (64). Virus-like particles expressed and self-assembled in insect cells also possess replicase activity (64). The conversion of exogenous mRNA to dsRNA by subviral particles has facilitated studies of (a) the specificity of viral proteins in recognition and replication of rotavirus mRNAs and (b) the effect of adding exogenous synthetic RNAs containing specific mutations on replication. Those cis-acting sequences required for replication are being identified (see Fig. 3). This system is unique for mammalian RNA viruses because it supports the *de novo* synthesis of genome-length negative-stranded RNA in the presence of only viral proteins and in the absence of host extracts. Adding viral mRNAs containing deletions of the 3'-consensus sequence, 5'-UGUGACC-3', to the replication system, has shown that this sequence contains a cis-acting signal that is essential for negative-stranded synthesis (268,381). Other studies identified parts of the 5'-untranslated region as containing sequences that stimulate RNA replication (268,381). Similarily, sequences upstream of the 3'-consensus sequence in the 3'-untranslated region contribute to the efficient synthesis of dsRNA by open cores. Based on the location of the cis-acting sequences and computer modeling, it has been hypothesized that the ends of the viral mRNAs interact in cis by complementary terminal sequences to form panhandle structures that promote the synthesis of dsRNA (60). An important feature of the predicted secondary structure of the rotavirus mRNA is that within the panhandle structure, the 3'-consensus sequence is either not base-paired or only partially base-paired to the 5'-terminus. It appears that the 3'-consensus sequence of the mRNA must be single-stranded for efficient dsRNA synthesis (60). Work is proceeding to enhance the efficiency of this *in vitro* replication system and to develop a cell-free system to support rotavirus RNA replication and the assembly of RNA into particles. To date, there is no evidence of RNA encapsidation in this system.

This *in vitro* template-dependent replication system is surprising because it does not absolutely require the nonstructural proteins for replicase activity (64). However, the nonstructural proteins may play a role in increasing the efficiency of replication or play other roles in the replication cycle. Clearly, much remains to be learned about the NSPs and RNA binding, transport, replication, and assembly. It is anticipated that these studies will be of general interest because the

rotaviruses contain a larger number of NSPs than some of the other viruses within the *Reoviridae* family.

RNA Encapsidation and Virion Assembly

The selective packaging mechanism that leads to the presence of equimolar genome segments within rotaviruses, or any of the other members of the *Reoviridae* family, remains a challenging puzzle. Several models have been proposed (reviewed in 266). Based on the characterization of replication intermediates from rotavirus-infected cells, one model proposes the formation of precore replication intermediate precursor complexes composed of viral mRNA, the viral RNA-dependent RNA polymerase, and the capping enzyme that would serve as nucleation sites for the binding of the VP2 core protein and the assembly of the T=1 core. Based on the ability of the rotavirus capsid proteins to self-assemble into empty virus-like particles (84,194), and on data on the dsRNA bacteriophage phi 6 (235), a second model suggests that empty cores are first made and that mRNAs would be inserted into these cores. A third model is based on structural data indicating that the core represents a collection of functionally separate pentameric units, with each unit containing its own RNA-dependent RNA polymerase activity and capping enzyme complex and being responsible for transcription of one of the genome segments (200). In this model, this enzymatic complex would associate with a specific mRNA and attract the VP2 core protein and assemble into pentamers. RNA–RNA interactions between the mRNAs of the distinct pentameric units would then drive the assembly of the icosahedral core from the pentameric units. Structural changes in the core lattice protein, as a consequence of pentamer–pentamer binding, may activate the RNA-dependent RNA polymerase and stimulate negative-stranded RNA synthesis to form the genome. Future work will determine which of these models, if any, may be correct.

A distinctive feature of rotavirus morphogenesis is that subviral particles, which assemble in cytoplasmic viroplasms, bud through the membrane of the ER, and maturing particles are transiently enveloped (see Fig. 8). This is one of the more interesting aspects of rotavirus replication differing from members of other genera in the *Reoviridae* family. The envelope acquired in this process appears to be lost as particles move toward the interior of the ER, and the envelope is replaced by a thin layer of protein that ultimately constitutes the outer capsid of mature virions.

The sites of synthesis or localization of the viral proteins have been examined by ultrastructural immunocytochemistry using polyclonal monospecific or monoclonal antibodies (273,302) and by studying the distribution of proteins by immunofluorescence or by subcellular fractionation (172,333). Taken together, the morphologic and biochemical data are consistent with rapidly assembling double-layered particles serving as an intermediate stage in the formation of triple-layered virions. Most of the rotavirus structural proteins and the nonstructural proteins are synthesized on the free ribosomes, although the nascent proteins on free ribosomes have not been analyzed. Instead, this conclusion has been drawn based on the absence of signal sequences that would indicate targeting to the ER and lack of protection from digestion in *in vitro* protease protection studies (102,171). In contrast, the glycoproteins VP7 and NSP4 are synthesized on ribosomes associated with the membrane of the ER and are cotranslationally inserted into the ER membrane as a result of signal sequences at their amino-termini.

The sites and precise details of RNA replication remain unclear. However, electron-dense viroplasms are probably the sites of synthesis of the double-layered particles that contain RNA (270,302). This conclusion is based on the localization of several of the viral proteins (VP2, NSP2, NSP5, NSP6) to viroplasms and of VP4 and VP6 to the space between the periphery of the viroplasm and the outside of the ER (133,274), and on the observation that particles emerging from these viroplasms often seem to bud directly into the ER that contains VP7 and NSP4. A fraction of VP4 has recently been detected in a filamentous array (133) and at the plasma membrane associated with microtubules, although the function of this protein in viral morphogenesis remains unclear (247).

Two pools of VP7 that can be separated biochemically by fluorocarbon extraction or by immunologic methods using two classes of antibodies are detectable in the ER of SA11-infected cells (172). One pool is found only on intact particles and is detected only by a neutralizing monoclonal antibody. The second pool of VP7 is unassembled, remains associated with the ER membrane, and is detected by a polyclonal antibody made to denatured VP7 (172). Distinction of these two forms of VP7 permitted a kinetic study of the assembly of VP7 and of other structural proteins into particles. The incorporation of the inner capsid proteins into double-layered particles was found to occur rapidly, whereas VP4 and VP7 appeared in mature triple-layered particles with a lag time of 10 to 15 minutes. Kinetic analyses of the processing of the oligosaccharides on the two pools of VP7 showed the virus-associated VP7 oligosaccharides had a 15-minute lag compared with that of the membrane-associated form, suggesting that the latter is the precursor to virion VP7. This lag appears to represent the time required for virus budding and outer capsid assembly (172).

Rotavirus maturation is a calcium-dependent process, based on the observation that virus yields are decreased when virus is produced in cells maintained in calcium-depleted medium (323). Viruses produced in the absence of calcium are often found to be exclusively double layered, and budding of virus particles into the ER is not observed (322). Among the viral proteins, reduced levels of VP7 are observed, owing to the preferential degradation, not the impaired synthesis, of VP7 (322). An interesting finding of these studies is that unglycosylated (but not mature) VP7 made in the presence of tunicamycin is relatively stable in a calcium-free environment. Although the role of calcium in morphogenesis was not completely revealed in these studies, it is possible that calcium stabilizes or modulates folding or compartmentalization of the newly glycosylated VP7 for subsequent assembly into particles. The budding process occurs in the absence of calcium, but VP7 is retained within the ER (5). VP7 does not fold properly unless it is expressed with other rotavirus proteins, and calcium must be present in cells for correct epitope formation (92,93). Calcium deprivation may destabilize the ER or ER proteins required for the stable association of glycosylated VP7 with the membrane. Outer capsid assembly also requires proper formation of disulfide bonds on VP7 (342).

Understanding viral morphogenesis has been facilitated by the expression of the rotavirus structural proteins individually or in combinations in insects cells using recombinant baculoviruses (84,194,299,398). This approach first showed that the single-layered VP2 particle shell self-assembles when VP2 is expressed alone, and all of the other capsid proteins have been shown to self-assemble into virus-like particles when coexpressed in the proper combinations (Fig. 10). Virus-like particles composed of VP2, VP1/2, VP1/2/3, VP2/3, VP2/6, VP2/6/7, VP2/4/6/7, and VP1/2/3/6 can be made (84,307,398). The outer and inner capsid proteins of different virus strains have also been shown to reassociate and be

able to be transcapsidated onto other virus strains (62,63). These results demonstrate that the structural proteins contain the intrinsic information required to form particles and that coexpression of mutant proteins is a feasible approach to analyze the domains responsible for the structural interactions between the proteins composing the virus particles. These particles have been useful to analyze the role of cleavage sites in the spike protein in infectivity (127) and the role of individual structural proteins in inducing protective immunity (67,83,167,249,250), to probe the inner structure of particles by analyzing difference maps of particles with distinct protein compositions (307), and to analyze RNA transcription and replication (64,258) and RNA packaging and assembly. These latter studies offer hope that a reverse genetics system will be established for the rotaviruses. Future studies should delineate what mechanisms control packaging of the viral genome and virus assembly.

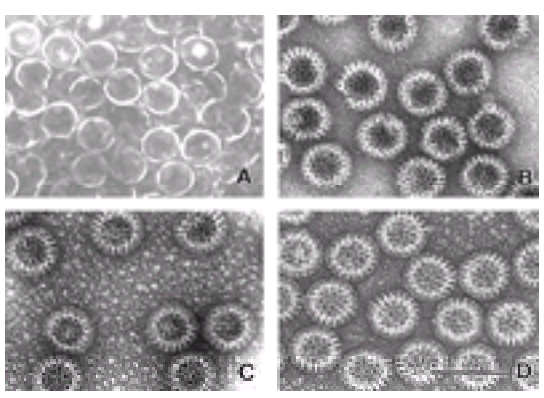

FIG. 10. Rotavirus-like particles self-assemble when the capsid proteins are coexpressed in eukaryotic cells. **A:** VP2. **B:** 2/6-VLPs. **C:** 2/6/7-VLPs. **D:** 2/4/6/7-VLPs. VPL, virus-like particle. (Modified from ref. 84, with permission.)

Virus Release

Electron microscopy studies have shown that the infectious cycle ends when progeny virus is released by host cell lysis in nonpolarized cells (8,55,229). Extensive cytolysis late during infection and drastic alterations in the permeability of the plasma membrane of infected cells result in the release of cellular and viral proteins (240). Despite cell lysis, most double-layered and many triple-layered particles remain associated with the cellular debris, suggesting that these particles interact with structures within cells. Interactions with cell membranes and the cell cytoskeleton have been suggested (240). Whether the cytoskeleton provides a means of transport of viral proteins and particles to discrete sites in the cell for assembly or acts as a stabilizing element at the assembly site and in the newly budded virions, or if particles are simply trapped by the cytoskeleton, remains to be determined.

GENETICS (TYPES OF MUTANTS AND VARIANTS)

Genetic studies have been exploited to understand rotavirus gene structure and function. Temperature-sensitive (ts) mutant collections have been isolated from several rotaviruses, including SA11, UK, and RRV (116,143,294,296,297). The most extensive mutant collection is for SA11, in which 10 of the expected 11 reassortment groups have been identified and six assigned to genome segments. The phenotypes of some of these mutants are consistent with functions inferred from biochemical data or recent sequence information (see Table 4). For example, mutant groups B and C, which mark genome segments 3 and 1, respectively, are defective in the synthesis of single-stranded and double-stranded RNA (58). Because these proteins are likely candidates to be part of the transcriptase particle, further biochemical and sequence analyses of these mutants or establishment of complementation systems with expressed gene products may permit dissection and localization of specific enzymatic domains on VP1 or VP3, or both.

Characterization of the genetic interactions between ts mutants has shown that recombination between pairs of ts mutants of SA11 occurs in the all-or-none fashion expected for recombination by a mechanism of reassortment of genome segments (294). Genetic interactions of rotaviruses are influenced by factors similar to those that affect such interactions of other segmented viruses, including multiplicity of infection and the time of infection (295). Maximal, or near maximal, reassortment frequency is obtained at the earliest times when reassortment can be detected. This suggests that reassortment occurs early in the infectious cycle and that there is a single round of mating, an observation consistent with the particle association of progeny dsRNA. Reassortment is efficient at nonpermissive temperature and mutants from all reassortment groups can interfere with the growth of wild-type virus at both permissive and nonpermissive temperatures. These results are important to consider when trying to produce reassortants.

Reassortment of genome segments among the progeny of *in vitro* mixed infections has been exploited to derive reassortant viruses with desired subsets of genome segments from each parent. Such reassortants have been used to assign ts mutants to specific genome segments and to correlate biologic properties with specific genome segments. For example, the genetic approach has been used to show that genome segment 4 is associated with the properties of hemagglutination, restriction of growth in tissue culture cells, protease-enhanced plaquing, reactivity with neutralizing antibody, and induction of diarrhea in a mouse model (131).

This approach will continue to be used to probe rotavirus gene function; therefore, it is worthwhile to note the criteria needed to reach firm conclusions. The early studies to examine the genome segments responsible for inducing neutralizing antibody identified one member of the genome segment 7, 8, or 9 complex with this property. However, in these studies, no reassortants containing genome segment 4 from a human virus was cultivated and characterized (142). Subsequent studies revealed that genome segment 4 also contains neutralization determinants, highlighting the importance of analyzing reciprocal reassortants in such studies, and the usefulness of selection with specific neutralizing

antibodies to obtain reassortants with desired combinations of VP4 and VP7 (187).

In addition, unexpected properties of parental viruses may influence the interpretation of studies of reassortant viruses. For example, two bovine viruses (UK and NCDV) have been found to share highly related VP7 antigens, but their VP4 antigens are distinct. Reassortant analyses between these two viruses will only allow examination of properties related to the distinct genome segment 4. Because the common properties of parental viruses may not be known in advance, it may be useful to confirm results from reassortment studies using different parental virus strains. The identity of naturally occurring intertypic rotavirus strains (158,159) and the direct demonstration that rotaviruses can undergo reassortment *in vivo* (129) highlight the fact that the genetic background of any rotavirus isolate cannot be assumed.

Naturally occurring rotavirus variants have also been found to be useful probes of gene function. Studies of a variant of the simian rotavirus SA11, which lacked a glycosylated VP7, showed that glycosylation was not essential for virus infectivity or hemagglutination (109,270) and that glycosylation of NSP4 was required for removal of the transient envelope from maturing particles (271). Further studies with neutralization escape mutants made from this variant have shown that glycosylation can affect rotavirus antigenicity (50). Studies with another variant of SA11 that has an altered genome segment 4 have also shown that the phenotype of certain reassortant constellations of genome segments depends on the parental strains used for analysis (56). This phenomenon has been confirmed with other reassortants containing specific combinations of VP4 and VP7, and this is beginning to explain some unexpected antigenic properties of some virus strains (57,95,181,188,241,387,402).

EFFECTS ON HOST CELLS

Rotaviruses have a rather limited tissue tropism, in most cases only infecting the villus epithelium of the small intestine. In addition, the virus generally shows a substantial host range restriction, and symptomatic infection is usually restricted to the young of a species (see Chapter 55). Previously, it was thought that the diarrheal disease resulting from infection is a consequence of a cytocidal infection. Increasing evidence indicates that disease may also result from the actions of viral proteins (e.g., the enterotoxin) as well as other proteins produced by infected epithelial cells (209,401). In nonpolarized tissue culture cells, rotaviruses are normally cytocidal viruses that rapidly kill the permissive cells they infect. Adaption of rotaviruses to culture can be difficult and may require initial passage of virus in primary monkey kidney cells before adaption to growth in continuous monkey kidney cell lines. In permissive cells, cytopathic effect is generally evident, and cell death is preceded by the shutoff of host RNA, DNA, and protein synthesis (48,101,225). Cell death probably results from the function of a viral gene on a specific target rather than from cumulative effects on host metabolism because certain ts mutants (ts groups F, G, H, and I) do not efficiently shut off host cell protein synthesis (104,298). NSP4 is one protein that mediates cell death by causing intracellular calcium levels to increase as well as by affecting the plasma membrane permeability and tight junctions of cells (234,248,345,360). NSP3 reduces host protein synthesis (277).

Upon infection of nonpolarized cells, rotavirus induces early alterations in vimentin (375), in the cytoskeleton organization (376), and in the biochemistry of the host cell (277). A dramatic decrease in the level of host cell proteins and a high level of viral proteins is observed (101). Four proteins that are specifically up-regulated in rotavirus-infected cells have been identified, two of which correspond to the ER chaperones BiP and endoplasmin; however, the role of these proteins in the replication and morphogenesis of rotavirus particles remains to be established (385). In general, little is known about the involvement of host proteins in the diverse steps of the rotavirus replication cycle. It can be expected that a cascade of signals that the virus elicits in the host cell, the cytoskeletal changes induced, and the temporal program of cell gene expression after virus infection will be dissected with the future application of microarray analysis to infected cells *in vivo* or in cultured cells.

Rotavirus infection in polarized human intestinal epithelial (CaCO-2) cells has been studied to determine whether infection occurs asymmetrically from either the apical or basolateral membranes. Initially, rhesus rotavirus was reported to infect polarized Madin-Darby canine kidney (MDCK) and CaCO-2 monolayers in a symmetric manner as does influenza virus (343). However, a reanalysis of rotavirus replication in polarized CaCO-2 cells grown on smaller pore—sized (0.4-µm) membrane filters (where cells do not grow through the filters) shows that RRV only infects polarized cells efficiently through the apical surface (68). Additional studies have shown that rotavirus infection induces microvillar F-actin disassembly (168), and an increase in $[Ca^{2+}]i$ is associated with release of a viral protein or peptide from infected polarized cells that induces a Ca^{2+}-dependent microvillar F-actin alteration in uninfected CaCO-2 cells (43). In addition, rotavirus infection causes alterations in tight junctions independent of virus-induced cytoskeletal rearrangements (251). MDCK cells support only restricted replication of rotavirus, but in both CaCO-2 and MDCK cells, infected cells show transmembrane leaks or opening of tight junctions before the development of cytopathic effect and extensive virus release. Additional studies have shown that RRV rotavirus is released through the apical surface of polarized cells by a nonclassic Golgi-independent vesicular route (170). Attachment and entry of virus into CaCO-2 cells does not cause any measurable transmembrane leak during the first hour of infection. These results suggest that infected epithelial cells could be sloughed from the intestine owing to the loss of tight junctions during the viral replication cycle and before development of cytopathic effect.

The epithelial cell response to rotavirus infection has also been examined in nonpolarized human intestinal cell lines, CaCO-2 and HT-29 cells (49,327). Interleukin-8, growth-related peptide-alpha, and RANTES (*r*egulated upon *a*ctivation, *n*ormal *T*-cell *e*xpressed and *s*ecreted) are secreted from rotavirus-infected cells, and chemokine production is time and dose dependent. Interferons and granulocyte-macrophage colony-stimulating factor have also been detected in mice infected with rotavirus (305). The chemokine secretion response by enterocytes is an active component of the host response to rotavirus infection and may play a role in viral pathogenesis and the in immune response to rotavirus infection.

Finally, rotavirus replication has been examined in polarized neuronal cells, where cytopathic effect again is not obvious. These infections are of interest because expression of VP7 is targeted to axons and dendrites by a pathway that does not involve the Golgi apparatus (374). In contrast, NSP4 is localized solely in the cell body. Rotavirus infection stimulates

alterations of specific neuronal proteins. Thus, the distribution of microtubule-associated protein 2, which is normally restricted to nerve cell bodies and dendrites, is altered and found in axons of cultured dorsal root ganglia and spinal cord neurons. This study indicates that selective interactions between certain viral and neuronal cytoskeletal proteins can occur and that noncytolytic viral infection can cause alteration in the polarized sorting of neuronal proteins (376). These studies are of interest because they highlight the usefulness of monitoring rotavirus protein transport to understand new intracellular trafficking and signaling pathways. It is not known if rotaviruses naturally infect neurons or if such infections might result in activation of the enteric nervous system, which is reported to be a component of the pathogenesis of diarrheal disease (209).

Rotaviruses also have the ability to establish persistent infections in cell cultures (18,65,106,238) and in mice with severe combined immunodeficiency disease (SCID) (303). Persistently infected cells that release low levels of virus for multiple passages have been established in semipermissive (simian rotavirus in rabbit kidney cells) and in fully permissive (bovine rotavirus in bovine kidney and simian rotavirus in MA104) cells. Characterization of these carrier cultures showed that a small percentage of cells were infected and that host–cell resistance to infection was partially overcome by the addition of trypsin to the media (65,106). In another case, a particular bovine rotavirus (strain 2352) that failed to destroy infected BSC-1 cells was apparently important in inducing persistence. Most of these cells (more than 80%) synthesized viral antigen during the S phase of the cell cycle, and the number of cells expressing antigen decreased as the cells moved into mitosis and cell division (18). Permissive MA104 cells that became persistently infected produced higher titers of virus, most of the cells expressed viral antigen, and virus was released from cells without producing cell lysis (238). Analysis of these cells showed that both viruses and the cells coevolved, and mutations in the virus were shown to confer trypsin-independent growth, suggesting that a protease requirement for efficient viral growth is a point of balance for viral growth and cellular survival during maintenance of persistent infection (238). Recognition that rotaviruses cause persistent infections in SCID mice suggests that the *in vitro* observations of carrier cultures may have clinical relevance but that normally such infections are cleared by immune mechanisms. The prolonged excretion and occasional extraintestinal spread of rotaviruses in immunocompromised children indicates that cell-mediated immunity is important in limiting and clearing virus infections (128,164,269,384). However, antibody also plays a role in overcoming persistent infections in animals and children (46,146,147). The detailed mechanisms that regulate persistent virus–cell interactions remain to be elucidated.

INHIBITORS OF REPLICATION AND THEIR MECHANISM OF ACTION

Relatively few studies have evaluated inhibitors of rotavirus replication. Replication is inhibited by fetal bovine serum, most probably owing to its antiprotease activities (110,332) or to other components in serum, such as lecithin, whose mechanism of action is uncharacterized (370).

Evaluations of the effects of antiviral agents as inhibitors of rotavirus replication have shown the following:

1. Nucleoside analogs are effective, probably owing to inhibition of *S*-adenosylhomocysteine hydrolase, a key enzyme regulating methylation of viral mRNA (185).
2. Ribavirin and 3-deazaguanine prevent rotavirus polypeptide synthesis and may exert their antiviral effect by inhibiting the production or modification (capping) of viral-specific mRNA. Polymerase activity is not affected (331).
3. 9-*S*-2,3-dihydroxypropyl adenine is an effective inhibitor of replication with low toxicity *in vivo,* but its mechanism of action is not known (331).
4. Clioquinol, an 8-hydroxyquinoline derivative, may reduce infection of mice if given at frequent intervals, possibly by removing the outer virus capsid (24).
5. Mucin, protease inhibitors, and human milk reduce infectivity in cultured cells or animals (98,99,151,369,392), and some evidence suggests that treatment with probiotic bacteria, live bacteria that survive passage through the gastrointestinal tract and have beneficial effects on the host, may shorten the diarrheal phase of rotavirus infection (87).

Recently, racecadotril, an enkephalinase inhibitor that decreases intestinal hypersecretion but not motility in animals and humans, has been shown to reduce rotavirus-induced diarrhea in hospitalized children (275,316). This is of interest because it supports the new evidence in animals that the pathophysiology of rotavirus diarrhea involves secretory pathways mediated by enterotoxins or neurotransmitters in addition to malabsorption. Additional inhibitors can be expected to be found as the structure and function of the unique rotavirus proteins are characterized and as the molecular mechanisms controlling virus–cell interactions are better understood.

PERSPECTIVES

Our knowledge of rotavirus structure, classification, evolution, and replication continues to increase rapidly. Despite these advances, the world remains without a rotavirus vaccine; the first live attenuated rotavirus vaccine, which was licensed in 1998, was withdrawn from the market in 1999 owing to possible association with induction of intussusception (100). This vaccine failure emphasizes our need to better understand the mechanisms of rotavirus pathogenesis, including details of the molecular interactions between rotaviruses and cells of the intestinal tract. It remains unknown if intussusception will be a problem with all live rotavirus vaccines or whether this adverse event will only be associated with the rhesus rotavirus tetravalent vaccine. If intussusception is found to be associated with all rotavirus infections as a rare event, one might expect to uncover other rare outcomes of rotavirus infection with further epidemiologic studies. As pathogens that infect the gastrointestinal tract, rotaviruses will continue to serve as useful models to understand mucosal virus–cell interactions. The interactions of viral and cellular proteins that influence virus entry into cells, trigger epithelial cell responses, and modulate function of the differentiated epithelial cells or neighboring cells in the complex architecture of the intestine remain to be understood. This requires new studies of virus pathogenesis *in vivo,* and such studies require multidiscliplinary efforts to understand fully the complex biochemical and physiologic consequences of infection. Analysis of cellular responses using microarray analysis will be a start to defining pathways of cellular signaling that may influence the outcome of infection as well as cellular genes important for replication. Future studies on rotaviruses that represent the majority of human and animal rotavirus strains that do not require sialic acid to initiate infection and that

replicate less efficiently in cultured cells clearly are needed to understand how these viruses differ from the well-studied sialic acid–dependent viruses. Will study of these viruses discover that they have other unusual properties that affect their stability or transmission? Development of a reverse genetics system for the rotaviruses, and other double-stranded RNA viruses, remains an important goal that will facilitate future probing of viral gene function in great detail. Achieving this goal may require more knowledge about the unique processes by which rotaviruses inhibit host cell translation as well as enhance virus-specific RNA replication and encapsidation that may be linked and compartmentalized within the cytoplasm of cells. Expanded studies that apply new techniques in cell biology to understand the structural and biochemical basis of the unusual processes of rotavirus entry into and exit from cells can be expected to unravel new mechanisms of protein and vesicle trafficking in cells. Structural knowledge of rotavirus and its proteins should help lead to new methods and inhibitors to interrupt virus transmission, prevent virus replication, and treat disease.

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CHAPTER 55 Rotaviruses

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Rotaviruses are the single most important cause of severe diarrheal illness in infants and young children in both developed and developing countries worldwide (26,385). Although diarrheal diseases are one of the most common illnesses in this age group throughout the world, they assume a special significance in developing countries, where they constitute a major cause of mortality among the young.

The scope of the problem in the United States was addressed in the Cleveland Family Study, in which infectious gastroenteritis (considered nonbacterial) was the second most common disease, accounting for 16% of approximately 25,000 illnesses over a period of about 10 years and averaging 1.52 episodes per person per year (111). This incidence has remained remarkably consistent in two other family studies conducted about 20 or 30 years later (1.2 to 1.9 enteric illnesses per person per year) (188,343,344). Although diarrheal diseases are not a prominent cause of mortality in infants and young children in the United States, a review of vital statistics indicated that between 1973 and 1983, 5,539 children 1 month to 4 years of age died from diarrheal disease (average, 504 deaths per year) and 79% of these deaths occurred in the less-than-1-year age group (206).

The toll from diarrheal diseases in developing countries is staggering. It is estimated that 1 billion diarrheal illnesses and 3.3 million deaths from diarrhea occur annually in the under-5-year age group in developing countries of Africa, Latin America, and Asia (excluding China) (26). Furthermore, diarrheal illnesses consistently rank as one of the top six causes of all deaths, one of the top three causes of death from an infectious disease, and one of the top two causes of death when considering years of life lost (352,353,523,524).

HISTORY

Despite the magnitude of the problem of infantile diarrhea, the search for important etiologic agents—bacterial, viral, or parasitic—was unrewarding until the 1970s. This was especially disappointing for virologists, because with the advent of tissue culture technology, scores of enteric viruses were discovered in the 1950s and 1960s, but none was found to be an important etiologic agent of infectious diarrhea (539).

This frustration ended with the discovery in 1972 by Kapikian et al. (262) of the 27-nm Norwalk virus and its association with epidemic viral gastroenteritis in older children and adults, followed by the discovery in 1973 by Bishop et al. (38) of the 70-nm human rotavirus and its association with severe endemic diarrhea in infants and young children. Both of these fastidious agents were discovered without the benefit of tissue culture technology; by necessity, their identification relied on direct visualization by electron microscopy (EM). The Norwalk virus was identified in feces by immune electron microscopy (IEM), whereas the first human rotavirus was visualized by thin-section EM of duodenal mucosa. Shortly afterward, rotavirus was identified in feces by EM by Flewett et al. (130), Bishop et al. (39), and others (261,333). The Norwalk and related viruses described in Chapter 27 cause over 50% of community outbreaks of nonbacterial gastroenteritis in older children and adults, and although these viruses are associated with mild gastroenteritis in infants and young children, they have not been established as an important cause of severe diarrhea in this age group (242).

It soon became apparent that the 70-nm particle (Fig. 1), subsequently designated rotavirus, was an important etiologic agent of diarrhea of infants and young children, causing about 35% to 50% of the hospitalizations for this form of gastroenteritis during the first 2 years of life (245). In a relatively short period, investigators from many countries reported

the detection of rotaviruses in feces of pediatric patients with diarrheal illness, and it was soon established that rotaviruses were the long-sought-after major viral etiologic agents of severe diarrhea of infants and young children in both developed and developing countries, consistently outranking in importance other known etiologic agents of severe diarrhea (Fig. 2).

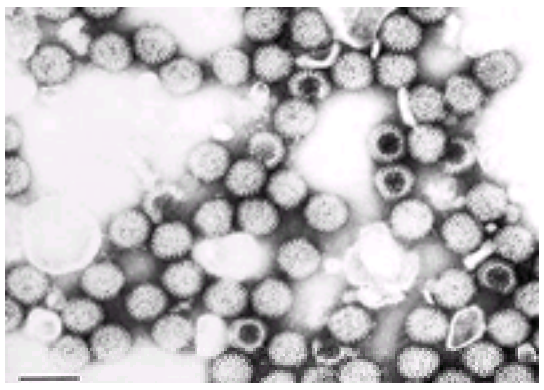

FIG. 1. Human rotavirus particles observed in a stool filtrate (prepared from a stool of an infant with gastroenteritis) after incubation with phosphate-buffered saline and further preparation for negative-stain EM. The particles appear to have a double-shelled capsid. Occasional “empty” particles are seen. Scale bar: 100 nm. (From ref. 261, with permission.)

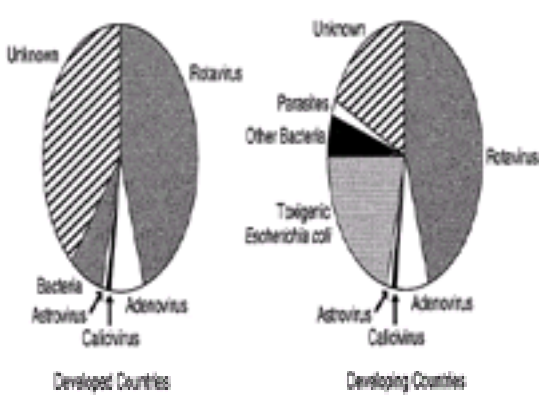

FIG. 2. An estimate of the role of etiologic agents in severe diarrheal illness requiring hospitalization of infants and young children in developed countries and in developing countries. (From ref. 245, with permission.)

Although the human rotaviruses were discovered in 1973, it should be noted that in 1963, Adams and Kraft, using thin-section EM, described virus-like particles in intestinal tissue of mice infected with epizootic diarrhea of infant mice (EDIM) virus (2). These particles were similar to those observed by Bishop et al. (38). In 1963, Malherbe and Harwin (311) described the isolation of a 70-nm virus designated SA11 (simian agent 11). This virus, derived from a rectal swab obtained from a healthy vervet monkey, was recovered in vervet monkey kidney cell culture. In 1967, Malherbe and Strickland-Cholmley (312) described another virus similar to SA11, the O (offal) agent, which was isolated in vervet monkey kidney cell culture from the mixed washings of intestines of cattle and sheep. In addition, in 1969, Mebus et al. (327) demonstrated the presence of 70-nm virus particles in stools from calves with a diarrheal illness and showed that this agent could be passaged serially in calves with production of disease. In 1971, Mebus et al. (328) reported successful cultivation of the Nebraska calf diarrhea virus (NCDV) in primary fetal bovine cell cultures; in 1972, Fernelius et al. (129) reported that the NCDV resembled the reoviruses morphologically but was distinct antigenically. The murine, simian, O, and bovine agents were later found to exhibit characteristic rotavirus morphology and to share a group antigen with other rotaviruses (131,250).

CLASSIFICATION

Rotaviruses are classified as a genus in the family *Reoviridae*, which contains eight other distinct genera (408). The unifying features of the rotaviruses that are responsible for their classification into a separate genus are detailed in Chapter 54.

INFECTIOUS AGENTS

Morphology and Morphogenesis

Rotaviruses have a distinctive morphologic appearance by negative-stain EM. Complete particles measure about 70 nm in diameter and have a distinctive double-layered icosahedral protein capsid that consists of an outer and an inner layer when viewed by transmission EM (see Fig. 1). Within the inner capsid is a third layer, the core, that contains the virus genome consisting of 11 segments of double-stranded (ds) RNA. The complete particles are also designated smooth particles because the outermost margin of the outer capsid layer has a well-defined, smooth circular appearance. Particles lacking the outer capsid layer measure about 55 nm in diameter and are designated rough particles because without the smooth outer layer, the capsomeres of the inner capsid project to the periphery, giving a circular, “bristly” appearance. The core, which appears to be hexagonal in outline in certain negative-stain preparations, measures about 37 nm in diameter. The term *rotavirus* is derived from the Latin word *rota*, which means wheel, and it was suggested because the sharply defined circular outline of the outer capsid gives the appearance of the rim of a wheel placed on

short spokes radiating from a wide hub (131).

Studies of the three-dimensional structure of unstained, unfixed, rotavirus particles using cryoelectron microscopy and image processing techniques have yielded important new information on the ultrastructure of rotaviruses. These and other findings relating to ultrastructure and morphogenesis are described in Chapter 54.

Physicochemical Properties and Genetics

The physicochemical properties of rotaviruses, and their genetics (including genome organization and coding assignments of the 11 RNA segments) are presented in detail in Chapter 54.

Genome

The rotavirus genome contains 11 segments of dsRNA, which have a size range of 0.6 to 3.3 kilobase pairs (319). The RNA segments fall into four size classes based on contour length measurements by EM and confirmed by nucleotide sequence analysis of each of the 11 genes (see Chapter 54). The distribution of the 11 segments into these four size classes is also evident by polyacrylamide gel electrophoresis (PAGE) of RNA as shown for representative human serotypes in Figure 3. Characteristically, these RNA segments are numbered in order of migration during PAGE, with the slowest RNA segment designated gene 1, and so on. For group A rotaviruses, there are four large segments, two medium-sized segments, three smaller segments, and the two smallest segments. This pattern contrasts with that of the reoviruses, which contain 10 RNA segments that are distributed into three size classes—large, medium, and small. These size designations are relative, because the tenth (i.e., the smallest) RNA segment of reovirus is larger than the five smallest segments of rotavirus (208).

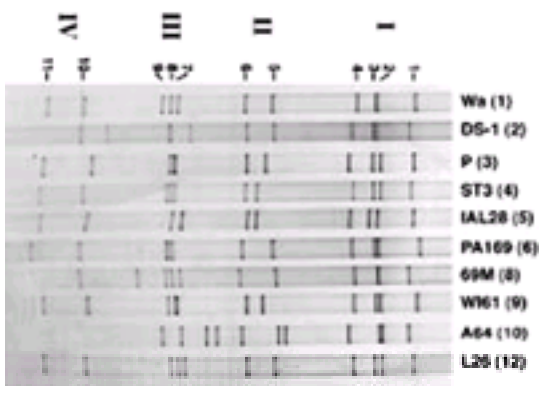

FIG. 3. Electrophoretic migration pattern (electropherotype) of RNAs of the 10 human rotavirus G serotypes. The viral RNAs were analyzed by electrophoresis in a 10% polyacrylamide gel and visualized by staining with silver nitrate. The *number in parentheses* shows G serotype. The strains DS-1 (G serotype 2) and 69M (G serotype 8) demonstrate the so-called short and supershort electropherotypes, respectively. The A64 strain (G serotype 10), which was isolated from a chronically infected immunocompromised child (228), carries rearranged RNA segments 8, 10, and 11.

Some group A rotaviruses do not display the characteristic RNA migration pattern just described (425). For example, murine rotavirus segment 11 migrates close to segment 10, whereas avian rotavirus segment 5 migrates close to segment 4, and segments 10 and 11 are difficult to resolve (Fig. 4). A lapine rotavirus that had a pattern of 4,2,4,1 instead of the usual 4,2,3,2 distribution is also shown in Figure 4. In some human rotavirus strains, the 11th (cognate) RNA segment migrates more slowly than usual and is thus located between the 9th and 10th (cognate) segments, yielding a “short” pattern characteristic of almost all antigenic subgroup I human rotaviruses [which are almost always viral protein 7 (VP7) serotype 2 strains] (see Fig. 3) (209,216). Similarly, a “long” 10th and 11th RNA segment pattern of human rotaviruses is characteristic of almost all subgroup II human rotaviruses. Moreover, several “super-short” patterns of RNA migration in which the 11th (cognate) gene segment migrated even more slowly than the “short”-pattern rotaviruses have been observed for viruses recovered from humans, bovines, porcines, and lapines (320) (see Fig. 3 and Fig. 4). These strains also exhibit subgroup I antigenic specificity. In addition, several human, porcine, bovine, ovine, caprine, canine, rodent, and avian rotavirus strains that do not share the common group antigen of conventional (group A) rotaviruses (but also have a genome with 11 double-stranded segments of RNA) display quite distinct migration patterns (Fig. 5) (49,424). Such strains, which belong to group (i.e., serogroup) B, C, D, E, F, or G, are referred to as non–group A rotaviruses. They were formerly termed pararotaviruses. Because of the segmented nature of the rotavirus RNA genome, individual gene segments reassort at relatively high frequency following co-infection *in vivo* or *in vitro* (401). However, reassortment between members of different groups (e.g., between groups A and B viruses) has never been documented, suggesting that each group may represent a separate gene pool.

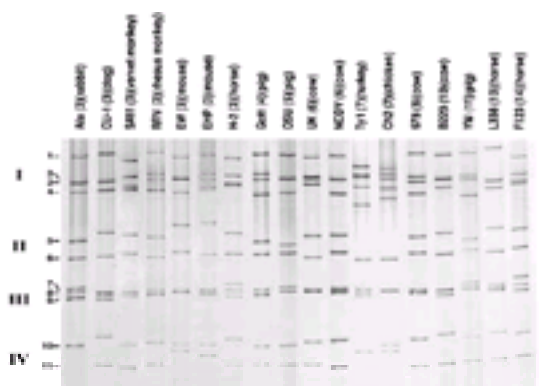

FIG. 4. Electrophoretic migration pattern of RNAs of 10 of the 13 animal rotavirus G serotypes. The viral RNAs were analyzed by electrophoresis in a 10% polyacrylamide gel and visualized by staining with silver nitrate. The *number in parentheses* shows G serotype followed by the animal species from which the virus was isolated.

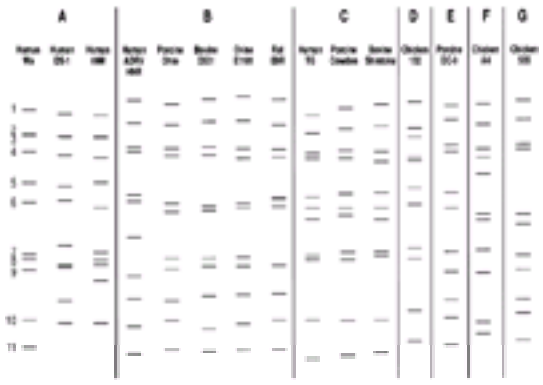

FIG. 5. Schematic diagram of electrophoretic migration patterns of RNAs of representative rotaviruses belonging to groups A to G.

Because human rotaviruses could not be propagated initially in tissue culture, comparison of the migration patterns of RNA during PAGE became an important laboratory and epidemiologic technique for characterization of strains. The term *electropherotyping* was applied to this method of differentiating strains (123,209). It soon became apparent that human rotaviruses exhibited a wide variety of electropherotypes and that these patterns differed from those observed with animal rotavirus strains. However, it was not known whether differences or similarities in electrophoretic pattern had any relationship to antigenic specificity of the human strains as defined by neutralization (i.e., serotype). Subsequent studies indicated that viruses of the same serotype can exhibit different electropherotypes, and that viruses of the same electropherotype can belong to different serotypes.

The extent of genetic relatedness among rotavirus strains has also been studied using labeled (+) single-stranded (ss) RNAs transcribed from viral cores as hybridization probes (Fig. 6) (356). The (+) ssRNAs represent messenger RNAs that are transcribed from the negative strand of genomic RNA by the viral polymerase. These (+) mRNAs appear to be colinear and coterminal with genomic (–) RNA. Early studies established that human, simian, and bovine rotaviruses were completely distinct when analyzed by this technique using hybridization conditions of high stringency (135).

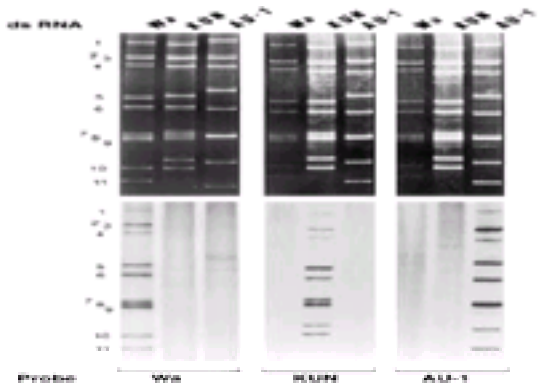

FIG. 6. RNA–RNA hybridization as used for study of genetic relatedness among three human rotavirus genogroups (families). ^{32}P-labeled RNA transcripts (probe) of the strain Wa, KUN, or AU-1 were hybridized to denatured genomic double-stranded (ds) RNAs of the Wa, KUN, or AU-1 virus. After hybridization at 65°C for 14 hr, the hybridized RNAs were pelleted and subjected to polyacrylamide gel electrophoresis. The resulting pattern was visualized by staining with ethidium bromide (**top panel**) and then by autoradiography (**lower panel**). Note that there was no significant homology among the three strains, as demonstrated by lack of formation of significant genomic length hybrids as observed by autoradiography. The homologous reaction yielded hybrids of 11 bands. (From ref. 355, with permission.)

Examination of rotaviruses obtained on a single day from children with gastroenteritis in Venezuela revealed numerous electropherotypes by PAGE (135). However, when these rotaviruses were tested with hybridization probes prepared from the human Wa (serotype 1) or DS-1 (or an equivalent serotype 2 strain KUN) virus, the RNA segments of each rotavirus exhibited significant hybridization to either the Wa probe or the KUN probe, but not to both. This observation led to the suggestion that there are at least two distinct families of human rotavirus (135). Subsequently, the existence of a third genetic family of human rotavirus designated AU-1 was demonstrated (see Fig. 6) (356). Additional studies revealed that gene segments with similar migration patterns by PAGE did not necessarily exhibit homology by hybridization; on the other hand, some gene segments that exhibited homology by hybridization varied in their electrophoretic migration (135).

Rearranged genome segment(s) have been observed in strains recovered from both immunocompetent and immunodeficient hosts (106). As noted, the 11th (cognate) segment of the “short” (DS-1) or “supershort” (69M and Ala) strains (see Fig. 3 and Fig. 4) is an example of such a rearranged genome that possesses a partially duplicated genome

structure (106). A rotavirus strain isolated from an immunodeficient, chronically infected child often displays multiple forms of genome rearrangements. The A64 strain (see Fig. 3) exemplifies such a virus with rearranged RNA segments 8, 10, and 11 (228). In addition to partial gene duplication, partial gene deletion also occurs in ature (106). Furthermore, genome rearrangement can be generated readily *in vitro* by passaging a rotavirus strain at high multiplicity of infection in cell cultures (227). Of note are the observations that (a) a VP7 serotype 2 human rotavirus strain that occurs worldwide consistently displays a rearranged “short” pattern (see Fig. 3), suggesting that a rotavirus with rearranged gene 11 is stable and “fit” in both *in vivo* and *in vitro* replication; (b) genome rearrangements occur exclusively in genes encoding nonstructural proteins, except for a rare example of a VP6 gene rearrangement (445); and (c) a rearranged genome segment encoding VP1, VP2, VP3, VP4, or VP7 has never been detected. Genome rearrangements (concatemerization and/or deletion) have been proposed to be a third mechanism of rotavirus evolution in nature, besides genomic point mutations and accumulations, as well as genetic reassortments (472).

Gene Coding Assignments

The gene coding assignments and rotavirus proteins are described in detail in Chapter 54.

Analysis of the gene coding assignments for each of the 11 genes established that there are six structural and six nonstructural proteins (408). The proteins of SA11 have been studied more thoroughly than those of other rotaviruses, in part because this virus was among the first of this group of agents to be propagated efficiently in cell culture (311). As shown in Figure 7, RNA segment 1, 2, or 3 of SA11 encodes a core or subcore structural VP, designated VP1, VP2, or VP3, respectively, whereas segment 6 encodes the major inner capsid protein VP6. Segments 4 and 9 encode the outer capsid structural proteins VP4 and VP7, respectively (319). The six nonstructural (NS) proteins, which are found in infected cells but not in virions, are encoded by segments 5, 7, 8, 10, and 11 and are designated as NSP1, NSP2, NSP3, NSP4, NSP5, and NSP6.

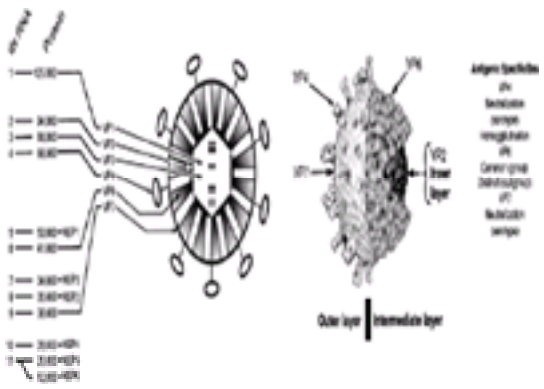

FIG. 7. Left: Schematic representation of the rotavirus particle. **Right:** Surface representations of the three-dimensional structures of the outer layer of the complete particle (*left*) and a particle (*right*) in which the outer layer and a small triangular portion of the intermediate layer have been removed, exposing the inner layer. (Modified from ref. 260, with permission. The three-dimensional figure on the right is courtesy of B. V. V. Prasad.)

VP4 is cleaved specifically by the proteolytic enzyme trypsin to form cleavage products designated VP5* [molecular weight (MW) 60,000] and VP8* (MW 28,000) (319). Proteolytic cleavage of VP4 potentiates infectivity by enhancing penetration of virus into cells. VP4 is a multifunctional protein, as discussed later in this chapter. The sixth gene encodes VP6, which contains the subgroup antigen as well as the antigen that acts as the main determinant of group reactivity (319). The ninth gene (or the seventh or eighth gene in certain rotavirus strains) encodes the neutralization protein VP7, which is largely responsible for serotype specificity defined by neutralization with hyperimmune antiserum (318).

Propagation and Assay in Cell Culture

Various animal rotaviruses, such as SA11 and the O agent, grow readily in cell culture. However, the human rotaviruses are rather fastidious agents that were not grown efficiently in tissue culture until relatively recently (437,484,527). Initially, they were observed to induce an abortive infection of cells in culture; viral antigens were produced, but infectious virus could not be passaged serially (526). The first human rotavirus to be grown efficiently and reproducibly in cell culture was a mutant of the Wa human rotavirus (VP7 serotype 1) that emerged during 11 serial passages of this strain in gnotobiotic piglets (527). Efficient growth of the mutant required pretreatment of virus with trypsin.

Because early attempts to propagate the noncultivatable human rotavirus DS-1 (VP7 serotype 2) in piglets were not successful, another strategy to cultivate this virus was adopted (179). This strategy took advantage of the well-known property of the *Reoviridae* to undergo genetic reassortment with high efficiency during co-infection in cell culture. Cell cultures were co-infected with a “noncultivatable” human rotavirus (such as DS-1) and a cultivatable bovine rotavirus temperature-sensitive (ts) mutant. Subsequently, the growth yield at restrictive temperatures from the co-infected cultures was analyzed for cultivatable reassortants (i.e., viruses with a mixed genotype) that were antigenically similar to the human rotavirus parent. Rescue of noncultivatable human rotavirus was achieved using two types of selective pressure that favored cultivatable reassortants with the neutralization specificity of the human rotavirus parent: (a) the bovine rotavirus ts mutant parent and reassortants bearing ts genes were selected against by incubating cultures inoculated with the growth yield at restrictive temperature; and (b) viruses with bovine rotavirus neutralization specificity were selected against by incubating the growth yield with bovine rotavirus antiserum. The reassortants that were selected in this manner grew efficiently in cell culture, produced plaques at the restrictive temperature, had the neutralization specificity of the noncultivatable human rotavirus parent, and were of mixed genotype. With this technique, 33 of 52 human rotaviruses were successfully rescued, and the serotypic diversity of these viruses was demonstrated by conventional

neutralization assays (181).

Later, efficient techniques were developed for the direct cultivation of most human rotaviruses in cell culture; these methods have undergone slight modifications as presented later (437,484). Cultivation is achieved by pretreatment of virus with trypsin (5 to 10 µg/mL), incorporation of trypsin (0.5 to 1 µg/mL) in the maintenance medium, the use of roller-tube cultures of simian MA104 cells, and incubation of inoculated cultures at 37°C. Following growth in roller-tube cultures, most human rotavirus strains also produced plaques under an overlay medium containing 0.6% agarose with 0.5 µg/mL trypsin. Virus present in positive fecal samples can be isolated successfully in roller-tube cultures with over 75% efficiency. However, cultivation of rotaviruses from rectal swab specimens is not nearly as efficient. Primary African green or cynomolgus monkey cell cultures, as well as continuous human colon adenocarcinoma cell lines (CaCo-2 and HT-29), appear to be more efficient than MA104 cultures for isolation of rotaviruses from clinical specimens (93,461,508). The non–group A rotaviruses have also proven to be quite fastidious. For example, only a single porcine group B strain and only a few porcine, human, or bovine group C strains have been successfully cultivated (426,429,446,469,477).

ANTIGENIC COMPOSITION

Designation of Serotypes and Subgroups

Rotaviruses have three important antigenic specificities: group, subgroup, and serotype. The group antigen is shared among all rotaviruses of a particular group. At present, groups A, B, C, D, E, F, and G are recognized (49,424). These cross-reactive (group) epitopes are present on all the structural proteins and most likely on the nonstructural ones, but in diagnostic assays VP6 is the predominant group antigen that is detected, perhaps because it constitutes 51% of the virion (319). VP6 also mediates subgroup specificity, which serves as an important epidemiologic marker because most strains belong to either subgroup I or subgroup II (249,319). The group and subgroup antigens do not induce neutralizing antibodies, although *in vivo* protection by selected anti-VP6 IgA monoclonal antibodies (MAbs) was recently described in a “backpack tumor” murine model (59). These antibodies were not active when applied directly to the intestinal lumen. It is speculated that this activity is caused by *in vivo* intracellular viral inactivation during transcytosis. In addition, it is of interest that a VP6 DNA vaccine induced protection in mice from rotavirus challenge (199).

Distinct serotypes among the group A rotaviruses have been identified by neutralization assay using hyperimmune antisera raised in various animals. Although neutralization assays measure antibodies to VP4 and VP7, the major reactivity of such sera is a function of antibodies to the VP7 outer capsid protein. However, a serotype scheme for VP4 based on neutralization was recently developed (166,216). Thus, in designating the antigenic composition of a rotavirus, four antigenic specificities must be considered: group, subgroup, and serotype mediated by VP7 (designated a G serotype because VP7 is a glycoprotein), and serotype mediated by VP4 (designated a P serotype because VP4 is protease sensitive) (319). For example, the human Wa virus is group A, subgroup II, G1, and P1A, whereas the DS-1 virus belongs to group A, subgroup I, G2, and P1B. Roman letters are used to designate group; Arabic numbers, both G and P serotypes; and Roman numerals, subgroup (13). A VP4 classification system based on genotype was recently adopted and is discussed later in this and the VP4 sections.

Because of the increasing number of “exceptions to the rule,” the tidy distinction between subgroup I and II strains by PAGE is not as clear-cut as presumed from earlier studies when human viruses with the “short” electrophoretic pattern formed a homogeneous group, VP7 serotype 2 by neutralization, whereas human viruses with the “long” pattern belonged to VP7 serotypes 1, 3, or 4 (216). It should also be noted that several human or animal rotaviruses have been detected that cannot be classified into either subgroup I or subgroup II, or that have both subgroup I and subgroup II specificities (216).

The development of techniques for cultivation of human rotaviruses in cell culture made it possible to identify virus serotype by conventional assays, which include (a) neutralization of cytopathic effect (CPE), (b) reduction of virus yield in roller-tube or multi-well plate cultures, (c) plaque or focus reduction, and (d) viral interference (216). The criterion for establishing a distinct VP7 serotype is a reciprocal, greater-than-20-fold difference in serum antibody titer when a candidate serotype is tested against prototype viruses representing established serotypes (225). With this standard among the group A rotaviruses, 14 VP7 (G) serotypes have been identified, 10 in humans and 13 in animals (Table 1) (216). Two or possibly three serotypes of group C rotaviruses have been reported (476).

TABLE 1. *Serotypic and genotypic classification of group A rotavirus VP7 and VP4*

Although the sharing of subgroup specificity by certain animal and human rotaviruses was surprising, the sharing of VP7 neutralization specificity by certain human and animal viruses was completely unexpected (225) (see Table 1). Initially, rhesus rotavirus and SA11 were shown by plaque reduction to be similar, if not identical, to human rotavirus serotype 3.

Further studies that involved extensive reciprocal cross-neutralization tests among human and animal rotaviruses revealed that (a) only one of the 10 human rotavirus serotypes (i.e., serotype 12) is restricted to humans; (b) serotype 3 viruses have the broadest host range, which includes humans, monkeys, dogs, cats, horses, pigs, mice, rabbits, and sheep; and (c) serotypes 11, 13, or 14 strains are restricted to horses or pigs, whereas serotype 7 appears to be restricted almost exclusively to birds (216). Although there is extensive sharing, certain serotypes occur almost exclusively in either humans or animals—for example, serotypes 1 and 2 are found often in humans but rarely in animals. It is of interest that the porcine rotavirus strains Gottfried and SB-2, which share serotype specificity, belong to different subgroups (216). This was the first example of shared neutralization but not subgroup specificity. It is surprising that this dissociation had not been observed more often, since serotype and subgroup specificities are encoded by different genes.

A system for categorizing rotaviruses according to VP4 has been proposed based on nucleic acid hybridization and sequence analysis of the VP4 gene (122). Currently, 21 VP4 genotypes have been described (216, 403). A recently developed VP4 classification scheme based on neutralization assay, using the same criteria as described previously for VP7, identified 13 distinct VP4 serotypes, including three subtypes (see Table 1) (216; and Hoshino et al., unpublished studies). The results of this classification were concordant, in general, with the genotypes established by hybridization or sequence analysis. To integrate the VP4 serotype and genotype designations, the letter *P* followed by an open Arabic number is reserved for serotype as determined by neutralization assay, whereas the VP4 genotype is designated by an Arabic number shown in brackets following the serotype designation (e.g., Wa virus is designated P1A [8]) (216).

Antigenic Specificities of Various Proteins

The VP6 Protein

VP6, which is located on the inner capsid of the virus, has a molecular weight of 41,000 and constitutes 51% of virion protein. Murine MAbs to the VP6 of human Wa (VP7 serotype 1, subgroup II) or rhesus rotavirus (VP7 serotype 3, subgroup I) react with almost all mammalian group A rotaviruses in various immunoassays (176). Although other structural proteins may show group reactivity under selected conditions, VP6 specifies the major group reactivity measured by such assays. However, two MAbs that immunoprecipitate VP6 have a more restricted activity (176). One of these antibodies, induced by rhesus rotavirus, reacted only with subgroup I viruses; the other antibody, induced by Wa rotavirus, reacted only with subgroup II viruses. When reassortant viruses of known genotype derived from co-infection of cells with Wa (a subgroup II rotavirus) and UK (a subgroup 1 rotavirus) were studied, it was observed that the MAb with subgroup II specificity reacted only with reassortants whose sixth gene was derived from Wa virus, whereas the subgroup I–specific MAb reacted only with reassortants whose sixth gene was derived from the UK rotavirus. None of the other rotavirus genes segregated with MAb reactivity. Thus, a domain of the sixth rotavirus gene product, VP6, specifies subgroup reactivity, whereas a separate domain(s) of this protein contains the major determinant of group A rotavirus antigenic reactivity (319). The site of attachment of VP6 subgroup MAbs on rhesus rotavirus (RRV) has been visualized clearly by IEM (248). These antibodies attach to the inner but not the outer capsid of the rhesus rotavirus.

The amino acid composition of the VP6 protein of several group A, B, and C rotavirus strains has been determined. Immunodominant sites of VP6 that contain group-specific epitopes for the group Arotaviruses have been localized in four regions on VP6 (amino acid residues 32 to 64, 155 to 167, 208 to 294, and 380 to 397) (280). In addition, amino acid residues 172, 305, and 315, and region 296 to 299 were reported as contributing to subgroup epitopes (302,466).

The VP6 protein is a target of both G serotype-specific and G cross-reactive cytotoxic T lymphocytes (CTLs) in mice (372). The serotype cross-reactive CTL epitope on the VP6 has been mapped to amino acid residues 376 to 384 (144). The VP6 protein has also been shown to carry an epitope that stimulates proliferation of cross-reactive helper T cells of $L3T4^+$ phenotype (53).

It was assumed until recently that all human and animal rotaviruses share common antigenic determinants present predominantly on VP6. However, several human and animal rotavirus strains do not share these determinants. Rotaviruses that share the group antigen are now classified in group A, whereas rotaviruses that lack this antigen are classified as non–group A viruses (49,424,468). The latter viruses also do not share subgroup antigens with group A viruses. The non–group A viruses have been given various names, such as pararotaviruses, rotavirus-like viruses, novel rotaviruses, antigenically distinct rotaviruses, and adult diarrhea rotaviruses. Currently, these viruses are classified as belonging to group B, C, D, E, F, or G. Group B viruses have been recovered from humans, pigs, cattle, sheep, goats, and rats; group C viruses from humans, pigs, cattle, dogs, and ferrets; group D viruses from chickens, pheasants, and turkeys; group E viruses from pigs; group F viruses from chickens and turkeys; and group G virus from chickens (49,108,187,380,424). A group B rotavirus was associated with (a) several large outbreaks of severe gastroenteritis in China that predominantly involved adults, (b) a mild outbreak of diarrhea in neonates in China, and (c) an outbreak of diarrhea in adults in India (97,287,308,468). Moreover, group C rotaviruses that have been detected in various locations of the world have not assumed epidemiologic importance (308,379). The non–group A rotaviruses appear to be of minor importance outside China.

The VP7 Protein

The VP7 glycoprotein is the major neutralization antigen of rotaviruses detected by hyperimmune antiserum and has served as the basis for identification of serotype (216). VP7, which has a MW of 38,000, is located on the outer capsid, forming the smooth external surface of the outer shell, and it constitutes 30% of the virion protein, thus making it the second most abundant rotavirus protein as well as the major constituent of the outer capsid (see Fig. 7) (319). VP7 is a glycoprotein that contains one to three potential sites for N-linked glycosylation, but carbohydrate side chains are not required for infectivity because a fully infectious mutant of simian rotavirus SA11 that lacks a glycosylation site has been described.

Early studies in which reassortant rotaviruses were analyzed for genotype and antigenic specificities indicated that most

neutralizing antibodies in hyperimmune antiserum were directed against the VP7 protein, which is encoded by the seventh, eighth, or ninth gene, depending on the strain (178,319). Also, many rhesus rotavirus MAbs that react with the outer capsid VP7 glycoprotein neutralize rhesus rotavirus to high titer (180). The site of attachment of these VP7 MAbs has been identified by IEM as the outer capsid of the virus (248). Thus far, group A rotaviruses have been classified into 14 distinct serotypes in reciprocal neutralization tests with hyperimmune antisera (see Table 1). Serotype identification of rotaviruses has been facilitated by the isolation of VP7 MAbs that are specific for the epidemiologically important human serotypes (1, 2, 3, or 4) as well as various other serotypes (215,216). Strains within a serotype that vary in their capacity to be neutralized by serotype-specific MAbs have been termed monotypes (88). VP7 has also been proposed to be responsible for attachment of virus to host cells, but most studies suggest that VP4 functions in this capacity (319).

The amino acid sequence of the VP7 of strains belonging to each of the 14 serotypes has been deduced from the nucleotide sequence of the VP7 gene. A comparison of the deduced amino acid sequences for all 14 serotypes indicates that there are nine regions of the linear amino acid sequence of VP7 that are highly divergent (Fig. 8) (172,363). Each of these regions is highly conserved in rotavirus strains within the same serotype. There is sufficient conservation of sequence of human rotavirus strains within a serotype that it is possible to predict the serotype of an isolate by direct sequence analysis of two of these variable regions (VR5 or A, and VR8 or C) of VP7 (174).

FIG. 8. Comparison of the deduced amino acid sequences of the VP7 protein from rotavirus strains of 14 different G serotypes. The conserved regions are *shaded*; hydrophobic regions are denoted by ▬; the hydrophilic regions are denoted by ⋀⋀⋀; variable regions (VRs) are denoted by [ ]; potential glycosylation sites are enclosed in o; conserved prolines are indicated by ▼; conserved cysteines are indicated by n; and the signal peptide cleavage site (amino acids 51) is indicated by an *arrow*.

Representative levels of identity of the nucleotide sequence of the VP7 gene as well as the deduced amino acid sequence of the VP7 protein among homotypic and heterotypic strains of VP7 serotypes 1 to 14 are shown in Figure 9. Within each serotype, amino acid homology was high (91% to 100%); however, between strains of different serotypes, conservation of sequence was significantly less. Thus, there was complete concordance between relationships among rotaviruses as determined by neutralization or sequence analysis of VP7. Conservation of VP7 amino acid sequence has been examined in more detail within serotype 3; this serotype has the broadest host range, encompassing ten different host species, including humans. Comparison of the deduced VP7 amino acid sequence of 27 strains recovered from eight of ten species indicated an overall homology of 85% or greater (363). With the exception of two feline strains, which had 90.8% deduced VP7 amino acid homology, conservation of VP7 sequence was greatest among strains recovered from the same species, with homology being 95% or greater. For example, among 13 serotype 3 human rotavirus strains, there was 95.3% or greater identity of VP7 sequence.

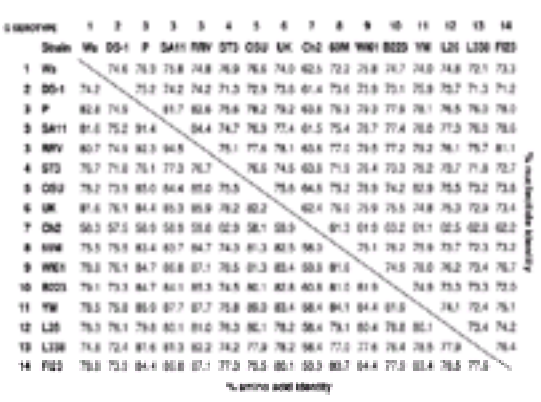

FIG. 9. The nucleotide and deduced amino acid identities among the VP7 genes of 14 rotavirus G serotypes. Three G serotype 3 rotavirus strains are included to show high nucleotide as well as deduced amino acid identities among them.

The major neutralization epitopes on VP7 have been identified by analysis of the deduced amino acid sequence of mutants that are resistant to neutralization by the VP7 MAb used for their selection (112). These were designated as antigenic region A (i.e., amino acids 87 to 99), B (amino acids 145 to 150), or C (amino acids 211 to 223) (Fig. 10). Each of the neutralization escape mutants analyzed sustained a single amino acid substitution in VP7, and these mutations occurred predominantly in VR5, 7, or 8, which corresponded to regions A, B, or C, respectively. The location of amino acid substitutions in mutants selected with hyperimmune sera were in general agreement with sites selected by MAbs (see Fig. 10). However, some additional sites in both conserved and variable regions on the VP7 were selected by MAbs

or hyperimmune antisera (167). It should be noted that monotype-specific as well as serotype-specific and serotype-cross-reactive epitopes have been mapped to the three major antigenic regions A (VR5), B (VR7), and C (VR8).

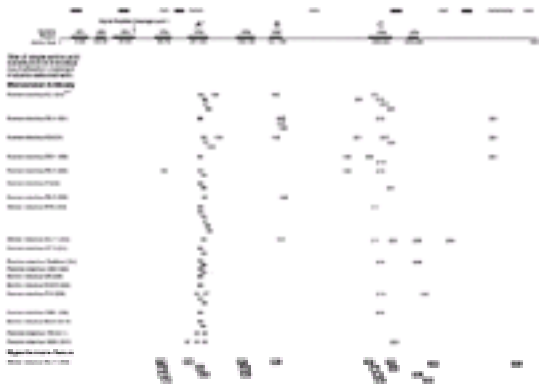

FIG. 10. Linear structure and antigenic organization of rotavirus outer capsid VP7. *Boldface numbers* indicate amino acid substitutions in mutants selected with monoclonal antibody exhibiting cross-reactive (i.e., heterotypic) neutralizing activity; *numbers not in boldface and not in boxes* indicate amino acid substitutions selected with MAb with homotypic neutralizing activity. *Numbers in boxes* indicate amino acid substitutions in mutants selected with hyperimmune serum to SA11 virus. At the *top* of the figure, hydrophobic regions are denoted by ▬, whereas hydrophilic regions are denoted by ⩘. (Adapted from ref. 215, with additions from refs. 73,74,89,159,219, and 293, with permission.)

The importance of glycosylation in the antigenicity of VP7 was shown by the effect of a single amino acid substitution at position 211 of simian virus SA11 (64). This mutation created a new glycosylation site, and the resulting mutant was rendered significantly resistant to neutralization by hyperimmune antiserum. Based on these observations, it was suggested that VR8*, which contains amino acid 211 (or a region contiguous to it in the folded protein), constitutes an immunodominant site that is rendered inaccessible to its corresponding neutralizing antibodies by the addition of a new carbohydrate side chain. This region appears to be the dominant antigenic site on SA11 VP7, but a generalization of its dominance to the whole virus is probably not valid because the role of VP4 in neutralization is not taken into consideration. Consistent with this view is the observation that the 211 mutant that bears a new glycosylation site is fully susceptible to neutralization by mouse postinfection sera, even though it is resistant to neutralization by hyperimmune antisera. This difference in susceptibility to neutralization probably reflects (a) the presence of sufficient VP4 neutralization antibodies in the former sera and (b) the low level of these antibodies in the latter sera.

Although various structural and nonstructural rotavirus proteins have been reported to elicit a CTL response in mice, the VP7 protein is a major target of rotavirus-specific CTLs. This CTL activity to VP7 is either G serotype specific or cross-reactive (109,372,374). The CTL epitopes on the VP7 have been mapped to amino acid residues 5 to 13, 8 to 16, and 31 to 40 (58,145,197).

The VP4 Protein

Study of rotaviruses by cryoelectron microscopy indicates that VP4 is present on the outer capsid as a series of 60 short spikes approximately 10 to 12 nm in length, with a knoblike structure at the distal end that comprises the region containing its proteolytic cleavage product, VP5* (see Fig. 7) (399,400). VP4 is nonglycosylated, has a MW of 88,000, and constitutes 1.5% of virion protein (319). Although it is a minor constituent of the outer capsid, VP4 performs a number of functions that have been defined by genetic and immunologic analyses (319). Analysis of rotavirus reassortants indicated that VP4 is encoded by the fourth gene (i.e., RNA segment 4) (319). Restriction of growth of human rotaviruses in tissue culture was assigned to VP4 during a study of reassortants produced during dual infection of cell cultures by a cultivatable bovine rotavirus and by a poorly cultivatable human rotavirus initially designated to be noncultivatable (179). Study of reassortants produced during dual infection of a rotavirus that has hemagglutination activity (RRV), and a rotavirus that lacks this property (bovine UK rotavirus) identified VP4 as the viral hemagglutinin (239). This assignment was confirmed when it was observed that MAbs to RRV that inhibited hemagglutination immunoprecipitated VP4 (180). This activity has been mapped to the region between residues 93 and 208 of the VP8* trypsin cleavage fragment (149); the sialic acid binding domain has also been mapped to this region (232). Trypsin cleavage of VP4 must occur for virus to become infectious and initiate penetration of the cell membrane (282,319). Furthermore, genetic mapping of various rotavirus reassortants indicated that VP4 is the major rotavirus cell attachment protein both *in vitro* and *in vivo* (306). More recently, both VP8* and VP5* proteins have been shown to mediate attachment of rotaviruses to cells (541). Rotavirus attachment and entry into cells involves a complex multistep process that requires initial interaction with various asialo- and sialoglycoplipids as well as gylcoproteins on the cell membrane (288,332).

Recently, VP4 and VP7 were shown to contain tripeptide sequences previously shown to act as recognition sites for integrins in extracellular matrix proteins, and to interact with different integrins in the early stages of infection of cells (90). Furthermore, two integrins a_2b_1 and a_4b_1 were shown to mediate simian SA11 attachment and entry into cells (200). It is suggested that binding of rotaviruses to cells involves initial carbohydrate recognition, particularly on integrins and integrin-associated glycosphingolipids, followed by a closer protein–protein interaction of virus with integrins via integrin ligand sequences on VP4 and possibly VP7. It was further proposed that rotavirus binding to integrin could facilitate virus–cell membrane interactions, including membrane permeabilization induced by VP5* or VP7 fusion and endocytosis (105,160,309,419).

Some RRV MAbs to VP4 were found to neutralize infectivity (180). This was unexpected because in earlier studies, VP4 had not been shown to play a role in neutralization, and it was thought that this function was mediated exclusively by VP7

(178). In these early studies, antiserum to bovine rotavirus strain NCDV was used to exert selective pressure on the bovine UK rotavirus in order to generate reassortants bearing genes of a cultivatable bovine UK rotavirus and a poorly cultivatable human rotavirus. However, the antiserum contained neutralizing antibodies directed only to the VP7 shared by the NCDV and UK strain, whereas neutralizing antibodies directed to UK VP4 were absent because these strains possessed disparate VP4s that did not induce cross-reactive neutralizing antibodies (see Table 1) (216). Hence, the role of VP4 in neutralization was not observed because all of the cultivatable reassortants recovered in this study bore the VP4 of the UK bovine rotavirus.

The ability of two outer capsid proteins (VP4 and VP7) to separately induce neutralizing antibodies necessitated a reevaluation of serotype classification that had been based on neutralization mediated primarily by VP7. The need for a reevaluation also became apparent when various strains were discovered that shared neutralization specificities with two distinct serotypes. The first such “intertypic” virus recognized was the M37 strain, which was recovered from an asymptomatic infant in a newborn nursery (222). Hyperimmune antiserum to strain M37 neutralized both serotype 1 and serotype 4 reference rotaviruses to high titer; similarly, antiserum to serotype 1 rotavirus neutralized M37 to high titer, whereas antiserum to serotype 4 virus neutralized M37 to low titer (222). Analysis of (a) M37 reassortants in which the genes coding for VP7 and VP4 had segregated independently and (b) antisera to these reassortants, demonstrated that the neutralization of serotype 1 virus by M37 antiserum was mediated by VP7, the ninth gene product, whereas the neutralization of serotype 4 virus by M37 antiserum was mediated by VP4, the fourth gene product. The serotype 4 reference strain ST-3, which was also recovered from an asymptomatic infant, has a VP4 sequence which is highly conserved among other nursery strains (165). A binary system of classification of rotaviruses is now available to identify the independent role of VP7 and VP4 neutralization specificities (see Table 1) (222,370). However, reagents for identification of VP4 neutralization specificity are available for only a few selected strains (84).

Although VP4 antigenic specificity had not been assigned for all rotavirus strains, RNA–RNA hybridization and/or sequence analysis of various human rotaviruses has defined the existence of at least 10 distinct VP4 gene groups (alleles) (see Table 1) (216). It is clear that independent segregation of VP7 and VP4 neutralization antigens occurs, and this has led to the confusing situation in which certain human strains with VP7 serotype 1, 2, 3, or 4 specificity can share a highly conserved VP4 (165). In addition, strains with identical VP7 specificities can possess antigenically distinct VP4 antigens (see Table 1).

The extent and distribution of VP4 polymorphism of human rotaviruses was recently elucidated when antisera to recombinant VP4 derived from strains exhibiting VP7 serotypes 1, 2, or 3 were used to analyze various human rotavirus strains by neutralization. Three distinct VP4 serotypes and one subtype were identified: serotype 1A (associated with VP7 serotypes 1, 3, 4, or 9); serotype 1B (associated with VP7 serotype 2); serotype 2A [associated with VP7 serotypes 1, 2, 3, or 4 (diverse VP7 serotypes recovered from asymptomatic neonates)]; and serotype 3 (which included a strain with VP7 serotype 1 specificity) (166). These newly assigned serotypes correlated with the VP4 amino acid homology of these strains.

Analysis of the recombinant-expressed VP5* or VP8* subunit of VP4 of three human rotavirus strains demonstrated that VP8* possessed the major antigenic site(s) for serotype specificity, whereas VP5* contained sites responsible for cross-reactivity among different VP4s (292). The VP8* subunit was analyzed further by expressing three peptides and studying them by immunoblot assay (291). These studies demonstrated that peptide B (amino acids 84 to 180) contained VP4 serotype and subtype specificities, whereas peptides A (amino acids 1 to 102) and C [amino acids 150 to 246 (and 247 to 251 from VP5*)] exhibited cross-reactivity among VP4s. This suggested that serotype and subtype specificities were located in a region extending from residues 102 to 149 of VP8*.

A significant role for VP4 in protective immunity was demonstrated during a study in which infection of newborn gnotobiotic piglets with a reassortant bearing the VP4 of one of its virulent porcine rotavirus parents and the VP7 of the other virulent porcine rotavirus parent was shown to induce resistance to disease caused by either virulent parental virus (221). This indicated that the VP4 and VP7 outer capsid proteins function as independent protective antigens *in vivo*. In addition, VP4 stimulated the same high level of neutralizing antibodies as VP7. Also, passively acquired neutralizing antibodies to VP4, as well as to VP7, independently conferred resistance to disease in infant mice in an experimental model of rotavirus illness (368). A MAb directed against VP4 of the porcine OSU strain that neutralized OSU as well as heterotypic simian RRV and bovine UK strains, passively protected suckling mice against challenge by either homotypic or heterotypic strains, indicating that cross-reactive antibodies to VP4 can also be protective (375).

Sera from animals convalescent from rotavirus infection usually contain significantly higher levels of VP4-neutralizing antibodies than do hyperimmune antisera to the same virus. Thus, serotype differences established with hyperimmune sera tend to be less clear when infection sera are employed in neutralization assays (521). In primary responses of gnotobiotic calves infected with bovine rotaviruses, significant serum antibody responses to VP4 or VP7 were approximately equal as determined by neutralization assay and epitope-blocking assay (451). It should be noted that infants vaccinated orally with a live attenuated RRV vaccine developed antibodies to neutralization epitopes on both VP7 and VP4, although the frequency of response to the latter was higher (175,442).

A comparison of the deduced VP4 amino acid sequences of a wide variety of rotaviruses revealed nine areas of divergence, the most prominent being the region including residues 92 to 192 (Fig. 11) (215). However, analysis of the sequence of single-amino-acid-substitution escape mutants selected with neutralizing MAbs identified certain variable and conserved regions on the linear sequence of VP4 that are involved in neutralization (see Fig. 11). Serotype-specific VP4 MAbs selected mutations predominantly in the VP8* subunit of VP4, whereas cross-reactive MAbs selected mutations predominantly in the VP5* subunit (see Fig. 11). Although the sites of mutation in VP8* ranged from residues 72 through 217, some of these sites, such as residues 89, 100, and 150, may be in close proximity on the folded protein because some mutants selected with a MAb directed to one of these regions were also resistant to neutralization by a MAb that selected a mutation at one of the other sites. One of the major sites at which a mutation was selected by cross-reactive antibodies is of special interest because it is within a hydrophobic region of 18 amino acids (384,385,386,387,388,389,390,391,392,393,394,395,396,397,398,399,400 and 401) that exhibits 45% identity with the putative fusion site of the E1 glycoprotein of Sindbis virus (309). Recently, a bacterium-expressed truncated recombinant

RRV VP5* containing this putative fusion region was shown to permeabilize liposomes (105).

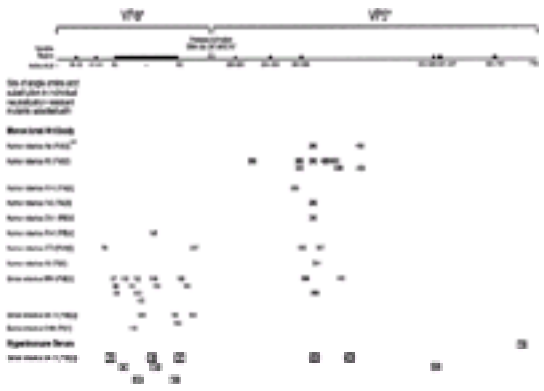

FIG. 11. Linear structure and antigenic organization of rotavirus outer capsid VP4. **VP4 serotype and genotype are shown in *parentheses. Boldface numbers* indicate amino acid substitutions in mutants selected with monoclonal antibody exhibiting cross-reactive (i.e., heterotypic) neutralizing activity; *numbers not in boldface and not in boxes* indicate amino acid substitutions in mutants selected with MAb with homotypic or limited heterotypic neutralizing activity. *Numbers in boxes* indicate amino acid substitutions in mutants selected with hyperimmune serum to SA11 virus. (Adapted from ref. 215, with additions from refs. 159,271,295,381, and 546, with permission.)

In addition to its other functions, VP4 appears to play a major role in virulence of rotaviruses (i.e., diarrhea, or loose, more frequent stools). When reassortant viruses derived from parental strains that varied in virulence for newborn mice were studied, it was observed that virulence segregated with the VP4 gene (371). Evidence for a role of VP4 in virulence has also been provided by RNA–RNA hybridization and sequence analysis of the VP4 gene of rotaviruses recovered from (a) infants and children with moderately severe to severe diarrhea, or (b) neonates infected in a nursery in which virus persisted for long periods of time and in which most affected newborns failed to develop significant symptoms (134,165). Virulence was not VP7 serotype specific in these instances, as each of the four major human rotavirus serotypes (1, 2, 3, and 4) was associated with both symptomatic and asymptomatic infections (224). In contrast, the VP4 gene of rotavirus strains from asymptomatic infections was highly conserved (95.5% to 97.5%) and was distinct from the VP4 gene of strains from symptomatic infections (25.4% to 27.1% divergence) and thus has now been classified as P2A [6] (165). In addition, the VP4 gene of strains of varying VP7 serotype that were recovered from symptomatic infections, exhibited significant conservation (87.4% to 97%). Thus far, long-term persistence of a uniform strain of rotavirus that does not cause significant symptoms has been documented in seven different nurseries in Africa, Europe, South America, and Australia (224,459). Each of these seven nursery strains possessed the conserved VP4 gene, suggesting that this gene is associated with reduced virulence (165). Furthermore, none of the other 10 rotavirus genes was conserved among the nursery strains. It should be noted, however, that in recent studies, rotavirus strains with a VP4 genotype [6] similar to that of strains from avirulent infections described previously, have been recovered from an increasing number of infants with diarrhea, some of which was severe (155,430). In addition, as noted later, the M37 nursery strain P2A [6] was evaluated as a vaccine candidate and found to induce a febrile response in 18% of infant vaccinees but was not associated with diarrhea (499). Thus, the presence of this genotype does not ensure an asymptomatic or attenuated rotavirus infection.

Further data regarding virulence were generated in the gnotobiotic porcine model, in which it was found that the VP3, VP4, VP7, and NSP4 encoding genes of a virulent porcine rotavirus each played an independent role in virulence: Substitution of any one of these genes by the corresponding gene from a human rotavirus avirulent for piglets resulted in an asymptomatic piglet infection (220). Moreover, all four genes in concert were required for the induction of illness in this model.

The NSP4 Protein

The NSP4 is a nonstructural, endoplasmic reticulum (ER)-specific glycoprotein encoded by genome segment 10 of group A rotavirus and has a MW of 28,000 (319,471). The NSP4 protein, which plays a unique role in rotavirus morphogenesis in infected cells, functions as an intracellular receptor by mediating the conversion of double-layered particles in the cytoplasm to triple-layered particles in the ER. Recently, the baculovirus-expressed recombinant NSP4 protein of the simian rotavirus SA11 strain was reported to act as an enterotoxin by inducing an age-dependent diarrhea in mice when administered by intraperitoneal or intraluminal routes but not when given intramuscularly (14,125). In addition, an SA11 synthetic peptide that contains amino acid residues 114 to 135 of NSP4 was also capable of inducing diarrhea in suckling but not adult mice. Furthermore, tyrosine at residue 131 of the protein was reported to be a key amino acid that was associated with the toxigenic activity of NSP4. It is hypothesized that NSP4 interacts with a putative cellular receptor of the gut epithelium and stimulates a calcium-dependent signal transduction pathway that increases plasma membrane chloride permeability and potentiates chloride secretion, thereby inducing a secretory diarrhea. The potential of antibodies to the NSP4 protein to protect against virus-induced diarrhea was examined by challenging mouse pups born to dams that were immunized with the SA11 NSP4 114-to-135 peptide or a control peptide. Pups born to dams immunized with the key NSP4 peptide had less severe diarrhea of shorter duration than controls. In an homologous system, an *Escherichia coli*-expressed NSP4 of murine rotavirus EW had similar toxigenic activity in mouse pups (213).

The nucleotide and deduced amino acid sequence of NSP4 from pairs of virulent and attenuated rotaviruses were compared in an attempt to further define the potential role of NSP4. Such sequence comparisons of the virus pairs yielded varying results. For example, comparison of the NSP4 sequences from two pairs of porcine rotavirus (strains OSU and Gottfried) as well as from symptomatic and asymptomatic human rotavirus strains suggested that mutations in NSP4, particularly those between amino acids 131 and 140, were associated with altered virus virulence of the

attenuated strain (272,542). However, comparison of the NSP4 amino acid sequence of human rotavirus strains Wa, M, and 89-12 as well as murine rotavirus strains EW, EC, and EHP that had been attenuated by tissue culture passage, with that of their respective virulent strains failed to identify any consistent or significant change (9,68,510).

Sequence analysis of over 90 NSP4 genes from strains from humans and nine different animal species indicated that there are four genetic NSP4 groups or alleles: NSP4A (genetic group I or DS-1 allele), which includes human DS-1 genogroup rotaviruses, bovine and equine rotaviruses, and simian SA11; NSP4B (genetic group II or Wa allele), which includes human Wa genogroup rotaviruses and porcine rotaviruses; NSP4C (genetic group III or AU-1 allele), which includes human AU-1 genogroup rotaviruses as well as rhesus, feline, canine, and lapine rotaviruses; and NSP4D (genetic group IV or EW allele), which includes murine rotaviruses (75,95,212,213,273,275). However, the relevance of such genetic diversity has not been determined.

Serum and intestinal humoral immune responses of mice to the NSP4 protein were measured by an immunocytochemistry assay that employed recombinant baculovirus-expressed SA11 NSP4 (NSP4A or DS-1 allele) as antigen. The mice that were studied were infected with a homologous murine rotavirus [EHP strain, (NSP4D or EW allele)], or a heterologous simian rotavirus [RRV, (NSP4C or AU-1 allele)] (234). Titers to NSP4 were low in both groups during the 28 weeks of observation, with no significant difference in titer between the two groups. Recently, humoral and cellular immune responses of naturally infected and/or vaccinated children and/or adults were evaluated by IgG and IgA enzyme-linked immunosorbent assay (ELISA) using recombinant baculovirus-expressed SA11 NSP4 and/or a synthetic peptide corresponding to residues 114 to 135 of SA11 NSP4. Most of the children and adults developed a low or modest immune response to the NSP4 protein and/or peptide after infection or vaccination, and 8 of 10 adults developed a cellular immune response (238). The role of the NSP4 enterotoxin in the natural history and pathogenesis of rotavirus infection in humans needs further clarification to assess its application to human disease.

INFECTION OF EXPERIMENTAL ANIMALS AND HOST RANGE

Group A rotaviruses have a wide host range, as indicated by their recovery from the newborn of almost all animal species that have been studied, except the guinea pig and rat (425). Many of these strains were detected in newborn animals with diarrhea. In contrast to human rotaviruses, most animal rotaviruses are not as fastidious in their requirements for cultivation *in vitro* (425). Under experimental conditions, some human rotavirus strains induce a diarrheal illness or subclinical infection in newborn animals such as gnotobiotic calves, gnotobiotic or conventional piglets, rhesus or cynomolgus monkeys, gnotobiotic lambs, puppy dogs, and newborn mice (24,330,340,427,453,479,530).

The gnotobiotic piglet has been particularly useful as an animal model, offering several advantages over other animal model systems (427): (a) prolonged susceptibility to infection and illness caused by certain strains of human rotavirus, (b) a close resemblance to humans in gastrointestinal physiology and development of mucosal immune system, (c) no influence of maternally derived antibodies in colostrum-deprived gnotobiotic pigs, (d) a relatively large litter size (average, five to eight piglets per litter), and (e) studied under gnotobiotic conditions to eliminate certain confounding variable factors.

In China, a human rotavirus was shown to induce a severe diarrheal illness in *Tupaia belangeri yunalis*, a nonhuman primate, following administration by the alimentary route (384). In limited studies, the ability of human rotaviruses to spread to animals has not been demonstrated. Movement of rotaviruses in the opposite direction has been investigated more extensively, and hybridization and sequence analyses indicate that certain naturally occurring animal rotavirus strains can infect humans on rare occasion (355). Moreover, the use of certain animal rotavirus strains as human vaccine candidates has been evaluated extensively under experimental conditions, as described later.

PATHOGENESIS AND PATHOLOGY

The following lesions have been identified in the jejunal mucosa of infants and young children hospitalized with rotavirus disease: shortening and atrophy of the villi, mononuclear cell infiltration in the lamina propria, distended cisternae of the ER, mitochondrial swelling, and sparse, irregular microvilli (210,345,463). Denudation of microvilli was also noted. Virus particles were visualized in dilated cisternae of ER and in lysosomes of columnar epithelial cells. Virus particles were also detected in goblet cells and phagocytes in the lamina propria. Occasionally, tubular structures were visualized in dilated cisternae of the ER. Impaired *d*-xylose absorption has also been observed (321), as well as abnormal gastric motor function, as indicated by a delay in emptying of a liquid meal (16). In addition, some patients have depressed levels of disaccharidases (maltase, sucrase, and lactase) (38).

Direct information concerning the pathogenesis of rotavirus infection has been obtained from experimentally infected animals that developed diarrheal illness following administration of human rotavirus (345). In one study, the pathogenesis of human rotavirus strain D (VP7 serotype 1) was studied in newborn, gnotobiotic, colostrum-deprived calves that developed illness following intraduodenal administration of virus (329). Morphologic changes proceeded in a cephalocaudad direction in the small intestine. Within half an hour of experimentally induced diarrhea, morphologic changes such as denuding of villi and flattening of epithelial cells were observed in the upper small intestine, but rotaviral antigens were not detected by immunofluorescence (IF). At this time, the lower small intestine was intact, but rotavirus antigens were abundant in swollen epithelial cells. Seven hours after onset of diarrhea, the lower small intestine showed morphologic changes similar to those seen earlier in the upper small intestine; however, rotavirus antigens were not detectable by IF. Forty hours after onset of diarrhea, the intestine appeared relatively normal. Examples of some of these changes are shown in scanning electron micrographs in Figure 12.

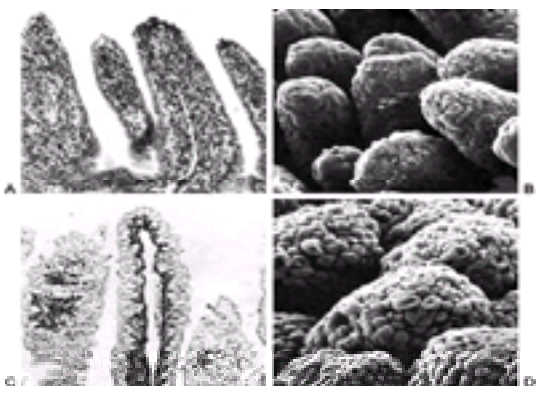

FIG. 12. Scanning electron and light micrographs of intestinal tissues from a gnotobiotic calf sacrificed 0.5 hr after onset of diarrhea induced by human rotavirus. **A:** Proximal small intestine with shortened villi and a denuded villus tip (second from right) (H&E, ×85)**. B:** Appearance of the same level of intestine shown in (A), depicting denuded villi by scanning electron microscopy (SEM) (H&E, ×125). **C:** Distal small intestine with normal vacuolated epithelial cells and normal villi (H&E, ×50). **D:** Same area as in (C), seen by SEM. Epithelial cells appear round and protruding (H&E, ×85). (Adapted from ref. 329, with permission.)

Piglets that developed rotavirus diarrhea following administration of human rotavirus demonstrated functional alterations in small intestinal villous epithelial cells (101,151,501). Glucose-coupled sodium transport was impaired, sucrase activity was decreased, and thymidine kinase activity was increased. However, adenylate cyclase and cyclic AMP were not altered. Absorption of the macromolecule human a-lactalbumin was found to be increased during rotavirus acute gastroenteritis in young children (207). In addition, studies in malnourished or well-nourished suckling mice infected with a murine rotavirus, or piglets infected with porcine rotavirus indicated that malnutrition increased the severity of symptoms and mucosal damage (177,411,482,548).

Although maladsorption is a generally accepted mechanism of rotavirus-induced diarrhea, which is characterized by viral replication in villus enterocytes in the small intestine with subsequent cell lysis and attendant villus blunting, depressed levels of mucosal disaccharidases, watery diarrhea, and dehydration, it may not be the sole explanation of rotavirus-induced diarrhea. For example, watery diarrhea was observed in homologous and heterologous piglet models before the detection of extensive intestinal histopathologic damage (322,470,501). Also, in a homologous piglet model, diarrhea subsided by 9 days after infection in nourished but not in malnourished piglets, whereas the degree of intestinal structural damage was comparable between these nutritional groups at that time after infection (547). In addition, in a heterologous mouse model, inactivated RRV induced diarrhea in the absence of viral replication (444). Diarrhea prior to obvious intestinal cell damage has also been reported in humans. In Germany, small intestinal mucosal biopsies were taken for histopathologic examination from 40 children aged less than 18 months during the acute phase (between days 2 and 10) of rotavirus diarrhea. Only two biopsies (5%) showed histopathologic evidence of damage (279). Thus, a clear distinction between intestinal mucosal damage and diarrhea exists in some cases. A secretory type of diarrhea similar to bacterial enterotoxin-induced diarrhea was observed recently in a homologous piglet model; it was characterized by inflammatory responses in the small intestine, including local T-lymphocyte expansion, enhanced intestinal major histocompatibility complex (MHC) class I and class II gene expression, and elevated tissue concentrations of prostaglandin E_2 (548). Intestinal inflammation induced by bacterial enterotoxin has been known to evoke fluid secretion by activation of the enteric nervous system (ENS). More recently, in a heterologous mouse model, four different drugs that inhibit ENS functions have been shown to attenuate the rotavirus (RRV)-induced secretory responses in the small intestine (307). This observation suggests that the ENS participates in the rotavirus-induced electrolyte and fluid secretion in the small intestine. Rotavirus nonstructural protein NSP4, which has recently been suggested to have a toxin-like function, may participate in inducing intestinal inflammation, activation of the ENS, and thereby a secretory-type diarrhea as discussed earlier.

The RRV strain MMU18006, which is present in a vaccine licensed for use in humans, can spread beyond the intestines in mice with severe combined immunodeficiency (SCID) and cause hepatitis (481). A similar response has been noted in malnourished mice or normal suckling mice (480). However, extensive clinical studies have failed to detect involvement of the liver during immunization of infants with RRV. Involvement of the liver has not been observed with a bovine (WC3) rotavirus strain that is currently a vaccine candidate. In addition, RRV, SA11, the canine CU-1, and the Ty1 turkey strains (and the UK bovine strain to a lesser degree) grow efficiently in HepG2 cells, a well-differentiated human cell line derived from a hepatoblastoma (276,440). The ability of SA11 to grow in these cells was assigned to the VP4 gene by segregation analysis (402). Most, but not all, human rotavirus strains grow poorly in these cells (276). However, most animal and human strains grow efficiently in MA104 cells (derived from monkey kidney) and CaCo-2 and HT29 cells (derived from a human colon carcinoma) (276). Additional data regarding pathogenesis were previously reviewed under individual viral proteins (e.g., VP4, VP7, NSP4).

EPIDEMIOLOGY

Morbidity and Mortality

Until the discovery of the rotaviruses, only a small proportion of severe diarrheal illnesses of infants and young children could be linked to an etiologic agent (240,539). However, as data from epidemiologic studies in the developed and developing countries have accumulated, it has become clear that rotaviruses are the major etiologic agents of serious diarrheal illness in infants and children under 2 years of age throughout the world (see Fig. 2) (34).

Although rotavirus diarrhea occurs with high frequency in the developed countries, mortality is low. In the United States, rotaviruses cause about 5% to 10% of all diarrheal episodes in infants and children under 5 years old; however, these

viruses account for 30% to 50% of severe diarrheal episodes (66). In this age group, it is estimated that rotaviruses are responsible annually for approximately 2.7 million episodes of diarrheal illness, 410,000 visits to a physician, 160,000 emergency room visits, 50,000 hospitalizations, and 20 deaths. Thus, during the first 5 years of life in the United States, approximately 4 of 5 children will develop rotavirus diarrhea, 1 of 7 will require a visit to a clinic or emergency room, 1 of 78 will be hospitalized, and 1 in 195,000 will die from rotavirus diarrhea (66,478). In addition, rotavirus diarrhea results in over $264 million in direct medical costs (primarily for hospitalization) and over $1 billion in total societal costs (primarily for loss of work time of parents or caregivers) (478). A high morbidity but low mortality rate can be attributed to the development during the 1940s of effective means for replacement of fluid and electrolytes lost during disease and their routine use in developed countries (116). This improvement in therapy has brought about a marked decrease in mortality from all forms of infantile diarrhea.

In developing countries, rotaviruses are the leading cause of life-threatening diarrhea in infants and young children. The burden of rotavirus diarrheal disease in infants and children under 5 years of age in developing countries has been estimated to be 130 million cases; over 18 million of these were considered moderately severe or severe (231). In addition, it has been estimated that 873,000 infants and children under 5 years old die from rotavirus diarrheal illness each year in developing countries (231). Thus, during the first 5 years of life in the developing countries, as in the developed countries, almost every child will experience an episode of rotavirus diarrhea but the consequences will be quite different in the developing countries: 1 of 8 will develop moderately severe illness and 1 in 160 will die (162).

In developed countries, the widespread distribution of rotaviruses in the community is indicated by the universal acquisition of serum antibodies to these viruses at an early age. For example, in the Washington, D.C., area, over 90% of infants and young children acquire rotavirus antibodies by the end of the third year of life, a pattern similar to that observed for respiratory syncytial and parainfluenza type 3 viruses (258,269,386). The high prevalence of rotavirus antibodies is maintained into adult life, suggesting that subclinical rotavirus reinfection occurs. A similar pattern of acquisition and maintenance of rotavirus antibody has also been observed in many other parts of the world (25).

Cross-sectional studies of hospitalized infants and young children with diarrheal illnesses have yielded the most compelling evidence for the importance of rotaviruses as etiologic agents of severe diarrhea of early life. For example, during a period of over 8 years, 34.5% of 1,537 infants and young children admitted with diarrhea to a Washington, D.C., hospital were shedding rotavirus in their feces on admission (Fig. 13) (47). Moreover, in a similar study in Japan of over 6 years' duration, 45% of 1,910 pediatric patients with diarrhea were rotavirus positive (281). In two similar shorter-term studies (1 year), one in Australia and the other in the United Kingdom, 52% of the infants and young children admitted with diarrhea were rotavirus positive (100,297). Also, in a 1-year study of hospitalized infants with diarrhea in Finland, 49% of the illnesses were associated with rotavirus infection (494).

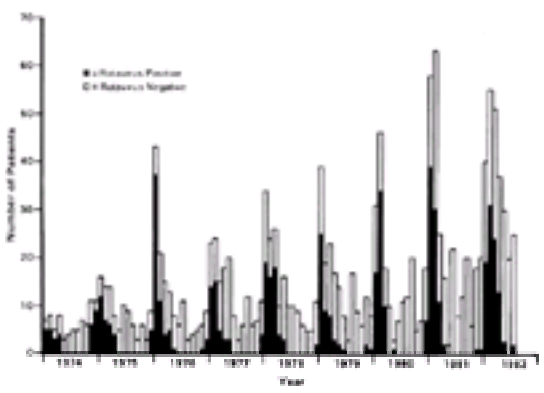

FIG. 13. Rotavirus infections in patients hospitalized with gastroenteritis at Children's Hospital National Medical Center, Washington, D.C., January 1974 to July 1982, as demonstrated by EM, IEM, and rotavirus confirmatory ELISA. (From ref. 47, with permission.)

The largest and longest cross-sectional study of hospitalized children with gastroenteritis was reported more recently from Australia (20). During a survey covering a period of 13 years, 39.6% of 3,785 children were shedding rotavirus in feces on admission. Notably, 55% of children (12 to 23 months old) with gastroenteritis were rotavirus positive. Rotavirus gastroenteritis was also often observed in the under 6-month age group (18.7% of cases) as well as the 5- to 13-year age group (16% of cases).

Rotaviruses have also been documented consistently as the most important etiologic agents of diarrheal illnesses in developing countries (25). For example, in a comprehensive study of the etiology of diarrheal illnesses for which treatment was sought, which included 6,352 patients treated at the Matlab Treatment Center in Bangladesh over a 1-year period, rotaviruses were the most frequently detected pathogen in children under 2 years of age, as 46% of the study group were rotavirus positive (43). The next most important agent was enterotoxigenic *E. coli*, detected in 28%. In the over-2-year age group, bacterial agents were recovered more frequently than rotavirus. The importance of rotaviruses in severe dehydrating diarrheal illnesses was demonstrated in another cross-sectional analysis in a hospital in Cairo, Egypt, in which 145 infants and young children under 18 months of age with fatal or potentially fatal diarrheal illness were studied (447). Rotaviruses were the pathogen most frequently detected, accounting for 34% of the cases. Enterotoxigenic *E. coli* ranked second (27% for LT and ST strains combined).

Prospective studies in developing areas of the world have demonstrated the magnitude of the problem of diarrheal diseases in general, and most studies have highlighted the unique capacity of rotaviruses to induce dehydrating illness with unusually high frequency. In a 30-month village-based study in northeastern Brazil, early childhood mortality exceeded 14% during the first 5 years of life. Furthermore, in over 52% of recorded deaths, diarrhea was listed as either

the primary or an associated cause of death (189). A sample of specimens from diarrheal episodes was tested for enteric pathogens, and 53% yielded a recognized etiologic agent. The most commonly detected pathogens were enterotoxigenic *E. coli* and rotaviruses, 21% and 19%, respectively. The poorest families had the highest diarrhea attack rates, which reached almost 10 per child per year in the 6- to 11-month age group. When a cohort of 45 children was studied prospectively over a 3-year period in Santa Maria Cauque, Guatemala, the incidence of diarrhea was 7.9 per child per year, and rotavirus was associated with 10% of such episodes (314,315). Significantly, dehydration was 14 times more frequent among children with rotavirus diarrhea than among individuals with diarrhea of bacterial, acute parasitic, or unknown etiology (531).

Rotavirus Serotypes

The development of MAbs that identify the VP7 of most clinical isolates and the widespread application of molecular biologic techniques have helped refine our understanding of the epidemiology of rotavirus serotypes with regard to infection and disease (155,217). Previously, the serotype of a rotavirus strain could be identified only by techniques, such as virus neutralization assays, not readily adaptable to large-scale epidemiologic surveys (13). Importantly, there has been complete concordance between serotype determined by ELISA using MAbs and by neutralization with hyperimmune antiserum (216).

MAb-based ELISA has been used to determine the distribution of VP7 serotypes of rotavirus isolates from children with acute gastroenteritis over a 6-year period in various countries of Europe, North and South America, Africa, and Asia. This analysis indicated that (a) 94.9% of 907 rotavirus isolates belonged to G serotypes 1, 2, 3, or 4 (5.1% were VP7 positive but could not be typed with MAbs to serotypes 1 through 4); (b) rotavirus serotype 1 was detected most often (54%), followed by serotype 2 (18%), serotype 3 (12%), and serotype 4 (11%); (c) each of the four serotypes had a worldwide distribution, with no single serotype being found exclusively in one location; (d) differences in the distribution of serotypes were observed year by year and in different countries during the same years; and (e) differences were not observed in the distribution of serotypes detected in developed and developing countries (23).

Information derived from 28 studies that included 5,419 rotavirus isolates obtained between 1979 and 1989 from North and South America, Europe, Asia, and Africa confirmed the results of earlier investigations: Serotype 1 was the most prevalent (61%), whereas serotypes 2, 3, and 4 were evenly distributed (10% to 16%), except that serotype 4 was not detected in Africa (522). A consistent difference in virulence of rotaviruses of varying VP7 serotype has not been observed (19,27). Serotype G 1 through 4 strains were also predominant in a long-term, 16-year study of children hospitalized with acute rotavirus diarrhea in Australia (40). In another large serotyping study, 91.5% of 3,756 rotavirus-positive stool specimens collected in seven regions of Japan from 1984 to 1997 were found to belong to serotype 1 (70.5%), 2 (9.6%), 3 (6.2%), or 4 (5.2%) (545). The need for continued surveillance is evidenced by the emergence of strains with VP7:5, 8, 9, or 10 specificity in several selected locations (85,94,98,155,171,217,358,383).

Serotype analysis of clinical isolates for their VP4 specificity has proceeded more slowly because of the scarcity of suitable reagents. In various parts of the world, P1A [8] strains appear to predominate in frequency. This was anticipated because VP7 serotype 1, the most frequently detected VP7 serotype, shares VP4 P1A specificity with two other epidemiologically important VP7 serotypes (i.e., 3 and 4) (216). As noted earlier in Table 1, an extensive VP4 genotyping classification has been developed (216).

Rotavirus Infections in Adults

Adults appear to undergo rotavirus reinfections quite commonly but characteristically with minimal or no clinical manifestations. For example, 22 (55%) of 40 adult household contacts of children hospitalized with rotavirus gastroenteritis developed serologic evidence of rotavirus infection, predominantly subclinical, at or about the time of their child's admission to the hospital (270). Although subclinical rotavirus infection is the most common outcome in adults, rotavirus gastroenteritis in adults has been described in army recruits in Finland as well as in patients (some geriatric) and staff in hospitals in various countries (34,226). Several outbreaks in geriatric groups had a high attack rate, and there were some fatalities (34). Nonetheless, these outbreaks are unusual because of the high level of rotavirus immunity that most adults have acquired from previous infections.

An unusual outbreak of rotavirus gastroenteritis was reported involving adults associated with children in a play group (414). Nine children 15 months to 5 years of age, three of five mothers who participated in play-group activities, four of five fathers, and each of two grandparents developed gastroenteric illness that could be traced to the play group. Overall, 18 of 21 individuals developed gastroenteritis, and 10 of 11 persons tested demonstrated evidence of rotavirus infection. Other sharp outbreaks of rotavirus-associated acute diarrheal illness have been described in which individuals of all ages were affected (140,300). During an extensive outbreak in an isolated South American Indian community in northern Brazil, the attack rate in adults over 41 years of age was 80% (300). However, these must be considered unusual, isolated events.

Rotavirus infections have also been associated with traveler's diarrhea in certain settings (41). However, these viruses are not considered to be major etiologic agents of this disease.

Group B rotaviruses have been implicated in several large outbreaks of severe gastroenteritis in adults in various parts of China involving 12,000 to 20,000 individuals. Affected individuals developed cholera-like, severe, watery diarrhea, and a few elderly patients died (468).

Nosocomial Infections

Nosocomial rotavirus infections occur frequently, as demonstrated in several studies (34). For example, 10 (17%) of 60 children hospitalized for nondiarrheal disease during a period of rotavirus prevalence developed a diarrheal illness associated with a rotavirus infection (420). Also, in another hospital study, about one of every five rotavirus infections

appeared to be hospital acquired (334).

Nosocomial infections have been described in various neonatal nurseries around the world (34). These are usually subclinical, although recently a nosocomial outbreak of rotavirus gastroenteritis was described in newborn nurseries in Italy (158).

Transmission

Rotaviruses are transmitted by the fecal-oral route. Oral administration of rotavirus-positive stool material induces diarrheal illness in volunteers (263,506). Nevertheless, speculation continues as to whether rotaviruses are transmitted also by the respiratory route (83,164,190). Evidence for this is circumstantial and rests on the following: (a) the rapid acquisition of rotavirus antibodies in the first few years of life in all settings, regardless of hygienic standards (385); (b) a few large outbreaks in which a fecal-oral spread could not be documented (140); and (c) the occurrence of respiratory symptoms in a proportion of patients with rotavirus gastroenteritis (190). There have been scattered reports of detection of rotavirus in the upper respiratory tract; however, the predominance of data from other studies suggests that this is not the usual mode of transmission (464,500,543,544).

The source of infection for the young infant who is normally not in contact with other infants and young children with gastroenteritis is not well documented. Most likely, infection is acquired from an older sibling or parent with subclinical infection (121,182). Shedding of rotavirus from the intestinal tract prior to onset of diarrhea or following cessation of diarrhea has been well documented (395).

Resistance to physical inactivation (along with the large number of viral particles shed in feces) may contribute to the efficient transmission of the human rotaviruses. This inference is drawn from the observed stability of various human and animal rotaviruses at ambient temperatures (10,124,267,341). For example, calf rotavirus present in feces retained infectivity for 7 months when kept at room temperature (347). Other observations that also suggest environmental contamination as a source of infection include (a) the persistence of rotavirus infection in certain newborn nurseries and (b) the high frequency of nosocomial rotavirus infection in hospitals (as described earlier). The ability of rotaviruses to survive on various surfaces under different conditions may contribute to the rapid spread of these agents (516). Although rotaviruses have been detected in raw or treated sewage, it is unlikely that contaminated water plays an important role in transmission of group A rotaviruses (449). High relative humidity (about 80%) results in a rapid loss in human rotavirus infectivity (10). Effective disinfection of contaminated material and careful handwashing constitute important measures to contain rotavirus infection, especially in a hospital or institutional setting (42,285,428,452,465,485,516).

Rotavirus has been detected on moist surfaces in day care centers, and in one controlled study rotavirus transmission was prevented in volunteers if a surface contaminated with rotavirus had been sprayed with a disinfectant (60,503). This approach may have applicability in such centers or in hospitals in an attempt to reduce the high rate of nosocomial infection, but it would not obviate the need for the development of immunoprophylaxis (301).

There has been speculation on the role of animals as a source of rotavirus infection of humans. This speculation has been intensified by the following observations: (a) certain animal rotaviruses share a neutralization antigen with human rotaviruses (see Table 1), and (b) certain naturally occurring animal rotavirus strains may infect humans or form reassortants with human rotaviruses (355). Nonetheless, this type of interspecies transmission appears to be a rare event in most parts of the world. However, it should be noted that human–bovine reassortant strain I321 or a human–porcine reassortant strain appears to be endemic in selected areas in India and Brazil, respectively (98,171).

Incubation Period

The incubation period of rotavirus diarrheal illness has been estimated to be less than 48 hours (100). The onset of experimentally induced rotavirus diarrhea in adult volunteers occurred 2 to 4 days after challenge (263,264). One of the volunteers who developed diarrhea 3 days after virus administration developed a fever and vomited on the first day after challenge, indicating that the incubation period of the illness under experimental conditions was 1 to 4 days.

Distribution

Geographic Distribution

Rotaviruses have been detected throughout the world wherever they have been sought (34,385). Furthermore, these viruses consistently constitute major etiologic agents of severe infantile diarrhea in every country where this disease has been studied by appropriate techniques.

Seasonal Patterns

Rotaviruses display a seasonal pattern of infection in developed countries, with epidemic peaks occurring in the cooler months of each year (34,385). This recurring pattern was clearly documented in the Children's Hospital National Medical Center (Washington, D.C.) study of infants and young children admitted with diarrheal diseases over an 8-year period (see Fig. 13) (47). The absence of rotavirus infections from July through October (1974 to 1982) was a consistent finding. In contrast, during January, 67% of 250 hospitalized diarrhea patients shed rotavirus, and during February, 58% of 219 patients were rotavirus positive. Outpatients with diarrhea exhibited a similar pattern. In a Japanese study extending from December 1974 through June 1981, rotaviruses were detected in the feces of 66% of 785 patients hospitalized with diarrhea during the cooler months of December, January, and February and in the feces of 56% of 549 children admitted in the spring (March, April, and May) (281). In contrast, only 5.6% of 576 pediatric patients admitted during the summer and autumn months were rotavirus positive. Recently, in a 13-year Australian study of children hospitalized with gastroenteritis, an annual winter admission peak was attributed to the increase in total incidence of rotavirus

gastroenteritis during this season, although rotaviruses were detected in 135 of the 156 months studied (20).

The cause for this striking seasonal pattern is not known, but the influence of low relative humidity in the home has been suggested as a factor facilitating the survival of rotaviruses on surfaces (46). However, when this factor was studied, a correlation with the temporal pattern of infection was not observed in every epidemiologic setting (281,341). A recent analysis of hospitalizations for diarrhea in the postneonatal period and the temporal distribution of rotavirus isolations from various centers suggests that rotavirus diarrhea spreads from the west to the east in the United States, appearing in the fall in the southwest and spreading progressively across the country, so that by late winter and spring it has reached the northeast (205,294). The usual seasonal pattern of rotavirus infection observed in the temperate climates does not occur uniformly in other settings (83,201).

Age, Sex, Race, and Socioeconomic Status

Rotavirus gastroenteritis severe enough to require hospitalization characteristically occurs most frequently in infants and young children from approximately 6 months to 2 years of age (25,35). Infants under 6 months of age experience the next highest frequency of such illness. In certain studies, this age group had the highest frequency (92,121,349,396,460,483). In one U.S. study, the age distribution of patients admitted to the hospital with gastroenteritis of any etiology was different for black and nonblack patients; 59% of all black patients admitted for gastroenteritis were younger than 6 months of age (45). This difference was also reflected in admissions for rotavirus diarrhea. Since the black children were predominantly from less affluent, inner-city areas, these differences probably reflect the effects of crowded living conditions, which may have allowed earlier and more efficient transmission of the virus.

An Australian study, which extended over a 13-year period, of hospitalized children with gastroenteritis deviated somewhat from this pattern (20). Although the peak incidence of rotavirus gastroenteritis was in the 12- to 23-month age group, in which 55% had rotavirus infection, rotaviruses were a frequent cause of gastroenteritis (18.7%) in children under 6 months of age. These viruses were also associated with 16% of the gastroenteric illnesses in the 5- to 13-year age group and were identified as the sole pathogen in 45 of 282 patients in this age group.

The low frequency of clinical illness in most (but not all) normal neonates who shed rotavirus remains an intriguing paradox that is being actively investigated because of its implications for immunoprophylaxis (34). As noted earlier, the gene encoding the outer capsid protein VP4 of strains recovered from asymptomatic neonatal infections is highly conserved and differs from the corresponding viral protein of strains from symptomatic infections (134,165). Further study is required to determine whether the highly conserved VP4 of strains from asymptomatic neonatal infections plays an important role in the ability of these viruses to persist over a long period of time in newborn nurseries without causing significant disease. As noted earlier, strains with a VP4 similar to that of strains recovered from asymptomatic (P2A [6]) infections have also now been observed in a small proportion of ill infants, indicating that the presence of this genotype does not ensure a silent infection (155,430,459).

Malnutrition is thought to play an important role in increasing the severity of clinical manifestations of human rotavirus infection (52,96,313). This phenomenon has been reproduced in an experimental mouse and piglet model (411,482,547). It has also been suggested that repeated diarrheal infections may be a precipitating factor in the development of malnutrition by damaging intestinal mucosa so that absorptive cells are compromised over an extended period (313,314).

Molecular Epidemiology

Initial investigations of the molecular epidemiology of human rotaviruses utilized gel electrophoresis of segmented genomic dsRNAs for strain identification (electropherotype) and comparison (123,209). For example, analysis of rotaviruses derived from 116 children hospitalized with gastroenteritis in Melbourne, Australia, between 1973 and 1979 identified 17 different electropherotypes (412). Distribution of electropherotypes was not random during the 7-year study period but showed, instead, a sequential pattern of appearance and prevalence. Only a limited number of electropherotypes was detected at any one time. Characteristically, a single electropherotype was predominant, often accompanied by less common types. In contrast, only two closely related electropherotypes could be identified among rotaviruses from 72 newborn babies studied in seven different hospitals from 1975 to 1979. It was remarkable that these two electropherotypes had similar RNA patterns and that neither was ever identified in viruses from children hospitalized for acute gastroenteritis. One of these electropherotypes was detected in nurseries in five different hospitals and persisted in one nursery for 4 years. This adds to the mystery of neonatal rotavirus infection, because the year-round occurrence of rotavirus infection in the nurseries by viruses of a single distinctive electropherotype differed from the seasonal pattern involving multiple electropherotypes in the general population.

Similarly, in France, an epidemiologic survey over a 16-month period in a maternity unit revealed that all 184 rotavirus fecal samples obtained from 3- and 6-day-old newborns exhibited the same electropherotype. This pattern was in sharp contrast to the variable electropherotypes found in postneonatal infections (152). In a similar study in Venezuela, 62 (57%) of 108 children shed rotavirus within the first few days of life; furthermore, a similar gel migration pattern was observed for each of the 52 samples examined. Six of the 62 children had diarrhea, but only one required oral rehydration therapy (390).

In addition to providing evidence for genetic diversity of human rotaviruses as well as heterogeneity of circulating rotaviruses, analysis of electropherotypes has also provided a method for tracing the spread of rotavirus through a population group (123). However, the electropherotype cannot be used to predict virulence (11,12,22,157).

Early RNA–RNA hybridization studies employing labeled ssRNA viral transcripts as probes for genomic RNA identified two major families of human rotaviruses (135,138). The 11 genes of most human rotaviruses exhibit significant homology to the corresponding genes of either Wa (VP7 serotype 1) virus or DS-1 (VP7 serotype 2) virus. On several occasions, human rotaviruses were identified that had a mixed genotype, with some genes being DS-1-like while others were Wa-like (135,138). This was interpreted as evidence for gene reassortment during co-infection *in vivo*. Hybridization

analysis also indicated that the corresponding genes of most animal viruses and human viruses lacked substantial homology. However, human rotavirus strains have been detected that possess certain gene segments with a high degree of homology with various animal rotavirus genes, indicating the likelihood of genetic reassortment between human and animal rotaviruses, but there is no evidence that this is of clinical or epidemiologic importance (355). In this regard, however, a third family of human rotavirus was described when a human rotavirus strain (AU-228 or AU-1) failed to hybridize to either Wa or a DS-1-like virus (KUN) (see Fig. 6) but did have a high degree of homology with a feline rotavirus (355). It was proposed that the term *genogroup* be adopted in lieu of *family* to conform to taxonomic hierarchy (355).

IMMUNITY

The mechanisms responsible for immunity to rotavirus infections and illness are not completely understood, especially in humans, in whom studies of the importance of rotavirus serum antibodies have yielded conflicting results. Animal models, however, have been particularly instructive in elucidating the role of antibodies and in dissecting the relative importance of systemic and local immunity (369,425,502). Initially, it was observed that newborn calves frequently developed rotavirus diarrhea despite a moderately high level of circulating rotavirus antibodies derived from colostrum (520). This observation was confirmed in a later experimental study in calves (51,520).

Subsequently, the relative roles of local and systemic rotavirus antibodies were examined in newborn gnotobiotic lambs (450). Two lambs were fed colostrum that contained rotavirus antibodies on day 1; two other lambs were fed the same preparation of colostrum containing rotavirus antibodies on days 1 through 4; and an additional 2 lambs were fed serum containing rotavirus antibodies on days 2 through 4. Following challenge with lamb rotavirus at 2 days of age, the 2 lambs that were fed colostrum only on day 1 developed diarrhea despite the acquisition of serum antibodies to rotavirus from the colostrum, whereas, the other 4 lambs did not become ill. These observations suggested that antibodies in the lumen of the small intestine were the primary determinant of resistance to rotavirus illness, whereas circulating serum antibodies failed to protect. Support for this interpretation was provided during a study of experimental heterologous rotavirus disease in newborn mice (373). It is also of interest that systemic rotavirus antibodies are present in the lumen of the gastrointestinal tract of neonatal calves if the level of circulating antibodies is sufficiently high. Such serum-derived mucosal antibodies can provide protection against experimentally induced infection and diarrhea (32).

In later studies of immunity in experimental animals, additional evidence for a role of rotavirus antibodies was obtained, but the antigenic determinants of resistance have not been clarified completely. Although serotype-specific immunity has been demonstrated convincingly in several experimental animal models, this could not be confirmed in other studies (369,425). For example, two avirulent bovine strains were evaluated in gnotobiotic calves for their ability to protect against disease caused by a virulent strain (50). Protection was conferred by prior infection with either avirulent strain, although one of these strains did not exhibit a significant antigenic relationship to the virulent challenge virus in cross-neutralization tests.

Information regarding correlates of rotavirus immunity was obtained during a volunteer study in which human rotavirus VP7 serotype 1 (D strain) was administered to 18 individuals by the oral route (263,264). Five of the 18 volunteers shed rotavirus, and four of those five developed diarrheal illness. A critical preexisting level of serum neutralizing antibodies to the homotypic virus or to a heterotypic VP7 serotype 2 human rotavirus correlated with resistance to diarrheal illness. The relationship of neutralizing activity in intestinal fluid to resistance was less clear. Volunteers with a high level (>1:100) of neutralizing activity in intestinal fluid to the homotypic virus tended to develop fewer diarrheal illnesses than those with lower levels (<1:100), but the difference was not statistically significant. The five volunteers who shed rotavirus developed an increase in intestinal fluid-neutralizing activity for the homotypic virus that ranged from 1.9- to 13.9-fold or greater. Each of the ill volunteers also developed a serum neutralizing antibody response to the challenge strain and also to heterotypic human rotavirus DS-1 (serotype 2), as well as to bovine rotavirus NCDV (serotype 6). More recently, the prechallenge sera from this study were reexamined by a competitive binding immunoassay (epitope-blocking assay) for antibody levels against several well-defined VP7 and VP4 neutralization epitopes (173). Volunteers who possessed prechallenge serum antibodies that blocked binding of a serotype 1 VP7-specific MAb (that mapped to residue 97) at a titer of more than 1:20 were resistant to infection and illness. A similar protective effect was observed for serum antibodies that blocked binding of a corresponding serotype 3 VP7-specific MAb that mapped to residue 94 on the serotype 3 VP7. This study not only confirmed the association of serum antibodies with resistance but also identified a specific protective epitope on VP7. Confirmation of the importance of antibodies was provided during a later series of challenge and rechallenge studies in which serum rotavirus IgG or jejunal neutralizing antibodies were observed to correlate with protection against infection and/or illness, whereas in a third series of studies, serum and jejunal rotavirus antibodies did not appear to correlate with resistance (504,505 and 506,509).

In related studies, salivary IgA antibodies as measured by ELISA were proposed as a surrogate for the level of intestinal rotavirus antibodies (512). Rotavirus serum IgA levels were also shown to be correlated with resistance to severe rotavirus illness (204). However, during a study of 1- to 24-month-old infants and young children residing in an orphanage, a firm correlation was observed between homotypic serum neutralizing antibodies and resistance to disease (70). A serum neutralizing antibody titer of 1:128 or greater to human VP7 serotype 3 was associated with resistance to gastroenteritis due to that serotype, whereas serum antibody levels of 1:64 or less were not associated with significant homotypic protection. The protective role of serum neutralizing antibodies in calves was also recently described (278). Nonetheless, serum antibodies may correlate only with, and hence reflect, local intestinal antibodies that are produced locally and/or enter the lumen of the intestinal tract by transudation (475).

The association between fecal (IgA) and serum (IgG) anti–rotavirus antibody titers and protection against infection and illness was also investigated in 100 children under 18 months of age attending day care centers in the United States for one or two rotavirus seasons (317,366). During the two seasons, seven outbreaks of G1 serotype and one of G3 serotype were detected. These studies concluded that (a) a rotavirus-specific fecal IgA titer of at least 1:80 or at least 1:20 correlated with protection against infection or illness, respectively; (b) preexisting serum anti–rotavirus IgA titer of greater than 1:200 or IgG titer of greater than 1:800 was associated with protection against infection; and (c) a high level of preexisting G type-specific blocking antibody was associated with protection against infection. Similarly, in Australia, a

direct correlation between high levels of anti–rotavirus intestinal IgA antibody and a significant degree of protection of children against natural rotavirus infection and illness was observed (86). Similar observations were made in animals; in a heterologous gnotobiotic piglet model, a direct association between the degree of protection and the level of intestinal IgA antibody-secreting cell response as well as serum IgA, intestinal IgA, and intestinal IgG titers was observed (473,540). In addition, in mice, a significant correlation was observed between anti–rotavirus intestinal and/or serum IgA and protection against homologous or heterologous rotavirus infection (126,324).

It should be noted, however, that in a recent study in a mouse model involving challenge with virulent murine rotavirus and IgA normal and IgA knockout mice, the correlation of protection with serum and intestinal immunoglobulin isotypes was investigated (365). This study showed that in the absence of IgA, other compensatory antibody isotypes, IgG in particular, were able to mediate protection against homologous rotavirus challenge (365).

The protective effect of prior rotavirus infection against severe rotavirus diarrhea was demonstrated in an Australian study (36). In a study of 81 neonates, 44 were rotavirus positive and 37 were rotavirus negative during the first 14 days of life; all were kept under clinical and serologic surveillance for 3 years. The frequencies of subsequent postnatal rotavirus infection were similar for the two groups. However, when postnatal rotavirus infection occurred, the children in the neonatal rotavirus-positive group developed significantly fewer rotavirus diarrheal illnesses when compared to the neonatal rotavirus-negative group (20% versus 46%). However, the protective efficacy of neonatal rotavirus infection was 100% against severe rotavirus diarrhea. Thus, neonatal rotavirus infection did not protect against reinfection but induced partial resistance against any rotavirus diarrheal illness and complete protection against severe rotavirus diarrhea during reinfection. The rotavirus strains responsible for neonatal infection in this study were of particular interest because these viruses exhibited the same electropherotype. One of the 20 neonatal strains was identified as serotype 3. The unusual electropherotype of the neonatal strains was not encountered among the strains that induced illness in the study group during the postneonatal period. Thus, infection at birth with a rotavirus strain that persisted in a newborn nursery without detectable modification, conferred partial protection against disease during reinfection with other rotavirus strains that most likely included a variety of serotypes. This finding, as described later, has been applied to the development of a candidate vaccine. Recently, neonatal infection with another unique rotavirus strain 116E, which shares VP7 specificity with the human WI61 (G9) strain and VP4 genotype with the bovine rotavirus B223 strain P [11], induced significant partial protection (46%) against subsequent rotavirus diarrhea during the first year of life (33). Also, rotavirus diarrhea in the neonatally infected group tended to be less severe.

Although there is a paucity of information from human studies, it appears that predominantly homotypic immunity is induced following the first naturally occurring or vaccine-induced rotavirus infection (502). This immunity is mediated by antibodies to VP7 and VP4 (221,244,475). Although in challenge studies the neutralizing antibody response to primary human rotavirus infection in piglets is primarily homotypic, a lower-level heterotypic response commonly occurs involving VP4 serotypes 1, 3, and 4 (223). Subsequently, in both infants and experimental animals, the predominantly serotype-specific response is expanded to include other serotypes following subsequent natural or vaccine-induced reinfection with the same or another serotype (55,56 and 57,175,451,454,467,513). For example, following vaccination with live RRV (VP7 serotype 3), adults develop a significantly greater number of heterotypic responses than do infants less than 6 months of age (59% versus 1.2%), as determined by an epitope-specific serum-blocking assay (175). It should be noted that one or more neutralization epitopes on VP4 or VP7 are shared among viruses of serotype 1, 3, or 4 (see Fig. 10 and Fig. 11). Thus it is not surprising that reinfection with a virus of the same serotype amplifies the cross-reactive neutralizing antibody response. Serotype-specific immunity has been demonstrated under experimental conditions in lambs and rabbits (82,454). Furthermore, immunization of cows with a single rotavirus serotype induced neutralizing antibodies to that strain and to heterotypic strains against which these animals had preexisting neutralizing antibodies but not to strains the animals had not encountered previously (454). In other studies, immunization of cows with a single rotavirus serotype induced a marked antibody response to numerous serotypes (56).

As noted earlier, the VP4 and VP7 proteins independently induce neutralizing antibodies, and as a consequence each outer capsid protein plays a role in resistance to disease (221,368,509). In a recent study employing an epitope-blocking assay, it was observed that a majority of infants and young children fed the live RRV vaccine developed antibodies to the major serotype-specific neutralization epitope on RRV VP7, and almost half of the vaccinees also responded to a major neutralization epitope on VP4 (442). This indicates that infection with an attenuated vaccine strain that is restricted in its capacity to replicate in the intestine can induce antibodies to neutralization epitopes on each of these protective antigens. In another study comparing serum antibody responses to VP4 or VP7 following oral administration of bovine rotavirus WC3 vaccine to infants not previously infected with rotavirus, VP7 appeared to be more efficient than VP4 for the induction of neutralizing antibodies by vaccination (507). However, the reverse was found in adults who possessed rotavirus antibodies and who were challenged with a wild-type human rotavirus (509). It is currently unclear which surface protein and which region of that surface protein is most efficient for the induction of protective immunity.

Although serotype-specific antibodies have been demonstrated in breast milk, the effect of breast-feeding on the occurrence of infantile gastroenteritis requires additional study (78,163,361). In any event, if breast-feeding does transfer immunity to newborns and young infants, its lasting protective effect appears to be modest.

External factors are being investigated for their influence on the immune response. For example, vitamin A deficiency impaired the serum antibody and the cell-mediated immunity responses in infant mice that were given the murine rotavirus strain EDIM orally (4).

The role of cell-mediated immunity in the immune response to rotavirus infection is also under investigation in humans and in animal models (367,415,475,502). Mice are susceptible to rotavirus-induced illness only during their first 15 days of life, a period too short to evaluate the active immune mechanisms involved in rotavirus-induced diarrhea. However, because mice of all ages are susceptible to infection with selected strains of murine rotavirus, an adult mouse model has been extensively used to study the factors and mechanisms involved in protective immunity (127,142,502). Such studies have shown that (a) rotavirus-specific antibodies are of primary importance in protection from rotavirus reinfection; (b) in general, homologous rotavirus strains are far more potent than heterologous rotavirus strains in inducing protective local humoral immune responses; (c) MHC class I–restricted CD8+ abT cells play a major role in resolution of primary rotavirus

infection, but rotavirus-specific antibodies, CD4+ T cells, and uncharacterized mouse strain–specific innate mechanisms are also involved in this role; (d) after primary infection, CD8+ T cells mediate almost complete protection (up to 2 weeks) or partial protection (3 months or less) from reinfection, however, this protection diminishes 8 months after primary infection; (e) perforin, Fas, or interferon-g are not essential in an anti-rotavirus activity mediated by CD8+ T cells; and (f) CD8+ T cells expressing a_4b_7 marker (an intestinal mucosal homing receptor integrin) are more efficient in mediating rotavirus clearance than the a_4b_7 negative cells (110,126,141,143,146,147 and 148,323,324,325 and 326,348,416). A central role played by CD8+ T cells in the control of primary rotavirus infection was also demonstrated in a homologous gnotobiotic calf model (378). In the same study, the importance of CD4+ T cells in the development of mucosal as well as systemic antibody responses was also shown. The relevance of these findings to understanding immune responses to rotavirus in humans remains to be established.

CLINICAL FEATURES

Rotavirus infection produces a spectrum of responses that vary from subclinical infection to mild diarrhea to a severe and occasionally fatal dehydrating illness. In one study, a comparison was made between (a) the clinical manifestations of 78 patients hospitalized with rotavirus diarrhea and (b) 72 patients hospitalized with a diarrheal illness that could not be associated with rotavirus (Table 2) (413). The majority of both rotavirus and nonrotavirus patients had a temperature of 37.9°C or above. However, the rotavirus group vomited and became dehydrated significantly more often than the rotavirus-negative group. The mean duration of vomiting as determined from history and hospital records was longer in the rotavirus group than in the rotavirus-negative group (2.6 days versus 0.9 days). Rotavirus diarrhea started later than vomiting but lasted longer (mean duration, 5 days versus 2.6 days). After infants and children were hospitalized, diarrhea continued for a mean of 2.6 days (range, 1 to 9 days) in the rotavirus group and 3.8 days (range, 1 to 16 days) in the rotavirus-negative group. The duration of hospitalization ranged from 2 to 14 days, with a mean of 4 days for the rotavirus group. It is of interest that in Japan, rotavirus infection has been associated in some instances with severe diarrhea characterized by a milky white stool (Hakuri) (346,354).

Clinical finding	Percent having each clinical finding	
	Rotavirus infection detected (72 patients)	Rotavirus infection not detected (78 patients)
Vomiting	90*	58*
Fever		
37.8–39°C	46	29
>39°C	31	33
Total	77	61
Dehydration	83*	40*
Hypertonic	5	16
Isotonic	95	77
Hypotonic	0	6
Irritability	47	40
Lethargy	36	27
Pharyngeal erythema	49	32
Tonsillar exudate	3	3
Rhinitis	26	22
Red tympanic membrane with loss of landmarks	19	9
Rhonchi or wheezing	8	8
Palpable cervical lymph nodes	18	9

*p < .01 (two children not included).
†p < .04.
Source: From ref. 413, with permission.

TABLE 2. *Clinical characteristics of 150 children hospitalized with acute gastroenteritis*

Laboratory findings reflect the high frequency of vomiting and dehydration associated with rotaviral illness (413). A BUN value of greater than 18 mg/dL was observed in 58% of the rotavirus-positive group, and a urine specific gravity of 1.025 or greater was noted in 71%. These rates were significantly higher than in the nonrotavirus group. The frequency of acidosis and electrolyte imbalance did not differ in the two groups.

Over a 5-year period in a Toronto hospital, rotavirus gastroenteritis was responsible for the death of 21 infants and young children between 4 and 30 months of age (62). On arrival at the hospital, ten of these children were dead, and 10 were moribund and could not be resuscitated. One of the children was already hospitalized when he acquired the disease; this patient had been hospitalized with congestive cardiomyopathy, which also contributed to his death. With the exception of this patient and one other child, each of the children who died had been healthy previously. Death occurred within 1 to 3 days of onset of symptoms. The major factor causing death was believed to be dehydration and electrolyte imbalance in 16 individuals and aspiration of vomitus in three. In the remaining two patients, seizures were a contributing factor. Each of the 16 patients tested had a sodium level (serum or vitreous humor) in excess of 150 mEq/L, and sodium was in excess of 160 mEq/L in 11 of this group. The rapidly fatal course of untreated severe rotavirus gastroenteritis was underscored by the fact that the parents of 16 of the 20 children who were brought to the hospital had had some contact with a physician during the course of the illness.

Rotaviruses can produce a chronic symptomatic infection or serious illness in immunodeficient children (236,376,438,518). For example, chronic diarrhea associated with prolonged shedding of rotavirus was described in children with primary immunodeficiency, T-cell immunodeficiency, or SCID. An unusual aspect of illness in certain individuals in the former group was the occurrence of rotavirus antigenemia. In addition, a child with SCID developed a transient hepatitis during rotavirus infection (376). Also, in four 4- to 8-month-old infants with laboratory-proven rotavirus infection who had severe immunodeficiency disease, rotavirus antigen was detected in sections of the liver and kidney examined after death (161). During chronic infection in immunocompromised children, rotavirus can undergo marked changes in its genome, as indicated by abnormal electrophoretic migration patterns of rotavirus RNAs (see Fig. 3., A64 strain) (106,228).

Rotaviruses pose a special threat to individuals who are immunosuppressed for bone marrow transplantation. In one study of gastroenteritis in a bone marrow transplant unit, 8 of 78 patients (average age, 20 to 21 years) shed rotavirus as the sole pathogen, and five of these individuals died (536). Also, rotavirus infections acquired nosocomially have been associated with severe diarrhea in adult renal transplant recipients (387). Rotaviruses do not appear to play an important

role in diarrhea occurring in adults infected with human immunodeficiency virus (HIV) (184).

A temporal association of rotavirus infection with a variety of other disease conditions, both isolated illnesses and single outbreaks, has been described (191,196,248,255,266,448,514). Because the occurrence of these conditions is extremely rare in comparison to the very high incidence of rotavirus infection, it appears that with the exception of severe disease manifestations in immunocompromised patients, necrotizing enterocolitis and hemorrhagic gastroenteritis in neonates, and pneumatosis intestinalis in infancy, the association of rotavirus infection with most of the reported conditions is most likely temporal and not etiologic (61,104,417,418,533).

However, because of the recent reports of intussusception after oral administration of a quadrivalent rhesus rotavirus–based vaccine in infants (see later), the significance of the temporal association of wild-type rotavirus infection with intussusception deserves further attention (198). Rotavirus was detected in the stools of 11 (37%) of 30 infants and young children in Japan with intussusception; 5 of 7 of the rotavirus-positive group developed a serologic response, suggesting that wild-type rotavirus might have been the cause of intussusception (283,284,357). Furthermore, expansion of this study indicated that 20 (33%) of 61 cases of intussusception (including the 30 patients just mentioned) had evidence of rotavirus infection (265). Three later studies in Australia, France, and Germany failed to find a significant association of intussusception with rotavirus infection (350,362,457).

Rotavirus genomic RNA has been detected by polymerase chain reaction (PCR) in serum and/or spinal fluid of certain patients with rotavirus gastroenteritis and convulsions (364). Further studies are needed to determine the significance of these findings.

DIAGNOSIS

The clinical manifestations of rotavirus illnesses are not sufficiently distinctive to permit diagnosis on this basis alone. Therefore, diagnosis requires detection of virus or viral antigen and/or demonstration of a serologic response. The epidemiologic pattern of rotavirus disease at any one time may suggest this diagnosis, but laboratory confirmation is required.

Many assays have been developed for the detection of rotavirus in stools (535). Specimens from the 1st to the 4th day of illness are optimal for virus detection; however, shedding may continue for up to 3 weeks, depending on the duration of symptoms. Viral shedding usually coincides with the duration of diarrhea, but diarrhea can continue for an additional 2 to 3 days (410). Initially, direct visualization of stool material by EM was employed for rotavirus detection (39,45,130). It had the advantage of high specificity because rotaviruses have a distinctive morphologic appearance. The EM continues to be important in the diagnosis of rotaviral diseases and is frequently used to resolve discrepancies in results from other techniques. When only a few specimens are to be examined for rotavirus, EM is the most rapid diagnostic method because fecal specimens can be stained with phosphotungstic acid and examined directly within a few minutes of collection. Direct EM examination of stools permits detection of rotavirus in 80% to 90% of the virus-positive specimens (45). IEM is not necessary for the detection of rotaviruses because the particle has such a distinct morphologic appearance that it can be readily identified without the use of an immune serum. However, modified methods have been developed that may prove useful under selected conditions (359,462,525).

Various automated methods are available for the detection of rotaviruses in stool specimens (535). The method of choice in many laboratories is the confirmatory ELISA, because it is highly sensitive, does not require specialized equipment, and has a built-in control for nonspecific reactions (535). For example, a preimmunization or postimmunization animal serum is employed as the solid-phase precoat; the preimmunization serum acts as the control because a fecal specimen must exhibit a significantly greater reactivity in the well that is coated with the postimmunization animal serum in order to be considered positive. Some laboratories have routinely employed other methods for virus detection, such as counterimmunoelectroosmophoresis, gel electrophoresis of rotavirus RNA, reverse passive hemagglutination assay (RPHA), latex agglutination, or an ELISA in which polyclonal and/or MAbs are used (535). Commercial kits are available for ELISA, latex agglutination, RPHA, and immunoblot enzyme assay, but assays that do not include confirmatory reagents may yield false-positive results. An enzyme immunoassay has also been developed for detection of group B or group C rotavirus and for measurement of antibodies directed against these viruses (150,308,534).

A dot hybridization assay for detection of rotaviruses has been developed based on *in situ* hybridization of labeled rotavirus ssRNA transcripts to heat-denatured rotavirus RNA immobilized on a nitrocellulose membrane (128,132). The method is highly specific, yielding results that are concordant with those obtained with other tests such as EM, RNA analysis, and ELISA (118,132). In a comparative study of ELISA and dot hybridization for the detection of rotavirus in various dilutions of fecal specimens, the dot hybridization method was 10- to 100-fold more sensitive than the confirmatory ELISA (132). The limit of detection for purified viral RNA by the dot hybridization procedure was 8 pg in a homologous reaction. A variety of other research laboratory techniques are now used for the detection of group A rotaviruses (535). The most promising is PCR, which is 100,000 times more sensitive than the standard electropherotype method and 5,000 times more sensitive than hybridization (517,532,535). For example, using a PCR-based assay, virus shedding was detected from 4 to 57 days after diarrhea onset, and 30% of the children shed virus detectable by the assay for 25 to 57 days (409). PCR has also been applied to the detection of group B and C rotaviruses (119,169). Flow cytometry is described as another sensitive method for rotavirus detection (15).

It is also possible to recover human rotaviruses from stool specimens directly in cell culture with reasonable efficiency. This method appears to be approximately 75% as efficient as antigen detection test procedures (195). The efficiency of recovery of virus from rectal swabs using cell culture is considerably less. Growth of rotavirus in tissue culture makes possible the determination of virus serotype by neutralization assay; however, serotype is most often determined by ELISA (for which commercial kits are available) and less often by solid-phase IEM using absorbed antisera or serotype-specific (VP7) MAbs for each of the four major serotypes (535). It may be necessary to use several MAbs directed at different epitopes of the same serotype because of epitope polymorphism within a serotype (88,511). Serotype-specific MAbs are employed in a sandwich procedure together with one or more polyclonal antisera. Rotavirus

serotype can also be predicted with considerable accuracy by nucleotide sequence analysis of the VP7 gene because there is a high degree of conservation of sequence in variable regions among rotaviruses belonging to the same serotype (174). VP7 serotype can also be predicted by a variety of other methods, including PCR, hybridization with single gene substitution (VP7) reassortants used as probes, and hybridization with oligonucleotide probes prepared from the VP7 sequence of human rotavirus serotype 1, 2, 3, or 4 or additional strains (170,215,233). The use of PCR has enabled the genotyping of rotavirus-positive specimens that could not be typed by ELISA. Also, VP4 genotype can be identified by PCR or PCR-derived probes, and VP4 serotype can be determined by neutralization or ELISA using monoclonal or other serotype specific antibodies (84,154,215,216).

There are a variety of techniques for measuring a serologic response to rotavirus infection, such as IEM, complement fixation (CF), IF, immune adherence hemagglutination assay, ELISA, neutralization, hemagglutination inhibition, inhibition of reverse passive hemagglutination, ELISPOT assay, and immunocytochemical staining (234,246,443). The CF method is about as efficient as the other methods for detecting an antibody response in patients between 6 and 24 months of age, but it is not as efficient in adults or in infants under 6 months of age. In these age groups, IF, ELISA IgG, ELISA IgM, and ELISA IgA are more efficient. Because IgA does not cross the placenta, a rotavirus IgA ELISA has been particularly effective in demonstrating serologic responses in young infants who possess passively acquired maternal IgG antibodies (304,305). This assay can also be used for measuring antibodies in saliva, duodenal fluids, and stools (305). Fecal rotavirus IgA antibody levels exhibit a direct relationship with duodenal IgA antibody levels (183). In addition, a rise in fecal rotavirus IgA antibodies measured by ELISA reflects an intestinal neutralizing antibody response (91). It has been suggested that copro-IgA conversion in feces obtained weekly would be a more sensitive indicator of rotavirus reinfection than seroconversion or detection of virus (37,87). Based on observations from a clinical study in infants and children, it was suggested that rotavirus IgA in serum reflects the immunologic status of the intestinal lumen with respect to this virus (102,203,204). Early diagnosis can also be made by ELISA, which can detect a specific serum IgM response as early as 5 or 6 days after onset of illness.

Neutralizing antibodies are detected by plaque reduction, neutralization of IF foci, neutralization of virus as determined by quantitation of viral antigen by ELISA, or inhibition of cytopathic effect in roller-tube cultures (246). Neutralization assays yield the most meaningful information about the identity of the infecting rotavirus and the development of a serotype-specific antibody response. The plaque reduction neutralization assay is more sensitive than tube neutralization for detection of antibody, although the latter is slightly more efficient for detecting a seroresponse (248). A competition-solid-phase immunoassay that measures epitope-specific immune responses to individual rotavirus serotypes has proven to be especially useful in evaluating immune responses at the level of individual epitopes. This technique employs the test serum as the blocking reagent and uses individual MAbs as the detecting reagent (442).

Detection of rotavirus and/or the demonstration of a serologic response to rotavirus in an individual patient does not necessarily establish an etiologic association of rotavirus with that patient's illness. This is important in all age groups but especially in newborns and adults, who commonly undergo inapparent infection.

TREATMENT

The primary aim of treatment of rotaviral gastroenteritis is the replacement of fluids and electrolytes lost by vomiting and diarrhea. Intravenous fluid administration has been used successfully for many years in treating dehydration from diarrhea. Because facilities for parenteral administration of fluids and electrolytes are not readily available in many parts of the world, intensive efforts have been made to evaluate the efficacy of oral fluid replacement therapy (3,268,421,423,433,439). Various formulations of oral rehydration salts (ORS) solutions have been shown to be effective in the treatment of dehydration caused by rotavirus diarrhea, with some minor variations in their efficacy. For example, in a double-blind study, oral rehydration therapy with electrolyte solutions containing either glucose or sucrose has been shown to be highly effective for rotavirus gastroenteritis (422). In another study of oral rehydration of infantile diarrhea of mixed etiology, all of the patients who were fed glucose electrolyte solution and 92% of those fed sucrose electrolyte solution were treated successfully (360). The sucrose group experienced a slower correction of electrolyte abnormalities, and a larger proportion of patients required more than 24 hours of therapy. It was concluded that sucrose could be employed as a substitute for glucose, but that glucose electrolyte solutions were preferable.

The standard World Health Organization (WHO) oral glucose electrolyte formula is composed of the following: sodium, 90 mmol/L; chloride, 80 mmol/L; potassium, 20 mmol/L; citrate, 10 mmol/L; and glucose, 111 mmol/L, resulting in an overall osmolarity of 310 mOsm/L (5,6,423). Bicarbonate, 30 mmol/L, may be substituted for citrate, 10 mmol/L (6). Recently, an ORS solution containing a reduced sodium concentration has been found to be more effective than the standard WHO solution. Thus, a solution containing sodium, 75 mmol/L; chloride, 65 mmol/L; potassium, 20 mmol/L; citrate, 10 mmol/L; and glucose, 75 mmol/L, resulting in an overall osmolarity of 245 mOsm/L (reduced osmolarity ORS solution), was compared to the standard WHO ORS solution in infants with noncholera diarrhea in a mulicenter trial (71). The proportion of infants who required unscheduled intravenous therapy was significantly lower in the group receiving the reduced osmolarity ORS (required in 10%) than among those receiving the WHO ORS solution (required in 15%). After correction of the initial calculated fluid loss by oral rehydration solution, either water or fluids without added electrolytes (such as breast milk or some other form of low-solute feeding) should be administered orally in addition to the oral rehydration solution. When this regimen is used, both continued diarrheal fluid and electrolyte losses will be replaced and normal daily fluid requirements will be maintained (432,433).

Rice-based solutions have been compared with glucose-based oral rehydration solutions in infants and young children hospitalized with mild to moderately dehydrating diarrheal illnesses associated with various pathogens, including rotavirus (63,397). Each was found to be effective, but the rice-based solution was more effective in decreasing total stool output and increasing absorption and retention of fluid and electrolytes. Later, glucose-based and rice-based oral rehydration solutions were each found to be effective in treating mild to moderate dehydration caused by various pathogens, including rotavirus (342). Although a rice-based solution was associated with less stool output during the first 6-hour period, this effect did not persist after the initial 12 hours of therapy, and it was concluded that this difference was

of minor clinical importance (63,139,342,397).

If oral rehydration does not correct the fluid and electrolyte loss, or if the patient is severely dehydrated or in shock, intravenous fluids must be given immediately. Oral rehydration therapy should not be given to patients with depressed consciousness because of the possibility of fluid aspiration.

With regard to the use of various medications for symptomatic treatment of the acute diarrhea in children 1 month to 5 years of age, the American Academy of Pediatrics (AAP) does not recommend the use of loperamide, anticholinergic agents, bismuth subsalicylate, adsorbents, or lactobacillus-containing compounds and, in addition, the use of opiates as well as opiate and atropine combination drugs for treatment is contraindicated (6).

Human milk, containing rotavirus antibodies, has been used successfully for treatment of immunodeficient children with chronic rotavirus infection and illness (99,303,438). In contrast, colostrum or milk concentrate from cows immunized with human rotavirus was not effective for the treatment of children with acute rotavirus gastroenteritis, although a decrease in the duration of virus shedding was observed (115,202). However, daily oral administration of rotavirus antibody–containing bovine colostrum appeared to exert a protective effect during an orphanage outbreak of rotavirus diarrhea (115). The effect of a single oral dose (300 mg/kg body weight) of gammaglobulin in infants hospitalized for gastroenteritis (70% rotavirus associated) was evaluated in a double-blind placebo-controlled trial. The treatment group had a significantly shorter duration of diarrhea, viral excretion, and hospital stay (185). In addition, in another study, the administration of human serum immunoglobulins by the alimentary route to two infants with prolonged rotavirus diarrhea of 4 or 7 months duration was effective in clearing the virus and the associated diarrhea (186). More recently, bovine colostrum prepared by immunization of pregnant cows with human rotavirus strains of G1, 2, 3, or 4 specificity was given orally to rotavirus diarrhea patients 4 to 24 months of age and was found to significantly reduce the duration and frequency of diarrhea as well as the consumption of oral rehydration solution (193,436)

The efficacies of several broad-spectrum antiviral agents have been examined as inhibitors of rotavirus replication *in vitro*. In this survey, various adenosine analogs were found to have antirotavirus activity, and it was suggested that this activity resulted from inhibition of S-adenosylhomocysteine hydrolase, an enzyme involved in regulating methylation required for maturation of viral mRNA (277).

Bismuth subsalicylate (BSS) (20 mg/kg) given orally five times a day for 5 days as an adjunct to rehydration therapy shortened the course of disease in children 4 to 28 months of age hospitalized with acute rotavirus diarrhea in a prospective double-blind placebo-controlled treatment trial (455,456). Because of the association between the use of salicylates and Reye's syndrome, the authors reviewed the possibility of such an association with BSS or other nonacetylsalicylic acid salicylates and could find none. In addition, blood salicylate and bismuth levels attained in the treatment group were well below those considered toxic. However, as noted before in considering the use of various medications for symptomatic treatment of acute diarrhea in children 1 month to 5 years of age, the AAP does not recommend the use of bismuth subsalicylate (6).

PREVENTION AND CONTROL

General Principles

Observations made during epidemiologic and hospital-based studies worldwide clearly indicate the need for prevention of rotavirus disease (25,244). The aim of a rotavirus vaccine should be to prevent severe rotavirus gastroenteritis during the first 2 years of life, the period when rotavirus disease is most serious and takes its greatest toll (34,244). This is a realistic goal because natural infection does not provide complete protection against a subsequent reinfection or mild disease (36,316,486). Considerable evidence from studies in animals indicates that local intestinal immunity plays the most important role in resistance to rotavirus disease. These observations suggest that the effectiveness of a rotavirus vaccine will depend, in large part, on its ability to stimulate intestinal IgA antibodies and other forms of local immunity. The most effective means of stimulating local immunity is thought to be infection involving the local site. For this reason, most current efforts in experimental rotavirus immunoprophylaxis are directed toward the development of live attenuated vaccines. Although progress has been made during the past few years in developing various live virus vaccine candidates, a number of important issues have been raised concerning the most effective strategy to be pursued.

Longitudinal studies have shed some light on the resistance to disease induced by prior, naturally acquired rotavirus infection. Reinfection within the first few years of life appears to be a common event in certain settings, but in most circumstances an infant who has had a severe bout of rotavirus diarrhea usually experiences a milder illness during reinfection (30,36,486). However, in most instances, in these studies it was not ascertained whether reinfection involved the same or a different serotype. In one study it was possible to demonstrate the effectiveness of homotypic antibodies. Residents of an orphanage who had a preoutbreak serum homotypic neutralizing antibody titer of greater than 1:128 were resistant to rotavirus disease (70). In another study, neonates who were infected in a newborn nursery during the first 14 days of life with what appeared to be a single strain of rotavirus experienced almost 50% fewer rotavirus diarrheal episodes during the next 3 years than did a cohort of infants who had not been infected with rotavirus when they resided in the nursery (38% versus 85%) (36). In addition, none of the 24 children infected neonatally developed severe diarrheal illness on reinfection, whereas 8 of the 20 who had not been infected neonatally developed severe diarrheal illness associated with rotavirus infection. During the 3-year surveillance period, participants in the study were infected with rotavirus strains that exhibited at least two different electropherotypes, neither of which corresponded to that of the strain responsible for neonatal infection.

Finally, in a cohort study in Mexico in which 200 infants were followed from birth to 24 months of age, 96%, 69%, 42%, 22%, and 13% of them experienced 1, 2, 3, 4, or 5 rotavirus infections, respectively. Although seemingly discouraging from a vaccinology perspective, the good news was that no child developed a moderate-to-severe diarrheal illness after the second infection. This suggests that a controlled vaccine-induced infection might have the same protective effect

(486). With regard to serotype, second infections tended to be caused by a different G serotype (p = 0.054).

If homotypic immunity proves to be relatively effective against severe diarrheal illness, the next issue that must be resolved involves the protective efficacy of attenuated rotaviruses. Rotavirus produces a superficial infection that primarily involves the epithelium of the small intestine. Experience with mutants of other viruses that cause a mucosal infection, such as influenza A virus and respiratory syncytial virus, indicates that in many instances attenuation is a reflection of decreased viral replication in the target organ, which is also the site at which infection is initiated (69). Experience with attenuated mutants of mucosal viruses also indicates that decreased viral replication is associated with a diminished immune response. This means that a delicate balance must be achieved between satisfactory attenuation and satisfactory immunogenicity.

Again, observations made during the longitudinal surveillance of children infected during the neonatal period are instructive (36). These neonatal infections were attenuated, primarily asymptomatic or associated, at most, with mild diarrhea. Nonetheless, neonatal infection induced immunity to severe rotavirus diarrhea for at least 3 years. This suggests that a naturally occurring attenuated rotavirus infection can induce significant resistance.

It is clear from epidemiologic studies worldwide that a highly effective vaccine is needed to produce protection against each of the four most prevalent serotypes that account for more than 95% of rotavirus illnesses (522). This means that effective coverage against disease could be provided most efficiently by a quadrivalent vaccine (VP7 serotypes 1, 2, 3, and 4), unless sufficient heterotypic immunity to protect against severe rotavirus disease could be induced by one or two VP7 serotypes. Epidemiologic studies have not yielded conclusive information in regard to this question.

In gnotobiotic calves and piglets, heterotypic immunity has been demonstrated between an animal rotavirus (bovine, VP7 serotype 6) and a human rotavirus (VP7 serotype 1) (528,529,549). Calves infected *in utero* with bovine rotavirus resisted challenge with human rotavirus at birth. However, in other studies in piglets and calves, only homotypic immunity was demonstrated, although in certain instances the severity of diarrhea was reduced following heterotypic challenge (44,153,351,519,521). Whether effective heterotypic immunity to each of the four major serotypes of human rotavirus could be induced in humans by infection with a single rotavirus was one of the early issues that needed to be addressed, because generally, heterotypic immunity to viruses is more transient than homotypic immunity, and this could limit the usefulness of monotypic rotavirus immunoprophylaxis, as described later in this section (253).

Current Strategies

Current strategies for development of a rotavirus vaccine range from cell culture cultivation of strains obtained from humans or animals to the application of recently developed molecular biologic techniques. The most extensively evaluated approach is based on the method first reported by Edward Jenner in 1798 for vaccination of humans against smallpox (290). Jenner used a live, antigenically related animal virus, cowpox, to immunize humans against variola virus, the etiologic agent of smallpox. The usefulness of this strategy for human rotavirus vaccination was suggested by the early observation that human and animal rotaviruses shared a major common antigen (250,251,520). Thus, infants and young children who developed illness following rotavirus infection developed a serologic response detectable by CF not only to the infecting human rotavirus strain but also to various animal strains (250). The feasibility of this approach was shown during animal studies in which calves administered a bovine rotavirus (serotype 6) *in utero* were protected from illness following challenge at birth with a human rotavirus of VP7 serotype 1 specificity (529).

Initially, a high tissue-culture passaged, cold-adapted, serotype 6 bovine rotavirus [strain NCDV (RIT 4237)] vaccine was developed and was studied extensively for reactogenicity, antigenicity, dose-requirement, and protective efficacy (487,488,491). This nonreactogenic, orally administered vaccine virus induced over 80% resistance to clinically significant diarrhea in infants and young children in two separate efficacy trials in Finland (489,490). In addition, vaccination of neonates appeared to modify the severity of rotavirus diarrhea during the 16-month follow-up period (496). Following two doses—one in the neonatal period and a second dose 7 months later—the vaccine had a protective efficacy of 43% against any rotavirus diarrhea and 89% against severe diarrhea (497). However, it failed to induce protection against rotavirus diarrhea in trials in several developing and developed countries and, as a consequence, further evaluation of the candidate vaccine was not pursued (48,103,107,194,338,434,441).

A related VP7 serotype 6 bovine rotavirus strain, designated WC3, has also demonstrated variable protective efficacy in various studies in the United States and overseas (48,76,77,107,338). For example, in 3- to 12-month-old infants in Philadelphia, its protective efficacy (when serotype 1 strains were predominant) was 76% against any rotavirus diarrhea and 100% against moderate-to-severe rotavirus diarrhea (76,77). In contrast, during later studies in Cincinnati and the Central African Republic, the vaccine exhibited a protective efficacy against moderate-to-severe rotavirus diarrhea of 35% to 41%, and in China, 50% against any rotavirus diarrhea (31,77,156,492). Because of the perceived low level of protection, the vaccine was withdrawn from further study in its monovalent formulation.

Another animal rotavirus strain, RRV strain MMU 18006, which is antigenically similar to human rotavirus VP7 serotype 3 by neutralization, has been studied intensively (48,107,241,259,260,338). The RRV vaccine has been evaluated in both developed and developing countries. Although in some settings it induces a mild, transient, febrile response in about one third of the target population of infant vaccinees under 6 months of age, it appeared to be more antigenic than the NCDV (RIT 4237) bovine rotavirus attenuated strain, as determined by the frequency of serum antibody responses following vaccination (304,493). However, the RRV vaccine has been nonreactogenic in neonates (133). This may be of some importance because vaccination soon after birth may be necessary in developing countries, where immunization is needed most. In these locations, 10% to 40% of neonates do not have contact with a health care provider beyond the newborn period (192). The vaccine is taken orally, usually following ingestion of buffered formula, or buffer alone, to protect the virus from partial or total inactivation by stomach acid (394). In addition, breast-feeding is usually withheld prior to immunization because of the possibility of virus inhibitors in the milk (393,394).

In a field trial in 1- to 10-month-old infants in Venezuela, the vaccine's overall protective efficacy rate was 64% against

any rotavirus diarrhea (137,391). Moreover, in the 1- to 4-month age group, vaccine efficacy was 82% against any rotavirus diarrhea, whereas its efficacy was 90% against the most severe illnesses encountered in the entire study group. Results of efficacy trials were also encouraging in Maryland, Sweden, and Finland, but the vaccine failed to protect young infants in Rochester, NY or Arizona; furthermore, it induced only a low level of protection (29%) in 2- to 5-month-old infants in Maryland (72,168,252,256,257,405,434,495). The basis for protective efficacy in these studies became apparent after the strains recovered during various trials were serotyped. The predominant strains responsible for disease in the Venezuelan study were serotype 3, the same serotype as the vaccine strain; whereas, in the Rochester and Maryland studies, almost all the strains recovered from ill infants were serotype 1. Moreover, in the Arizona study almost all typed strains belonged to serotypes other than serotype 3. These observations suggested that serotype-specific immunity was required for protection against rotavirus diarrhea in young infants not primed by previous rotavirus infection (304). Protection against heterotypic strains in Finland and Sweden was observed in older infants and young children who may have been primed by previous rotavirus infection (168,495). Nonetheless, the effectiveness of a rotavirus vaccine must ultimately be judged by its ability to induce protective antibodies to all of the epidemiologically important rotaviruses in very young infants.

Failure of the RRV vaccine to induce protection in young infants against heterotypic rotavirus strains suggested that the initial strategy for development of a rotavirus vaccine required modification. As a consequence, the Jennerian approach to rotavirus immunoprophylaxis was altered to include the use of a quadrivalent vaccine that incorporated the VP7 specificity of each of the four epidemiologically important VP7 serotypes coupled to the attenuation phenotype of RRV (241,243,254,256,257,259,335,337). This approach took advantage of the segmented genome of rotaviruses and the ability of these viruses to undergo gene reassortment with high efficiency during co-infection (181). Virus reassortants were isolated from tissue cultures co-infected with a cultivatable, wild-type animal virus (RRV or bovine UK rotavirus) and a poorly cultivatable human rotavirus (335,337). Monospecific antiserum directed against the animal rotavirus parent, or a set of MAbs directed against the VP7 of the animal rotavirus parent, was used to select for virus reassortants with human rotavirus VP7 neutralization specificity. With this selective pressure, it was possible to isolate reassortants that derived only the gene segment coding for VP7 from the human rotavirus parent, whereas the remaining 10 genes were derived from the animal rotavirus parent. In this manner, single human rotavirus gene substitution reassortants were isolated that possessed the human rotavirus gene for VP7 of serotypes 1, 2, 3, or 4 on a background of 10 animal rotavirus genes. As expected, these reassortants exhibited the neutralization specificity of the human rotavirus parent (335,337). Because RRV belongs to serotype 3, it was not necessary to prepare an RRV-human rotavirus serotype 3 reassortant. Phase I studies indicated that each of the RRV-human rotavirus reassortants was similar to its RRV parent with respect to attenuation and immunogenicity (259). Subsequently, a quadrivalent rotavirus vaccine formulation, which contained single VP7 gene substitution human-RRV reassortants with serotype 1, 2, or 4 specificity and RRV itself as the serotype 3 representative, was shown to be as safe and as attenuated as RRV alone (388,389). Simultaneous administration of monovalent or quadrivalent RRV vaccine and trivalent oral poliovirus vaccine did not appear to influence the “take” rate of either vaccine (66,230,235,339,407).

Individual reassortants or the quadrivalent vaccine were evaluated for efficacy in over 10,000 infants and young children worldwide (243). The individual reassortants, DxRRV (serotype 1) or DS-1xRRV (serotype 2), each induced 67% protection against VP7 serotype 1 diarrhea in Finland (498). In Rochester, the DxRRV vaccine induced 77% protection and the RRV vaccine 66%, both against predominantly VP7 serotype 1 diarrhea (310). Protection against serotype 1 induced by RRV or the serotype 2 reassortant suggested that heterotypic immunity can be effective in some circumstances. Also, a reanalysis of two efficacy trials, one in Peru and one in Brazil, yielded promise that the quadrivalent vaccine may be effective in developing countries if the end point is the prevention of severe diarrheal illness (289,298,299).

In a large study of the quadrivalent vaccine and a monovalent DxRRV vaccine in the United States, the protective efficacies during the first rotavirus season against predominantly VP7 serotype 1 rotavirus diarrhea were 64% and 69%, respectively (28). During the second season, when only about two thirds of the strains had VP7 serotype 1 specificity, the monovalent vaccine provided only 7% protection, whereas the quadrivalent vaccine achieved a protective efficacy of 48%, but the number of infections were too few to achieve statistical significance. However, the trend was consistent with the importance of serotype-specific immunity. In a large-scale follow-up efficacy study of these same vaccines (but at a 10-fold higher dose of each component), a protective efficacy of 49% and 54% was observed for the quadrivalent and monovalent vaccines, respectively, over a single season when the most common serotype was serotype 1 (71%) (404). However, when efficacy was evaluated according to severity score, the quadrivalent vaccine achieved a protective efficacy against severe diarrhea of 80% (100% against dehydration), while the monovalent vaccine had an efficacy of 69% (85% against dehydration) against this form of diarrheal disease. Moreover, the quadrivalent vaccine tended to protect more effectively against the diarrhea caused by G3 serotypes when compared to the monovalent vaccine (77% versus 45%) (404).

In three additional large-scale efficacy trials with the higher dose of vaccine in Finland, Native Americans in the United States and a poor population in Venezuela, vaccine efficacy ranged from 48% to 68% against any rotavirus diarrhea. However, against severe rotavirus diarrhea, it reached 91% in Finland (97% against dehydration and 100% against hospitalization), 88% in Venezuela (75% against dehydration), and 69% in Native Americans (too few cases of dehydration to evaluate) (237,237a,392,435). In the Native American study also, the quadrivalent vaccine induced a higher level of protection than did the monovalent G1 vaccine against a non–serotype 1 strain that was predominant (53% versus 20%). A transient low-grade fever of greater than 38°C (rectally) was observed in up to 29% of the vaccinees in these large clinical trials within the first 5 days of the first dose of vaccine.

Progression Toward Licensure of RRV-based Vaccine

From the evidence presented in June 1998, the Advisory Committee on Immunization Practices (ACIP) recommended routine immunization with three oral doses of the RRV-based vaccine at 2, 4, and 6 months of age for all full-term infants (66). Shortly thereafter, the U.S. Food and Drug Administration granted a biologics license to Wyeth Laboratories for the manufacture and distribution of the rotavirus vaccine (RotaShield) for the immunization of infants at 2, 4, and 6 months of

age. Later, in 1998, the AAP also recommended the vaccine (7).

Withdrawal of ACIP Recommendation for Rotavirus Vaccine

In July 1999, the Centers for Disease Control (CDC) reported that between September 1, 1998, and July 7, 1999, 15 cases of intussusception had been reported to the Vaccine Adverse Events Reporting System (VAERS) following administration of rotavirus vaccine to almost 1 million infants (65). Although this was consistent with the expected number of cases of intussusception per week (background rate, 51/100,000/yr in the first year of life), there was concern that because VAERS is a passive reporting system, there were likely to be additional cases; also, the observation that 11 of the 15 cases occurred during the first week after the first dose was of further concern. Thus, the CDC and the AAP recommended that administration of rotavirus vaccine be postponed pending availability of more data (8,65).

In a follow-up meeting in October 1999, the CDC reported to the ACIP that 102 confirmed or presumptive cases of intussusception had been reported to VAERS, 46 of them with onset within 7 days of receipt of the first dose of vaccine and, in addition, that case-series and case-control studies supported the observation that the highest risk was during the first week (3 to 7 days) after the first dose of vaccine. They estimated an attributable risk of intussusception following vaccination to be 1 in 2,500 to 1 in 5,000 infants. [These attributable risk estimates were recently revised in April 2000 by the CDC to be 1 case in 12,274 infants who were vaccinated, or 1 case in 20,100 doses, from expanded cohort studies (286).]

With the initial data, the ACIP “concluded that intussusception occurs with significantly increased frequency in the first one-to-two weeks following vaccination with RRV-TV, particularly following the first dose” and withdrew its recommendation that rotavirus vaccine be given at 2, 4, and 6 months of age (67).

It should be noted that in pre-licensure studies of RotaShield (as later licensed), 2 of 8,240 infants developed intussusception, one case on day 51 after the second dose, and one case on day 7 after the third dose (406).

Other Vaccine Approaches That Have Been in Clinical Trials

A bovine rotavirus WC3 strain–based reassortant vaccine designated WI79-9, which contains the gene-encoding VP7 of a human serotype 1 strain and the remaining 10 genes from WC3, was nonreactogenic and had 64% protective efficacy against any rotavirus diarrhea and 87% against severe rotavirus diarrhea following three doses given to infants and young children (474). Furthermore, a four-component WC3-based vaccine containing a single gene from a human rotavirus of G1, 2, or 3 or P1A specificity and the remaining genes from the bovine strain was shown to induce about 70% protection against all disease and against moderate to severe disease (77). A double-gene substitution reassortant, WI79 (4 and 9) with P1A [8] and G1 specificity in a background of nine WC3 genes was found to have a low immunogenicity (77). However, a mixture of each single gene reassortant, WI79-4 and WI79-9, was highly immunogenic.

The UK strain of bovine rotavirus has also been evaluated in clinical trials sequentially in adults, older children, and infants, using the individual strains of human–bovine rotavirus reassortants with VP7 specificity for serotype 1, 2, 3, or 4 in a background of 10 bovine rotavirus genes (80). Each component was nonreactogenic, and immunogenic in phase I trials. The four strains were combined into a quadrivalent formulation and administered sequentially as described previously and proved to be safe and immunogenic in phase I trials (79).

In addition, (a) a single gene substitution reassortant, WaxUK, with a single human rotavirus gene from the Wa strain that coded for VP4 P1A in a background of 10 bovine rotavirus genes, and (b) a double-gene substitution reassortant, WaxDS-1xUK, with a gene from the Wa strain as earlier and a VP7 gene from the human rotavirus strain DS-1 that encoded G2 specificity in a background of nine bovine rotavirus UK genes, have been evaluated sequentially as described (214). Each candidate vaccine has been shown to be safe and immunogenic in phase I trials (117).

Another approach to rotavirus immunoprophylaxis involves the use of a live, orally administered, neonatal human rotavirus strain that appears to be naturally attenuated. The impetus for evaluating this strain came from the observation that neonates who experienced a subclinical rotavirus infection in an Australian nursery during the first 14 days of life were protected against clinically significant rotavirus diarrhea for up to 3 years (36). The neonatal rotavirus strain M37 (G1, P2A), derived from a Venezuelan neonate, appeared to be satisfactorily attenuated and immunogenic during a phase I trial in infants (136,336,499). However, in an efficacy trial in Finland, the M37 strain failed to induce protection against VP7 serotype 1 illness (388,499).

Phase I studies with another neonatal strain, RV3 (G3, P2A), which was obtained from the Australian nursery noted before, have also been initiated (18). This strain was attenuated and induced an antibody response in certain vaccinees after a single dose. A cold-adapted strain of human rotavirus D (G1, P1A) has also undergone early clinical studies and needs further evaluation (218; and Treanor, unpublished studies).

A live, orally administered, human rotavirus strain (89-12) with VP7:1, VP4:1A [8] specificity, which was attenuated by 33 passages in monkey kidney cells, has recently been evaluated in a clinical trial of efficacy in infants enrolled at 10 to 16 weeks of age. The vaccine was safe, inducing a low grade fever (38.1°C or greater, rectal) in about 15% of the vaccinees, and immunogenic. It had a protective efficacy of 89% against any rotavirus diarrhea and approximately 83% against severe or very severe diarrhea over a single season during which a VP7:1 strain was predominant (29).

Another candidate vaccine undergoing early clinical trials is a live oral vaccine in which a lamb rotavirus (G10, P [12]) is the immunogen (48).

Other Approaches to Vaccination

Other strategies to immunization are also being explored. Among these is the use of molecular biologic approaches

including (a) synthetic viral proteins, (b) viral proteins expressed by cloned rotavirus cDNA, (c) empty capsids expressed by a baculovirus recombinant, (d) synthetic peptides, (e) DNA vaccines, and (f) an inactivated G1, P [4] strain (AU64) for parenteral administration (48,81,199,229,338).

Passive Immunization

Finally, passive immunization has been shown to be effective in preventing or modifying rotavirus illness in animals (113,193,436). Similar studies have now been carried out in infants and young children, with promising results in most, but not all, instances (17,54,55,99,114,115). Various sources of passively administered rotavirus antibodies (such as eggs from immunized hens, or milk formula containing bovine milk immunoglobulin from cows hyperimmunized with human rotaviruses) have been considered (99,537,538). However, except for special circumstances such as in immunocompromised individuals, passive immunization is not practical for protection against rotavirus illness in humans, because it requires repeated administration of antibodies.

THE FUTURE

It is apparent that major advances made in the quest for a rotavirus vaccine have yielded encouraging signals that severe rotavirus disease can be prevented or modified. Although there are numerous questions regarding future rotavirus vaccines (e.g., formulation, nature of antigens, need for VP4 component, need for additional serotypes, role of enterotoxin), they all appear minor when compared to the issue of the risk of intussusception following administration of any vaccine candidate. Can any rotavirus vaccine in pre-licensure studies be ensured to be free of the risk of inducing intussusception? How large must studies be before proposing licensure? What are the ethical implications of withholding the use or development of a rotavirus vaccine for a developing country where mortality from rotavirus diarrhea is up to 870,000 per year in infants and young children (120,247,296,331,515,524a)? How does one weigh risk versus benefits of rotavirus vaccine?

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CHAPTER 56 Orbiviruses

Polly Roy

Orbiviruses constitute a genus within the *Reoviridae* family. They are transmitted between their vertebrate hosts by hematophagous arthropods (ticks, *Culicoides* species flies, or mosquitoes, depending on the viral species), and replicate in both vertebrates and arthropods. No orbivirus is considered an important human pathogen. However, orbiviruses can cause infections and diseases of economic importance in ruminants in many parts of the world, including Africa, Australia, Southeastern Asia, South and Central America, and the United States. Although orbiviruses were recognized as early as 1900, and the initial morphologic and biochemical characterizations were reported in 1969, detailed studies of the molecular biology, genome structure, and encoded products have been obtained only recently. Because of its economic importance, most analyses have focused on the prototype virus of the genus, bluetongue virus (BTV). Significant advances have been made in understanding the structure–function relationships of BTV proteins, their roles in virus replication and assembly processes, and the three-dimensional (3D) x-ray–resolved structure of BTV proteins and virus particles. Because BTV is the best studied member of the *Orbivirus* genus, this chapter predominantly discusses BTV, and other viruses are mentioned only if they demonstrate important differences or have unique features. However, for certain sections, such as Classification, Genetics and Orbivirus Evolution, and Pathogenesis and Disease, all members are discussed. Coltiviruses are considered as a separate genus at the end of the chapter, although many of the principles of their structure and replication strategies appear to be quite similar to those of Orbiviruses.

CLASSIFICATION

The *Orbivirus* genus is one of at present nine genera within the *Reoviridae* family. The *Reoviridae* family includes vertebrate, arthropod, and plant pathogens. Despite their basic similarity with certain other members of the family (e.g., reoviruses and rotaviruses), orbiviruses differ greatly in details of their structure, physicochemical properties, replication cycle, pathogenesis, and epidemiology. The members of the *Orbivirus* genus, unlike the reoviruses and rotaviruses, are arthropod borne. Based on serologic cross-reactivities, 19 groups of orbiviruses are distinguished; in addition, there are several unclassified isolates (134). Within each serogroup, multiple serotypes are distinguished by neutralization tests. The orbivirus serogroups, serotypes, principal vectors, and main hosts are listed in Table 1 with their assigned abbreviations. The BTV group, which consists of at least 24 different serotypes (BTV-1, -2, etc.), infects *Culicoides* species and a number of wild and domestic ruminants (e.g., sheep, cattle), occasionally causing high morbidity and mortality, but often with no apparent clinical symptoms (38). Other orbiviruses that can cause disease in animals include African horse sickness virus (AHSV; nine serotypes, AHSV-1, -2, etc.) and epizootic hemorrhagic disease virus of deer (EHDV; nine serotypes, EHDV-1, -2, etc.).

TABLE 1. *Orbiviruses: Serogroups, serotypes, vectors and vertebrate hosts*

VIRION STRUCTURE

Like all members (reoviruses and rotaviruses) of the *Reoviridae* family, orbiviruses are nonenveloped viruses with two protein shells and a genome consisting of double-stranded RNA (dsRNA) segments (Fig. 1**A**, Fig. 1**B**, Fig. 1**C**, Fig. 1**D**, Fig. 1**E**, Fig. 1**F**). The inner shell of BTV, termed the *core,* is composed of two major proteins: VP3 and VP7. The inner shell in turn encloses three minor proteins, VP1, VP4 and VP6, and the genome of 10 segments of dsRNA. The outer shell, termed the *outer capsid,* consists of two other major proteins: VP2 and VP5 (225).

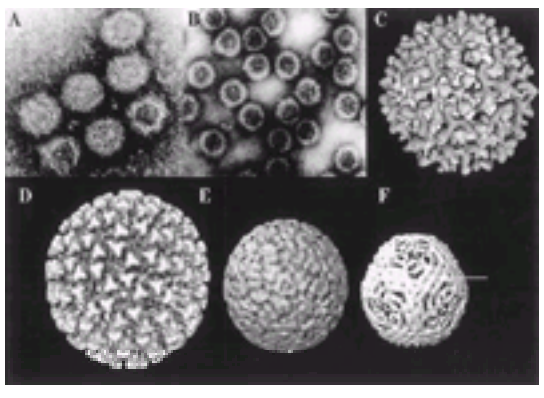

FIG. 1. A: Electron micrographs of negatively stained bluetongue virus (BTV) particles and core. **B:** Surface representations of the 3D cryoelectron microscopy structures of BTV virion (40 Å resolution) (**C**), showing sail-shaped VP2 spikes (trimers) and globular VP5 trimers; and the core of BTV viewed along the icosahedral threefold axis, showing the whole core (**D**), the middle layer of VP3 (210 to 290 Å) molecules cut away from the core structure (**E**), and the innermost density of three minor proteins in association with genome (**F**). Ordered strands of genomic RNA *arrow*) are visible at 2.3 Å resolution. Only the upper hemisphere is displayed for clarity. (Courtesy of B.V.V. Prasad, Baylor College, Houston, TX; from ref. 71, with permission.)

The morphologic features of unstained and unfixed, complete virus particles and the cores have been determined by 3D analysis at 23 to 40 Å resolution using cryoelectron microscopy and computer image reconstruction methods (63,71,167). More recently, the atomic structure of the core particle has also been resolved by x-ray crystallography at a resolution of 3.6 Å (62).

The 3D structure of the BTV core (sedimenting at 470 S), with a diameter of 69 nm (radius, 34.5 nm), has an icosahedral symmetry with a triangulation number of 13 (T=13) in a left-handed configuration. The core consists of two concentric layers of density surrounding a central density (see Fig. 1). The outer layer of the core is made up of clusters of VP7 trimers, which exist as prominent triangular protrusions, and are located at all the local and strict threefold axes (see Fig. 1D). These trimers are about 40 Å in length and 40 Å in diameter at the surface of the core and are arranged as six-membered rings, with five-membered rings at the vertices of the icosahedron. These ring structures are alluded to in the genus name *Orbis* (ring or circle). There are a total of 780 VP7 molecules per particle, forming 260 trimers, or triangular capsomers, that are arranged around distinctive 132 channels, which are present at all 5- and 6-coordinated positions of the T=13 lattice (see Fig. 1D). The VP7 trimers extend 8 nm outward from an inner density (at a radius of 28 nm), and the protruding capsomers give a bristly appearance to cores when examined by cryoelectron microscopy. The aqueous channels that are surrounded by these trimers are about 8 nm wide at the outer surface and 8 nm deep. Some of these channels extend inward, penetrating through to the inner layer. These channels probably provide pathways for transportation of metabolites and messenger RNA (mRNA) species during the transcription processes of core particles. The inner layer, which is made up of the second major protein, VP3 (see discussion VP3 structures, later), is relatively featureless and exhibits a smooth bed of density, with almost spherical shape (see Fig. 1E), but it has an angular appearance owing to variations in thickness of the protein shell. The density in the central portion of the core structure is within a radius of less than 21 nm (see Fig. 1F). It represents the structures involving the VP1, VP4, and VP6 proteins and the genomic RNA (see later).

The 3D reconstruction of BTV double-capsid particles by cryoelectron microscopy has shown a well-ordered morphology of the complete virion (71). This is in contrast to the morphology deduced by negative-staining methods, which gives a fuzzy appearance to the outer capsid (225). The outer capsid has an icosahedral configuration, and the two proteins of the capsid have distinctive shapes, one globular and almost spherical, the other sail-shaped (see Fig. 1C). The globular

structures, 120 in number, sit neatly in the spaces formed by each of the six-membered rings of VP7 trimers. The sail-shaped spikes, which project 4 nm beyond the globular proteins, are located above 180 of the VP7 trimers and form 60 triskelion-type motifs that cover nearly all the VP7 molecules. These spikes are believed to be the hemagglutinating protein VP2 that also contains the neutralizing epitopes. The underlying globular proteins are probably composed of VP5. The two proteins form a continuous layer around the core, except for the holes at the fivefold axes. A similar well-ordered morphology has also been obtained by cryoelectron microscopy reconstruction analysis of the tick-borne Broadhaven virus (BRDV) outer capsid, indicating that indeed orbiviruses are icosahedral particles (197). Moreover, since the two outer capsid proteins, VP4 and VP5, of BRDV differ in their sizes from BTV VP2 and VP5, it was possible to confirm the identity of the triskelion shape as trimers of VP4 of BRDV (equivalent to VP2 of BTV). The shape of the BRDV VP5 (VP5 of BTV) indicates that VP5 is also a trimer, thus implying that there are 360 copies of VP5 or 120 trimers. The current understanding of the topography of the seven structural proteins in virions is illustrated in a schematic diagram (Fig. 2).

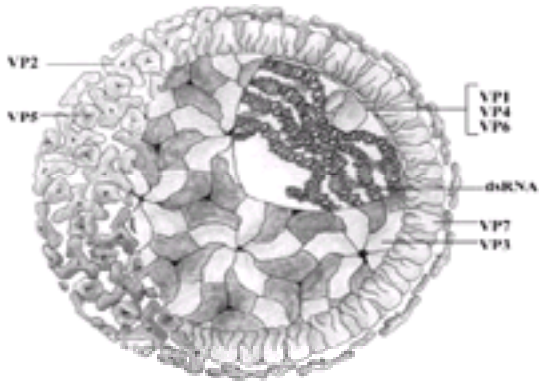

FIG. 2. Schematic diagram of the bluetongue virus (BTV) particle showing the positions and structures of BTV components. The diagram is based on data obtained from cryoelectron microscopic and x-ray crystallographic analyses and on data obtained from baculovirus recombinant expression systems.

GENOME STRUCTURE AND ORGANIZATION

The orbivirus genome is composed of 10 dsRNA segments, ranging in size from 0.56×10^6 to 2.7×10^6 daltons (48). The segments can be separated by polyacrylamide gel electrophoresis (PAGE) into patterns distinctive for each group and within groups between serotypes. For some isolates, certain segments co-migrate.

The RNA, when extracted under acidic conditions (pH 4.0) with sodium docecyl sulfate (SDS) and phenol, occasionally appears as an unfragmented continuous structure. Under the electron microscope, this structure has a rosette configuration with 10 varying-length loops that emanate from a central region resembling a doughnut. The relative size of each of the 10 loops is about equivalent to the sizes of the individual gene segments. The RNA segments are numbered 1 to 10 in order of migration (Fig. 3) and also may be referred to as large, medium, and small segments (i.e., L1 to L3, M4 to M6, and S7 to S10) (206). Their relative order of mobility may vary according to the electrophoretic conditions used. The dsRNA profiles of members representing each orbivirus serogroup are generally distinctive by PAGE and also differ from those of other members of the *Reoviridae* family (105).

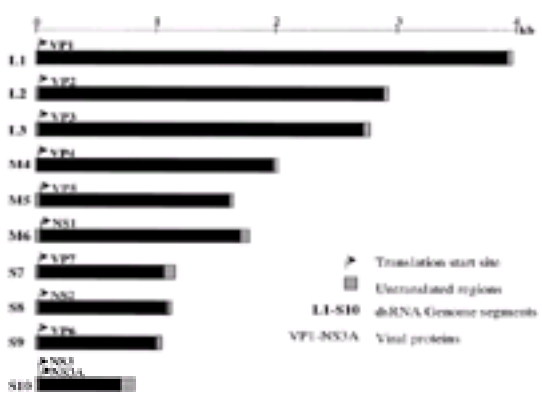

FIG. 3. Schematic diagrams of the 10 RNA segments of BTV-10 and their relative lengths and other features revealed by sequence analysis.

The complete sequences of all 10 dsRNA segments have been determined initially of BTV-10 (48) but subsequently for a number of other BTV serotypes as well as for EHDV, AHSV, the tick-transmitted BRDV, and more recently the Chuzan virus of the Palyam serogroup (134,143,144,189,241,244).

The 10 dsRNA segments of BTV have conserved terminal sequences (Table 2; see Fig. 3). Both strands of each of the 10 dsRNA segments are entirely complementary, forming complete duplexes. However, the 5' terminus of the coding strand of each duplex is capped and methylated, as are the BTV mRNA transcripts (84). The opposing, noncoding strands in the virion dsRNA species are neither capped nor methylated. For the mRNA sense strand of all segments, the conserved terminal sequences involve eight nucleotides at the 5' ends and six nucleotides at the 3' ends (175). For BTV-10, the total genome is 19,220 base pairs (bp) in length, representing a molecular weight ratio (M_r) of a 13×10^6

daltons (Table 3; see Fig. 3). The sizes of the BTV-10 RNAs range from 3,954 bp (L1, $M_r \sim 2.7 \times 10^6$) to 822 bp (S10, $M_r \sim 5 \times 10^5$). The base distribution total of the genome is 28.1% A, 28.1% U, 21.9% C, and 21.9% G. The base ratios of the individual segments are similar (i.e., A or U range from 29.3% to 25.4%, G or C from 24.6% to 20.7%). The 5'-noncoding regions vary in length from eight (segment M4) to 34 (M6) nucleotides. The 3'-noncoding sequences are generally longer, varying from 31 (M5) to 116 (S10) nucleotides (see Table 2).

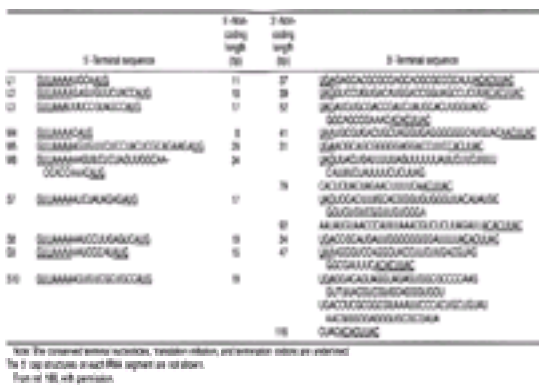

TABLE 2. *Noncoding terminal sequences of the bluetongue virus viral RNA segments*

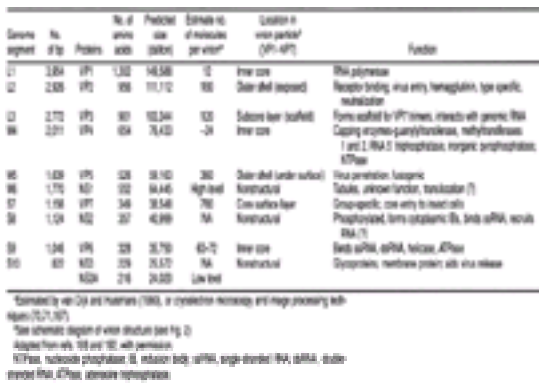

TABLE 3. *Bluetongue Virus (BTV-10) coding assignments*

The 5'- and 3'-terminal sequences of the 10 mRNA strands of BTV, EHDV, and AHSV have partial inverted complementarity and are thus capable of forming secondary end-to-end (panhandle) structures and exhibiting some common features (e.g., a looped-out sequence proximal to the 3'-terminus) (21,95). Overall, each mRNA appears to have the possibility to form a complex secondary structure involving stem-loop configurations that are similar not only between corresponding segments of various BTV serotypes but also to those of EHDV and AHSV (21,187). Although this attribute may be of particular relevance to mRNA stability (on the premise that secondary structure limits degradation by endonucleases, or exonucleases), it is potentially also relevant for mRNA recognition by the viral polymerase and for the association of RNA segments. Interestingly, whatever the intrastrand complementary sequence arrangement may be, parts of the conserved 5'- and 3'-hexanucleotides remain unpaired and, by virtue of their actual sequence as well as the presence of a capped, methylated structure, provide a means to distinguish the ends of the coding from the noncoding strands. Thus, both the sequences and lengths of the BTV noncoding regions may be relevant for mRNA packaging into precursor particles, for strand selection for mRNA synthesis, and for determining translation efficiencies of different mRNAs.

Apart from the BTV-10 L1 segment, the first AUG initiation codon in the mRNA strand of each segment initiates a single open reading frame (ORF) and product characteristic of that mRNA. For L1, the second AUG (positions 12 to 14) codon but not the first (positions 7 to 9) is the initiation codon (see Table 2). There are two methionine codons in the same reading frame in the S10 RNA sequence, one at residues 20 to 22, the other at residues 59 to 61 (48,115). These are used to synthesize the NS3 and NS3A products of S10 (see Fig. 3).

Extensive data on the genome sequences of the different BTV serotypes and of other orbiviruses are discussed in the section on Genetics and Orbivirus Evolution. The data demonstrate that, apart from RNA segments L2 and M5, the other RNA segments are highly conserved within each serogroup.

GENE CODING ASSIGNMENTS AND VIRUS PROTEINS

Each genome segment of BTV codes for the synthesis of one major protein, except segment S10, which codes for two related proteins: NS3 and NS3A (43,130). The structural proteins are numbered VP1 to VP7 in order of their decreasing sizes on SDS-PAGE. In addition to the seven structural proteins, four nonstructural proteins (NS1, NS2, NS3, and NS3A) are also synthesized in infected cells (188). The coding assignments, sizes, and locations of the gene products are summarized in Table 3. When individual gene products are compared with these values, several striking variations are evident. These variations are discussed later, with other data, for each gene product.

The Two Outer Capsid Proteins

The outercapsid proteins VP2 and VP5 are encoded by RNA segments L2 and M5, respectively (130) and exhibit the least conservation between virus serotypes (188).

The BTV-10 VP2 protein consists of 956 amino acids (M_r 111,000; see Table 3), rich in cysteines and residues, which may indicate that it has a highly ordered, disulfide-bonded structure (46,47,49,56,169,240,241). Comparisons of BTV-10 VP2 sequence with VP2 sequences of other BTV serotypes and with that of EHDV and AHSV, the two other closely related Orbiviruses (Table 4) indicate that it is the most variable of BTV proteins. Nevertheless, some BTV serotypes are more closely related than others (e.g., BTV-10 and -17 than BTV-10 and BTV-1, -2, and -13). In general, the differences in amino acids among the seven serotypes that have been sequenced to date are distributed evenly throughout the protein (243), although there are a number of highly variable regions that can be identified (Fig. 4). Conversely, there are a number of highly conserved regions that are evident. The C-terminus and the middle region of VP2 are relatively well conserved, with the absolute conservation of an octapeptide (see Fig. 4) among all seven serotypes (NPYPCLRG; amino acids 360 to 367 in BTV-10), which may reflect a functionally important region of the protein. The common phylogenetic origin of all these viruses is shown by other conserved features, such as six conserved cysteine residues, similar hydropathic profiles, the formation of perfect diagonal lines when the BTV VP2 proteins are aligned, and the indicated secondary structures, such as a-helices, b-strand, and turns (188).

Serotype	BTV-10A	BTV-2	BTV-3	BTV-10	BTV-11	BTV-13	BTV-17	BTV-20Au	EHDV-1	AHSV-3	AHSV-4	AHSV-6
BTV-10Au	87	69	58	51	51	57	52	64	39	41	40	39
BTV-10SA		68	57	49	59	56	50	63	37	41	37	39
BTV-2			60	52	59	59	52	62	37	33	39	35
BTV-3				52	51	79	51	58	38	36	38	36
BTV-10					78	51	80	51	37	35	36	38
BTV-13						51	81	51	36	36	38	35
BTV-17							52	59	41	41	41	38
BTV-20Au								59	34	45	42	32
EHDV-1									30	36	36	35
AHSV-3										60	62	59
AHSV-4												62

Note: Comparisons made with residues belonging to the same functional group. Numbers indicate percent homology at amino acid level.
Au, Australian serotype; SA, South African serotype.
From ref. 243, with permission.

TABLE 4. *Homologies among nine bluetongue virus (BTV), one epizootic hemorrhagic disease virus (EHDV), and three African horse sickness (AHSV) VP2 proteins*

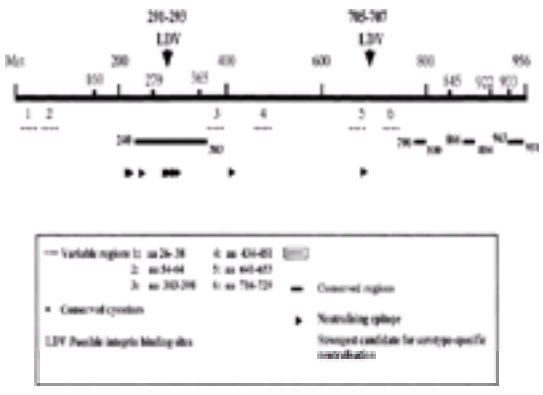

FIG. 4. Schematic diagram of VP2 protein. Structural and antigenic properties of VP2 based on the analyses of amino acid sequences of different bluetongue virus serotypes.

The VP2 protein of BTV is a hemagglutinin and is directly involved in the attachment of virus to cells (30,67,75). When VP2 is removed, the hemagglutinin, infectivity, and binding of the derived particles are significantly reduced for vertebrate cells (20,44,135,162). However, *in vitro*–generated intermediate subviral particles (ISVP) of BTV that still contain the chymotrypsin cleavage products of VP2 are fully infectious (135). The VP2 protein is the principal antigen that determines serotype specificity (79,102,191), and VP2 elicits serotype-specific neutralizing antibodies that confer protection against homologous virus infection in vertebrate hosts (80,94,191). The neutralizing epitopes of VP2 are predominantly conformation dependent, and VP2 purified from SDS-PAGE fails to induce neutralizing antibodies (87,116,180,236). VP2-specific monoclonal antibodies neutralize virus infectivity and provide passive protection against homologous virus challenge. Antisera raised in rabbits and mice after immunization with insect cell lysates expressing recombinant VP2 proteins of different serotypes efficiently neutralized the infectivities of homologous and, to a lesser extent, certain other heterologous BTV serotypes (94,217). These data suggest that not all BTV serotypes are equally distinct.

A number of conformationally dependent neutralization epitopes on VP2 have been identified (27,56,57,69,116,236) (see Fig. 4). Five epitopes for BTV-1 (amino acids 211, 267, 328 to 335, 491, and 539) and for BTV-10 (amino acids 208, 211, 327, 402, and 654) at similar positions and an additional one for BTV-13 (amino acids 642 to 651) have been mapped. These neutralizing epitopes of BTV are probably not unique to each serotype (166,178,180). Some data for AHSV VP2 neutralization sites have also been generated recently that are similar to those of BTV (127).

The second outer capsid protein, VP5, of BTV-10 has 526 amino acids (M_r 59,163) (53,139,170). The protein is variable to a certain extent, but not as variable as the VP2 protein (74,162,246). The protein also shares strong sequence homology with the VP5 proteins of EHDV-1, AHSV-4, and Chuzan virus but not with the VP5 of tick-borne BRDV VP5

(95,96,143,242,244). It is noteworthy that after the initiating methionine at the N-terminus, a glycine residue is present that is conserved in the VP5 proteins of all BTV and EHDV analyzed so far (95). However, the protein is not myristoylated (P. Roy, unpublished data). VP5 may be glycosylated containing short and unbranched N-linked carbohydrate moieties and possibly O-linked sugars (246). It has recently been shown that VP5 has a role in virus penetration of endosomal membrane and is responsible for the release of virus core into cell cytoplasm (68).

Based on the amino acid sequence, VP5 is divided into an aminoterminal coiled-coil domain and a carboxyl-terminal globular domain with a flexible hinge region in between (Fig. 5). The aminoterminus of VP5 could also potentially form two amphipathic helices (helix 1 and helix 2), each with about equal separation of hydrophobic and hydrophilic faces followed by a putative fusion peptide (see Fig. 5). Such structural features support the concept that VP5 is capable of inducing membrane destabilization and permeabilization (68).

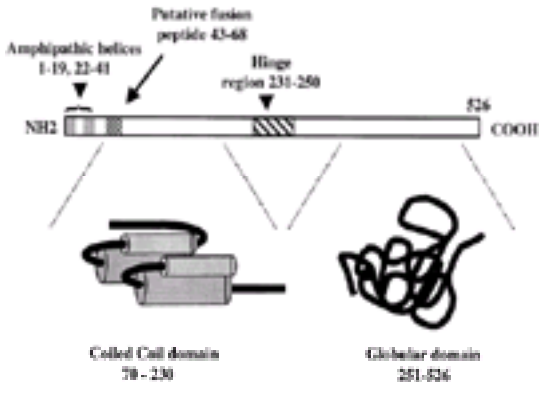

FIG. 5. Schematic organization of VP5 protein showing the putative fusogenic coiled-coil domain and the globular domain that are linked by a hinged region.

Clearly, the two outer capsid proteins VP2 and VP5 are responsible for virus entry and penetration processes. The predicted structures of these proteins correlate with such functions. Like VP5 of BTV, both rotavirus and reovirus have similar structural organization in the outer capsid protein (Fig. 6). It is interesting that in rotavirus and reovirus, a coiled-coil structure is present in the VP4 and s1 proteins, which are the hemagglutinin proteins and the viral attachment proteins to cellular receptors, unlike the situation in BTV, in which it is the VP5 protein that has the coiled-coil structure and not VP2. The positions of the domains within the rotavirus VP4 are inverted in relation to the BTV VP5 protein. The function of virus attachment and the penetration for orbiviruses are therefore performed by two different proteins.

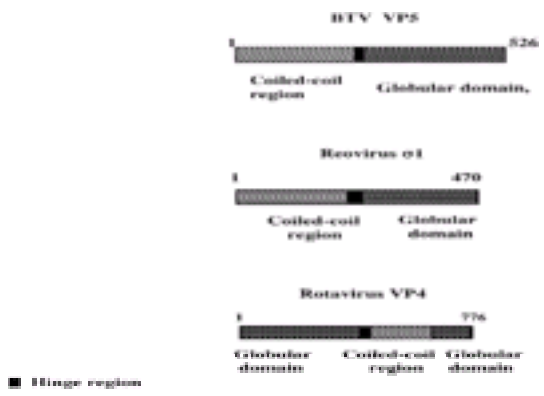

FIG. 6. The overall organization of VP5 primary structure shows the similar organization with rotavirus outercapsid protein, VP4, and reovirus outer capsid protein, s1. Note that all three proteins consist of coiled-coil and globular domains.

VP5 may also play a role in virus neutralization activity. In BTV, VP5 appears to enhance the protective neutralizing activity of VP2 (191), whereas in AHSV and the Kemerovo serogroup, VP5 appears to carry neutralization epitopes itself (128,154).

The Five Core Proteins

In virus-infected cells, parental BTV virions (550S) are converted to core particles (470S) containing VP1, VP3, VP4, VP6, and VP7 and exhibiting RNA-dependent RNA polymerase activity. This enzymatic activity allows the virus to synthesize mRNA from virion RNA templates (83). Recently, much progress has been made in dissecting enzymatic functions provided by the core proteins. This has been possible owing to the availability of highly purified recombinant baculovirus-expressed functional proteins and of subvirion structures.

The three minor proteins VP1, VP4 and VP6, together with 10 dsRNAs, form the innermost part of the core. All three proteins are highly conserved across the different BTV serotypes that have been analyzed to date. The largest viral protein, VP1, with an M_r of 149,588, is encoded by the dsRNA segment 1, or L1 (184). The molecule is a highly basic protein and is present in a low molar ratio in the virion (see Table 3). Based on its size, location, molar ratio in the core, and predicted amino acid sequence, this protein is the prime candidate for the virion RNA-dependent RNA polymerase (184). The primary sequence of VP1 contains a GDD motif (positions 287 to 289), characteristic of other RNA

polymerases. In addition, VP1 exhibits some sequence homology with a number of other RNA and DNA polymerases (184). Indeed, extracts of cells that were infected with a recombinant baculovirus expressing VP1 protein showed a poly(A) polymerase activity in an *in vitro* assay (218). Recent data have shown directly that indeed VP1 is the viral polymerase protein and has the capability to synthesize *in vitro* complementary BTV RNA on a BTV single-stranded (ssRNA) template, generating RNase-resistant dsRNA duplexes (172).

The second minor protein of the core, VP4 (M_r 76,433), is encoded by M4 RNA (250). The protein has a high content of charged amino acids and exists as a dimer in solution and within the core. A leucine zipper in the center of the molecule is responsible for the dimerization of VP4 (173). The dimeric form of VP4 is essential for encapsidation of the protein within the core. Highly purified recombinant VP4 has been demonstrated to possess various enzyme activities (nucleotide triphosphatase, RNA 5'-triphosphatase, inorganic pyrophosphatase, guanylyltransferase, methyltransferase 1 and 2) that are necessary for the modification (capping) of 5'-termini of positive stranded RNA species (18,111,171,174). Thus, complete BTV RNA capping appears to be associated with this one viral protein alone (171).

The third minor protein, VP6, is encoded by S9 RNA. VP6 has an M_r of 35,750 and is very rich in basic amino acids (Arg, Lys, and His), suggesting that VP6 may bind to nucleic acids (181). Baculovirus-expressed and BTV-derived VP6 have both been shown to have a strong affinity for binding ssRNA and dsRNA species, even in the presence of SDS, indicating that its nucleic acid–binding ability is independent of its tertiary structure and suggesting its close association with the viral genome in virions (181). Subsequent studies demonstrated that VP6 protein is highly active as a helicase enzyme (199). VP6 hydrolyzes adenosine triphosphate (ATP) in the presence of divalent cations. Both ssRNA and dsRNA stimulate the ATPase activity of VP6. Like most helicases, in the presence of ATP, the VP6 enzyme unwinds dsRNA substrates with 3' overhangs. Interestingly, it also unwinds substrates with 5' overhangs, which has been reported for only a few other helicases, and, as expected, it unwinds blunt-ended dsRNA (Fig. 7). Like other helicase proteins, VP6 appears to form hexameric oligomers in the presence of RNA. Oligomer formation can be monitored by size-exclusion chromatography after chemical cross-linking. The nucleic acid–binding and helicase capabilities might allow VP6 to participate in mRNA transcription, but also in virus assembly by interacting with the 10 mRNA species and assisting the process of rendering them into dsRNA segments. The fact that VP6 binds dsRNA (as well as ssRNA) may be relevant to the encapsidation process and sequestering of dsRNA species into precursor cores and virions (194,195).

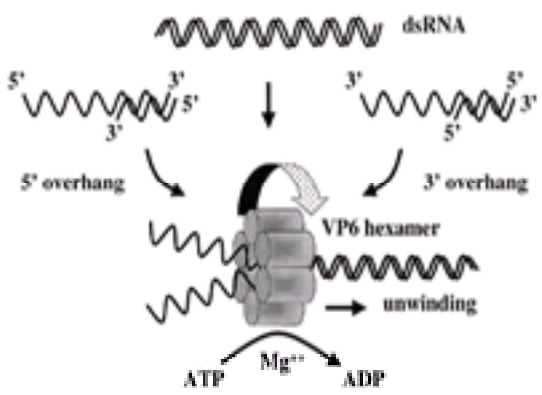

FIG. 7. Schematic of helicase activity of hexameric VP6 on various duplex RNA templates.

The VP6 protein is abundant in basic residues, particularly in two regions of the carboxy half of the molecule. A series of aminoterminal and carboxy-terminal deletion mutants when transiently expressed revealed that VP6 has nuclear transport signals that may be correlated with positively charged domains of the molecule (247). In the intact molecule, though, these signals are masked, and the protein is retained in the cytoplasm. The sequences near the amino acids (33 to 80) of the 328–amino acid protein are required for the retention of VP6 within the cell cytoplasm, whereas a region rich in basic residue (amino acids 303 to 308) near the carboxy terminus appears to possess nuclear localization signals (247).

The VP3 and VP7 proteins are the two major proteins of the core; VP7 is the more abundant (see Table 3). The larger protein, VP3, forms an inner scaffold for the deposition of the surface layer of VP7 in the core. VP3 also interacts with the three minor proteins. The L3 RNA encodes the VP3 protein (M_r 103,344) (50,168). The protein is hydrophobic and for BTV-10 consists of 901 amino acids. As far as analyzed to date, VP3 is highly conserved among BTV serotypes and other orbiviruses (e.g., EHDV and AHSV). For example, only nine amino acids are different in the VP3 proteins of U.S. serotypes BTV-10 and BTV-17 (50). A similar conservation is seen by comparing the VP3 sequences of other U.S. and Australian BTV serotypes (60). The protein is also remarkably similar to that of EHDV-1 and AHSV-4 (59,110), reflecting its very specific role in determination of virus capsid morphology and in the organization of the virus genome (Table 5). Recombinant VP3 protein is recognized by all anti-BTV antisera and reacts weakly with anti-AHSV and anti-EHDV antisera (93,110), suggesting that there are conserved functions in VP3 as well as conserved amino acid sequences.

BTV-10	VP2		VP7	
Viruses	Similarity (%)[a]	Identity (%)[b]	Similarity (%)[a]	Identity (%)[b]
BTV-10A	ND	ND	98	98
BTV-10Ag	ND	ND	98	98
BTV-2	98	96	98	98
BTV-11	98	96	ND	ND
BTV-13	98	96	ND	ND
BTV-15	ND	–	98	93
BTV-17	98	96	98	98
EHDV-1	87	79	77	53
EHDV-2	87	79	ND	ND
AHSV-4	68	58	53	43
AHSV-6	68	58	53	43
AHSV-9	ND	ND	53	43

[a]Similarity: Residues belonging to the same functional group.
[b]Identity: Identical amino acid residues.
ND, not done; BTV, bluetongue virus; EHDV, epizootic hemorrhagic disease virus; AHSV, avian herpesvirus.
From ref. [56], with permission.

TABLE 5. *Comparisons of the two core proteins of three orbiviruses*

The second major protein, VP7, has an M_r of 38,548 and is highly conserved among the various BTV serotypes (106,107,229,249). The VP7 protein of BTV is also closely related to those of EHDV and AHSV (up to 80%; see Table 5) (108,221,279) but is only distantly related to the equivalent protein of the tick-borne BRDV, only 21% amino acids being identical in type and position (144). The BTV-10 VP7 protein is deficient in charged but rich in hydrophobic amino acids. VP7 has only one lysine, which is remarkable because all the other BTV gene products are lysine rich.

The recombinant BTV-10 VP7 protein reacts with antibodies raised to different BTV serotypes and, to a much lower level, with antibodies raised to AHSV and EHDV, indicating that, like VP3, it possesses group-specific determinants (161). Similarly, recombinant VP7 of AHSV-4 reacts with antisera raised to nine different AHSV serotypes and, at a much lower level, with BTV antisera (15). The high-level synthesis of BTV VP7 has facilitated the purification and crystallization of the protein (5). Three different crystal forms (I through III) suitable for x-ray diffraction analyses were generated. Type I crystals are hexagonal rods, whereas type II and III crystals are monoclinic. From x-ray and light-scattering studies, it was deduced that VP7 forms trimers in crystal forms and in solution. These crystals diffracted at up to 2 Å resolution (further details given later).

The Nonstructural Proteins

Four different nonstructural proteins have been identified in BTV-infected cells. The two major nonstructural proteins, NS1 and NS2, are synthesized abundantly, whereas the two minor, closely related proteins, NS3 and NS3A, are barely detectable (31,43,85). The sequences of each NS protein are highly conserved (96%) among BTV serotypes (reviewed in 188). The synthesis of NS1 and NS2 in BTV-infected cells coincides with that of two virus-specific structures, tubules, and the granular inclusion bodies (IBs), that are characteristic for orbivirus-infected cells (31,85). IBs can be both granular and fibrillar and are found throughout an infected cell, but predominantly near the nucleus (31). Virus tubules are present in large numbers, mostly in perinuclear locations. So far, no function has been ascribed to BTV tubules, although they may be involved in the translocation of virus particles to the cell plasma membrane, whereas the NS3 proteins facilitate their egress (see Release of Virions). NS1-specific monoclonal antibodies have been shown to react with virus particles that are either in close proximity to IBs or leaving infected cells.

Tubules are composed of the M_r 64,445 NS1 protein, the product of RNA M6 (219) (see Table 3). This protein is synthesized more abundantly than any other BTV protein (78). The protein may be used as a group-specific diagnostic reagent (179). The BTV-10 NS1 protein has a low content of charged amino acids but is rich in cysteines (114,188), suggesting a highly ordered structure. Interestingly, six of the BTV-10 NS1 cysteines are clustered in pairs, with two or five amino acids in between; they may form disulfide bridges. When the NS1 gene is expressed in insect cells by a recombinant baculovirus, tubules similar to those found in BTV-infected mammalian cells are formed, confirming that the tubules are multimeric forms of the NS1 protein (219). Cryoelectron microscopy analyses have shown that the BTV tubules are on average 52.3 nm in diameter and up to 1,000 nm long with a helical configuration (73). The NS1 protein, which is about 5.5 nm in diameter, forms a dimer. The tubular structure consists of a helically coiled ribbon of NS1 dimers, with about 21 or 22 dimers per turn. A ribbon of dimers may be seen unwinding from the ends of tubules under certain conditions.

The nature of the NS1 interactions involved in tubule formation have been analyzed using a series of N- and C-terminal deletion and site-directed mutagenesis targeting cysteine residues (139). When expressed by recombinant baculoviruses, the data demonstrated that both the N- and C-termini and only one pair of cysteines, at amino acid positions 337 and 340, are essential for tubule formation. Using a panel of monoclonal antibodies, together with a series of NS1 mutants containing deletions and substitutions, an antigenic site has been identified at the carboxy end of the molecule (139). Interestingly, extension of the C-terminus with a peptide of at least 250 amino acids had no effect on the assembly of the tubules and on their morphology (137). Recombinant chimeric NS1 tubules are highly immunogenic, eliciting protective B- and T-cell responses (185).

The second nonstructural protein, NS2, with an M_r of 41,000 (215) (see Table 3), is the only virus-specific phosphoprotein that is present in BTV-infected cells (28,85). NS2 is encoded by RNA segment S8 and is rich in charged amino acids (48,130). The BTV-10 NS2 protein has seven cysteine residues, four of which are located near the carboxy terminus (48). The protein is generally hydrophilic, with pairs of positively charged amino acid (K, R) domains located near both ends of the protein. NS2 is synthesized to a high level and is mostly present in cytoplasmic IBs (90,215). NS2 expressed by baculovirus vectors forms multimeric IBs in insect cells in the absence of other BTV gene products, and the morphology is similar to that found in BTV-infected cells (215).

NS2 protein has been shown to bind ssRNA (82,212,215), but not dsRNA. This property is similar to that of the rotavirus NS3 protein, which also forms oligomeric structures, binds ssRNA, and exists in complexes with viral RNA species in

infected cells (104). The phosphorylation of NS2, which involves specific serine residues, does not appear to be important in RNA-binding activity (213,215). Similarly, the C-terminus (including the last 130 amino acids) is not required for ssRNA binding or for oligomerization (251). The N-terminus of NS2 was not required for oligomerization (up to residue 92) either, but was found to be essential for ssRNA binding. In particular, the arginines at amino acid positions 6 and 7 and a lysine at position 4, but not a glutamic acid at position 2, are required for ssRNA binding (251). Similarly, for NS2 of EHDV, a short N-terminal region (amino acids 75 to 83) have also shown to be essential for RNA-binding activity (212). It is believed that NS2 is involved in recruiting mRNA species into IBs during assembly of virus components.

Unlike the NS1 and NS2 proteins, the two smallest nonstructural proteins, NS3 and NS3A, are synthesized in low abundance in BTV-infected BHK cells (43,85). However, in contrast to vertebrate cells, high-level expression of these proteins can be detected in invertebrate cells in which virus release is nonlytic (65). This suggests that NS3 or NS3A may have a significant functional role in invertebrate cells. Both NS3 and NS3A proteins are encoded by BTV RNA S10 (43,130,221). Biochemical and immunologic analyses on expressed S10 gene products have shown that NS3 and NS3A are related and derived from alternative translation initiation sites (43,115). Deletion of the first AUG abolishes synthesis of NS3 but not that of NS3A (238).

The NS3 proteins of all BTV serotypes have two conserved hydrophobic domains (amino acids 118 to 141 and 162 to 182) (115). In addition, there are two potential glycosylation sites (amino acids 63 and 150). Using a transient expression system, NS3 and NS3A proteins made in mammalian cells are glycosylated and converted into heterologous polylactosaminoglycans (238). In contrast to BTV NS3 sequences, NS3 of different AHSV serotypes are not highly conserved, and the variation in the NS3 sequence has been shown to influence the virulence characteristics of the progeny virus strain (126). Based on mutational analysis of NS3, it appears that like rotavirus NSP4 (a nonstructural glycoprotein), both hydrophobic domains span the cell membrane, with both termini being cytoplasmic (Fig. 8). Only the site at amino acid position 150 but not at position 63 may have the potential for modification by N-linked glycosylation (2). The NS3 proteins are transported through the Golgi apparatus to the cell plasma membrane in the presence or absence of tunicamycin, suggesting that carbohydrate chains are not required for transportation (238). These proteins are found to be associated with intracellular, smooth-surfaced vesicles as well as the plasma membrane (91). The data strongly suggest that the NS3 proteins are integral membrane glycoproteins. By using recombinant baculoviruses, it has been shown that NS3 or NS3A facilitates the release of virus-like particles (VLPs; as surrogates of virions) from host cells, and that these proteins are present at the site of the membrane from which VLPs are released (92). This indicates that the integration of NS3 or NS3A into the plasma membrane probably helps the release of mature virions. The function is similar to that of NSP4 of rotaviruses, which interacts with viral bilayered particles to facilitate their transport across the rough endoplasmic reticulum of infected cells (1,136).

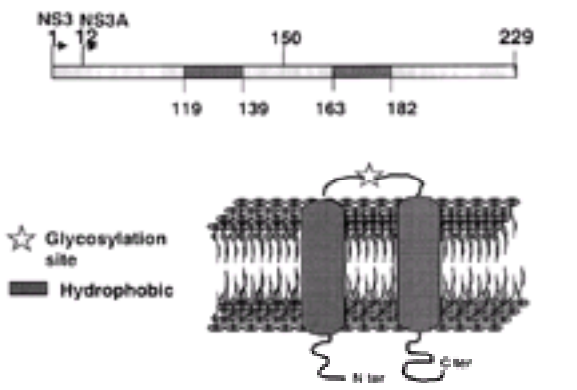

FIG. 8. Diagram of NS3 protein (*top*) and its organization in membrane (*bottom*). Note the positions of the two hydrophobic domains (amino acids 119 to 139 and 163 to 182) and the single glycosylation site (amino acid 150). The two start codons (amino acids 1 and 12) are indicated at the top schematic.

From recent studies, it appears that the release of mature virions aided by NS3 takes advantage of cellular exocytic pathway. NS3 forms a complex with cellular protein p11, which is the smaller component of the p36/p11 heterotetrameric calpactin II complex (22). Further, although the first 13 residues of NS3 interact with this p11, the carboxy end of NS3 interacts with VP2 of mature virions. Because calpactin II complex is involved in cellular exocytosis, it is assumed that p11 binding by NS3 provides a mechanism for the virus to use the cellular exocytic pathway for nonlytic virus release (7).

X-RAY CRYSTALLOGRAPHIC STRUCTURES AND THREE-DIMENSIONAL RECONSTRUCTION OF PROTEINS AND PARTICLES

The Atomic Structure of VP7

BTV-10 VP7 is the first capsid protein of members of the *Reoviridae* family that has been crystallized, and the structure has been solved at atomic level. The data show some striking features in comparison to the protein structure of other viruses. The VP7 structure revealed a unique molecular architecture of trimeric nature (61). Each monomer consists of two distinct domains, the upper and lower, which are twisted such that the top domain of one monomer rests on the lower domain of a threefold-related subunit in a clockwise direction (Fig. 9). The interactions between subunits are extensive both between the top and bottom domains, involving both hydrophobic and specific hydrogen bond interactions. The smaller upper domain of VP7 forms the “head region” of the trimer, which appears as a knobby projection on the outer surface of the core by cryoelectron microscopy and contains the central one third of the polypeptide chain of the molecule (amino acids 121 to 249). In the trimer, the three upper domain subunits, each of which is folded into an antiparallel b-sandwich, are intimately associated through two extended loops from each subunit, both with each other and with all possible threefold related subunits. The x-ray structure of the upper domain of AHSV-4 VP7, which was

solved subsequently, also revealed similar structural arrangement, indicating that it is likely that the VP7 of all orbiviruses may have similar structures (6). The characteristic feature of the upper domains of both molecules is the presence of an Arg-Gly-Asp (RGD) motif (although for AHSV, the RGD site is located at a slightly different position), which may be responsible for binding of the orbivirus core to a cellular integrin receptor. The lower domain of the VP7 trimer is composed of nine a-helices and long extended loops and is formed by both the N-terminus (amino acids 1 to 120) and the C-terminus (amino acids 250 to 349) of the molecule (see Fig. 9). The interjunction region between the two domains is exposed and is easily accessible to proteolysis. The single lysine residue of BTV-10 VP7 is situated at this junction between the two domains, which explains why mutation at this site had deleterious effect on core assembly (112). The helices in the bottom domain pack together tightly. The mutational studies obtained indicate that the interactions between VP7–VP7 monomers are rigid and that the compact nature of VP7 trimer could easily be destabilized by a single point mutation at the key positions (112,117).

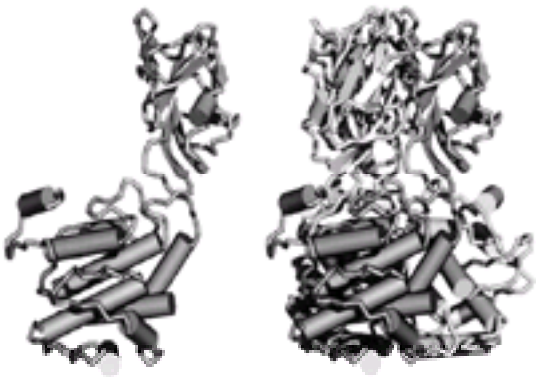

FIG. 9. The structure of a monomer (*lower*) and trimer (*upper*) of VP7. Note the aminoterminus in the *right foreground* and the carboxyl terminus in the *left* on the monomer. The view is shown from the side, and the extensive rather flat base of the molecule lies in a horizontal plane in this view (61,62). (Drawn by J. Diprose, J. Grimes, and D, Stuart, using Bobscript [40], POV-ray and Photoshop.)

Core Particle

X-ray crystallographic structures at 3.5 Å resolution for core particles of two different BTV serotypes (BTV-1 and BTV-10) have been determined (55,62,63). Combining the atomic data with high-resolution (23 Å) cryoelectron microscopy of BTV core, an atomic model has been constructed for the structure and organization of VP7 and VP3 within the core particle.

The organization of 260 VP7 trimers follows precisely a T=13 icosahedral lattice, in accordance with the theory of quasi-equivalence (63). The threefold axis of the trimer (85 Å long) is perpendicular to the core surface, and the broader base of the trimer (65 Å) contacts the internal network of the VP3 of the subcore (see Fig. 9A and Fig. 9B). The VP7 layer occupies between 295 and 365 Å from the center of the core. As shown in Figure 10A, five VP7 trimers at different distances from the fivefold axis form a protomeric unit (labeled P to T). These trimers show relatively little variation in their structure throughout the core surface layer (see Fig. 10B). The 260 trimers of VP7 adhere to a scaffold that is made up of only 120 VP3 molecules, indicating a symmetry mismatch in the interactions and that these trimers interact in 13 different relative positions. The interactions between the different trimers are facilitated by a thin band of hydrophobic patch on the contacting surfaces, which leads to some small deviations between the 13 copies in the contact patches. The interface between VP7 and VP3 trimer layer is principally formed by the side-to-side packing of short hydrophobic side chains from helix 4 and its twofold or quasi-twofold partner in the neighboring trimers. The interaction between various VP7 (T13) trimers and underneath VP3 (T2) layer is extensive and relatively well filling; the shape of the various VP7 trimers remains essentially the same. The T trimers appear to be the most tightly attached to the VP3 subcore and are likely to act as nucleation points, and the P trimers probably constitute the last series of trimers that attach to the VP3 subcores.

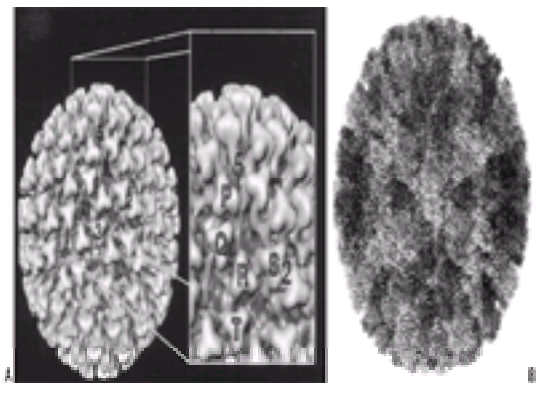

FIG. 10. Surface representation of the bluetongue virus (BTV) core. **A:** The cryoelectron microscopic three-dimensional (3D) reconstruction of BTV-10 core showing the trimers of VP7, with an enlarged portion highlighting the protomeric unit of the viral capsid. The 260 trimers of VP7 are arranged in laevo T = 13 lattice on the VP3 shell. The icosahedral axes are marked, as are channels II and III in the layer of VP7. In the enlarged section, the five quasi-equivalent trimers are marked P (closest to the fivefold axis) and S (closest to the twofold axis) through to T (the trimer on the icosahedral threefold axis. (From ref. 63, with permission.) **B:** The structure of the BTV-1 (*SA*) core (62). The structurally equivalent trimers have been given the same color (shade of *gray*). The trimers cover most of the pores through the VP3 layer, leaving a single pore on the icosahedral fivefold axes as the likely site of exit of nascent mRNA (for details, see text).

(Drawn by J. Diprose, J. Grimes, and D. Stuart, using Bobscript [40], POV-ray, and Photoshop.

The inner protein shell, known as the subcore, is composed entirely of 120 copies of the VP3 protein, which are organized as an icosahedral lattice with T=2 symmetry (62). The VP3 subcore shell has a maximal diameter of 59 nm, forming a central space with an internal diameter of 38 nm, that contains both the genome and the minor core proteins. The 120 copies of VP3 that are arranged as two sets (A and B) of 60 subunits form the subcore layer (Fig. 11A). Five of the A-group molecules are arranged as a five-pointed star around each fivefold axis. Five additional B-group molecules are positioned one between each of the points of the star, but at a further distance from the fivefold axis. Thus, the VP3 molecules form convex disk-shaped decamers. Twelve decamers, positioned one at each of the fivefold axes, are arranged end to end, forming the complete VP3 shell.

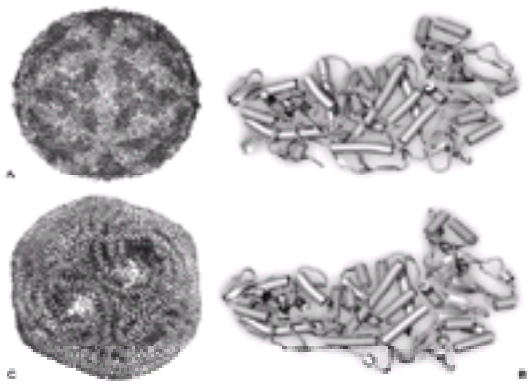

FIG. 11. A: The structure of the VP3 layer showing the arrangement of 120 copies of two types of VP3 molecules. **A** is shown in *light gray* and *B* is shown in *dark gray*, as seen in bluetongue virus serotype 1 (BTV-1). Sixty copies of each of the two molecules (A and B) pack together to form a thin closed shell of protein that encapsulates the viral genome. An analysis of the interactions between the molecules suggests that the A–B (and to a lesser extent the A–A) interactions forming a flower-shaped decamer around each fivefold axis are the strongest, with weaker A–B pseudo-twofold and B–B pseudo-threefold interactions connecting the decamers (62). **B:** The structure of two types of VP3 molecule, *A* (*left*) and *B* (*right*), as observed in the BTV-1 core. The molecules are similar in overall shape of thin triangular plates, with slight differences in conformation. Note some curling up of the B molecule, which facilitates it packing around the icosahedral threefold axes. The details of the fold within the two molecules differ at several places. Note the differences at the top of the molecules (closest to the icosahedral fivefold axes), where a compact structure is seen in *A* and a more extended structure in *B* (62). **C:** The ordered genomic dsRNA in the core of BTV. The diagram shows the model that has been built into the four layers of electron density, accounting for about 80% of the genome. The empty volume close to the icosahedral fivefold axes is the putative location for transcription complex (*TC*) (55). (Drawn by J. Diprose, J. Grimes, and D. Stuart, University of Oxford, UK, using Bobscript [40], POV-ray, and Photoshop.)

Both the A and B VP3 subunits have similar overall shape, and the individual VP3 molecule forms a flat, elongated triangular wedge shape (13 to 35 Å thick × 75 Å wide × 130 Å long) which contains many a-helices as well as b-strands and is made up of three distinct domains (see Fig. 11B). The three domains have quite different folds from each other. The *apical* domain (amino acids 298 to 587) is situated closest to the fivefold axes in the intact particle. The *carapace domain* (amino acids 7 to 297, 588 to 698, and 855 to 901) forms a rigid plate that represents most of the surface of the subcore shell. The *dimerization domain* (amino acids 699 to 854), which forms quasi-twofold interactions between individual A-group molecules, is situated farthest away from the fivefold axes (62). The dimerization domain is also responsible for interactions of A and B subunits between different decamers.

The inner surface of the VP3 shell interacts directly with the minor protein components (VP1, VP4, and VP6) of the transcriptase complexes (TCs), which are located at the fivefold axes, within the central space. The inner surface of the VP3 subcore shell has relatively few charged residues and has a series of shallow grooves. These features may be essential to organize the dsRNA molecules of the genome and to allow them to be transported rapidly and simultaneously through the active sites of the TCs. The TCs (probably 12 in total) appear to be present as a single complex at the vertices of the icosahedral particle and therefore do not have strict icosahedral symmetry. It has not yet been possible to resolve their structure or their interactions with the dsRNA in atomic detail. It is clear, however, that activation of the core associated enzymes in the presence of magnesium ions and nucleoside triphosphate (NTP) substrates causes conformational changes around the fivefold axis of the core and results in outward movement of VP3 and VP7, which allows opening of a pore in the VP3 layer at the fivefold axes (55). The mRNAs synthesized by the core-associated TCs are extruded through this pore, then pass out through the central space in the pentameric rings of VP7 trimers. There are also pores in the VP3 layer at the threefold axes, but in the intact core particle, these are completely blocked by the T trimer of VP7.

Much of the genomic RNA can also be detected by x-ray crystallography as electron density within the central core space. The dsRNA is highly ordered, and about 80% of the entire genome can be modeled as four distinct concentric layers that have center-to-center spacing between RNA strands of 26 to 30 Å (see Fig. 11C). As a consequence of the spacing of the A and B forms of VP3, the grooves in the inner surface of the subcore shell form a spiral around the fivefold axis. The outer dsRNA layer appears to interact with these grooves and can also be modeled as a spiral extending outward from the TC. It is uncertain how the RNA molecules pass inward to the next and subsequent concentric shells. The density detected in the inner layers of RNA gets progressively weaker, suggesting that its organization and icosahedral symmetry is progressively less rigid, although each layer maintains an overall spiral

organization (55).

REPLICATION

The replication cycle of orbiviruses has not been studied in detail. It is presumed that the basic features, including the transcription processes and the conservative mode of genome replication, are similar to those of reoviruses. Unlike reoviruses or rotaviruses, however, orbiviruses multiply in arthropods as well as in vertebrates. They are also structurally different, with protein components that bear little or no primary sequence similarities to those of the reoviruses or rotaviruses. In view of this, it is likely that some stages of the orbivirus multiplication and morphogenesis processes are unique. The overall information available on the BTV replication cycle and assembly has been generated primarily from immunoelectron microscopic studies and the use of baculovirus expression systems and is summarized in a schematic diagram (31,194) (Fig. 12). In the following subsections, the various steps of replication cycle of BTV and distinct features of the viral multiplication and assembly processes are reviewed and discussed.

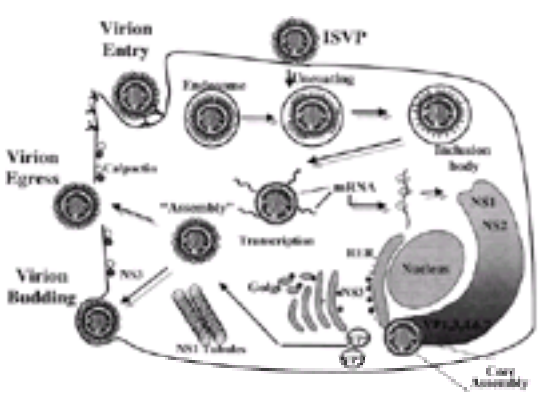

FIG. 12. Schematic diagram of an orbivirus replication cycle (deduced from bluetongue virus data).

Adsorption

Field isolates of orbiviruses often replicate poorly in cultured cells; however, most adapt readily to growth *in vitro* as reported by passage in embryonated chicken eggs or in varieties of cultured cells of mammalian and insect origins, including BHK-21, Vero, MDBK, HeLa, *Aedes* species, and *Culicoides* species cell lines (129,233).

BTV adsorbs rapidly to susceptible cells at both 4°C and 37°C, with the maximal adsorption occurring within 20 minutes (83). The cell receptors involved in orbivirus infection have not been identified, although some information is available suggesting their nature. In early studies, analysis of BTV–erythrocyte interactions indicated that the virus binds to a sialoglycoprotein, the glycophorin component of human and a number of animal erythrocytes (30). The binding to erythrocytes and vertebrate cells appears to be mediated by the BTV outer capsid protein VP2. Virion particles lacking VP2 but retaining the second outer capsid protein, VP5, exhibit a reduced capacity to bind to vertebrate cells (81). Using reassortants of BTV-20 (which agglutinates sheep erythrocytes only) and BTV-21 (which agglutinates sheep, bovine, human, or goose erythrocytes), it has been demonstrated that hemagglutinating ability segregates with VP2. ISVPs of BTV, from which the outer capsid has been modified by digestion with trypsin or chymotrypsin but not completely removed, also bind cells (131). In a recent study using a purified recombinant VP2 protein, it was demonstrated that VP2 alone is responsible for virus entry into mammalian cells and indeed is the viral hemagglutinin (67). Purified VP2 also had a strong affinity for binding to glycophorin A, confirming that VP2 is responsible for BTV transmission by the *Culicoides* species vector to vertebrate hosts during blood feeding. Further, by various enzymatic treatments of BTV-permissive mammalian cells, it has been shown that the BTV receptor molecule is likely a protein component of the glycoprotein; alternatively, a second protein molecule could also serve as a co-receptor for BTV infection of mammalian cells (67). In contrast, BTV-derived core particles bind efficiently to invertebrate cells (e.g., *Culicoides* species), but only at a low level to mammalian cells (133,239). BTV cores are highly infectious for invertebrate cells. The available data suggest that the RGD motif of VP7 is involved in the core entry mechanism for *Culicoides* species cell culture (148,200).

Entry and Uncoating

Like some reoviruses, BTV is subject to endocytosis after binding to a receptor on a cell plasma membrane through VP2 of the outer capsid (67). After invagination and detachment from the cell surface, a coated vesicle containing the virus is formed (31). The clathrin coat of this vesicle is rapidly lost, leading to the formation of a large translucent, endocytic vesicle (see Fig. 12). Shortly after infection, virions are found in vesicles in the vicinity of the nucleus. During these initial events, the morphology of BTV is probably drastically altered owing to degradation of VP2 and to an altered conformation of VP5. It seems likely that the C-terminal globular domain of VP5 molecule somehow attaches to endosomal membranes, allowing the extension of N-terminal coiled-coil domain, which is likely to destabilize the endosomal membrane (68). ithin 1 hour of infection, BTV is converted to core particles that appear to lack all the VP2 and most of the VP5 of the outer capsid layer (83). Removal of both outer capsid proteins appears to be required to activate the virion transcriptase, analogous to rotavirus transcription mechanism. In the cytoplasm, the partially or completely uncoated particles bind to the cell cytoskeleton (specifically the intermediate filaments) and initiate transcription of viral mRNA species (221).

Electron microscopy using a variety of detection procedures indicates that soon after the initiation of transcription, a matrix structure (similar to IBs) accumulates near the core and eventually spreads to surround it (81,90). These structures are probably formed by condensation of viral mRNA and newly synthesized viral proteins. Particles with bi-shelled morphology are often observed at the periphery of these matrix bodies beginning 6 hours after infection (88,89). It is

suggested that such progeny virus particles are assembled within or in juxtaposition to the IBs.

Transcription and Replication

In vitro, BTV mRNA synthesis can be activated by proteolytic digestion of the outer capsid of the virus (131,221,222 and 223). This facilitates access of NTPs to the genome within the core and the extrusion of the newly synthesized mRNA to the cytoplasm through the channels along the fivefold symmetry axes. The BTV transcriptase has a low *in vitro* temperature optimum of 28°C, lower than the 47° to 52°C optimum reported for reovirus transcriptase (103,225). However, BTV mRNA synthesis is efficient at 37°C, particularly at low core concentrations (223). The structural integrity of the core particle appears to be essential for maintaining an efficient transcriptional activity. Intracellular core particles eventually lose their surface VP7 layer to generate subcores that, at least *in vitro,* fail to transcribe RNA (83). It is noteworthy that the *in vitro* transcription activity of the core could be completely blocked by certain VP7 monoclonal antibodies (P.P.C. Mertens unpublished observation). Recent studies using individual recombinant proteins in *in vitro* assay systems have allowed delineation of specific roles of the proteins of the transcription complex within the core and the tentative cascade of RNA synthesis as shown in Figure 13. It appears that VP6 helicase unwinds the genomic duplex RNA molecules, whereas VP1 polymerase initiates the synthesis of positive-stranded RNA species from the 3' end of each negative-stranded RNA segment of the dsRNA molecule (172,199). It is not known, however, whether all the *de novo* RNA molecules are synthesized simultaneously or consecutively, or whether VP1 alone is sufficient for synthesis of positive-stranded RNA species on the newly generated negative strands. It is most likely that dsRNA templates are required for the synthesis of positive-stranded RNA species, and the RNA synthesis is more efficient when VP1 and VP6 are present as TC complex within the subcore. Nevertheless, it is accepted that the negative-stranded RNA species are synthesized from the 3' end of the positive-stranded RNA molecule, and VP1 alone is able to initiate the replicase activity. Once the positive-stranded RNA molecules are synthesized, the 5'-termini of these molecules are modified by the enzymatic activities of VP4. The class I methylated cap structure is formed solely by VP4, and thus both genomic positive-stranded RNAs and the 10 mRNAs possess the 5'-methylated cap structure (18,132,171). Various stages of RNA synthesis are depicted in Figure 13.

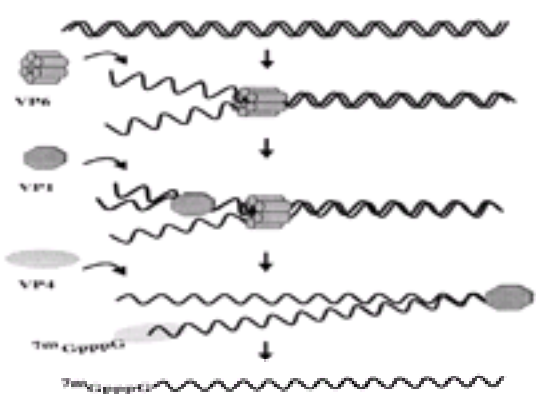

FIG. 13. Mechanisms of viral RNA synthesis and the role of each of the three minor proteins in RNA synthesis.

It is presumed that the overall strategy of orbivirus mRNA transcription is similar to that of reoviruses and rotaviruses. The mRNA species are capped and methylated during the transcription process. They are extruded from subviral particles into the cytoplasm and program the synthesis of virus proteins. The secondary structures of the mRNAs may facilitate mRNA egress from particles and may also contribute to the overall stability of the mRNA species within the cytoplasm of infected cells as well as to their interactions with ribosomal components for the purpose of translation.

The relative frequency of transcription of the respective genome segments remains unaltered during the course of infection (84). The transcripts are not produced at equimolar amounts for all 10 segments of BTV (84). The smaller genome segments are generally the most frequently transcribed, although M6 RNA (encoding NS1) is synthesized more abundantly than the smallest segment, S10 RNA (encoding NS3). The molar ratios of the 10 different BTV mRNAs appear to remain the same throughout the infection cycle. The ratios of mRNAs synthesized *in vivo* and *in vitro* are similar. Equivalent data have been obtained for other orbiviruses (76).

Nothing is known about the *in vivo* RNA replication mechanisms of orbiviruses. It is believed that like reoviruses and rotaviruses, the packaged positive strand serves as a template for synthesis of a negative strand, and once the negative strand is synthesized, it remains within the nascent progeny particle. As discussed previously, VP1 acts as the replicase enzymes, but the roles of other proteins in negative strand synthesis remain undefined.

Protein Synthesis

Infection of cells with BTV leads to a rapid inhibition of cellular macromolecular synthesis. Components of the infecting virion or its interaction with cellular structures may be involved in producing a cytotoxic effect. The first virus-specific polypeptides are detected 2 to 4 hours after infection, and the rate of protein synthesis increases rapidly until 11 to 13 hours after infection, after which it slows down but continues until cell death (84). Most proteins are synthesized throughout the infection cycle and accumulate during infection until cell lysis. In infected cells, VP6 is often resolved into two closely migrating bands in PAGE, one of which may be a modified form of the other (85,130). Recent data have shown that the two proteins are derived from initiation of protein synthesis at two distinct initiation codons in the S9 mRNA and not by posttranslation modification (226). The properties of the viral proteins were discussed previously (see Gene Coding Assignments and Virus Proteins).

Cytoskeleton in Bluetongue Virus–Infected Cells

During BTV infection of cells, a reduction in the number of microtubules occurs with some fragmentation of the microtubule network near the cell membrane. Microfilaments also change during infection (31).

The involvement of the cytoskeleton in BTV multiplication has been indicated by its close association with BTV antigens and virus-specific structures, including IBs, tubules, and 55-nm diameter, virus-derived particles (32). The 10-nm diameter filaments, rather than the 25-nm filaments, are involved. Analyses indicate that intermediate filaments probably provide sites for particle binding (32). Also, although most of the IBs are cytoskeleton associated, only some tubules are associated with the cytoskeleton (32,88).

Assembly

During infection, a fibrillar network develops around the infecting core particle in the cytoplasm. This resembles the IBs that are so pronounced later during the infection course. IBs are rich in mRNA and the phosphorylated NS2 protein (10,215). In addition, viral capsid proteins, such as core proteins VP3 and VP7, are also present in the IB matrix, indicating that this may be the assembly site of core particles either within or at the periphery of the IBs (88,90). By contrast, outer capsid proteins VP2 and VP5 are present either at the periphery of the IBs or at other locations (90). The presence of electron-dense virus-derived immature particles inside IBs has led to the belief that core particles may be synthesized within IBs and that the further stages of mature virion development occur either in the cytosol or after binding of the particles to the cytoskeleton. A hallmark of orbivirus infections is the presence of tubules throughout the cytoplasm (113). These tubules are of indeterminate length, contain no RNA, and appear to be empty. The tubules of each orbivirus appear to have distinct width and morphology, implying that they may have some specific role for each type of virus (78). NS1, which forms these tubules, is also found associated with corelike structures within IBs, implying that NS1 before multimerization may have certain roles in virus assembly. In BTV-infected cells, IBs, tubules, and intracellular particles are associated with vimetin-rich, intermediate filament networks of the cell cytoskeleton (31). The components requirements and temporal order of assembly of cores and virus particles have mostly been generated using baculovirus expression system in which VLPs and subviral corelike particles (CLPs) are produced by coexpression of certain BTV proteins (Fig. 14). The formation of CLPs and VLPs was neither dependent on the presence of the IBs or tubules nor on the other nonstructural proteins (NS3 proteins), the three minor proteins, or the dsRNA genome. The results from a series of studies could be summarized as follows: coexpression of VP3 and VP7 yields CLPs that are visually identical to authentic core morphology (see Fig. 14) (45,70). Generation of CLPs, combined with cryoelectron microscopy studies, revealed that of the 260 trimers, some 200 are tightly bound, with the remaining 60 trimers more loosely attached. In the absence of VP3, VP7 forms trimers, but these trimers do not assemble as particles. However, VP7 of certain orbiviruses (e.g., AHSV) can self-assemble to flat sheets of hexagonal rings (13). These rings are similar to the six-membered rings of VP7 trimers seen in the core surface layer. Similar assembled structures were seen in insect cells when AHSV VP7 was expressed by recombinant baculoviruses (15). In contrast, VP3 alone does assemble as icosahedral subcore-like structures (see Fig. 14, *lower panel*), but such subcores are relatively unstable, unlike the subcore structure formed by equivalent protein of the tick-borne BRDV orbivirus or of rotaviruses (146). Nevertheless, by low-resolution cryoelectron microscopy analysis, VP3 subcore exhibits similar organization of VP3 molecules (see Fig. 14), as seen by subsequent atomic structures of authentic VP3 within the core, indicating that VP3 is probably assembled first, and it serves a primary role in formation of the virus capsid (reviewed in 194). The VP7 layer not only provides increased rigidity and stability to the core particle but also can play a role in cell entry by the core and initiation of infection, particularly in insect cell systems (vector), as well as in protection against virus infection (227).

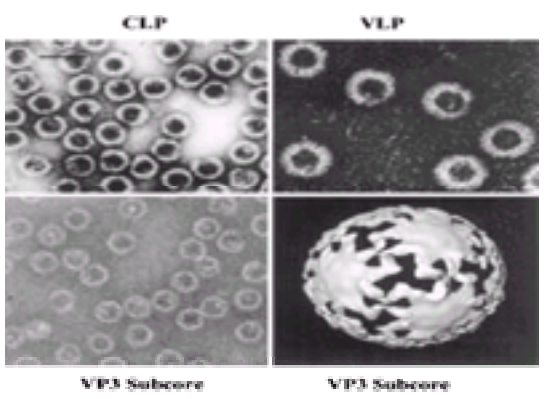

FIG. 14. Electron micrographs of baculovirus-expressed corelike particles (CLPs) and virus-like particles (VLPs) (*upper panel*). The appearance of expressed CLPs composed of VP3 and VP7 (*left*). The double-shelled VLPs containing VP2, VP3, VP5, and VP7 (*right*) (44,45). The *lower panel* shows images of spherical subcores (VP3 alone) in amorphous ice (*left*) and reconstructed inner shell only, with a cutoff radius of 28.2 nm (subcores), viewed down a twofold axis (*right*) (From ref. 70, with permission.)

Expression of BTV VP3 and VP7 in the presence of VP1, VP4, or VP6, or any combination of these, leads to the encapsidation of these minor proteins. After dialysis, they remain with the subcore, indicating that they interact specifically with VP3 (110,120). Further, high-resolution cryoelectron microscopy analysis (Fig. 15) indicates that the localization of TC within the core is highly specific.

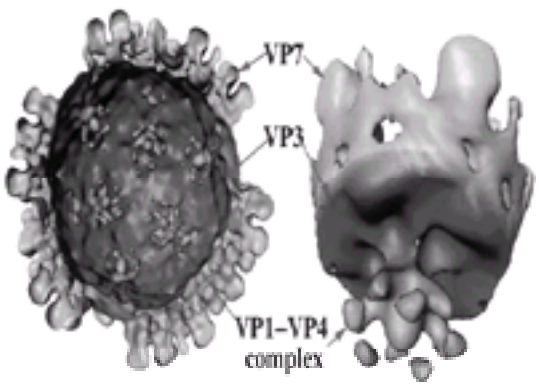

FIG. 15. High-resolution cryoelectron microscopy of corelike particles (CLPs) showing the localization of VP1 and VP4 within the CLPs. (Courtesy of B.V.V. Prasad, Baylor College, Houston, Texas.)

The combination of CLP assay and site-directed mutagenesis has allowed mapping of the domains within VP3 and VP7 that are essential for core assembly even before availability of x-ray structure (110,211). For example, specific VP3 residues (methionine at amino acid 500, arginine at amino acid 502) have been shown to be crucial to core assembly. Similarly, the C-terminus, but not the N-terminus, of VP7 is essential for assembly of VP7 trimers onto the VP3 subcore. Other internal residues on VP7 are also identified that are either dispensable or essential for protein–protein interactions during core assembly (110). In addition, by making domain-switched chimeric VP7 molecules using BTV and AHSV, the regions and residues responsible for protein folding and oligomerization have been mapped, confirming the role of these residues on overall structure of the VP7 molecules and their role in virus capsid assembly (140).

Further, based on subsequent x-ray structure of VP7, an extensive range of targeted VP7 mutations have been generated that not only precisely identify the critical residue responsible for core assembly but also revealed that core assembly depends on the trimer formation and the overall precise shape of the trimers, which eventually generate tight lattice of 260 VP7 trimers within the core. The data also indicate that there is a clear sequential order of trimer attachment on VP3 scaffold. The T trimers, which are at the threefold axis of the icosahedron, act as nucleation, whereas P trimers that are farther from the threefold axis and near to the fivefold axis, are the last to attach (117).

In contrast to assembly of VP7 trimers on VP3 subcore, nothing is known about how and in what order the viral genome and the three minor proteins interact with each other to form the innermost core structure in association with VP3.

Once the stable core is assembled, the two outercapsid proteins VP2 and VP5 are acquired by interaction with the core surface, both proteins being attached to the VP7 layer of the core. The assembly of VP3, VP7, or VP5 and VP2 is easily mimicked both by baculovirus expression systems and by the *in vitro* transcription-translation system (see Fig. 14) (44,110,118,120). The 3D reconstruction of these VLPs showed an icosahedral structure of 86-nm diameter with essentially the same features as for the native BTV virus particles (72). After VP2 and VP5 are attached, the particles are no longer transcriptionally active. Instead, they are available for release from the infected cell.

VLP technology has also given an opportunity to determine the structural compatibilities of VP2 and VP5 of various BTV serotypes (119). When assembled particles were examined, it was clear that despite the high level of sequence variation among the different serotypes, the VP2 and VP5 proteins of different BTV serotypes formed VLPs with the VP3 and VP7 of another source (119). Some combinations (e.g., the VP2 of BTV-2 and the VP5 of BTV-10) did not form VLPs, suggesting that VP2 may require the VP5 of the same or a closely related serotyping (e.g., BTV-10 or BTV-17) but may not form VLPs efficiently with the VP5 proteins of a more distant serotype.

Although the viral proteins have an intrinsic ability to self-assemble, a specific mechanism must exist for the recognition and incorporation of mRNA species and their use to generate dsRNA. This may involve the virus-induced nonstructural proteins (e.g., NS2), the core proteins, and particular sequences and structures of the viral RNA species themselves. It is hypothesized that the viral mRNA species are selectively assembled so that particles each acquire all 10 mRNA species. The role of the NS2 protein, which binds ssRNA but not dsRNA, may be crucial to the success of encapsidation. The conservation of the terminal regions of the mRNAs may be of relevance to the question of their recruitment to the sites of particle morphogenesis and encapsidation into cores and eventually virions.

Release of Virions

The mechanism whereby BTV is released from infected cells has been the subject of some speculation and appears to depend on the cell type. In most vertebrate cell cultures, a high proportion of virus remains cell associated, although some may appear in the culture medium after cell death and subsequent lysis. However, other mechanisms are operative for virus release as shown by infection of MDBK cells with BTV-8. In this system, by 4 to 5 hours after infection, progeny virions are released, well before cell (23). Such a process is more pronounced in insect cell cultures, such as *Culicoides* species or *Aedes aegypti*–derived C6/36 cells.

Virus particles have been observed to leave infected cells in either of two ways. First, particularly early in infection, particles can be seen to bud through the cell membrane and acquire, at least temporarily, an envelope (10,31,34). However, no virus antigen has been detected so far at the surface of such enveloped particles. Second, egress of virus particles from infected cells is accomplished by a process of extrusion in which individual or groups of virus particles move through a locally disrupted plasma membrane (89). This appears to happen without a significant effect on host cell viability. Both types of particles have been observed to be released at least from certain cell surfaces infected with BTV as discussed previously (89). The nonstructural, NS3 protein has been identified at the sites of the plasma membrane where virus particles leave, suggesting a role for this protein in this process (89). In contrast to mammalian cells, in

Culicoides species or C6/36 cells, where NS3 is synthesized at a significant level (in comparison to the mammalian cells), more than 90% of particles are released in the culture medium (13,65) (Fig. 16). Released particles and those remaining on the cell surface often appear to retain an association with an underlying cortical layer of the cell cytoskeleton (89).

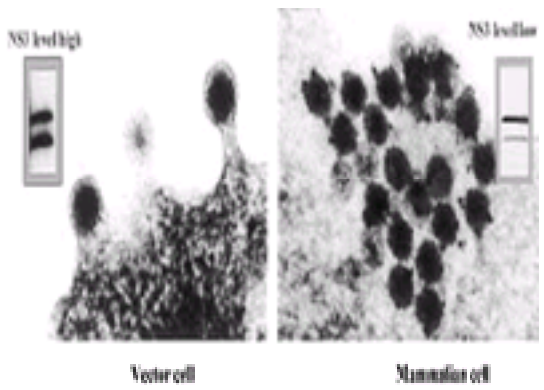

FIG. 16. Electron micrograph of two types of cells infected with bluetongue virus and expression level of NS3 and NS3A in these cells.

The involvement of NS3 in virus release had been further substantiated by an elegant study using recombinant baculoviruses expressing VLPs and NS3. The data demonstrated that VLPs are generally associated with the cell cytoskeleton upon ssembly. However, assembled particles were released from infected cells in the presence of the recombinant NS3 proteins (92). Thus, clearly, NS3 proteins are required for budding and subsequent release of VLPs and BTV particles from infected cells. The current hypothesis is that NS3 mediates virus release through the cellular exocytic pathway.

GENETICS AND ORBIVIRUS EVOLUTION

Because of their segmented genomes, and depending on genetic compatibility, orbiviruses may reassort their genes during co-infection. This has been analyzed using both wild-type and temperature-sensitive (ts) mutant viruses, although unlike with reoviruses and rotaviruses, only limited studies have been carried out with orbivirus ts mutants. Reassortants have been widely used to define the gene coding assignments and the biologic functions of the viral proteins (19,20,102,145,156). The opportunities for virus evolution by a step-wise process of reassortment as opposed to a process of drift through the accumulation of point mutations, deletions, and duplications are well known for a number of viruses with segmented genome (e.g., arenaviruses, bunyaviruses, orthomyxoviruses, certain plant viruses, and members of the *Reoviridae* family).

Reassortment Capability and Genetic Diversity of Genome Segments

High-frequency genome segment reassortment between different but compatible orbiviruses has been demonstrated not only in the natural vertebrate and invertebrate hosts but also in tissue culture systems (16,52,102,124,151,153,154 and 155,158,176,177,196,203,206). For BTV, experimentally generated reassortment in insect vectors (e.g., *Culicoides variipennis*) is reportedly higher (42%) than in a vertebrate host (e.g., 5% in sheep) (196). Genome segment reassortment may be an important factor in the generation of genetic diversity in orbivirus populations in nature.

It is believed that many field isolates of orbiviruses consist of mixtures of RNA segments from populations of virus strains. When virus was recovered from a BTV outbreak in Washington State and fingerprinted, it exhibited a mixed population of RNA segments, representing both BTV-10 and BTV-11 serotypes, the two circulating viruses of that region (17). Similarly, BTV isolates from *Culicoides* species vectors have been shown to have mixed populations of genome segments from two BTV serotypes (176). Another feature of natural orbivirus infections is that multiple serotypes of the same serogroup or different serogroups (e.g., BTV and EHDV) have been recovered from individual hosts (152,157,204), indicating that antigenically distinct viruses can be present in a single host. Under optimized conditions in tissue culture systems, reassortment between compatible serotypes occurs at such a high frequency that the parental viral genotypes are present as only a minority of the progeny virus population.

The coding assignment for certain genome segments of BTV and Kemerovo serogroup viruses were initially determined using reassortant viruses (64,102,130,142,165). In addition, reassortant analysis has been used to study genes that affect virus virulence in the Kemerovo serogroup and the genes involved in virus neutralization (151,153,155). Similar studies involving BTV and AHSV serotypes have also been reported (14,156).

Although reassortment has been demonstrated within orbivirus serogroups (e.g., Wallal, Kemerovo, and BTV, as discussed previously), no evidence has been obtained for reassortment between members belonging to different orbivirus serogroups.

Studies of the genetic diversity and variation in RNA segment sequences within a serotype have been determined, initially by using RNA oligonucleotide fingerprint analyses of BTV field samples and more recently by using cDNA cloning or polymerase chain reaction (PCR) (reviewed in 188,192, and 193). For example, genotypic variation of BTV-11 isolates obtained in the same year (1973) and from different regions of the United States, or from the same region (e.g., Colorado) but in different years (1963 to 1975), have been reported. The results indicated that although all the BTV-11 isolates were related, albeit to various extents, to the United States prototype BTV-11 strain, all showed some sequence differences, indicating that considerable evolution of the BTV-11 genome has occurred in the United States (207). In

summary, both genetic drift and genetic shift contribute in orbivirus evolution.

Genetic Relationships Within Serogroups

P>In early studies, hybridization techniques were applied to determine the genetic relationship of the dsRNA genome segments between different orbivirus serotypes, in particular for BTV (77,179,190). The results of such studies showed that the L2 and M5 segments (encoding the two outer capsid proteins) exhibited little or no cross-hybridization between serotypes, whereas studies of the other eight RNA segments, which gave strong hybridization signals, indicated that they were more conserved. Of these eight segments, some segments gave stronger cross-hybridization signals than did others. For example, cross-hybridization with M6 and S8 that code for two nonstructural proteins (NS1 and NS2, respectively) were usually the strongest, indicating that these two genes are highly conserved across the serotypes. The S7 segment appears to be less well conserved among BTV serotypes than were the remaining segments that code for core proteins. Apart from the S10 segment of AHSV, which appears to be more variable than that of BTV (25,224), similar results have been obtained for AHSV.

Although the available data suggest that the BTV serotypes evolved from a common ancestor, the gene pools of viruses on different continents may have diverged significantly (reviewed in 52 and 192). This may reflect the accumulation of mutations and reassortment and coevolution of gene pools at different locations, such as the Far East, Australia, Africa, or North America. It has been proposed that the term *topotype* should be applied to each distinct group (58). Cross-comparisons using PCR and RNA sequence data indicate that BTV isolates can be grouped within a single topotype if their L3 segments show less than 8% sequence difference. For BTV or EHDV strains that contain greater than 11% but less than 23%, variants can be grouped within different topotypes (58).

Phylogenetic Relationships Between Different Serogroups Based on Sequence Analyses

Although relationships between viruses from different serogroups cannot be assessed by cross-hybridization techniques, phylogenetic relationships can be assessed by cDNA and protein sequence analyses of corresponding genes of different orbiviruses. By such means, a close genetic relationship has been demonstrated among BTV, EHDV, and AHSV (Fig. 17). Sequence comparisons indicate that VP3 is the most conserved protein, whereas VP2 is the most variable (see Table 4 and Table 5). Some 57% to 58% of the aligned BTV-10 and EHDV-1 VP3 amino acids are identical to those of AHSV-4 VP3. This compares with an identity of 79% between the BTV and EHDV VP3 sequences. For the VP7 proteins, 64% of the aligned amino acids of BTV-10 and EHDV-1 are identical, whereas 44% to 46% of amino acid residues are identical to the aligned AHSV-4 sequence (95,96,187,193). As expected, the VP2 proteins of the three viruses are only 17% to 23% identical. However, various other comparative analyses of the proteins indicate that the VP2 species of the three orbiviruses are similar. Unlike VP2, the other outer capsid protein VP5 is more conserved among the three viruses. On alignment, the AHSV-4 VP5 is 42% to 45% identical in amino acid sequence to VP5 proteins of BTV-10 and EHDV-1 (193,237). The identity between BTV and EHDV VP5 is 62% (95,96). Clearly, these viruses share strong phylogenetic relationships.

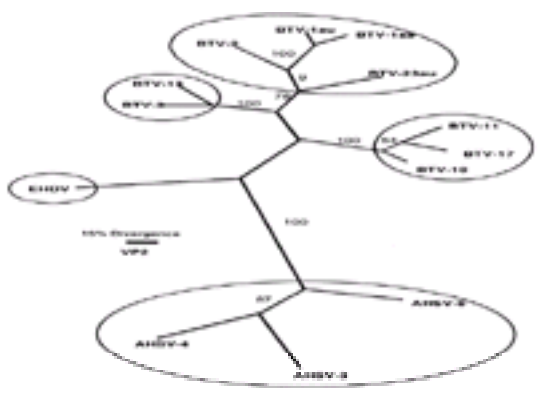

FIG. 17. Phylogenetic relationships of gnat-transmitted orbiviruses based on the VP2 sequences of different African horse sickness virus (AHSV), bluetongue virus (BTV), and epizootic hemorrhagic disease virus (EHDV) serotypes. An unrooted maximal likelihood tree for the VP2 protein data is shown, with branch lengths proportional to the number of amino acid changes. The BTV serogroup is illustrated as a monophyletic group of viruses with 100% bootstrap support and maximum parsimony branches as indicated by values above branches. (Modified from ref. 141, with permission.)

PATHOGENESIS AND DISEASE

Orbiviruses produce a spectrum of consequences from infection, reaching from severe and fatal disease to subclinical infection, depending on virus or host strain. Virus is transmitted to vertebrate hosts through blood-feeding insect vectors, and infectious particles migrate to lymph nodes where they initially replicate and subsequently spread to spleen, thymus, and other lymph nodes, becoming associated with circulating blood cells, where virus may persist for weeks or months (reviewed in 4 and 122). A distinct feature of orbivirus infections is their capacity to induce prolonged cell-associated viremias in vertebrate hosts, including both the species involved in virus transmission cycles and clinical hosts (122,138,205).

Prolonged bluetongue viremias are particularly notable in cattle, which thus serve as virus reservoirs. After experimental inoculation of calves, infectious virus can be detected for up to 8 weeks, and viral nucleic acid can be detected in erythrocytes for up to 20 weeks (122). BTV has been shown to bind to glycophorins on the surface of bovine and ovine erythrocytes (30), where it may remain as invaginations of the erythrocyte cell membrane in an infectious state for prolonged periods, possibly for the life time of the red blood cell (11). The location of orbiviruses in erythrocyte

membrane invaginations precludes contact with antibody and T cells and protects against immune elimination, thus providing multiple opportunities for infection of blood-sucking midges over an extended time period.

The role of leukocytes in cell-associated viremias is less certain. BTV has been recovered from bovine mononuclear cells during the early stage of infection (4,205,234). However, active replication of virus in these cells appears to be restricted to proliferating cells. *In vitro* studies of bovine lymphocytes have shown multiple subpopulations to be susceptible to infection (201). Helper T-cell subsets replicate BTV to high titer and undergo cytopathic infection, whereas T-cytotoxic suppressor and null cells become persistently infected without lysis. Monocyte-macrophages appear to be targets for infection with AHSV.

Most orbiviruses are neurovirulent in infant mice or hamsters inoculated intracerebrally, and some viruses are neuroinvasive and neurovirulent after parenteral inoculation. Susceptibility decreases with advancing age, and fetal animals are the most sensitive to infection. Neurovirulence of BTV strains for bovine fetuses correlated with neuroinvasiveness in mice (230). Kemerovo complex viruses occasionally produce meningoencephalitis in humans (141).

Another feature of orbivirus pathogenesis appears to be the ability of viruses to replicate in and to damage endothelial cells, with tropisms for cells representing distinct anatomic sites. This phenomenon has been demonstrated in animals infected with BTV, EHDV, and AHSV (109,123,216). Infection of endothelial cells is followed by infection of vascular smooth muscle cells and pericytes, which undergo lytic infections, resulting in virus-induced vascular injury and a cascade of pathophysiologic events characterized by capillary leakage, hemorrhage, and disseminated intravascular coagulation. Clinically, these events are manifested by mucosal edema, hemoconcentration, pulmonary edema, hydrothorax, hydropericardium, serosal hemorrhages, other hemorrhagic and thrombotic phenomena, hypotension, and shock (reviewed in 163 and 164). A curious feature of BTV infections in sheep is hemorrhage at the base of the pulmonary artery. The differential susceptibility of endothelial cells to BTV infection also may explain markedly distinct features of pathogenesis in sheep and in cattle. Generally, sheep develop potentially fatal hemorrhagic disease and have extensive endothelial cell infections, whereas cattle typically develop subclinical infections and have only minimal endothelial infections (38,122,163,164). Thus, BTV and other related orbiviruses such as AHSV and EHDV in their respective large animal hosts have pathophysiologic features resembling other viral hemorrhagic fevers.

Congenital infection is a feature of orbiviruses in ruminant species. Infection of pregnant cattle and sheep with BTV or EHDV can result in fetal infection, abortion, or congenital anomalies (99,121,122,159,163,164,248). A variety of anomalies have been reported, including runting, blindness, deafness, hydranencephaly, arthrogryposis, campylognathia, and prognathia. Although wild-type BTV infection of pregnant ewes can lead to maternal death, abortion, and fetal death, a number of studies have implicated live attenuated BTV vaccines as a principal cause of teratogenesis (99,163,248). Congenital BTV infection occurring in late gestation usually does not result in congenital anomalies, although newborn animals may circulate low levels of virus in their blood and have delayed immune responses. The interval between maternal infection and last detectable viremia in offspring has been as long as 20 weeks, suggesting a mechanism for long-term survival of BTV. However, such congenitally infected animals eventually clear the infection and are not immunologically tolerant or persistently infected (122). Congenital infection of calves with Kasba (Chuzan) virus, a Palyam group virus, results in a syndrome of hydranencephaly and cerebellar hypoplasia, distinct from BTV disease (54). Palyam group viruses also cause arthrogryposis and other malformations in the animal model, such as chicken embryos, similar to those seen in BTV-infected ovines (235).

As for other viral infections, orbivirus pathogenesis is influenced by a variety of host factors, including age, genetics, immunologic function, and other underlying diseases (38,39,109). Some of the key features (Table 6) of orbivirus disease are summarized later. For detailed information, see other reviews (3,38,231).

Genus	Serogroup	Disease hosts	Syndrome	Geographic distribution
Orbivirus	African horse sickness	Equidae, zebra, dog	Cardiopulmonary disease, hemorrhagic fever	Africa, Middle East, Asia, Europe
	Bluetongue virus	Sheep, cow, goat, deer	Rhinitis, stomatitis, coronitis	Africa, Asia, Australia, Americas
	Changuinola	Human	Febrile illness	Panama
	Epizootic hemorrhagic disease	Deer	Similar to bluetongue	Americas, Australia, Africa
	Equine encephalosis	Cow, horse	Similar to bluetongue, encephalitis	Asia, Australia, Japan, South Africa
	Kemerovo	Human	Febrile illness, encephalitis	Russia
	Lebombo	Human	Febrile illness	Africa
	Palyam	Cow	Abortion, congenital anomalies	Japan, South Africa
	Orungo	Human	Febrile illness	Africa
Coltivirus	Colorado tick fever	Human	Febrile illness (encephalitis, hemorrhagic fever)	China, Europe, North America

Adapted from ref 141, with permission.

TABLE 6. *Orbiviruses and coltiviruses that cause disease in humans and domesticated animals*

African Horse Sickness

African horse sickness (AHS) differentially affects members of the Equidae family; horses are the most susceptible, and donkeys and mules show mild symptoms. Laboratory adopted AHSV may cause disease in dogs and humans (141). Zebras, which are thought to be the only indigenous animal known to act as a natural reservoir for the virus, exhibit viremia but do not manifest clinical signs of infection. The disease caused by AHSV was first recognized as a distinct entity after the introduction of horses into Central Africa in 1569 (39). In South Africa, AHSV caused outbreaks as early as 1719 and gradually was introduced to other areas of sub-Saharan Africa, demonstrating its wide geographic distribution (39). From time to time, AHS has spread beyond its usual distribution, causing devastating epizootics in many countries, including the Middle East, Pakistan, Afghanistan, India, North Africa, and the Iberian Peninsula, due to

importation of infected animals (e.g., zebra into Spain in 1987).

There are four recognized forms of the disease in horses, reaching from a 100% mortality rate to mild symptoms. The *pulmonary form* is characterized by a short incubation time of 3 days with death occurring (100% mortality rate) 2 to 4 days after the initial symptoms. A body temperature of up to 41°C, dyspnea, paroxysms of coughing, and discharge of frothy fluid from the nostrils are the main clinical signs. Edema of the head and neck and bulging of the supraorbital fossae can also be seen. This form of the disease is most common in outbreaks in susceptible animals. In the *cardiac* or *subacute edematous form*, initial symptoms include fever and edema of the neck, supraorbital fossae, eyelids, lips, cervical and sternal regions, and ventral abdomen. The incubation period is 7 to 8 days with 63%. The cardiac form of AHS is usually common in horses in enzootic areas. The *mixed form* is an intermediate form of the pulmonary and cardiac disease. *African horse sickness fever*, the mildest form of disease, is characterized by a 10-day incubation period, with high temperature (40.5°C for 1 to 3 days), but 100% recovery. This form of the disease is typical within enzootic areas.

Bluetongue

Bluetongue disease was first detected in South Africa as early as 1900 and subsequently spread to all parts of sub-Saharan Africa where nonindigenous breeds of sheep were introduced (38). In the 1940s, the disease was recognized to occur outside of Africa (in Cyprus and in North America), and in the 1950s, it appeared in Israel, the Iberian peninsula, and the Indian subcontinent (38). It is now clear that BTV is present on all continents, except for Europe, where it has been periodically introduced but has not persisted. Sheep, white-tailed deer, and pronghorn antelopes infected with BTV may develop severe disease as discussed previously, whereas cattle generally experience asymptomatic or only mild infection (38). Cattle sustain prolonged viremias and thus serve as silent hosts. The expression of clinical signs in sheep depends on the strain of virus, breed of sheep, and possibly other factors; attack rates have been as high as 80%, with mortality rates as high as 50%.

Epizootic Hemorrhagic Disease of Deer and Ibaraki Virus Disease

Epizootic hemorrhagic disease of deer was recognized clinically in North America in the 19th century. In 1955, a virus was recovered from white-tailed deer during an epizootic in New Jersey, and the following year a distinct virus (EHDV serotype 2) was isolated during an outbreak in South Dakota. In 1974, EHDV was recovered from *Culicoides* species in Nigeria. EHDV causes disease in white-tailed deer in North America but is not pathogenic for cattle or sheep. However, a subtype of EHDV, named Kawanabe virus (138), was recovered from the brain of a calf with central nervous system signs in Japan in 1984. In 1959, an epidemic of a BTV-like disease affecting cattle in Japan was shown to be due to Ibaraki virus, a close relative of EHDV. Ibaraki virus also occurs in other Southeast Asian countries and in the Philippines (141).

Equine Encephalosis

This virus was first isolated in 1967 from South African horses with fatal peracute illness resembling AHSV (36). Outbreaks have been recognized in South Africa and are associated with infection by four of the seven serotypes known.

Palyam Group Virus Disease

Two orbivirus strains isolated from sentinel calves and *Culicoides oxystoma* in Japan in 1984 and 1985 were provisionally named Chuzan and Kagoshima virus respectively (138) but were later shown to be the same virus. Chuzan virus was subsequently implicated in an epizootic of congenital anomalies (hydranencephaly–cerebellar hypoplasia) in cattle in Japan in 1985 and 1986 (138). An inactivated, whole-virus vaccine is marketed in Japan against Chuzan virus disease. Recently, Chuzan virus was shown to be antigenically indistinguishable from Kasba virus, a Palyam group agent recovered from *Culex vishnui* mosquitoes in India in 1956 (101). Another member of Palyam virus group, Nyabira virus was recovered repeatedly from aborted cattle fetuses in Zimbabwe in the 1970s (208). The medical importance of this agent is presently uncertain.

IMMUNITY AND VACCINE

Orbivirus infection is followed by the appearance of neutralizing antibodies within 8 to 14 days (41). Antibodies also play a central role in protection against reinfection. There is a good correlation between the induction of neutralizing antibodies against the major outer capsid protein VP2 and protection of sheep against homologous challenge (80,191). Orbivirus infection also results in the production of antibodies to other structural and nonstructural viral proteins.

Infection of sheep and cattle with two distinct serotypes confers protection against other serotypes that had not been included in inoculum (205). Similarly, antibodies raised with a mixture of recombinant VP2 proteins of various serotypes not only neutralized the infectivity of the homologous strains but also cross-neutralized certain heterologous viruses (217). It is possible that cross-reactive epitopes exist on VP2, but other mechanisms may help to protect as well (see later).

In addition to neutralizing antibodies, cell-mediated immunity (CMI) also plays an important role in recovery from infection and protection against reinfection. Cytotoxic (CD8+) cells responsive to BTV antigen *in vitro* were demonstrated in blood of cattle and sheep during the first week of infection and peaked at 2 weeks (34). The role of CMI in protection against BTV infection was demonstrated in sheep that conferred protection on challenge with live virus (205). Lymphocyte stimulation by *in vitro* BTV antigens correlated with protection in these animals. Immunity was major histocompatibility complex (MHC) restricted and thus host specific. Further evidence for CMI in sheep was obtained by demonstrating cytotoxic T-lymphocyte (CTL) responses and protection of recipient twins by passive transfer of immune lymphocytes depleted of B cells (205). These data are also supported by *in vitro* studies. BTV antigen-specific ovine T-cell lines have been shown to become stimulated by a number of different BTV serotypes (210). Also, sheep vaccinated with recombinant BTV CLPs containing only VP3 and VP7 (without neutralizing determinants) were shown to cross-protect

against virulent virus challenge (98). However, the specificity and location of antigenic sites for T cells are still poorly understood. Studies of BTV in mice identified CTL responses that were principally cross-reactive, but other studies suggest that serotype-specific CTL responses may occur as well (202,205). Ovine T-cell lines established from sheep immune to BTV-1 showed serotype specificity and were directed against antigenic sites on the VP2 outer capsid protein (209), but other T-cell lines reacted with antigens localized on VP5 and inner core proteins. Antibody-dependent cell-mediated cytotoxicity may also play a role in protective immunity (202).

Using vaccinia virus recombinants that individually express each of the 10 BTV gene products, it has been shown that both the structural and nonstructural proteins are capable of inducing CTL responses (100). Further, CTL response mediated by NS2 appears to confer protective immunity in mice against challenged virus (100).

Despite the high morbidity and mortality associated with BTV and other orbiviruses, little attention has been given to developing efficacious vaccines for these viruses. The only vaccines that are currently used to protect against BTV and AHSV are live attenuated vaccines. Attenuated BTV vaccines have been developed by serial passage in embryonated chicken eggs; because of the plurality of serotypes (24 serotypes), multivalent vaccination has generally been used. Similarly, live virus vaccine for nine serotypes of AHSV has been developed in South Africa. Polyvalent live attenuated vaccines are presently produced in South Africa (37) and administered as two quadravalent vaccines consisting of serotypes 1, 3, 4, and 5 and serotypes 2, 6, 7, and 8. These vaccines are considered to induce cross-protective immunity to AHSV-9. Optimal immunity and protection from disease, however, is best provided by a serotype monospecific vaccine (109). This was recently demonstrated in an AHSV outbreak in Spain, where polyvalent vaccines failed to give protective immunity against AHSV-4, the serotype responsible for the disease (109). A commercial live attenuated virus vaccine against Ibaraki disease is also available in Japan. Problems associated with these live virus vaccine use include teratogenicity, insect transmission, interference by maternal antibody, and the potential for reversion and gene reassortment with wild-type viruses (232,248). Various whole-virus inactivated vaccines have been tested but are not yet available commercially.

Considerable clinical trials have been carried out in recent years with baculovirus-generated recombinant BTV proteins, CLPs, and VLPs (183,191). Initial studies involving unpurified VP2 and VP5 proteins of BTV-10 have demonstrated that VP2 alone at high doses (>100 µg/dose) could afford protection in sheep against virulent virus challenges, whereas much less VP2 was needed for complete protection when mixed with a low amount of VP5 (~20 µg/dose), suggesting that the two proteins are probably sufficient for vaccine development. Subsequently, baculovirus-synthesized VLPs consisting of VP2, VP3, VP5, and VP7 were used in vaccination trials with the rationale that VLPs are biologically and immunologically similar to virions and elicit high titers of neutralizing activity against the homologous and also heterologous BTV serotypes. The protective activities of VLP vaccination have been assessed in a number of vaccination trials in large numbers of susceptible Merino sheep. The data demonstrated that long-lasting protection against homologous BTV challenge could be achieved by vaccination with VLPs. Some cross-protection was also achieved depending on the challenge virus and the amounts of antigen used for vaccination (183).

VLPs are highly immunogenic, even at low doses. Sheep given only 10 µg of VLPs actually received only 1–2 µg of VP2 (10% to 20% of the VLP mass). This compared well with the much higher amounts required for VP2 alone or VP2 and VP5 mixtures in protection studies. There are several possible explanations. First, when assembled into VLP, the conformational presentations of the relevant epitopes on VP2 probably mimic those on the authentic virus. Second, both VP2 and VP5 are present. Third, any of the four BTV proteins might have a direct role in eliciting cell-mediated immunity induced by the BTV VLP.

The question of whether CLPs, containing only the two conserved proteins VP3 and VP7 would provide a measure of homologous and heterologous BTV protection was also investigated (98). The data obtained from clinical trails in sheep indicated that either partial protection (with only slight fever) or complete protection (depending on dose) against homologous and heterologous virus challenge was achieved by CLP vaccination. Animals showed strong group-specific antibody response but no neutralizing antibodies. Since CLPs are conserved across the various serotypes, CLPs could have potential for candidate vaccine, which may at least mitigate the bluetongue disease and inhibit the virus spread.

BTV CLPs and VLPs offer particular advantages as potential vaccines over more conventional systems. Large quantities of CLPs and VLPs can be produced as a result of the high-expression capabilities of baculovirus vectors (produced in serum-free medium) and can be purified using a one-step generic protocol based on the physical properties of the particle. More importantly, these particles are devoid of any detectable amount of insect or baculovirus proteins or nucleic acids and thus pose no potential adverse effects.

Baculovirus expressed subunit vaccines have also been generated for AHSV-4 in response to the recent outbreak of AHSV-4 in Europe (182). Insect cells infected with recombinant baculovirus expressing VP2 and VP5 were used for the vaccination of horses. These studies demonstrated that complete protection against AHSV infection occurred when horses were vaccinated with as little as 5 µg of recombinant AHSV-4 VP2. These trials have shown that vaccines based on synthesized recombinant proteins can give high immunogenicity while providing a safer alternative to modified live vaccines.

The immunodominant group-specific protein VP7, in the absence of other AHSV proteins, has also demonstrated its potential for use as a subunit vaccine. In a mouse model, T-cell immunity was shown to play an important role in the development of a protective immune response to AHSV in the absence of neutralizing antibodies. The results also demonstrated that a single inoculation of purified VP7 generated significant protection against a heterologous virus serotype (227).

In conclusion, both mixtures of recombinant proteins and multicomponent empty particles, such as CLPs and VLPs, have potential for development of efficacious and safe vaccines against orbivirus infections and disease.

COLTIVIRUSES

Coltiviruses, which have been isolated from ticks, mosquitoes, rodents, and humans, had previously been classified with the orbiviruses (42). However, viruses of this group have many distinctive features and have recently been recognized as a separate genus in the *Reoviridae* family (134). Only a brief account is presented here. To date, only three coltivirus serotypes are recognized. The type-specific virus, Colorado tick fever virus (CTFV), can cause a fatal human disease. It was originally isolated from ticks in North America. The other two serotypes, Eyach and S6-14-03 viruses, are not associated with human disease and show little, if any, cross-reaction in neutralization tests with CTFV. Published information concerning the molecular and genetic features of these viruses is limited.

The negatively stained coltivirus particles are about 80 nm in diameter with two capsids. The surface of the core has an icosahedral symmetry, although the overall structure appears to be slightly different from orbivirus cores by conventional electron microscopic analysis (147). Neither the capsid organization nor the protein components have been fully characterized. Unlike the genomes of orbiviruses, coltivirus genomes consist of 12 dsRNA segments with estimated sizes ranging from 2.53×10^6 to 0.24×10^6 daltons (total genome size about 18×10^6 daltons). All isolates of CTFV have similar dsRNA PAGE profiles. Their similarities have been substantiated by data obtained from hybridization studies (8,12). By such analyses, RNA segments 4 and 6 appear to be more variable than other segments, with RNA segment 6 exhibiting the highest degree of variation. By analogy with other members of the *Reoviridae* family, RNA segments 4 and 6, therefore, probably code for the outer capsid proteins of virions. Coltiviruses exhibit high frequency of RNA segment reassortment both in tissue culture and in tick vectors (12).

The coltivirus multiplication cycle and replication processes in cell culture have not been studied in detail. It is possible that they are similar to those of members of the *Orbivirus* genus because CTFV-infected cells are characterized by a large number of striated filaments equivalent to the orbivirus NS1 tubules as well as the presence of large numbers of inclusion bodies.

CONCLUDING REMARKS

Since the early 1990s, significant advances have been made in orbivirus research. The ability to synthesize CLPs and VLPs representing distinct stages in the virus life cycle in eukaryotic cells by the use of expression systems has opened up a novel approach for defining the mechanisms and steps involved in the intracellular protein–protein interactions that lead to virus assembly. The recent data on x-ray crystallographic structures of BTV core have revealed how a complex particle with multiple proteins at different molar ratio can be assembled. There is little doubt that expression systems, together with crystallographic data of various orbivirus proteins and the corelike structures that are now being prepared, will eventually allow a complete understanding of the virus capsid assembly process. The function of most of the structural and nonstructural proteins have also been defined, in relation to the replication cycle and virus particle assembly. These have been possible mainly due to the availability of highly purified individual proteins. However, some of the most intriguing questions, specifically, the packaging mechanism that encapsidates the exact number of 10 discrete RNA molecules into the newly synthesized viral capsid, remains elusive and a challenging problem, but this area too may yield to experimental systems in the near future. A reverse genetics system involving exact-copy BTV RNA transcript vectors and BTV protein expression vectors is under investigation. It is likely that such systems, in combination with assembled protein components in eukaryotic cells, will allow the dissection of the mechanism by which viral RNA species are incorporated into cores to serve as the viral genome. The opportunity then to build infectious BTV *de novo* from its component parts becomes very real and should allow a comprehensive analysis of many biologic properties of the orbiviruses.

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CHAPTER 57 *Retroviridae*: The Retroviruses and Their Replication

Stephen P. Goff

The retrovirus family, the *Retroviridae*, consists of a large and diverse group of viruses found in all vertebrates. These viruses replicate through an extraordinary and unique life cycle, differentiating them sharply from other viruses. The virion particles generally contain a genomic RNA, but upon entry into the host cell, this RNA is reverse transcribed into a DNA form of the genome that is integrated into the host chromosomal DNA. The integrated form of the viral DNA, the provirus, then serves as the template for the formation of viral RNAs and proteins that assemble progeny virions. These features of life cycle—especially the reverse flow of genetic information from RNA to DNA, and the establishment of the DNA in an integrated form in the host genome—are the defining hallmarks of the retroviruses. This life cycle also accounts for many of their diverse biologic activities. The creation of the proviral DNA confers on the viruses a powerful ability to (a) maintain a persistent infection despite a host immune response, and (b) enter the germ line, permitting the vertical transmission of virus.

The retroviruses have played a unique role in the history of molecular biology. They have attracted attention on several grounds.

- Biochemistry: The viral replication enzymes, including the reverse transcriptase (RT) and integrase (IN), are extraordinarily useful tools in manipulating nucleic acids *in vitro* and *in vivo*. Through the preparation of cDNAs, RT has been crucial for studies of mRNA synthesis and gene regulation.
- Pathogenicity: Retroviruses are known as major pathogens affecting nearly all vertebrates. Human immunodeficiency virus type 1 (HIV-1), the agent of the acquired immunodeficiency disease syndrome (AIDS) pandemic, will probably cause more human death and suffering than all but a handful of pathogens in recorded history.
- Markers of evolutionary history: The insertion of a provirus into the germ line provides a Mendelian tag that marks an event at a particular time in evolution. The inheritance of that tag can then be used to follow speciation, migrations, and divergence of species.
- Insertional activation of oncogenes: The integration of retroviral DNA is inherently mutagenic, and retrovirus replication thus causes gross alterations of host genes. When insertions lead to tumor formation, the locations serve to identify new oncogenes.
- Transduction: Retroviruses can acquire host sequences in the formation of acutely transforming genomes. The identity, structure, and expression of these genes has provided much of our current knowledge of the routes by which normal growth control can be subverted by genetic alterations.
- Gene delivery vectors: The structure of transforming viruses provided a model for the use of retroviruses to deliver therapeutic genes efficiently and cleanly into cells. Retroviruses now serve as major tools in the medical black bag of gene therapists.

This chapter will describe the replication and molecular biology of the retroviruses, concentrating on the most broadly conserved aspects of the life cycle. Because of the magnitude of the retroviral literature, citations here cannot be comprehensive, and referencing has been selective and concentrated on more recent publications. The distinctive features of the human retroviruses, especially the lentiviruses and spumaviruses, will be addressed in much more detail in later chapters. A comprehensive review of retroviral biology [*Retroviruses* (130)] has recently been published and should be consulted for additional details of almost all aspects of their replication.

TAXONOMIC CLASSIFICATION

The retroviruses were originally classified by the morphology of the virion core as visualized in the electron microscope. Examples of the appearance of the virions in these micrographs are presented in Figure 1. The virion particles are spherical and surrounded by an envelope consisting of a lipid membrane bilayer. The surface is studded by projections of an envelope glycoprotein. There is a spherical layer of protein under the membrane, and an internal nucleocapsid (or nucleoid) whose shape varies from virus to virus. The shape and position of the nucleocapsid core was historically used as the major classifying feature of the retroviral genera. A-type viruses were defined as those forming intracellular structures with a characteristic morphology, a thick shell with a hollow, electron-lucent center. These particles are now appreciated as representing an immature capsid on route toward the formation of other structures. This term is thus no longer in use to denote a virus classification, although it is used to describe the structures formed by some virus-related intracellular retrotransposons (the intracisternal A-type particles, or IAPs) (400,444). B-type viruses show a round but eccentrically positioned inner core. C-type viruses assemble at the plasma membrane and contain a central, symmetrically placed, spherical inner core. The D-type viruses assemble in the cytoplasm, via an A-type intermediate, and on budding they exhibit a distinctive cylindrical core.

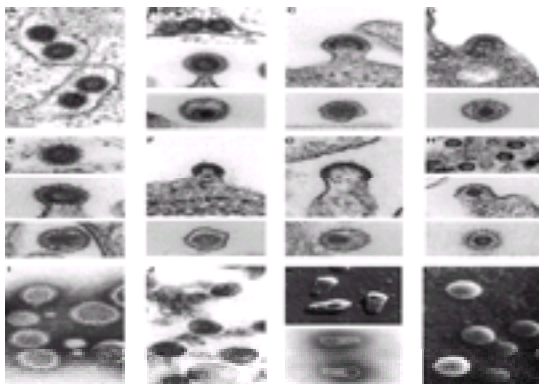

FIG. 1. Electron micrographs of representative virion particles. The diameters of all the particles are approximately 100 nm. **Panel A:** Type A particles. Intracisternal A particles in the endoplasmic reticulum. **Panel B:** Betaretrovirus. Mouse mammary tumor virus (MMTV), type B morphology (*top*, intracytoplasmic particles; *middle*, budding particles; *bottom*, mature extracellular particles). **Panel C:** Gammaretrovirus. Murine leukemia virus (MLV) type C morphology (*top*, budding; *bottom*, mature extracellular particles). **Panel D:** Alpharetrovirus. Avian leukosis virus, type C morphology (*top*, budding; *bottom*, mature extracellular particles). **Panel E:** Betaretrovirus. Mason Pfizer monkey virus (MPMV), type D morphology (*top*, intracytoplasmic A-type particles; *middle*, budding; *bottom*, mature extracellular particles). **Panel F:** Deltaretrovirus. Bovine leukemia virus (BLV) (*top*, budding; *bottom*, mature extracellular particles). **Panel G:** Lentivirus. Bovine immunodeficiency virus (*top*, budding; *bottom*, mature extracellular particles). **Panel H:** Spumavirus. Bovine syncytial virus (*top*, intracytoplasmic particles; *middle*, budding; *bottom*, mature extracellular particles). **Panel I:** Betaretrovirus. Mouse mammary tumor virus, type B morphology, visualized by negative staining with phosphotungstic acid. **Panel J:** Gammaretrovirus, visualized as pseudoreplica stained with uranyl acetate. **Panel K:** Lentivirus. Purified cone-shaped cores of equine infectious anemia virus (*top*, cores visualized by shadow casting technique; *bottom*, cores visualized by negative staining with phosphotungstic acid). **Panel L:** Budding retroviral particles visualized by scanning electron microscopy. (Micrographs are courtesy of Dr. Matthew Gonda and reproduced from ref. 708, with permission.)

These older classifications have been useful in partially defining the various genera of the family *Retroviridae*, but the number of genera has now been increased on the basis of new criteria. The genera have recently been formalized and given new names by the International Committee on Taxonomy of Viruses. The alpharetroviruses, betaretroviruses, and gammaretroviruses are considered “simple” retroviruses, whereas the deltaretroviruses, epsilonretroviruses, lentiviruses, and spumaviruses are considered “complex.” The simple viruses encode only the Gag, Pro, Pol, and Env gene products; the complex viruses encode these same gene products but also an array of small regulatory proteins with a range of functions. The properties of the viruses belonging to each of these genera will be summarized briefly here. Representative members of each genus are listed in Table 1.

New name	Examples	Morphology
Alpharetrovirus	Avian leukosis virus (ALV) Rous sarcoma virus (RSV)	C-type
Betaretrovirus	Mouse mammary tumor virus (MMTV) Mason-Pfizer monkey virus (MPMV) Jaagsiekte sheep retrovirus (JSRV)	B-, D-type
Gammaretrovirus	Murine leukemia viruses (MuLV) Feline leukemia virus (FeLV) Gibbon ape leukemia virus (GaLV) Reticuloendotheliosis virus (RetV)	C-type
Deltaretrovirus	Human T-lymphotropic virus (HTLV)-1, -2 Bovine leukemia virus (BLV) Simian T-lymphotropic virus (STLV)-1, -2, -3	–
Epsilonretrovirus	Walleye dermal sarcoma virus Walleye epidermal hyperplasia virus 1	–
Lentivirus	Human immunodeficiency virus type 1 (HIV-1) HIV-2 Simian immunodeficiency virus (SIV) Equine infectious anemia virus (EIAV) Feline immunodeficiency virus (FIV) Caprine arthritis encephalitis virus (CAEV) Visna/maedi virus	Fullerene core
Spumavirus	Human foamy virus (HFV)	Immature

TABLE 1. *Retrovirus genera*

Alpharetroviruses

The alpharetroviruses are simple retroviruses characterized by a C-type morphology, and are typified by the avian leukemia viruses (ALV). The genome contains *gag*, *pro*, *pol*, and *env* genes, with no additional known genes; *pro* is at the 3' end of *gag* and in the ame reading frame. The tRNA primer is tRNATrp. The viruses are widespread in many avian host species. The ALV members are classified into 10 subgroups (termed A through J) by their distinct receptor utilization. The first four subgroups represent exogenous viruses of chickens; the subgroup E includes a family of endogenous chicken viruses; and subgroups F and G include endogenous viruses of pheasants.

Betaretroviruses

The betaretroviruses are simple retroviruses characterized by either a B-type morphology, with a round eccentric core, or a D-type morphology, with a cylindrical core. The best-known examples are the mouse mammary tumor virus (MMTV) and the Mason-Pfizer monkey virus (MPMV). Assembly occurs in the cytoplasm via an A-type intermediate, and the completed immature particle is then transported to the plasma membrane and budded. The genomes contain *gag*, *pro*, *pol*, and *env* genes, and the *gag*, *pro*, and *pol* genes are all in different reading frames. The genome of MMTV contains an additional gene termed the *sag* gene, for superantigen. The viruses also contain a dUTPase region as part of the *pro* open reading frame (ORF) (204). The tRNA primer is tRNALys-3 or tRNALys-1,2. There are both exogenous and

endogenous viruses in this genus. Examples are found in mice, primates, and sheep.

Gammaretroviruses

The gammaretroviruses are simple viruses characterized by a C-type morphology. This genus has the largest number of members known, including the murine leukemia viruses (MuLVs), the feline leukemia viruses (FeLVs), and the gibbon ape leukemia virus (GALV). The genome contains only *gag*, *pro*, *pol*, and *env* genes; the *gag*, *pro*, and *pol* sequences are in the same reading frame, and the Gag-Pro-Pol protein is expressed by translational readthrough of a stop codon at the end of *gag*. The genome primer is tRNAPro or tRNAGlu (132,514). The murine viruses are divided into subgroups by their distinct receptor utilization. Many exogenous and endogenous viruses are found in diverse mammals, and examples have been isolated from reptiles and birds.

Deltaretroviruses

The deltaretroviruses are complex viruses characterized by a C-type morphology. The most famous examples are the human T-lymphotropic viruses (HTLVs) and the bovine leukemia virus (BLV). The genome contains *gag*, *pro*, *pol*, and *env* genes; the *gag*, *pro*, and *pol* genes are present in three different reading frames, and expression of the Gag-Pro-Pol protein requires two successive frameshifts. In addition, the genomes contain regulatory genes termed *rex* and *tax* that are expressed from an alternatively spliced mRNA. These gene products control the synthesis and processing of the viral RNAs. The tRNA primer is tRNAPro. No closely related endogenous viruses are known, and the exogenous viruses are only rarely found in a few mammals.

Epsilonretroviruses

The epsilonretroviruses are complex viruses characterized by a C-type morphology. The prototype is the walleye dermal sarcoma virus (469). The genomes contain *gag*, *pro*, *pol*, and *env* genes; the *gag*, *pro*, and *pol* genes are in the same reading frame. They also contain one to three additional genes termed *orfs A, B,* and *C*. The *orfA*gene is a viral homolog of the host cyclin D gene and so may regulate the cell cycle (410). The viruses use tRNAHis or Arg as the primer. The only known examples are exogenous viruses in fish and reptiles.

Lentiviruses

The lentiviruses are complex viruses characterized by a unique virion morphology, with cylindrical or conical cores. The most important example is HIV-1, but nonprimate viruses in the genus include the caprine arthritis encephalitis virus (CAEV) and visna virus. The genomes express *gag*, *pro*, *pol*, and *env* genes; *gag* is in one reading frame, and *pro-pol* in another. A single frameshift is used to express Gag-Pro-Pol. The Pol region of the nonprimate lentiviruses includes a domain for deoxyuridine triphosphatase (dUTPase). A number of accessory genes are also expressed. In HIV-1, these genes are *vif*, *vpr*, *vpu*, *tat*, *rev*, and *nef*; these genes control transcription, RNA processing, virion assembly, host gene expression, and a number of other replication functions (150). The tRNA primer is tRNALys1,2. A large number of exogenous viruses in this genus have been found in diverse mammals, but the only endogenous sequences are relatively distant from these viruses.

Spumaviruses

The spumaviruses are complex viruses with a unique virion morphology, containing prominent spikes on the surface and a central but uncondensed core. The prototype example is the human foamy virus (HFV). The virion is assembled in the cytoplasm and budded into the endoplasmic reticulum (ER) and the plasma membrane. There is probably only a single cleavage of the Gag protein near the C-terminus (797), and no major change in morphology during maturation. The genomes express *gag*, *pro*, *pol*, and *env* genes, and also at least two accessory genes known as *tas*/*bel-1*, and *bet* (242,483). The *tas* gene encodes a transcriptional transactivator. Unique features are the separate expression of the Pol protein from a spliced mRNA and the presence of large amounts of reverse transcribed DNA in the virion (493). The genome contains a second transcriptional start site near the 3' end of the *env* gene. The tRNA primer is tRNALys1,2. A number of exogenous viruses have been found in diverse mammals, and distantly related sequences are present as endogenous elements in the human genome (141).

Evolutionary Relationships

The sequences of the various retroviral genomes have been compared and used to determine the relatedness of any pair (475). A number of phylogenetic trees can be constructed using *gag*, *pro*, *pol*, or *env* genes, and in most aspects these trees are similar. A tree based on comparisons of the *pol* gene (Fig. 2) shows the clustering of viruses within each of the main genera. However, it is important to realize that a phylogenetic tree is not necessarily identical to an evolutionary history, and that the history that led to the formation of the known genera is not necessarily simple. It is noteworthy that there is no obvious clustering of all the simple viruses into a group apart from all the complex viruses. Thus, complex viruses probably arose from the simple ones more than once, with many evolving through the independent acquisition of separate genes.

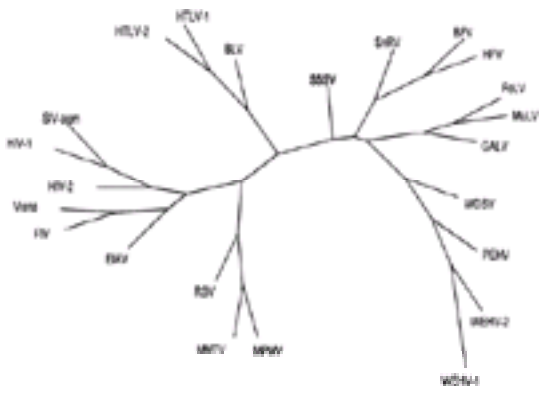

FIG. 2. Phylogenetic relationships within the retrovirus family. An unrooted neighbor-joining phylogenetic tree, constructed with the PHYLIP package based on an alignment of the amino acid residues of reverse transcriptase genes of several retroviruses (777). (Provided courtesy of S. Quackenbush and J. Casey.)

The retroviruses are related to viruses of other families. The retroviral RTs show close sequence similarity to the polymerases of the hepadnaviruses and the caulimoviruses, which also replicate by reverse transcription. The retroviruses also show extensive similarity in both *gag* and *pol* gene sequences to the retrotransposons, endogenous mobile elements with long terminal repeats (LTRs), and to retroposons, elements without LTRs. Retroviral RTs show more distant similarity to the group II mitochondrial introns; to the retrons, elements in myxobacteria and rare isolates of *Escherichia coli*; and to telomerase, an RT responsible for maintenance of the chromosomal termini in eukaryotes; and they show a slight similarity to the DNA polymerases of viruses and hosts (474).

Transforming Viruses

During the replication of any retrovirus, replication-defective variants can arise through deletion or recombination events. Such mutants or variants can be propagated as a mixed virus culture along with the wild-type parent. In these mixtures of two genomes, the replication-competent parent acts as a helper virus to provide the missing replication functions in *trans* for the replication-defective transforming virus. In extremely rare events, the genomes of a replication-competent virus can acquire novel host sequences that provide significant new biologic activities to the virus. If the new gene product is mitogenic or antiapoptotic for the host cell, or in more subtle ways alters the growth of the cell, the recombinant may become a potent oncogenic virus. A large number of such transducing viruses have been isolated and characterized as derivatives of one or another of the replication-competent parent viruses. In each case, the morphology and host range of the virus is determined by the helper virus that provides the replication functions to the defective genome. A partial listing of the most intensively studied of these viruses is presented in Table 2.

Parental virus	Transforming virus	Transduced gene
Avian leukosis virus (ALV)	Rous sarcoma virus	v-*src*
	Avian myelocytomatosis virus	v-*myc*
	Avian erythroblastosis virus	v-*erbA,B*
	Avian myelocytomatosis virus 29	v-*myc*
	Avian sarcoma virus CT10	v-*crk*
	Fujinami sarcoma virus	v-*fps*
	Y73 avian sarcoma virus	v-*yes*
	Avian sarcoma virus 17	v-*jun*
Moloney Murine leukemia virus (MoLV)	Abelson MuLV	v-*abl*
	Harvey sarcoma virus	H-*ras*
	Kirsten sarcoma virus	Ki-*ras*
	Moloney murine sarcoma virus	v-*mos*
	FBJ murine sarcoma virus	v-*fos*
	3611-MSV	v-*raf*
Feline leukemia virus	Synder-Theilen feline sarcoma virus	v-*fes*
	Gardner-Arnstein feline sarcoma virus	v-*fes*
	McDonough feline sarcoma virus	v-*fms*
Simian sarcoma-associated virus	Woolly monkey sarcoma virus	v-*sis*

TABLE 2. *Examples of acute transforming retroviruses*

VIRION STRUCTURE

Retrovirus virions are initially assembled and released from infected cells as immature particles containing unprocessed Gag and Gag-Pol precursors of the proteins that eventually make up the mature virus. The immature virion morphology is spherical, with a characteristic electron-lucent center. The virions have been described as a protein vesicle, to suggest some fluidity in the interactions between the individual Gag proteins that make up the particle. At maturation, the precursor proteins are cleaved, and the structure and morphology of the virion changes drastically (784). The mature retrovirus particle is a spherical structure, roughly 100 nm in diameter. The size of the virions in a given preparation is not highly homogeneous but rather varies over a fairly wide range, suggesting that a discrete, highly ordered structure may not exist. After processing of the Gag precursor during virion maturation, the capsid (CA) protein collapses to form a more ordered paracrystalline core, but even then the overall diameter of the virion is heterogeneous and suggestive of considerable disorder. The virions exhibit a buoyant density in sucrose in the range of 1.16 to 1.18 g/mL. The sedimentation rate of the particles is typically about 600 S. The virions are sensitive to heat, detergent, and formaldehyde.

Virion Proteins

The stoichiometry of the various viral gene products in the virion are not very firmly established, but estimates suggest that about 1,500 to perhaps 2,000 Gag precursors are present per particle. After processing, all cleavage products are

thought to be retained, suggesting equimolar presence of these proteins in the mature virions. The levels of the Pol proteins are typically about 1/10 to 1/20 of the Gag proteins, corresponding to about 100 to 200 molecules per virion. The levels of the Env proteins are quite variable among the viruses. For the gammaretroviruses, the levels of Env are close to that of Gag; perhaps 1,200 monomers, or 400 trimers, are present per virion. For the lentiviruses, the levels of Env per virion are much lower, possibly as low as 10 trimers per virion.

Nomenclature

The cleavage of Gag, Pol, and Env precursors forms the products in the mature infectious virions. These proteins are named by convention by a two-letter code: MA for matrix or membrane-associated protein, CA for capsid, NC for nucleocapsid, PR for protease, DU for dUTPase, RT for reverse transcriptase, IN for integrase, SU for surface protein, and TM for transmembrane protein (416). The localization of these proteins in the mature virion is not known with great precision, but a highly schematic version of the generic retrovirion can be drawn (Fig. 3).

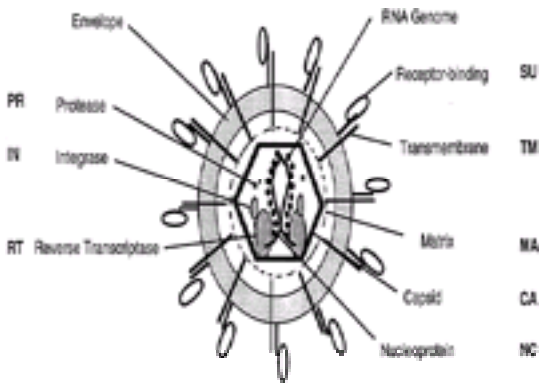

FIG. 3. Generalized retrovirion structure and components. A highly schematic view of the arrangement of viral gene products within the virion particle. The two-letter nomenclature for each protein is indicated.

Arrangement of Virion Components

The genomic RNA is highly condensed in the virion by its association with the nucleocapsid protein, NC. The complex is contained within a protein core largely composed of the CA protein, another *gag* gene product. The shape of the core differs among the various retroviral genera, and indeed it is a distinguishing feature of each genus. In most of the viruses, the core is roughly spherical, but in some cases it is either conical or cylindrical. In all the viruses, the core is surrounded by a roughly spherical shell consisting of MA, which in turn is surrounded by the lipid bilayer of the virion envelope. The virion membrane contains the envelope glycoprotein, with the TM subunit present as a single-pass transmembrane protein anchor, and the SU subunit as an entirely extravirion protein bound to TM. The envelope proteins for those viruses examined closely have been found to reside in the membrane as trimers.

ORGANIZATION OF THE RNA GENOME

The viral genome is a dimer of linear, positive-sense, single-stranded RNA, with each monomer 7 to 13 kilobases (kb) in size. The viral genomic RNA is present as a homodimer of two identical sequences, and thus the virions are functionally diploid. The dimer is maintained by interactions between the two 5' ends of the RNAs in a self-complementary region termed the dimer linkage structure (DLS). The RNA genome is generated by normal host transcriptional machinery and thus exhibits many of the features of normal mRNA. The RNA is capped at the 5' end, using the common $_{m7}$G5'ppp5'G$_{m}$p structure; and it contains a string of poly(A) sequences, about 200 long, at the 3' end.

A number of sequence blocks are so important that they have been named to facilitate descriptions of their functions in the life cycle (Fig. 4). These key sequences are clustered at the termini of the RNA. A short sequence, the R (for repeated) region, is so called because it is present twice in the RNA: once immediately after the cap at the 5' end and again at the 3' end, just before the poly(A) tail. Downstream of the 5' R lies another sequence, termed U5 for unique 5' sequence, which includes one of the *att* sites required for proviral integration. The U5 region is followed by the primer binding site (pbs), an 18-nt sequence at which a host tRNA is hybridized to the genome and the site of initiation of minus-strand DNA synthesis.

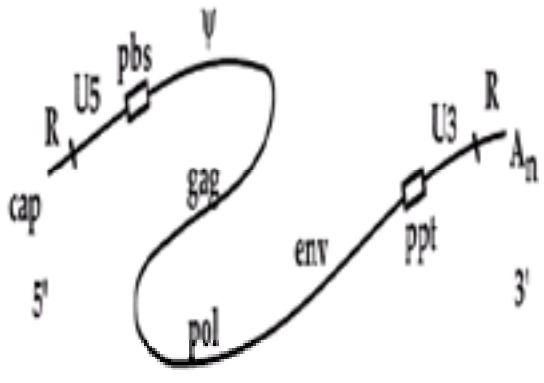

FIG. 4. The organization of the retroviral RNA genome. The single-stranded RNA genome is depicted as a *curved line.* From 5' to 3' along the RNA, the features include a 5' cap structure; *R*, a sequence block repeated at both 5' and 3' ends; *U5*, a unique 5' sequence block; *pbs*, the primer binding site and site of initiation of minus-strand DNA synthesis; *Y*, the

major recognition site for the packaging of the viral RNA into the virion particle; the *gag*, *pol*, and *env* genes; *ppt*, the polypurine tract and site of initiation of the plus-strand DNA synthesis; *U3*, a unique 3' sequence block; the second copy of the R sequence; and finally, a 3' poly(A) sequence.

The region downstream from the pbs often contains the major signals for the encapsidation of the viral RNA into the virion particle, in sequences called the Psi element. The region also often contains a major splice donor site for the formation of subgenomic mRNAs. The bulk of the RNA sequences that follow are coding regions for the viral proteins. The genomes of all the replication-competent retroviruses contain at a minimum three large genes, or ORFs: from 5' to 3' along the genome, the genes are termed *gag*, for group-specific antigen; *pol*, for polymerase; and *env*, for envelope. The three genes in the simple retroviruses occupy nearly all the available space in the center of the genome.

Downstream of the genes lies a short polypurine tract (ppt), a run of at least nine A and G residues. The ppt is the site of initiation of the plus-strand DNA. The ppt is followed by a sequence block termed U3 for unique 3' sequence; this region contains a number of key cis-acting elements for viral gene expression, and one of the *att* sites required for DNA integration. The U3 abuts the 3' copy of the R region, which is followed by the poly(A) tail. As we shall see, the R, U5, U3, pbs, and ppt sequences all play important roles in reverse transcription. The lengths of these sequence blocks in various retroviruses are summarized in Table 3.

Virus (example)	U3	R	U5
Alpharetrovirus (ALV)	150–250	18–21	80
Betaretrovirus (MMTV)	1200	15	120
Gammaretrovirus (MLV)	450	60	75
Deltaretrovirus (HTLV)	250–350	120–240	100–200
Lentivirus (HIV-1)	450	100	60–80
Spumavirus (HFV)	800	200	150

TABLE 3. *Size of sequence blocks in various retroviral RNAs (nt)*

OVERVIEW OF THE LIFE CYCLE

The retroviruses replicate through a complex life cycle. A short summary of the steps of the cycle is as follows (a schematic view is shown in Fig. 5):

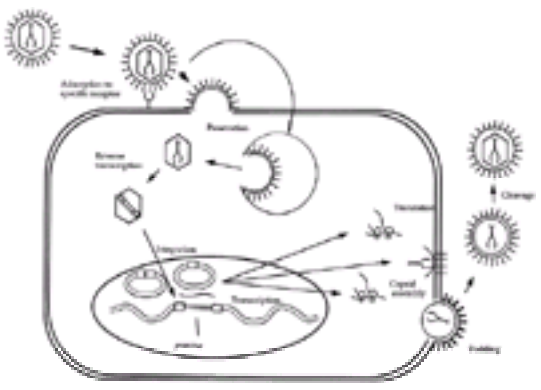

FIG. 5. A schematic view of the retrovirus life cycle. The major steps in the replication of a typical retrovirus are indicated, including those in the early phase of the life cycle, extending from the infecting virion (*top left*) to the formation of the integrated provirus, and those in the late phase of the life cycle, extending from the provirus to the formation of mature progeny virus (*right*).

- Receptor binding and membrane fusion
- Internalization and uncoating
- Reverse transcription of the RNA genome to form double-stranded linear DNA
- Nuclear entry of the DNA
- Integration of the linear DNA to form the provirus
- Transcription of the provirus to form viral RNAs
- Splicing and nuclear export of the RNAs
- Translation of the RNAs to form precursor proteins
- Assembly of the virion and packaging of the viral RNA genome
- Budding and release of the virions
- Proteolytic processing of the precursors and maturation of the virions

Changes in the Viral Genome

A quick perusal of this list reveals that the life cycle begins with an RNA genome, passes through an intracellular DNA intermediate, and is completed with a return to an RNA form in the progeny virus particle. An overview of the structures of the genome at various times in this cycle is presented in Figure 6. The RNA genome of the virion contains short terminal repeats (the R region) at its termini. During reverse transcription, as we shall see later, sequence blocks termed U5 and U3 are duplicated, so that the resulting double-stranded DNA is longer at both ends than the RNA template. This DNA thus contains LTRs (consisting of sequence blocks U3, R, and U5) at both ends. The next step is the integration of the DNA to form the provirus; the integrated provirus is collinear with the preintegrative DNA, and it retains the LTRs [except for one or two base pairs (bps) lost during the course of integration]. Finally, the DNA is forward transcribed by the RNA polymerase II system to produce the progeny RNA genome. Transcription is initiated at the U3-R boundary of the 5' LTR, and the transcripts are processed and polyadenylated at the R-U5 boundary of the 3' LTR, recreating the exact structure of the input RNA, complete with its short terminal repeats. This RNA is packaged and exported in virion particles. Each step will be described in more detail later.

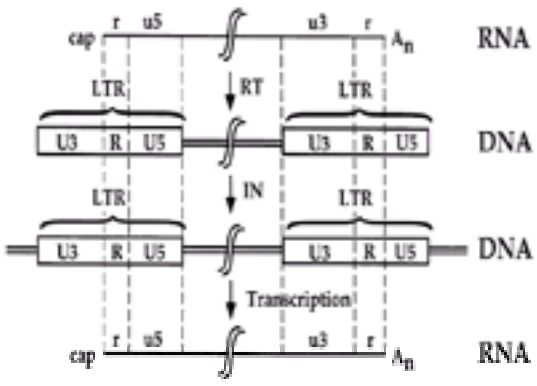

FIG. 6. Structures of the termini of the viral RNA and DNA genomes at various stages of the viral life cycle. Sequence blocks in RNA are indicated by lower case, and those in DNA by upper case. The structure of the RNA genome in the virion particle is indicated at the top. Reverse transcription of the RNA soon after infection involves the duplication and translocation of u5 and u3 sequence blocks, and it results in the formation of a double-stranded DNA molecule containing two terminal LTRs. The integration of the DNA genome occurs at the terminal sequences, establishing a provirus that is collinear with the preintegrative DNA. The forward transcription of the provirus is initiated at the U3/R border in the provirus; the resulting RNAs are cleaved and polyadenylated at the r/u5 border, recreating a viral RNA genome (*bottom*) identical to the infecting RNA.

THE VIRUS RECEPTORS

To enter a cell and initiate infection, all retroviruses require an interaction between a cell surface molecule—a receptor—and the envelope protein on the virion surface. The interactions are complex, involving an initial binding, drastic conformational changes in the envelope protein, an induced fusion of the viral and cellular membranes, and the internalization of the virion core into the cytoplasm. The SU subunit of Env is thought to make the major initial contacts with the receptor, and the TM subunit is thought to be most important for membrane fusion. The reorganization of the two lipid bilayers—one on the virion and one on the cell—to join them and evert the core into the cell is a remarkable process. The details of these complex processes are not understood for any retrovirus, and the whole Env protein is likely to be involved in efficient entry. However, there is a great deal of information about the identity and structures of the receptors used by various retroviruses. It is apparent that these viruses utilize an extraordinarily diverse set of cell surface molecules as receptors (see refs. 60 and 754 for reviews).

An important tool in the analysis of receptor utilization is the phenomenon of virus interference, or superinfection resistance. Cells chronically infected by a particular virus can be challenged for their susceptibility to infection by the same or different viruses. Such cells cannot be infected by any virus that must enter by the same receptor as used by the first virus, but they are readily infected by viruses that utilize a distinct receptor from that utilized by the first. The reason is that the expression of the Env protein by the first virus binds to the receptor intracellularly, preventing its export to the cell surface or its function as a receptor for newly applied virus (148,369). The phenomenon allows for the rapid classification of those viruses that use a common receptor.

The properties of the receptors of the major retroviral genera will be summarized later.

Alpharetrovirus Receptors

Three genes that encode functional receptors for various members of the ASLV family have been characterized. The receptor for the A subgroup was identified by selecting for the transfer of susceptibility to resistant mouse cells by genomic DNA fragments (787). The active gene was identified as encoding a membrane-anchored glycoprotein with sequence similarity to the ligand-binding repeat of the low-density lipoprotein receptor (LDLR) (50), and its identity as the true receptor has been confirmed by correlating its genetic map position with the *tv-a* locus (49). A soluble form of the receptor binds virus and blocks its infectivity (135). Functional versions of the protein can be anchored into the membrane either by a transmembrane segment or by a glycosylphosphaticlylinositol (GPI) anchor, and the receptor can even be fused to a foreign membrane protein, the epidermal growth factor (EGF) receptor, to permit efficient virus entry (667). The key determinants of the receptor for virus recognition and utilization have been identified (627).

The *tv-b* locus, encoding the receptor for both the B and D subgroups of the ALV, was similarly cloned and shown to encode a protein termed CAR1, unrelated to *tv-a* but with sequence similarity to the receptors for tumor necrosis factor (TNF) and the Fas death receptors (95). The intracellular portion of the molecule contains the sequence of a “death domain,” present on other cytotoxic receptors, and can trigger the apoptotic death of the cell upon ligand binding. This response is likely to mediate the toxic effects of the cytopathic ASLVs. However, noncytopathic viruses seem also able to bind and trigger the same receptors, and so the basis for the distinct effects of the two virus classes remains uncertain.

The *tv-e* locus is present in turkey but not chicken, and it allows infection by the subgroup E viruses. The gene was cloned by its sequence similarity to the chicken *tv-b* locus (5). The cloned gene product was shown to confer susceptibility to subgroup E but not B viruses, and to allow binding of subgroup E envelopes to the cell surface.

Betaretrovirus Receptors

A receptor for MMTV has been identified by gene transfer of cDNA libraries (278). The gene product allows cells to bind virus, and it allows infection by pseudotypes carrying the MMTV envelope. A second receptor for the betaretroviruses was also recently identified. The type D simian viruses, including MPMV and SRV-1, -2, -4, and -5, show cross-interference with three type C viruses: feline endogenous virus (RD114), baboon endogenous virus (BaEV), and avian reticuloendotheliosis virus (REV), suggesting that they all utilize a common cell surface receptor. Gene transfer of a human cDNA library into nonpermissive mouse cells was used to identify a gene that conferred susceptibility to infection by RD114 (694). The cDNA encoded a protein of 541 amino acids nearly identical to the previously cloned human Na^+-dependent neutral-amino-acid transporter named Bo (371,596). Consistent with this similarity, expression of the RD114 receptor in NIH 3T3 cells resulted in enhanced cellular uptake of L-^{3}H-alanine and L-^{3}H-glutamine.

Gammaretrovirus Receptors

A total of four receptors have been characterized for various subgroups of the MuLVs and their relatives, although there are others not yet identified (488). The ecotropic murine viruses are the most studied of this group, including the Moloney MuLV (MMLV), Friend MuLV, and Rauscher MuLV, as well as endogenous viruses from the AKR mouse, the AKR MuLV (AKV). The ecotropic receptor used by all these viruses is expressed on all strains of laboratory and wild mice of the species *Mus musculus*, mapping to the Rec1 locus on chromosome 5; a functional receptor is also expressed by rats. The ecotropic receptor was identified by gene transfer to nonpermissive human cells, selecting for susceptibility to MuLV infection (13). The gene encodes a membrane glycoprotein of 67 kd containing a total of 14 membrane-spanning domains; critical portions have been identified by mutational analyses. The corresponding human gene is highly homologous, but it cannot serve as a functional receptor by virtue of a small number of amino acid changes in the third extracellular loop of the protein. Other subtle mutations in this region of the receptor of other strains of mouse and of other rodents can cause varying changes in the susceptibility of the cell to infection by different ecotropic viruses (12,197). The SU subunit of the envelope protein binds to the receptor *in vitro* using a relatively small pocket region (162). The normal function of the protein has been identified as a transporter or permease for cationic, basic amino acids (379). The receptor, termed mCAT-1, was shown to be identical to y+, the previously characterized transporter in mammalian cells. The gene for mCAT-1 is now known as *Atrc1.* There is no strong evidence for a coreceptor or for any additional molecules besides the receptor being required; however, it has been noted that the infectivity of various cell lines does not correlate with the levels of receptor (747). Thus, it is possible that other molecules may modulate virus utilization of the receptor.

The amphotropic receptor is utilized by a group of MuLVs derived from wild mice and able to infect a wide range of mammalian species, including humans. The receptor was cloned by selection for susceptibility to virus infection after transfection of cDNA libraries into nonpermissive Chinese hamster ovary (CHO) cells (195,490), and by its homology to the gene for the previously identified GALV receptor (734). The gene, known variously as *Ram1* or GLVR2 or rPiT-2, encodes a 652-amino-acid protein that functions as a sodium-dependent phosphate symporter (368). The protein is predicted to contain 10 membrane-spanning domains, and residues in extracellular loops 2 and 4 have been shown important for virus entry (418,446). The synthesis and stability of the receptor is regulated by phosphate levels, and its down-regulation by virus infection results in substantial reduction in phosphate uptake by cells.

The receptor utilized in common by the GALV, the simian sarcoma–associated helper virus, and the FeLV subgroup B (698) is widely expressed in many mammals, including primates, cat, dog, mink, rabbit, and rat (but not mouse), as well as in some avian species. The human receptor, termed GLVR1 or hPiT-1, was cloned by gene transfer into nonpermissive mouse cells (346,517). The sequence of the gene predicts the existence of 10 membrane-spanning segments, and a large third intracellular loop. The protein shows similarity to that of a *Neurospora* phosphate permease gene, pho4+, and has been shown directly to serve as a sodium-dependent phosphate symporter (368,521). Specific amino acid changes introduced into the fourth extracellular loop can block FeLV-B and simian sarcoma–associated virus (SSAV) infection without affecting GALV, suggesting that these various viruses interact in slightly different ways with the receptor. GALV cannot use the human amphotropic receptor GLVR2, but a single amino acid change in the receptor will permit its use (198).

The xenotropic MuLVs are viruses present as proviruses in the mouse germline but unable to infect inbred mouse cells. These viruses can infect some wild mice and mice of other species, as well as other mammals. The polytropic MuLVs are also endogenous viruses with a wide host range that includes many mammalian species. These viruses are often detected by the appearance of portions of the envelope sequences in recombinant polytropic viruses called mink cell focus-forming (MCF) viruses that arise during the replication of ecotropic viruses in mice. These MCF viruses can infect a wider range of cells than the parental viruses and can accelerate the course of leukemogenesis induced in mice by the ecotropic viruses. Xenotropic and polytropic MuLVs cross-interfere to various extents in nonmurine species and in wild Asian mice, suggesting that they might use a common receptor for infection. The mouse receptor for the polytropic viruses was cloned by gene transfer and was identified with the *Rmc1* gene (783). The human xenotropic receptor was cloned similarly (51,693). The human xenotropic receptor mediates infection by both the xenotropic and the polytropic

viruses, whereas the homologous protein of inbred mice mediates only polytropic MuLV infections. The gene encodes a membrane protein related to the yeast Syg1p protein (suppressor of yeast G alpha deletion). Its function is unknown, but its multiple membrane-spanning segments and its sequence suggest that it may act as a G-coupled receptor.

Very recently, the isolation of a distinct receptor utilized by the subgroup C FeLVs (FeLV-C) for entry into heterologous cells (590). The cDNA recovered was predicted to encode a protein with 12 membrane-spanning domains with significant sequence similarity to the D-glucarate transporters of bacteria and nematodes. The binding of virus to this receptor may be responsible for the pathogenicity, a block in erythroid differentiation.

Additional receptors for other gammaretroviruses are known to exist. Three newly characterized porcine endogenous retroviruses (PERV-A, -B, and -C) have been tested in interference assays with each other and with murine viruses using the known receptors, and all three apparently utilize distinct and novel receptors (697). The identity of these molecules is presently unknown.

Deltaretrovirus Receptors

The receptors for the delta-retroviruses are not well characterized yet. A candidate gene has been identified as a possible receptor for BLV (526,579). A determinant of the HTLV-1 receptor has been mapped to human chromosome 17 (671).

Lentivirus Receptors

The first receptor identified for any retrovirus was the CD4 molecule, established as essential for infection by HIV-1 (153,385,451). Soluble forms of CD4 can bind to virions and inhibit infection; in some strains of virus, CD4 induces release or shedding of the SU subunit from the virion. However, other strains are quite resistant to inhibition by soluble CD4. CD4 is an important surface protein on T cells, and with few exceptions it serves to define the helper subset of T cells. CD4 is also expressed at significant levels on dendritic cells, on macrophages, and on certain cells in the brain, which may be related astrocytes rather than cells of neural origin. The limited distribution of expression of CD4 accounts well for the tropism of HIV-1, largely restricted to helper T cells and macrophages. There may be other routes of entry utilized at lower efficiency: Antibody to virus, for example, can promote virus entry into cells by the Fc receptor (696).

Early work established, however, that although CD4 was sufficient to mediate virus binding to a cell surface, it was not sufficient to mediate virus infection and entry. For example, rodent cells and other cells of nonprimate origin could not be successfully infected by HIV-1 even if they were engineered to express human CD4. Searches for genes that would render such cells sensitive to virus infection ultimately led to the identification of various members of the chemokine receptor family, notably CCR5 and CXCR4, as coreceptors that were needed to mediate the postbinding step of membrane fusion and virus entry (194,233,268,660). The regions of the human coreceptors that are needed for virus entry have been identified (186,268,593,659), although, surprisingly, some HIV-1 isolates can utilize the murine CXCR4 (551). Antibodies to the coreceptor as well as the natural ligand for these molecules, the chemokines themselves, can block virus entry. Variants of simian immunodeficiency virus (SIV) and HIV-1 have been identified that are CD4-independent, needing only a chemokine receptor for infection; these viruses suggest that a chemokine receptor might have been the primary receptor for a primordial virus. A further proof of the importance of the chemokine receptor is the existence of a mutant allele of the gene encoding CCR5 in the human population, a 32-bp deletion, that confers dramatic virus resistance to homozygous individuals. More discussion of the roles of CD4 and the coreceptors in virus entry will be presented in the following chapters on HIV-1.

PENETRATION AND UNCOATING

Once virus particles have bound to the receptor, the virion and host membranes fuse together, and the virion core is delivered into the cytoplasm of the infected cell. For most retroviruses, the processes of fusion and entry are thought to be pH independent; that is, they are not dependent on an endosomal acidification step to induce a pH-dependent change in the conformation of the envelope (see, e.g., refs. 467,476, and 677). Thus, fusion can occur at the cell surface. However, there may be exceptions; early tests suggested that the ecotropic MuLVs could be inhibited from infection of some cells by drugs that block the acidification (476). This property has been mapped to the TM portion of the envelope protein (515).

It is not known whether specific uncoating steps are required before reverse transcription can begin. It is clear that the processing of the Gag precursor to the mature Gag proteins is required; immature virions are uninfectious and cannot initiate reverse transcription, and mutants that prevent particular cleavages of the Gag protein are similarly blocked. A large number of mutant viruses with other alterations in the *gag* gene have been shown to be defective in early steps of infection, before reverse transcription, but the functions of Gag proteins at this stage remain uncertain. Some of these mutations affect CA, suggesting that a mutant core may not properly open or allow the initiation of reverse transcription. Other mutations lie in NC (280), suggesting that the nucleic acid itself is bound inappropriately by the mutant protein, or again, is not properly unfolded for reverse transcription. There are also indications that host factors are important in these early stages (263). In the case of HIV-1, the host protein cyclophilin is required for the efficient initiation of reverse transcription, and Gag mutants that do not package cyclophilin cannot initiate reverse transcription (90). A plausible role for this protein is to facilitate virion disassembly. Recently, inhibitors have been used to demonstrate a role of the cytoskeleton in virus entry, and furthermore to suggest that viruses may utilize different entry pathways in different cell lines (384). Biochemical analyses of these early events are made difficult by the presence of large numbers of defective particles that are probably not on the infectious pathway and that tend to obscure the properties of the rare particles that are on this pathway.

REVERSE TRANSCRIPTION

The reverse transcription of the viral RNA genome into a double-stranded DNA form is the defining hallmark of the

retroviruses and the step from which these viruses derive their name. The course of reverse transcription is complex and highly ordered, involving the initiation of DNA synthesis at precise positions, and translocations of DNA intermediates that result in duplication of sequence blocks in the final product (reviewed in refs. 271 and 708). The major steps in the reaction are relatively well established, largely through the analysis of reactions carried out *in vitro* in purified virion particles (the so-called endogenous reaction).

Reverse transcription normally begins soon after entry of the virion core into the cytoplasm of the infected cell. The reaction takes place in a large complex, roughly resembling the virion core, and containing Gag proteins including NC, RT, IN, and the viral RNA (87). The signal that triggers the onset of DNA synthesis is not known, although it may be as simple as the exposure of the viral core to the relatively high levels of deoxyribonucleotides present in the cytoplasm. This notion is consistent with the observation that simply stripping or permeabilizing the virion membrane with detergents in the presence of deoxyribonucleotides is sufficient to induce DNA synthesis. This may also be at least part of the explanation for the difficulty HIV has in completing reverse transcription and infection in quiescent cells (794). In some cells, notably cells arrested by starvation, triphosphate levels may be low and limiting for RT, so that addition of exogenous nucleosides can stimulate viral DNA synthesis (286).

It has been recently appreciated that DNA synthesis can be initiated “prematurely” during virion assembly and release, such that virion preparations can be shown to contain small amounts of the early DNA intermediates, such as minus-strand strong-stop DNA (437,718,795,804). In most cases, the levels of these DNAs are very low, indicating that only a very small minority of the virion particles have carried out any significant synthesis. However, some circumstances affecting the rate of production and release of virions may enhance this synthesis (183). In addition, in some particular retroviruses, notably the spumaviruses, substantial DNA synthesis may occur during assembly, such that the major form of the genome found in mature virions is a partially or even completely reverse transcribed DNA molecule (493,790). These viruses thus resemble the hepadnaviruses more closely than the conventional retroviruses in the relative timing of assembly and reverse transcription.

Steps in Reverse Transcription of the Retroviral Genome

The course of reverse transcription is complex. The reaction can be broken down into a series of discrete steps (271), as presented in Figure 7.

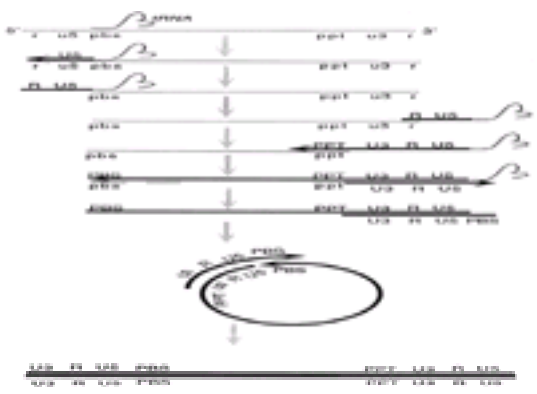

FIG. 7. The reverse transcription of the retroviral genome. *Thin lines* represent RNA; *thick lines* represent DNA. See text for details. Drawing courtesy of A. Telesnitsky.

Formation of Minus-Strand Strong-Stop DNA

The process of reverse transcription is initiated from the paired 3' OH of a primer tRNA annealed to the viral RNA genome at a complementary sequence termed the pbs. DNA is first synthesized from this primer, using the plus-strand RNA genome as template, to form minus-strand DNA sequences. Synthesis occurs toward the 5' end of the RNA to generate U5 and R sequences. The intermediate formed in this step is termed minus-strand strong-stop DNA. The primer tRNA remains attached to its 5' end.

First Translocation

The next step involves the translocation, or “jump,” of the strong-stop DNA from the 5' to the 3' end of the genome. This translocation requires the degradation of those 5' RNA sequences that were placed in RNA:DNA hybrid form by the formation of strong-stop DNA. The degradation is mediated by the RNase H activity of RT; mutants with altered RNase H activity do not mediate the translocation (71,702). This step exposes the single-stranded DNA and facilitates its annealing to the R sequences at the 3' end of the genome. Normally, a full-length strong-stop DNA, synthesized by copying to the 5' cap of the RNA, performs the translocation (404), although incomplete molecules can jump at low efficiency. Specific sequences or structures in or near the R region are required for efficient jumping (14,716). The NC protein may facilitate the transfer step. Although there have been reports that jumping is always in *trans,* from one RNA template to the other RNA in the virion, the best evidence is that minus-strand strong-stop jumping goes randomly to either RNA (788).

Long Minus-Strand DNA Synthesis

The annealing of minus-strand strong-stop DNA recreates a suitable primer-template structure for DNA synthesis, and RT can now continue to elongate the minus-strand strong-stop DNA to form long minus-strand products. Synthesis ends in the vicinity of the pbs. As the genome enters RNA:DNA hybrid form, the RNA becomes susceptible to RNase H action

and is degraded.

Initiation of Plus-Strand DNA Synthesis

The primer for plus-strand synthesis is created by the digestion of the genomic RNA by RNase H. A particular short, purine-rich sequence near the 3' end of the genome, the ppt, is relatively resistant to the activity of RNase H. The oligonucleotide remains hybridized to the minus-strand DNA and serves as the primer for synthesis of the plus-strand DNA, using the minus-strand DNA as template. Sequences upstream of the ppt, an AT-rich region called the T-box, are also important for proper priming (333,621). The primer, once it has served to initiate DNA synthesis, is quickly removed from the DNA, presumably by RNase H action. Synthesis proceeds toward the 5' end of the minus strand, first copying the U3, R, and U5 sequences, and then extending to copy a portion of the primer tRNA still present at its 5' end. Elongation stops at a modified base normally found at position 19 of the tRNA. The resulting intermediate is termed plus-strand strong-stop DNA.

In some viruses, secondary plus-strand initiation sites are used. There may be multiple RNA primers generated from the RNA genome by the nuclease action of RNase H that can initiate DNA synthesis at dispersed heterogeneous sites. In the case of the lentiviruses and spumaviruses, a second copy of the ppt sequence near the center of the genome is used at high efficiency and is important for proper completion of reverse transcription (112).

Removal of tRNA

In the next step, the primer tRNA at the 5' end of the minus-strand DNA is removed by RNase H. Its removal may occur in two stages, with an initial cleavage near the RNA–DNA junction and a second one within the tRNA. The cleavage need not occur exactly at the RNA–DNA junction, and a single ribonucleotide base (A) is normally left on the 5' terminus of the minus strand (588,646,664,758) without affecting subsequent processes. The sequence of bases near the junction can significantly modulate the cleavage by RNase H (663). Recent experiments indicate that the posttranscriptional modifications present in the natural tRNA may be important for proper recognition by RT and for plus-strand strong-stop translocation (37).

The Second Translocation

The removal of the tRNA exposes the 3' end of the plus-strand strong-stop DNA to permit its pairing with the 3' end of the minus-strand DNA. The sequences anneal via the shared pbs sequences. This annealing forms a circular intermediate, with both 3' termini in a suitable structure for elongation.

Completion of Both Strands

Both strands are now elongated. The final extension of the minus-strand DNA is coupled to displacement of the plus-strand strong-stop DNA from the 5' end of the minus strand; as minus-strand elongation occurs, the plus-strand strong stop is peeled away and transferred to the 3' end of the minus strand. At the end of this elongation, the circle is opened up into a linear DNA. The plus strands are all extended, and displacement synthesis may occur to remove short DNAs and make longer plus-strand DNAs (372). Any internally primed plus-strand DNAs may be prevented from participating in strong-stop translocation simply by kinetics; the normal plus-strand strong-stop DNA may simply form first, and elongation to transfer it to the minus strand may occur before it could be displaced by any plus-strand elongation (88). Finally, the plus-strand strong-stop DNA is extended to complete the plus strand.

When multiple plus-strand initiation events occur, the completed plus strand will consist of adjacent fragments and so contain nicks or discontinuities. Displacement synthesis by an upstream fragment can slowly displace downstream RNAs and DNAs, leading to longer plus strands (372). However, some nicks or gaps may persist in the final double-stranded product. These breaks may be at heterogeneous positions, although strong sites of plus-strand initiation, such as the one at the central ppt of lentiviruses, can lead to specific sites for such discontinuities. Sequences near the central ppt of the lentiviruses cause termination of synthesis during elongation from upstream primers, thus ensuring the maintenance of a discontinuity at this site (113). This site retains a partially displaced sequence or overlap of a few nucleotides—99 nt in the case of HIV-1. The structure has been shown to persist even to the time of integration of the DNA into the cell. Host DNA repair processes ultimately correct all such discontinuities.

Although most of the viral DNA is made in the cytoplasm, it may not always be completed in the cytoplasm. For some viruses, completion of the two DNA strands may occur only after entry into the nucleus (415). Specific mutants with alterations in the Cys-His residues of the NC protein show an interesting phenotype: the formation of linear DNA with heterogeneous and truncated ends (282). These experiments suggest that NC plays a role in the completion, or the stabilization of the ends, of the viral DNA.

A key consequence of the two translocation events that occur during reverse transcription is the duplication of sequences—duplication of U5 during minus-strand strong-stop DNA translocation, and of U3 during plus-strand strong-stop DNA translocation. The resulting DNA contains two LTRs that are assembled during reverse transcription. Each LTR consists of the sequence blocks U3-R-U5. The positions of the LTR edges—the left edge of U3 and the right edge of U5—are determined by the sites of initiation of DNA synthesis for the two DNA strands. Thus, the terminal sequences of the complete DNA molecule are also determined by these sites of initiation. These sequences for most viruses are perfect or imperfect inverted repeats, and they serve an important role during integration of the DNA (see later).

Biochemistry and Structure of Reverse Transcriptase

The enzyme that mediates the complex series of events outlined above is RT, one of the most famous of the viral polymerases (44, and reviewed in ref. 661). All RTs contain two separate activities present in two separate domains: a

DNA polymerase able to incorporate deoxyribonucleotides on either an RNA or a DNA template, and an RNase H activity able to degrade RNA only in duplex form. These two activities are responsible for the various steps of reverse transcription. Two distinct domains of the enzyme contain these two activities: an aminoterminal domain contains the DNA polymerase, and a carboxyterminal domain contains the RNase H activity (700). Whereas isolated domains can be shown to exhibit either one of the two activities separately, an intact enzyme is required for full activity and specificity. However, the two functions can be provided by two mutant RT molecules so long as they are co-incorporated into a single virion (707).

DNA Polymerase

The DNA polymerase activity is similar to that of all host and viral DNA polymerases in requiring a primer, which can be either RNA or DNA, and a template, which can also be either RNA or DNA. RTs incorporate dXTPs to a growing 3' OH end with release of inorganic pyrophosphate (PPi), and they require divalent cations, usually Mg^{2+}. The primer must contain a 3' OH end that is paired with the template. RTs cannot perform nick-translation reactions, but they can efficiently perform strand displacement synthesis. The only fundamental way in which RTs are unusual among the DNA polymerases is that they exhibit comparable specific activity on either DNA or RNA templates.

RTs are readily obtained from purified virion particles, and they can be even more easily prepared as recombinant proteins expressed in bacteria. RTs are relatively slow DNA polymerases, under standard conditions incorporating only 1 to 100 nucleotides per second, depending on the template. Further, they exhibit poor processivity, and tend to release primer-template frequently *in vitro*. The enzyme must then rebind to the substrate to continue synthesis. Secondary structures in RNA templates can strongly enhance the pausing of RT and its tendency to release from the template (298). The enzyme also exhibits low fidelity, and although the values of the error rate vary widely with the primer (533), template (374,378), and type of assay, the misincorporation rate of most RTs under physiologic conditions is on the order of 10^{-4} errors per base incorporated. This rate suggests that during replication there would be approximately one mutation per genome per reverse transcription cycle. Indeed, the mutation rate observed *in vivo* is roughly consistent with this high error rate (101,184,380,496,654), although fidelity *in vivo* may be somewhat better than *in vitro* (461). Drug-resistant variants that do not incorporate chain-terminating analogs are often found to exhibit higher fidelity, perhaps because they require a more precise fit for the correct incoming triphosphate to allow for discrimination against the analog. A wide range of types of mutations are created by RT errors, and both the type and the frequency of appearance of each type of mutation exhibit a complex dependence on sequences and structures in the template (54,553,554).

RTs do not generally exhibit a proofreading nuclease activity (52), and misincorporated bases are not removed efficiently by most RTs as they are by host DNA polymerases. Very recently, however, mutants of the HIV-1 RT resistant to azidothymidine (AZT) have been shown to exhibit an enhanced ability to remove the incorporated AZT moiety at the 3' end through a pyrophosphorolysis reaction (486). Thus, it is possible for RT to remove some such analogs and rescue a terminated chain for continued elongation.

RNase H

The RNase H of RT is an endonuclease that releases oligonucleotides with a 3' OH and a 5' PO_4. This property allows the products of RNase H action to serve as primers for initiation of DNA synthesis by the DNA polymerase function of RT. There is an obligate requirement that the RNA be in duplex form, normally an RNA–DNA hybrid. However, retroviral RTs are also able to degrade RNA–RNA duplexes (57,70), an activity termed RNaseH* (318). The RNase H enzyme is capable of acting on the RNA of a template in concert with the polymerase as it moves along a nucleic acid, and as it does so its active site is located about 17 to 18 bp behind the growing 3' end (279). RNase H can also act independently of polymerization. All RNase H activity requires a divalent cation.

Subunit Structures

RT is incorporated into the virion particle during assembly in the form of a large Gag-Pol precursor (see later), and it is released by proteolytic processing of the precursor during virion maturation. Different viruses make somewhat different cleavages in the precursor, and thus the RTs exhibit several different subunit structures (see later). In the gammaretroviruses, RT is a simple monomer in solution, corresponding only to the aminoterminal DNA polymerase and the carboxyterminal RNase H domains. These two domains can be expressed separately, and the isolated proteins exhibit their respective activities (700), although the specificity of the RNase H is affected by this separation. There is some evidence, however, that while bound to the substrate and carrying out DNA synthesis, this RT may act as a dimer (706). In the avian viruses, the RT is present as an ab heterodimer, composed of a smaller a subunit containing the DNA polymerase and RNase H domains, and a larger b subunit containing these two domains but also retaining the IN domain. In the lentiviruses, RT is again a heterodimer with a larger subunit (p66) containing the DNA polymerase and RNase H domains, and a smaller subunit (p51) lacking RNase H. The properties of the different enzymes as DNA polymerases are very similar in spite of these different subunit structures, and so the significance of these various compositions for RT function is unclear.

Crystal Structures

The three-dimensional structures of a number of RTs have been determined by x-ray crystallographic studies. Structures of the unliganded HIV-1 RT (320,622), RT bound to nonnucleoside RT inhibitors (160,174,391,605), RT bound to an RNA pseudoknot inhibitor (343), and RT bound to a duplex oligonucleotide (28,324,341,342), as well as the isolated RNase H domain (163), have all been reported. The two subunits are folded quite differently, so the overall structure is highly asymmetric. The structure of the p66 is similar to that of a right hand, with fingers, palm, and thumb domains all named on the basis of their position in the structure (Fig. 8). The nucleic acid lies in the grip of the hand, held by the fingers and thumb. The YXDD motif present at the active site for the DNA polymerase lies at the base of the palm. The RNase H domain is attached to the hand at the wrist. The p51 subunit, while made up of the same domains as the aminoterminal part of p66, is folded differently and lies under the hand, not making direct contact with the nucleic acid and so not

thought to participate in the chemistry. The structure of p66 with and without a liganded nucleic acid is quite different, with the thumb domain flexing to allow substrate binding. Theoretical considerations suggest the thumb may move during elongation (41). A structure of the fingers and palm subdomain of the MMLV RT at very high resolution (269) has also been determined.

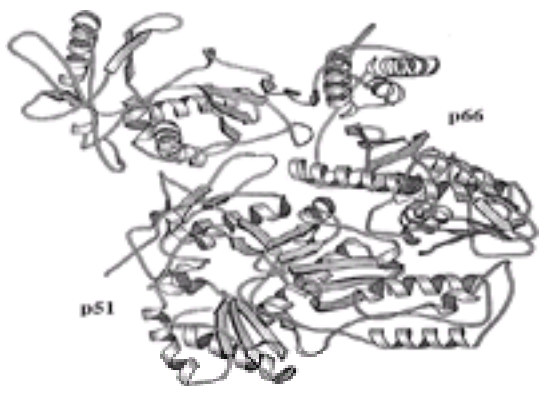

FIG. 8. A ribbon diagram of the heterodimeric reverse transcriptase of HIV-1, showing the p66 (*top*) and p51 (*bottom*) subunits. The molecule is arranged in the conventional orientation to show its similarity to the human right hand, palm up. The image was generated by Hans-Erik G. Aronson (Department of Biochemistry and Molecular Biophysics, Columbia University) using MOLSCRIPT (396). The HIV RT coordinates used to draw the image were retrieved as file 1VRT.pdb from the Research Collaboratory for Structural Bioinformatics (RCSB) Protein Data Bank (PDB) (http://www.rcsb.org/pdb). HIV RT secondary structural elements were defined according to the header (HELIX and SHEET records) of the coordinate file. The structure as solved was originally a complex of RT and the inhibitor nevirapine (604).

Inhibitors

RT is a major target of antiviral drugs useful in the treatment of retroviral diseases such as AIDS. All such drugs used to date are inhibitors of the DNA polymerase activity of RT, and they fall into two classes: nucleoside analog inhibitors (chain terminators) and nonnucleoside RT inhibitors (NNRTIs). The nucleoside analogs are typically precursors and need to be activated by phosphorylation to the triphosphate form. These are then incorporated by RT into the growing chain, and they serve to block further elongation. Examples include AZT, ddC, ddI, d4T, and 3TC. The NNRTIs are a group of compounds that are structurally diverse but nevertheless interact with a common binding pocket in RT to prevent its normal activity (724). There are indications that the binding may inhibit the enzyme's flexibility. For both classes of inhibitors, monotherapy with a single drug selects for drug-resistant variants that quickly predominate in the virus population, and for each drug, a pattern of mutations has been identified that serves to indicate the appearance of drug resistance (411). In many cases, these mutations alter the binding site for the nucleoside or NNRTI such that the drug cannot bind and so cannot inhibit the enzyme. In the case of AZT, however, the mutations do not prevent the binding and incorporation of AZT-triphosphate into the growing chain but rather seem to activate a reverse reaction in which the AZT nucleotide is removed from the chain, subsequently permitting normal elongation (486). Combination therapy, typically involving the simultaneous treatment with three different drugs, can suppress virus replication to such an extent that variants resistant to all the drugs do not appear, at least for months or years.

RECOMBINATION

The process of reverse transcription could in principle take place using a single template RNA molecule. In fact, however, retrovirions contain two copies of the RNA genome copackaged into one particle, and the course of reverse transcription typically makes use of both RNAs (322,686). Recombination occurs between homologous sequences in the two RNAs (170,562), and it happens normally at a surprisingly high frequency, perhaps once per replication event per genome on average (799). Normally the two RNAs in a virion are identical, so that homologous recombination events are invisible and without consequence. When the two RNAs are distinct, however, as when they derive from two viruses or viral strains, the result is a very high frequency of recombination between them among the resulting proviral DNAs. Thus, physical markers and genetic markers recombine rapidly whenever the two genomes are copackaged into one virion and so are coextant during a single round of reverse transcription. The frequency is highly dependent on the sequence and structure of the RNA in the region undergoing recombination (19). Similar recombination does not occur at high frequency when cells are co-infected simultaneously with two separate virus preparations, suggesting that each virus particle performs its own reverse transcription reaction in the cytoplasm in *cis*, and does not freely exchange RNAs with other reactions happening in the same cell.

Models for Recombination

Two mechanisms can provide for recombination between two genomes. In one, the copy choice model, recombination occurs during minus-strand synthesis. As RT proceeds along an RNA, it has the potential to carry out a template switch in which an incomplete DNA copied from one template serves to prime further elongation on the other RNA molecule (447,561). Pausing may enhance this transfer, and structural features in the RNA may act as hot spots for such recombination. A break in an RNA genome, which may be encountered often, would cause a “forced copy choice”; transfer to the other RNA would rescue an otherwise dead virus, and this may represent the major evolutionary basis for high-frequency recombination in the viruses. The RNase H activity of RT may help release an incomplete DNA and so promote its serving as primer on the new template, and NC may also facilitate the reaction (510).

In the other model, the strand-displacement assimilation model, recombination may occur during plus-strand synthesis.

When multiple plus-strand fragments are elongating on the minus-strand template, strand displacement can expose the 5' end of such fragments. If a second minus-strand DNA has been synthesized, these can then pair with this other minus-strand DNA to form a bridged “H” structure as intermediates; further synthesis and repair would lead to the transfer of sequences to the new DNA (353). These H structures are visible in electron micrographs of the nucleic acids from reverse transcription reactions. However, it is not clear how often this mechanism is used. The general inefficiency of reverse transcription dictates that two minus-strand DNAs may not always be available in one virion to permit this latter process, so it may be infrequent.

When a recombination event occurs, there is a nonrandom increase in the probability that another recombination will occur nearby—a phenomenon called negative interference (20,323). This suggests that RT or the genomes may become recombination prone at specific times. When multiple recombination events occur, the resulting DNA is a patchwork of the sequences derived from the two input RNAs.

The translocation of the strong-stop DNAs provides a special opportunity for recombination between the two viral genomes. When the minus-strand strong-stop DNA is formed, it has the potential to translocate from the 5' end of its template to the 3' end of either RNA molecule; although this event has been reported to occur strictly in *cis*, or strictly in *trans*, it most likely occurs randomly. Similarly, when plus-strand strong-stop DNA is formed, it too could in principle translocate to the 3' of either minus strand. However, this translocation seems most often to occur in *cis*, perhaps simply because the frequency with which two long minus-strand DNAs are formed, and so are available to serve as acceptors, is low.

Recombination between two RNAs during reverse transcription can also occur between nonhomologous sites at lower frequency. Reconstructions suggest that these events are perhaps 100 to 1,000 times less frequent than homologous recombination. These events can result in duplications or deletions in the DNA product of the reaction. Furthermore, if nonviral RNAs or chimeric RNAs containing viral and nonviral sequences are packaged into virions, such nonhomologous recombination events can create new joints and link a viral sequence to the nonviral sequences. These events are thought to play a central role in the process of transduction of cellular genes, and most importantly during the formation of acute oncogenic retroviral genomes (see later).

INTEGRATION OF THE PROVIRAL DNA

The integration of the linear retroviral DNA, like reverse transcription, is a crucial feature of the retroviral life cycle. Integration is required for efficient replication of most retroviruses; mutants that are unable to integrate do not establish a spreading infection. The orderly and efficient integration of viral DNA is also unique to the retroviruses. Although infection by some DNA viruses can result in the integration of viral DNA fragments into the host genome at low efficiency, these events are not the result of specific viral functions. Further, the establishment of the integrated provirus is responsible for much of retroviral biology. It accounts for the ability of the viruses to persist in the infected cell, for their ability to permanently enter the germline, and for the mutagenic and oncogenic activities of the leukemia viruses.

Once the provirus is established, the DNA is permanently incorporated into the genome of the infected cell. There is no mechanism by which it can be efficiently eliminated. At very low frequencies, homologous recombination between the two LTRs can delete most of the provirus, but even here a single (“solo”) LTR remains (736). As the host cell divides, the provirus is transmitted to daughter cells as a new Mendelian locus. Thus, it is likely to persist in the cell for its normal life span, and to convert the cell permanently to a chronic producer of progeny virus.

Unintegrated DNA Forms

The product of the reverse transcription reaction, as outlined earlier, is a full-length double-stranded linear DNA version of the genome, flanked at each end by copies of the LTR. The next step is the movement of the DNA into the nucleus, and the appearance two new DNA forms: closed circular molecules containing either one or two tandem copies of the LTR (Fig. 9). A small amount of the one-LTR circle may be formed during reverse transcription (see preceding), but the bulk is thought to be formed by homologous recombination between the two LTRs of the linear DNA. Little is known about the requirements for the formation of circles, although recently, mutants with alterations in portions of the MuLV Gag p12 protein have been shown to be blocked after synthesis of the linear DNA and before formation of circles (793). The tandem two-LTR circles are apparently formed by the blunt-end ligation of the termini of the linear DNA. This event creates a unique sequence, termed the LTR–LTR junction, that is often used as a hallmark of nuclear entry of the viral DNA. The joints are often imperfect, with loss of nucleotides from one or both termini at the joint (664,758). There are also some circles that arise by autointegration of the ends of the linear DNA into internal sites, forming DNAs with deletions or inversions (658); these circles are generally nonfunctional in terms of generating progeny virus.

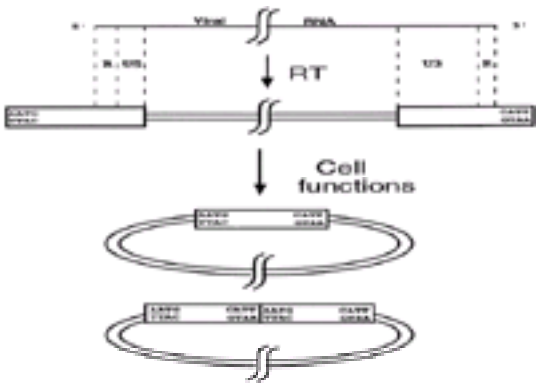

FIG. 9. Unintegrated DNA structures formed after retroviral infection. The incoming RNA genome (*top*) is converted by RT to a double-stranded linear DNA containing two LTRs (*boxes*) in the cytoplasm. The termini of the DNA consist of short inverted repeats and always contain a conserved CA dinucleotide near the 3' ends; the 3' terminal sequences of the

MLVs (CATT) are shown. The linear DNA is then localized to the nucleus, and two circular double-stranded DNAs are formed: a circle containing one LTR, and a circle containing two tandem LTRs. The LTR–LTR junction contains a unique inverted repeat sequence.

Because three distinct unintegrated DNA forms coexist in the nucleus, it was uncertain for many years which form might serve as the precursor for establishment of the integrated provirus. In spite of prejudices based on such precedents as phage lambda, it is now clear that circles are not efficient substrates in the integration reaction (206,427), and that the immediate precursor for the integration reaction is the linear duplex DNA. The circles are apparently dead-end products of a side reaction, formed by host enzymes acting on linear DNAs that have failed to integrate. There are settings and cell types in which unintegrated viral DNAs are observed to accumulate to high levels; various tissues in human HIV disease show considerable circular DNAs. While this DNA may reflect some unusual processing of the DNA, much of it is probably formed simply by massive infection occurring shortly before the DNA is harvested (620).

The unintegrated DNA is not a good substrate for forward transcription (636), perhaps because it is still retained in a complex that is poorly accessible to RNA polymerase. Mutant viruses that cannot integrate are unable to establish an efficient spreading infection, although low levels of virus can be produced (649). A very small subset of cells infected with such integration-defective mutants do integrate viral sequences through nonviral means, creating oligomeric tandem repeats similar to those formed after naked DNA-mediated transformation (294).

Entry into the Nucleus

A key step that must take place before integration can occur is the entry of the viral DNA into the nucleus. The mechanisms of nuclear entry are largely unknown, but there are probably at least two distinct routes used by different retroviruses. The simple retroviruses show a profound requirement for passage through mitosis for successful establishment of the integrated provirus (420,489,624,735), and the block in nondividing cells is at or close to the step of nuclear entry. Tests of the state of the viral DNA in nondividing cells are consistent with the notion that the preintegration complex must await the breakdown of the nuclear membrane to have access to the cellular DNA. Infection of nondividing cells results in the accumulation of linear double-stranded DNA in the cytoplasm, and no further signs of infection. The viral DNA will persist in the cell for some time, and if the cell is stimulated to undergo division, the viral DNA will integrate and infection will proceed. However, the DNA loses its capacity to become activated in this way fairly rapidly (23,489). The restriction is quantitatively very significant, and it profoundly limits the utility of simple retroviral vectors for gene therapy.

In contrast, the lentiviruses and spumaviruses are able to successfully infect nondividing cells, and thus there must be an active transport of the viral DNA through an intact nuclear membrane (100,419,635,752). This capability has made lentiviruses very attractive as gene delivery vectors for gene therapy applications. The molecular basis for this capability is a subject of great controversy. The lentiviral MA and Vpr proteins have been argued as essential for the infection of nondividing cells (257,507,513,743), and the phosphorylation of MA has been argued as necessary to promote dissociation from the membrane and allow nuclear import (99,106,258,259). These findings have not been universally confirmed (247,248,392), however, and the role of MA in these steps is uncertain. Similarly, it has been shown that the Vpr protein is present in the preintegration complex and can bind to nucleoporin components that may mediate nuclear import. However, here too universal support for this model has not been forthcoming. DNA structures present at the second internal copy of the ppt have also been suggested as important for infection of nondividing cells (798). Another attractive model is that the IN protein might be involved in the nuclear import of the complex. IN itself contains nuclear localization signals (257,401), and these can function to target ectopically expressed IN to the nucleus, but it is not clear whether this is related to preintegration complex (PIC) nuclear import or nuclear retention. The foamy viruses may have a distinctive route of nuclear entry involving microtubular transport and centrosomal association, but the mechanism is not well understood (635).

Structure of the Provirus

An important aspect of retroviral integration that distinguishes the process from nonviral or other viral mechanisms of DNA integration is the fact that the insertions create a consistent provirus structure. The integrated provirus is collinear with the product of reverse transcription, and it consists of a 5' LTR, the intervening viral sequences, and a 3' LTR, inserted cleanly into host sequences. The joints between host and viral DNA are always at the same sites, very near the edges of the viral LTRs. Compared to the unintegrated linear DNA, there is a loss of a small number of bps, usually two, from each terminus of the viral DNA; and there is a duplication of a small number of bps of host DNA initially present once at the site of insertion that flank the provirus (Fig. 10). The number of bps duplicated is characteristic of each virus and ranges from four to six.

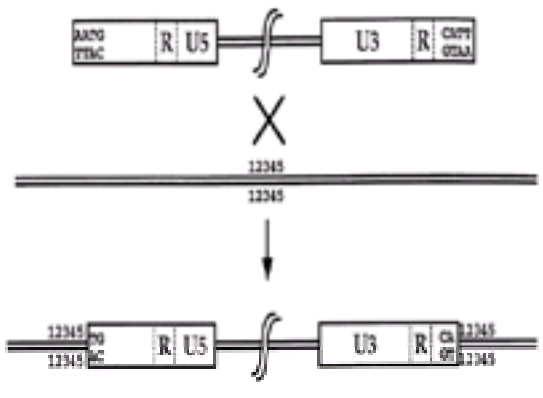

FIG. 10. Integration of the viral DNA to form the provirus. The precursor for the formation of the provirus is a linear double-stranded DNA containing two LTRs (boxes) and with inverted repeat sequences at the termini. The target site in the host DNA is indicated by the arbitrary sequence block denoted 12345. Integration occurs by joining the 38 CA dinucleotides near the termini to the target DNA. The reaction is associated with loss of two bps at the termini of the viral DNA, and with duplication of a small number of bps (five shown here) initially present only once in the target DNA.

Biochemistry of Integration

The actual integration of the viral DNA into a target is mediated *in vivo* by the viral IN protein (540,591,649), which is brought into the cell inside the virion and acts to insert the linear DNA into the host chromosome. Some IN functions have been studied by analysis of viral DNA formed *in vivo* (630). Most of our understanding of IN function, however, has been obtained through analysis of *in vitro* integration reactions, first using complexes extracted from infected cells (96,256), and later using recombinant IN protein. The reaction proceeds in two steps: 3' end processing and strand transfer. A schematic view of these reactions is shown in Figure 11.

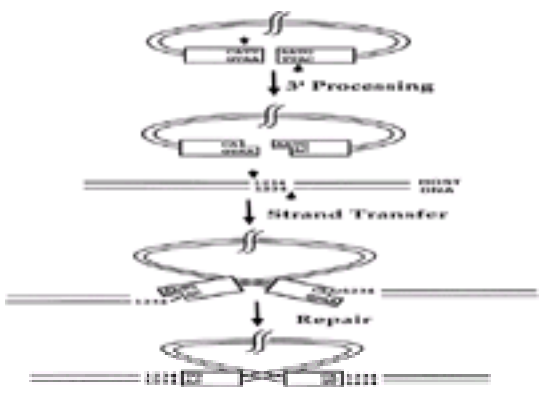

FIG. 11. Steps in the integration of the viral DNA. The full-length linear DNA (*top*) is processed by the viral integrase by the endonucleolytic removal of dinucleotides at the 3' termini. The resulting DNA is then used in a strand transfer reaction in which the 3' OH ends make staggered breaks in the two strands of the target site DNA. The resulting gapped intermediate is presumably repaired by host enzymes.

3'-End Processing

In the first step, the two terminal nucleotides at the 3' ends of the blunt-ended linear DNA are removed by the IN to produce recessed 3' ends and correspondingly protruding 5' ends. This cleavage occurs endonucleolytically at a highly conserved CA sequence, and it releases a dinucleotide. For most viruses, the terminal sequence is such that a TT dinucleotide is released, although this rule has exceptions. The ends do not remain covalently bound to protein, and the energy of the hydrolyzed phosphodiester bond is not retained.

Strand Transfer

In the second step, the 3' OH ends created by processing are used in a strand transfer reaction to attack the phosphodiester bonds of the target DNA (256). The attack occurs by an Sn2-type reaction, with inversion of the phosphorus center as detected with chiral labeling of the phosphate (213). The formation of the new phosphodiester bond between the viral end and the host DNA displaces one of the phosphodiester bonds in the host DNA, leaving a nick. The protruding 5' end of the viral DNA is not joined to the host DNA by IN. The reaction is a direct transesterification, and so no ATP or other energy source is required. Mutational studies strongly suggest that the two activities—processing and joining—utilize the same active site residues. In fact the two steps involve similar chemistry: 3'-end processing is an attack on DNA by a hydroxyl residue of water, while joining is an attack on DNA by a 3' hydroxyl residue of another DNA. It should be noted that other hydroxyl residues can participate; alcohols such as glycerol can be utilized (740), and the 3' OH of a DNA can even attack a phosphodiester bond on the same DNA, forming a cyclic product (213).

Disintegration

The IN protein exhibits a third enzymatic activity *in vitro*: a reversal of the integration reaction known as disintegration (118). This activity releases DNA from a branched structure and seals the nick at the site of the branch. The significance of the activity *in vivo* is uncertain.

Target Site Duplication

In a wild-type virus, when the two ends are joined to the two strands of the target DNA, the two sites of attack are staggered by a few bps. After the joining, the resulting structure contains short gaps in the host DNA and unpaired bases from each 5' end of the viral DNA. The 5' ends of the viral DNA are not joined to host DNA by any known activities of IN. However, the 5' ends are very quickly repaired *in vivo*, almost as quickly as the initial integration reaction (623). These discontinuities are presumed to be repaired by the host repair enzymes, although it is possible that the viral RT or IN

could participate. Indeed, the IN protein has been suggested to manifest a DNA polymerase activity that might fill in the gaps. The processing and filling in the gaps creates a short duplication of sequence that was present only once at the target site; these duplications flank the integrated provirus. The number of bases duplicated is characteristic of each virus. Thus, the murine and feline viruses cause a four-bp duplication; HIV-1 causes a five-bp duplication; and the avian viruses cause a six-bp duplication.

Viral *att* Sites

The sequences at the termini of the viral DNA, the *att* sites, are recognized by the viral IN protein and are important for end processing and joining (103,125,131,739). These terminal sequences are imperfect inverted repeats. The most conserved residues are a CA dinucleotide pair that lies near the 3' terminus and determines the site of 3' processing. Although these bases are absolutely conserved among all the retroviruses, and among many transposable elements, small changes can be tolerated, and mutants can utilize TA or CG relatively well (134). Sequences upstream from the CA for perhaps 10 to 12 bp are needed for efficient integration, but these sequences are different for different viruses, with no indication of broadly conserved sequence motifs. Since the two termini of any given virus are somewhat different, they usually show differential efficiency of utilization in various assays (472). The fact that two distinct ends are bound together in a complex may be important for the concerted integration of these ends into the target (744).

The sequence-specific binding to the *att* site is probably performed by the core domain of IN (367). The nonspecific DNA binding activity of IN has made it difficult to detect sequence-specific binding to these regions, although under some conditions preferential binding to the authentic sequences can be demonstrated (217). A demonstration that an IN mutation can compensate for a mutation in the DNA termini provides nice evidence for the delicate interaction between IN and the DNA termini (188).

Both 3'-processing and strand-transfer reactions are concerted reactions *in vivo*. The processing step occurs simultaneously at both termini of the viral DNA and requires the correct sequences at both termini. Thus, a mutation altering the sequence at one end of the viral DNA blocks the processing reaction at both ends (504). This result suggests strongly that the reaction requires both termini to be loaded into a complex before hydrolysis proceeds. Similarly, the strand transfer reaction normally occurs so that both ends are joined to the target DNA, and at a fixed spacing between the two sites along the DNA helix. The 3'-processing and strand-transfer reactions can both be carried out *in vitro* using native PICs, extracted from recently infected cells, and these reactions reconstruct the concerted nature of the *in vivo* reactions. Alternatively, integration can be performed using artificial DNA constructs and recombinant IN protein. However, these systems typically mediate only a half-reaction—that is, the uncoupled processing of one viral terminus and its joining into a single target DNA. Recent efforts have led to the identification of conditions and factors that can mediate formation of a complex and enhance concerted joining (8,240,745). Once such a protein–nucleic acid complex is formed, it is quite stable.

Structure of the Integrase

The IN protein consists of three distinct domains: an N-terminal region containing an HHCC zinc-finger motif; a central catalytic core containing the so-called D,D-35-E motif; and a less well conserved C-terminal region. The IN protein is a multimer: It readily dimerizes, and at high concentration it forms tetramers as well. All three regions may be involved in the multimerization of IN (21) and in DNA binding (448,503). Many of the residues important for enzymatic activities have been identified by mutagenesis. The most crucial residues for catalysis are the acidic amino acids in the D,D-35-E motif, a highly conserved array of three residues in the core region of many INs and transposases (403). Mutants indicate that both the N-and the C-terminus are also important for function. Surprisingly, pairs of IN mutants with alterations in different regions of the molecule can complement to restore normal function (212,732). The separate N-terminal domain can even complement a nonoverlapping fragment, suggesting that these domains can still coassemble into a functional oligomeric complex (780).

The structures of the HIV-1 and avian virus IN cores have been determined by x-ray crystallography (98,192,275,452), revealing a compact dimer with similarity to prokaryotic recombination enzymes and RNase H (781). The structures of the N- and C-terminal domains have been determined by nuclear magnetic resonance (NMR) (105,199,200,433). The structures can be partially merged into a complete molecule (22), but the models do not yet provide a clear picture of the binding sites for viral or host DNAs or the mechanism of the reaction. Indeed, the likely active sites are not located at positions in the dimeric molecule that could mediate concerted integration of two termini into a single target DNA. Cross-linking studies have helped the development of some models for the interaction of IN with DNA (309). We note that the avian IN is phosphorylated at a carboxyterminal serine (316), but the significance of this modification is not clear.

Preintegration Complex

IN does not normally act alone, and a large complex of proteins and nucleic acid is responsible for mediating the formation of the provirus *in vivo* (87,115,224). The nature and components of the PIC, or intasome, are not known in any detail for either the simple or the complex viruses. The PIC of the simple viruses seems to contain CA, RT, and IN, but other viral proteins may be present (87). Many of these proteins probably stay with the DNA even after entry into the nucleus (617). The PICs of the complex viruses do not contain detectable CA but instead contain MA, NC, Vpr, RT, and IN (492). Thus, the PICs of these viruses may be very different, consistent with their very distinctive ability to infect nondividing cells. Normal PICs apparently contain a large structure covering the two ends of the DNA, and perhaps holding them in proximity. The formation of this structure, detected as a footprint in a modified nuclease sensitivity assay (750), requires both IN and the correct sequences at the termini of the DNA (751).

Host Proteins and Integration

A number of host proteins have been recently identified as potentially involved in the establishment of the provirus. One such protein, Ini1, was recovered in a yeast two-hybrid screen as interacting with the HIV-1 IN protein (356). This protein

binds to IN and can enhance its activity *in vitro*. Ini1 is the human homolog of the yeast Snf5 protein, a component of the SNF/SWI complex involved in chromatin remodeling, and the interaction of IN with this complex raises the possibility of several contributions it may make to the course of integration. Ini1 is not present in the virion but associates with the PIC in the early phase of the life cycle. Another such protein is BAF-1, a low-molecular-weight protein recovered from the MuLV PIC for its ability to inhibit autointegration of the LTR edges into internal sites in the viral DNA (413,414). By inhibiting this reaction, BAF-1 can enhance normal integration into target DNAs in trans. The structure of BAF-1 has been recently determined by NMR, leading to models for its ability to bind and condense DNA (104). Other proteins include the high mobility group (HMG) proteins, important in transcriptional regulation at many promoters and found to dramatically stimulate concerted integration of the two LTRs into a target DNA *in vitro* (311); and HMG I(Y), similarly important in forming protein complexes on promoters, and stimulating both overall and concerted integration *in vitro* (223,421). The viral NC protein also exhibits similar activity (109). The relative importance of these and other proteins in modifying the integration reaction remains uncertain.

Recently, the integration of retroviral DNA has been shown to activate an apoptotic program in cells deficient in the DNA-stimulated protein kinase (DNA-PK), an enzyme implicated in the DNA damage response and required for the proper rearrangement of immunoglobulin gene and T-cell receptor gene segments in lymphocyte precursors (156). Although it is not clear whether DNA-PK plays any direct role in integration, the enzyme must be involved in sensing the products of active IN and responding to the damage. Its absence leads to substantial cell death in cells exposed to an active IN.

Distribution of Integration Sites

An important issue affecting the ability of the retroviruses to create mutations is the distribution of integration sites in the host genome. Proviruses are probably inserted at nearly random locations in the genome, with no or little sequence specificity, and thus have the opportunity to create mutations in any gene. Various studies, however, have suggested some deviations from a completely random distribution. Some potential targets for insertional mutagenesis seem to be less often disrupted after infection than predicted for truly random distribution of integration sites (383), suggesting that there may be “cold spots.” There may be some modest preference for transcriptionally active genes (498,643), for DNase I hypersensitive sites (625,738), or for open or accessible chromatin, but other studies have not detected such biases. Similarly, there have been reports of highly preferred integration sites or hot spots (655), but these observations have not been reproducibly confirmed (770). DNA in chromatin, such as simian virus 40 (SV40) DNA covered with nucleosomes, is a good substrate for integration *in vitro* (584), so nucleosomes probably do not need to be removed to allow efficient targeting; the presence of nucleosomes can, however, induce a 10-bp periodicity onto the pattern of integration, presumably reflecting the accessibility of the outside of the DNA helix to the PIC. DNA bound by other more sequence-specific binding proteins can be protected from integration (586). Heterochromatic satellite repeat DNAs seem to be disfavored sites of integration (110). The sites that are selected can have profound position effects on the ability of the provirus to be expressed, with some sites silent and some expressed at high levels, but on average the transcription of proviruses arising by natural infection is more efficient than when the same DNA is introduced artificially by transfection (331).

EXPRESSION OF VIRAL RNAs

The integration of the provirus signals a dramatic change in the life style of the retroviruses: It marks the end of the early phase of the life cycle and the beginning of the late phase. The early phase is driven by viral enzymes performing abnormal events such as reverse transcription and DNA integration, whereas the late phase is mediated by host enzymes performing such relatively normal processes as transcription and translation. This late phase of gene expression may begin immediately with the synthesis of viral RNAs and proteins, and the assembly of progeny virions (see Fig. 12 for an overview). For many viruses, the transcriptional promoters that drive this expression are constitutively active and cause the production of virions in a relatively unregulated way. In other viruses, the activity of the promoter may be regulated, either by viral or host factors. This section will review the basic phenomena of proviral gene expression and mention briefly the regulation exhibited by the complex retroviruses.

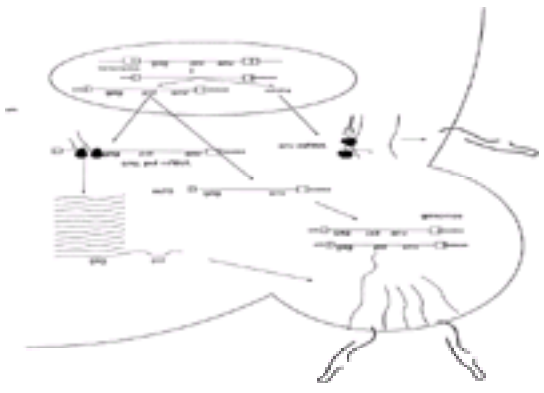

FIG. 12. The late stages of the retroviral life cycle. The integrated provirus is used as the template (*top*) for the expression of viral RNAs. A subset of the transcripts are spliced, and the unspliced and spliced mRNAs are exported to the cytoplasm. The unspliced RNA is used to make Gag and Gag-Pol proteins and also serves as the genome; spliced mRNA is used to make Env proteins. The proteins and RNA associate under the membrane to form the budding progeny virion.

Overview of Viral RNA Synthesis

The synthesis of viral RNA from the viral DNA leads to the formation of a long primary transcript, which is then processed and may be spliced to form a small number of stable transcripts. The U3 region of the LTR contains a promoter recognized by the RNA polymerase II system, and these sequences direct the initiation of transcription starting at the U3-R border. Cellular machinery then caps the 5' end of the RNA with $_{m7}$G5'ppp5'G$_{m}$p. The first G residue after the cap is a templated base in the provirus. Transcription proceeds through the genome, and continues through the 3' LTR and into the downstream flanking host DNA. Finally, the RNA is cleaved and polyadenylated at the R-U5 border of the 3' LTR, generating a complete, unspliced viral genomic RNA suitable for incorporation into the virion particle. Most genomes contain an AAUAAA sequence acting as the signal for this 3' processing. The sequence normally lies in the R region (159), but the complete sequence needed for recognition can be complex, lying upstream or downstream (78,171,730), and it may even be discontinuous, brought together by RNA folding to create the functional signal (6,46). The exact site of polyadenylation is not critical for virus replication; mutants in which the polyadenylation signal is inactivated generate longer RNAs that extend into downstream flanking sequences (800), and these RNAs are quite efficiently able to mediate normal replication (689). A subset of the RNA is spliced to give rise to one or more subgenomic RNAs. The patterns of spliced mRNAs can be simple or exceedingly complex. Both the unspliced and the spliced RNAs are then exported from the nucleus for translation.

Initiation of Transcription

The efficiency of initiation of transcription at the 5' LTR is the major determinant of the levels of viral RNA formed in the cell. The promoter in the LTR is typically a very potent one, and the levels of viral RNA are often constitutively high. However, the cell type, the physiologic state, and the integration site (10,229) can all result in substantial variation in the efficiency of transcription. In some viruses, the promoter is not constitutively active but depends on the activity of specific transcription factors such as the glucocorticoid receptors.

Positive Regulatory Elements in U3

The transcriptional elements in the U3 region of the simple viruses contain both core promoter sequences and enhancers. The core promoters contain a TATA box, bound by TFIIB; a CCAAT box, bound by CEBP (633); and sometimes an initiator sequence near the U3-R border. The U3 regions of even closely related retroviruses are very diverse and can evolve rapidly during viral replication. The enhancers are similar to those found in many host promoters, in that they contain multiple short sequence motifs, arranged in very close packing; often there are tandemly repeated copies of some of these motifs. These short sequences are the binding sites for a large number of host factors that regulate transcription (e.g., see ref. 674). Different cells and cell types will make use of distinct arrays of these factors to mediate transcription from a given viral LTR (284,287,505,606). The factors are not simply additive but may interact in complex ways on particular viral sequences.

Examples of the identity and distribution of known binding motifs in a few LTRs are shown in Figure 13. A partial list of these factors used by various retrovirus LTRs includes Sp1; USF-1; the Ets family of factors, which includes more than 20 members in vertebrates; the core-binding factor (CBF), consisting of an a-b heterodimer; nuclear factor 1 (NF1); and a mammalian type C retrovirus enhancer factor (MCREF-1). Specific viruses often contain recognition sites for other, more specific positive regulatory factors. Major examples of such factors include the glucocorticoid receptors, driving expression of the MMTV genome (25,140), and to a much lesser extent, other MuLVs; NF-kB, important for expression from the HIV-1 LTR in certain cell types (122,628); the GATA factors for Cas-BR-E and other viruses (47); and the myb protein (161). Evidence has recently been obtained that the STAT factors, DNA binding proteins normally activated the Janus kinases (Jaks) may also be important for MMTV transcription (589).

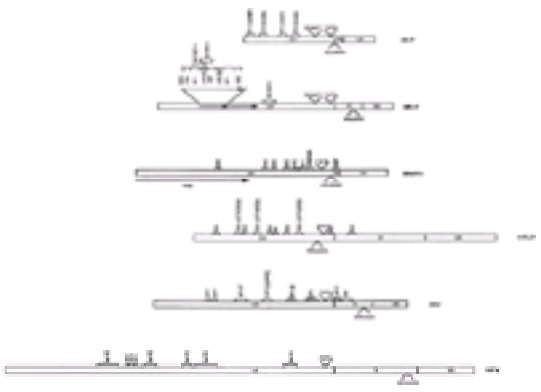

FIG. 13. Structures of the LTR regions of various representative retroviruses. The U3, R, and U5 sequence blocks are indicated for each virus. The binding sites for several of the most important transcription factors are indicated above the LTRs, and the signals for polyadenylation are indicated below. The imperfect direct repeats in the MLV LTR are indicated by the *arrows.* The open reading frame (ORF) for the *sag* gene of MMTV is indicated by the *bold arrow*.

Negative Regulatory Elements

A number of negative regulatory factors that reduce viral expression have been identified. Embryonic carcinoma cells, and true embryonic cells, are the best-characterized examples of cell types that strongly represses LTR-mediated transcription through expression of negative regulatory proteins (237,373,737). These factors include a cellular embryonal LTR-binding protein (ELP) (721,722); and a stem-cell specific repressor that binds, curiously, to the tRNA pbs (373,435,436,567,778). Viruses that lack the recognition sites for these proteins can escape the repression (310). Other

negative factors include one known variously as UCRBP, NF-E1, or YY1 (241); and others less well characterized (111).

Trans-acting Viral Regulatory Factors

The complex retroviruses encode an array of small regulatory proteins that can activate transcription from the viral LTR in trans. Examples of these transactivators include the HTLV-1 Tax protein (168) and the HIV-1 Tat protein (149). The Tax protein acts in concert with a complex of host proteins, the activating transcription factor/CRE-binding protein (ATF/CREB), and binds to three cAMP response elements in the viral LTR. Tax thus sets up a positive feedback loop that results in high levels of viral transcripts. The Tat protein is unusual among transcriptional activators in that it binds to a structure in the 5' end of nascent viral RNA (175, 652), rather than to DNA. Tat binds to a bulged hairpin structure, the TAR element, and recruits a pair of host proteins, cyclinT/cdk9, to the RNA. These proteins enhance the ability of RNA polymerase to elongate beyond the LTR and down the genome with high processivity, probably by phosphorylation of the C-terminal repeat domain (CTD) of the polymerase. Again, the result is a strong positive feedback loop that results in high levels of viral RNA. (For more detailed discussion of *tat* function, see Chapter 59 and refs. 149 and 150).

Beginning and Ending the RNA

Because there are two identical LTRs in the provirus, it is possible that transcription can be initiated at both the 5' LTR and the 3' LTR. However, the 5' LTR is generally much more efficiently utilized than the 3' LTR (306). One possible mechanism is promoter interference, in which the upstream promoter being active suppresses the utilization of the downstream promoter (151). It is possible that elements near the 3' LTR may restrict use of the downstream LTR, so that generally transcripts initiating at the 5' LTR predominate. These restraints may be lost in tumors, in which transcription from the 3' LTR can be significantly enhanced (76). Similarly, since there are two LTRs, transcripts might be subject to 3'-end processing at either the 5' LTR or the 3' LTR. For those viruses with a long R region, there are signals for RNA cleavage and polyadenylation at both ends of the RNA, and so these events could occur at both 5' and 3' sites. However, it is likely that sequences near the 5' end of the RNA (or the lack of sequences) restrict processing at the 5' LTR. The RNA that begins at the 5' LTR is so short when the site for cleavage is reached (the length of the R region) that structural constraints may limit its recognition by the processing enzymes (337). Finally, there may be signals promoting this recognition that are present only at the 3' sequences. Thus, most of the RNAs formed extend from the 5' LTR to the 3' LTR (306). There is at least one exception to this rule: The foamy viruses include a second internal promoter required for the formation of the Tas and Bet regulatory proteins (429, 430, 482). The separate promoter for formation of these proteins presumably reflects a need for their temporally ordered and regulated synthesis.

RNA Processing

The full-length transcript of the retroviral genome is directed into several pathways. A portion of the transcripts is exported directly from the nucleus and serves as the genome to be packaged into the progeny virion particle, assembling either at the plasma membrane or in the cytoplasm. Another portion with identical structure is also exported and used for translation to form the Gag and Gag-Pol polyproteins. It is not yet clear if these two subsets are truly distinct, whether there can be interchange between the pools, or whether there is a single pool of such molecules used for both purposes. It has been proposed that the translated mRNAs are selectively packaged in *cis* (370). A third portion is spliced to yield subgenomic mRNAs. For the simple retroviruses, there is a single spliced mRNA encoding the Env glycoprotein. For the complex viruses, there can be multiple alternatively spliced mRNAs, encoding both Env and an array of auxiliary proteins (150, 619). Examples of the complicated array of mRNAs that are formed for both simple and complex viruses are shown in Figure 14. The protein products of these multiply spliced mRNAs will be discussed in the following chapters.

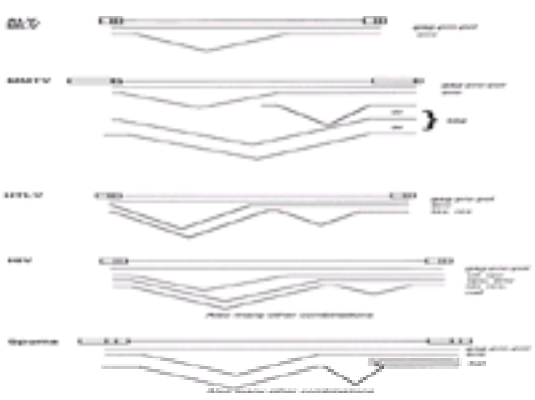

FIG. 14. Splicing patterns of representative retroviral RNAs. All retroviruses direct the synthesis of an unspliced RNA transcript, as well as a variable array of subgenomic mRNAs. Examples of the splicing patterns of the mRNAs of various retroviruses are show. The complex viruses such as HIV-1 also encode a larger array of mRNAs containing various combinations of exons.

The splicing and subsequent export from the nucleus of only a portion of an initially transcribed RNA is an extraordinary process; normally, splicing goes to completion, and only then is the mRNA exported. The export of a precursor mRNA is prevented until splicing is complete. At least three aspects of the retroviral genome may promote the export of unspliced mRNAs. First, the splice sites of the viral RNA may have quite poor overall efficiency of utilization by the splicing machinery in the cell (365). Indeed, the sequences at the splice donor and acceptor regions are often poor matches to the consensus sequences for splice sites, and mutations that make the sites better matches increase splicing and are actually deleterious to virus replication (31, 364). These mutations can be suppressed by secondary mutations that reduce splicing efficiency. The overall folding of the RNA may affect the efficiency of splicing, and thus sequences at

some distance, as in the *gag* gene, may modulate splicing (32,680).

Second, studies of ASLV have identified specific sequences that act as negative regulators of splicing (NRS) through their interaction with host factors (17,137,480,481). These elements can be important for the expression of transduced genes in some viruses (666). Similar signals may exist in other viruses; mutations in the Gag region of MuLV can affect RNA processing in complex ways (27).

In addition, unspliced mRNAs contain *cis*-acting elements that promote the export of the RNA out of the nucleus, the so-called constitutive export elements (CTEs) (92). These sequences are located near the 3' end of the genomic RNA of MPMV (216), between *env* and the LTR, and possibly in similar regions of ASLV (520). CTE function of the MuLVs may be present in the Gag region (382), or possibly in the R region of the LTR (719). The CTE is recognized by one or more host proteins that assemble a complex onto the RNA to mediate its export, although which proteins are most important is controversial (293,552,703). In the complex viruses, RNA export is regulated through complex interactions of both *trans*-acting proteins, the Rex or Rev gene products, and *cis*-acting sites, the Rev-responsive elements (RREs) that promote RNA export and the *cis*-acting repressive sequences (CRS) elements that prevent it (127,152,154,173,208,230,313,455,456 and 457). A key aspect of this process is the recruitment of Crm1, a cellular nuclear export factor, to the RNA (see Chapter 59 for detailed discussion of the mechanism of Rev action). The Rev/RRE pathways of export are probably distinct from those used by the CTEs, because the two are differentially sensitive to a competitive block acting through Crm1 (77,634).

Viral RNAs are subject to other modifications common to cellular mRNAs. Like cellular mRNAs, the N6 position on specific A residues can be methylated (357), and other sites can be modified by double-stranded RNA adenosine deaminase (295). The significance of these modifications is uncertain.

TRANSLATION AND PROTEIN PROCESSING

All retroviral genomes, at a minimum, contain ORFs designated the *gag*, *pro*, *pol*, and *env* genes. These genes are expressed by complex mechanisms to form precursor proteins, which are then processed during and after virion assembly to form the mature, infectious virus particle. The expression of the various proteins as large precursors that are subsequently cleaved provides several advantages: It allows for many proteins to be made from one ORF, it ensures that the proteins are made at proper ratios, and it allows many proteins to be targeted to the virion during assembly as a single entity. The *gag*, *pro*, and *pol* genes are expressed in a complex way from the full-length unspliced mRNA. The arrangement of these genes, and especially the way *pro* is expressed, are different in different viruses. A summary of the arrangement of the ORFs of various viruses is shown in Figure 15.

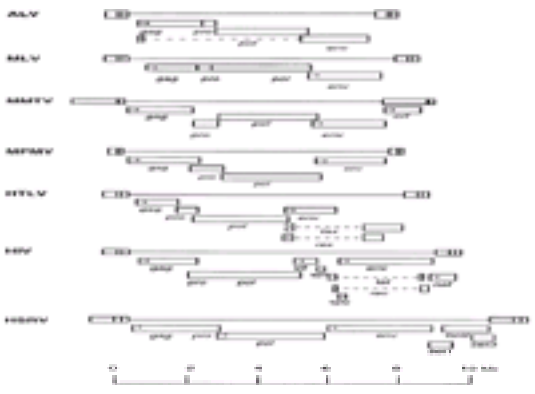

FIG. 15. Arrangements of the ORFs encoded by various retroviruses. The major ORFs of each virus are indicated by the *open boxes.* ORFs in the same reading frame are in the same line, and ORFs in different frames are on different lines. Translational starts are indicated by the *small arrows.* Spliced introns are indicated by the *dashed lines*.

Gag Gene Expression

The *gag* gene is present at the 5' proximal position on all retroviral genomes. A full-length mRNA, identical in sequence to the genomic RNA, is translated in the cytoplasm to form a Gag precursor protein, in the 50- to 80-kd range. Translation begins with an AUG initiator codon and proceeds to a terminator codon at the 3' end of the ORF. The viral RNA typically contains a relatively long 5' untranslated region (UTR), and it has been uncertain whether ribosomes could scan from the 5' cap to the start codon for Gag translation. These 5' RNA sequences are predicted to contain stable secondary structures that would inhibit scanning. Furthermore, the long 5' UTRs often contain AUG codons in contexts that are favorable for translation, that are not in frame with the *gag* ORF, and that presumably would inhibit successful translation of Gag (178). Recent experiments suggest that for the MuLVs, an internal ribosome entry site (IRES) is present near the start of the *gag* ORF and is used to initiate translation in a cap-independent mechanism (63,729). Thus, at least in these viruses, ribosomes can bind directly near the *gag* gene and do not need to scan the mRNA. However, other viruses, such as HIV-1, may not use such sequences (487).

Some retroviruses encode an additional Gag protein besides the major product. This Gag protein is longer than the major product and derives from translational initiation at a nonconventional CUG codon upstream from the initiating AUG codon. Translation beginning at this codon first forms an N-terminal leader sequence and then proceeds in the same reading frame through the normal AUG and the rest of the Gag protein. Thus, where the proteins overlap, their sequences are identical. The leader sequence contains a functional signal peptide directing the translation machinery to the ER, and specifying that the Gag protein be cotranslationally inserted into the secretory pathway. The Gag become glycosylated at several sites, is transported via the Golgi to the cell surface, and persists for some time as a

membrane-bound glycoprotein, with the carboxyl-terminal domain exposed on the cell surface (570). The protein is processed into several fragments and has a relatively short half-life. The function of the protein is uncertain. It is not required for virus replication, and mutant viruses lacking the protein show no gross defects (648). The glycosylated Gag is a prominent immunogen in infected animals, however, and it may thereby or in some other way modulate the replication and the leukemogenicity of the viruses (139). The neuropathogenesis associated with some of the MuLVs may depend on sequences in glycosylated Gag (255,580).

The major Gag product is often modified by the addition of myristic acid, a relatively rare 14-carbon fatty acid, to the penultimate aminoterminal residue, a glycine (304). The addition is mediated by a myristyl CoA transferase that cotranslationally transfers myristate from a myristyl CoA donor to the amino group of the glycine residue, forming an amide bond. The fatty acid is important for the membrane localization and binding of the Gag precursor, increasing the hydrophobicity of the aminoterminal domain. Mutant Gags in which the glycine is altered are not modified, and these Gags do not associate with membrane properly and do not aggregate to form virions (97,285,599). It should be noted that although myristate is important, it is not sufficient for membrane targeting; hydrophobic residues in the MA domain are also required. Furthermore, basic residues further downstream in the MA of some viruses form a patch of positive charge that may interact with negatively charged phospholipids in the membrane.

An aminoterminal myristate is not found on the Gags of bovine immunodeficiency-like virus (BIV), equine infectious anemia virus (EIAV), visna, or ASLV. For the avian retroviruses, the aminoterminus is not myristylated but rather acetylated. The Gag protein of these viruses is apparently sufficiently hydrophobic to be targeted to the membrane without the fatty acid in avian cells, although, curiously, not in mammalian cells. Alteration of the avian Gag to allow its myristylation permits virion assembly in mammalian cells (765) and does not block its function in avian cells (215).

Pro Gene Expression

The relative position of the *pro* gene on retroviral genomes is always similar—in between *gag* and *pol*. However, the *pro* gene is expressed in very different ways in different viruses. Sometimes it is fused in frame onto the 3' end of *gag*, sometimes it is fused to the 5' end of *pol*, and sometimes it is present as a separate reading frame. These various patterns have led to considerable confusion in the literature; sometimes *pro* is considered a portion of *gag*, or sometimes of *pol*. Because of these different patterns of expression, it is best to consider this ORF as a separate gene.

The various arrangements of the *pro* gene and its mode of expression are as follows. For the alpharetroviruses, *gag* and *pro* are fused and expressed as a single protein; *pol* is in a different reading frame, and a frameshift is used to express the Gag-Pro-Pol polyprotein. For the beta- and deltaretroviruses, *gag*, *pro*, and *pol* are all in different frames, and successive frameshifts are used to express Gag-Pro and Gag-Pro-Pol polyproteins. For the gamma- and epsilonretroviruses, *gag* and a *pro-pol* fusion are in the same reading frame and separated by a stop codon, and translational readthrough is used to make Gag-Pro-Pol. For the lentiviruses, *gag* and a *pro-pol* fusion are in different reading frames, and frameshifting is used to make Gag-Pro-Pol. Finally, for the spumaviruses, *pro* is fused to *pol*, and the Pro-Pol protein is expressed without Gag, from a spliced mRNA. More will be said about these varied mechanisms of expression in the following section.

Pol Gene Expression

The *pol* gene encodes several proteins needed at lower levels for the replication of the virus, including the RT and IN enzymes. The *pol* ORF is not expressed as a separate protein in most retroviruses, but rather is expressed as a part of a larger Gag-Pro-Pol fusion protein. The Gag-Pro-Pol protein must be made at the correct abundance, in proportion to the amount of Gag protein, for efficient assembly of infectious virus; expression of only Gag-Pro-Pol does not result in virion assembly (231,548). The formation of this protein is mediated by either one of two mechanisms, depending on the virus.

Translational Readthrough

In the gamma- and epsilonretroviruses, the Gag and Pro-Pol ORFs are in the same reading frame and are separated by a single UAG stop codon at the boundary between Gag and Pro-Pol. The translation of Gag-Pro-Pol in these viruses occurs by translational readthrough—that is, by suppression of termination—at the UAG stop codon (786). Most of the time, translation of the RNA results simply in the formation of the Gag protein. But approximately 5% to 10% of the time, ribosomes translating the RNA do not terminate at the UAG codon but instead utilize a normal aminoacyl tRNA, usually a glutamine tRNA, to insert an amino acid at the position of the stop codon. Translation then continues, in frame, through the entire long *pro-pol* ORF, resulting in the formation of a long Gag-Pro-Pol precursor protein. The high-level suppression of termination is specified by a specific structure in the RNA immediately downstream of the UAG stop codon (314,539). The precise features of this structure that are required for suppression are not completely known, but they include a purine-rich sequence immediately downstream of the stop codon, and a pseudoknot formed from the next 60 or so nucleotides (11,232,236,273,766,767). The structure may slow translation, and it may also in some other way alter the balance between termination, which requires binding of termination factors eRF-1 and eRF-3 by the ribosome, as opposed to incorporation of an amino acid, which requires misreading of the codon by an aminoacyl tRNA. No changes in the tRNA pool occur during infection (234). The signals in the RNA can operate to mediate suppression of both UAA and UGA termination codons as well (235).

Translational Frameshifting

In the alpharetroviruses and lentiviruses, the *gag* and *pol* ORFs lie in different reading frames, and the formation of the Gag-Pro-Pol fusion is mediated by a translational frameshift mechanism (340). Most of the time, translation again results in the simple formation of the Gag protein. But approximately 10% of the time, as the translation approaches a specific site near the end of the *gag* ORF, the ribosome slips back one nucleotide (a –1 frameshift) and proceeds onward in the new reading frame. The ribosome passes through the stop codon out of frame and so continues to synthesize protein using the codons of the *pol* ORF. As for readthrough, the determinants of frameshifting lie in the RNA sequence and

structure near the site of the event (338,462,550,769). The requirements for frameshifting include a “slippery site,” a string of homopolymeric bases where the frameshift occurs; these are oligo-U or oligo-A in different viruses. In addition, the frameshifting requires either a very large and near-perfect hairpin or stem-loop structure; or a large pseudoknot structure, similar to those used in readthrough, although apparently containing a distinctive bend at the junction of the two paired sequences (117,187,358,653). As for readthrough, the proper frame-shifting efficiency is crucial for normal virus replication.

In the betaretroviruses (e.g., MMTV) and deltaretroviruses (e.g., BLV, HTLV-1), the *pro* gene is present as a separate ORF, in a different reading frame from that of *gag* or *pol*. Two successive frameshifts are utilized to make the long Gag-Pro-Pol fusion protein (339,497,508). Near the 3' end of the *gag* ORF, ribosomes carry out a first (–1) frameshift and continue into the pro ORF; near the 3' end of the pro ORF, they perform a second (–1) frameshift and continue on into the *pol* ORF. These two frameshifts occur at extremely high frequencies—as much as 30% of the time that the ribosome transits through each site—so that the overall frequency of formation of the Gag-Pro-Pol protein is perhaps 10% that of formation of ag.

Separate Pol Expression

The spumaviruses are unique among the retroviruses in that the synthesis of the Pol protein is not mediated by the formation of a Gag-Pol fusion protein. Instead, a separate subgenomic spliced mRNA (352) is translated directly to form a separate Pro-Pol protein (214,428). This protein must be directed to the assembling virion by distinct domains rather than by the Gag portion of a Gag-Pol fusion.

Env Gene Expression

In all retroviruses, the *env* gene is expressed from a distinct subgenomic mRNA. The *env* message is a singly spliced mRNA, in which a 5' leader is joined to the coding region of *env*. Thus, the bulk of the *gag* and *pol* genes are removed as an intron from the mRNA. The resulting message is exported to the cytoplasm and translated from a conventional AUG initiator codon. In the alpharetroviruses, the AUG is actually the same one used for Gag translation; it lies in the leader, and the splicing brings this AUG and the first six codons into frame with the *env* coding region. The first translated amino acids constitute a hydrophobic signal peptide, and they direct the nascent protein to the rough ER. The leader is removed by a cellular protease (the signal protease) in the ER, and the protein is heavily glycosylated by transfer of oligosaccharide from a dolichol carrier to asparagine residues on Env. These residues lie in the conventional Asn-X-Ser/Thr motifs recognized by the modification enzymes. Near the end of the cotranslational insertion of Env into the ER, a highly hydrophobic sequence acts as a stop transfer signal to anchor the protein in the membrane. The remaining C-terminal portion of the protein stays on the cytoplasmic side of the membrane.

Before the Env proteins are transported to the cell surface, they are folded and oligomerized in the ER. The formation of oligomers is required for stable expression of the protein, and it is sensitive to overall conformation; many mutants of Env show defects in oligomerization (723). There is considerable controversy about the oligomeric state of the Env protein in different viruses and at different times during their transport (193,201,202,573,753). The most-studied envelope proteins (ASLV and HIV-1) may pass through dimeric or tetrameric intermediates, but the nature of these intermediates is not clear. Although some laboratories disagree (see e.g., refs. 193,479,642, and 713), ultimately these envelopes probably form trimers in the mature virus (202,441). The folding of the protein is presumably catalyzed by chaperone proteins in the ER, and the formation of disulfide bonds between various pairs of cysteine residues is similarly catalyzed by disulfide interchange enzymes.

The Env protein is then exported to the Golgi, and it is cleaved by furin proteases to form the separate SU and TM subunits. This cleavage is essential for the normal function of the Env protein. The cleavage occurs at a dibasic pair of amino acids (177), and it produces a hydrophobic N-terminus for the TM protein that is required to mediate fusion of the viral and host membranes during virus entry. In the Golgi, the sugar residues are modified by the sequential removal of mannose residues and addition of N-acetyl glucosamine and other sugars to many of the oligosaccharide. O-linked glycosylation and sulfation of Env glycoproteins have also been documented (572,656). The pathway by which Env is transported to the cell surface is not fully understood, but presumably host vesicular transport systems are utilized. There is evidence that clathrin adaptor complexes interact with the cytoplasmic tail of Env and direct its movement to the plasma membrane (64). The protein typically becomes a prominent cell surface protein on the infected cell.

In polarized epithelial cells, Env proteins are often restricted to the basolateral surface of the cell (534). This localization is mediated by a tyrosine-based motif, Yxxp, present in the cytoplasmic tail of Env (x, any amino acid; p, hydrophobic residue) (432,535). Remarkably, this targeting of Env can redirect the budding of Gag proteins to this surface.

Other Viral Gene Products

The complex retroviruses express a number of small proteins with a range of functions. The proteins are translated from subgenomic mRNAs, usually resulting from multiple splicing events that join a 5' LTR to a number of small exons encoding the protein. These gene products will be discussed in greater detail in later chapters, but a short summary follows (see refs. 149,150,290,485, and 577 for reviews).

Deltaretroviruses

Tax

The Tax gene product is a positive regulator of transcription from the viral LTR. Tax functions in association with the ATF/CREB by binding to three cAMP response elements in the viral LTR. Tax also plays a role in transformation, perhaps through E2F-1 activation or through effects on the cell cycle.

Rex

The Rex gene product facilitates the export of unspliced and singly spliced viral mRNAs from the nucleus. Its action is probably similar to that of the lentiviral Rev protein.

Epsilonretroviruses

ORF A

The ORF A product of the piscine retroviruses is a cyclin D homolog that functions as a cyclin in yeast (410). The function of the protein in virus replication or tumor formation is uncertain.

ORFs b and c

The function of these ORFs is unknown.

Lentiviruses

Tat

The Tat protein is a potent transactivator of transcription from the viral LTR. The protein acts by binding to a hairpin structure, the TAR element, encoded in the R region of nascent viral RNA, and recruiting host factors cyclinT and Cdk9 to the RNA. Tat does not increase the rate of RNA polymerase II initiation but seems to enhance its processivity or elongation, perhaps by phosphorylation of the CTD of pol II.

Rev

The Rev protein mediates the export of the unspliced and singly spliced viral RNAs from the nucleus, thus permitting the expression of the Gag, Pol, and Env gene products. Rev binds to the RRE present in the HIV-1 *env* gene, and by interacting with the importin Crm1, it acts to export the viral RNAs through the nuclear pore.

Nef

The Nef protein is a multifunctional protein not essential for replication in some cells in culture, but important for replication *in vivo*. Nef-defective viruses do not induce high-level viremia in infected animals, and progression to disease is delayed or prevented (376). Nef down-regulates the CD4 receptor from the cell surface, facilitating virus release, probably by bridging CD4 to adapter proteins (APs) (569). Nef also down-regulates MHC class I levels, thereby inhibiting the cytotoxic T-lymphocyte–mediated lysis of HIV-1–infected cells. Finally, Nef may enhance virion assembly and release through unknown mechanisms.

Vpr

The Vpr protein, as noted later, is packaged at high levels into virion particles through an interaction with the p6 domain of Gag (39,651). Vpr may facilitate the import of the PIC into the nucleus in nondividing cells. Vpr also causes a strong cell cycle arrest in the G2 stage of the cell cycle, perhaps through an indirect inhibition of Cdc25 phosphatase activity.

Vif

The Vif protein is expressed at high levels in the cytoplasm, and it is packaged into virion particles of both homologous and heterologous viruses. Vif enhances infectivity of virus produced in certain nonpermissive cells, perhaps by blocking the action of a cell-specific inhibitor.

Vpu

The Vpu gene product, found only in HIV-1, is a membrane protein that enhances virion production and also mediates the degradation of CD4 by the ubiquitin-conjugating pathway (644,762).

Spumaviruses

Tas

The Tas (or Bel1) protein is a transactivator of transcription from the viral LTR, acting at sequences near the 5' end of the genome. Its mechanism of action may be similar to that of the lentiviral Tat protein.

Bet, Bel2, and Bel3

The functions of the *bet* (and the overlapping *bel2*) and the *bel3* genes are unknown, although there is some evidence that the Bel3 protein may be a negative regulator of replication.

VIRION ASSEMBLY

As the Gag, Gag-Pro-Pol, and Env proteins are synthesized, they come together to form progeny virions (reviewed in

refs. 246,330,637,691, and 764). The assembly of the retrovirus particle is driven primarily by the Gag precursor protein. Gag is required for the formation of a virion, and indeed it is sufficient to mediate the assembly and release of a “bald” particle—lacking infectivity and the “hair” of the Env protein. The Gag protein that is responsible for assembly is the uncleaved Gag precursor. This form of the protein is thus targeted for assembly and export—the “way out” of the cell. Once the Gag proteins are processed, changes in virion structure can occur to promote entry—the “way in” to the next cell.

There are two major routes by which the various retroviruses assemble their virions.

Assembly of C-type Virions

For most of the retroviruses, those with the C-type morphology, assembly occurs at the plasma membrane; in these cases, the Gag precursor protein is targeted to the cytoplasmic face of the plasma membrane by hydrophobic sequences, by basic residues, and sometimes by a myristic acid moiety (304) present at the aminoterminus. It is not clear if monomeric or dimeric or higher-order structures of Gag are transported to the membrane to begin assembly. The Gag proteins then aggregate, presumably by side-to-side contacts, and create a patch under the membrane. As the patch of protein grows, curvature is induced in the membrane, causing the nascent virus to bud outward. The bud eventually grows to a complete sphere, attached to the cell by a narrow stalk. The stalk is then pinched off, the virion is released, and the host membrane is sealed. The various steps are depicted in Figure 16.

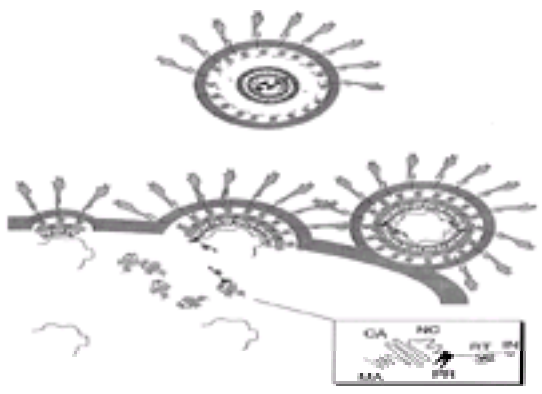

FIG. 16. A schematic diagram of the process of virion assembly. The Gag precursor, containing the MA, CA, and NC domains, and the Gag-Pol precursor, containing the MA, CA, NC, PR, RT, and IN domains (see *blowup*), are transported to the inner leaflet of the plasma membrane. The proteins bind the viral genomic RNA (*thin line*). Curvature is induced in the membrane as the virion grows, and the roughly spherical particle is finally pinched off and released from the cell. The virion proteins are reorganized upon processing by the viral protease to form the mature, infectious virus (*top*).

Assembly of B- and D-type Virions

In the alternative lifestyle of the viruses with B- and D-type morphology, the betaretroviruses and the spumaviruses assemble in the cytoplasm and are subsequently transported to the plasma membrane for envelopment and release (610). These two pathways would seem relatively distinct, and one might suppose that the two groups of viruses would have evolved very different requirements for assembly, and that the details of the Gag-Gag interactions would be different. But these two mechanisms are not so far apart. Indeed, a single amino acid substitution in the MA protein of MPMV can change the morphogenetic pathway of the virus from a cytoplasmic site of assembly to a membrane site of assembly (612). Thus, the main difference may be the timing of exposure of determinants for membrane transport: In the C-types, such a determinant might be constitutively available, whereas for the B- and D-types, the determinant may not be exposed until assembly occurs. For both mechanisms, the nascent virions consist of a spherical particle surrounded by a lipid bilayer, which is pinched off from the cell and then released into the extracellular space.

Assembly of Intracisternal A-type Particles

There is a third route by which retroviral-like particles can assemble, although it is not known to be important for any infectious agent. The endogenous retroviral elements known as IAPs direct the synthesis of virus-like particles with an immature morphology in the cytoplasm of cells expressing these elements. The particles are budded into the lumen of the ER and are not released efficiently outside the cell. These steps may be most closely related to the formation of hepatitis B virions.

Gag and Virion Assembly

For most retroviruses, the expression of the Gag precursor is sufficient to mediate virion assembly and release, earning the protein the name, the particle-making machine. [An exception to this rule is the foamy viruses, which also require the presence of the Env glycoprotein for efficient budding (568).] Because of its central role in virion assembly, the Gag proteins have been subjected to intense mutational analyses to define the domains required for various steps in the process (296,764). Surprisingly, small portions of Gag, containing only a few critical regions, can still assemble virions (746). Three domains seem to be crucial: a membrane-binding (M) domain; an interaction (I) domain; and a late assembly (L) domain. It is important to remember that the form of the Gag protein that is mediating assembly is the precursor; thus, the assembly domains need not lie neatly within any of the cleavage products that form later, and they can span cleavage sites.

The M Domain

The M domain, or membrane-binding domain, ranging from 30 to 90 residues in length, is located at the aminoterminus of Gag, in MA. Mutations affecting this domain abolish assembly, but M mutants retain their ability to interact with other Gags and can be rescued into particles by the coexpression of a wild-type protein. The region seems to contain both hydrophobic and basic residues that are needed for proper interaction with lipid and with the acidic moieties of phospholipids. Structural information for the isolated M domain is consistent with this role (478).

For many retroviruses, myristylation of Gag, along with specific residues in MA, is required for membrane binding. This interaction with membrane, in turn, is important for virion assembly of the C-type viruses, and for their proper subsequent Gag processing (645). Mutational studies have led to the notion of a “myristyl switch,” in which the myristic acid is exposed to mediate plasma membrane binding during virion assembly, but then can be sequestered in the compact globular core of MA after Gag processing (524,536,672,803). Although this region is generally considered important for virion assembly, surprisingly, much of the Rous sarcoma virus (RSV) MA (511), and the entire HIV-1 MA domain (597) can be deleted from Gag without preventing assembly, so long as a functional membrane binding signal is retained. In the latter case, there are some effects on assembly: Virions are budded indiscriminately into both intracellular membranes as well as at the cell surface. The aminoterminal sequences of Gag can be replaced with a heterologous membrane binding signal, such as that present at the aminoterminus of the Src kinase. It should be noted that the interaction of Gag with membrane is not required for assembly of the B- or D-type viruses per se. For these viruses, mutations in the myristate addition signals do not affect the cytoplasmic assembly of the virions, but rather block the transport of the assembled particles to the plasma membrane (611).

The I Domain

The I, or interaction, domain is defined as a major region of Gag-Gag interaction, largely contained in the NC region. Although the major I domain has been suggested to lie in NC, some analyses have suggested that the C-terminal half of CA and NC are equally important for normal assembly (1,85). Mutations in the I domain block or reduce assembly, and those particles produced by these mutants have aberrantly low density, indicating fewer and poorly packed Gag proteins. The key feature of the I domains is not the zinc-binding residues of the Cys-His box, but rather basic residues flanking the boxes (89). The assembly function could involve the binding of RNA—either the genomic RNA or other RNAs—but it is not completely clear that RNA is necessary. The assembly functions of NC can be replaced by foreign proteins, and the key activity seems to be the formation of protein–protein contacts (801). Mutations in this region can also affect particle size and yield (397). Recently, the I domain of HIV-1 has been proposed to be involved in the membrane association of the Gag precursor (640), but other studies suggest that NC is not required (523).

The L Domain

The third domain is the L, or late assembly, domain (763,776). Mutants affected in this function fail to produce and release particles efficiently, and although the mutant Gag proteins form spherical structures, they accumulate under the membrane and do not progress normally. The buds remain tethered to the cell surface by a membrane stalk, suggesting that the function of the L domain is to mediate virus–cell separation. Recently, the domain has been shown to also be important in determining the size of the virion: The structures formed by L domain mutants are often large and heterogeneous in diameter (264). L domains lie at different locations in the Gag proteins of different viruses. In ASLV, MPMV, and the MuLVs, the L domain lies in the aminoterminal third of the protein and its critical residues are the PPPY motif. In HIV-1, the domain lies in p6, at the C terminus, and instead contains the motif PTAP. Remarkably, many of these L domain motifs are interchangeable among the various retroviruses and show a substantial position independence for their function (544). It is likely that these motifs represent the binding site for some protein important for the late stages of budding, and indeed the PPPY motif has been identified as the recognition sequence for the WW-domain family of proteins, including the Yes-associated protein YAP, NEDD45, and many others (265). Recently, the L domain of the EIAV Gag has been shown to associate with a cellular adapter protein, AP-2, which could mediate transport or localization steps (587). Which of these (or other proteins that may interact with the L domain) are most crucial for virion release remains uncertain. A curious additional link between two of these proteins is provided by the observation that both HIV-1 p6 and the MuLV p12 proteins are modified by the addition of ubiquitin at low levels (529). It is possible that a PPPY-binding host protein is responsible for this modification.

Virion Assembly *In Vitro*

Recently, Gag proteins and fragments of Gag have been shown competent to assemble *in vitro* to form various structures that more or less closely resemble virion cores (196,386,638,673). The CA-NC portions of ASLV and HIV-1 expressed as recombinant proteins can assemble to form particles or long hollow tubes; the formation of these structures is dramatically enhanced by the addition of RNA, and the length of the tubes is determined by the length of the RNA (108). Larger Gag fragments that include more aminoterminal regions can assemble into spherical particles (292), and this assembly is stimulated by RNA and host cell extracts (107). Although HIV-1 Gag interacts with cyclophilin A *in vitro* and *in vivo*, addition of cyclophilin A to assembly reactions has only little effect (288). HIV-1 Gag CA-NC fragments can assemble into conical structures (262), with a pitch that falls into discrete values. Image reconstruction of these cones has allowed the formation of a model for the packing of the protein into hexagonal arrays. Virus-like particles have also been formed with the Gag proteins of MPMV in cell-free protein synthesis systems.

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Virion Size

The number of Gag proteins per virion particle is estimated to be in the range of 1,200 to 1,800, although this number may vary somewhat from virus to virus. The number of Gag-Pol proteins is roughly 10 to 20 times lower, approximately 100 to 200 per virion. It is unlikely that these proteins form a completely homogeneous, ordered crystalline array, but rather they may form a “protein micelle” that is somewhat fluid like a lipid micelle. The diameter of even wild-type virus preparations is not tightly homogeneous but shows a distribution that suggests some flexibility in the structures during assembly. However, the average size of the particle is determined by the Gag protein, and mutants with alterations in Gag often show abnormal or excessively heterogeneous diameters (397). Mutations in the CA domain commonly show this phenotype. Thus, CA-CA contact may play a role in determining the angle between Gags during their packing into a spherical shape. The heterogeneity of virions has frustrated efforts to determine the detailed structure, although chemical cross-linking has provided some clues (297).

Gag proteins of one virus are sometimes able to interact with the Gags of another virus to coassemble and form mixed virion particles. Various mutants with alterations in the Gag proteins of the MuLVs can coassemble into particles that show phenotypes of both parental Gags (361,598). Viruses of very different genera can even form mixed particles in some cases (59). The physical interactions between Gags can be monitored in other assay systems as well (243,442).

Incorporation of Other Proteins into Assembling Virions

During assembly, other proteins are incorporated into the particle by contacts to Gag; these include Gag-Pol, Env, and auxiliary proteins encoded by the complex viruses. The Gag-Pol precursor is thought to be incorporated into the assembling bud by virtue of the Gag protein present at the aminoterminus. Gag to Gag-Pol contacts can in some cases lead to the incorporation of mutants of Gag-Pol that do not retain the myristate modification to the aminoterminus (549), suggesting that the interaction is quite strong. Gag fusions to foreign proteins can be similarly incorporated into particles formed by Gag (351); this process can even be used to target antiviral proteins into virions. Consistent with this notion, many mutations that block assembly of Gag, when tested in the context of Gag-Pol, are found have similar effects on the incorporation of Gag-Pol (146,650). However, some mutations in HIV-1 Gag have also been identified that specifically affect the incorporation of Gag-Pol, suggesting that Gag-Pol utilizes some distinctive contacts not important for Gag-Gag interactions (662). Further, in the spumaviruses, Pol is incorporated without an appended Gag region, suggesting that distinct interactions must be utilized for its incorporation (214).

The Env protein is thought to be concentrated at the sites of budding and incorporated into the virions by virtue of contacts between the cytoplasmic tail of Env and the aminoterminal portion of Gag. These interactions have been difficult to document directly, although there is some biochemical evidence (143) and cross-linking studies (267) in support of these contacts. Genetics has provided good evidence for this interaction. Selected mutants of MA show defects in Env incorporation (181,250,791), and some mutants of the cytoplasmic tail of TM are not efficiently incorporated (792). In addition, Env proteins that are specifically directed to the basolateral surface of polarized epithelial cells can redirect the sites of budding of Gag from a nonspecific assembly on both membranes to the exclusive assembly at basolateral membranes (432,535), and they can similarly redirect Gag in neurons (749). Finally, mutants and revertants of these mutants with second-site suppressors in the binding partner have provided strong evidence for these interactions (249,250). However, it should be noted that the envelope proteins of viruses very distant from retroviruses, including vesicular stomatitis virus (VSV) and influenza, can be functionally incorporated into retrovirus particles without any obvious sequence similarity in their cytoplasmic tails. Furthermore, truncating the tail of ASLV Env does not prevent its incorporation or function (563). Thus, there may be mechanisms to direct Env proteins to assembling virions without these specific contacts to Gag—a default pathway, or a pathway using other interactions. Indeed, other distinct parts of Gag, including the p6 region of HIV-1 Gag, have been implicated in Env incorporation (528).

The HIV-1 protein Vpr is efficiently incorporated into assembling virions at very high levels, approaching equimolarity with Gag. This incorporation requires the presence of the p6 domain of Gag (651) and may be mediated by a direct interaction (39). The binding can be used to direct foreign proteins into the particle; a fusion between Vpr and a foreign protein will be targeted to virions. Furthermore, Vpr can be used to direct separately expressed versions of RT or IN to particles in a functional form, to complement mutations in the RT or IN domains of the Gag-Pol fusion (402).

Host Proteins in the Virion

A number of host proteins have been shown to be present inside the virion particle; in most cases, the significance of the protein is unknown. Prominent among the virion-associated factors are a number of cytoskeletal proteins. These include actin (33,512,530,760) and various members of the Ezrin-Radixin-Moesin (ERM) family, specifically ezrin, moesin, and cofilin (530). The Gag, and especially the nucleocapsid protein of HIV-1 have been shown to directly bind to actin (426,609,760), perhaps offering a mechanism for its incorporation into the particle. A complication in analyzing virion-associated proteins is that virion preparations tend to be contaminated with substantial amounts of microvesicles, entities released by cells that exhibit a density and size very similar to that of virions, and containing an array of host proteins (66).

The virions of HIV-1 contain substantial levels of cyclophilin A, a proline isomerase of uncertain function but implicated in protein folding and signal transduction (244,443,712). The incorporation of cyclophilin A is not required for assembly, but it is important for efficient entry of HIV-1 into newly infected cells, before the process of reverse transcription is initiated

(90). Its exact role is uncertain. Several other proteins have also been found in virions: a translational elongation factor, eIF-1a (121), and a protein known as H03 (408), with similarity to histidyl tRNA synthetase, are additional examples.

Host proteins may also be attracted into virion cores by mechanisms other than Gag. Recently, the host uracil DNA glycosidase, responsible for removing uracil bases from DNA, was shown to be incorporated into virions by contacts to IN (761). Another protein, the RNA transporter staufen, was shown to be incorporated into virions, probably through contact to viral RNA (502).

There are also substantial levels of host proteins in the virion envelope. The mechanism of incorporation of these proteins into the virion is not clear, and in most cases again the significance is uncertain. However, one such molecule, major histocompatability complex (MHC) class I, is present at levels approaching those of the Env protein and can be functionally significant, in that xenogeneic antibodies targeted to MHC can neutralize the infectivity of viruses such as HIV-1 (33,34).

RNA PACKAGING

The RNA genome is incorporated by virtue of interactions between specific RNA sequences near the 5' end of the genome, termed the packaging or Psi sequences, and specific residues in the NC domain of Gag (see refs. 30 and 61). Both partners in this interaction have been intensively studied.

Gag Sequences Important for Packaging

The Gag precursor is probably the form of the protein that is responsible for packaging the RNA (519), and the NC portion of the precursor plays the largest role. Mutations affecting the NC protein often reduce the incorporation of the genomic RNA into the virion particle (see ref. 61 for review). The most crucial sequences are the Cys-His boxes, short sequence blocks resembling zinc fingers and containing the motif Cys-X2-Cys-X4-His-X4-Cys, but basic residues elsewhere in the NC molecule are also important (319,578). The NC protein of various viruses contains either one or two copies of the Cys-His box. When two copies are present, they are not equivalent or interchangeable, suggesting that they mediate distinct interactions with RNA (281). Some viral cores can cross-package heterologous viral RNAs, suggesting good binding to the heterologous Psi region, and sometimes there is a strong preference for the homologous RNA. Exchanging the NC domains between viruses can sometimes transfer the preferential selectivity of a Gag protein for its cognate RNA, although the specificity of these hybrid Gags is often poor, and in some cases other sequences in Gag can determine the preference for RNA packaging by chimeric Gags.

RNA Sequences Important for Packaging

The packaging or Psi regions on the viral RNA genome that are recognized for incorporation are quite distinct in nucleic acid sequence among the various viruses. The key Psi regions lie near the 5' end of the RNA, generally between the LTR and Gag. However, other regions of the genome can affect RNA packaging, including sequences upstream in R and in U5, downstream in Gag coding regions, and even near the 3' end of the genome. In the case of ALSV, a region of 270 nt is necessary and sufficient to mediate the packaging of a foreign RNA (29,45,366). In the case of the MuLVs, sequences that are at least partially sufficient to mediate packaging have been similarly identified (3). These Psi regions are relatively autonomous; Psi can be moved to ectopic positions in the genome with at least some retention of function (459).

The various Psi sequences have been predicted or shown to form a number of stem-loops, often containing GACG in the loops. Reversion analysis of mutants with alterations in these loops confirms the importance of the stem-loop structure (182). Mutational studies show that several such loops may incrementally contribute to the efficiency of packaging of the RNA, although one or two are often found to be most important (238,501). Recently, one of the stem-loop structures of the HIV-1 Psi was replaced by a completely foreign sequence that was selected on the basis of its binding activity with NC; the resulting RNA was efficiently packaged and utilized for replication, strongly suggesting that the binding to Gag is the key function of Psi (124). A structure of the HIV-1 NC bound to one such stem-loop has been resolved by NMR, revealing specific contacts between both hydrophobic and basic residues of NC and nucleotides in the stem-loop of the RNA (165).

Dimerization of the Viral Genome

Mature virions contain a dimeric RNA that is highly condensed into a stable, compactly folded structure referred to as the 70S dimer on the basis of its sedimentation rate. Packaging of RNA is associated with the dimerization of the RNA, but it is not completely clear whether the genome is present as a dimer before it is incorporated into virions or is incorporated as a monomer and then dimerized after its incorporation. However, some ASLV mutants suggest that monomeric RNA can be packaged under some circumstances (527), implying that dimerization may follow packaging. There are specific sequences in the RNA, termed dimerization or dimer linkage structures (DLS), that are required for RNA dimerization *in vitro*, and for the formation of the dimeric virion RNA *in vivo*. These DLS elements are in close proximity or even intermingled with sequences required for packaging of the RNA, often making it difficult to determine their separate contributions to these processes. The dimerization of viral RNAs can be induced *in vitro*, and it is stimulated by the addition of NC or the Gag precursor. However, it is uncertain to what extent these reactions reflect dimerization *in vivo*.

The viral RNA in newly budded virions is present as an unstable dimer, dissociated by heat at relatively low temperatures, and it becomes condensed to a more stable dimer during virion maturation (254). This condensation requires the proteolytic processing of Gag (252) and may be mediated by the free NC upon its release from the precursor. A model for the process of dimerization, the “kissing loop” model, suggests that duplex formation between two RNAs is initiated between loops on the two RNAs and propagates outward through the stems to form a more stable duplex. It is possible, but by no means certain, that such changes in the duplex regions could account for the change in

thermal stability upon maturation.

Many cells contain vast arrays of endogenous proviruses and retrovirus-like elements, a subset of which can be expressed constitutively or under various conditions of stress to produce large amounts of genomic RNA. If such a cell is infected by an exogenous virus or has been engineered by expression constructs to produce virions, the particles will incorporate the endogenous RNAs along with the viral RNA (65,556). The endogenous retroviral RNAs, notably the VL30 RNAs of rodents, contain highly efficient Psi sequences (484,717), presumably because they were selected to compete with the homologous genomes of exogenous viruses for packaging.

Virions also contain a number of host RNAs of uncertain significance. There are substantial levels of 7S RNA, a low-molecular-weight RNA thought to function in host RNA splicing (116). In addition, there are low levels of host mRNA. Particles released without efficient packaging of the viral genome (as are produced by Psi-mutant genomes) may carry enhanced levels of host RNAs, and various mutants with alterations in NC can show selective enhancement of both endogenous viral and host RNAs (484). A variant avian leukosis virus (ALV), SE21Q1b, packages unusually high levels of host RNA (260,425) and is capable of transducing these host sequences into new cells by reverse transcription (445). This phenotype of high-efficiency transduction is associated with an unusually high level of proviral expression and particle production rather than any specific alteration in a viral protein (18).

Incorporation of tRNA Primer

A key aspect of RNA packaging is the incorporation of a host tRNA along with the genome to serve as the initiating primer for minus-strand DNA synthesis (for review, see ref. 453). Virions contain a substantial pool of free tRNA, perhaps 50 to 100 copies per particle. The bulk of these tRNAs are not associated with the genomic RNA and are present in virions that lack the genome. In some viruses, these tRNAs are largely representative of the pool of tRNAs in the cell, whereas in others they are highly enriched for the tRNAs needed for priming DNA synthesis, although even here many other tRNAs are present. Viruses prepared without the Pol proteins do not show this enrichment, suggesting that Pol, and most probably the RT protein, are responsible for bringing these tRNAs into the virion (566). In accord with this notion, the RT of ASLV has been shown to preferentially bind tRNATrp from a mixture of tRNAs, accounting for its enrichment in the virion (538). Similarly, HIV-1 RT preferentially binds tRNALys3, and the interaction domain has been shown to at least include the anticodon loop of the tRNA. However, no similar preference for the natural primer tRNAPro has been detected for the MuLV RT. It may be significant that the MuLVs have been shown to be able to utilize a range of different primer tRNAs when only the complementary sequence in the genome (the pbs) is altered to promote their use.

tRNA Primer Placement

A very small subset of the incorporated tRNAs—two per virion—are annealed to the pbs, an 18-nt sequence near the 5' end of the genome with perfect complementarity to the 3' sequences of a specific primer tRNA. The pbs sequences are, as one would expect, essential for normal reverse transcription of the virus (613). The sequence of the pbs can determine the primer tRNA that is utilized (789), but changes in the pbs tend to revert back to the wild type (759), suggesting that alternate tRNAs do not function well. An interesting aspect of reverse transcription provides an efficient mechanism for this reversion: The use of the original tRNA even once during replication will convert the pbs back to the original sequence, because the tRNA itself is the template for the DNA copy of the pbs. Other sequence blocks of the tRNA are also paired with complementary sequences in R and U5 to form a large, complex structure required for proper tRNA primer placement and utilization (7,126,205,328,334). These other sequences are presumably responsible for the selectivity for the natural tRNA primer. In the alpharetroviruses, *pol* gene products are required to mediate the placement of the tRNA on the genome, but in the gammaretroviruses, *pol* is not required (253). The Gag precursor, and especially the NC domain, are thought to play a major role in promoting the annealing of the tRNA to the genome. Although NC can promote annealing of complementary RNAs and DNAs *in vitro*, its role and the mechanism by which it may act *in vivo* remain uncertain.

PROTEIN PROCESSING AND VIRION MATURATION

As retrovirions are budded from the cell surface, the Gag and Gag-Pro-Pol precursor proteins are proteolytically cleaved to release the smaller proteins present in the infectious virions (for review, see ref. 741). The cleavage of Gag and Gag-Pro-Pol is mediated by the viral protease PR, which is expressed either in Gag, Gag-Pol, or Gag-Pro-Pol fusion proteins. Thus, PR is responsible for cleaving itself out of a precursor protein and then making a number of other cleavages in these proteins.

Activation of the Protease

The processing of Gag and Gag-Pro-Pol precursors is intimately linked to assembly and budding, and it is controlled so that the precursors are not cleaved until they are assembled. It is not certain how PR is regulated during assembly to begin cleaving its substrates. The structure of PR has revealed that the active enzyme is a homodimer (see later), and thus its activation could be promoted by dimerization of the Gag or Gag-Pro-Pol precursor associated with assembly. As the virions form, one could imagine the high concentrations of the protein generating an active PR that would begin to cleave Gag and Gag-Pro-Pol, and that would release the mature PR dimer as well. However, for the betaretroviruses such as MPMV, this mechanism cannot explain the delay in processing. For these viruses, assembly occurs in the cytoplasm and should result in the establishment of a high concentration of Gag-Pro-Pol at that time. Yet cleavage does not begin in the cytoplasm, but rather is restrained until budding and export of the preformed virion particle. Thus, other unknown mechanisms, perhaps coupled to membrane association, must be responsible. Similar conclusions were reached from analysis of IAP virions, in which assembly in various intracellular locations did not result in processing—membrane association was required (755).

Various domains of Gag have been suggested to inhibit PR (266), and conformational changes could relieve this inhibition. In the alpharetroviruses, a cleavage at the NC-PR boundary is required to release active PR, so activating this

cleavage could serve as a trigger (102). Similar cleavages at the p6*-PR boundary are important for full activation of the HIV-1 PR (711). Another possibility is the activation of the PR by a drop in the pH associated with virion release. It should be noted that the overexpression of PR in many artificial settings, both in bacteria and in animal cells, as a Gag-PR fusion or alone, can result in formation of highly active enzyme (24). The high-level expression of PR is often toxic for cells, presumably because of its inappropriate action on many host proteins.

Protease Structure and Function

The retroviral proteases are aspartyl proteases with clear sequence similarity to members of the cellular family of aspartyl proteases (362,434,742). The three-dimensional structure of many proteases, including those from ALSV, HIV-1, HIV-2, SIV, feline immunodeficiency virus (FIV), and EIAV, have been determined by x-ray crystallography (406,409,491,509,582,616,748,772). The viral enzymes are small, typically containing about 100 amino acids, and are homodimers as isolated from virions. Each subunit contributes to the active site a single aspartate residue, lying in a loop near the center of the molecule. There is a long cleft at the interface between the subunits where the substrate lies; there are pockets to interact with each of the side chains of the substrate, conferring specificity to the enzyme. Each subunit has a flap consisting of an antiparallel sheet with a b-turn that covers the cleft, and this flap moves out of the way to permit the binding of the substrate into the active site.

Retroviral proteases have a complex specificity for substrate peptides (560). The enzyme makes contact with approximately seven or eight side chains on the substrate and thus can select its cleavage sites on the basis of at least these amino acids. The cleavage sites tend to be within hydrophobic sequences and yet must lie in accessible and extended conformations. Some analyses of the various sites in Gag and Gag-Pol that are recognized by PR suggest that either one of two sequence motifs constitutes a consensus site: One set has an aromatic residue or proline flanking the cleavage site, and the other set has aliphatic residues at these positions. Mutational analyses have allowed further definition of the residues on PR that make specific contacts to the substrate.

Protease Inhibitors

Studies of mutant viruses lacking PR demonstrated that the protease is essential for virus replication. Viruses lacking a functional PR can still express Gag and Gag-Pol precursors, and they can mediate the assembly and release of immature virion particles. Thus, PR is not required for the process of virion assembly per se. However, these particles are noninfectious and are blocked at an early step prior to the initiation of reverse transcription (147,363,390). Because of its essential role in virus infectivity, PR was appreciated early in the course of the AIDS epidemic as an attractive target for antiviral therapy. A number of molecules have been generated that can bind and inhibit PR, including peptide mimetics with uncleavable, nonsessile bonds at the cleavage site (158,494). Some are transition state analogs, and may have inhibition constants (K_i) in the nanomolar or subnanomolar range. These inhibitors have been extremely effective antiviral agents, and because they target a distinct enzyme and a distinct step in the life cycle from the RT inhibitors, they have been particularly effective in combination with earlier drugs targeted at RT. The combination of three drugs that include a protease inhibitor is now the standard of treatment for AIDS, and such highly active antiretroviral therapy (HAART) can keep virus loads below detectable levels in some patients for many years. Ultimately, however, point mutations in PR that confer resistance to the drugs can arise (210), allowing some virus replication in spite of therapy.

Processing of the Gag Precursor

During and after release from the cell, the Gag precursor is cleaved by the protease into a series of products present at equimolar levels in the virion. The number and size of the products vary considerably among the various viruses; the spumaretroviral Gag is exceptional in undergoing the fewest cleavages. A summary of the Gag products of some representative viruses is shown in Table 4. There are many features of these products common to most of the retroviruses.

Protein	ALV	MLV	MMTV	MPMV	HTLV-1	HIV	HFV
MA	p19	p15	p10	p10	p19/15	p17	–
?	p10	p12	p24	p24	–	–	–
CA	p27	p30	p24	p27	p24	p24	p33
NC	p12	p10	p14	p14	p12	p7	–
DU	–	–	p46	–	p15	–	–
PR	p15	p14	p13	?	p14	p14	p16
RT	~p9	p80	?	?	?	p66/51	p86
IN	pg32	p46	?	?	?	p31	p46
SU	gp85	gp70	gp52	gp70	gp46	gp120	gp130
TM	gp37	p15E	gp36	gp22	gp20	gp41	gp48

ALV, avian leukemia virus; MLV, murine leukemia virus; MMTV, mouse mammary tumor virus; MPMV, Mason-Pfizer monkey virus; HTLV-1, human T-lymphotropic virus type 1; HIV, human immunodeficiency virus; HFV, human foamy virus.
Proteins: MA, matrix; CA, capsid; NC, nucleocapsid; DU, deoxyuridine triphosphatase; PR, protease; RT, reverse transcriptase; IN, integrase; SU, surface; TM, transmembrane.

TABLE 4. *Virion proteins found in mature particles of various retroviruses*

The Matrix Protein

Beginning at the aminoterminus, most Gags are processed to form a membrane-associated or matrix protein (MA). The MA protein is thought to remain bound to the inner face of the membrane as a peripheral membrane protein, and it can be cross-linked to lipid. MA may make contacts with the cytoplasmic tail of the envelope protein. When the precursor Gag is myristylated at the aminoterminus, the corresponding MA protein retains that myristate and so is presumably bound tightly into the membrane. The compact structure of the MPMV MA protein has been elucidated by NMR (136,473). The MA proteins of HIV-1 and SIV have been shown to form trimers in crystallization studies (56,595) and can contribute to

the ability of a larger Gag precursor to form trimers in solution (500). The protein can form extended sheets of trimers, with a large opening in the network. If similar structures were to form in a sphere, the surface could have openings into which the envelope tail may fit.

The Capsid Protein

Gag proteins are cleaved to generate a large product serving as the major capsid protein (CA) in the virion core. The CA protein is relatively well conserved among Gags and contains the only highly conserved motif among Gags, the major homology region (MHR). The function of this motif remains uncertain; although mutations in the region affect virion assembly in some viruses (145,458,685), it is not absolutely required for this process, as the entire CA domain of ASLV can be deleted without blocking assembly. CA is thought to form the shell of the condensed inner core of the mature virus, and thus it makes either a spherical, a cylindrical, or a conical structure, depending on the virion morphology. The protein has proved difficult to crystallize, and the structure of the complete protein is uncertain (495), but the structures of both the N-terminal and C-terminal fragments of the HIV-1 CA have been determined (261,272). Very recently, the analogous structures of the RSV capsid (393) and of the EIAV CA protein (345) have been determined and used to model a structure of the virion core. The CA protein can form dimers in solution, and recombinant proteins containing CA, or CA plus NC, can assemble to form higher-order structures consisting of either tubes, spheres, or, in the case of HIV-1, cones (262). CA has also been studied after tethering sheets of the protein to membrane (48). Image reconstruction of electron micrographs, coupled to the subdomain structures, have led to models for the packing of CA to form these large assemblies. The major CA-CA contacts must form after processing during the condensation of the virion core, and they may be very different from any contacts that exist in the immature virion particle (211).

The Nucleocapsid Protein

All Gag proteins except for those of the spumaviruses are cleaved to produce a nucleocapsid protein, NC, located near the carboxyl terminus of the precursor. NC proteins are small, highly basic proteins containing one or two copies of the Cys-His motif, Cys-X2-Cys-X4-His-X4-Cys. These sequences bind a single Zn^{2+} ion avidly, and they fold around the ion into a characteristic structure that is smaller and rather different from the better-known zinc-finger structure. The structures of NC proteins in solution have been studied by NMR, which revealed a tightly folded knuckle with disordered flanking sequences (389,499). The interaction with zinc results in the incorporation of substantial levels of Zn^{2+} into all retrovirus virion particles (67).

The NC protein in virions is closely associated with the viral RNA, probably coating the entire RNA molecule; the stoichiometry of binding is such that each NC molecule can bind to about six nucleotides of RNA. NC proteins bind nonspecifically to heteropolymeric single-stranded nucleic acid with moderate affinity (728). However, NCs also exhibit specificity. Tests of binding to nucleic acids of defined sequence have shown that NCs bind poorly to poly(A), and most tightly to nucleic acids containing GT dinucleotides, especially alternating (GT)n polymers (239). In addition, NC has been shown to exhibit sequence-specific binding activity *in vitro* for nucleic acids containing the Psi region, required for packaging of the viral RNA (62,123). The interaction can also be assayed in reconstructed systems in yeast via the so-called three-hybrid method (40,412). This binding presumably reflects at least some aspect of its role in packaging the genome. A specific complex of the HIV-1 NC with a stem-loop derived from Psi has been studied by NMR, and the resulting structure shows a number of specific contacts between hydrophobic residues of NC and bases in the four-nucleotide loop, and between basic residues and specific phosphates in the stem and loop (165).

NC proteins change the base-pairing properties of nucleic acids and thus can have profound effects on the kinetics and thermodynamics of annealing (166,720). Under various conditions *in vitro*, NC can stimulate the dimerization of RNAs, and duplex formation between tRNA and its complementary sequences at the pbs (81). Thus, NC can help promote primer tRNA placement during virion assembly (603). NC can also help melt out secondary structures, and it may facilitate the movement of RT along the template during reverse transcription (775). In addition, it is clear that NC can bind to double-stranded nucleic acid, and thus it is probably retained on the viral DNA after its synthesis by RT. NC mutants have been found that affect the course of DNA synthesis or DNA stability during the early stages of virus infection, suggesting a role in the processing of the DNA and protection of DNA from degradation (282,699). Finally, NC has been shown to promote the concerted integration of the two termini of the viral DNA into a target sequence (see earlier, and ref. 109).

An important class of inhibitors of virus infectivity and replication that act by targeting the NC protein has been identified (615,725). These compounds, disulfide-substituted benzamides (DIBAs), eject the zinc ion from NC and cross-link the cysteines via disulfide bonds. Virions treated with these compounds are potently inactivated without disrupting the virion structure, and the course of virion assembly in infected cells is similarly blocked (531,601,629,727). Drug-resistant variants are not readily recovered.

Other Gag Products

Some retroviral Gag proteins, including those of the alpha-, beta-, and gammaretroviruses, contain one or more poorly conserved domains of 10 to 24 kd lying between MA and CA. The functions of these proteins is unclear. The ASLV p2 protein, the MuLV p12 protein, and the MPMV p24 protein contain a PPPY motif that plays an important role in late stages of virion assembly (see earlier). The MuLV p12 protein has also been shown to play a role in the early stages of infection (146,793).

In the lentiviruses, a p6 domain is present at the carboxyl terminus. The role of p6 is unclear, although it contains the L domain and thus may be important in virion release; it also is required to mediate the incorporation of Vpr into virion particles, perhaps by providing a direct docking site. Proteins can be targeted to virions by generating Vpr-X fusions, which are incorporated into lentiviral virions in a p6-dependent manner.

Processing of the Gag-Pro-Pol Precursor

At the same time that the Gag precursors are cleaved during virion maturation, the Pro and Pol region of the Gag-Pro-Pol precursor is also cleaved, giving rise to the PR, RT, and IN products. The Pro and Pol–containing precursors of different viruses are cleaved in diverse patterns (Fig. 17). In the gammaretroviruses, the Pol region is processed by complete digestion to form PR, RT, and IN (321,701). In the alpharetroviruses, the Pol region is cleaved to produce a heterodimeric RT with a larger b subunit and a smaller a subunit. The larger b subunit contains both RT and IN domains. It is not clear whether the IN domain in the context of this subunit performs an important function, although it is responsible for a weak nuclease activity associated with RT (277). A portion of the Pol precursor undergoes an additional cleavage to produce the a subunit of RT, an aminoterminal fragment of the b subunit, and the separate IN protein. In the lentiviruses, Pol is processed to give rise to PR, a heterodimeric RT, and IN. However, the RT of these viruses is not identical to the heterodimeric RT of the alpharetroviruses. Here, the IN domain is fully removed from RT. One RT subunit remains intact (for HIV-1, this is the p66 subunit), and the other subunit undergoes an additional cleavage to remove a carboxyl-terminal domain (to form the p51 subunit). The functional significance of the different subunit structures of these various RTs is unclear, as they all perform a very similar set of reactions during virus replication. The processing of Pol precursors may be associated with the activation of the DNA polymerase of RT. In the alpharetroviruses, the immature Gag-Pol protein has very low DNA polymerase activity, and its maturation results in a large increase in activity (144,679). However, the immature Gag-Pol protein of MuLV and HIV-1 has high DNA polymerase activity, and there is only a very modest increase upon maturation (147).

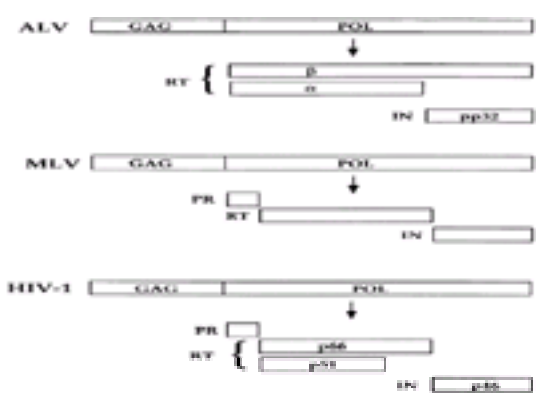

FIG. 17. Cleavage patterns during the processing of the Gag-Pol fusion proteins of various retroviruses. The structure of the mature cleavage products found in the virion particles are shown aligned with their location in the precursor.

The Gag-Pro-Pol precursor of the betaretroviruses and the nonprimate lentiviruses is also processed to produce the dUTPase protein, DU. In the betaretroviruses, the *pro* ORF encodes both DU and PR; in the nonprimate lentiviruses, the enzyme is encoded in the *pol* ORF, and DU lies in between RT and IN in the polyprotein. This enzyme acts to reduce the levels of dUTP that could otherwise be incorporated into viral DNA. Mutants of FIV lacking the function indeed show increased rates of mutation during replication (417), and similar mutants of CAEV tend to accumulate G-to-A substitution mutations (726), presumably as a result of incorporation of deoxyuridine (dU) residues that are subsequently read as deoxythymidine (dT). The FIV enzyme has been crystallized, and the structure of the protein has been determined by x-ray diffraction (583).

Processing of the Env Precursor

The major proteolytic cleavage of the Env protein to form the SU and TM subunits is performed during its transport through the ER and Golgi by host proteases termed furins. This cleavage is essential for virus infectivity (477,564), and it is thought to induce substantial rearrangements of the polypeptide chain. The TM subunit remains embedded in the membrane and contains an extracellular domain, a membrane-spanning segment, and a cytoplasmic tail. The SU subunit lies wholly outside the cell, and after its incorporation into the virion particle, wholly on the extravirion surface. It is held to the virion by contacts to TM, and most often by noncovalent bonds (302), although disulfide links may occur in some viruses (574). SU is heavily glycosylated, and the presence of at least some of these sugars is important for virus infectivity. Perhaps the most important function of this heavy glycosylation is to hide the peptides on the surface of Env from neutralizing antibodies that would otherwise have access to the virion surface.

The Surface Subunit (SU)

For most viruses, the major receptor-binding site is located in hypervariable sequences on the SU subunit, so that SU is a major determinant of host range. Chimeric SU proteins can be generated to demonstrate that the receptor utilization function maps to specific regions of the protein. The key regions of the avian Env proteins have been similarly defined by selecting for changes in host range *in vivo*; these studies show that very small changes can result in the use of new receptors (704). The structures of two SU proteins have been recently determined at high resolution: a fragment of MuLV SU (225), and a fragment of the HIV-1 SU bound to its receptor CD4 (405,618). These structures suggest that the receptors make contacts to the envelope in shallow pockets that may not be readily bound by antibodies.

The Transmembrane Subunit (TM)

The TM subunit contains the so-called fusion peptide at its aminoterminus, and TM is thought to play the major role in fusion of the virion and host membrane (167,251,303,308,639). Many TM mutations are defective for membrane fusion (189,805). However, mutations blocking fusion can lie in SU as well (796). The entire Env protein probably acts as a

unified machine to mediate fusion, with complex interactions between the subunits (155), and with major movements of the subunits during the process of fusion. The fusion peptide of TM may simply insert into the host membrane, or it may make contacts to proteins. The major contacts for oligomer formation of Env are thought to lie in TM (203); isolated TM proteins form trimers in solution (227) and in crystals (225,226,387,454). The trimer is held together by a modified leucine zipper motif that bridges the monomers via hydrophobic interactions. This zipper region is crucial for virus infectivity (422,594,757).

It is possible to separate the two major functions of the Env protein onto two different molecules that cooperate to mediate these steps. Thus, the receptor binding function can be mediated by one Env protein, and the membrane fusion function can be mediated by another Env. This is apparent in the ability of two Env proteins to complement in mixed oligomers (602,802). It is also demonstrated by the ability of a wild-type Env to provide the membrane fusion function for a chimeric Env that on its own can mediate only cell surface binding.

The TM subunit of the MuLVs undergoes a second cleavage during virion assembly (359) that is mediated by the viral protease, PR. This step removes a short sequence called p2E, or the R peptide, from the carboxyl terminus of TM (289). The cleavage step may require presentation of the tail to the protease, or some conformational change in the tail, that is mediated by Gag proteins; alterations in the MA or p12 Gag proteins can modulate the cleavage of TM (377,793). Astonishingly, the cleavage is necessary to activate the fusogenic activity of the envelope protein and thus for virus entry (94,600). Mutants in which the tail is truncated at the site of cleavage are constitutively activated for fusion, and these viruses induce dramatic syncytia in receptor-positive cells. Mutants in which the tail is not removed are inhibited for fusion, and particular residues can be shown to be required (779). How the cytoplasmic tail inhibits the fusogenic activity of Env is very much unclear.

In a similar way, the cytoplasmic tails of the TM of MPMV (670) and EIAV (614) are processed by the protease. In the case of MPMV, the presence of the intact tail is necessary for efficient incorporation of Env into the virion. The majority of the retroviruses, however, do not carry out cleavage of TM, and for these viruses an intact tail is needed for infectivity (190). The replication of some viruses in host cells of foreign species can select for alterations and truncations of the TM tails (388,614,657). The selective advantage conferred by this truncation is not well understood, although various aspects of Env function seem to be enhanced by this truncation (806).

Morphologic Changes upon Virion Maturation

The maturation of retrovirus particles is a complex process that is required for the formation of an infectious virus. The particles that are initially assembled either at the plasma membrane (by most retroviruses) or in the cytoplasm (by the betaretroviruses) have a characteristic immature morphology: The particles are round and stain with an electron-dense ring and a relatively electron-lucent center. After release from the cell, the morphology changes to a more condensed structure, with a central core largely detached from the surrounding envelope. In the alpha-, gamma-, and deltaretroviruses, the core is spherical and concentric with the envelope; in the beta-retroviruses, the core is spherical but eccentrically placed within the envelope; in the lentiviruses, the core is cylindrical or conical, with thin connections to the surrounding shell. In the spumaviruses, the morphology does not change dramatically after assembly.

Mutant viruses lacking the protease show little change in morphology. Thus, cleavage of Gag and Gag-Pol is required for the restructuring of the virion into the mature form (363). The changes in morphology visible in electron micrographs are probably associated with major rearrangements of the Gag proteins. Indeed, the physical properties of the virus change dramatically upon maturation. Whereas the immature core is quite stable to nonionic detergents and harsh conditions, the mature virion core is relatively labile. This change may reflect the inability of the immature virion, and the acquired ability of the mature virion, to uncoat upon infection of new cells and initiate reverse transcription.

RETROVIRAL DISEASES

The Varied Effects of Retroviral Infection

Retroviruses cause an extremely wide range of responses in infected animal hosts. The discussion of retroviral pathogenesis will begin with a little-appreciated but important point: Retroviruses in general are surprisingly benign. The vast majority of the replication-competent retroviruses are not cytopathic, and the infection of cells causes remarkably little impact on their replication or physiology. The morphology, the control of cell division, and the doubling time of cells in culture are not significantly changed after infection. Once a chronic infection is established, only a relatively small amount of the cellular metabolism is committed to virus expression: Typically, a small percentage of the cellular mRNA and protein is viral, and so the cell can perform its normal functions and survive for its normal lifespan. Indeed, animals show few acute affects upon infection. The animals do become viremic, and a vigorous immune response is often mounted that can reduce the levels of virus production. However, infected mice or birds may live relatively normal lives for many months or years, and so it is appropriate to consider the viruses as relatively benign parasites. It is noteworthy that the virus is not eliminated but only suppressed by the immune response, and low-level viremia usually persists in infected animals for life.

Retroviruses often do, however, cause disease. The chronic viremia of the replication-competent retroviruses is tantamount to high-level mutagenesis of the infected cells, for each infection event is associated with a proviral insertion that constitutes a mutation. Eventually, the odds are that a cell will suffer an insertion that alters the normal control of cell division or cell survival, and abnormal proliferation of this cell results in tumorigenesis. Many retroviruses cause disease in this way, including the so-called slow leukemia viruses and agents such as MMTV. A few retroviruses, however, are more pathogenic: A small minority of the retroviruses are directly cytopathic, and many of the infected cells are killed. These agents can thus destroy the infected tissues and directly impair their function. These include the cytopathic avian viruses and, probably, the AIDS virus, HIV-1. Finally, a special class of retroviruses exists, the so-called acute transforming viruses, that can induce a rapid tumor formation. These viruses were among the first filterable oncogenic agents ever discovered, and their dramatic effects were a major motivation for the intense study of all the tumor viruses

throughout the 20th century. We now understand that these agents are transducing viruses; the replication of retroviruses allows recombination events between viral and host sequences that move genes onto the viral genome. These viruses carry and express host genes at inappropriate levels, in inappropriate cells, and often with drastic alterations in gene structure. If the gene product so expressed by the virus is mitogenic or antiapoptotic, the result can be a potent alteration in the physiology of the infected cell. These acute transforming viruses can thus initiate a highly aggressive tumor very efficiently and with minimal latency, because each infection of a cell has the high potential to initiate an oncogenic transformation event. Most often, the acquisition of the host gene comes with a loss of a viral gene essential for its replication; as a result, these viruses are often replication-defective and depend on a helper virus, usually a replication-competent leukemia virus, for their transmission to new cells. Each of these various classes of pathogenic viruses will be discussed in turn.

Diseases Caused by the Replication-Competent Retroviruses

The typical pathology of many of the simple replication-competent retroviruses is the development of leukemia or lymphoma after a very long latency. For this reason, these agents are often called the slow leukemia viruses. Examples are found in rodents, including the many MuLVs, and in birds, including the avian leukemia-leukosis viruses. The symptoms eventually begin with a lymphoid hyperplasia (681), which may be directly attributed to the immune response; not all the affected cells are infected, and the proliferating cells may be stimulated by cytokines that are released in response to the infection (93). These cells may include a preleukemic state of partially transformed cells (164). There may be some cell killing resulting from enhanced apoptosis in these early times (82), although the mechanism of the apoptosis and the relationship to tumorigenesis is unclear. A subset of these expanding cells progress to frank leukemia, which ultimately can be fatal in susceptible animals. These observations strongly suggest that leukemogenesis is a multistep phenomenon, and it is also likely that the virus plays a role in more than one of these steps (221). The cell type transformed by the virus can be very narrowly defined, or more broadly variable, but will depend strongly on viral determinants. For example, the ASLV group of viruses typically cause a bursal or B-cell lymphoma in birds; the MMLV causes a T-cell leukemia; the Friend helper MuLV causes an erythroleukemia; MMTV causes a mammary epithelial tumor.

In some species and settings, the infecting virus is the proximal agent of disease; such is the case with infection of rats by the MMLV. However, the course of leukemogenesis in mice and other animals is often associated with the appearance of recombinant retroviruses derived from the parental infecting virus and endogenous sequences present in the germline (114,218,471). The recombinant viruses are often the true or proximal pathogens. These viruses are heterogeneous in structure and phenotype, but most contain substitutions of the *env* gene and LTR that confer novel properties to the initial virus (172). Some of the viruses arising in mice can be detected through an expanded host range, as an ecotropic virus acquires *env* sequences that allow infection through the xenotropic or dual-tropic receptors; these viruses are the MCF viruses (312). The range of cell types and the replication ability of the input virus can be expanded by recombination to significantly enhance the incidence of leukemia and shorten the latency period to disease. The donor sequences for these recombination events are not all universally present in a given species but are highly variable from strain to strain (16,407,592). The presence or absence of suitable endogenous proviruses in the germline that provide the sequences needed for recombination can control the severity and course of disease.

Leukemogenesis by Insertional Activation

The most common mechanism of action of the replication-competent viruses in initiating tumors is termed proviral insertional mutagenesis, and it leads to the activation of endogenous proto-oncogenes (300,516,559). During replication in the infected animal, an enormous number of cells are infected and so acquire new proviral DNA insertions at near-random sites. Each of these insertions constitutes a somatic mutation, and thus retrovirus infection can be thought of as similar to a massive exposure to a potent mutagen. The vast majority of the insertions are harmless, causing no significant change in host gene expression, and the majority of those that do disrupt genes simply create a recessive mutation at one allele out of two present in the cell, again causing no significant change in the overall pattern of gene expression. But very rarely, a provirus insertion can create a dominant-acting mutation that profoundly alters the physiology of the cell. When a provirus integrates near a gene that controls growth and so alters its expression, the cell may proliferate and ultimately form a clonal tumor in which all cells contain the provirus integrated at the same site.

A large number of cellular genes have been identified as potential targets for insertional activation in retrovirus-induced tumors. Among the most notable are an array of transcription factors, including *c-myc, N-myc, c-myb, Fli1, Fli2, Ets1 (Tpl1), Evi1 (Fim3), Bmi1 (Flvi2),* and *Spi1* (PU.1); a number of secreted growth factors, such as *Wnt1 (Int1), Wnt3 (Int4), Int2 (Fgf3),* and *Fgf8;* growth factor receptors, including *c-erbB, Int3 (Notch4), Mis6 (Notch1), c-fms (Fim2),* the prolactin receptor, and Fit1; and genes implicated in intracellular signal transduction pathways, such as the serine/threonine kinases *Pim1* and *Pim2.* Many of these genes are also known to be involved in or implicated in tumorigenesis in other settings, either when transduced on retroviral genomes or when activated by more conventional mutations. However, a number of the proto-oncogenes have only been identified by virtue of their having served as target sites during tumorigenesis by leukemia viruses, and thus this route has made important contributions to our list of known proto-oncogenes. Many of these genes would not have been obvious candidates to control cell division or transform cells, such as the translation initiation factor eIF3 (35).

The patterns of activation of these proto-oncogenes by retroviral insertion are quite varied. At least four distinct mechanisms have been observed (Fig. 18).

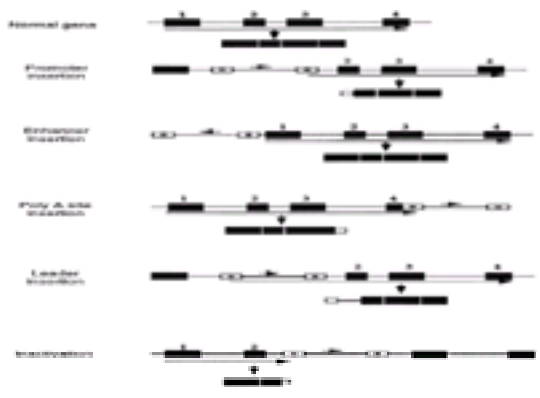

FIG. 18. Genetic alterations in target gene expression induced by retroviral insertional mutagenesis. Various changes in normal gene expression that have been observed upon insertion of retroviral DNA are diagrammed. A target gene containing four exons is used in these examples (*top*). *Promoter insertion:* Insertion of the provirus in the same transcriptional orientation in the first intron is shown to result in the formation of a new mRNA initiated in the 3'LTR and extending into the downstream exons. *Enhancer insertion:* Insertion upstream of the gene, in this case in reverse orientation, is shown enhancing the expression from the natural promoter. *Poly(A) site insertion:* Insertion at the 3' end of the gene in the forward orientation is shown providing a poly(A) addition signal and thereby increasing the levels of a prematurely truncated mRNA. *Leader insertion:* Insertion of the provirus in the same transcriptional orientation is shown to result in the formation of an RNA initiating in the 5' LTR, extending through the provirus and into downstream exons. Splicing results in the retention of only the viral leader on the chimeric mRNA. *Inactivation:* Insertion is shown causing premature end formation of the mRNA, resulting in the formation of an inactive fragment.

1. Promoter insertion. The provirus may insert upstream of the gene or within the gene, and in the same transcriptional orientation as the gene. Transcription beginning in the 3' LTR reads into the gene and results in high-level expression of a transcript with R-U5 sequences at the 5' end. The resulting transcripts may be similar to the natural transcripts, but they may be longer or truncated relative to the normal mRNAs.
2. Enhancer insertion. The provirus may insert either upstream or downstream of the gene, and in either orientation relative to the gene. The insertion brings the powerful transcriptional enhancers present in the U3 regions of the two LTRs into close proximity of the gene, and so activates the endogenous promoter elements. Although the levels are inappropriately high, the structure of the resulting transcript is normal.
3. Posttranscriptional stimulation of expression. The provirus may insert downstream of the coding region and stabilize the formation of an mRNA. The provirus may provide a polyadenylation signal that enhances the formation of stable transcripts, or the insertion may remove RNA destabilization signals in the 3' UTR that would normally mediate the rapid turnover of the RNA. These mechanisms can result in inappropriately high steady-state levels of the mRNA and protein products.
4. Readthrough transcription. The provirus inserts upstream or in the gene, but transcription initiates in the 5' LTR, reads through the provirus, and continues into the gene. The formation of such transcripts is often enhanced by mutations in the provirus, such as loss of the 3' LTR. The transcripts may be spliced aberrantly in complex patterns.

Insertional activation of a proto-oncogene by a provirus is not sufficient on its own to fully transform a cell but represents only one step in a progression to a frank leukemia or tumor. Other mutations are usually required; these mutations can be point mutations in other proto-oncogenes or loss of function mutations of tumor suppressor genes. In some retroviral tumors, more than one oncogene can be activated by insertion of separate proviruses. Similarly, an acute transforming genome is usually not sufficient to transform a cell in one step, and additional mutations must arise. In some tumors induced by a replication-defective transforming virus, the helper virus may provide such mutations by its own insertional activation event (576).

Gene inactivation, as opposed to gene activation, is also an important event in some tumors. Retrovirus insertion can frequently disrupt gene expression to effectively produce a null or hypomorphic mutation (see Fig. 18). These mutations are normally silent, as a second allele would be expected to continue to express a functional gene product. However, if the host animal is already heterozygous due to an inherited germline mutation in one allele, or if the insertional inactivation is coupled to a loss of the other allele by other means, the net result can be homozygous loss of function. When the target gene is a tumor suppressor, the consequence is the promotion of tumorigenesis.

Viral Determinants of Pathogenicity

Several viral genes and sequences can affect the incidence and severity of retroviral disease. The viral LTR contains the most important determinants of leukemogenicity and of the cell tropism for transformation. The enhancer and promoter elements of the LTR are responsible for proto-oncogene activation, and their relative transcriptional activity thus controls the transforming ability of many viruses (220,222,276,335). If these elements are strongly tissue or cell type specific, the virus will be most competent for transformation of those cells in which the LTR is most active. A variety of viruses show profound tropisms for transformation that are controlled in this way (36,84,438,541). For example, the promoter of the MMLV is most active in T cells, and the virus shows strong tropism for the formation of T-cell leukemias. The Friend helper virus LTR contains an enhancer that is most active in erythroid cells, and the virus is correspondingly highly tropic for erythroid cells (86). The promoter of MMTV contains glucocorticoid response elements that provide high-level expression only in cells with high levels of the glucocorticoid receptor and only when exposed to glucocorticoids; as a result, MMTV is specific for mammary tumors (291). Variant betaretroviruses, such as the thymotropic DMBA-LV virus, show selectivity for T cells that is probably attributable to changes in the LTR (42). Determinants of leukemogenicity have also been mapped to *gag*, *pol*, and *env* genes, although in most cases it is not clear what aspects of their functions are required. It may be that vigorous replication *in vivo* is the simple key feature of a highly transforming leukemia virus. There may also be trans-acting functions encoded by the leukemia viruses that modulate expression of specific host genes (219,283). The MuLV and FeLV LTRs encode short RNAs that can trans-activate host genes, apparently through

activation of an AP-1-like activity (270).

Other aspects of infection, distinct from the genetic makeup of the virus or host, can modulate the pathology associated with infection. For example, the route of entry of the virus can affect the disease course (55). Presumably, the route can determine the initial cell types infected and the route of virus spread.

Other Retroviral Diseases

Cytopathic Viruses

Some viruses show distinctive pathogenicity mediated by specific gene products. Cas-Br-E MLV is a well-studied murine virus that induces a hind-limb paralysis with significant neuronal loss in the absence of an inflammatory response (581). Both neurons and glial cells accumulate vacuoles. The virus targets endothelial cells and microglial cells (450) in the brain, and it is likely that the infection of the microglial cells is most crucial to disease induction. Infection may impair or block the neuronal support function of these cells, resulting in loss of neurons, although the mechanism of neuronal cell death is unclear. It is possible that the expression of the Env protein is toxic. The major determinant of pathogenicity is in the SU subunit of the Env protein (449,542). A number of other MuLVs, such as the ts1 mutant of the MMLV TB strain (774), can cause neurologic symptoms, including hind-limb paralysis and spongiform encephalomyelopathy (69), and in these cases too the SU protein is thought to be important (692). TR1.3, a Friend-related MuLV, is a neuropathogenic virus that induces fusion of capillary endothelial cells, leading to a hemorrhagic stroke syndrome (545). The crucial determinant in the virus is a tryptophan residue at position 102 of the SU protein (120,546). In some viruses the LTR is also likely to play a role in disease induction (169,543), perhaps by determining the level of expression and the ability to spread efficiently and access the primary target cell.

A number of the ASLV group of viruses are cytopathic (756) and can cause an acute wasting disease characterized by poor growth, anemia, and immunosuppression associated with atrophy of the bursa and thymus (607). The disease probably reflects the ability of these viruses to lyse infected cells (375,756). Recently, the isolation of the ALV receptor for the subgroup B viruses and its identification as a member of the TNF receptor family suggests the possibility that the binding of Env to the receptor is directly triggering an apoptotic response. The cytopathic and the noncytopathic viruses both seem to be able to trigger similar responses, however, so that it is not clear at this time what aspect of the interaction might be necessary and sufficient for cell killing. The vigorous replication of the virus, and an ability to mediate high-level reinfection before superinfection resistance appears, may also be significant determinants of cytopathology.

Yet another disease caused by a variant virus is the feline acquired immunodeficiency syndrome, or FAIDS. This disease was originally associated with a complex mixture of FeLV isolates. The agent responsible was shown to be an FeLV with mutations affecting the SU subunit of the *env* gene. The mutant FeLV is incapable of establishing superinfection resistance, and so large amounts of unintegrated viral DNA accumulate during superinfection, ultimately leading to high expression of viral gene products and causing cell lysis.

The lentiviruses cause an array of important diseases in animals and humans, most notably AIDS. The major cause of disease is probably cell killing, but the most important target cells and the mechanism by which infection leads to cell death are not clear. The very high level of gene expression mediated by HIV-1 infection in some cell types may be a crucial aspect of the cell killing (668), but the key viral gene products remain obscure. HIV-1 infection eventually leads to depletion of CD4-positive cells and thus to immunodeficiency, culminating in severe opportunistic infections. The lentiviruses also cause a number of other pathologies, including neurologic disease, that are poorly understood. These diseases will be discussed in later chapters.

Stimulation of Host Cell Proliferation

MMTVs lead to the formation of mammary tumors through the insertional activation of a number of proto-oncogenes. However, unlike other simple retroviruses, MMTVs carry an additional gene termed *sag*, for superantigen, that is important for disease induction (see ref. 129 for review). Sag proteins bind to MHC class II molecules in regions that are common to molecules with many different binding specificities, and so they can activate as many as 10% of all T cells. The *sag* gene is located in the U3 region of the MMTV LTR and encodes a low-abundance glycosylated membrane protein (91). The protein must be proteolytically processed for proper export to the cell surface (547). Importantly, expression of a functional Sag protein by MMTV is required to establish infection in an animal. The virus is normally transmitted in mother's milk to newborn mice, infects B cells in the Peyer's patch, and induces a vigorous Sag-mediated stimulation of T cells. There follows a B-cell response that provides a large pool of susceptible B cells for the virus (26), and it is these cells that then carry the virus to the mammary gland. The infection of the mammary epithelial cells ultimately leads to transformation of these cells by insertional activation. This pattern of viral spread through one intermediate cell type, ultimately leading to disease in another cell type, is a paradigm for complex viral pathologies, such as that exhibited by polioviruses.

The *sag* gene of MMTV was also appreciated as acting as a host gene important in disease progression when a number of mouse genes, termed Mls for minor lymphocyte-stimulating antigen, were shown to map to endogenous MMTV proviruses, and were ultimately identified as the *sag* genes. The expression of Mls results in the clonal deletion of many T cells in mice carrying the gene (2). Thus, mice carrying endogenous proviruses will often lose T cells needed for virus replication, and so will be resistant to exogenous MMTV disease.

A number of other viruses carry variants of the normal replication genes that cause specific pathologies in the infected host. One spleen focus-forming virus, SFFV-P, causes a severe polycythemia; infection leads to a massive expansion of erythroid precursors (BFU-E and CFU-E) and a concomitant loss of mature red cells. This agent consists of a complex of a replication-defective variant and a Friend MuLV helper virus to propagate it. The defective genomes carry a mutant *env* gene encoding a shorter SU molecule, termed gp55, that no longer functions to mediate virus entry. However, gp55 can

bind directly to the erythropoietin receptor (EpoR) and stimulate the mitogenic and differentiative responses normally triggered by ligand binding to the receptor. This activity allows the virus to infect these dividing pre-erythroid cells, and the continued expression of the envelope protein in these cells promotes their factor-independent growth and expansion in an autocrine loop (632). Ultimately, a frank erythroleukemia results and may be associated with proviral activation of proto-oncogenes occurring as a result of the continuing infections. It is clear that the *env* gene of the virus is sufficient to cause the disease (773). A very similar virus, the SFFV-A, causes a severe splenomegaly and anemia. This virus is closely related to SFFV-P and also activates the EpoR to expand immature cells. Variation in the envelope between these two strains alters the target cell and the consequences of its expansion (354,631). It should be noted that this mitogenic activity of gp55 is not a completely novel property of the deleted Env. The parental F-MuLV helper Env protein has a weak ability to bind and send mitogenic signals through the EpoR, presumably resulting in expansion of EpoR-positive cells. This increase in target cell number is presumably able to enhance virus spread and so serves as a positively selected trait for the virus. Other Env proteins, including those of the MCF viruses, may activate the interleukin 2 (IL-2) receptor.

Another replication-defective variant, the murine acquired immune deficiency syndrome or MAIDS virus, causes a relatively acute hyperproliferation of B-lineage cells in infected mice (38,327,381). There is a subsequent proliferation of macrophages and CD4+ T cells. The expansion of these cells displaces many other cell types, including T cells, and the animals eventually show a significantly defective immune response. The mechanism of the immunodeficiency is not fully clear, and there is some indication that an antigen-driven stimulation leads to an anergic state. However the immunosuppression occurs, the disease is in reality a lymphoproliferative disorder and is quite distinct from human AIDS in its pathology. The causative agent is again a replication-defective variant carried by a replication-competent helper virus (326). The defective genome encodes a mutant Gag precursor in which the central portion, including the p12 region, is replaced by a foreign Gag derived from endogenous retrovirus sequences (325,398,399). The altered Gag has been shown to interact with the c-Abl protein, a tyrosine kinase first identified as the transduced oncogene of the Abelson MuLV, in that virus expressed as a Gag-Abl fusion protein. Thus, the MAIDS virus seems to act by forming a noncovalent interaction with the c-Abl protein as an approximate mimic of the Gag-Abl protein formed by transduction on the Abelson virus (191).

An ovine disease has recently attracted attention as a potential model for human lung cancer. Sheep pulmonary adenomatosis (SPA) is a contagious bronchiolo-alveolar carcinoma of sheep associated with an exogenous type D/B retrovirus, Jaagsiekte sheep retrovirus (JSRV) (785). The etiology of the disease is unclear, although the epithelial tumor cells are sites of large amounts of viral DNA (537).

Two of the epsilonretroviruses, the piscine (fish) retroviruses, cause a dermal sarcoma that shows a remarkable seasonal appearance and regression. The mechanism of transformation of cells by the virus is not totally clear, but it eems to reflect the activity of a cyclin D homolog encoded by the viral genome. This gene, the *ORF a* gene, may induce inappropriate entry of the cells into cycle by activation of a cyclin-dependent kinase (cdk).

Host Determinants of Retroviral Disease

A number of genes have been identified that determine sensitivity or resistance to retroviral diseases. Some of these genes act at the level of virus replication, directly controlling the ability of the virus to spread. The Fv1 locus is a good example of a gene that acts in a cell-autonomous way to restrict the replication of various MuLVs (348). Fv1 was identified in the early 1970s as mediating resistance to leukemogenesis by the Friend MuLV (424,571). Two naturally occurring alleles provide resistance: the $Fv1^{b}$ allele (in Balb/c mice) allows replication of B-tropic viruses but blocks N-tropic viruses, whereas the $Fv1^{n}$ allele (in NIH Swiss mice) allows replication of N-tropic viruses but blocks B-tropic viruses. Resistance is dominant in heterozygous animals. The tropism of the MuLVs can be characterized by their ability to replicate in cells of particular genotypes: N-tropic viruses grow only on $Fv1^{nn}$ cells, B-tropic viruses grow only on $FV1^{bb}$ cells, and NB-tropic viruses grow on both. The determinants of viral tropism lie in the *gag* gene and affect a small sequence of the CA protein (83,315,532,626). The block to infection in resistant cells is at an interesting stage: largely, it occurs after reverse transcription and before nuclear entry and provirus integration (349,350,782). Curiously, the block to integration that is observed for a particular virus and cell combination *in vivo* is lost when the PIC is extracted and tested for its ability to integrate *in vitro* (585). Recently, the Fv1 gene was cloned and identified as a unique member of an endogenous retrovirus gene family, showing similarity to the *gag* genes of the human endogenous retrovirus (HERV)-L family (68). This family is widely distributed in mammals (58). The two alleles differ by a few point mutations and a different carboxyl-terminal region. Thus, the intracellular expression of this variant Gag protein can somehow interact with the incoming viral DNA and its associated CA protein to block infection. Another gene in this series has an even simpler mode of action: The Fv4 gene (687) restricts virus replication by blocking the receptor. The Fv4 locus is a defective endogenous provirus that encodes an Env protein fragment; the product down-regulates the receptor and renders mice resistant to infection by exogenous viruses (332,394).

A large set of those genes that affect sensitivity to viral disease modify the availability of target cells for virus growth, or the immune response to virus infection, and therefore indirectly control the levels of viremia. The Fv2 gene is an example of such a gene (423). Virus susceptibility is dominant over virus resistance at this locus. The virus-susceptible allele encodes a truncated form of the stem-cell kinase receptor (Stk) that promotes virus-induced erythroleukemia (565); expansion of the Fv2s-expressing cells may provide increased cells for virus replication. The $Fv2^{rr}$ homozygous mice are resistant because of a limited expansion of BFU-E clones and a reduced ability of the Friend MuLV to find sensitive targets (79,688). Finally, mutations in certain genes can sensitize or predispose organisms to oncogenic transformation by retroviruses. Because transformation is almost always a multistep process, mutations in one of the genes in a transforming pathway can increase the frequency with which a virus-mediated loss of another gene becomes manifest as a frank tumor. Thus, knock-out mutations of such tumor suppressors as the p53 or p73 genes can sensitize to transformation by a number of oncogenic retroviruses (53,714). Similarly, a preexisting transgene such as a regulated version of the *myc* oncogene, Eμ-Myc, can predispose particular cells expressing the gene to insertional activation of other proto-oncogenes in viremic animals (733). New integration sites that would not normally be detected in wild-type

mice are often utilized in such mice.

ACUTE TRANSFORMING RETROVIRUSES: TRANSDUCTION OF CELLULAR PROTO-ONCOGENES

There exist more potent transforming retroviruses than the slow leukemia viruses, and these can initiate rapid tumor formation with a quickly fatal outcome. These viruses are recombinant transducing viruses that have acquired portions of cellular genes that are responsible for the transforming activity. The prototype of these viruses is the Rous sarcoma virus, which carries a transforming version of the c-*src* gene. In the exceptional case of RSV, the viral replication functions, including the coding regions for the Gag, Pol, and Env proteins, are all intact so that the resulting transducing genome is replication competent. In nearly all other cases, the acquisition of the transforming gene from the host has occurred with a loss of one or more of the viral replication functions, so that the resulting virus is replication defective. However, these genomes retain all the cis-acting elements needed for their replication, and thus can be transmitted from one cell to another by a replication-competent helper virus. The concerted replication of two viral genomes in a complex—a replication-competent helper virus and a replication-defective acute transforming virus—is a common feature of most of the transforming viruses.

Transforming viral genomes exhibit a range of different structures, but they have some features in common. Those segments required in cis for viral replication are always retained: The LTRs, the pbs, and the ppt are present because they are required for reverse transcription and forward transcription. The RNA packaging signals are retained. Many of the regions required in trans are often deleted, since these functions can be provided by the helper, and are replaced with the host sequences. The host gene may be expressed separately, or, more often, it is fused to Gag, Pol, or Env sequences to form a fusion protein.

The formation of a transducing virus is thought to involve a complex series of events that results in the acquisition of the coding regions of a host gene by the replication-competent parental virus (690). Several models have been proposed to account for the observed structures, including DNA-based events (e.g., see ref. 647) but more often RNA-based events (summarized in ref. 716). The most commonly accepted model includes the following steps (Fig. 19):

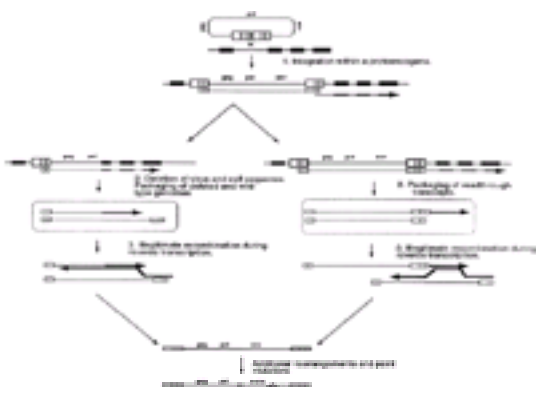

FIG. 19. Two pathways for the acquisition of host oncogenes by a replication-competent retroviruses in the formation of an acute transforming genome. Integration is shown establishing a provirus within a proto-oncogene in the same transcriptional orientation (*top*). Either of two processes then occurs. In one mechanism (*left*), a deletion of the chromosomal DNA fuses the 5'half of the provirus to the downstream portion of the gene. The fused DNA then encodes a fused RNA, which may be spliced and packaged into virion particles along with wild-type helper RNA. During reverse transcription, RT switches from the helper to the fusion RNA to append the 3' portion of the helper onto the hybrid RNA. The completed reverse-transcribed DNA is integrated and transmitted thereafter as a replication-defective viral genome. In the other mechanism (*right*), a readthrough RNA extending from the 5' LTR through the provirus and into the downstream portion of the gene is formed. The RNA is packaged into virion particles along with wild-type helper RNA. During reverse transcription, RT switches from helper to host and back to helper RNAs to form the hybrid genome. As before, the completed reverse transcribed DNA is integrated and transmitted thereafter as a replication-defective viral genome. In either scenario, the transducing genome may undergo additional rearrangements and mutations under selective pressure for more efficient transforming activity and transmission.

- The process begins with the insertion of a provirus upstream of the gene to be transduced. An insertion in the middle of a gene can initiate the transduction of the downstream portion of that gene.
- Next, readthrough transcription beginning in the 5' LTR generates a large RNA containing viral sequences fused to downstream sequences. This event can be enhanced by lesions in the 3' LTR that prevent normal RNA processing and polyadenylation at this site. Alternatively, a deletion in the DNA could fuse the 5' half of the provirus to downstream sequences, again leading to the expression of a fusion RNA.
- In either mechanism, the chimeric RNA can be spliced and is then packaged into virions along with the RNA of a helper virus (307,689).
- Finally, nonhomologous recombination occurs during reverse transcription to append 3' viral sequences to the chimeric genome. A template switch by RT from the helper to the chimeric RNA during minus-strand synthesis can mediate such a nonhomologous event at low but easily detected frequencies (274,710,799). The completion of reverse transcription on this template would result in the generation of a provirus with host sequences flanked by viral termini, similar to those seen in transforming retroviral genomes. Consistent with this model is the appearance of poly(A) sequences at the 3' junction between host and viral sequences in some viruses; if the translocation by RT from viral to host RNA occurs in the host poly(A) sequences, a portion will be retained in the final genome.

A key feature of the resulting genome is the presence of only the mRNA sequences—that is, only the exons—and not the

introns of the host gene. Thus, very large genes can be transduced by retroviruses because they carry only the exonic coding regions of the gene. Most transforming retroviral genomes not only are a result of these relatively simple recombination events but also have undergone multiple rearrangements thereafter. The RNAs encoded by these genomes often exhibit complex patterns of splicing, which can involve cryptic splice sites in both virus and host sequences. Several of the known rodent viruses carry segments of endogenous retroviral or virus-like sequences, especially the virus-like 30S (VL30) elements.

The genes that have been identified on the many acute transforming viruses are wildly diverse in their sequence and functions. These genes are among the most intensively studied of all known genes; their clear involvement in oncogenesis has focused enormous attention on their structures and function. The genes include growth factors (v-*Sis*); growth factor receptors (v-*erbB*); intracellular tyrosine kinases (v-*src*, v-*fps*, v-*fes*, v-*abl*), members of the G protein family (H-*ras*, Ki-*ras*); transcription factors (v-*myc*, v-*erbA*); and many others. The genes are now appreciated as playing major roles in mitogenic signalling pathways, in the control of the cell cycle, and in antiapoptotic pathways that act to limit cell survival. There is no indication that all such genes have been identified, and it is likely that new transforming viruses will continue to provide new examples of genes that can be activated by transduction to initiate tumor formation.

The acquisition of these genes, as noted earlier, is often associated with fusion of the coding region to Gag, Pol, or Env sequences. Thus, the expression of the oncogene results in a fusion protein that may exhibit dramatically altered biochemical activity, intracellular localization, or stability. These changes are often a key aspect of the activation of the normal function of the proto-oncogene to create the fully transforming viral oncogene. In other cases, or in addition to these alterations, there may be specific mutations that arise during or after the transduction process. These mutations, which may be as simple as a point mutation, or as drastic as a frameshift or deletion mutation, can be the major cause of activation of the oncogene. Presumably, the high rate of mutation during viral replication and a selection for tumor formation in enhancing virus spread are responsible for the appearance of these mutations.

ENDOGENOUS VIRUSES AND VIRUS-LIKE SEQUENCES

Virtually all cells contain a large number of retroviral or retrovirus-like DNA elements integrated into the germline (for comprehensive reviews, see refs. 75,439,558, and 682). These endogenous retroviral elements can represent a substantial fraction of the total DNA in a genome; whereas the sequences most closely related to the exogenous retroviruses may represent only a percent or so of the total DNA in many species, the retroelements in total can occupy 10% or more of the genome (709). These elements have presumably accumulated over evolutionary times, with no mechanism by which they can be removed and no strong selection against individuals that acquire them.

The retroviral provirus is closely related in structure and mode of replication to transposable elements—the retroelements—found in the genomes of all living things, from bacteria to humans (74). Many of these elements are remarkably similar to proviruses, with LTRs that function similarly and with sequence similarity to *gag* and *pol* genes (715). The retroviruses have probably existed as parasites of cells from very ancient times and have evolved together with transposable elements (179,180).

Endogenous Elements in Chickens, Mice, Pigs, and Humans

Endogenous retroviruses have been characterized for many avian species. Large numbers of sequences have been characterized in chickens and other birds (329) and can be grouped into at least four families. The ALV-related elements were among the first to be discovered, including the replication-competent provirus RAV-0. Most of the other family members are replication defective and lack *env* sequences. The newer families of such viruses continue to be characterized (665).

There is a vast literature describing the endogenous retrovirus sequences in inbred mice (58,80,138,336,395,683). At least eight families have been described, although only four have been studied in detail. The VL30 (for virus-like 30S) elements are present in the genome at a copy number of perhaps 100 to 200 (4); these elements encode a 30S RNA that is packaged efficiently into the virion particles of exogenous viruses and so contaminates most virion RNAs. They presumably represent a parasitic RNA that spreads by exploiting exogenous viruses. Second, the IAP elements are present at a level of about 1,000 to 2,000 copies in the genome (444,518,525). These elements can express intracellular particles containing RT, but the particles are budded into the ER and not released from cells. Most lack *env* genes and so cannot form infectious particles. However, a few members o contain *env* sequences (608). Some IAPs can transpose intracellularly at low frequencies (301). Third, there are a small number of proviruses (0 to 4 per genome) closely related to MMTV that encode functional B-type viruses. Fourth, there are proviruses related to the exogenous MuLVs, present at 50 to 100 copies per genome (683). These proviruses are all very similar to one another, but they can be divided (according to their similarity to exogenous viruses that utilize particular receptors) into four groups: the ecotropic, xenotropic, polytropic, and modified polytropic. The distribution of these sequences among different murine species and subspecies can help reveal their evolution and spread. For example, xenotropic MLV-related proviruses are present only in *M. musculus* subspecies, whereas polytropic MLV-related proviruses are found in both *M. musculus* and *M. spretus.* Replication-competent members of the family are found in many, but not all, inbred mice (128). (For reviews of the properties of the murine endogenous retrovirus genomes, see refs. 245 and 344).

Recently, the potential to use pig organs or cells in xenotransplantation into humans has raised considerable interest in the presence of endogenous retroviral elements in the pig (522,684). Although viruses can be rescued from porcine cells (9), and although these viruses can infect human cells quite efficiently (557,768), preliminary studies suggest they are not easily transmitted to humans in transplant settings (305,555). There remains a real possibility for their transfer to humans, however, and the consequences could be significant.

Retroviral elements are also abundant in the human genome (73,439,463,468). These elements are collectively termed HERVs, and subgroups are denoted by a letter indicating the amino acid specificity of the tRNA primer. Most are defective, but a very small number of these elements are still actively transcribed in somatic cells and are capable of

transposition to new sites (176). The distribution of the HERV-K family in various primates has been surveyed to help build evolutionary trees of these species (347,678). There are also provirus families distantly related to the lentiviruses (317); some have the potential to encode rev-like elements that could, in principle, be pathogenic (440). A detailed review of the HERVs is presented in Chapter 62.

Properties of the Endogenous Provirus-like Elements

The distribution of elements in a given strain is relatively stable over the course of a few generations. Thus, most individuals in an inbred population show a constant, characteristic pattern of endogenous elements. The rate of loss of a given provirus is very low, and the appearance of new proviruses is rare in most animals. However, the pattern is quite different in different species, and even in different strains of animals, suggesting that rearrangements happen often over longer evolutionary times. It is known that new copies can appear at higher frequency if newborn females are viremic (431,675). Thus, early infection of germ cells can introduce new proviruses into the germline. This route can even be used to create mutations *de novo* in laboratory mice at reasonable frequencies.

Most endogenous proviruses are transcriptionally silent; the DNA is often heavily methylated and so repressed. These may reflect the mechanisms by which the transcription of many exogenous viruses are repressed *in vivo*. Expression of many of the endogenous viral RNAs is induced by agents causing DNA damage, such as ultraviolet light and bromodeoxyuridine, and the expression of others is stimulated by glucocorticoids (207). The IAPs are often induced during the differentiation of various cell types (695), and even more often in immortalized tumor cell lines.

The bulk of the endogenous retroviruses are fossil DNAs, grossly defective, and no longer capable of encoding proteins; the ORFs contain numerous stop codons and frameshifts that would preclude the formation of any functional viral gene products. However, these elements can often give rise to RNAs, and these RNAs can be packaged efficiently by virions encoded by exogenous viruses and thus can give rise to new proviruses. Furthermore, these copackaged RNAs can then recombine with the exogenous viral RNAs and so contribute small sequence blocks to these viruses, potentially altering the host range and replication properties of the virus. The continuous contribution of endogenous sequences to virus evolution is a fact that needs to be considered whenever genetic selections are imposed on a virus. In addition, a few of the elements are functional and can transpose intracellularly (705) or even give rise to replication-competent viruses. Even when viruses are induced from the elements, however, the viruses are most often not highly pathogenic for the host in which they reside. Thus, many of the inducible elements in the mouse are xenotropic and cannot spread in the animal; and those that can, do not cause an acute disease. The LTRs of the endogenous elements are often quite weak as transcriptional promoters as compared to those of exogenous viruses. This may reflect selections against highly pathogenic agents either before or after their introduction into the germline.

The creation of a new provirus in the germline by necessity creates a mutation, and although most such insertions probably have no significant effect, occasionally deleterious germline mutations occur. A number of ancient, “spontaneous” mutations, upon analysis, have been found to have been caused by a proviral insertion. These include such classic mutations as the rd1 allele, which causes a slow retinal degeneration, and which includes an insertion affecting the beta subunit of the retinal cGMP phosphodiesterase; the hr mutation, which causes a hairless phenotype; the dilute coat-color allele d; and a mutation termed Slp (for sex-limited protein) in the C4 complement gene, in which an insertion of a viral LTR renders the gene androgen responsive.

Many of the endogenous elements may be positively selected in their host species. This may be the result of advantageous mutations that are created by the insertion, or antiviral effects mediated by the gene products encoded by the endogenous proviruses. The Fv1 and Fv4 genes are examples of such elements. These virus-like elements confer resistance to exogenous viruses and so may serve to protect the host from leukemia induced by infection. The MMTV *sag* gene, if present on an endogenous provirus, acts to delete T cells that would respond to the superantigen; thus, subsequent infection by an exogenous MMTV cannot use *sag* to induce a proliferation of cells needed for its vigorous replication. Thus, the inherited provirus protects the host from MMTV disease.

RETROVIRAL VECTORS, PACKAGING LINES, AND GENE THERAPY

The structure and mechanism of transmission of the naturally arising replication-defective transforming viral genomes provide a clear model for the directed use of retroviruses to mediate gene transfer. Retroviral vectors that mimic the structure of the transforming viruses can readily be generated, and these vectors can be engineered to carry the cDNA sequences of virtually any gene. These genomes can then be propagated with wild-type virus as helper. However, it is also possible to generate helper-free preparations of particles that transduce the vector genome via the early steps of the life cycle, without delivering the helper genome, thereby preventing subsequent spread of the vector. These helper-free particles are generated in packaging cell lines: cells engineered to express the *gag*, *pol*, and *env* genes but not expressing packageable helper viral RNAs. The first such lines simply carried a provirus lacking the Psi site, the RNA packaging signal (460). These cells produce virions deficient in the helper genome, and introducing a Psi+ vector construct into these cells results in the encapsidation and release of the vector RNA into those particles. These particles can then be harvested and used to deliver the vector and its gene into susceptible cells. It is also possible to generate transducing virus preparations by transiently transfecting cells with DNAs that encode the helper functions and DNAs that encode the vector. This approach is preferable in instances where the viral gene products are toxic and therefore difficult to express stably in a packaging cell line.

A limitation of these packaging systems is that small amounts of the Psi-minus helper RNA are encapsidated along with the vector. Endogenous retroviral genomes, such as VL30 RNAs (4), are also encapsidated efficiently, and recombination events between these RNAs during reverse transcription can recreate a replication-competent virus (556,641). These events are probably similar to recombinational repair of mutations in genomes that occur during growth in cell culture (132,133,470). This issue has raised considerable fears that gene therapy vectors intended for therapeutic use could initiate a viremia, and perhaps a viral leukemia, in patients. More elaborate cell lines, in which the *gag*, *pol*, or *env* genes are expressed via separate RNAs, can reduce the frequency with which such recombination events occur to

very low levels (142,157,465,466).

Retrovirus particles transducing a desirable gene can be directed to target cells through the use of many distinct envelope proteins. This method is possible because retrovirus particles can readily form pseudotypes; that is, they can incorporate and use the envelope proteins of a wide array of different viruses (209,299,676,771). The wide range of pseudotypes that can be formed presumably reflects the flexibility of the core–envelope interaction. The host range can be further expanded or restricted by the engineering of envelopes with new binding specificities (731). Chimeric envelope molecules have been particularly popular tools in targeting virions to new receptors (119,360,464,669). Another approach is to engineer animals that express a foreign receptor in a tissue-specific manner, and deliver genes with a virus envelope that recognizes only the transgenic receptor (228). The envelope–receptor interaction can even be reversed: It is possible to express a particular virus receptor molecule on the virion surface, and thereby to target the virus to those cells expressing the corresponding viral envelope (43).

A major limitation of early retroviral gene therapy efforts is the inability of most helper viruses to mediate the infection and transduction of nondividing cells. The major block is during the early stages of infection, when there is a strong requirement for cell division for infection by most viruses (624). However, the lentiviruses have the ability to infect nondividing cells, and thus gene therapy based on lentiviral packaging systems could overcome this limitation (for review, see ref. 15). Recent efforts have substantiated these expectations: Delivery to nondividing neurons, and to poorly dividing primary lymphocyte cultures, has been demonstrated with vectors based on HIV-1 (72,185,355,506,507) as well as FIV (575).

PERSPECTIVES

The study of retroviruses has led to a detailed characterization of many steps of virus replication but also to important fundamental discoveries concerning host physiology and genetics. The viruses have served as entrés into such phenomena as cell surface receptors, cell division, DNA synthesis, the cell cycle, mechanisms of gene expression, and intracellular transport. The value of focusing on retrovirus functions in unraveling cellular functions is clear: These agents have evolved over huge periods of time to exploit key aspects of the cell, and we should make use of their success to help identify those aspects. There is every reason to believe that their continued study will yet reveal new aspects of cell physiology.

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CHAPTER 58 Human T-Cell Leukemia Virus Types 1 and 2

Patrick L. Green and Irvin S. Y. Chen

For nearly two decades, the human T-cell leukemia viruses (HTLVs) have been intensely studied because of their association with neoplasia and neuropathology, and their capacity to transform primary human T-cells in cell culture. The study of HTLV also represents an attractive model that has allowed investigators to dissect the mechanism of various cellular processes, several of which may be critical steps in HTLV-mediated leukemogenesis. This chapter will review the molecular biology of HTLVs, including the organization of their genome, the structure and function of their gene products, and the pathogenic features both in cell culture and in infected patients. Given the enormous amount of material published on HTLV, we apologize to colleagues whose data could not be cited because of space limitations.

HISTORY OF HUMAN T-CELL LEUKEMIA VIRUS DISCOVERY AND ASSOCIATION WITH DISEASE

Human T-Cell Leukemia Virus Type 1

Adult T-cell leukemia (ATL) was first described in 1977 in Japan (346). Since then, ATL has also been found in most other parts of the world. The etiologic role for HTLV-1 in ATL was established by (a) seroepidemiologic surveys associating the virus with disease in patients with ATL and close contacts; (b) the clonality of ATL tumor cells, which correlates with a monoclonal or oligoclonal pattern of integration of the viral genome in the tumor cell DNA; (c) infection of lymphocytes *in vitro*, resulting in immortalization or transformation of the same cell type (CD4+ or CD8+ T-cells) as the tumor cell; and (d) showing HTLV-1 to be oncogenic in animal models.

HTLV-1 was first identified in a T-lymphoblastoid cell line (HUT 102) established from a patient with a cutaneous T-cell lymphoma (269). C-type retrovirus particles were identified in this cell line. In 1981, an independently isolated cell line (MT-1) derived from a patient with ATL also harbored a retrovirus and produced antigens that reacted with sera from patients with ATL (121). The viruses produced from these two distinct cell lines were subsequently shown to be the same and were given the name HTLV-1 (270). Since the initial description of ATL and discovery of HTLV-1, the virus has been recognized as being associated with human diseases other than ATL. The most notable of these is a neurologic degenerative disorder known as tropical spastic paraparesis (TSP) or, more commonly, HTLV-1-associated myelopathy (HAM).

Human T-Cell Leukemia Virus Type 2

HTLV-2 was first identified in a T-cell line established from a patient with hairy-cell leukemia (294). This cell line (Mo-T) was derived from splenic tissue in 1978 and subsequently shown to harbor a retrovirus. Based on serologic cross-reactivity, this virus was related to (but was distinct from) HTLV-1, and it was termed HTLV-2 (150). Since then, HTLV-2 infection has been documented in a number of population groups. Like HTLV-1, HTLV-2 infects and transforms T-lymphocytes *in vitro*. A few HTLV-2-infected patients have been characterized in detail, providing limited molecular evidence supporting an etiologic role of HTLV-2 in a form of atypical hairy-cell leukemia (281). In addition, there have been a few cases of neurologic disease associated with HTLV-2 infection (125,199). The limited number of individuals shown to harbor HTLV-2 in association with specific diseases has, to date, precluded convincing epidemiologic demonstration of a definitive etiologic role for HTLV-2 in human disease.

GENETIC STRUCTURE

The overall genetic structure of HTLV is similar to but distinct from that of other retroviruses (Fig. 1). In addition to the usual structural and enzymatic genes of *gag*, *pol*, and *env*, there is a unique region at the 3' end of the genome not found in other replication-competent retroviruses. Because of its unknown function, this portion of the genome was initially referred to as the X region. There are four (I through IV) open reading frames (ORFs) contained within the X-region of HTLV-1/-2. Two of these ORFs (III and IV) encode well-characterized *rex* and *tax* genes, respectively. The *tax* gene (previously referred to as pX, c, x-lor, tat-I/tat-II, and TA-1/TA-2) encodes a protein responsible for transcriptional activation of the long terminal repeat (LTR). The *rex* gene is necessary for expression of structural and enzymatic proteins, and it partially overlaps *tax* in an alternative reading frame. Three major messenger RNA (mRNA) species have been identified for HTLV (see Fig. 1). Like other retroviruses, full-length RNA is utilized for synthesis of *gag* and *pol* gene products and is also the genomic RNA packaged into virions. The primer used for reverse transcription of the genomic RNA is the cellular tRNAPro. A single-spliced subgenomic mRNA encodes the *env* gene product. A second subgenomic mRNA has two introns removed (doubly spliced or completely spliced) and encodes the *tax* and *rex* gene products. The *tax* gene initiation codon is the same as that used for the *env* gene and is contained within the second exon of the *tax*/*rex* mRNA. The initiation codon for the Rex protein is also located within the second exon of this mRNA, a further 59 nucleotides upstream. Several minor alternatively spliced mRNA species have been detected by reverse transcriptase–polymerase chain reaction (RT-PCR) or RNase protection assays in cells isolated from patients with leukemia or HAM/TSP. These RNAs encode proteins from ORFs I, II, and III of the pX region. Figure 1 shows the arrangement of the HTLV genome, gene products, and ORFs, and the three major and four minor mRNA species. Table 1 gives a comparison of the entire HTLV-1/-2 genomes based on the sequence of HTLV-1 ATK and HTLV-2 MO isolates (301,312).

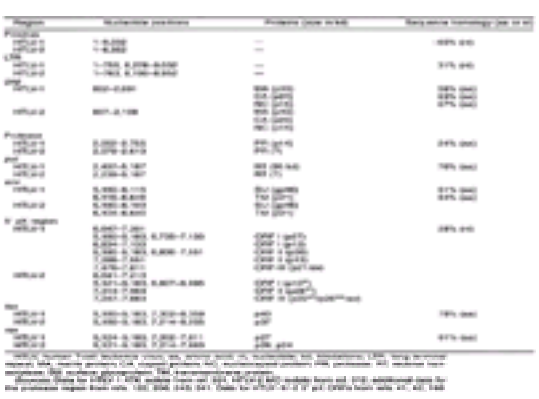

TABLE 1. *Comparison of the HTLV-1 and HTLV-2 genomes*

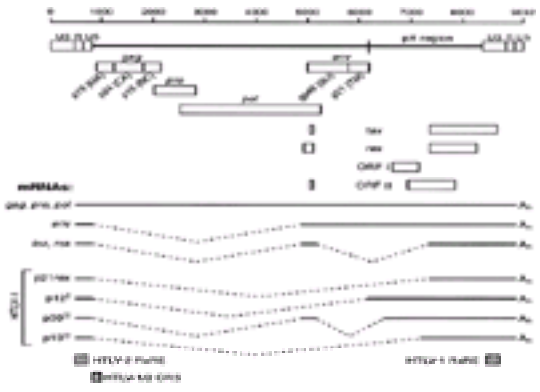

FIG. 1. Structure and organization of the HTLV genome. The HTLV provirus genome is shown at the *top* of the figure. Location of the proteins encoded by the provirus, the RxRE, and cis-acting repressive sequence (CRS) are shown beneath the provirus. The structures of the three major messenger RNA species and four minor RNA species produced are shown at the *bottom* of the figure. [From refs. 168 and 301 (HTLV-1) and ref. 312 (HTLV-2).]

HTLV GENE PRODUCTS

The *gag* Gene

As in other retroviruses, the *gag* region is initially translated as a polyprotein precursor, which is subsequently cleaved to form the mature Gag polypeptides, the 19-kd matrix (MA), 24-kd capsid (CA), and 15-kd nucleocapsid (NC) proteins (259). Like other retrovirus *gag* gene products, p19 is posttranscriptionally modified and contains myristic acid at the NH_2-terminus (259). Myristoylation targets the p55 Gag precursor polypeptide to the inner surface of the cell membrane (114). As is the case with other retroviruses, it is likely that this association is important in the assembly and budding of virus particles from infected cells.

A variety of other Gag-related polypeptides are found in HTLV-infected cells (296), including species of 70, 55, 37, 29, and 28 kd. Peptide mapping of these proteins indicates that some are immature precursors of the three major Gag proteins (e.g., p55 and p29, both of which have determinants related to the mature p24 and p19 peptides), but pulse-chase experiments suggest that p55 is the genuine precursor molecule. At its 3' end, the *gag* ORF overlaps the 5' end of the reading frame that encodes the protease (see Fig. 1).

Protease

In HTLV, a reading frame that spans the 3' part of the *gag* region and the 5' part of the *pol* region encodes the protease. Synthesis of the protease as part of the Gag polyprotein precursor is accomplished by ribosomal frameshifting (243). The function of the HTLV-1 protease has been examined *in vitro*, and these studies have shown that the protease is responsible for processing the mature Gag products and is responsible for generating a mature form of itself by autocatalyzed self-cleavage (243).

The *pol* Region

The polymerase region is potentially able to encode an 896-amino-acid product in HTLV-1 and a 982-amino-acid product in HTLV-2 (301,312). However, a second ribosomal frameshifting event is necessary to express the *pol* gene (243), resulting in polypeptides from the *pol* reading frame of 863 and 864 amino acids in length for HTLV-1 and HTLV-2, respectively. The 5' portion of the *pol* gene encodes the reverse transcriptase protein and sequences further downstream encode the integrase and RNaseH functions. HTLV reverse transcriptase functions most efficiently using Mg^{2+} as the required divalent cation (275).

The *env* Gene

The product of translation of the envelope ORF is a glycoprotein of 61 to 69 kd, depending on the laboratory and cell line studied (186,296,297). This precursor protein is detected on the surface of HTLV-infected cells. Treatment with tunicamycin or endoglucosamine H results in a nonglycosylated precursor species of 54 kd (186,297). Some higher-molecular-weight Env-related species have been shown to be the result of abnormal Env-Tax fusion proteins (219,332). The envelope protein is cleaved into the mature products, the 46-kd surface glycoprotein (SU) (gp46) and the 21-kd transmembrane protein (TM) (p21). Culture media from HTLV-transformed cells contain large amounts of free gp46, which is shed from the surface of the cells (296,371).

The *tax* Gene

Two unique transregulatory genes are found in HTLV, *tax* and *rex* (see Fig. 1). Both genes are essential for viral replication and cellular transformation. The HTLV-1/-2 *tax* genes encode phosphoproteins of 40 and 37 kd, respectively. There is approximately 77% to 85% amino acid homology between HTLV-1 Tax (Tax-1) and HTLV-2 Tax (Tax-2). Both Tax-1 and Tax-2 co-localize primarily in nuclear bodies with RNA polymerase II, splicing complexes, and specific transcription factors (19,88,304,320). Structure/function analysis has revealed at least four functional domains (Fig. 2). The amino-terminal amino acid residues 2 to 58 contain an activation domain and also comprise a nuclear localization signal distinct from the highly basic amino acid sequence that performs this function in most nuclear proteins. The amino-terminal residues 23 to 49 contain the zinc-binding domain (zinc finger) that very likely plays a critical role in protein–protein interactions. The central amino acid residues 161 to 211 constitute the structural domain, and the carboxyl-terminal 289 to 322 residues contain an activation domain. The transactivation phenotypes of specific mutations within these regions will be discussed later. It has been reported that two adjacent serine residues at the carboxyl terminus of Tax-1 (amino acids 300 and 301) are the major sites of phosphorylation. Phosphorylation of at least one of these sites is required for Tax-1 localization to nuclear bodies and Tax-mediated activation of gene expression (20).

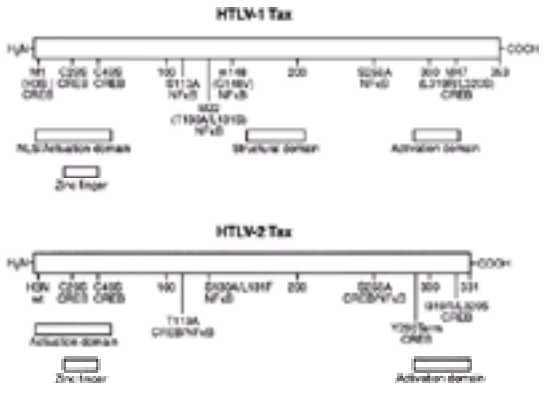

FIG. 2. Structural and functional domains of HTLV-1 Tax and HTLV-2 Tax. Specific mutations and the transcriptional activation pathway interrupted are indicated. [From refs. 305,306,321,323,373, and 376 (HTLV-1 Tax) and ref. 283

(HTLV-2 Tax).]

The Tax protein is a trans-acting transcriptional activator, and it increases the rate of transcription initiation from the promoter in the 5' LTR of the provirus genome. It was originally found that transcription of the HTLV LTR was greatly stimulated in virus-infected cells compared with that in noninfected cells (39,324). The role of the *tax* gene was investigated further in assay systems involving co-transfection of cells with *tax* cDNA and reporter plasmids. By determining what genetic information is necessary to stimulate transcription in trans from the reporter plasmid (trans-activation), it was confirmed that the *tax* gene was responsible for this activity.

The *rex* Gene

The *rex* gene encodes proteins of 27 or 21 kd for HTLV-1, and 26 or 24 kd for HTLV-2. The larger p27 and p26 products are both phosphorylated, which most likely plays a role in their function. Antisera directed against peptides from the carboxyl-terminal portion of the *rex* ORF immunoprecipitate both proteins (see Fig. 1). Originally, a study utilizing *in vitro*-synthesized RNA and selective mutations within the *rex* ORF suggested that deletion of an internal methionine codon (amino acid 79) resulted in loss of the smaller HTLV-1 p21 product (238). It was later shown that a novel, alternatively spliced mRNA transcript was responsible for p21 production in HTLV-1-infected cell lines (see Fig. 1) (258). Experiments have shown that the internal methionine codons of the HTLV-2 *rex* gene are not required for p24 production, and that the p26 product is actually a phosphorylated form of p24 (97,98). Subsequently, in HTLV-2 infected cells, an alternatively spliced mRNA transcript was shown to have the capacity to produce protein products (p22XIII/p20XIII) analogous to HTLV-1 p21 (41).

The larger Rex protein is involved in the control of HTLV gene expression, but the function of the smaller HTLV protein (p21 or p22/p20) has not been established and will not be discussed further here (41,116). Like the Tax protein, the Rex protein is essential for HTLV replication. Unlike Tax, Rex acts at a posttranscriptional level to regulate viral gene expression. Rex has been shown to increase the ratio of the incompletely spliced mRNA to completely spliced mRNA in the cytoplasm of infected cells (138,174). The ultimate effect of expression of the *rex* gene products in HTLV-1-/-2-infected cells is therefore to regulate the levels of expression of genes encoding virion components, and thus determining whether infectious virions are produced.

The effects of Rex are mediated through specific cis-acting sequences in the LTR. Augmentation of 5' LTR-linked gene expression by Rex requires two distinct cis-acting sequences, one termed the Rex-responsive element (RxRE), which allows Rex to overcome the inhibitory effect of a second, termed the cis-acting repressive sequence (CRS). In HTLV-1, the RxRE was first localized to the 3' LTR of the virus (338), but it has subsequently been shown to be present, at least in part, in the 5' LTR as well (5). The U5 region of the LTR contains sequences functionally defined as the CRS (see Fig. 1) (303). The HTLV-2 RxRE is located in the R/U5 region of the 5' LTR, and the CRS is contained within the RxRE downstream of the splice donor site (see Fig. 1) (23,377).

The phosphorylated *rex* gene products of HTLV-1/-2 are localized in the nucleus (and particularly in the nucleoli) of infected cells (1,98,99). Functional analyses have revealed that Rex-1 contains at least three domains. One domain corresponds to an arginine-rich region that mediates RNA binding by direct interaction with the RxRE (27,91). A second domain close to the RNA-binding domain is required for assembly of multimeric Rex complexes onto the RxRE (27). A third activation domain, represented by a short leucine-rich sequence, interacts with Rab and other nucleoporin-like cofactors to mediate nuclear export (28,129,265,362). Dominant negative mutations have been identified, some of which reside in the multimerization domain of the protein (27).

The mechanism by which the Rex/RxRE interactions culminate in the synthesis of viral structural and enzymatic proteins are not yet clear, but the experimental data suggest it may involve stimulation of nucleocytoplasmic transport, increased stability, inhibition of splicing, and/or facilitation of translation of the incompletely spliced RNAs. Studies investigating the intracellular distribution of RNAs transcribed from indicator plasmids containing RxRE DNA demonstrated that Rex-1 stimulates the nucleus-to-cytoplasm transport of incompletely spliced HTLV-1 and human immunodeficiency virus (HIV)-1 RNA (110,137). Another study demonstrated that Rex-1 activity resulted in large increases in the amounts of unspliced viral RNA by reducing the rates of intron excision and degradation in the nucleus (103). A more recent study investigating Rex-2 function in T-cells following transfection of full-length proviral clones indicated that Rex-2 increases the stability of the unspliced *gag*/*pol* RNA and promotes the nucleus-to-cytoplasm transport of the incompletely spliced RNAs (174). The increase in incompletely spliced RNAs was associated with a slight reduction in completely spliced *tax*/*rex* RNA, a result consistent with the possibility that Rex inhibits pre-mRNA splicing, which has been reported *in vitro* (9), but which may also be caused by a redistribution of the completely spliced RNA pools. It has been suggested that Rex-1 stabilizes human interleukin-2 receptor (IL-2R)-a chain mRNA and may therefore contribute to cell transformation (151). Prevented from accumulating in the nucleus, Rex-1 fails to stabilize IL-2R mRNA (365). Because Rex is critical for viral replication, it has been difficult to directly assess its role in cellular transformation.

pX Region ORFs I and II

A region of approximately 600 nucleotides is present between the end of the *env* gene and the beginning of the third exon of the *tax* and *rex* genes in HTLV-1, HTLV-2, and the related bovine leukemia virus (BLV). These pX sequences were originally referred to as the nontranslated or untranslated region, because expression of the ORFs within this region had not been documented in virus-infected cells. This region of the genome has been evolutionarily conserved in the HTLV family of viruses. However, the nucleotide sequence homology of the 5' pX sequences between HTLV-1 and HTLV-2 is relatively low compared to the rest of the genome (see Table 1).

Reverse transcriptase-PCR and RNase protection analyses have been used to identify viral RNAs in HTLV-1- and

HTLV-2-infected cells that splice into ORFs contained within the pX region (18,42,169). These RNAs have been detected in *in vitro* infected cells, in cells isolated from infected asymptomatic individuals, and in cells from patients with ATL or HAM/TSP. Characterization of three of these alternatively spliced HTLV-1 RNAs revealed that they have the potential to encode proteins from pX sequences termed ORF I and ORF II (ORF III and ORF IV encode Rex and Tax, respectively) (see Fig. 1). Using mRNAs from ORF I and ORF II, cDNAs were constructed to express hemagglutinin 1 tagged proteins in COS-1 and HeLa-tat cell lines (169). It appears that the preferential product expressed from ORF I is a 12-kd protein, $p12^{I}$, although a protein of 27 kd has also been reported. This $p12^{I}$ is highly hydrophobic and localizes to cellular endomembranes (168). It contains four minimal SH3 binding motifs (PXXP) and when overexpressed it associates with the vacuolar H^{+} ATPase, and it appears to bind the b and g chains of the IL-2 receptor complex (69,169,233). It was demonstrated that $p12^{I}$ had similar structural features to and cooperated with the E5 oncoprotein of bovine papillomavirus type 1 in transformation of mouse C127 cells (69). In HTLV-2, the protein product expressed from ORF I is $p10^{XI}$ (41).

ORF II-containing HTLV-1 RNAs encode two proteins, a full-length $p30^{II}$, and a truncated $p13^{II}$ that is derived from initiation at the first internal methionine codon in ORF II and corresponds to the carboxyl-terminal 87 residues of $p30^{II}$ (see Fig. 1). ORF II, in an alternate reading frame, extends into the last exon of *rex* and *tax* (ORF III and ORF IV, respectively). The $p30^{II}$ and $p13^{II}$ proteins are localized to the nucleus/nucleolus and mitochondria, respectively (43,168). The cellular segregation of ORF II gene products suggests that these proteins may serve a regulatory function. $p30^{II}$ has distant homology to transcription factors Oct-1 and 2, Pit-1, and POU-M1. Cells transformed by HTLV-1 molecular clones with mutations in $p30^{II}$ and $p13^{II}$ have differential patterns of phosphorylation of the signal transduction adaptor protein Vav, suggesting their role in alteration of T-cell signaling (202). However, ORF II sequences from ATL patients frequently contain premature stop codons, suggesting that this ORF may not be important for the long-term maintenance of the transformed phenotype. In HTLV-2, the protein product expressed from ORF II is $p28^{XII}$ (41).

Several studies indicated that pX sequences contained between *env* and the last exon of *tax*/*rex* of HTLV-1 and HTLV-2 are not required for viral replication and cellular transformation of human T-lymphocytes *in vitro* (96,277). However, further characterization using an infectious rabbit animal model revealed that these ORF-containing sequences or their expression are important for maintaining high viral load and a long-term productive infection *in vivo* (15,48,49). Additional studies are necessary to determine the precise contribution of the protein products of ORFs I and II to HTLV pathogenesis.

Structure of the LTR

Like those of other retroviruses, the HTLV LTR is divided into three regions, U3, R, and U5. The U3 region contains sequences that control transcription of the provirus (Fig. 3). In particular, three imperfect 21-nucleotide repeats, termed Tax response element 1 (TRE-1), are necessary for trans-acting transcriptional activation by Tax (31,74,313). The U3 region also contains sequences responsible for termination and polyadenylation of mRNAs. The polyadenylation signal in U3, AATAAA, is an unusually long distance away from the polyadenylation site in R (about 250 nucleotides), and it is hypothesized that it is brought into proximity of the polyadenylation site by the secondary structure of the RxRE (13). The 5' part of the U3 region also encodes the carboxyl terminus of the Tax protein. Comparison of the LTRs of HTLV-1 and HTLV-2 (see Table 1) reveals that those elements that are found to be critical for viral gene expression (e.g., the 21-nucleotide repeats, polyadenylation signal, and TATA box) are well conserved, whereas other regions of the LTR are not. The R and U5 regions are unusually long in comparison to those of other retroviruses. These regions form the leader sequence encoded at the 5' end of the messenger RNAs. Their role in posttranscriptional control of gene expression was discussed earlier.

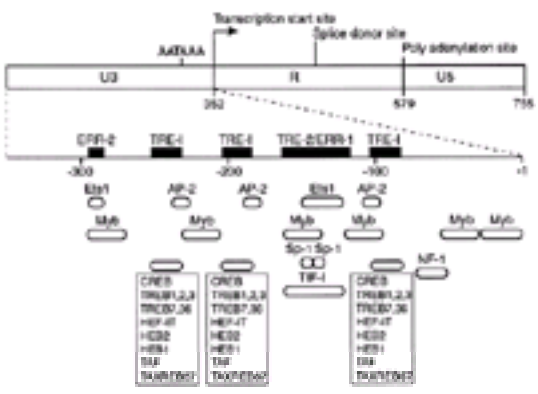

FIG. 3. Structure of the HTLV-1 long terminal repeat (LTR). The total sizes of the provirus LTRs are 755 nucleotides for HTLV-1 and 763 nucleotides for HTLV-2. The sizes of the U3, R, and U5 regions are 352 and 314; 227 and 249; and 176 and 200 nucleotides, for HTLV-1 and HTLV-2, respectively. [From refs. 300 and 302 (HTLV-1), refs. 311 and 312 (HTLV-2), and refs. 87,95, and 252 (nuclear factor binding factors).]

The interaction of the HTLV LTR with proteins involved in regulation of transcription has been investigated (87), and it was shown that multiple factors bind to different regions of the LTR in a complex pattern (see Fig. 3). DNase-protected regions are evident over each of the three 21-nucleotide repeats, as well as other regions within U3, and binding of these factors is critical for regulation of HTLV transcription. The DNase protection pattern of U3 is the same in the presence or absence of Tax, indicating that transcriptional activity of Tax is indirect and does not involve direct binding to the LTR (251). The most important functional interaction appears to be that of the cyclic AMP response element binding protein/activating transcription factor (CREB/ATF) family of DNA-binding proteins within the 21-nucleotide repeats or TRE-1. TRE-1 contains three conserved domains, designated (from 5' to 3') as A, B, and C. Tax responsiveness requires domains A and B or domains B and C. Domain B consists of the first five of the eight-base cAMP-response element (CRE), TGACGTCA, found in many promoters (68). Several techniques have been used to identify cellular proteins that

interact with the TRE-1 elements and that include formulated complexes of proteins composed of CREB homodimer, CREB/ATF-1 heterodimer, and ATF-1 homodimer, as well as others (see Fig. 3). Although the Tax protein does not bind directly to the LTR, it is predicted to stabilize binding of these complexes to the weak viral CRE sequences (328,382). A second Tax response element (TRE-2) or Ets response region 1 (ERR-1) is located between the two proximal TRE-1 domains. TRE-2 contains binding sites for Sp1, Ets1, Myb, and Tif-1 (30,250). An additional Ets binding region has also been described, ERR-2 (see Fig. 3). The cooperative binding of Tax to several of these proteins to the promoter region may position Tax to interact with basal transcription factors CBP, TFIIA, and TFIID (59,176).

Genetic Variation of HTLV

HTLV-1 and HTLV-2 share about 65% overall similarity at the nucleotide sequence level. The homology is lowest in the LTR and 5' pX region and highest in the *tax* and *rex* genes (see Table 1). Among different HTLV-1 isolates, the genome shows little nucleotide sequence variation. Isolates from Japan show 97% to 99% homology, and even isolates obtained from geographically diverse regions such as the Caribbean basin, Japan, and Africa show 96% to 99% homology (92,119,206,298,301,341).

Phylogenetic analysis of nearly 300 HTLV-1 strains originating from nearly all known endemic areas has revealed four distinct HTLV-1 molecular subtypes (A through D) or clades (for extensive review, see ref. 83). Subtype A (Cosmopolitan) is the most widespread and is found in many geographic areas and populations. Subtype A is the least divergent (<2%) and comprises four distinct molecular groups (Japanese, Transcontinental, North African, and West African). The global distribution of this subtype can be traced to large-scale human migration of infected populations such as the slave trade from Africa to the New World. Subgroups also exist in subtype B (central African clade) specific to the geographic origin of the strain. Subtype C, or the Australo-Melanesia clade, was shown to be present in Papua New Guinea and the Solomon Islands. Subtype D (central African Pygmies) was shown to be present in some individuals living in Cameroon, Central African Republic, and Gabon. In spite of considerable effort, there is no indication that specific subtypes of HTLV-1 are associated with particular pathologic consequences. For example, there are no consistent differences between strains from patients with ATL and those from patients with HAM/TSP. Epidemiologic and phylogenetic analyses indicate that HTLV-1 and the related simian virus (STLV-1) have been present in Africa and Asia for tens of thousands of years. The picture of the origin of HTLV-1 is not entirely clear, but some evidence suggests that it has arisen from interspecies transmission between STLV-1-infected monkeys and humans, followed by evolution in the human host (83,203).

Sequence homology among different HTLV-2 isolates is equally high. There have been two major but closely related molecular subtypes of HTLV-2, designated HTLV-2A and HTLV-2B, that differ in the p21 *env* gene by 4% (81,108). HTLV-2A is mainly present in U.S. intravenous drug abusers (IVDA), whereas HTLV-2B is found in most of the Amerindian groups in North, Central, and South America. Recently, a divergent variant of subtype A, labeled by one group as HTLV-2C, was found in Amerindian tribes and IVDA in Brazil (61). Because of the low incidence of HTLV-2 worldwide, it had been difficult to draw firm conclusions about the origins and spread of the virus. HTLV-2 was thought to be a New World virus brought from Asia to the Americas some 10,000 to 40,000 years ago by migration of populations infected with the virus (81,351). However, there does not appear to be an endemic population in Asia to support this. In addition, both HTLV-2 subtypes have recently been found in Africa. HTLV-2B was found in remote Pygmy populations, considered to be the oldest inhabitants of Africa (90). Interestingly, the discovery of STLV-2 in African monkeys might suggest interspecies transmission similar to what has been proposed for HTLV-1.

The lack of genetic variability in HTLV may be the result of the relatively low levels of viral replication in infected individuals. The genome replicates primarily by clonal expansion of infected cells rather than by reverse transcription (361). Replication of the provirus in dividing cells would result in a mutation frequency much lower than that of reverse transcription (189). In addition, cell-associated replication (i.e., passive replication of integrated viral genomes in dividing cells) may allow the HTLV to escape a degree of evolutionary pressure from antibody responses. Compared with that of HIV, the HTLV *env* gene appears to be unable to tolerate many mutations without becoming nonfunctional. This might provide an additional basis for conservation of what might otherwise be a highly variable region of the virus genome (267).

HTLV INFECTIVITY

Cells Susceptible to HTLV Infection

Direct cell-to-cell contact is usually required for efficient HTLV infection; however, infection with cell-free virus preparations can occur, but with low efficiency. *In vitro* infection of cells with HTLV is usually accomplished by co-cultivation of target cells with gamma-irradiated or mitomycin-C-treated virus-producing cells. Compared with that by other retroviruses, infection of susceptible cells by HTLV is inefficient, with a slow course. Usually, only a small proportion of cells express viral antigens, and this number may gradually increase over a course of weeks. The reasons for the slow kinetics may relate to positive and negative regulation by *tax*/*rex* genes or other unknown processes of infection. Infection of human cord or peripheral blood mononuclear cells by HTLV-1/-2 results in virus production and efficient transformation of the cells. Only T cells have been shown to be transformed by HTLV. Productive infection with HTLV has been demonstrated only in a few cells of nonlymphoid origin (44,127,130,364,366).

HTLV-1 appears to infect but not transform a number of different cell types *in vivo*. Epstein-Barr virus (EBV)-transformed B-cell lines productively infected with HTLV have been established from patients (370). B cells can also be infected in culture. There is also evidence that HTLV is capable of infecting immature cells from human bone marrow that do not have a T-cell phenotype (210). Infection of macrophages and cells of neural origin has been documented *in vitro* but not *in vivo* (3,128). Although cases of ATL with central nervous system (CNS) involvement are rare, direct infection of the CNS may account for such symptoms in a few patients. There is evidence of infection of dendritic cells *in vivo*, which may play a key role in antigen presentation, immune activation, and subsequent neurologic disease (205). From 90% to 99% of the HTLV-1 DNA in peripheral blood from infected patients is found in CD4+/CD8+ T cells (276). This is consistent

with one *in vitro* study indicating that HTLV-1 transcription is enhanced in primary CD4+ T lymphocytes (248). In contrast, HTLV-2 is found predominantly in CD8+ cells (136,271).

The HTLV Receptor

The cellular receptor for HTLV has not been identified, but it has been demonstrated by various assays to exist on a wide variety of human and animal cells, including primate, canine, feline, and rodent cells (45,170,369). The presence of a cell-surface receptor for HTLV has also been demonstrated by virus-induced cell fusion, leading to syncytium formation (131,239). The absence of syncytium formation when HTLV-1- and HTLV-2-infected cells are mixed has been cited as evidence that the two viruses utilize the same receptor molecule, whereas the receptor for BLV is distinct (325). Other experiments showed that interference to vesicular stomatitis virus (VSV) pseudotypes of HTLV-1/-2 were established when cells were preinfected with HTLV-1. Thus, it is likely that HTLV is able to penetrate and infect a number of cell types. However, productive infection is observed in only relatively few cells, and the block to productive infection by HTLV is still not understood. The most specific evidence about the HTLV receptor comes from experiments with human–mouse somatic cell hybrids (325). In these experiments, the only human chromosome common to all cell hybrids susceptible to HTLV infection was chromosome 17, and the gene responsible was localized to the 17q region. However, it is formally possible that this gene does not encode the actual receptor but may be required for effective display.

T-CELL IMMORTALIZATION/TRANSFORMATION BY HTLV

Both HTLV-1 and HTLV-2 will immortalize primary human peripheral blood T cells *in vitro*. Immortalization is defined as IL-2-dependent, long-term growth in culture. Subsequently (2 months to years in culture), cells become fully transformed as defined by continuous cellular proliferation in the absence of exogenous IL-2. Cellular transformation is an early step in the pathogenic process and is distinct from oncogenesis or malignancy. In addition to human T cells, T cells from monkeys (223), rabbits (226), cats (133), and rats (336) have been transformed *in vitro* by HTLV-1. *In vitro* transformation by HTLV-1/-2 of human peripheral blood lymphocytes (PBL) or cord blood lymphocytes results primarily in transformation of CD4+ or CD8+ T cells. However, CD4+/CD8+ cells, and immature CD4–/CD8– cells from bone marrow can also be transformed by HTLV-1 or HTLV-2 *in vitro*. Following co-cultivation of PBL or cord blood lymphocytes with irradiated HTLV-producing cells, proliferating immortalized/transformed cells predominate in the cultures after 4 or more weeks. Initially (4 to 8 weeks after co-cultivation), the population of transformed cells shows a polyclonal pattern of integration of HTLV proviruses. With continued long-term passage, a few dominant clones of cells often arise, resulting in an oligoclonal pattern of integration. In patients with ATL, tumors are almost invariably of the CD4+ type, although occasional CD8+ tumors have been reported.

HTLV-transformed cells typically display phenotypes and surface markers associated with T cells functionally activated by specific antigens or lectin. In addition to proliferation, this includes (a) production of a wide spectrum of lymphokines, including g-interferon, granulocyte-macrophage colony-stimulating factor (GM-CSF), CSF-1, or M-CSF and IL-3; (b) altered surface marker expression, including markers associated with an activated state, such as IL-2R, transferrin receptor, and major histocompatibility complex class II molecules (e.g., HLA-DR) (Table 2). Transformed cells not only display an activated phenotype but in some cases also retain T-cell functional properties. Among the functional activities of HTLV-transformed cells that have been reported are (a) the ability to induce suppressor-cell activity (although not directly functioning as suppressor cells) (232), (b) the ability to retain antigen-specific responses (217), and (c) cytotoxic function (65). However, in other cases, aberrant function such as loss of accessory cell requirement is evident (217).

Continuous proliferation
Surface marker expression
Usually CD4+
Occasionally CD4−CD8+
Invariably IL-2R+
Express transferrin receptor (101)
May show altered HLA expression (208)
Increased expression of adhesion molecules (75)
Jak/STAT constitutively activated, HTLV-1 only (215)
Expression of cytokines
IL-1 (358)
IL-2 variable
IL-3 (249)
IL-5 (7)
IL-6 (353)
IL-9 (159)
γ-IFN (34)
CSF-1 (M-CSF) (158)
GM-CSF (158)
TNF-α and -β (164)
TGF-β (161)
NGF (93)
EGR-1 (269)
GCPF (17)

HTLV, human T-cell leukemia virus; IL, interleukin; HLA, human lymphocyte antigen; IFN, interferon; CSF, colony-stimulating factor; M, macrophage; G, granulocyte; TNF, tumor necrosis factor; TGF, transforming growth factor; NGF, nerve growth factor; EGR, epidermal growth factor receptor; GCPF, glial growth promoting factor.

TABLE 2. *Properties of HTLV-transformed cells*

Cell types other than T cells transformed by HTLV have not been reported. Cells transformed *in vitro* actively transcribe HTLV RNA, produce virions, and can themselves be used to transform other normal cells. This contrasts with the lack of HTLV gene expression in ATL tumor cells, suggesting that the process of *in vitro* transformation by HTLV does not directly parallel formation of a leukemic clone *in vivo*, but probably represents the first step in the malignant process. The polyclonal pattern of transformation *in vitro* is reminiscent of B-cell transformation by EBV and may be related to events, which occur *in vivo* in the early stages of HTLV-associated disease, prior to the formation of a leukemic clone. It has been shown that HTLV-1-transformed cells are associated with constitutive activation of Jak/STAT pathway, whereas in HTLV-2-transformed cells Jak/STAT is not activated (215,235). The significance of this difference is not clear, but it might suggest that the mechanism of *in vitro* transformation is distinct between HTLV-1 and HTLV-2.

The HTLV genome does not contain an oncogene (i.e., a viral homolog of a normal cellular sequence); therefore, HTLV does not fall into the category of acutely transforming retroviruses. In ATL, a common integration site for HTLV is not found in different tumors. Therefore, promoter insertion mechanisms are not operative, and the mechanism of HTLV transformation must also differ from that found for chronic-transforming retroviruses. Several lines of evidence strongly

implicate the Tax protein as the transforming component of HTLV. These include induction of tumors in Tax transgenic mice (104,246), morphologic transformation of cells by Tax in traditional *in vitro* rodent cell transformation assays (322,334), and immortalization of human T cells using various Tax viral transduction systems (2,282). More recent studies using infectious HTLV-1 and HTLV-2 molecular clones have directly shown that HTLV-mediated cellular transformation requires expression of the Tax oncoprotein (278,285). The precise mechanism by which Tax initiates the malignant process is unclear, but it most likely involves several points of transcriptional and posttranscriptional dysregulation in the infected T lymphocyte.

Mitogenic Stimulation Mechanisms

HTLV virions are mitogenic for quiescent human T cells (80,379). Both CD4+ and CD8+ T cells are stimulated to divide, and unlike T-cell activation by specific antigens, accessory cells (monocytes/macrophages) are not required. This mitogenic activity of the virion preparations does not require infection by HTLV, as heat-inactivated virions show similar properties. Mitogenic stimulation is probably a consequence of interaction between a virion surface molecule and a specific receptor on the surface of the T cell. Neither the virion protein responsible nor its receptor has as yet been definitively identified. Likely candidates are the virus *env* gp46 (SU) protein and receptors on the surface of the cell that normally participate in T-cell activation responses. Several reports have indicated that this mitogenic activity operates via the CD2 receptor activation pathway with an important contribution by the adhesion molecule, leukocyte function-associated antigen 3 (LFA-3) (58,105,162).

One important function for the mitogenic activity induced by HTLV virions might be to enable the virus to establish infection once it has penetrated the cell. Previously activated and dividing T cells are more easily transformed by HTLV than are quiescent T cells (214). Retroviruses are generally blocked from productive infection unless the cells have traversed through the S phase of the cell cycle. By inducing its target cell to divide, HTLV is thus able to overcome this block to productive infection. This may be of particular importance to HTLV, because 95% of the T cells in the peripheral circulation are in a quiescent state. The mitogenic activity of HTLV virions suggests a model for HTLV transformation whereby continual stimulation of the T cell by a virion component via a T-cell surface receptor results in continuous cell proliferation. Continued virion stimulation does not allow continued proliferation of the cells. Transformation of T cells most likely requires (a) mitogenic activity of the virion at the cell surface and (b) the pleiotropic effects of the *tax* gene product within the cell. This process may be analogous to that of transformation of primary rodent fibroblasts *in vitro*, where coexpression of two oncogenes, one acting in the nucleus and the other acting at the cell membrane, are required to effect transformation of the cell.

TAX MECHANISMS OF ACTION

Transcriptional Control

The discovery that the *tax* gene positively regulates HTLV gene expression gave rise to the idea that it may aberrantly regulate cellular gene expression. Indeed, Tax is capable of trans-activating many cellular promoters in addition to its own LTR. Much of the data on this subject has been compiled for Tax-1 and is summarized in Table 3. The promoters of IL-2 and the IL-2R-a chain, two genes centrally involved in the control of T-cell proliferation, are activated by Tax (10,52). This observation provides support for a possible autocrine model of T-cell transformation whereby activation of IL-2 and IL-2R-a genes leads to continuous proliferation of HTLV-infected cells. Although the majority of HTLV-transformed T cells possess an activated phenotype and express surface markers characteristic of T-cell activation (including IL-2R), not all HTLV-transformed T cells express IL-2.

TABLE 3. *Promoters whose expression is influenced by the Tax protein*

In addition to the IL-2 and IL-2R genes, T cells transformed by HTLV-1 constitutively express a high proportion of early response or serum-responsive genes normally transiently induced following antigenic or mitogenic activation of T cells (160). Some of these include proliferating cell nuclear antigen, *erg*-1, and *erg*-2, and proto-oncogenes c-*fos*, c-*myc*, c-*jun*, and c-*sis* (72,144,191,274,339). Again, the mechanism responsible for the activation of these genes is an increased rate of transcription rather than a change in mRNA stability. Tax has also been shown to repress transcription of b-polymerase, a host-cell DNA repair enzyme, and the *bax* gene, whose gene product accelerates apoptosis (32,145).

The mechanism of Tax protein action on various promoters is indirect through activation of a number of different transcription factors including CREB/ATF (328,381), NFkB/Rel (10,191), serum response factor (SRF) (73), NF-AT (89), and basic helix-loop-helix proteins (bHLH) (348) (see Table 3). Tax activates HTLV gene expression via interaction with CREB/ATF proteins and the transcriptional coactivators CREB binding protein (CBP)/p300, resulting in increased binding of these factors to three 21-bp repeats present in the LTR (21,86,113,176). Likewise, Tax activates NFkB/Rel-responsive

genes by interacting with members of the NFkB family of proteins and their inhibitors including p50, RelA, p100/NFkB2, and p105/NFkB1. NFkB subunits are sequestered in the cytoplasm through high affinity binding with the labile cytoplasmic inhibitors IkBa and IkBb (85). The mechanism of activation of NFkB by Tax involves site-specific phosphorylation of the inhibitors IkBa and IkBb, followed by their ubiquitination and degradation and the nuclear translocation of the active NFkB subunits. Phosphorylation involves a large multiprotein complex containing Tax; IkB kinases (IKK) IKKg/NEMO, IKKa, and IKKb; mitogen-activated kinase kinase 1 (MEKK1); NFkB-inducing kinase (NIK); NFkB/Rel; and IkBa or IkBb (40,56,148,184,213,245,273,375).

A number of mutations in both Tax-1 and Tax-2 proteins have been described that selectively abrogate the ability of Tax to activate transcription through the CREB/ATF or NFkB/Rel signaling pathways (283,306,321) (see Fig. 3). These mutants have been invaluable tools in dissecting cellular signaling and in determining the interplay between Tax and cellular transcription factors and cellular transformation. Several approaches have been used to evaluate domains of Tax-1 required to immortalize or transform rodent cell lines or primary human T lymphocytes. These analyses have yielded conflicting results about whether Tax-1 activation of the CREB/ATF or the NFkB/Rel pathway is critical for cellular transformation (2,140,278,282,322). A recent study has indicated that Tax-2 activation of both NFkB/Rel and CREB/ATF are required to induce IL-2-independent T-lymphocyte transformation. It appears that the activation of NFkB/Rel provides a critical proliferative signal early in the cellular transformation process, whereas CREB/ATF activation promotes sustained cell growth and IL-2 independent cellular transformation (284).

Posttranscriptional Control and Cell Cycle

Progression through the cell cycle and apoptosis is controlled through the interplay between key positive and negative regulators. Viral oncoproteins interfere with this regulation by increasing the expression of growth-promoting genes as described earlier and/or by altering the function of cell cycle regulatory proteins through direct physical association. This probably contributes to uncontrolled cell growth early, followed by subsequent cell damage or mutations, and ultimately malignancy. For example, Tax can inhibit apoptosis via its effects on the p53 tumor suppressor and *bax.* Tax stabilizes p53 and inhibits its transactivation function while repressing *bax* gene expression (32,111,234,268). Tax induces unscheduled transition of cells from G_0/G_1 into S phase by altering the activity of cyclin D, cyclin-dependent kinases cdk4 and cdk6, and the cdk inhibitors $p21^{waf}$ and $p16^{INK4A}$ (198,247,295,327). The dysregulation of these important cell cycle regulatory pathways may represent a key step in T-cell transformation.

Tax has also been shown to directly bind the mitotic checkpoint protein, MAD1, interfere with the function resulting in the induction of multinucleated cells and chromosomal abnormalities (147). A complete understanding of the mechanism of T-lymphocyte transformation and malignancy will require correlation of Tax domains important in modulating gene transcription, cell cycle control, and induction of DNA damage with those required for cellular transformation.

ANIMAL MODELS FOR HTLV INFECTION

The development of a small animal model for HTLV infection and disease manifestation would be extremely useful. HTLV has successfully been transmitted to rats and to rabbits via inoculation of *in vitro*-infected T cells. Infection of rats with HTLV-1 has been shown to be a potential model for HAM rat disease (173). Recently, rats infected with HTLV-1 were shown to develop a chronic progressive myeloneuropathy with paraparesis of the hind limbs after an incubation period of 15 months (156). The microglia/macrophages were determined to be one of the dominant viral reservoirs in the spinal cord and lymph nodes in these animals, suggesting that pathology may result from neurotoxic cytokine synthesis. Rabbits have been used to examine HTLV-1 infection, replication, cellular transformation, transmission, induced immune responses, disease manifestations, vaccine candidates, and treatment methods (reviewed in ref. 272). The use of the rabbit model has been more recently used in the characterization of molecular clones of HTLV-1/-2. Most notable is its use in determining that the pX region sequences containing ORF I and ORF II are important for viral infectivity and maintenance of viral load *in vivo* (15,48,49).

Technical advances in recent years have allowed new approaches to animal studies beyond direct infection of experimental animals with virus. Transgenic mice containing the HTLV-1 genome or the HTLV-1 *tax* gene have been generated by *in vitro* manipulation and selective cross-breeding. However, although these animals became symptomatic, they did not develop a syndrome similar to ATL. Different genetic lines of mice transgenic for HTLV have shown distinct patterns of pathology dependent on the promoter/enhancer driving gene expression and the composition of the HTLV transgene. In one case, mice carrying the HTLV-1 genome or HTLV Tax developed chronic arthritis resembling rheumatoid arthritis, suggesting the possibility that HTLV-1 is one of the etiologic agents of chronic arthritis in humans (106,139). Subsequent clinical reports have borne out the hypothesis that chronic inflammatory arthropathy does occur in HTLV-infected individuals as a rare complication of infection. LTR Tax transgenic mice have been shown to develop neurofibromas (246) as well as an exocrinopathy resembling Sjögren's syndrome (94). A more recent experiment targeting expression of the *tax* gene to the mature T-lymphocyte compartment using the human granzyme B promoter resulted in the development of large granular lymphocytic leukemia (104). The common link to pathology by the different transgenic approaches is the expression of the *tax* gene.

Severe combined immunodeficient (SCID) mice can support the survival of human lymphoid cell populations by reconstituting their immune systems with human tissues. Reconstituted SCID mice have been infected with HTLV-1, and many of these mice became persistently infected with the virus (67). Lymphoblastic lymphomas of human origin developed in several animals injected with PBL from two patients with ATL. The tumor cells represented an outgrowth of the original ATL leukemic clone with monoclonal or oligoclonal integrations of the HTLV-1 provirus identical to the original leukemic clone. In contrast, cell lines derived by HTLV transformation of T cells *in vitro* did not persist or form tumors when inoculated into SCID mice. These distinct growth properties indicate differences between *in vitro* transformed cells and ATL leukemic cells and may relate to differential sensitivities to NK cells (66).

DISEASES OF HTLV-1 INFECTION

ATL and HTLV-1

Several categories have been proposed as stages in ATL (315). These have been termed as follows: (a) asymptomatic carrier state, (b) preleukemic state (pre-ATL), (c) chronic/smoldering ATL, (d) lymphoma type, and (e) acute ATL (Table 4). In some cases, these categories may be temporally related. The majority of HTLV-1-infected individuals are asymptomatic carriers of the virus. These infected individuals are capable of transmitting the virus because the proviral genome is integrated into host-cell DNA sequences. An infected individual has about a 1% chance of developing a tumor over a lifetime of infection (167), although the cumulative lifetime chance of developing any HTLV-associated condition is slightly higher, approximately 5% to 10%.

Stage	Monoclonal proviral	Skin lesions	Visceropathy	White blood count
Carrier Clinical status: no clinical signs.	+	-	-	Normal (<4 × 10⁹/L)
Pre-ATL Clinical status: variable-uncertain category.	+	-	-	Slightly elevated? (>4 × 10⁹/L)
Smoldering ATL Clinical status: 5% abnormal lymphocytes, no hypercalcemia, variable course, may progress to ATL.	+	+	-	Normal (<4 × 10⁹/L)
Chronic ATL Clinical status: 5% abnormal lymphocytes, no hypercalcemia, variable course, may progress to ATL.	+	+	-	>4 × 10⁹/L
Lymphoma type Clinical status: <1% abnormal lymphocytes, lymphadenopathy, median survival ~10 months.	+	+/-	+	Normal (<4 × 10⁹/L)
Acute ATL Clinical status: >5% abnormal lymphocytes, hypercalcemia, median survival ~6 months.	+	+	+	>4 × 10⁹/L

These clinical phases may be but are not necessarily temporally related (264).

TABLE 4. *Clinical Phases of HTLV-1 infection*

ATL generally occurs in adulthood, at least 20 to 30 years following infection. Some patients manifest a disorder known as pre-ATL, which is usually asymptomatic. This condition was originally diagnosed by incidental detection of leukocytosis and/or morphologically abnormal lymphocytes, but more recently it is diagnosed as a result of serologic screening. HTLV-1 can be identified in the cells of these individuals by PCR amplification, and the provirus can be found to be integrated in abnormal T lymphocytes in a monoclonal or oligoclonal pattern by Southern blot hybridization. These findings indicate the beginnings of the development of a clonal population of cells, some of which may progress to the malignancies found in acute ATL. Approximately 50% of patients with pre-ATL undergo spontaneous regression of lymphocytosis. In the remaining group, T-cell lymphocytosis persists, and some of these patients progress to the acute stage.

About 30% of patients with clinical manifestations of HTLV-1 infection have chronic or smoldering ATL (317). These forms of ATL are less aggressive than the acute stage of the disease and are characterized by skin lesions, low levels of circulating leukemic cells, and an absence of visceral involvement. Patients in the smoldering ATL category present with skin lesions and marrow involvement, whereas patients with chronic ATL generally have elevated numbers of circulating ATL cells, accounting for an increased leukocyte count. Patients in both the smoldering and chronic stages of ATL can progress into acute ATL within a period of months. Pre-ATL, smoldering ATL, and chronic ATL may represent transitional states in the development of the malignant clone that is evident in acute ATL.

In acute ATL (315,317), a dominant clone of malignant cells is present, as evidenced by the presence of (a) a single rearrangement of T-cell antigen receptor gene and (b) one or a few proviruses arranged in an oligoclonal fashion in the population of tumor cells. These patients have an elevated white blood cell count, with many morphologically abnormal T lymphocytes (Fig. 4). Also, there is often prominent eosinophilia and neutrophilia. Findings in acute ATL include lymphadenopathy that does not involve the mediastinum, hepatosplenomegaly, and skin lesions caused by infiltration of leukemia cells (Fig. 5). Interstitial pneumonitis, also caused by leukemic cell infiltration, is sometimes present. Patients manifest abnormalities in certain serum chemistries, including elevation of lactate dehydrogenase (LDH), hyperbilirubinemia, and hypercalcemia. Hypercalcemia is associated with the presence of lytic bone lesions detected on the skull (Fig. 6) and long bones (26). Recent work has shown that hypercalcemia in acute ATL may involve production of IL-1a and parathyroid hormone–related protein by HTLV-1-infected cells (62,230,360). Patients with ATL are also immunocompromised and may present with opportunistic infections, including disseminated fungal infections, *Pneumocystis carinii* pneumonia, cytomegalovirus pneumonia, and various bacterial infections. The only significant prognostic factor in acute ATL is the presence of ascites, which is associated with shorter survival. The median survival in acute ATL is approximately 6 months (142,319), despite aggressive intervention with chemotherapy.

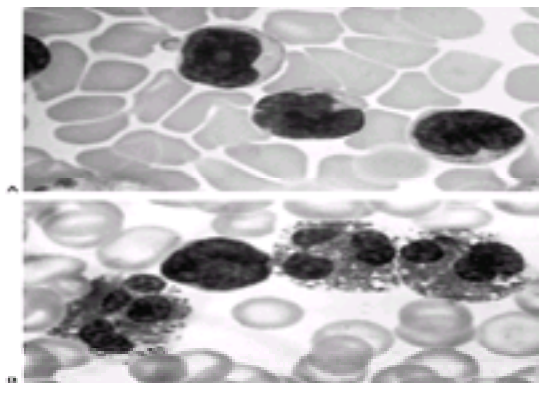

FIG. 4. A: Typical morphology of ATL tumor cells, showing convoluted nuclei. (From ref. 280, with permission.) **B:**

Prominent eosinophilia in ATL.

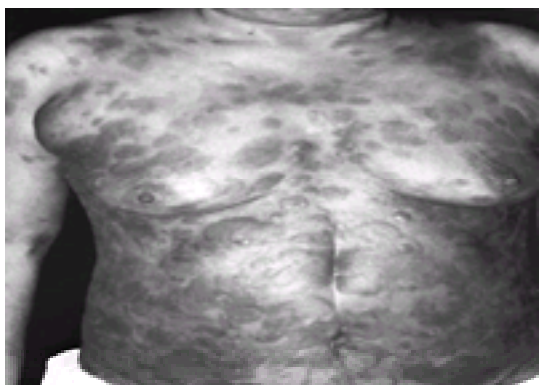

FIG. 5. Skin lesions on a patient with acute ATL.

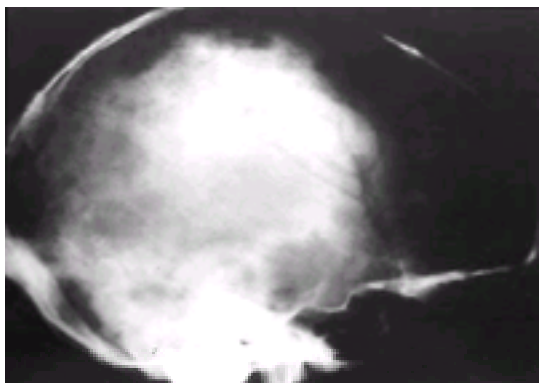

FIG. 6. Lytic bone lesions in the skull of a patient with ATL.

The morphology of peripheral blood cells in ATL is variable. Typical ATL cells have highly lobulated or flower-shaped nuclei, but the degree of nuclear irregularity is variable (253) (see Fig. 4). This aberrant nuclear morphology is consistent with the observed pleiotropic effects of Tax, including induction of micronuclei and chromosomal abnormalities (147,204). The histopathology of lymph nodes from patients with ATL is also heterogeneous, but it most frequently falls into the large-cell, immunoblastic classification. Nodal histopathology has no correlation with the patient's prognosis or therapy. Immunologic studies of ATL cells have revealed phenotypic heterogeneity for T-cell subset markers (232). owever, virtually all ATL cells have markers for CD4 antigen, with rare exceptions having CD8 markers. Although these markers usually indicate a helper/inducer phenotype, assays revealed that these cells have suppressor activity for immunoglobulin synthesis (354). Further studies suggested that this suppression is not directly caused by ATL cells but is, instead, mediated via suppressor CD8+ cells that are activated by the ATL cells (232). One characteristic marker of ATL tumor cells is high levels of expression of the IL-2R-a chain, Tac antigen (345), both attributed to the transactivation effects of Tax.

The form of disease in some patients with ATL is predominantly that of a T-cell lymphoma rather than a leukemia (315). These non-Hodgkin's lymphomas have differing histologic patterns and can involve any organ. Lymph node biopsy specimens from these patients contain oligoclonally integrated HTLV-1, indicating a clonal malignancy. The differential diagnosis of ATL includes other T-cell malignancies such as non-Hodgkin's lymphoma, mycosis fungoides, Sezary's syndrome, and T-cell chronic lymphocytic leukemia. However, most cases of these secondary conditions are not associated with HTLV infection. A variety of laboratory studies can help establish a diagnosis of ATL, including a HTLV-1 serum positivity, hypercalcemia, elevated serum LDH, staining for terminal deoxynucleotidyl transferase (typically negative in ATL), and immunologic typing of ATL cells for CD4 and Tac antigen (IL-2R-a chain). Definitive evidence for the disease is obtained if monoclonally or oligoclonally integrated HTLV-1 genome is present.

HTLV-1–Associated Myelopathy/Tropical Spastic Paraparesis

In 1985, a group of West Indian patients with a neurologic disease known as tropical spastic paraparesis were found to be seropositive for HTLV-1 (84). Subsequently, a report from Japan described a number of patients with a slowly progressing myelopathy and pyramidal disturbances, all of whom had elevated HTLV-1 antibody titers (262). This neurologic disease was termed HTLV-associated myelopathy, now considered to be identical to TSP. HAM/TSP has been described in all areas of the world known to be endemic for HTLV-1, and the risk factors are the same as those for HTLV infection. The lifetime risk for development of HAM/TSP has been estimated at below 1% (154).

Neurologic findings in HAM/TSP include weakness and spasticity of the extremities, hyperreflexia, Babinski sign, urinary/fecal incontinence, and mild peripheral sensory loss (260). The cerebrospinal fluid (CSF) of these patients contains anti-HTLV-1 antibodies and may show a lymphocytic pleocytosis as well as elevated protein levels. Patients with HAM/TSP generally have normal lymphocyte numbers, but morphologically atypical lymphocytes resembling ATL cells can be seen in peripheral blood or in the CSF (77). Magnetic resonance imaging has demonstrated lesions in both the white matter and the paraventricular regions of the brain in patients with HAM/TSP (216). However, other diseases such

as multiple sclerosis show similar lesions. Autopsy studies show significant abnormalities in the thoracic spinal cord, including demyelination, capillary proliferation, and perivascular cuffing with lymphocytic infiltration (4).

Other Diseases Associated with HTLV-1

A number of clinical reports have suggested that hematologic malignancies other than ATL are associated with infection with HTLV-1. Cases of T-cell non-Hodgkin's lymphoma, T-prolymphocytic leukemia, Sezary's syndrome, mycosis fungoides, small cell carcinoma, and large granular lymphocytic leukemia have been described to be serologically or molecularly linked to HTLV-1 infection. However, most of these studies base their association on limited data, making it difficult to determine the role played by HTLV-1 in these conditions. HTLV-1 may play an indirect role in certain cases of chronic lymphocytic leukemia of B-cell origin (B-cell CLL). In Jamaica, where HTLV-1 infection is endemic, HTLV seropositivity was significantly higher in patients with B-cell CLL than in the general population (24). It has been proposed that B-cell CLL is derived from the clonal outgrowth of a B-cell clone reactive against an HTLV-1 antigen (207). If HTLV-1 infection is associated with B-cell CLL, then additional factors must also be involved, because B-cell CLL is not common in Japan.

There is evidence that chronic inflammatory arthropathy and uveitis may occur in a few HTLV-infected individuals as a rare complication of infection (228,292). Certain lines of mice transgenic for HTLV have developed chronic arthritis resembling rheumatoid arthritis, suggesting that HTLV-1 is one of the etiologic agents of chronic arthritis in humans (139). Uveitis has also been observed in a rabbit experimentally infected with HTLV-1 (329).

There is some evidence for functional impairment of the cellular immune response in patients with ATL as well as among some HTLV-1 carriers (37). Defects in cell-mediated immunity may result in particular problems with parasitic infections. Immunosuppressive effects may lead to opportunistic infections. Several epidemiologic surveys have indicated the existence of groups of individuals who are concurrently infected with both HTLV-1/2 and HIV. This is particularly common among IVDA who become infected by sharing contaminated needles and syringes, a major route of transmission of both viruses. There is evidence that coinfection may accelerate progression to AIDS. HTLV can form enveloped pseudotypes with HIV particles, which broadens the cell tropism of HIV *in vitro*, potentially increasing the spread of virus through the body (183,200), although this has not been formally demonstrated *in vivo*.

HTLV-2 DISEASE

HTLV-2 infection has been reported in a number of locations worldwide, primarily among IVDA (185,279,337), in the Amerindian population of North, Central, and South America (115,126,179,192), and in Africa (82,90,351). However, the absolute role of HTLV-2 in the few cases of leukemia and neurologic disease associated with viral infection remains unclear, since insufficient cases are available for clear epidemiologic studies of transmission. A clearer understanding of the etiology of HTLV-2 in T-cell malignancies may emerge over the next decade as further isolates of the virus are made in association with malignancy. At the present time, the evidence is growing that HTLV-2 is a cause of human disease. Comparative studies between HTLV-2 and HTLV-1 will probably be essential to understand the different pathobiologies of these two distinct but related viruses.

PATHOGENESIS OF HTLV INFECTION

Oncogenesis

When HTLV-infected lymphocytes are present in individuals at a level sufficiently high to allow detection of viral sequences by Southern blotting, the viral sequences are always present as integrated forms and are generally oligoclonal or monoclonal with respect to integration sites (300). An exception is in some healthy carriers and in some patients with smoldering ATL, where polyclonal integration can be detected. Similar observations have been made for BLV in the early phase of the disease known as persistent lymphocytosis, where polyclonal integration of BLV is evident (36). Rare cases of cancer similar in pathology to ATL have been identified in which HTLV-1 infection is not present (316). In these cases, the HTLV-1 sequences may have been lost, or, alternatively, other agents or factors may give rise to a related disease.

These observations strongly suggest that the initial infection by HTLV results in transformation of a polyclonal population of cells (dependent on the Tax oncoprotein), and that subsequent selection processes result in the evolution of clones that may ultimately develop into the malignant cells. Chromosomal abnormalities commonly exist in ATL cells (76,290), and the degree of cytogenetic aberration is often related to the severity of the disease (i.e., abnormalities are frequently found in acute ATL but are less likely to be seen in chronic or smoldering ATL, suggesting the possibility of clonal evolution during disease progression). Viewed in this context, HTLV transformation of T lymphocytes results in a pool of proliferating cells that are not oncogenic themselves but that provide a population from which a malignant clone may subsequently arise. This accounts for the long latent period between infection and manifestation of a tumor in HTLV disease. Therefore, Tax may also contribute to these later events as a result of its pleiotropic effects on proteins regulating mitosis, apoptosis, and DNA repair.

During the acute phase of ATL, when patients have an elevated leukocyte count with many malignant lymphocytes, the viral DNA can be easily detected and is generally monoclonal with respect to integration sites. However, examination of viral integration sites in ATL cells from several patients demonstrates that these are distinct and present on different chromosomes (300). Unlike some animal retroviruses, where common integration sites are found about particular cellular oncogenes, the integration of HTLV is random. Therefore, integration sites provide little clues as to the mechanism of oncogenesis. As discussed earlier, it is likely that trans-activation mechanisms are responsible for this process.

One of the distinguishing features of the virus in the acute phase, and perhaps also in pre-ATL and chronic/smoldering ATL, is that, although the viral genome is present as a provirus in all of the tumor cells, there is no (or a very low level of) detectable expression of viral genes within the cells. No viral RNA transcripts can be detected by Northern hybridization

or S1 nuclease analysis (70). If fresh ATL cells are placed into culture *in vitro*, virus expression can be detected shortly (130), indicating that the block to expression is not a result of an intrinsic genetic defect in the virus genome. Most isolates of HTLV-1/-2 have been obtained in this manner, wherein virus is expressed from leukemic cells and then infects other cells in the culture. Most HTLV-infected cell lines derived from patients are not the same clone as the original tumor cells (130). It is unclear whether there is any viral expression during the pre-ATL or chronic/smoldering phase of the disease. Lack of viral expression may relate to either a latent state of the virus or an immune selection against those cells that express recognizable viral antigens on their surface. Evidence supports the role of the immune system in suppressing the emergence of oncogenic clones of cells during the asymptomatic phase of the disease (11,343). The immunosuppression seen in HTLV-infected individuals may also be implicated in the development of leukemia. Presumably, the virus is expressed in some cells of the body. However, the source and frequency of expression and factors that may induce expression are not known.

HTLV-Associated Myelopathy/Tropical Spastic Paraparesis

The pathogenesis of HAM/TSP remains poorly understood. In contrast to ATL, which generally occurs 20 to 30 years following HTLV-1 infection, HAM/TSP may develop in some patients within a few years of infection, often after transfusion of infected blood. The presence of intrathecal antibody titers to HTLV-1 indicates that the virus has infected the CNS in addition to the blood (124,260). Immune complexes have also been detected in the blood of patients with HAM/TSP (310). A number of studies have demonstrated the presence of HTLV-1 DNA in blood and lymphocytes of the CSF (22,378). One study provided evidence that HTLV-1 Tax RNA is expressed in astrocytes of HAM/TSP patients (187). It is noteworthy that polyclonal integration of the virus is detected in patients with HAM/TSP, as opposed to oligoclonal or monoclonal integration in ATL. Thus, development of HAM/TSP is not a consequence of the development of a malignant clone of cells. Characterization of viral isolates from patients with HAM/TSP does not show any distinguishing features from HTLV-1 from those with ATL and provides no evidence that a variant virus is involved in the disease. Some investigators have concluded that persistent active replication of HTLV-1 is an important factor in the pathogenesis of HAM/TSP, compared with the mostly quiescent state of the virus in ATL. Indeed, one recent comparative analysis of proviral load in the peripheral blood of HAM/TSP patients (compared to asymptomatic HTLV-1 carriers) has indicated that high proviral load strongly predisposes to HAM/TSP (237).

The mechanisms underlying the neurologic damage observed in HAM/TSP patients remain to be determined. CNS damage may directly result from HTLV invasion of resident cells, indirectly via autoimmune or neurotoxic cytokine-mediated mechanisms, or a combination of both. In support of these models are (a) the ability of HTLV to infect cells of nervous system origin *in vitro*, and its detection in cells of neuro origin *in vivo* (see preceding); (b) the finding that expression of cytokines (including TNF-a, GM-CSF, INF-g, and IL-1a) is higher in HAM/TSP patients then in asymptomatic carriers (359); and (c) the detection of high levels of cytotoxic T-lymphocytes (CTLs), which predominantly recognize HTLV-infected cells, in patients with HAM/TSP (102,141). Primary demyelination and remyelination by oligodendrocytes has been shown to occur in the spinal cord lesions of patients with HAM/TSP (254). Of note is the observation that patients with HAM/TSP tend to have higher levels of circulating antibody to HTLV-1 antigens than do those with ATL (260). There is also an association between development of HAM/TSP and certain HLA haplotypes (146,350), whereas there is no association with the development of ATL.

IMMUNE RESPONSE TO HTLV

All patients with ATL make humoral antibodies to various HTLV-1 antigens. The major viral gene products recognized by sera from infected individuals are those of the *gag*, *env*, and *tax* genes. As in all retroviruses, the Gag proteins are the major immunogens and are responsible for the earliest antibodies to appear. Sera from infected individuals usually recognize all three Gag proteins, p15, p24, and p19. There is considerable cross-reactivity between HTLV-1 and HTLV-2, particularly in the region encoding p24 (182) (see Table 1). This similarity was initially responsible for the discovery of HTLV-2, but it has also resulted in considerable confusion over serodiagnosis of HTLV-1/-2 infection, and in a common diagnosis of HTLV-1/-2 positive. However, the presence of endogenous retroviruses may also provide a possible explanation for the HTLV-1 Gag-reactive autoantibodies that have been noted in some cases (12).

Antibodies are also directed against both the external envelope glycoprotein, gp46, and, to some extent, the transmembrane protein, p21 (45,64,130,372). These antibodies react with the envelope precursor protein, gp61, in infected cells. The amino-terminal regions of HTLV-1 and -2 gp46 contain type-specific, linear, neutralizing determinants (263). A recent study has found that antibodies to conformational epitopes of gp46 comprise the majority of the immune responses to HTLV-1 Env, and the epitopes are distinct from previously recognized immunodominant linear epitopes (107). Antibodies directed against the envelope proteins may be an important means of modifying the course of virus infection, but seroconversion does not lead to elimination of virus infection, as individuals infected with HTLV (both patients with ATL and HAM/TSP and apparently healthy carriers) all typically possess such antibodies.

Many individuals also have antibodies against nonstructural viral proteins, in particular Tax. This antigen may be released from the nucleus of infected cells and recognized by the immune system on lysis. Some antibodies against HTLV-1 have been found to be present in antigen–antibody complexes (225,299). Rare antibody-negative cases of ATL have also been reported (314,318). The time course of seroconversion has been examined in patients infected by receiving HTLV-contaminated blood transfusions (209). In the early posttransfusion period, antibodies to the Gag proteins were predominant, with anti-p24 appearing before anti-p19. Envelope protein p21(TM) was the earliest Env reactivity detected, while anti-gp46 and anti-gp61 appeared later. Anti-Tax antibodies were the last to appear (several months after infection).

The serologic profile of HTLV-infected individuals varies considerably and includes some individuals who display a virtually monospecific pattern of antibodies. Natural immunity to HTLV appears to be different from that to other retroviruses, in that human complement–mediated virolysis was not effective for HTLV virions, using either normal human serum or human serum from an HTLV-antibody carrier. Thus, the virions may have an intrinsic resistance to humoral

immune mechanisms (132).

Cellular immunity against HTLV-infected cells has also been described. However, there has been only limited mapping of specific determinants. CTL lines have been established from patients with ATL that are specific for HTLV-1-infected cells (152,218), but the majority of information regarding HTLV-specific CTLs has come from the study of CTLs in HAM/TSP. In HAM/TSP patients, some HTLV-1-specific CTLs recognized the envelope region, but the majority were predominantly restricted to the *tax* gene product (63,141,165). Further study indicated that HTLV-specific CTLs that recognize Tax are specific for a peptide at the amino terminus (amino acids 11 to 19). The presence of these CTLs as well as alterations in immune recognition may play a key role in HTLV-induced pathogenesis. Antibody-dependent cellular cytotoxicity (220,380) and natural killer cell activity (287) have also been described. As in HIV, the immune response induced by HTLV is not capable of clearing the viral infection resulting in long-term viral persistence.

EPIDEMIOLOGY OF HTLV INFECTION

HTLV-1

HTLV-1 virus infection was originally discovered in Japan but has now been found worldwide. In general terms, HTLV-1 predominates in south-western Japan, the Caribbean basin, equatorial Africa, parts of South America, the Middle East, and parts of Melanesia. The number of people around the world infected with HTLV-1 has been estimated as between 15 and 25 million.

Initial data demonstrating that patients with ATL in southwestern Japan were seropositive for HTLV-1 provided the first evidence for an etiologic association (79,121). Since then, a large amount of data has been compiled regarding HTLV-1 infection in Japan. It is currently estimated that the number of infected individuals is over 1 million (119), among a population of approximately 121 million. Rates of seropositivity in different regions vary widely: 35% in Okinawa, 8% to 10% in Kyushu Province, and 0% to 1.2% in nonendemic areas (120,211,330). Infection with HTLV-1 generally occurs quite early in life, probably perinatally, but it has been estimated that 30,000 to 50,000 Japanese have also been infected through blood transfusions (291).

The incidence of HTLV-1-infected individuals appears to be increasing worldwide, especially in western Europe and the United States, particularly among IVDA and homosexuals. The estimated numbers of HTLV-1 infection in different parts of the world are as follows: the Kyushu district in Japan, 8%; Uganda, 8%; Ghana, 9%; Zaire, 3.2%; Dominican Republic, 2%; Mashhad, Iran, 3%; and the United States, 0.003% to 0.04%. However, specific pockets or regions are higher. For example, one published study of IVDA in New York City reported a prevalence of 9%, 18%, and 41% for HTLV-1, HTLV-2, and HIV, respectively (279). Also, in Trinidad, 15% of homosexuals were seropositive for HTLV-1, as opposed to 2.4% of the general population (14). Although HTLV-1 is endemic in Trinidad and HIV was only relatively recently introduced into the country, approximately 40% of homosexuals were found to be infected with HIV, compared with less than 1% of the general population, suggesting that the same populations are at risk for infection with HTLV and HIV, but that HTLV is spread less efficiently than HIV.

HTLV-2

HTLV-2 infection appears to be more common than previously thought. However, the distribution of HTLV-2 tends to be localized to certain population groups. HTLV-2 clusters in IVDA worldwide; in Amerindian populations in North, Central, and South America; and in parts of Africa. The absolute incidence of HTLV-2 infection is low compared with the worldwide incidence of HTLV-1, but HTLV-2 infection has been reported throughout the world. More recent pockets include Spain and Venezuela (57,326).

TRANSMISSION OF HTLV

HTLV transmission occurs by three different modes. Although much of the experimental data has been compiled for HTLV-1, transmission for HTLV-2 is similar. The overall mode of HTLV-1 transmission is similar to that of the AIDS virus, with the exception that the virus is apparently not readily transmitted by cell-free body fluids. These observations are consistent with the *in vitro* studies of HTLV, where infection of target cells requires co-cultivation of the infected with the uninfected cells.

First, mothers infected with HTLV-1 can transmit the virus to the fetus or newborn (163,166,331). The mode of transmission here is either through transplacental passage of infected maternal lymphocytes or through infected lymphocytes in breast milk. In one study, the overall prevalence of HTLV-1 among children born to infected mothers was 16%. The prevalence of HTLV-1 among children breast-fed for over 3 months was significantly higher (27%) than that of those breast-fed for under 3 months (5%). Of 78 bottle-fed children, 13% were positive for HTLV-1, suggesting that about 13% of bottle-fed children born to carrier mothers are infected with HTLV-1 by routes other than breast milk (123). Polymerase chain reaction amplification has detected HTLV proviral DNA in the peripheral blood and milk of all carrier mothers examined, but only rarely in cord blood from carriers' neonates, indicating that transplacental infection with HTLV-1 is rare and that postpartum infection via breast milk is the major perinatal transmission route (288). These observations have produced recommendations that carrier mothers should refrain from breast-feeding to reduce the incidence of HTLV-1 transmission to their offspring.

Second, HTLV-1 can be transmitted from man to woman during sexual intercourse via HTLV-1-infected cells in semen (242,331). Similar results were obtained in studies of the related STLV-1 in Japanese macaques maintained in captivity (222). It is possible that that female-to-male sexual transmission also occurs, but only at a very low rate.

The third route of transmission is through infected blood or blood products via transfusion. However, unlike for HIV, only blood products that involve passage of whole lymphocytes from donor to recipient can transmit the virus (143,180,201,221). Studies of HTLV-1/-2 among United States blood donors indicated that a significant proportion of

blood samples are infected. HTLV seroprevalence is about three times greater than that for HIV-1 (0.043% versus 0.013%); 52% of these cases are the result of HTLV-2 infection and 43% the result of HTLV-1. Up to 2,800 individuals per year may have been infected in the United States through blood transfusion before routine donor testing began in 1988. In Europe, the general incidence of HTLV infection appears to be lower than in the United States, although the virus is found at higher frequencies in populations with known risk factors—in particular, in people of Caribbean origin and in IVDA as a result of passage of infected blood lymphocytes in shared needles (352). In the United Kingdom, a survey of nearly 100,000 blood donors indicates an overall seroprevalence of approximately 1:20,000 (33). Although a few European countries have started to screen blood supplies for HTLV, this has not yet been started in all countries, as the low seroprevalence rate probably does not justify the cost of screening. For example, it was recently reported that Sweden has an HTLV seroprevalence of 2:100,000 blood donors; their current screening policy is to test only new blood donors (344).

DIAGNOSIS OF HTLV INFECTION

Antibodies

The diagnosis of HTLV-1 or HTLV-2 infection requires the ability both to detect and to discriminate between infection by either virus. Several methods have been utilized. The development of suitably rapid and sensitive assays has been complicated by the relatively low antibody titers in individuals infected with HTLV-1/-2, as compared to individuals infected with HIV. Several commercial assays based on enzyme-linked immunosorbent assay (ELISA) or particle agglutination formats are licensed for screening of HTLV antibodies. An ELISA/agglutination assay is used as the primary screen, followed by confirmatory assays using Western blotting, radioimmunoprecipitation, or PCR. In spite of recommendations on interpretation of test results from the United States Public Health Service and World Health Organization, there is still some concern over problems with testing. False-positive results mean that use of a second confirmatory technique is absolutely essential. These difficulties arise from the variability in the serologic response to HTLV infection and the relatively low antibody titers in some infected individuals. Screening of blood supplies since 1988 has reduced the overall rate of HTLV-1 transmission via blood transfusions to an extremely low level. Initial ELISA kits had difficulty detecting HTLV-2-infected blood, but newer modified versions have been improved dramatically (78). However, these kits have not been adopted for use by the Federal Drug Administration because of questions about the cost/benefit ratio.

Nucleic Acid

Another complication in antibody screening methods is an age-dependent increase in seropositivity (25,46,175,255), indicating that certain individuals may not develop antibodies until some time after infection. Detection of HTLV genetic material provides an alternative. In patients with ATL, the majority of lymphocytes harbor the provirus, so detection of this DNA by Southern blotting is relatively straightforward and reliable. However, asymptomatic carriers are more problematic, as only a small proportion of cells are infected with the virus. One means formerly employed was to first culture the cells for 3 to 5 weeks, allowing (a) replication and spread of the virus and (b) consequent amplification of viral genetic material. The virus can then be detected in these cultured cells by Southern hybridization or *in situ* hybridization to HTLV RNA. The disadvantage of this method is that *in vitro* culture of cells is time consuming and expensive and is therefore not suitable as a rapid clinical screening assay.

These problems have been solved by the application of PCR amplification of specific sequences in the virus genome (177). PCR can be used to detect a single HTLV-1/-2 provirus and is now the method of choice for detection of HTLV DNA directly from blood and many other tissues. Commercial PCR kits for HTLV are available. However, the unmatched sensitivity of PCR also has drawbacks. False-positive results from inadvertent contamination of samples are a major problem. Target inactivation protocols are available to circumvent this problem in diagnostic laboratories. Although PCR is now a common research tool, its use still presents problems of cost in large-scale screening operations, particularly in underdeveloped countries where HTLV infection may be endemic.

Differential Diagnosis of HTLV-1 and HTLV-2

Earlier serologic methods for diagnosis of HTLV infection did not conclusively distinguish between HTLV-1 and HTLV-2. Competition radioimmunoassays or competition ELISAs have been developed to distinguish between HTLV-1 and HTLV-2. Most monoclonal antibodies to p19 and p24 core antigens detect both HTLV-1 and HTLV-2 and do not discriminate between the viruses. However, some monoclonals are capable of distinguishing between HTLV-1 and HTLV-2 (264) and allow positive identification to be made. Western blotting can distinguish between the two (286), but the use of PCR amplification provides the ideal tool for the differential diagnosis. In addition to its unrivaled sensitivity, the nucleotide sequence of the amplified products can easily be determined, allowing rapid, sensitive, and definitive diagnosis of HTLV infection.

TREATMENT OF HTLV DISEASE

Therapeutic Agents

Adult T-cell leukemia is a highly malignant disease; median survival in subacute or acute ATL is measured in months. However, as only a small proportion (1%) of infected individuals progress from an asymptomatic to a disease state, treatment is reserved for the subacute and acute forms of ATL. Standard combination chemotherapy regimens, particularly those designed for treatment of aggressive non-Hodgkin's lymphoma or acute lymphoblastic leukemia, have not proved valuable for ATL (35,47,158,197,342,367). Some reports have suggested that deoxycoformycin is an effective drug, whereas other trials have shown variable therapeutic efficacy (53,196,368). There has been one report of successful treatment of chronic ATL with ubenimex (257) and one successful report of treatment with chlorodeoxyadenosine (347). Other agents that have been used to treat ATL include b-interferon, g-interferon (212,333), and administration of anti-Tac antibody, because high levels of IL-2R are generally present on ATL tumor cells

(100,333,356). This latter treatment has been shown to induce remission in at least some patients with ATL (357). More recently, combination therapy using zidovudine and a-interferon and the two in combination with etretinate have proven successful (16,38).

Treatment of HAM/TSP has been attempted with oral corticosteroids with variable clinical efficacy (109,112,240,261,355). High-dose intravenous gammaglobulin has shown beneficial effect but only for a short duration (172). The use of a-interferon in patients with HAM has been also been investigated and shown to be effective in a minority of patients (171,308,374). The data from one study suggested that the sustained clinical benefits of long-term a-interferon therapy is causd by immunomodulation and the reduction of HTLV-1 proviral load (374). A recent study showing clinical benefits using anti-Tac antibody also supports this theory (188). However, it is likely that significant progress will not be made in the treatment of HAM/TSP until the pathogenesis of this condition is better understood.

Vaccine Development

The number of people around the world infected with HTLV-1 is estimated to be between 15 and 25 million. The cumulative lifetime incidence for developing ATL or HAM/TSP is approximately 5%, which places HTLV-1 in the category of viruses for which efficient vaccines are made and used. There are several factors favoring the feasibility of a vaccine against HTLV-1: The virus displays relatively low antigenic variability, natural immunity occurs in humans, and experimental vaccination with envelope antigens has been shown to be successful in animal models (54). Using recombinant DNA methods, several groups have begun the development of a vaccine against HTLV-1. HTLV-1 envelope proteins synthesized in bacteria were used to immunize cynomolgus monkeys (241). Several of the monkeys developed adequate antibody titers and were able to withstand an infectious challenge with HTLV-1. Similar results were obtained using a recombinant vaccinia virus expressing *env* (134).

Trials of subunits vaccines in monkeys have also shown that it is possible to achieve protection against STLV infection (55). Marmosets have been infected with HTLV-1 by feeding with breast milk from infected women (135). This might in the future provide a useful model for testing vaccines designed to prevent neonatal infection. Miyoshi and others (224,307) have demonstrated that (a) rabbits can be successfully infected with HTLV-1; (b) transmission of the virus can be achieved using rabbit cells; and (c) tumors will develop in some cases. The rabbit model has been used to show that passive immunization can protect rabbits against HTLV infection (157) and milk-borne transmission to offspring (293). Investigators have introduced the *env* gene into recombinant vaccinia viruses and reported successful immunization of rabbits (309). Some studies have shown that rabbits can be protected against HTLV-1/-2 infection with synthetic peptides corresponding to HTLV-1/-2 *env* neutralization regions, whereas others have shown no protection (8,155,181). One report has shown that the humoral immune response to HTLV *env* is directed primarily against conformational epitopes (107). It is likely that long-term protection form HTLV-associated diseases will require a vaccination approach that induces both neutralizing antibodies and specific cell-mediated immunity. This will require both *env* and *gag* coding sequences in the vaccine preparation. It appears that the rabbit model is an excellent one for testing candidate HTLV vaccines prior to human clinical trials.

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CHAPTER 59 HIVs and Their Replication

Eric O. Freed and Malcolm A. Martin

In the late 1970s and early 1980s, previously healthy patients with symptoms of immunologic dysfunction sought advice and treatment from physicians in the United States and Europe. This new and unusual syndrome was characterized by generalized lymphadenopathy, opportunistic infections (typically *Pneumocystis carinii* pneumonia, but also *Toxoplasma gondii* encephalitis, cytomegalovirus-associated retinitis, and cryptococcal meningitis), and a variety of unusual cancers (non-Hodgkin's lymphoma and Kaposi's sarcoma). A common accompanying laboratory finding in affected individuals was marked depletion of the CD4+ T-lymphocyte subset in the peripheral blood. The disease was first brought to the attention of the general medical community in June 1981, when the Centers for Disease Control described five California men with severe immunodeficiency in the *Morbidity and Mortality Weekly Report* (79). This notification was followed by several reports describing male homosexuals and intravenous drug users with impaired immune systems and T lymphocytes that responded poorly to antigen and mitogen stimulation in functional assays. Within several months, it became clear that a similar immunodeficiency disease was also affecting other groups, including hemophiliacs, blood transfusion recipients, recent Haitian immigrants, and, most significantly, sexual partners and/or children of members of the various risk groups.

The emerging epidemiologic pattern suggested that the new disease was transmitted by a novel pathogen in contaminated blood or following sexual intercourse with an affected individual. Between late 1981 and early 1983, numerous microorganisms were proposed as possible etiologic agents for the acquired immunodeficiency syndrome, or AIDS, as the disease was soon called. The presence of lymphadenopathy in many affected individuals was reminiscent of the clinical course associated with several different human viral pathogens, and it focused attention on virus families known to infect cells of the immune system. A strong case was made for members of the human herpesviruses, particularly cytomegaloviruses (CMVs), which were frequently isolated from AIDS patients and known to replicate efficiently in human lymphocytes (514). Others favored a retroviral etiology based on the presence of antibodies purported to react with human T-cell leukemia virus type-I (HTLV-I), and/or the actual isolation of HTLV-I from AIDS patients (166,216).

In 1983, scientists at the Pasteur Institute recovered an agent from the lymph nodes of an asymptomatic individual who presented with generalized lymphadenopathy of unknown origin (21). During its replication in cultured cells, the lymphadenopathy-associated virus, or LAV as it was subsequently named, released high titers of progeny virions that contained magnesium-dependent reverse transcriptase activity and exhibited electron microscopic (EM) features typical of retroviruses. However, unlike the commonly studied retroviruses of diverse vertebrate origin such as the avian leukosis viruses (ALVs) and the murine and feline leukemia viruses (MuLV, FLV), LAV was highly cytopathic in human peripheral blood mononuclear cells (PBMC), specifically killing the CD4+ T-lymphocyte subset in the cell cultures (441). Gallo and colleagues at the National Institutes of Health subsequently reported the isolation of retroviruses from AIDS patients, which they named HTLV-III to distinguish them from the noncytopathic HTLV-I, and obtained the first convincing serologic evidence linking exposure to LAV-like retroviruses and immunodeficient individuals from the various groups at risk (507,551). Contemporaneously, Levy and associates (371) recovered a similar retrovirus from both AIDS patients and healthy individuals from the various risk groups, which they named the AIDS-associated retrovirus (ARV). This latter report suggested that the newly discovered human pathogen could induce both asymptomatic and symptomatic infections. The new retrovirus, associated with AIDS in the United States, Europe, and central Africa and exhibiting morphologic and genetic characteristics typical of the *Lentivirus* genus, was named human immunodeficiency virus, or HIV (102) (and subsequently HIV-1). In 1986, a related, but immunologically distinct and less pathogenic human retrovirus (now called HIV-2), was recovered from individuals residing in several west African countries such as Senegal, Ivory Coast, and Guinea-Bissau (97).

CLASSIFICATION OF THE HUMAN IMMUNODEFICIENCY VIRUSES

One of the first features the Pasteur Institute group noted about HIV-1 was its particle-associated reverse transcriptase, a property that placed the new agent in the retrovirus family. This was consistent with EM analyses of particles released from infected cell cultures, which revealed 100- to 120-μm enveloped virions, similar in size and morphology to previously studied retroviruses. The mature HIV-1 particles contained a cone-shaped cylindrical core or nucleoid reminiscent of that previously described for visna virus (Fig. 1A) (242,441). The cloning and sequencing of proviral DNA, initially purified from productively infected cultures of T-lymphocytes/T-cell leukemia lines (T-cell lines), indicated that

HIV-1 not only possessed a genomic organization related to other replication-competent retroviruses but placed it, taxonomically, in the *Lentivirus* genus (517,648). This relationship is shown diagrammatically in Figure 1 of Chapter 57. As their name suggests, lentiviruses were known to cause slow, unremitting disease in sheep, horses, and cattle, and to target various lineages of hematopoietic cells, particularly lymphocytes and differentiated macrophages.

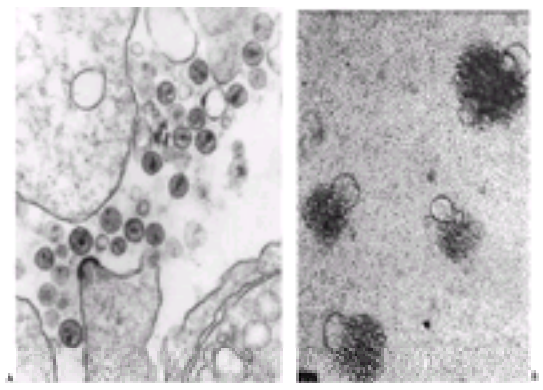

FIG. 1. Primate lentiviruses have a distinct morphology and can induce syncytia during productive infections. **A:** Electron micrograph showing a single HIV-1 particle in the process of budding from an infected cultured human PBMC, and several mature virions containing the characteristic conical/bullet-shaped nucleoid (×100,000). (Photomicrograph kindly provided by Dr. Jan Orenstein.) **B:** Typical ballooning syncytia induced by primate lentiviruses as visualized by inverted light microscopy at the time of peak virus production.

Following the isolation, molecular cloning, and initial classification of HIV-1, several genetically distinct primate lentiviruses were discovered and their phylogenetic relationships to HIV-1 were ascertained. For example, viruses isolated from captive macaques or feral monkey species in Africa were shown to possess particle morphologies and genomic organizations similar to those of HIV-1. Because inoculation of Asian macaque species, such as rhesus monkeys, with these newly discovered agents frequently induced an AIDS-like illness, these viruses were named simian immunodeficiency virus (SIV) to distinguish them from the human viruses, HIV-1 and HIV-2.

The phylogenetic relationships of the human lentiviruses are shown in Figure 2; the detailed genetic interrelationships of all members of the *Lentivirus* genus are presented in Figure 2 of Chapter 61. HIV-2 is more closely related to SIV_{smm} (285), a virus isolated from sooty mangabey monkeys in the wild, than to HIV-1. It is currently believed that HIV-2 represents a zoonotic transmission of SIV_{smm} to man (224). A series of lentiviral isolates from captive chimpanzees, designated SIV_{cpz}, are close genetic relatives of HIV-1. Unlike the other SIVs, which are endemic to African monkeys, the SIV_{cpz} genome contains a *vpu* gene (see below), also present in HIV-1 (298).

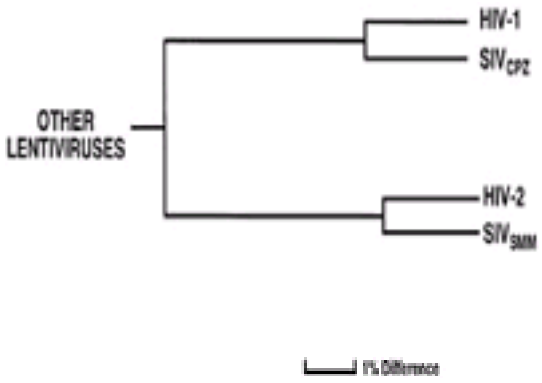

FIG. 2. Phylogenetic relationships of HIV-1 and HIV-2 based on identity of *pol* gene sequences. SIV_{cpz} and SIV_{smm} are subhuman primate lentiviruses recovered from a chimpanzee and sooty mangabey monkey, respectively. (Adapted from ref. 454, with permission.)

It was originally thought that HIV-1, like previously studied replication-competent retroviruses (e.g., MuLVs, ALVs, HTLVs), would be genetically homogeneous. However, as proviral DNAs corresponding to HIV-1 isolates from Europe, North America, and Africa became available and were compared, initially by restriction enzyme digestion, their extensive genetic heterogeneity became apparent (28). No two HIV-1 isolates were identical. When subjected to nucleotide sequence analysis, even HIV-1 samples recovered from a single individual exhibited significant heterology (545). Some of the nucleotide changes (nonsynonymous) resulted in amino acid substitutions, whereas others (synonymous) did not alter the protein sequence. Although nucleotide changes were distributed throughout the HIV-1 genome, the greatest variability occurred in the *env* gene when either intra- or interpatient virus specimens were compared. Hypervariable regions (designated V_1 to V_5), present in the gp120 envelope coding sequences, were sandwiched between relatively conserved (C_1 to C_5) sequences (see Fig. 27 for their locations) (596,666). The term *quasi-species* was subsequently coined to describe the heterogeneous and changing populations of virus present in an HIV-1-infected individual (435). Despite the heterologous nature of different HIV-1 isolates and, in particular, their envelope glycoproteins, a highly conserved and immunodominant domain, located within the gp41 envelope protein, still elicited antibody in infected individuals, which was reactive with (and diagnostic for) HIV-1 samples of diverse geographic origin (238).

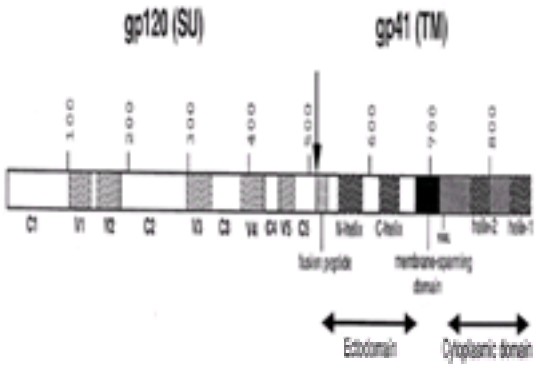

FIG. 27. Linear representation of the HIV-1 Env glycoprotein. The *arrow* indicates the site of gp160 cleavage to gp120 and gp41. In gp120, *cross-hatched areas* represent variable domains (V_1 to V_5) and *open boxes* depict conserved sequences (C_1 to C_5). In the gp41 ectodomain, several domains are indicated: the N-terminal fusion peptide, and the two ectodomain helices (N- and C-helix). The membrane-spanning domain is represented by a *black box.* In the gp41 cytoplasmic domain, the Tyr-X-X-Leu (YXXL) endocytosis motif and two predicted helical domains (helix-1 and -2) are shown. Amino acid numbers are indicated.

The earliest phylogenetic analyses of HIV-1 isolates focused on samples from Europe/North America and Africa; discrete clusters of viruses were identified from these two areas of the world. Distinct genetic subtypes or clades of HIV-1 were subsequently defined and classified into three groups: M (major); O (outlier); and N (non-M or O) (Fig. 3A). The M group of HIV-1, which includes over 95% of the global virus isolates, consists of at least eight discrete clades (A, B, C, D, F, G, H, and J), based on the sequence of complete viral genomes (384,453,673). Members of HIV-1 group O have been recovered from individuals living in Cameroon, Gabon, and Equatorial Guinea; their genomes share less than 50% identity in nucleotide sequence with group M viruses (604). The more recently discovered group N HIV-1 strains have been identified in infected Cameroonians, fail to react serologically in standard whole-virus enzyme-linked immunosorbent assay (ELISA), yet are readily detectable by conventional Western blot analysis (580).

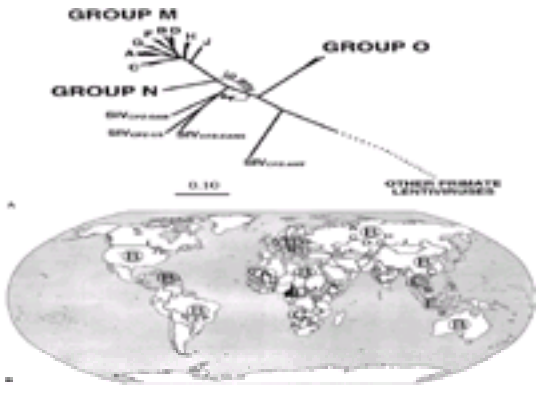

FIG. 3. HIV-1 genetic subtypes and their worldwide distribution. **A:** Phylogenetic relationships of HIV-1 groups M, N, and O with four different SIV_{cpz} isolates. This maximum likelihood tree, based on full-length *pol* gene sequences (115,344) from the indicated primate lentiviruses, was constructed by Bette Korber. The *bar* indicates a genetic distance of 0.1 (10% nucleotide divergence) and the *asterisk* positions group N HIV-1 isolates based on *env* sequences. **B:** The 1998 global prevalence of HIV-1 subgroups is shown with the predominant clade in each geographical region indicated by the *large circled letters.* Based on data collected by the Joint United Nations Programme on HIV/AIDS (UNAIDS). The location of Cameroon on the west coast of Africa is *shaded*.

Most current knowledge about HIV-1 genetic variation comes from studies of group M viruses of diverse geographic origin (383,384,604). Data collected during the past decade indicate that the HIV-1 population present within an infected individual can vary from 6% to 10% in nucleotide sequence. HIV-1 isolates within a clade may exhibit nucleotide distances of 15% in *gag* and up to 30% in gp120 coding sequences. Interclade genetic variation may range between 30% and 40% depending on the gene analyzed. HIV-1-like isolates from chimpanzees (SIV_{cpz}) are positioned at two locations in the maximum likelihood tree shown in Figure 3A. $SIV_{cpz\text{-}ant}$ is not closely related to any of the three HIV-1 groups, whereas $SIV_{cpz\text{-}gab}$, $SIV_{cpz\text{-}us}$, and $SIV_{cpz\text{-}cam3}$ cluster between group N and group O isolates, based on *pol* gene sequences (115,261).

All of the HIV-1 group M subtypes can be found in Africa (Fig. 3B). Clade A viruses are genetically the most divergent (362) and were the most common HIV-1 subtype in Africa early in the epidemic. With the rapid spread of HIV-1 to southern Africa during the mid to late 1990s, clade C viruses have become the dominant subtype and now account for 48% of HIV-1 infections worldwide (465). Clade B viruses, the most intensively studied HIV-1 subtype, remain the most prevalent isolates in Europe and North America. It should be emphasized, however, that in the context of the different HIV-1 clades, there has been no demonstrated association between antigenic serotypes and HIV-1 genetic subtypes (711).

Approximately 1% to 6% of the HIV-1 isolates in Cameroon are members of group O (424). A somewhat variable immunodominant gp41 region accounts for a 20% failure rate in identifying HIV-1 group O infected individuals by ELISA

assays, capable of detecting virtually all persons exposed to HIV-1 group M isolates. Interestingly, unlike group M viruses, group O strains do not require cyclophilin A for biologic activity (see the Gag protein section later) (52).

High rates of genetic recombination are a hallmark of retroviruses. It was initially believed that simultaneous infections by genetically diverse virus strains were not likely to be established in individuals at risk for HIV-1. By 1995, however, it became apparent that a significant fraction of the HIV-1 group M global diversity included interclade viral recombinants. It is now appreciated that HIV-1 recombinants will be found in geographic areas such as Africa, South America, and Southeast Asia, where multiple HIV-1 subtypes coexist and may account for more than 10% of circulating HIV-1 strains (222). Molecularly, the genomes of these recombinant viruses resemble patchwork mosaics, with juxtaposed diverse HIV-1 subtype segments, reflecting the multiple crossover events contributing to their generation. Most HIV-1 recombinants have arisen in Africa and a majority contain segments originally derived from clade A viruses. In Thailand, for example, the composition of the predominant circulating strain consists of a clade A *gag* plus *pol* gene segment and a clade E *env* gene (76). Because the clade E *env* gene in Thai HIV-1 strains is closely related to the clade E *env* present in virus isolates from the Central African Republic, it is believed that the original recombination event occurred in Africa, with the subsequent introduction of a descendent virus into Thailand (223). Interestingly, no full-length HIV-1 subtype E isolate (i.e., with subtype E *gag*, *pol*, and *env* genes) has been reported to date.

Recombination very likely occurred during the evolution of the three HIV-1-related chimpanzee isolates ($SIV_{cpz\text{-}gab}$, $SIV_{cpz\text{-}us}$, and $SIV_{cpz\text{-}cam3}$) based on their different tree locations when *pol* and *env* genes are used to establish phylogenetic relationships (see asterisk, Fig. 3A). When *env* sequences are used, these nonhuman lentiviruses cluster with group N HIV-1 isolates, whereas they map between groups N and O on the basis of their *pol* genes. This discordance points to an ancestral recombinational event between this group of SIV_{cpz} and group M HIV-1 strains (221). An intergroup (groups M and O) recombinant virus has recently been described (496), but no recombination between HIV-1 and HIV-2 has been reported, although mixed *in vivo* infections involving the latter are known to occur (254).

The origin of HIV-1 as a human pathogen remains an unsolved enigma of the AIDS epidemic. As noted earlier, HIV-2 appears to have been transmitted to man from SIV_{smm}-infected sooty mangabeys, which are endemic to west Africa, the same geographical region where HIV-2 is prevalent (224). Previous studies have shown that distinct phylogenetic lineages of SIV_{agm} are associated with different substrains of African green monkeys, each with nonoverlapping geographical ranges (447). This type of association implies exogenous infections hundreds of thousands of years ago and subsequent virus–host coevolution. In this regard, three of the four HIV-1-like viruses recovered from chimpanzees ($SIV_{cpz\text{-}gab}$, $SIV_{cpz\text{-}us}$, and $SIV_{cpz\text{-}cam3}$) were isolated from the central African *Pan troglodytes troglodytes* chimpanzee substrain, whereas the fourth ($SIV_{cpz\text{-}ant}$) was recovered from a completely different chimpanzee lineage, *P. t. schweinfurthii*, whose natural range is in eastern Africa (221). HIV-1 phylogenetic relationships based on *pol* genes (see Fig. 3A) indicate that not only do the viruses recovered from *P. t. troglodytes* subspecies cluster with the three main HIV-1 groups but also their *env* genes are very closely related to the group N virus strains. In contrast, the *P. t. schweinfurthii*–associated virus, $SIV_{cpz\text{-}ant}$, is the outlier, mapping to a highly divergent phylogenetic lineage. These results imply that, as in the case of SIV_{agm} and African green monkeys, host-dependent HIV-1 diversification occurred in discrete chimpanzee substrains. Given the fact that the natural range of *P. t. troglodytes* overlaps geographically with the region in Africa (Cameroon/Gabon) where all three HIV-1 groups are endemic, it seems likely that this chimpanzee subspecies is the source of a zoonosis to humans. A survey of HIV-1 prevalence in feral chimpanzees and in humans living in the same locale will be needed to verify this association.

GENOMIC ORGANIZATION OF THE HUMAN IMMUNODEFICIENCY VIRUSES

The nucleotide sequencing of the original HIV-1 isolates revealed an unexpected result. In contrast to prototypical retroviruses such as ALV and MuLV, whose genomes contain only three genes that encode the three groups of structural proteins [group-specific antigen (*gag*), polymerase (*pol*), and envelope (*env*)], the HIV-1 genome included several additional and overlapping open reading frames (ORFs) of unknown function (Fig. 4). Although an extra ORF, now known to encode a superantigen, had been previously identified within the 3' long terminal repeat (LTR) of mouse mammary tumor virus proviral DNA (136), the existence of additional, functionally important retroviral genes was generally unappreciated until the discovery and genomic analysis of HTLV-I (270). The sequencing of HTLV-I proviral DNA revealed the presence of three short, overlapping ORFs, located between the *env* gene and the 3' LTR. One of these encoded a regulator of transcription, the Tax protein. Not only did HIV-1 and HIV-2 contain multiple additional ORFs, but superficially, their genomic organizations appeared to be identical. However, further analyses demonstrated that HIV-1 contains the distinguishing *vpu* gene (107,602), and HIV-2 encodes the unique *vpx* (321) gene product (see Fig. 4).

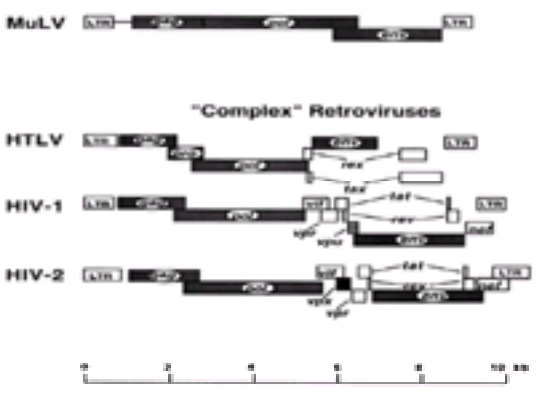

FIG. 4. Genomic organization of simple and complex retroviruses. The genes of Moloney murine leukemia virus (MuLV), HTLV-I, HIV-1 and HIV-2 are depicted as they are arranged in their respective proviral DNAs. The sizes of the different proviral DNAs are shown in proportion to the 9.7-kb HIV provirus.

As is the case for all replication-competent retroviruses, the three primary HIV-1 translation products, all encoding structural proteins, are initially synthesized as polyprotein precursors, which are subsequently processed by viral or cellular proteases into mature particle-associated proteins (Fig. 5). The 55-kd Gag precursor $Pr55^{Gag}$ is cleaved into the matrix (MA), capsid (CA), nucleocapsid (NC), and p6 proteins. Autocatalysis of the 160-kd Gag-Pol polyprotein, $Pr160^{Gag\text{-}Pol}$, gives rise to the protease (PR), the heterodimeric reverse transcriptase (RT), and the integrase (IN) proteins, whereas proteolytic digestion by a cellular enzyme(s) converts the glycosylated 160-kd Env precursor gp160 to the gp120 surface (SU) and gp41 transmembrane (TM) cleavage products. The remaining six HIV-1-encoded proteins (Vif, Vpr, Tat, Rev, Vpu, and Nef) are the primary translation products of spliced mRNAs.

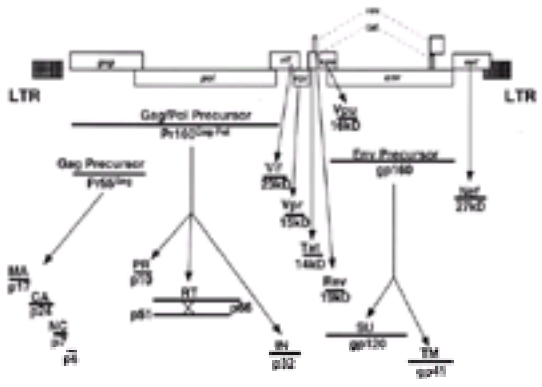

FIG. 5. HIV-encoded proteins. The location of the HIV genes, the sizes of primary translation products (in some cases polyproteins), and the processed mature viral proteins are indicated.

HIV-1 and HIV-2 have incorporated multiple sequence elements into their genomic RNAs that are required for the balanced and coordinated production of progeny virions. Most of these so-called cis-acting RNA elements (Fig. 6) are present in other retroviral genomes, but a few are unique to the primate lentiviruses. The $tRNA^{Lys}$ primer binding site (pbs), the major splice donor sequence (MSD), the encapsidation element (y), and an RNA dimerization initiation sequence (DIS), are all located within the 5'-terminal 400 nt (nucleotides) of the HIV-1 genome. In addition to the pbs, the HIV-1 genome contains other elements that help stabilize the encapsidated $tRNA^{Lys3}$ primer. These include an A-rich region located upstream of the pbs, which binds the anticodon loop of the tRNA primer, and a series of complex structural U5 motifs (301,319,374). Furthermore, the $Pr55^{Gag}$ precursor polyprotein appears to play the role of chaperone by catalyzing the annealing of the primer tRNA to the pbs/U5 region during virus assembly (178), a subject discussed in further detail in the Gag section of this chapter. The principal packaging signal for HIV-1 genomic RNA consists of three stem-loop structures, SL1, SL3, and SL4 (Fig. 6, inset A), which span the MSD (99,425). Elements located (a) at the 5' terminus of the genome (231), (b) within the so-called poly(A) stem-loop (125), and (c) at the base of the TAR stem (98) are also required for efficient RNA encapsidation. The DIS contains complementary and exposed sequences (GCGCGC in Fig. 6, insets A and B) located at the crown of SL1. It participates in the formation of viral RNA dimers, which are recruited into progeny particles by first forming “kissing loop” intermediates (35,484). Although two polyadenylation signals are present in HIV-1 genomic RNA, only the 3' AAUAAA hexameric motif directs poly(A) addition. A polyadenylation enhancer, situated upstream of the 3' polyadenylation signal, stabilizes the binding of the cleavage-polyadenylation-specifying factor to the AAUAAA hexamer (236,631), whereas the upstream poly(A) stem-loop structure has been reported to block poly(A) addition at the 5' end of the viral RNA (124).

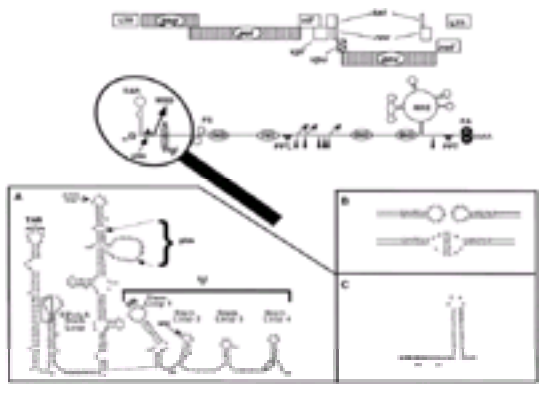

FIG. 6. Cis-acting elements present in the HIV-1 genome. Elements associated with HIV-1 genomic RNA include the methyl capped terminal G residue at the 5' terminus of viral RNA (*mG*); the Tat-responsive stem-bulge-loop structure (*TAR*); the binding site for the $tRNA^{Lys}$ primer (*pbs*); the major splice donor (*MSD*); the major RNA packaging site (y); the frameshifting motif (*FS*); RNA instability/nuclear retention elements (*INS*); the central polypurine tract (*PPTc*); splice acceptors (*arrowheads*); internal splice donors (*small arrows*); the Rev-responsive element (*RRE*); the canonical 3' polypurine tract (*PPT*); and the polyadenylation signal (*PA*). **A:** The putative secondary structure at the 5' terminus of HIV-1 mRNAs. The positions of the TAR stem-loop, the poly(A) stem-loop and the boxed poly(A) addition signal, the pbs, the dimerization initiation sequence (*DIS*), the major splice donor (*MSD*), and the translation initiation AUG codon for Gag are indicated. (Adapted from ref. 98, with permission.) **B:** The self-complementary DIS sequences, located at the crown of stem-loop 1, participate in the formation of “kissing loop” intermediates, an initial step in the RNA dimerization reaction. **C:** RNA stem-loop structure downstream of the UUUUUUA HIV-1 frameshifting sequence.

The HIV-1 genome is unique in having a novel central polypurine tract (PPT) in addition to a traditional U3-proximal PPT, both of which are used for the synthesis of plus-strand viral DNA (81). The genomic RNA also contains a heptameric U UUU UUA shift sequence within the *gag* gene, which functions in conjunction with a downstream hairpin (see Fig. 6, inset C) to mediate –1 translational frameshifting (305). The viral RNA folds into two additional complex structures (TAR and RRE), which are involved in RNA synthesis and transport, respectively. The TAR element has also been reported to be required for the efficient initiation of reverse transcription (268). Multiple inhibitory sequences (designated INS in Fig. 6), associated with the instability/ nuclear retention of HIV transcripts, are scattered throughout genes encoding Gag, Pol, and Env proteins (403,535). Finally, although adenosine deoxyribonucleotide (A) is not strictly considered a cis-acting element, it is worth noting that HIV-1 genomic RNA contains a significantly higher A content (approximately 39%) than mammalian DNA; this bias contributes to an unusual codon usage (34).

THE BIOLOGY OF HIV INFECTIONS

A quintessential property of HIV-1 and the other primate lentiviruses is to sequentially use CD4 and a second cellular receptor during entry into susceptible cells, a subject discussed more extensively in the section dealing with the envelope glycoproteins. Consequently, the main cellular targets for HIV-1 are the CD4+ T-helper/inducer subset of lymphocytes, CD4+ cells of macrophage lineage, and some populations of dendritic cells. Because HIV-1 infections *in vivo* are limited to humans and chimpanzees and no tractable animal model for HIV-1 currently exists, our current understanding about virus replication derives primarily from retrospective analyses of clinical specimens collected from seropositive individuals and a voluminous body of work examining virus infections in tissue culture systems. The very earliest studies indicated that mitogen-activated human PBMC were the cells of choice for isolating virus from infected persons. Some early HIV-1 isolates were also able to infect continuous CD4+ T leukemia cell lines such as CEM, Jurkat, Hut 78, and the HTLV-I-containing MT-2 and MT-4. Human T-cell lines were clearly more logistically tractable than PBMC, and their use greatly facilitated molecular and genetic studies of HIV-1. Most human T-cell lines released large quantities of virions while undergoing a typical cytopathic infection and cell death, but a few (e.g., H9 cells) became persistently infected and exhibited only minimal cytopathic effects.

Many of the early HIV-1 isolates were classified on the basis of their replicative, cytopathic, and tropic properties. A majority of primary virus isolates, which characteristically replicate slowly and generate small amounts of progeny particles, were labeled “slow/low” to distinguish them from “rapid/high” HIV-1 strains, which exhibit faster infection kinetics and release high titers of virions (179). Some HIV-1 primary isolates, frequently those recovered from symptomatic individuals, induced syncytia formation following co-cultivation of virus-producing PBMC with MT-2 cells (see Fig. 1B) (343). These isolates were designated SI (syncytium-inducing) to distinguish them from non-syncytium-inducing (NSI) strains. Tropism was a third property used to classify HIV-1 primary isolates. For many retroviruses, the determinants of tropism reside in both the external envelope glycoprotein and the LTR. Although changes in the LTR may modulate the efficiency of replication in a variety of T-cell types, the principal determinant of HIV host range resides in the SU Env glycoprotein. All HIV-1 strains can productively infect activated PBMC; some are also able to replicate in cultures of monocyte-derived macrophage (MDM) and have been classified as macrophage- or M-tropic (112). Virus isolated from individuals shortly after they have been infected with HIV-1 are frequently M-tropic (533). Other isolates, often obtained late in the disease course, can establish infections in continuous human CD4+ T-cell lines but not in MDM, and they have been designated T-cell-line or TCL-tropic. Subsequent studies reported primary HIV-1 isolates able to infect both MDM and T-cell lines; such strains have been classified as dual tropic (111). Although exceptions exist, M-tropic isolates, in general, are NSI and “slow/low,” whereas TCL-tropic strains are frequently SI and exhibit the “rapid/high” replication phenotype. Forced passage of some primary isolates in continuous CD4+ human cell lines has resulted in T-cell-line adapted (TCLA) HIV-1 strains, which possess altered tropic properties and increased sensitivity to neutralizing antibodies and soluble CD4 (606). In molecular clones corresponding to one of the original TCLA strains, HIV-1_{IIIB}, which had been extensively propagated in Hut 78 cells, the reading frames for the *vpr*, *vpu*, and *nef* genes are not open, suggesting that these three genes are not required for efficient replication in certain human T-cell lines. This is discussed in greater detail in the section dealing with accessory proteins.

The discovery that a and b chemokine receptors function as coreceptors for virus fusion and entry into susceptible CD4+ cells (92,134,177) has led to a revised classification scheme for HIV-1 (Table 1). Isolates can now be grouped on the basis of chemokine receptor utilization in fusion assays in which HIV-1 gp120 and CD4+ coreceptor proteins are expressed in separate cells (43). As indicated in Table 1, HIV-1 isolates using the CXCR4 receptor [now designated X4 viruses (30)] are usually TCL-tropic SI strains, whereas those exclusively utilizing the CCR5 receptor (R5 viruses) are predominantly M-tropic and NSI. The dual-tropic R5/X4 strains, which may comprise the majority of patient isolates and exhibit a continuum of tropic phenotypes, are frequently SI (579). However, because the expression levels of chemokine receptor, CD4, or gp120 may be unphysiologically high in fusion assays for assessing coreceptor usage, the classification of a particular gp120 based on these criteria may not accurately predict coreceptor utilization during actual spreading virus infections of PBMC, T-cell lines, and MDM (91).

Chemokine coreceptor used	PBMC replication	Macrophage replication	T-cell-line replication	Replicative phenotype	Syncytium-inducing phenotype
X4	+	-	+	Rapid/high	++
R5	+	+	-	Slow/low	-
R5X4	+	+	+	Rapid/high	+

PBMC, peripheral blood mononuclear cells.

TABLE 1. *Tropic and biologic properties of HIV-1 isolates*

The exclusive use of tissue culture systems to classify HIV-1 isolates and to define their replicative phenotypes clearly fails to address a myriad of biologic issues attending viral infections in exposed humans. For example, *in vivo*, most T lymphocytes circulating in the blood or present in lymphoid tissues are resting in the G_0 phase of the cell cycle; potential tissue macrophage targets exist as nondividing cells in G_1 (263). Early studies demonstrated that resting human PBMC do not support productive HIV infections unless they are stimulated with mitogens and propagated in the presence of interleukin (IL)-2 (430). This could reflect the extremely small pool sizes of ribonucleotides, deoxyribonucleotide triphosphates, and ATP in quiescent T lymphocytes (467). Although it is still unclear whether the reverse transcription or the integration step of the HIV-1 replication cycle is dysfunctional in unstimulated T lymphocytes, there is general agreement that no viral proteins or progeny virions are produced (598,696). Activation of resting PBMC up to 14 days following an initial abortive infection is still able to resurrect a productive infection (696).

Growth factors, cytokines, and antigens are physiologic activators of T lymphocytes *in vivo*. Antigen-mediated activation of the T-cell receptor leads to increased expression of several regulatory factors and the transition of lymphocytes from the G_0 resting state to the G_1 phase of the cell cycle. Co-stimulation of CD28 results in increased production of IL-2, surface expression of the IL-2 receptor, and progression through G_1, S, and mitosis. The absence of a co-stimulatory signal, however, leads to the unresponsive anergic state and arrest at the G_1a/G_1b transition point of the cell cycle (235). G_1a cells have a 2N DNA content, produce higher levels of RNA than G_0 cells, but are not committed to progress to S phase; in G_1b, cellular RNA levels are equivalent to those present in S phase and cells pass through the G_1/S checkpoint. Cell-cycle studies have shown that progression into G_1b is required to achieve productive HIV-1 infections of circulating CD4+ T lymphocytes (345). Incubation of resting T lymphocytes with IL-2, IL-4, IL7, or IL-15, in the absence of other stimuli, has also been reported to confer susceptibility to HIV-1 (630).

One of the most prominent and earliest recognized clinical features of AIDS is the selective depletion of CD4+ T cells, a property that correlates with tropism of HIV-1 and its cytopathicity for this lymphocyte subset in tissue culture infections. More detailed studies, primarily using X4 HIV-1 strains, indicated that memory CD4+ cells, rather than naive CD4+ T cells, were preferentially infected and killed (96,557). However, selective loss of CD4+ memory cells is not commonly observed in HIV-1 seropositive individuals. More recent work, employing CD3/CD28 co-stimulation to activate CD4+ T lymphocytes, has confirmed the greater susceptibility of memory cells to X4 viruses but it has also shown that naive but not memory CD4+ cells are infectable by R5 strains of HIV-1 (528). When lymphocytes from HIV-1-infected individuals were, in fact, examined, integrated proviral DNA was detected and virus could be recovered from both memory and naive CD4+ T cells with no evidence of selective chemokine coreceptor usage for either lymphocyte subset (478).

In addition to quiescent CD4+ T lymphocytes, nondividing pleuripotent hematopoietic stem cells (HSC) in G_0 have attracted considerable attention as targets for HIV-1, in the context of both natural infections and development of HIV-1 vectors for gene therapy. HSC are a subset of CD34-positive bone marrow cells that express CD4; some HSC also produce functional cell-surface CXCR4 and CCR5 proteins (571). Unfortunately, conflicting results have been obtained about their susceptibility to HIV-1, most likely reflecting (a) the criteria used for selecting G_0 HSC and not more mature progenitor cells, (b) the purity of the fractionated HSC, and (c) the transduction/infection protocol employed (571,629).

Dendritic cells (DCs) are antigen-presenting cells that capture, transport, and present antigens to CD4+ and CD8+ T lymphocytes. They include Langerhans cells in skin, follicular DCs in germinal centers, interdigitating DCs in T-cell areas of lymph nodes, interstitial DCs in intestinal and genital mucosa, and circulating, blood-derived DCs. It is currently thought that DCs associated with mucosal surfaces may represent the first line of defense against sexually transmitted HIV-1, transporting recently inoculated virus particles from portals of entry to secondary lymphoid organs. Nonetheless, productive HIV-1 infections of different types of purified DCs have been difficult to demonstrate *in vitro* (71), although vigorous virus replication is observed when DCs are first pulsed with virus and then cocultivated with CD4+ T lymphocytes (627). Interestingly, the infectious virions released from such cell conjugates bear T-cell-, not DC-, specific surface proteins acquired during the budding process, implying that a complete virus replicative cycle occurs in the T lymphocyte and that DCs may, in fact, be refractory to HIV-1 infection (189). It has recently been reported that DCs express a C-type lectin (named DC-specific ICAM-3-grabbing nonintegrin, or DC-SIGN) that “captures” HIV by binding to virion-associated gp120 and transmits the virus to T cells expressing CD4 and the appropriate chemokine coreceptor (232). Intriguingly, the virus bound to dendritic cells via DC-SIGN retains infectivity even on long-term culture (>4 days), suggesting that the dendritic cells are able to sequester or “protect” bound virions from degradation. These findings have significant implications for the transmission and dissemination of HIV *in vivo* and may present a novel target for antiviral therapy.

Virtually all lentiviruses have retained the capacity to replicate in nonproliferating, terminally differentiated tissue macrophages. Thus, unlike the oncoretroviruses, which require mitosis and dissolution of the nuclear membrane to complete a cycle of replication, HIV-1 is able to productively infect these G_1- (not G_0-) arrested cells. This property, thought to be mediated by one or more of the viral-encoded proteins, requires that the HIV-1 preintegration complex be translocated through intact nuclear pores. *In vitro*, differentiation of blood-derived monocytes is required for vigorous replication by M-tropic viral strains (562). As is the case for productive infections of T lymphocytes, HIV-1 replication in MDM requires integration of the reverse transcript (163), but only minimal cytopathicity has been reported and morphologically mature virus particles may accumulate intracellularly within intracytoplasmic vacuoles (477).

A variety of approaches have been used to measure the kinetics of HIV-1 infections in cell cultures and to determine the intrinsic infectivity of a virus stock. For example, the end-point dilution of a virus preparation (ID_{50}) is commonly reported as the amount of inoculum having a 50% probability of initiating an infection during a defined observation period. For

many commonly used cell-free HIV-1 stocks, the number of physical particles released from 10^6 virus-producing cells in 1 mL of medium can be more than 10^9 physical particles or approximately 10^3 virions per cell (138,429). Such culture supernates will typically contain 10^5 to 10^6 tissue culture ID_{50} (or $TCID_{50}$) per mL. These values will vary depending on the cell type (PBMC versus T-cell line) used and the virus source (primary versus TCLA isolate), but the time required for one complete cycle of HIV-1 infection *in vitro* is usually 3 to 4 days. It is worth noting that the particle-to-infectivity ratio for HIV-1 calculated from these values is quite low (10^{-3} to 10^{-4}). In contrast, the infectivity of virus-producing cells, as measured in co-culture systems, is approximately 10^2 to 10^3 times higher than the infectivity of all of the cell-free particles present in the culture supernate from the same infected cells (138). Taken together, these data suggest that, like many other retroviruses, HIV-1 relies on cell-to-cell transmission for dissemination of virus *in vivo*. Certainly the high density and proximity of virus-infected cells in lymphoid tissue would favor this mode of virus spread.

Although HIV-1 causes the selective loss of cultured CD4+ cells and induces depletion of the same T-lymphocyte subset in virus-infected individuals, the mechanisms responsible for this cytopathic effect are still unknown. Cell death is currently thought to occur by either necrosis or apoptosis, processes that can be distinguished morphologically and biochemically. Direct cell killing by HIV-1 in tissue culture systems can occur as a consequence of single-cell lysis or syncytium formation and is frequently associated with the presence or expression of the viral envelope glycoproteins (74,339). Apoptosis of T lymphocytes, characterized morphologically by nuclear condensation and DNA fragmentation and the absence of an inflammatory response *in vivo*, has been observed in lymph node biopsies from HIV-1-infected individuals (451). It has also been reported that cultured PBMC from HIV-1 seropositive persons undergo spontaneous apoptosis at higher rates than cells from uninfected individuals (255). A critical issue, pertaining specifically to apoptosis, is whether HIV-1 and related primate lentiviruses kill only cells they infect or indirectly cause the death of uninfected bystander cells including CD8+ T lymphocytes. Although many studies have shown that HIV-1-infected cells undergo apoptotic death, the use of reporter virus systems indicates that in cultures of purified T lymphocytes, infected CD4+ cells, not bystander cells, undergo apoptosis (219). The specific apoptotic pathway responsible for cell killing in HIV-1-infected cells has not yet been elucidated.

HIV-1 ANIMAL MODELS

Chimpanzees

Because HIV-1 establishes infections only in humans and chimpanzees and, with rare exceptions (464), induces disease only in humans, multiple questions about the biology of HIV-1 and the capacity of this virus to induce immunodeficiency remain unanswered. These include (a) the mechanism(s) underlying the transient depletion of CD4+ cells that occurs immediately following exposure to the virus; (b) the functional reversibility of this initial CD4+ T cell loss; (c) the pathogenic process(es) leading to the gradual destruction or aberrant functioning of the immune system despite the rapid and nearly complete elimination of the virus from the blood after the acute infection; (d) the irreversible loss of CD4 positive T lymphocytes which accompanies clinical progression to disease onset; (e) the contribution, if any, of specific HIV-1 phenotypes (SI versus NSI; T-cell-line tropic versus macrophage tropic) to virus transmission and disease progression; and (f) the biologic role of “auxiliary” viral genes such as *vpr*, *vif*, and *nef* during the establishment of the initial infection and its subsequent spread to other sites in the body.

In the search for an HIV-1 animal model during the very early phase of the AIDS epidemic, cell suspensions from virus-infected individuals were inoculated into a variety of animals including nonhuman primates, but only chimpanzees consistently became infected. It was later shown that chimpanzees could be reliably infected with relatively low doses of HIV-1 (13), but no longstanding impairment to the immune system was observed. Like the acute infection in man, a primary HIV-1 infection of chimpanzees is characterized by a brief period, lasting several weeks, during which virus can be readily recovered. Unlike in humans, however, the primary HIV-1 infection in chimpanzees is usually asymptomatic, and virus is infrequently recovered from the plasma of inoculated animals (209). Antibodies directed against several of the viral proteins can be detected within several weeks of inoculation, and a rapid decline of the virus load, typically measured by the number of PBMCs needed for HIV-1 isolation or containing viral DNA, is commonly observed. In most instances, virus isolation from persistently infected chimpanzees becomes intermittent after resolution of the primary infection (13,234).

The absence of virus-induced immunodeficiency in any of the more than 150 chimpanzees inoculated with HIV-1 led to a variety of hypotheses to explain the failure of the infection to progress to clinical disease. These included (a) the predominant use of clade B T-cell-line tropic virus isolates; (b) the generally poor replicative capacity/cytopathicity of HIV-1 in chimpanzee PBMC; (c) a failure of HIV-1 to induce apoptosis of CD4+ chimpanzee cells; and (d) the ability of the chimpanzee to mount a potent, long-lived protective response to HIV-1, similar to that reported for SIV-infected (and asymptomatic) sooty mangabeys, the natural host of SIV_{smm}. One chimpanzee, however, did develop high virus loads and a CD4+ T-lymphocyte depletion to the 100 cell/μL range nearly 10 years after documented exposure to two different virus strains ($HIV\text{-}1_{SF2}$ and $HIV\text{-}1_{LAV\text{-}1b}$) (464). These laboratory findings were associated with a nonregenerative anemia, chronic intermittent diarrhea, and the presence of opportunistic microorganisms (e.g., *Blastocytis hominis*, *Balantidium coli*, and *Cryptosporidium*). The transfusion of whole blood from this animal to a second chimpanzee resulted in extremely high plasma viral RNA loads ($>1\times10^7$ copies/mL) and a rapid decline of CD4+ T cells. Biologic and molecular analyses of the HIV-1 recovered from the two affected animals revealed (a) an augmented replication phenotype in chimpanzee PBMC/macrophages, and (b) that the pathogenic variant which had emerged was very likely a recombinant between the original $HIV\text{-}1_{SF2}$ and $HIV\text{-}1_{LAV\text{-}1b}$ input viruses (452).

The failure of chimpanzees to develop disease in a timely fashion following inoculation of tissue culture– adapted or patient-derived virus isolates coupled with their endangered species status has greatly dampened enthusiasm for their use as an animal model of HIV-1– induced immunodeficiency.

Transgenic Mice

When it became apparent, in the late 1980s, that HIV-1 infections could be established only in chimpanzees and humans, potentially more tractable and novel rodent systems were developed to model specific steps of *in vivo* HIV-1 infections. In one approach, transgenic mice, containing varying portions (individual viral genes/cis-acting elements up to the entire HIV-1 genome) of molecularly cloned viral DNA were constructed. Because HIV-1 transgenic mice contain copies of proviral DNA in every cell, they represent *in vivo* systems for modeling the postintegration phase of the virus life cycle. They provide a means for assessing possible deleterious effects of HIV-1–encoded proteins *in vivo* and for monitoring the mammalian responses to different viral gene products.

The earliest and simplest DNA constructs introduced into mice consisted of the HIV-1 LTR linked to a reporter gene [most commonly, chloramphenicol acetyltransferase (CAT)]. Animals bearing the viral promoter/enhancer of transcription usually expressed extremely low levels of CAT activity in specific tissues (skin, eye lens, lymph nodes, spleen, and thymus) (365). It was also reported (641) that HIV-1 LTR-directed expression of the viral *tat* gene induced dermal lesions in mice with features of Kaposi's sarcoma, although this result has not been independently confirmed. Animals harboring copies of full-length and potentially infectious HIV-1 DNA were also constructed and initially maintained in a biosafety level 4 (BL4) facility (367). In these transgenic mice, HIV-1 LTR-directed expression was barely detectable in 23 of 25 founder animals. In one of the two instances of high-level HIV-1 gene activity, heterozygous offspring, generated from a mosaic founder female, exhibited a spontaneous and fatal disease syndrome characterized by severe runting/growth retardation; splenomegaly; lymphadenopathy; dry, scaly skin; and perivascular pulmonary lymphoid infiltrates (367). The second transgenic line, also carrying a complete copy of the HIV-1 genome, has been used to assess the effect of opportunistic microorganisms on the expression of the HIV-1 transgene. Interestingly, *Toxoplasma gondii* or *Mycobacterium avium* infections of these transgenic mice resulted in marked elevations of HIV-1 RNA synthesis in multiple lymphoid tissues (142,230). In both cases, splenic macrophages represented a major target of increased HIV-1 expression.

To overcome the intrinsically low levels of HIV-1 LTR-directed expression in rodents, transgenic mice have also been generated that carry viral genes under the control of cell- or tissue-specific promoters. Mice harboring a transgene consisting of the mouse mammary tumor virus LTR-driving HIV-1 structural genes synthesized substantial amounts of HIV-1 Gag and Pol proteins in mammary and salivary glands, but no associated disease was observed (314). In another study, central nervous system disease developed in mice carrying HIV-1 gp120 coding sequences linked to the murine fibrillary acid protein promoter (621).

Several independent transgenic mouse lines have also been constructed to assess the *in vivo* function(s) of the Nef protein. These included animals in which *nef* expression was directed to T cells by using promoter/enhancers for the d subunit of the mouse CD3 gene (586), the human CD2 gene (54), and the mouse TCR b gene (375). Altered T-cell activation, CD4+ T-cell down-regulation, and immunologic dysfunction, respectively, were observed in these three lines of transgenic animals. More recently, transgenic mice bearing a complete copy of HIV-1 coding sequences, directed by a chimeric (human–mouse) CD4 promoter/enhancer, were constructed. These animals spontaneously developed muscle wasting, weight loss, severe depletion of lymphocytes from lymphoid organs, diarrhea, and death within 2 to 4 weeks of birth. When individual or combinations of HIV-1 genes within the full-length viral transgene were inactivated, only those animals expressing a functional *nef* gene exhibited the same debilitating clinical syndrome (266). Thus, targeting HIV-1 Nef synthesis to mouse CD4+ cells, in the absence of a spreading viral infection or the expression of other HIV-1 gene products, was sufficient to elicit a disease with some of the characteristics of human AIDS.

Severe Combined Immunodeficient Mice

In addition to their use for investigations of human immune responses, severe combined immunodeficient (SCID) mice, engrafted with human cells, have been used as a small animal model for HIV-1. In one protocol, 10^7 to 10^8 human PBMC are implanted intraperitoneally to create SCID-hu-PBMC animals (445). Human CD8+ T cells in these animals tend to remain in the peritoneal cavity, whereas the CD4+ T lymphocytes migrate to lymphoid tissue; both human T-cell subsets may persist for up to 6 months. The HIV-1-susceptible CD4+ T cells in SCID-hu-PBMC animals represent a population of mature, activated memory cells, functionally similar to those present in adult humans. In a modification of the SCID-hu-PBMC model, PBMC from HIV-1-infected individuals have been implanted into mice and the induction of virus from the transferred cells monitored.

In the second SCID mouse system used for HIV-1 studies (SCID-hu-thy/liv), fragments of human fetal thymus and fetal liver are engrafted under the renal capsule (457). The implanted organs increase in size for several months with CD4+/CD8+ double-positive T-lymphocytes predominating in the differentiating engrafted thymus. The SCID-hu-thy/liv system therefore models the fetal/neonatal human thymus and is relevant for studies of maternal–fetal virus transmission and pediatric HIV-1 infections.

In both SCID-hu systems, HIV-1 infections peak 3 to 4 weeks after inoculation and lead to variable (partial to complete) depletion of CD4+ T cells, depending primarily on the replication phenotype of the virus used (7,446). Any T-cell replacement that occurs is caused by proliferation of the implanted human cells. In general, both macrophage and T-cell tropic HIV-1 strains or primary viral isolates exhibiting rapid infection kinetics are more pathogenic in both SCID-hu mouse systems than more slowly replicating virus strains (311). Although the engrafted human tissue possesses demonstrable functional immunologic activity (299,426), the inoculation of HIV-1 fails to elicit a primary immune response in infected animals. Thus both of the SCID-hu systems model uncontrolled HIV-1 infections characterized by moderate to profound CD4+ T-cell depletions and no humoral or cellular responses to the virus. In some experiments, virus-specific antibodies and cytotoxic T lymphocytes have been adoptively transferred to SCID-hu-PBMC mice prior to virus challenge (489,632).

SIV–HIV Chimeric Viruses

A nonhuman primate lentivirus, initially isolated from a captive rhesus monkey in the mid 1980s, was shown to induce an

AIDS-like disease following inoculation of Asian macaques (123,368). As noted earlier, these SIVs establish asymptomatic chronic infections in their natural hosts, different species of African monkeys. The SIV genomic organization, while similar to that of HIV-1, can be distinguished by the presence of the *vpx* and the absence of the *vpu* genes (Fig. 7). When it became apparent that humans and chimpanzees were the only mammalian species that could be reliably infected by HIV-1, attention turned to the SIV–Asian macaque model with the idea of constructing chimeric SIV–HIV viruses (SHIVs) capable of infecting small, nonendangered, nonhuman primates. The earliest versions of SHIVs contained 3 to 4 kilobases (kb) of HIV-1 sequences, including the *tat*, *rev*, *vpu*, *env*, and, in some instances, portions of *vpr* and *nef* genes, which were inserted into the genetic backbone of SIV_{mac239} (see Fig. 7). The first generation of SHIVs successfully established chronic infections and elicited immunologic responses in inoculated macaques, but the virus did not replicate to high levels or cause clinical disease (372,573). Nonetheless, subsequent serial animal-to-animal transfers of blood, lymphoid cells, and/or bone marrow resulted in the emergence of pathogenic SHIV strains containing alterations affecting gp120-coding sequences (313,521). In contrast to SIV_{mac239} and HIV-1, which, on average, will induce immunodeficiency in 1 and 10 years following infection of macaque monkeys or man, respectively, some of the highly pathogenic SHIVs cause an unremitting and irreversible depletion of CD4+ T cells within 3 to 4 weeks of inoculation and death due to AIDS in 3 to 4 months. Because they possess and express the HIV-1 *env* gene, these new versions of SHIVs provide a disease endpoint for monitoring the effectiveness of HIV-1 envelope-based vaccine strategies.

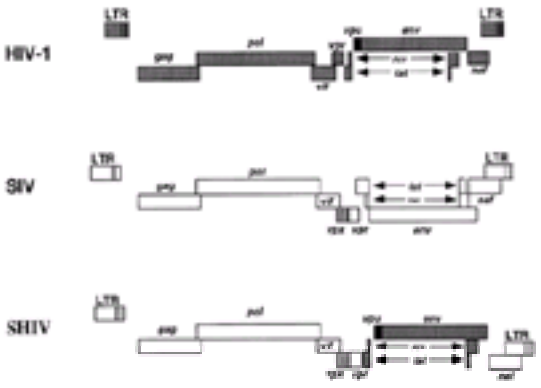

FIG. 7. Genomic organization of HIV-1, SIV, and SHIV lentiviruses. The *vpu* gene of HIV-1 and the *vpx* gene of SIV are highlighted. Some SHIV constructs contain an HIV-1–SIV chimeric *vpr* gene.

THE MOLECULAR BIOLOGY OF HIV-1 REPLICATION

Overview

More than 10,000 papers have been published on HIV-1 since its discovery as the cause of AIDS in 1984. A dizzying array of biochemical, genetic, and immunologic techniques, and biologic systems have been used to study the structure and function of HIV-1-encoded proteins and the host cell mechanisms that regulate virus replication. In addition to the production of HIV-1 proteins in bacterial, yeast, and insect cells, much of this effort has involved transfections of mammalian cells with vectors expressing individual viral proteins or cloned HIV-1 proviral DNA (Table 2). Transfection experiments have been important for elucidating (a) the intracellular location and trafficking of viral proteins, (b) the interaction of HIV-1 proteins with other viral or cellular proteins, (c) the binding of viral and cellular proteins to cis-acting elements associated with the viral genome or proviral DNA, (d) the fusion/entry steps of the virus life cycle, and (e) the assembly and release of progeny virions. In a majority of instances, the cells used for these transfection studies have been both CD4 negative and of nonhematopoietic origin, and they include HeLa, COS, and 293T.

TABLE 2. *Experimental systems used in HIV-1 research*

Transfection experiments are to be distinguished from HIV-1 infections, which are characterized by particle-mediated cell entry, in conjunction with the use of one or more surface receptors, and the spread of progeny virions through the culture, in successive cycles of replication. Thus, infections provide an opportunity to integrate the function(s) of an individual viral-encoded protein (or even a subdomain thereof) into the entire HIV-1 replicative cycle. For many studies, single-cycle infections, involving defective HIV-1 genomes, pseudotyped with a heterologous envelope protein, have been used to obtain answers to specific questions, even though the newly produced virus particles are incapable of sustaining a virus infection. It is worth keeping in mind that although transfection experiments and *in vitro* studies such as the yeast

two-hybrid system have yielded invaluable information about the function and interacting cellular partners of HIV-1-encoded proteins, the results obtained must always be verified in assays that monitor spreading viral infections.

The HIV-1 replicative cycle (Fig. 8) begins with adsorption of virus particles to CD4 molecules on the surface of susceptible cells. While binding of virions to CD4 is essential for HIV infectivity, their subsequent interaction with a coreceptor, one of which are now known to be the seven membrane-spanning CC or CXC families of chemokine receptors, is required for membrane fusion and entry (92,134,145,177). Although multiple chemokine receptors exhibit activity in fusion/entry assays with HIV-1 gp120 (see the Env section below), the two most important are CXCR4 (previously called fusin, or LESTR) and CCR5. Unlike other enveloped viruses, which utilize receptor-mediated endocytosis to enter cells, HIV-1 and most other retroviruses directly fuse with the plasma membrane of a susceptible cell. Following entry, subviral particles are partially uncoated in the cytoplasm and initiate the reverse transcription of their viral RNA genomes (see Fig. 8, steps 2 to 4). Investigations of these early events in the virus life cycle have been greatly hampered by the very high (>100:1) retrovirus particle-to-infectivity ratios noted earlier. Thus, simply monitoring the fate of tagged viral proteins during the entry and uncoating steps is unlikely to provide useful information about the composition and trafficking of biologically active virions.

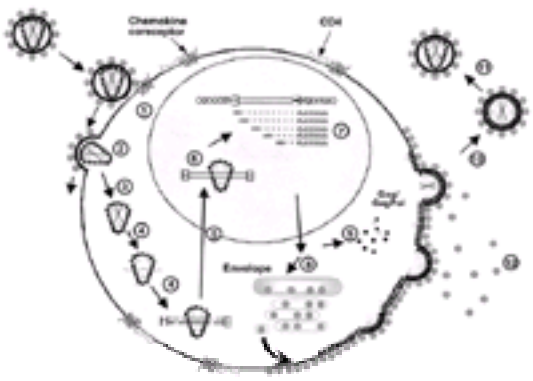

FIG. 8. The HIV-1 replication cycle. Productive HIV-1 infections begin with the adsorption of cell-free virions to cells and their interactions with both the CD4 and chemokine receptors (*step 1*). In the case of HIV, virus entry (*step 2*) is a pH-independent process that occurs following the fusion of viral and cellular membranes and results in the partial uncoating (*step 3*) of incoming virions. Reverse transcription occurs within subviral particles in the cytoplasm of infected cells (*step 4*), and the double-stranded DNA product is transported to the nucleus (*step 5*), where integration into chromosomal DNA (*step 6*) is mediated by the virus-encoded integrase (*open circles*), a component of a subviral preintegration complex. The integrated viral DNA serves as a template for DNA-dependent RNA polymerase (*Pol II*) and leads to the production of mRNAs (*step 7*) that are translated into viral proteins in the cytoplasm of infected cells. Envelope (*step 8*) and Gag plus Gag/Pol (*step 9*) polyproteins are transported via independent pathways to the plasma membrane, where progeny virus particles begin “budding” from cells and are released as immature particles (*step 10*). Subsequent proteolysis by the virion-encoded protease generates mature particles (*step 11*) containing a characteristic condensed core. Non-virion-associated gp 120 envelope protein is also released from cells (*step 12*).

As is the case for other retroelements (51,58,206), the partially double-stranded DNA reverse transcription product is transported through the cytoplasm and into the nucleus as a component of a nucleoprotein–preintegration complex (PIC) containing a subset of the Gag and Pol proteins (see Fig. 8, step 5). This poorly understood process very likely depends on host factors that actively transport the PICs from the plasma membrane to the nuclear pore. These “early” steps in the retrovirus replication cycle undoubtedly require the participation of multiple cellular factors for their successful completion. Following the import of the PIC into the nucleus, full length linear copies of the reverse transcript are integrated into the chromosomal DNA of the infected cell, a step required for the efficient viral RNA synthesis and infectious particle production (568). As noted earlier, the lentiviruses are unique among retroviruses in generating PICs that are actively transported by the nuclear import machinery into the interphase nucleus of nondividing cells arrested in the G_1 phase of the cell cycle. In activated T lymphocytes, integrated copies of HIV DNA serve as templates for RNA polymerase II (Pol II)-directed viral RNA synthesis (see Fig. 8, step 7). The coordinated interaction of the HIV-encoded Tat protein and the cellular transcriptional transactivator proteins NF-kB and Sp1 with the Pol II transcriptional apparatus ensures the production of high levels of viral RNA. Unspliced or partially spliced HIV transcripts are exported from the nucleus to the cytoplasm by a unique transport mechanism mediated by the shuttling virus-encoded Rev protein. The subsequent translation of the gp160 Env precursor occurs in the endoplasmic reticulum, whereas the Gag and Gag-Pol polyproteins are synthesized on free cytoplasmic ribosomes, and they are transported via independent pathways to the plasma membrane (see Fig. 8, steps 8 and 9). The Gag and Gag-Pol polyproteins, in association with dimers of genomic RNA, condense at the plasma membrane to form an electron-dense “bud” (see Fig. 1) that gives rise to a spherical immature particle containing the mature TM and SU envelope glycoproteins. Proteolytic processing of the Gag and Pol proteins by the HIV PR during or immediately after particle release generates the cone-shaped nucleoid characteristic of mature HIV virions (see Fig. 8, steps 10 and 11).

Thus HIV-1 uses the same replicative strategy as the so-called simple retroviruses, which encode only the Gag, Pol, and Env proteins. However, during its evolution, HIV-1 acquired six additional genes to carry out functions that are performed by cellular proteins that are either (a) already present in the cells infected by the simple retroviruses or (b) uniquely required for virus replication, transmission, and survival in hematopoietic cells targeted by the primate lentiviruses. Some of the HIV-1 accessory proteins (Vif, Vpr, Vpu, and Nef) are not required for replication in certain human T-cell lines, although virus infectivity may be affected up to several thousand-fold depending on the accessory gene and the type of infected cell. In view of their conservation, however, these additional genes must be required for functions unique to HIV-1 replication *in vivo*.

Because many of the HIV-1-encoded proteins individually mediate critical functions at both early and late times in the virus life cycle (see Fig. 8), the synthesis, processing, and utilization of the various viral gene products will be presented in the order of their location within the HIV-1 genome rather than the usual temporal format. The multiplicity of biologic functions is particularly true for the Gag and Env proteins, which play important roles during both the initial phases and the final steps of the infectious cycle. Thus, rather than sequentially discussing the production and subsequent spread of progeny virions (virus entry, uncoating, reverse transcription, integration, transcription, translation, virus assembly, particle release), and to minimize unnecessary redundancy, the following sections will integrate the synthesis, structure, and function of individual HIV-1 nucleic acids and proteins in a context of HIV-1 biology both *in vitro* and *in vivo*.

The HIV Long Terminal Repeat

The retroviral LTR is a useful starting point for understanding the complex interplay between HIV and its principal target, human CD4+ T lymphocytes. LTRs are generated during the process of reverse transcription and therefore exist only as "repeats" in viral DNA. During the replicative cycle, LTR sequences serve a multitude of functions in the context of both proviral DNA and genomic RNA (Fig. 9). DNA and RNA sequences, mapping to the R region of the LTR, participate in one of the early steps of reverse transcription by forming intermolecular DNA–RNA hybrid "bridges" linking the newly synthesized DNA strand ("minus" polarity) with the 3' end of the same or a different genomic RNA template ("plus" polarity). During the integration step of the virus life cycle, LTR elements (*att* sequences) located at the termini of full-length linear viral DNA molecules mediate their insertion into the chromosomal DNA of host cells (109). Other LTR sequences, located in the context of U5 RNA sequences, have been shown to contribute to the packaging of progeny HIV-1 RNA genomes during virus assembly (637).

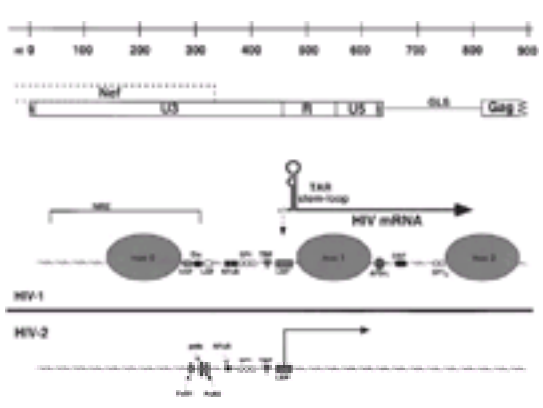

FIG. 9. Structure of the HIV-1 long terminal repeat (LTR). The HIV-1 LTR is a duplicated 630+ bp element located at the termini of integrated proviral DNA and has been divided into three functional subregions. The R (repeat) domain is defined as a 96-bp-nt repeat present at the 3' and 5' termini of HIV-1 genomic RNA and all viral mRNAs; U5 is an 84-nt segment located immediately 3' to the R region; and U3 is a 454-nt segment situated immediately 5' to R. The region of *nef* gene overlapping U3 and the position of LTR terminal *att* sequences (in the context of full-length viral DNA) are shown at the *top.* The 5' LTR and adjacent *gag* leader sequence (GLS) are aligned with the subdivided LTR (*middle*) to indicate functionally important binding sites for transcriptional regulatory proteins. For HIV-1, the "enhancer" domain contains three Sp1 binding elements and two NF-kB binding motifs. The transcription start site is located at map position +1, which is defined as the border between U3 and R. The NRE contains binding sites for USF and the Ets proteins. Sequence motifs that interact with the TATA binding protein (TBP), the leader binding protein (LBP), the LEF, AP3-like (AP3-L), the DBF-1, and two downstream Sp1 elements ($SP1_D$) are shown. The position of nucleosomes nuc0, nuc1, and nuc2 are indicated. An HIV-1 RNA transcript, which is initiated at the first nucleotide within the R region (designated +1) and contains the TAR element near its 5' terminus, is also shown. For HIV-2 (*bottom*), the unique binding sites for the PuB1, pets, and PuB2 transcriptional factors are indicated.

In the context of integrated viral DNA, the major function of the retroviral LTR is the regulation of viral RNA synthesis. The HIV-1 transcriptional promoter and adjacent regulatory elements, both of which are involved in recruiting the RNA Pol II holoenzyme to the start site of viral RNA synthesis, are located within the U3 region and function in the context of the 5' LTR (see Fig. 9). In primate lentiviruses, the *nef* ORF overlaps 60% to 70% of the U3 region, directly contributing to the relatively large size (400 to 500 nt) of the HIV-1 U3 compared to other retroviruses. Nef coding sequences of HIV-1, in fact, extend into the 5' 331 of the 454-nt U3 region (see Fig. 9). The polyadenylation signal (AATAAA) and polyadenylation addition site are also located in the R region but, as noted previously, are active only as components of the 3' LTR.

Like other eukaryotic cellular promoters, the integrated HIV LTR contains several elements that facilitate the loading of Pol II on the DNA template. The initial recognition of the HIV promoter requires the participation of several general transcription factors (GTFs), including TFIID, which specifically binds to the TATA element, located –29 to –24 nts upstream of the transcriptional start site, and to adjacent viral DNA sequences. This process generates a platform upon which a functional transcriptional complex can be assembled. The bound TFIID is then recognized by a single GTF, TFIIB, which recruits Pol II to the promoter, a critical interaction that definitively establishes the location of the transcription start site (373).

Another GTF (TFIIH), which modifies Pol II allosterically and allows the transcription complex to initiate RNA polymerization and "escape" from the promoter, must be incorporated into the Pol II complex (385,470). TFIIH is a multicomponent transcription factor that includes a kinase activity able to phosphorylate a unique region within the largest Pol II subunit, the C-terminal domain (CTD). The phosphorylated state of the CTD critically determines the functional activity of Pol II. Whereas hypophosphorylated Pol II (Pol IIa) is readily recruited into the preinitiation

transcription complex, the hyperphosphorylated form of the enzyme (Pol IIo) efficiently mediates elongation of nascent RNA transcripts (466). Three kinases, associated with the transcriptional apparatus, are known to phosphorylate the CTD. These include (a) the CDK7/cyclin H subunits of the cdk-activating kinase (CAK) complex associated with TFIIH, (b) the CDK8/cyclin C component of the Pol II holoenzyme, and (c) the CDK9/cyclin T subunits of the positive transcription elongation factor b (P-TEFb). Hyperphosphorylation of the CTD induces conformational changes in Pol II that allow the transcription complex to clear the promoter and begin elongating nascent RNA molecules.

In eukaryotic cells, regulatory transcriptional factors, which bind to DNA elements near the promoter, modulate the basal rate of RNA synthesis in response to physiologic cues. These regulatory factors function in a combinatorial fashion via protein–protein interactions to recruit GTFs, as well as the Pol II enzyme itself, to the promoter. This results in the more frequent assembly of stable transcription complexes that are able to readily escape the promoter and generate full-length primary transcripts. As is the case for other eukaryotic cellular promoter/enhancers, the HIV LTR contains its own ensemble of DNA elements to which transcriptional regulatory factors bind (see Fig. 9). Some of these were identified by mutagenesis of LTR-driven reporter gene constructs in transfection experiments, some were found as a result of gel-retardation or DNA footprinting studies, and others were discovered from searches of nucleotide sequence databases. Although early studies of HIV LTR-directed expression of reporter genes suggested that a large domain (–410 to –157) within U3, designated the negative regulatory element (NRE), might “silence” viral gene activity (534), there is little convincing evidence that the NRE plays a significant role during virus replication. Furthermore, mutations located in the region of the SIV *nef* gene that overlaps the NRE segment of the U3 LTR did not specifically affect viral replicative properties in infected rhesus monkeys (300). However, mutations altering the recognition sites for USF, Ets, and LEF-1 (572,575), which are located in the promoter-proximal portion of the *nef* overlap of U3, markedly impaired HIV-1 replication in activated PBMC (332). It is also worth noting that the U5 portion of the HIV LTR and *gag* leader sequences, both of which are situated *downstream* of the transcription start site, also contain cognate binding sites for cellular factors (see Fig. 9) that potentially regulate RNA synthesis during productive viral infections (see later).

The LTRs of HIV and other primate lentiviruses contain three tandemly arranged binding sites for the constitutively expressed Sp1 transcription factor (267); these DNA elements are situated upstream of a canonical Pol II TATA box (see Fig. 9). The Sp1 and TATA elements constitute the HIV-1 core promoter and must be present for basal levels of LTR-directed RNA synthesis. Sp1 was originally identified in HeLa cells and shown to bind to the multiple GGGCGG motifs (GC box) associated with the 21 base-pair (bp) repeats in SV40 DNA, activating both early and late SV40 transcription *in vitro*. Functional analyses of HIV-LTR-driven reporter constructs have shown that mutations of individual or pairs of Sp1 sites have little, if any, effect on the basal or Tat-transactivated levels of expression (267). Mutation of all three HIV Sp1 sites, however, markedly reduced the response to the HIV-1-encoded Tat transactivating protein (32,267). In the context of virus infections, the role of the Sp1 elements is cell type dependent (366,536). Mutations that functionally inactivate all three Sp1 motifs eliminated detectable replication in Jurkat cells, delayed progeny virus production in CEM and H9 cells, and had little effect on infectivity in activated PBMC.

Adjacent to the Sp1 binding sites, the two tandem recognition sites for the NF-kB/Rel family of transcription factors constitute an activatable enhancer for HIV-1 LTR-directed expression (see Fig. 9) (455). Although the LTRs of HIV-2 and SIV retain the triplicated Sp1 motifs, they contain only a single NF-kB binding site. NF-kB is a member of a large family of cellular transcription factors, which include the c-Rel protein of chickens and the dorsal gene product of *Drosophila*, a regulator of pattern development in embryos. Selected members of this NF-kB/Rel gene family are shown diagrammatically in Figure 10. Several contain a highly conserved N-terminal Rel homology domain (RHD), approximately 300 residues in size, that includes DNA binding and dimerization regions and a nuclear localization signal (NLS). A conserved protein kinase A site is present in several but not all RHDs. The p65/Rel A protein contains a potent transcription activation domain located downstream from its RHD. The p50 protein is derived from the p105 precursor, which contains a glycine-rich hinge linked to an ankyrin repeat domain; the p65/Rel A protein, in contrast, is a primary translation product. The inhibitory IkB proteins all contain multiple 30- to 34-amino-acid ankyrin motifs, which provide the interface for binding to various species of NF-kB.

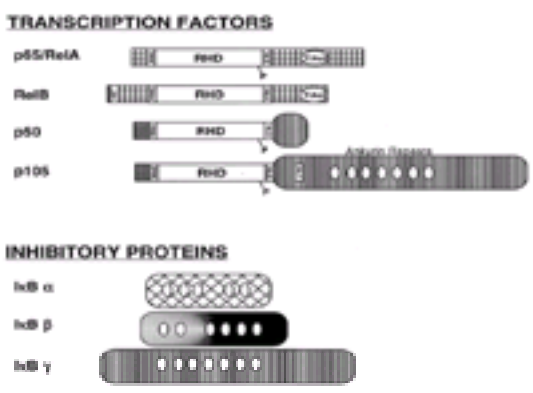

FIG. 10. NF-kB transcriptional regulatory proteins and IkB inhibitors. The NF-kB/Rel family of transcriptional factors shown may contain a Rel homology domain (*RHD*), DNA binding motifs (*DNA*), a leucine zipper (*ZIP*), a nuclear localization signal (*NLS*), a glycine hinge (*GLY*), a transactivation domain (*T/Act*), and a protein kinase A phosphorylation site (*P*). The ankyrin repeats are indicated by the *white ovals*.

NF-kB “binding activity” was originally identified as the p50-p65/Rel A heterodimer, the most abundant and functionally active form of NF-kB in human T cells (17). Because the various members of the c-Rel family are able to form dimeric NF-kB molecules, a heterogeneous population of homo- and heterodimers with different functional specificities could potentially bind to the two NF-kB sites present in the HIV-1 LTR. NF-kB functional activity is controlled by its intracellular location. In the cytoplasm, NF-kB dimers are complexed to IkB proteins or other Rel family members containing ankyrin repeats, such as the unprocessed p105 protein (see Fig. 10), and cannot be imported into the nucleus (326). The binding

of IkBa to the RHD of the p65 subunit of heterodimeric NF-kB results in its sequestration in the cytoplasm due, perhaps, to a masking of the p65 NLS (26). Nearly all of the T lymphocytes encountered by HIV-1 *in vivo* will be quiescent and lack NF-kB binding activity. Activation of mammalian T cells results in the phosphorylation of the bound IkB, the rapid degradation of IkB via the ubiquitin pathway, and the translocation of NF-kB into the nucleus where it can activate multiple eukaryotic genes (326,485). Convergent signal transducing pathways associated with T-cell activation, including those initiated at the IL-1 and tissue necrosis factor (TNF) receptors have been experimentally studied for their contributions to NF-kB activation (75,540).

The nuclei of activated human T lymphocytes and T-cell lines contain amounts of NF-kB capable of binding to cognate sites in the HIV-1 LTR and stimulating both basal and Tat-induced levels of expression, as monitored with reporter genes in transient transfection assays (455,574). When the NF-kB motifs are altered in the context of HIV, infectivity is affected, depending on the type of mutant constructed and the endogenous cellular levels of NF-kB. Deletion of both HIV-1 NF-kB binding motifs resulted in no (366) or modest (536) delays in peak virus production in activated human PBMC, whereas point mutations affecting specific nucleotides involved in NF-kB recognition but not the spatial organization of the NF-kB elements within the HIV-1 LTR, reduced progeny virus production by more than 10-fold (83). The replication of HIV-1 NF-kB mutants in several human T-cell lines was found to be inversely proportional to the basal levels of NF-kB. It is likely that the binding of other transcription factors present in these cell lines can compensate for the absence of NF-kB at the HIV-1 promoter in these virus mutants. The simultaneous mutation of the two NF-kB motifs and all three Sp-1 sites completely abolishes HIV-1 replication (366,536), consistent with the demonstration that the cooperative interaction of NF-kB and Sp-1 promotes the binding of both factors to the HIV-1 LTR and induces transcriptional activation (499). A synthesis of all of these results is that NF-kB mediates a more rapid and robust production of HIV-1 progeny in activated human T lymphocytes.

The HIV-2 LTR also contains three Sp1 binding sites adjacent to its TATA box, but the organization of its upstream enhancer region is different from that of HIV-1 (see Fig. 9). This portion of the HIV-2 U3 LTR contains only a single functional NF-kB motif as well as three other binding sites (PuB1, PuB2, and pets) not present in the HIV-1 LTR (415). The purine-rich PuB1 and PuB2 sequences are recognized by members of the Ets family of proto-oncogenes (mainly Elf-1) and the human autoantigen DEK binds to the pets site (203,361). Thus, while activation of HIV-1 LTR-directed expression following T-cell activation is mediated primarily through the binding of NF-kB to its two cognate sites, induction of HIV-2 gene expression depends on a different set of cellular transactivators.

Most of what is currently known about regulated HIV gene expression comes from experiments in which naked DNA was transiently transfected into mammalian cells. In eukaryotic cells, however, the interactions of transcriptional regulatory proteins with their cognate cis-acting elements is further modulated by the packaging of DNA into chromatin. This is clearly the case for HIV-1 proviral DNA, which becomes stably integrated into host cell chromosomal DNA during productive viral infections. DNA footprinting and restriction enzyme accessibility studies of integrated viral DNA have in fact revealed the presence of nucleosomes located both upstream and downstream of the HIV-1 promoter (designated nuc-0 and nuc-1 in Fig. 9). Nucleosome positioning defines two open chromatin regions encompassing (a) promoter/enhancer sequences [–250 to +11 (relative to the transcription start site)], and (b) a downstream segment that begins within the 3' portion of U5 and extends into the Gag leader (+150 to +250) (159,635). This chromatin organization is independent of the provirus integration site. The nucleosome-free region promoter-distal to nuc-1 and located downstream of the transcription start site contains recognition sites for three different transcription factors: Ap-3-like, DBF-1, and Sp1 (see Fig. 9). Mutagenesis of these binding motifs effectively abolishes the downstream nucleosome-free zone and markedly inhibits the transcriptional activity of the HIV-1 promoter in the context of both stably integrated LTR constructs and infections mediated by mutagenized cell-free virus (159,633). Taken together, these results are consistent with a model in which the positioning of nucleosome nuc-1 immediately downstream of the transcription start site sterically interferes with transcription, resulting in the characteristically low basal/uninduced levels of HIV-1 RNA synthesis. The positioning of nuc-1 is thought to reflect the binding of transcriptional regulatory factors to upstream promoter/enhancer motifs (viz. Sp 1 and NF-kB sites) and downstream recognition sequences situated in the U5/Gag leader region (Ap-3-like, DBF-1, and Sp1), a combination that creates two nucleosome-free regions. Transcriptional activation of cells containing the integrated HIV-1 LTR with TNF-a or inhibitors of histone deacetylation specifically results in the rapid disruption of nucleosome nuc-1 and generates a 500-bp open chromatin region (635,636). It is not presently known whether the chromatin associated with the HIV-1 template must be remodeled prior to the initiation of transcription or is an inevitable consequence of the RNA synthetic process.

Proteins encoded by several heterologous animal viruses have been reported to transactivate the HIV LTR. Although this effect has been touted by some as indicating that these agents contribute to disease progression, the co-infection of individual human T cells by HIV-1 and other viruses has been difficult to document. On the basis of tropism for CD4+ human mononuclear cells, viruses capable of directly or indirectly transactivating the HIV LTR would include HTLV-I, human CMV (HCMV), human herpes virus type 6 (HHV6), and possibly human adenoviruses (460). In the case of HTLV-I, it has been proposed that elevated levels of NF-kB induced by the viral-encoded Tax protein (18) activate HIV replication by binding to the two NF-kB motifs in its LTR.

HIV-Encoded Regulatory Proteins

Tat

Although the mechanism underlying Tat transactivation of HIV LTR-directed expression was hotly debated for several years, it is now generally agreed that Tat increases the steady-state levels of viral RNA several hundred fold by directing the formation of a more processive Pol II transcription complex in virus-infected cells. Tat is an indispensable viral protein; when the *tat* gene of an infectious molecular proviral clone of HIV is mutagenized, no detectable progeny virions are produced as monitored in either transfection or infectivity assays (128,182). HIV-1 Tat is a nuclear protein containing 101 amino acid residues encoded by two exons (Fig. 11). A shorter 72-amino-acid, “one-exon” HIV-1 Tat protein possesses all the transcriptional activating properties of full-length Tat, as measured in tissue culture infections or in LTR-driven reporter gene experiments. The termination codon following the first Tat exon is highly conserved among diverse HIV-1 isolates, suggesting that the “one-exon” and “two-exon” Tat proteins mediate different functions during

productive viral infections *in vivo*.

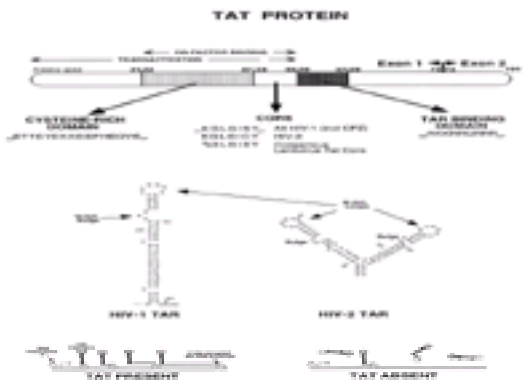

FIG. 11. Tat and its TAR response element. Schematic representation of the HIV-1 Tat protein (*top*) with the cysteine-rich activation, core, and TAR-binding domains indicated. The stem-bulge-loop configurations of HIV-1 and HIV-2 TAR elements are shown in the *middle*; the release of prematurely terminated HIV-1 transcriptional complexes from the DNA template in the absence of Tat is depicted at the *bottom*.

As previously noted, RNA synthesis in eukaryotic cells is usually modulated by transcription activator proteins, which bind to DNA motifs located *upstream* of their respective promoters and recruit GTFs and the Pol II holoenzyme to the transcription initiation complex. Visna virus, another member of the *Lentivirus* genus, in fact encodes a DNA-binding Tat protein, which binds to Ap-1 sites located in its LTR (283). In contrast, the immediate target of all primate lentiviral Tat transactivating proteins are sequences situated *downstream* of the start site for viral RNA synthesis (534). This unusually situated transactivation response region, or TAR element, also differs from typical binding sites for Pol II transactivators because it is inactive when present (a) in an inverted orientation, (b) 5' to the promoter, or (c) downstream of the transcription termination site (i.e., in the U5 region of the 3' LTR) (309). These unusual properties were subsequently reconciled when it was demonstrated that TAR did not function as a DNA recognition site but as an RNA element (33).

The HIV-1 TAR encompasses the 5'-terminal 59 nts of *all* viral RNAs and folds into a stable stem-loop structure (see Fig. 11, middle). It was subsequently shown that the minimal TAR element (mapping between bases +19 and +43) contains three critical components: a base-paired stem, a trinucleotide bulge (containing the sequence UCU at positions +23 to +25), and a hexanucleotide G-rich loop (176). Interestingly, the HIV-2 TAR forms a double stem-loop structure, each arm of which possesses a dinucleotide bulge and the hexanucleotide G-rich loop (see Fig. 11) (160). Sequences located in both the hexanucleotide loop and the bulge of the HIV-1 TAR are required for Tat function. *In vitro*, purified preparations of Tat bind to TAR RNA with high affinity and moderate specificity (140). HIV-1 Tat binds to both wild-type or “loop” mutants of TAR but *not* to TAR elements containing alterations affecting the bulge region (139,542). Modification of the invariant “bulge” U_{+23} nucleotide and elimination of the base pairs immediately above and below the bulge (see Fig. 11) significantly reduce the binding of Tat to TAR (650). Nuclear magnetic resonance (NMR) studies have shown that the binding of a large Tat peptide to TAR causes the major groove in the RNA duplex to widen, thereby generating multiple points of contact between Arg residues in the Tat-binding domain with critical nucleotides (e.g., U_{+23}) and several phosphate groups comprising the TAR backbone (1). This distortion of the TAR structure also brings the loop and bulge regions into close proximity, which could facilitate the interactions of proteins binding to both of these TAR domains.

The functional organization of the HIV-1 Tat protein has been deduced from TAR binding and transcriptional activation experiments using both wild-type and mutagenized derivatives of the Tat protein. The tripartite Tat activation (or effector) domain encompasses the amino-terminal 48 residues, which include (a) a string of highly acidic amino acids (residues 1 to 21), (b) a Cys-rich region (seven invariant, six of which are required for function) between positions 22 and 37, and (c) a hydrophobic core segment (amino acids 38 to 48), which is highly conserved among different HIV isolates (see Fig. 11). The activation domain is critical for recruiting an essential cellular cofactor to TAR; mutations affecting this region of the Tat protein drastically reduce or eliminate transactivation activity (543). The RNA binding domain of Tat has been mapped to a Lys/Arg-rich region between residues 49 and 57 (see Fig. 11); peptides from this Tat segment bind to the TAR bulge region as well as to a few base pairs surrounding the bulge, but with somewhat less affinity and specificity than do purified preparations of Tat protein (116). A nuclear/nucleolar localization signal ($_{48}$Gly-Arg-Lys-Lys-Arg_{52}) also overlaps the RNA-binding region of Tat.

At first glance, the functional organization of HIV-1 Tat is highly reminiscent of many other transcriptional regulatory proteins, which contain modular nucleic acid binding and activation domains. However, a growing body of work has revealed that Tat/TAR interactions are more complex *in vivo* and involve the participation of several cellular cofactors. This became apparent from experiments showing that the Arg-rich domain of the Tat protein did not function as an independent RNA-binding module *in vivo*. These experiments indicated three things: (a) The targeting of heterologous fusion proteins to TAR required *both* the RNA binding and activation regions of Tat (392). (b) The RNA binding domain of Tat failed to function as a transdominant negative inhibitor of wild-type Tat, as did analogous domains from other RNA binding proteins such as HIV Rev (see later). This result implied that the Tat RNA binding region was neither autonomous nor able to form functional Tat/TAR interactions *in vivo* (398). (c) Wild-type TAR, but not mutant TAR decoys containing changes limited to the G-rich loop, rendered CEM cells resistant to HIV infection (605). The failure of excess Tat protein to relieve the blockade induced by wild-type TAR decoys suggested that at least one loop-binding cellular cofactor, present in limiting amounts, was required for functional Tat/TAR interaction *in vivo*.

Other studies demonstrated that Tat chimeric proteins, which could be targeted to the promoter via heterologous (non-TAR) RNA binding sites, also exhibited high levels of transactivation (569,620). This implied that, if necessary, Tat could function in the absence of TAR and recruit cellular factor(s) to the transcription complex. It was also reported that

inefficient Tat transactivation in rodent cells (269) could be overcome if Tat was delivered to the promoter using a surrogate RNA binding site (9). This latter result indicated that a cellular cofactor required for Tat transactivation was indeed present in rodent cells but could not be recruited to or bind TAR. Collectively, these findings are consistent with a model in which Tat uses its transactivation domain to recruit a cellular cofactor (or cofactors) to TAR; in such a scenario, Tat would bind to the TAR bulge and the cofactor(s) to the TAR loop.

The hallmark of HIV LTR-directed RNA synthesis in the absence of Tat is the accumulation of prematurely and randomly terminated transcripts, which are converted to longer RNA species when Tat is expressed (see Fig. 11, bottom). This Tat-deficient phenotype has been observed in *in vitro* transcription experiments, following transient transfection of LTR-driven reporter gene constructs, and in cells harboring integrated HIV proviruses with mutated *tat* genes (173,355,622). Because Tat expression markedly stimulated transcriptional elongation in many of these experimental systems, attention shifted to the possible involvement of the CTD of Pol II, which, as noted earlier, is hyperphosphorylated in highly processive transcription complexes. This line of reasoning was also consistent with the reported inhibition of Tat-stimulated transcription by the adenosine analog DRB, which blocks Pol II–mediated elongation *in vitro* and *in vivo* by inactivating protein kinases (53). The demonstrated requirement of the Pol II CTD for Tat-mediated transactivation of viral RNA synthesis also supported such a model (487,682).

Reports describing the specific interaction of HIV-2 Tat [or just the effector domain of HIV-1 Tat (residues 1 to 48)] with a cellular kinase linked many of these disparate findings and provided the first mechanistic clues about Tat function (280,281). The novel enzymatic activity identified, initially named Tat-associated kinase or TAK, was able to phosphorylate the CTD of Pol II. Contemporaneous studies evaluating Tat-stimulated Pol II processivity in cell-free systems suggested that Tat interacted with components of the TFIIH complex to enhance transcriptional elongation and raised the possibility that TAK might be a TFIIH-associated kinase (227,487). However, it was subsequently shown that Tat binds with high affinity to a Pol II CTD kinase complex (710) related to the *Drosophila* multicomponent P-TEFb mentioned previously, which is catalytically independent of TFIIH (418,497). Moreover, Tat interaction with TFIIH is weak and not dependent on the integrity of its activation domain (84). P-TEFb shares no subunits in common with the TFIIH elongation factor complex, and it regulates the transition from abortive to fully processive transcriptional elongation (417). Additional experimentation revealed that TAK hyperphosphorylated the CTD of Pol II, independent of TFIIH (and its associated CDK7 kinase), and was more sensitive to DRB inhibition than was CDK7 (279,417,418). A recent study indicated that TFIIH activity is not required for HIV-1-mediated transcriptional elongation (84).

The Tat-related kinase component in human P-TEFb was subsequently identified to be PITALRE, a 42-kd CDC2-related kinase. When antibodies directed against PITALRE (now named CDK9) were used to deplete the kinase in nuclear extracts, the residual activity for Tat-simulated RNA chain elongation was reduced nearly 100-fold (498,710). Tat activity could be restored by adding purified P-TEFb to the cell-free system. Furthermore, the identification of novel Tat inhibitors, which specifically abolished CDK9 activity *in vivo* and *in vitro*, coupled with the demonstrated dominant negative effects of a CDK9 mutant on Tat transactivation (409,710), provided strong evidence that CDK9 was intimately involved in regulating Tat function. Taken together, these results suggested that TAK and P-TEFb were functionally indistinguishable transcription factors, sharing the common CTD kinase activity, CDK9. The subsequently demonstrated identity of the cloned catalytic subunit of P-TEFb and the kinase subunit of TAK provided conclusive evidence for this idea (681,710).

Although, as noted previously, earlier studies had shown that TAK/P-TEFb interacted with the activation domain of HIV-1 Tat, the CDK9 catalytic subunit itself failed to bind to Tat in *in vitro* assays. Equally perplexing was the identification of the TAK/P-TEFb kinase activity in nuclear extracts migrating not as the 42-kd CDK9 but as a 110-kd complex (682). This suggested the existence of a possible cyclin-related partner for CDK9, which might provide substrate specificity. These unresolved issues were clarified with the isolation of the “missing link”—an 87-kd protein from nuclear extracts, which bound to wild-type HIV-1 Tat but not to a mutant Tat protein containing a nonfunctional activation domain (651). The sequence of a cDNA clone encoding the 87-kd protein revealed the presence of an amino-terminal cyclin box that was nearly 40% identical to human cyclin C. The new protein, initially named cyclin T (because it bound to Tat), is encoded by a gene mapping to human chromosome 12. The CDK9 family of kinases are now known to interact with cyclin regulatory subunits, many of which have been cloned and characterized (497). Several human cyclins (T1, T2a, and T2b) can functionally partner CDK9, greatly increasing its kinase activity (498); of these, only cyclin T1 is able to directly bind to the HIV-1 Tat activation domain and is a component of P-TEFb (651).

The binding of Tat to TAR has traditionally been the centerpiece of models for Tat-mediated transactivation. Following the identification of CDK9/cyclinT1 as cellular cofactors of Tat, attention turned to determining how they interacted with Tat, TAR, and the cellular transcriptional machinery. A large body of work revealed the following information: (a) CDK9 and cyclin T1 are components of the large human P-TEFb transcription complex that promotes RNA chain elongation. (b) CDK9/cyclin T1 is present in the nucleus (possibly also the cytoplasm) of eukaryotic cells as an assembled heterodimer, and its recruitment into the Pol II complex is associated with highly processive transcriptional activity. (c) Recombinant cyclin T1 directly binds to the effector domain of wild-type Tat but not to Tat proteins containing mutations affecting this region. This is consistent with the reported interaction of Tat and purified human P-TEFb in the absence of TAR (681,710). (d) The binding of Tat to TAR RNA is markedly enhanced in the presence of cyclin T1. Several reports have, in fact, shown that the Tat/cyclin T1 heterodimer has greater affinity for TAR than does either Tat or cyclin T1 alone (39,225,651). As expected, the interaction of Tat with purified P-TEFb greatly increased the affinity of Tat for TAR (706). (e) The Tat–cyclin T1 complex will bind only to TAR RNA in which both the loop and the bulge are intact, whereas Tat alone requires only the trinucleotide bulge for stable TAR interaction. These results suggest that multiple pair-wise interactions are involved in stabilizing the binding of Tat to TAR: (a) Tat with P-TEFb, (b) Tat with the TAR bulge, (c) cyclin T1 with the TAR loop, and (d) the TAR bulge/TAR loop with Tat/P-TEFb.

As noted earlier, Tat transactivation of HIV-1 LTR-driven gene expression is quite low in rodent cells but can be greatly augmented in rodent somatic cell hybrids containing human chromosome 12 (269). This defect can also be corrected by overexpressing human cyclin T in rodent cells, which results in enhanced Tat transactivation of the HIV-1 LTR to levels measured in human cells (651). Recent studies have shown that the murine homolog of human cyclin T is able to bind to HIV-1 Tat, but the resulting Tat/murine cyclin T heterodimer is not efficiently recruited to TAR (204,225). Substitution of a

single amino acid residue in murine cyclin T with its human analog (Tyr to Cys at residue 260) also restores Tat activity in rodent cells (39,225). Thus, the extremely weak Tat transactivation in rodent cells is not caused by a failure to form the Tat/cyclin T heterodimer; rather, the cross-species heterodimer that does form is apparently unable to bind to TAR and deliver P-TEFb to a poorly processive transcription complex.

A model of HIV Tat transactivation is shown in Figure 12. Tat interacts, via its activation domain, with the cyclin T1 subunit of cyclin T1/CDK9, a component of the P-TEFb elongation complex present in the nuclei of virus-infected cells. The binding of human P-TEFb to Tat most likely induces a conformational change in the structure of Tat, which alters its affinity and specificity for binding to TAR RNA. Thus, the function of Tat is to recruit the critical P-TEFb elongation factor to a promoter-proximal location where it can hyperphosphorylate the Pol II CTD, thereby stimulating transcriptional processivity. It is worth noting that expression of both CDK9 and cyclin T1 increases following activation of resting PBMC with phytohemagglutinin (PHA), phorbol myristic acid (PMA) plus ionomycin, anti-CD3 antibody plus IL-2, or antibodies to both anti-CD3 and the CD28 receptor (278). Thus T-cell activation increases steady-state levels of both NF-kB as well as the two cellular cofactors of Tat, resulting in high levels of HIV-1 LTR-directed gene activity.

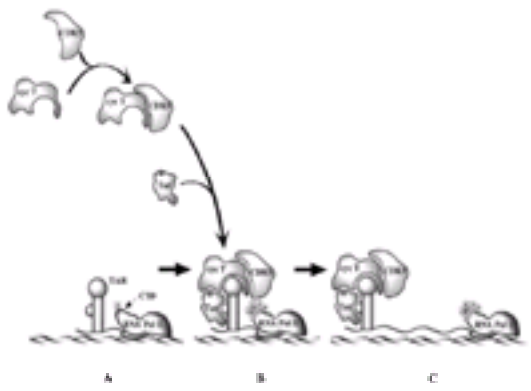

FIG. 12. Tat promotes the phosphorylation of the carboxy terminal domain (*CTD*) of RNA Pol II. **A:** In the absence of Tat binding to TAR, the processivity of the RNA Pol II complex is inefficient. **B:** When the activation domain (*) of Tat interacts with the cyclin T (*cyc T*)/CDK9 complex, the conformation of Tat changes and its affinity and specificity for TAR RNA increases. **C:** By recruiting cyc T and CDK9 (the TAK complex) to a promoter proximal location, Tat mediates the hyperphosphorylation of the CTD and promoter clearance (elongation) of transcriptional complex. (Drawing kindly provided by Dr. Michael Cho.)

Possible extracellular roles for Tat have been suggested from studies showing that Tat is taken up by cultured cells, enters the nucleus, and transactivates genes linked to the HIV LTR (191). Tat purified from *Escherichia coli* has also been reported to inhibit antigen-induced, but not mitogen-induced, proliferation of PBMC (639). In a similar vein, it has been reported that low concentrations of Tat modestly stimulate the growth of cultured Kaposi sarcoma cells from patients with AIDS (164). The significance of these extracellular Tat activities in HIV-infected individuals is currently unclear.

Rev

Like other primary RNA transcripts synthesized in eukaryotic cells, HIV pre-mRNAs undergo a series of modifications (capping, 3'-end cleavage, polyadenylation, and splicing) prior to their export to the cytoplasm. Retroviruses utilize the cellular posttranscriptional processing machinery to carry out these functions. With few exceptions, introns present in nascent cellular mRNAs must be removed prior to their export from the nucleus, presumably to prevent their translation into nonfunctional proteins. This requirement poses an obvious problem for retroviruses such as HIV, which must export a variety of intron-containing mRNAs into the cytoplasm (e.g., the unspliced 9.2-kbp primary transcript for encapsidation into progeny virions and production of Gag and Pol proteins, as well as several other partially spliced mRNAs such as those encoding Env, Vif, and Vpr proteins). In part, retroviruses have solved the requirement for the removal of all intronic sequences from pre-mRNAs prior to nuclear export by incorporating suboptimal splice sites into their genomes. These sites are inefficiently processed by splicing factors, resulting in the accumulation of unspliced viral RNAs in the nucleus. The nuclear export of intron-containing retroviral RNAs may be additionally impeded as a result of their interaction/retention with the cellular splicing apparatus (456).

All retroviruses must splice their primary RNA transcripts to generate *env* mRNAs, eliminating upstream *gag* and *pol* sequences in the process. For simple retroviruses such as the ALVs and MuLVs, this is the only splicing reaction that the viral pre-mRNA undergoes (Fig. 13, top). In contrast, the splicing of HIV RNA is far more complex because of the presence of both constitutive and alternatively used splice-donor and splice-acceptor motifs scattered through its genomic RNA. Three general classes of HIV mRNAs have been identified in productively infected cells: (a) unspliced genomic RNA, which serves as the mRNA for synthesis of Gag and Pol proteins; (b) partially spliced RNAs, approximately 4.3 to 5.5 kb in size, which are translated into Vif, Vpr, Vpu, and Env proteins; and (c) multiply or completely spliced viral mRNAs, ranging from 1.7 to 2.0 kb in size, which encode the Tat, Rev, and Nef proteins (see Fig. 13, bottom). The first two classes of viral mRNA contain several spliceable introns yet are efficiently exported from the nucleus into the cytoplasm.

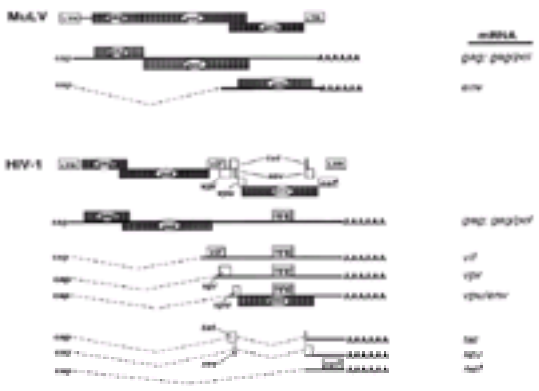

FIG. 13. Retrovirus splicing patterns. In contrast to murine leukemia virus (MuLV), which generates only two discrete mRNA species (the unspliced *gag*/*pol* and the singly spliced *env*), HIV-1 produces several alternatively spliced mRNAs ranging from the unspliced *gag*/*pol* to the multiply spliced *tat*, *rev*, and *nef* RNA transcripts. The genomic organization of the proviral DNA, the location of protein coding sequences, and the position of the Rev-responsive element (*RRE*) in intron-containing mRNAs encoding viral structural proteins are indicated. The *dashed lines* connect the major splice donor to a downstream splice acceptor; alternative forms of *tat*, *rev*, *vpu*/*env*, and *nef* mRNAs, some of which contain short upstream noncoding exons, are not shown.

Analyses of viral mRNAs by RT polymerase chain reaction (RT-PCR) have revealed the existence of more than 30 different HIV transcripts in virus-producing cells (513,566). These mRNAs are generated as a consequence of alternative selection of the five splice donors (sd) and the more than 10 splice acceptors (sa) embedded in the viral genome (some of which are shown at the top of Fig. 14). Variable usage of this ensemble of splicing signals gives rise to several sets of distinct, closely related RNAs that serve as alternative templates for translation into the same protein. For example, 12 different *rev*, 5 different *nef*, 8 different *tat*, and 16 different *env* mRNAs have been identified (513). This HIV mRNA diversity is caused, in part, by the variable inclusion of two upstream 50- and 74-nt noncoding exons (generated from sa2/sd2 and sa3a/sd3, respectively; Fig. 14) in many of these spliced RNA species. The functional significance of multiple spliced mRNAs encoding the same viral protein, their relative abundance, and the hierarchy of HIV splice site usage are not presently understood.

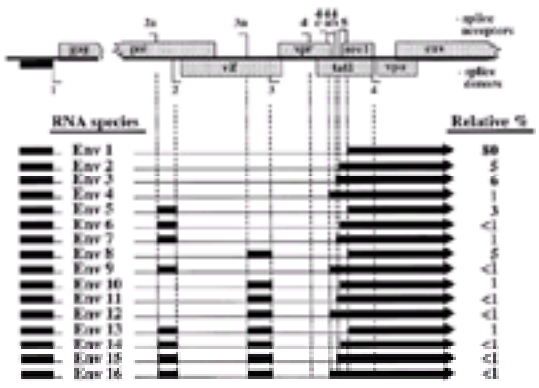

FIG. 14. Structure and relative abundance of differentially spliced HIV-1 *env* mRNAs. For each RNA species, the *dark bars* and associated 3' *arrows* represent exons included in the spliced transcript as determined by semiquantitative PCR analysis. The relative proportion of each mRNA is indicated at the right.

Complex retroviruses, such as HIV, have dealt with the restriction to the nuclear export of unspliced and incompletely spliced transcripts by incorporating a novel reading frame, encoding the Rev (which stands for regulator of expression of viral proteins) protein, into their genomes. In the absence of Rev, the unspliced *gag*/*pol* and the partially spliced *vif*, *vpr*, and *vpu*/*env* mRNAs fail to accumulate in the cytoplasm, thereby rendering the Rev mutant virus replication incompetent (174,264,588).

HIV-1 Rev is a 19-kd, predominantly nucleolar phosphoprotein containing 116 amino acid residues. Like Tat, Rev is encoded by two exons and contains two functional regions: (a) an Arg-rich domain that mediates RNA binding and nuclear localization, which is flanked by sequences that facilitate Rev multimerization; and (b) a hydrophobic segment, located between residues 73 and 84, that contains several Leu residues, now known to promote nuclear export (see Fig. 15, top) (404,406,407,698). Mutation of any one of three critical Leu residues (at position 78, 81, or 83) within this domain eliminates Rev activity. Unlike those of *tat*, both coding exons of *rev* are required for function (546).

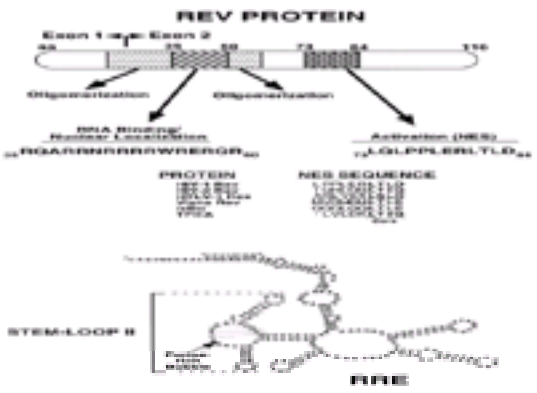

FIG. 15. Rev and its response element, the RRE. A schematic representation of the HIV-1 Rev protein with its RNA-binding, activation, and oligomerization domains is shown at the top. The amino acid sequence of the leucine-rich nuclear export signal (*NES*) present in retroviral Rev/Rex proteins and two cellular proteins are also shown. The core tetramer motif, LxLy, where y is usually a charged residue and x is highly variable, is indicated. The structure of the RRE is presented at the *bottom* with the high-affinity target of HIV-1 Rev (*stem-loop II*) shown. The "bulged" G residues in stem-loop II able to form noncanonical purine–purine base pairs are *circled.*

The HIV Rev protein regulates the expression and utilization of viral transcripts by binding to a cis-acting target, the Rev response element (RRE), present in all unspliced and partially spliced viral mRNAs (see Fig. 13). The RRE, located in a 250-nt segment spanning the junction between gp120 and gp41 coding sequences of the *env* gene, is a complex RNA structure containing multiple stem-loops branching from a large central bubble (174,405,535) (see Fig. 15, bottom). The RRE must be present *within* a Rev-responsive transcript and in the *sense* orientation for Rev responsiveness. Nuclease protection, chemical modification, and mutagenesis studies indicate that Rev specifically interacts with a 60+ nt portion of the RRE, designated stem-loop II (see Fig. 15) (129,288,407). This region of the RRE binds to Rev even when isolated from the complete RRE structure, and it mediates Rev responsiveness in functional assays (294). The determinants for high-affinity binding of Rev to RRE reside in the central purine-rich "bubble" in stem-loop II; this bubble contains unusual G:G and G:A base pairs that distort the duplex RNA structure and widen the major groove to accommodate the Rev protein (22,289,407). NMR analyses have shown that an alpha helical, Arg-rich, 17-residue peptide, from the RNA binding domain of Rev, burrows deep into the major groove of a stem-loop II oligonucleotide, stabilizing the non-Watson-Crick base pairs through specific interactions involving the Arg side chains (23). It is now believed that Rev initially makes high-affinity contact with nucleotides in the RRE loop II bubble as a monomer, thereby generating a nucleation point for the multimerization of additional Rev molecules through cooperative protein–protein and protein–RNA interactions (698,700). The functionally complete RRE is most likely 350 nt in size, and an individual element may accommodate eight or more Rev molecules (410). Although not yet developed into usable antiviral agents, some aminoglycosides are able to block the binding of HIV Rev to its RRE target and inhibit the production of progeny virions (699).

Mutations affecting either the RNA binding or the Leu-rich activation domains of the Rev protein result in loss of function. For example, activation domain mutants are able to bind to the RRE but are functionally inactive. They interfere with the transactivation mediated by wild-type Rev, exerting a trans-dominant effect in assays for Rev function (404). Rev also possesses the unique property of continuously shuttling between the nucleus and the cytoplasm (434). Mutations affecting the Rev activation domain abolish the shuttling and restrict Rev to the nucleus. Multimerization-defective Rev mutants can still bind to the high-affinity RRE site but are unable to form oligomeric complexes (399).

Although the total amount of viral RNA in cells infected with wild-type or Rev-deficient HIV mutants is quite similar, *gag-pol*, *env*, *vif*, and *vpr* mRNAs are either absent or markedly underrepresented in the cytoplasm of cells infected with Rev mutants compared to the relatively normal levels of multiply spliced *tat*, *rev*, and *nef* transcripts in the same cellular compartment (174,405,588). This pattern of RNA expression initially led to a plethora of proposed posttranscriptional functions mediated by Rev, including (a) the regulation of splicing, (b) nuclear to cytoplasmic transport, (c) RNA stabilization, and (d) stimulating the translation of RRE-containing HIV RNAs. As noted earlier, the HIV-1 genome contains multiple sequence motifs, located in *gag*, *pol*, and *env* genes (and designated INS in Fig. 6), which contribute to a requirement for Rev. These cis-acting AU-rich elements markedly impair the expression of fused reporter genes by impeding RNA transport to the cytoplasm and/or decreasing RNA stability (403,535). It is quite likely that cellular proteins bind to these inhibitory sequences, contributing to the nuclear retention and the intrinsic instability of intron-containing viral RNAs. Rev is able to reverse these effects, provided that an RRE is present in such transcripts.

Careful analyses of HIV RNA expression patterns in different cellular compartments indicated that the ratios of unspliced (or partially spliced) to completely spliced viral transcripts in the nucleus did *not* change in the presence or absence of Rev (174,405). Rather, as noted previously, the hallmark of insufficient Rev expression was a superabundance of the 1.7- to 2.0-kb, completely spliced RNAs. and greatly reduced or no detectable RRE-containing mRNAs *only* in the cytoplasm. Because splicing of viral RNA in the nucleus was not affected by the absence of Rev, attention shifted to the role that Rev might have on HIV RNA transport.

The first unambiguous demonstration that Rev could promote the nuclear export of intron-containing viral RNA came from experiments in which purified Rev protein and RRE-containing RNA molecules were microinjected into cell nuclei (180,656). These studies showed that the RRE-containing unspliced RNA substrates were transported from the nucleus into the cytoplasm only in the presence of Rev. Interestingly, the excised exon (containing the RRE element), derived from those transcripts that did undergo splicing, was also detected in the cytoplasm, indicating that Rev was able to mediate nuclear export *without* inhibiting RNA splicing (181). Other microinjection studies revealed that the Leu-rich Rev activation domain was indeed a nuclear export signal (NES) because, when fused to bovine serum albumin (BSA), it promoted the transport of NES/BSA molecules into the cytoplasm (180,656). In contrast, NES/BSA fusion proteins containing a mutated, nonfunctional Leu-rich Rev domain remained in the nucleus. The nuclear export mediated by the

Rev NES was also shown to be energy dependent and was blocked by high concentrations of NES/BSA, a result that implied that the amounts of intracellular nucleus-to-cytoplasm transporting proteins were limiting. Surprisingly, the nuclear export of 5S rRNA and spliceosomal U small nuclear (sn)RNAs, but not Pol II–derived cellular mRNAs, were also inhibited when high concentrations of NES/BSA conjugates were microinjected. In this regard, a growing list of viral and cellular proteins involved in RNA metabolism and signal transduction have been shown to contain a Rev-like, Leu-rich NES (see Fig. 15, middle), which mediates the transport of a variety of substrates from the nucleus to the cytoplasm (303,423). Taken together, these results are consistent with a model in which the Rev effector domain functions as a NES, directing RRE-containing viral RNAs to the cytoplasm via a pathway used by some cellular mRNAs.

The bi-directional passage of proteins between the nucleus and cytoplasm occurs through the nuclear pore complex (NPC) and depends on the presence of NLS and NES transport signals. The NPC is an extremely large (over 100 megadaltons) gated structure composed of 50 to 100 different proteins, called nucleoporins, only a few of which have been characterized (168,603). One class of nucleoporins, thought to directly participate in nuclear-cytoplasmic transport, contains Phe-Gly (FG) repeat domains and are located primarily at the periphery of the NPC. Proteins destined to be imported into the nucleus (e.g., bearing a typical basic-type NLS) initially associate with a member of a growing family of transport “receptors” in the cytoplasm. The first receptor of this type discovered, importin-b, shares structural features and interacting partners with other family members and is considered to be the prototypical transporter. Proteins bearing the basic-type NLS usually begin the import process by first binding to a related transport protein, importin-a. Importin-a serves as a bridge, linking the NLS-containing substrate to importin-b. However, in contrast to most other proteins that carry a classic NLS, Rev appears to bind directly to importin-b, and this heterodimeric complex is transported to the NPC where the importin-b subunit successively binds to and dissociates from the resident nucleoporins, translocating the Rev substrate through the pore and into the nucleoplasm (148).

It is now known that the reverse process, nuclear export, utilizes transport receptors that are also members of the importin-b family. The most well studied of these is named CRM1 (for chromosome region maintenance 1), or exportin 1 (184,595). Studies of HIV-1 Rev and related viral and cellular proteins bearing Leu-rich NES motifs (see Fig. 15) have led to the discovery and established the functions of many proteins involved in nuclear export. A critical experiment linking the Rev NES mechanistically to the nuclear export machinery was the demonstration that the antibiotic leptomycin B (LMB) blocked the nuclear export of Rev-dependent RNAs in HeLa cells and human PBMC (672). A follow-up study, using microinjected *Xenopus* oocytes, indicated that LMB inhibited the nuclear-to-cytoplasmic transport of both the Rev protein and several UsnRNAs (184). Completely unrelated experiments, examining the antimicrobial effects of LMB in yeast, had mapped LMB resistance to the antibiotic to the CRM1 gene (463), an observation reported prior to the time when the role of CRM1 in nuclear export was appreciated. Additional experiments demonstrating that LMB binds directly to *in vitro* translated CRM1 and that overexpression of CRM1 eliminates the LMB blockade of Rev NES-mediated nuclear export suggested that Crm 1 might be the long-sought “export receptor” for proteins containing the Leu-rich NES. This conclusion was also supported by studies showing that CRM1 shuttled between the nucleus and cytoplasm and interacted with the FG repeats present in nucleoporin proteins (184,461,595).

The small GTPase molecule, Ran (41), also plays a central role in nuclear-cytoplasmic transport and is intimately involved in the Rev-mediated nuclear export of intron-containing HIV-1 pre-mRNAs. Ran is a guanine nucleotide-containing enzyme that provides both the energy and the directionality cues for nuclear import and export. Intracellularly, Ran cycles between two forms, RanGTP and RanGDP, which are concentrated primarily in the nucleus and cytoplasm, respectively (304). The specificity of Ran activity in nuclear transport is regulated by the nature of the bound nucleotide. Several cellular proteins are able to modulate the nucleotide-bound state of Ran and, therefore, contribute to the asymmetric distribution of the two Ran forms (42). One of these, the chromatin-associated regulator of chromosomal condensation 1, or RCC1, stimulates the replacement of GDP with GTP when Ran is in the nucleus. On the other hand, two other factors, Ran GTPase activating protein 1 (Ran GAP1) and Ran binding protein 1 (Ran BP1) both augment Ran activity in the cytoplasm by promoting the hydrolysis of the Ran-associated GTP. The net effect of these interactions is the maintenance of the observed RanGTP/RanGDP intracellular gradient required for nuclear import and export functions.

The complete Rev nuclear transport cycle and its critical interacting partners are shown in Figure 16. During the early, postintegration phase of the virus life cycle, the HIV-1 mRNAs transported to the cytoplasm consist almost entirely of intronless, multiply spliced transcripts encoding the Tat, Rev, and Nef proteins. As noted earlier, Rev uses its NLS to bind directly to importin-b in the cytoplasm and is translocated through the nuclear pore as a Rev/importin-b heterodimer. When the complex reaches the nucleus, the binding of RanGTP triggers the Rev/importin-b heterodimer to dissociate and release the Rev subunit. In the nucleoplasm, Rev initiates its role as a nuclear exporter by binding to the high-affinity site on stem-loop II of RRE-containing HIV-1 pre-mRNAs and then multimerizes via cooperative protein–protein and protein–RNA interactions over the entire length of the RRE. When Rev oligomerizes on the RRE, its NES elements become aligned on the outside of an enlarging protein–RNA complex and are available to interact with other proteins (275). Although CRM1 is able to directly bind to Rev (14,184,207), this interaction is nonspecific because it does not require an intact NES and is unaffected by the antibiotic LMB. Instead, to form a functional complex, the multimerized Rev/RRE must interact with CRM1 in the presence of Ran in its GTP bound form. The formation of this complex (RRE/Rev/CRM1/RanGTP) is (a) blocked by the antibiotic LMB, (b) sensitive to Rev NES mutations, and (c) dissociated when RanGTP is hydrolyzed to the RanGDP state (14). The most likely scenario linking all available data is that the entry of RanGTP into a preexisting Rev/CRM1 heterodimer converts it into a functional export complex. This is supported by the results of *in vitro* protease protection experiments showing that the addition of RanGTP further stabilizes the Rev–CRM1 complex, making it more resistant to enzymatic digestion (14). Once a functional REV export complex is formed, it is transported to the NPC where the CRM1 subunit interacts with nucleoporins (185), translocating the entire complex into the cytoplasm. There, in the presence of Ran GAP1 and Ran BP1, the RanGTP component of the export complex is converted to the RanGDP form and the complex disassembles (245,462). Rev is released along with its unspliced and partially spliced HIV-1 RNA “cargo,” allowing the latter to be incorporated into progeny virions or be translated into viral proteins. It should be noted that microinjection studies utilizing *Xenopus* oocytes have shown that the export of Rev-responsive RNAs is blocked in the presence of a RanGTP mutant unable to undergo GTP hydrolysis (336). Rev therefore enables lentiviral intron-containing RNAs to elude the cellular splicing machinery and to be transported to

the cytoplasm via an export pathway not used by most cellular mRNAs.

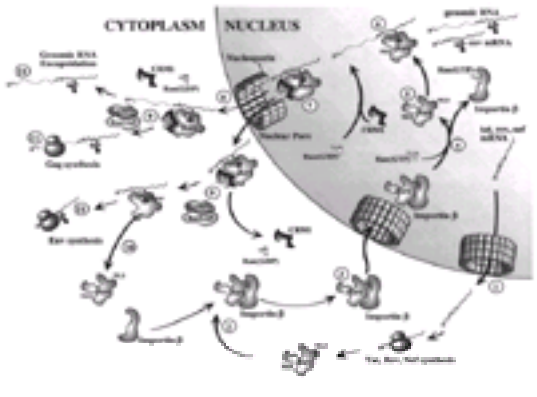

FIG. 16. Rev nuclear export pathway. Intronless multiply spliced HIV-1 mRNAs (*tat*, *rev*, and *nef*) use a Rev-independent pathway to exit the nucleus (*1*). Using its nuclear localization signal (NLS), newly synthesized Rev protein binds to importin b (*2*) and the resulting heterodimer (*3*) is translocated through the nuclear pore. In the nucleus, Rev/ importin b dissociation is mediated by Ran(GTP) (*4*) and the free Rev protein binds (*5*) to RRE-containing viral pre-mRNAs (*6*). The Rev/RRE RNA complex is converted to a functional export structure following the addition of CRM1 and Ran(GTP) (*7*) and is transported through the nuclear pore (*8*). In the cytoplasm, RanBP1/RanGAP mediate the conversion of Ran(GTP) in the export complex to Ran(GDP) (*9*), which then disassembles. Following the release of Rev from intron-containing HIV-1 transcripts (*10*), the full-length viral RNA may be encapsidated into progeny virions or, as is the case for the partially spliced mRNAs, may be translated into viral proteins (*11*). (Drawing kindly provided by Dr. Michael Cho.)

It is also worth noting that a constitutive transport element (CTE), isolated from the genomes of type D retroviruses such as Mason-Pfizer monkey virus (MPMV) and simian retrovirus-1, can functionally substitute for the Rev protein and facilitate the nuclear export of unspliced or partially spliced HIV RNAs (55,712). CTEs are likely to be present in the genomes of simple retroviruses that do not encode their own Rev-like transactivating proteins and presumably bind to cellular factors that mediate nucleocytoplasmic transport (433,469,585). One such candidate is the human TAP protein, which binds to CTE loop sequences and mediates nuclear export of CTE-containing RNAs, as monitored in the *Xenopus* oocyte system (256). Microinjection of the MPMV CTE blocks mRNA but not Rev/NES or tRNA nuclear export, suggesting that in *Xenopus* cells, at least, CTE-containing substrates and cellular mRNAs use a nucleus-to-cytoplasm transport system different from that employed by Rev (490).

Gag

The Gag proteins of HIV, like those of other retroviruses, are necessary and sufficient for the formation of noninfectious, virus-like particles. Retroviral Gag proteins are generally synthesized as polyprotein precursors; the HIV-1 Gag precursor has been named, based on its apparent molecular mass, $Pr55^{Gag}$. As noted previously, the mRNA for $Pr55^{Gag}$ is the unspliced 9.2-kb transcript (Fig. 5 and Fig. 13) that requires Rev for its expression in the cytoplasm. When the *pol* ORF is present, the viral protease (PR) cleaves $Pr55^{Gag}$ during or shortly after budding from the cell to generate the mature Gag proteins p17 (MA), p24 (CA), p7 (NC), and p6 (see Fig. 5). Two spacer peptides are also cleaved out of the Gag precursor: p2, located between CA and NC, and p1, situated between NC and p6. In the virion, MA is localized immediately inside the lipid bilayer of the viral envelope, CA forms the outer portion of the cone-shaped core structure in the center of the particle, and NC is present in the core in a ribonucleoprotein complex with the viral RNA genome (Fig. 17). The location of p6 in the virion remains to be precisely defined. Retroviral Gag proteins perform several principal functions during virus assembly, including (a) forming the structural framework of the virion, (b) encapsidating the viral genome, (c) targeting the nascent particle for export from the cell, and (d) acquiring a lipid bilayer and associated envelope (Env) glycoproteins during particle release (for review, see ref. 192, and Chapter 8). These processes require that Gag proteins participate in protein–protein, protein–RNA, and protein–lipid interactions. As described later, Gag proteins also play critical roles in the early stages of the virus replication cycle.

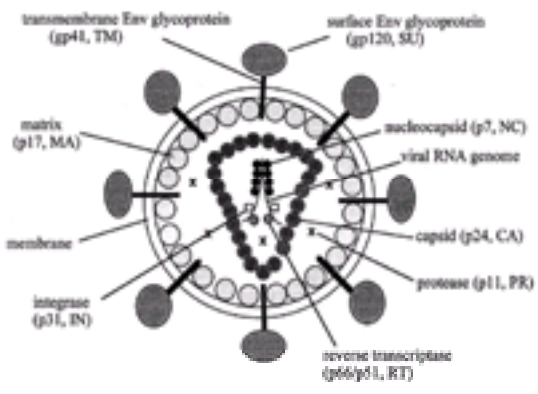

FIG. 17. Schematic representation of a mature HIV-1 virion. (Adapted from ref. 192, with permission.)

The HIV $Pr55^{Gag}$ precursor oligomerizes following its translation and is targeted to the plasma membrane, where particles of sufficient size and density to be visible by EM are assembled. Formation of virus-like particles by $Pr55^{Gag}$ is a self-assembly process, with critical Gag–Gag interactions taking place between multiple domains along the Gag

precursor. The assembly of virus-like particles does not require the participation of genomic RNA (although the presence of nucleic acid appears to be essential), *pol*-encoded enzymes, or Env glycoproteins, but the production of infectious virions requires the encapsidation of the viral RNA genome and the incorporation of the Env glycoproteins and the Gag-Pol polyprotein precursor $Pr160^{Gag\text{-}Po}$. Processing of the Gag and Gag-Pol precursor proteins by PR concomitant with budding results in a major structural and morphologic rearrangement, referred to as maturation, during which the core condenses into the cone-shaped structure characteristic of the mature HIV particle (see Fig. 1A). Disruption of this maturation process abolishes virus infectivity.

Detailed NMR and x-ray crystallographic data have provided significant insights into the structure–function relationship of HIV-1 MA, CA, and NC (see later). It remains to be seen, however, to what extent the structures of the mature Gag proteins will reflect those of the corresponding domains of $Pr55^{Gag}$. In some instances, for example at the N-terminus of CA, major refolding accompanies PR-mediated $Pr55^{Gag}$ cleavage. We can hope that future technologic advances will allow this issue to be addressed as structures of larger portions of $Pr55^{Gag}$ are solved.

Matrix Domain

The MA domain of $Pr55^{Gag}$ (Fig. 18A) performs several essential functions during the viral life cycle. Following Gag synthesis, MA directs $Pr55^{Gag}$ to the plasma membrane via a multipartite membrane binding signal. The affinity of the MA domain for membrane is provided in part by a myristic acid moiety covalently attached to the N-terminal Gly of MA. Mutation of this residue significantly impairs binding of Gag to membrane and abolishes virus assembly in most systems. Sequences in MA downstream of the myristate also contribute to membrane binding. NMR and x-ray crystallographic analyses of HIV-1 MA [as well as MA of SIV, bovine leukosis virus (BLV), HTLV-II, and MPMV] suggest that residues in a highly basic patch cluster on the face of MA predicted to juxtapose the plasma membrane (Fig. 19) (422; for review, see ref. 114). It has been proposed that these basic residues interact with the negatively charged acidic phospholipids on the inner leaflet of the lipid bilayer, thereby stabilizing membrane interactions (277,422,708). Mutations affecting these basic residues can be detrimental to virus assembly (194,695,707). The finding that $Pr55^{Gag}$ binds membrane more tightly than MA itself led to the suggestion that MA undergoes a conformational switch following Gag cleavage that triggers its partial release from membrane (708). An analogous switch in membrane binding potential has been described in detail for the retinal rod sensor protein recoverin, which upon binding calcium undergoes a conformation change, referred to as a “myristyl switch,” that exposes the myristate moiety and induces membrane binding. A prediction of this model is that mutations in MA downstream of the myristylation consensus signal might increase or decrease Gag membrane binding, perhaps by altering MA conformation; indeed, such mutations have been reported (330,474,483,593). The ability of MA to partially dissociate from membrane after PR-mediated Gag cleavage would in theory allow it to participate in post-entry steps in virus replication (see later).

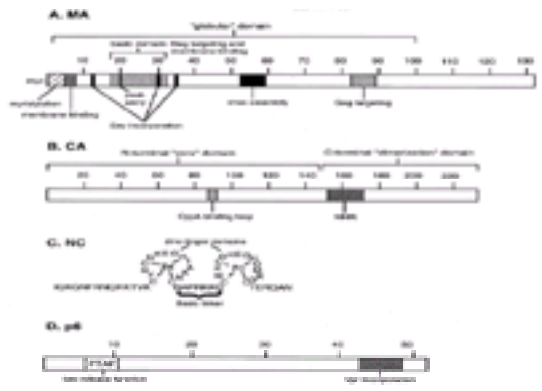

FIG. 18. Linear organization of the major HIV-1 Gag proteins. *Panel A*, matrix (MA); *panel B*, capsid (CA); *panel C*, nucleocapsid (NC); *panel D*, p6. Amino acid positions are indicated, as are major functional domains. (Adapted from ref. 192, with permission.)

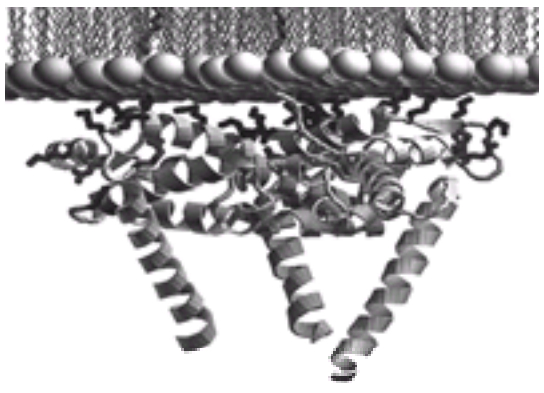

FIG. 19. Structure of a MA trimer bound to membrane. At the *top* is depicted the lipid bilayer; myristate moieties are shown embedded in the bilayer. The helical C-terminal tail of MA is shown projecting away from the globular core. (Reprinted from ref. 284, with permission.)

The mechanism by which Gag traffics specifically to the plasma membrane rather than to intracellular membranes is still under investigation. Deletion of a large portion of MA (169), as well as single amino acid changes between MA residues 84 and 88 (202,476), cause virus assembly to be redirected to cytoplasmic compartments. Thus, MA appears to contain

determinants not only of membrane binding, but, more specifically, of plasma membrane targeting. A role for MA in targeting is supported by the finding that substitution of MA with a heterologous membrane binding signal results in promiscuous assembly both at the plasma membrane and at intracellular sites (520). That a substitution in the MA highly basic domain corrected the mistargeting induced by a residue-86 mutation (475) supports the notion that the basic domain may be a determinant not only of membrane binding but also of plasma membrane targeting. This hypothesis is consistent with the finding that a basic domain deletion appeared to cause a defect in Gag transport (695), and that more subtle mutations in this region retargeted virus assembly to an intracellular site (476).

After Gag binds the plasma membrane, it assembles into particles that bud from the cell. During budding, the viral Env glycoproteins are incorporated into the nascent virions. MA plays a crucial role in this process; small deletions, insertions, and specific point mutations in MA block Env incorporation without perturbing virus assembly. Interestingly, the block to Env incorporation imposed by MA mutations can be reversed by pseudotyping virions with heterologous Env glycoproteins containing short cytoplasmic tails, or by removing sequences within the long cytoplasmic tail of the HIV-1 TM glycoprotein gp41 (196,197,408). These results imply that a specific interaction takes place between MA and gp41, or that MA has evolved to accommodate the long gp41 cytoplasmic tail. Although a direct interaction has been difficult to demonstrate in virus-infected cells, *in vitro* data supporting an HIV-1 MA–gp41 interaction have been reported (117). A direct interaction is also suggested by the ability of HIV-1 Env to induce basolateral Gag budding in polarized epithelial cells (380), and by the recent finding that a single amino acid substitution in MA reversed the Env incorporation defect imposed by a small deletion near the center of the gp41 cytoplasmic tail (448). X-ray crystallographic analysis of both SIV and HIV-1 MA reveals that MA may form trimers that assemble into a higher-order lattice containing holes into which the long cytoplasmic tail of gp41 could insert (284,516). Although this hypothesis provides an appealing model for Env incorporation that is consistent with mutational analyses, the biologic relevance of MA trimerization is currently unclear.

In addition to its function in virus assembly and Env incorporation, it has also been proposed that MA plays a role early in the virus life cycle after Env-induced membrane fusion. Early postfusion steps, which are collectively referred to as uncoating, are poorly understood, and it is not clear mechanistically how MA functions in this process. Nevertheless, it has been reported that mutations in MA impair the synthesis of viral DNA early postinfection, suggesting an early post-entry block (78,330,690). It has also been observed that MA mutants with this “early” defect display impaired endogenous RT activity and increased Gag membrane binding (330). A correlation between increased MA membrane binding and a post-entry defect is consistent with the hypothesis that an inability of MA to disengage from the lipid bilayer after membrane fusion results in an uncoating defect and destabilization of the subviral complex in which reverse transcription takes place (330).

Lentiviruses, including HIV-1, are unique among retroviruses in their ability to infect nondividing cells. It has been proposed that the basic domain of MA, discussed earlier in the context of membrane binding, plays a role in translocating the viral preintegration complex to the nucleus (63,274,645). It was also reported that phosphorylation of a Tyr residue at the C-terminus of MA is required for infectivity in fully differentiated (nondividing) macrophages (214). However, certain key aspects of these studies have been questioned (62,186,193,194 and 195,520). Currently, it appears that Vpr (see section on Vpr) and perhaps IN (213), but not MA, play the dominant role in transporting the preintegration complex to the nucleus. It has also recently been proposed that a central DNA “flap” that is a product of lentiviral reverse transcription (see section on reverse transcription) may promote nuclear import of the viral preintegration complex (701).

Capsid Domain

Like MA, CA functions in both early and late steps in virus replication. CA is composed of two structural and functional domains: the N-terminal two-thirds of the protein forms the so-called core domain, and the C-terminal one-third is referred to as the dimerization domain (see Fig. 18B). NMR and x-ray crystallographic data are available for both domains (217,237,440), but the structure of the entire protein has not yet been resolved. The core domain (residues 1 to 146) is highly helical, with an exposed, cyclophilin A–binding loop (217, and see later); the C-terminal domain (residues 148 to 231) is also highly helical and globular (218). The N-terminus of the dimerization domain contains the major homology region (MHR) (668), which, apart from the zinc-finger motifs in NC, is the only sequence in Gag that displays significant amino acid identity between divergent retrovirus genera. The structures of the N- and C-terminal domains of CA have been combined to create a hypothetical structure for the intact CA (Fig. 20) (218,674).

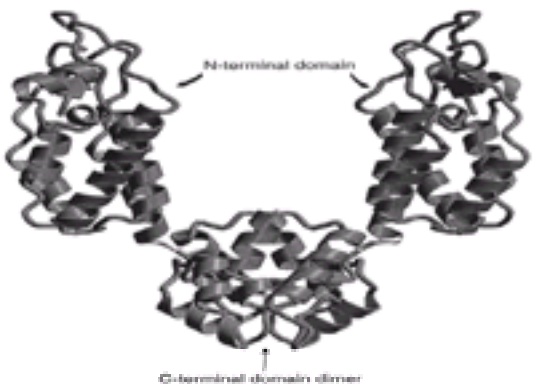

FIG. 20. Model of an HIV-1 CA dimer. The N-terminal core domain is shown at the *top*; the C-terminal dimerization domain is depicted at the *bottom.* The two domains are connected by CA residues 146 and 147. (Reprinted from ref. 674, with permission.)

Mutations within CA produce a range of phenotypes: Those in the C-terminal, dimerization domain often elicit assembly defects, whereas changes in the N-terminal core domain generally do not affect the efficiency of virus production, but

rather can inhibit proper virion maturation after release and severely impair infectivity. These results have led to the proposal that the C-terminus of CA promotes Gag multimerization, whereas the N-terminus plays a role in core condensation and morphogenesis. Mutations in the MHR disrupt virus assembly, maturation, and infectivity. Structural analysis of the dimerization domain revealed that MHR residues form a network of intramolecular hydrogen bonds that stabilize the structure of the entire domain (218). Residues 146 to 151 appear to promote the creation of a high-affinity CA interface, which, in conjunction with a more N-terminal b-hairpin interface, is postulated to drive the formation of higher-order CA interactions during assembly and maturation (218).

Using the yeast two-hybrid system, it was demonstrated that HIV-1 Gag binds members of the cyclophilin family of proteins, which function in the cell as peptidyl-prolyl cis-trans isomerases (389). Further investigation indicated that one of these proteins, cyclophilin A, is specifically incorporated into HIV-1 virions (190,618); this incorporation is mediated by an interaction between cyclophilin A and a Pro-rich region near CA amino acid 90 (190). It is noteworthy that the Gag–cyclophilin A interaction is unique to HIV-1; HIV-2 and SIV Gag proteins neither bind cyclophilins nor incorporate them into virions. Treatment of HIV-1-infected cells with cyclosporin A or its analogs, or mutation of CA residues that participate in the CA–cyclophilin A interaction, block the incorporation of cyclophilin A into virions and reduce virus infectivity. Mutant or drug-treated virions, which are impaired for cyclophilin A incorporation, are defective at an early step in the virus life cycle, apparently after membrane fusion but before the initiation of reverse transcription. The mechanism by which cyclophilin A incorporation enhances infectivity is currently unclear. The N-terminal domain of CA forms planar strips between which cyclophilin A intercalates at a Gag-to-cyclophilin A ratio of approximately 10:1; it has been proposed that cyclophilin A might destabilize CA-CA interactions, thereby promoting efficient uncoating after virus entry (217). Alternatively, cyclophilin A in the virion may function as a chaperone during maturation by promoting proper CA refolding and by minimizing unfavorable CA aggregation after Gag processing (250,658).

Several studies have reported that HIV-1 CA, when expressed *in vitro*, is capable of assembling into tubular or spherical particles (157,647). CA-NC fusion proteins form tubes *in vitro* at protein concentrations lower than those required to assemble into similar structures with CA alone; in the case of CA-NC, tube formation appears to depend on the presence of RNA (72). Interestingly, adding as few as four residues of MA to the N-terminus of CA converts *in vitro* particle assembly from tubes to spheres (647). This observation led to the hypothesis that PR-mediated processing at the MA-CA junction causes a major refolding of the CA N-terminus, and that this structural rearrangement plays a key role in promoting the morphologic changes that occur during core condensation. Recently, it was reported that core-like cones can be assembled *in vitro* using CA-NC fusion proteins and RNA; these cones are organized in a manner reminiscent of the fullerene structures formed by elemental carbon (220).

Nucleocapsid Domain

The third major domain synthesized as part of the Gag precursor is the NC (see Fig. 18C). A principal function of NC involves the specific encapsidation of full-length, unspliced (genomic) RNA into virions, although, as is the case with the other Gag proteins, NC serves multiple roles during the virus life cycle. With the exception of the spumaretroviruses, all retroviral NC proteins contain one or two zinc-finger motifs. Unlike most cellular zinc-finger domains, those of retroviruses are of the CCHC type (Cys-X_2-Cys-X_4-His-X_4-Cys). HIV-1 NC contains two zinc-finger motifs, which bind zinc tightly both *in vitro* and in virions (38,591). NMR data indicate that the two HIV-1 NC zinc fingers are brought together into a central globular domain by a highly basic linker domain (see Fig. 18C), which appears to be very flexible (443,608). Mutations that abrogate zinc binding abolish genome encapsidation and virus infectivity. Other mutations within the zinc-finger domains increase the level of spliced versus unspliced viral RNA encapsidated into virions. NC also contains two clusters of basic residues flanking the first zinc finger; mutations in these sequences impair the binding of NC to RNA *in vitro* and RNA encapsidation into virions. NC engages in both sequence-specific and sequence-nonspecific interactions with nucleic acid; in general, the sequence-specific interactions involve the zinc fingers in conjunction with the basic residues, whereas nonspecific interactions are driven largely by the highly basic nature of NC. The specificity of HIV genome encapsidation results from an interaction between NC and an approximately 120-nt sequence located between the 5' LTR and the Gag initiation codon. This sequence, variously known as the packaging signal, encapsidation element, or y-site, folds into a series of four stem-loops (see Fig. 6; for review on RNA encapsidation, see ref. 36). It appears that this secondary structure, rather than the primary nucleotide sequence itself, confers RNA encapsidation specificity. The structure of the HIV-1 NC protein complexed with stem-loop 3 of the y-site was determined by NMR spectroscopy (Fig. 21) (130); this structure indicates the involvement of both basic and zinc-finger residues in the NC–y interaction. In addition to this specific interaction with the y-site, NC may coat the viral RNA in the mature virus particle.

FIG. 21. Complex between NC and stem-loop 3 (SL3) of the HIV-1 packaging signal. The two zinc ions coordinated by the zinc-finger domains are shown as gray balls. (Reprinted from ref. 628, with permission.)

Many HIV-1 NC mutations cause defects in virus assembly and release. This observation suggests a role for NC in Gag

multimerization, a function mapping largely to the N-terminal basic domain, rather than the zinc fingers. NC appears to play a role in the tight packing of Gag in virions, leading to the production of particles with a density characteristic of retroviral particles (29). It is suspected that NC-RNA interactions are critical for promoting Gag multimerization; according to this model, RNA provides a template along which molecules of $Pr55^{Gag}$ can align and pack. Binding between the NC domain of $Pr55^{Gag}$ and components of the host cell cytoskeleton (F-actin in particular) may also facilitate Gag assembly and transport (376,524,660). It has been proposed that NC plays an important role in binding of HIV-1 Gag to membrane (503,550). However, using membrane flotation centrifugation methods, which effectively distinguish membrane-bound from non-membrane-bound protein, it was determined that a truncated Gag molecule containing only MA and the N-terminal domain of CA exhibited steady-state membrane binding properties comparable to those of $Pr55^{Gag}$ (473).

In addition to its role in RNA encapsidation and Gag assembly, other functions for HIV-1 NC have been observed. Many of these functions can be attributed to a “nucleic acid chaperone” (282) activity. This property of NC enables it to catalyze the refolding of nucleic acid molecules into structures with the most thermodynamically favorable conformation (i.e., the greatest number of base pairs) (for review, see ref. 522). NC, or the NC domain of $Pr55^{Gag}$, contributes to RNA dimerization, binding of the $tRNA^{Lys3}$ primer to the primer-binding site, stabilization or “maturation” of the intermolecular interactions between the dimeric RNAs in the virion, initiation of reverse transcription from the $tRNA^{Lys3}$ primer, and strand transfers during reverse transcription. The nucleic acid chaperone activity of NC appears to require that NC molecules coat nucleic acid at near-saturating levels of approximately one molecule of protein per seven nucleotides. The basic nature of NC plays a more critical role in this activity than the integrity of the zinc fingers (522).

Several additional functions for NC early in the virus replication cycle have been reported. Mutations in HIV-1 NC may impair infectivity by destabilizing newly reverse-transcribed viral DNA (37). Studies performed with MuLV demonstrated that changing the single CCHC zinc-finger motif to CCCC or CCHH had no effect on zinc binding, tRNA incorporation, RNA encapsidation, or RNA maturation but profoundly impaired virus infectivity (243). Detailed examination of viral DNA in mutant-infected cells revealed that full-length, circular DNA products were formed inefficiently and that a variety of mutations (deletions and insertions) were present at the DNA ends (243). Such aberrations, if present at the ends of linear DNAs, would render these DNAs unsuitable for integration. Similar mutations in the N-terminal zinc finger of the HIV-1 NC have been reported; these changes resulted in defects late in reverse transcription, leading to a lack of circular DNA production (612). It has also been reported that NC stimulates “coupled joining” (wherein both ends of the viral genome integrate into a target DNA) in *in vitro* integration reactions (77) (see section on integrase). Mutations in the NC zinc fingers modulated this stimulatory effect (77).

Because NC participates in multiple steps in the virus life cycle, and because the retroviral CCHC-type zinc-finger motif is relatively rare among cellular proteins, NC presents an attractive target for antivirals. Promising anti-NC compounds attack the sulfurs on the zinc-coordinating Cys residues and cause zinc to be “ejected” from the zinc fingers (526,527). These compounds effectively inhibit virus replication in culture without significant cytotoxicity. Fourteen compounds that demonstrated anti-NC activity *in vitro* were tested for their effect on MuLV replication *in vivo*. One of these compounds, Aldrithiol-2 (2,2'-dithiopyridine), delayed the onset of MuLV-induced disease and reduced virus loads in infected mice (480). Such compounds could also in theory be used to render stocks of HIV particles noninfectious for use in vaccine studies (538).

p6 Protein

In addition to the MA, CA, and NC proteins, retroviral *gag* genes encode a variety of additional ORFs that are generally unique to a particular genus of retrovirus (for review, see ref. 668). HIV-1 encodes a Pro-rich, 6-kd protein, known as p6, at the C-terminus of $Pr55^{Gag}$ (see Fig. 18D). Mutation of p6, specifically within a highly conserved Pro-Thr-Ala-Pro (PTAP) motif, blocks a late step in virus assembly such that virions accumulate at the plasma membrane but fail to release efficiently (248,293). Interestingly, mutation of PR largely reverses this defect (293), suggesting a functional interplay between p6 and PR function, and perhaps explaining why some groups, using Gag-only expression systems, failed to detect a requirement for p6 in virus production. p6 also functions to direct the incorporation of Vpr and Vpx into virions (494,678).

In addition to HIV-1 p6, domains encoded by other retroviral Gag proteins serve analogous “late” roles in virus release. These include p2b of Rous sarcoma virus (667), pp16 of MPMV (685), p9 of equine infectious anemia virus (EIAV) (511), and p12 MuLV (693). Interestingly, at least in certain cases, these late domains can function independently of their position in Gag and can be exchanged from one retrovirus to another (488). These late domain proteins all contain motifs that have been implicated in protein–protein interactions: Pro-X-X-Pro in HIV-1 p6; Pro-Pro-Pro-Tyr in RSV p2b, MPMV pp16, and MuLV p12; and Tyr-X-X-Leu in EIAV p9 (228). It has been proposed that these proteins interact with host cell plasma membrane proteins to facilitate the final release step during virus budding (228). Indeed, p2b of RSV binds proteins containing so-called WW motifs (228), and EIAV p9 has been reported to interact with the AP-2 adapter protein complex (512). Recent evidence implicates the host ubiquitination machinery in the function of retroviral late domains 642a).

Pol

Downstream of *gag* lies the most highly conserved region of the HIV genome, the *pol* gene, which encodes three enzymes: PR, RT, and IN (see Fig. 5). RT and IN are required, respectively, for reverse transcription of the viral RNA genome to a double-stranded DNA copy, and for the integration of the viral DNA into the host cell chromosome. PR plays a critical role late in the life cycle by mediating the production of mature, infectious virions. The *pol* gene products are derived by enzymatic cleavage of a 160-kd Gag-Pol fusion protein, referred to as $Pr160^{Gag\text{-}Pol}$. This fusion protein is produced by ribosomal frameshifting during translation of $Pr55^{Gag}$ (see Fig. 5 and Fig. 6C). The frame-shifting mechanism for Gag-Pol expression, also utilized by many other retroviruses, ensures that the *pol*-derived proteins are expressed at a low level, approximately 5% to 10% that of Gag. Like $Pr55^{Gag}$, the N-terminus of $Pr160^{Gag\text{-}Pol}$ is myristylated and targeted to the plasma membrane.

Protease

Early pulse-chase studies performed with avian retroviruses clearly indicated that retroviral Gag proteins are initially synthesized as polyprotein precursors that are cleaved to generate smaller products (643,644). Subsequent studies demonstrated that the processing function is provided by a viral rather than a cellular enzyme, and that proteolytic digestion of the Gag and Gag-Pol precursors is essential for virus infectivity (120,323). Sequence analysis of retroviral PRs indicated that they are related to cellular “aspartic” proteases such as pepsin and renin (for review, see ref. 126). Like these cellular enzymes, retroviral PRs use two apposed Asp residues at the active site to coordinate a water molecule that catalyzes the hydrolysis of a peptide bond in the target protein. Unlike the cellular aspartic proteases, which function as pseudodimers (using two folds within the same molecule to generate the active site), retroviral PRs function as true dimers. X-ray crystallographic data from HIV-1 PR (352,458,671) indicate that the two monomers are held together in part by a four-stranded antiparallel b-sheet derived from both N- and C-terminal ends of each monomer (Fig. 22). The substrate binding site is located within a cleft formed between the two monomers. Like their cellular homologs, the HIV PR dimer contains flexible “flaps” that overhang the binding site and may stabilize the substrate within the cleft; the active-site Asp residues lie in the center of the dimer. Interestingly, although some limited amino acid homology is observed surrounding active-site residues, the primary sequences of retroviral PRs are highly divergent, yet their structures are remarkably similar.

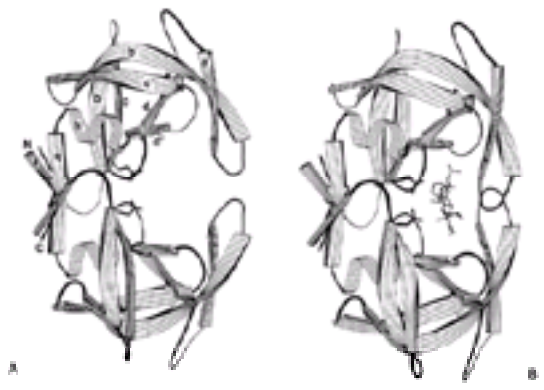

FIG. 22. Structure of the HIV-1 PR dimer uncomplexed (**A**) or complexed (**B**) with the PR inhibitor Ro-31-8558. The following domains are indicated in the first half of the molecule: b-strands *a* (residues 1 to 4), *b* (residues 9 to 15), *c*, and *d* (residues 30 to 35). The active-site residues are at positions 25 to 27. The second half of the molecule is structurally related to the first half, with the following domains indicated: b-strands *a′* (residues 43 to 49), *b′* (residues 52 to 66), *c′* (69 to 78),*d′* (83 to 85), and helix *h′* (86 to 94). The inner portion of the dimer interface is formed by b-strand *q* (residues 95 to 99). (Reprinted from ref. 670, with permission.)

The first cleavage events catalyzed by retroviral PRs during or immediately after virion release from the cell serve to liberate PR from the Gag-Pol precursor. It is still unclear whether this initial cleavage event takes place in cis (intramolecularly) or in trans (intermolecularly); in either case, following the release of PR from the Gag-Pol precursor, the dimeric enzyme cleaves a number of sites in both Gag and Gag-Pol (see Fig. 5). The efficiency with which PR cleaves the individual target sites in Gag and Gag-Pol varies widely and is influenced by two major factors: the amino acid sequence at the site of cleavage, and the context (i.e., the degree of exposure or accessibility) of the cleavage site. The proteolytic processing of model proteins and substrate analogs by the HIV-1 PR indicates that the binding cleft can accommodate a peptide of approximately seven residues in length, and synthetic peptides of this size are cleaved *in vitro* (40). Although a number of studies have attempted to define a consensus target sequence for HIV-1 PR, the rules governing which sites can be cleaved are fairly loose. As a consequence of the relatively divergent PR target sequences present in Gag, and the varying efficiencies with which these sites serve as substrates for PR activity, Gag cleavage takes place as an ordered, step-wise cascade. Processing at the amino terminus of NC is the most rapid *in vitro*, whereas the cleavage converting p25 to p24 CA is the slowest. Mutations in Gag that disrupt the ordered nature of PR-mediated processing severely disrupt virus assembly or subsequent maturation. Furthermore, HIV-1 mutants engineered to synthesize a linked PR dimer (i.e., a duplicated PR-coding region) exhibit rapid, premature processing of Gag and Gag-Pol polyproteins and a block in virus production (349).

PR appears to be most active during the budding process just prior to particle release from the cell (320). PR cleavage of the Gag and Gag-Pol precursors leads to a dramatic change in virion morphology, a process known as maturation. Immature retroviral particles (for example, those produced in the presence of PR inhibitors or an inactive PR) appear doughnut-shaped by EM. In contrast, mature virions contain condensed cores, which in the case of the lentiviruses are cone-shaped (Fig. 1A). Cryo-EM studies have suggested that in immature virions, Gag precursors are aligned in a rodlike fashion like spokes on a wheel, with the N-terminal MA domain associated with the viral lipid and the C-terminus projecting inwards toward the center of the virion (208,686). According to this model, cleavage of Gag by PR releases CA, NC, and p6 from their attachment (via MA) to the lipid and allows CA to form a shell around the NC–RNA complex. One would predict that numerous conformational changes take place within the Gag proteins upon cleavage that allow this structural reorganization to take place. In one case, a major, cleavage-induced refolding has been characterized (see section on CA); however, much remains to be learned about the molecular details of core condensation.

It is clear that premature Gag and Gag-Pol cleavage is severely detrimental to virus assembly. Thus, the activation of PR must be tightly controlled to prevent significant processing prior to the completion of assembly. It is not yet understood how premature PR activation is prevented. One model proposes that the concentration of Gag and Gag-Pol precursor proteins plays the key role in PR activation; sufficiently high concentrations are not achieved until assembly has taken place. This model fails to account for the fact that type B/D retroviruses assemble in the cytoplasm, yet the particles that

form maintain an unprocessed, immature state until budding and release have occurred. It has also been suggested that the plasma membrane may provide an environment uniquely compatible with Gag/Gag-Pol processing and virion maturation. However, MA mutations have been described that redirect assembly to the Golgi apparatus or Golgi-derived vesicles; the resulting redirected virus particles can undergo Gag processing and maturation at the intracellular site (202,476). In the case of HIV-1 PR, but not all retroviral PRs, some Gag cleavage can occur in the absence of membrane binding. This point is illustrated by the observation that mutations that block Gag myristylation, and consequently impair membrane binding, do not fully block Gag processing (249). The assembly of high-molecular-weight Gag complexes in the absence of, or prior to, membrane binding (360) suggests that processing of non-membrane-bound Gag may still take place within a large, multimeric Gag/Gag-Pol complex.

HIV-1 PR activity is not limited to cleavage of the Gag and Gag-Pol precursors. Nef is a substrate for PR cleavage, although the implications for Nef function remain unclear (see section on Nef). The cytoplasmic domains of some retroviral Env proteins (e.g., MuLV and MPMV) are also cleaved by PR; in such cases, the cleavage event is essential for Env function (515,523,590). Although no evidence exists that HIV PR cleaves the cytoplasmic tail of gp41, it efficiently cleaves the cytoplasmic domain of the MuLV TM Env protein in HIV-1 virions pseudotyped by MuLV Env (329). The finding that PR-mediated processing of the MuLV TM can be blocked by HIV-1 MA mutations (329) has implications for the use of HIV-based vectors pseudotyped with MuLV Env. In addition to cleavage of viral proteins, PR has been reported to act on a number of cellular proteins (for review, see ref. 642). The role of such events in virus replication and virus-induced cytotoxicity has not been fully elucidated.

Compounds that inhibit HIV-1 PR function have proven to be the most effective antiviral drugs presently available. The development of PR inhibitors represents a classic example of structure-based drug design; detailed knowledge of the topology of the substrate binding site, the enzyme–substrate interaction, and the chemistry of the cleavage reaction enabled investigators to synthesize a large number of inhibitors that potently inhibit PR in culture at nanomolar concentrations. The earliest PR inhibitors were small oligopeptide derivatives that mimicked PR target sequences but contained nonhydrolizable groups (e.g., hydroxyethylenes, hydroxyethylamines, and dihydroxyethylenes) at the P1 and P1' positions flanking the scissile bond that PR would normally cleave. The early, peptide-based inhibitors were subsequently modified to improve stability and bioavailability, and the structure of the PR active site was used to design novel inhibitory compounds (670). When PR inhibitors are used to treat HIV-infected patients, virus loads decline precipitously, but drug-resistant variants soon emerge. Sequencing of the PR coding region of drug-resistant variants often reveals consistent changes. For example, saquinavir escape mutants frequently contain changes at PR residue 48 and/or 90 (308); ritonavir resistance is often associated with mutations at PR residue 82 (439). The initial changes that confer drug resistance generally result in only partial resistance and may be accompanied by decreased “fitness” (i.e., impaired PR function relative to wild type in the absence of drug) (121). With time, however, additional changes take place within PR that increase resistance and improve fitness. The same secondary mutations are often observed during escape from different PR inhibitors; these changes increase resistance and allow adaptation to the original drug-resistance-conferring mutation. Unfortunately, mutations that arise in the presence of one inhibitor frequently induce at least partial resistance to others (113), a phenomenon known as cross-resistance. In addition to changes in PR, mutations that affect the natural PR targets (i.e., the Gag and Pol cleavage sites) have also been observed (149). Whereas monotherapy with PR inhibitors generally leads to rapid resistance, longer-lasting benefits are achieved with so-called triple therapy (also known as highly active antiretroviral therapy, or HAART), in which a PR inhibitor (e.g., indinavir or saquinavir) is combined with two RT inhibitors (e.g., AZT and 3TC).

Reverse Transcriptase

By definition, retroviruses possess the ability to convert their single-stranded RNA genomes into double-stranded DNA during the early stages of the infection process (20,615). The enzyme that catalyzes this reaction is RT, in conjunction with its associated RNaseH activity. Retroviral RTs have three enzymatic activities: (a) RNA-directed DNA polymerization (for minus-strand DNA synthesis), (b) RNaseH activity (for the degradation of the tRNA primer and genomic RNA present in DNA–RNA hybrid intermediates), and (c) DNA-directed DNA polymerization (for second- or plus-strand DNA synthesis).

The retroviral genome is packaged in the virion as two copies of single-stranded RNA. The two RNAs, which are identical or at least highly homologous, are held together in part by a sequence near their 5' ends known as the dimer initiation signal (see Fig. 6). Although each retroviral particle carries two strands of RNA, it appears that only one provirus is formed per virion (291). Retroviruses are therefore referred to as pseudodiploid. As with other DNA polymerase reactions, reverse transcription is dependent on the 3'-OH group of a primer to initiate polymerization. Retroviruses employ specific tRNAs for this function—$tRNA^{Lys3}$ in the case of HIV-1. The mechanism of tRNA selection and placement on the template is complex, involving interactions with RT and NC as well as with the 18-nt pbs near the 5' end of the viral genome. Although the interaction between $tRNA^{Lys3}$ and the HIV-1 genomic RNA involves primarily the tRNA 3' end and the pbs, more extensive base pairing between these two RNAs has been proposed (301).

Reverse transcription of the retroviral RNA genome to a double-stranded DNA copy proceeds via a series of steps that are outlined briefly later. (For a more detailed description of this process, refer to recent reviews in refs. 246 and 613; and see Chapter 57.)

1. Minus-strand DNA synthesis is initiated from the 3'-OH of the tRNA bound to the pbs. DNA synthesis then proceeds to the 5' end of the genome.
2. RNaseH digests the RNA portion of the newly formed RNA/DNA hybrid, releasing the resulting short, single-stranded DNA fragment (known as the minus-strand strong-stop DNA).
3. The minus-strand strong-stop DNA is transferred to the 3' end of the genome where it hybridizes by virtue of a short region of homology (the “repeated” or R region) present at both 5' and 3' ends of the RNA genome. Whether this first strand transfer is intermolecular, intramolecular, or both has been the subject of some debate.
4. Minus-strand synthesis, accompanied by partial degradation of the RNA in the resulting RNA–DNA hybrid by RNaseH, continues to the pbs at the 5' end of the genome.
5. Fragments of RNA that were not removed by RNaseH serve as primers for plus-strand synthesis. The major site of

such priming occurs at the PPT; however, residual RNA fragments that remain hybridized to regions outside the PPT can also be used for priming plus-strand synthesis. In the case of HIV-1, one such region, known as the central PPT, appears to be particularly important in this regard (81).

6. RNaseH removes the tRNA that initially served as the primer for minus-strand synthesis. This exposes the pbs at the 3' end of the plus-strand DNA, allowing the plus-strand DNA to transfer (this is the second-strand transfer) and hybridize with the homologous region at the 3' end of the minus-strand DNA.
7. Plus- and minus-strand syntheses proceed to completion. Plus-strand synthesis terminates at the end of the minus strand and, for HIV, at a sequence known as the central termination signal (CTS) (82). The position of the central PPT upstream of the CTS results in the displacement of approximately 100 nt of plus-strand DNA and the formation of a triplex DNA structure. Interestingly, it has been suggested recently that this triplex, or “flap” structure may play a role in nuclear import of the viral preintegration complex (701). The final product of reverse transcription is a double-stranded DNA molecule capable of serving as the substrate for integration. Some discontinuities may remain in the final product; these can be resolved following integration (436).

In addition to serving an essential function in the virus life cycle, the enzymatic activity of RT is routinely used in the laboratory to quantitatively monitor levels of virus present in the supernate of infected cultures and to elucidate details of replication at the molecular level. Two types of assays are most commonly employed to measure RT activity. In the “exogenous” assay, virions are disrupted with detergent and the presence of RT is detected by its ability to extend a short oligonucleotide primer (e.g., oligo-dT) using an exogenously provided template [e.g., poly-r(A)] and a radiolabeled deoxynucleoside triphosphate (dNTP) (e.g., a-[^{32}P]TTP). In the “endogenous” reaction, virions are gently disrupted with a mild detergent treatment and provided with dNTPs (one of which is generally radiolabeled), and RT activity is monitored using the viral RNA genome as a template. Depending on the assay conditions, endogenous reactions result in the synthesis of discreet reverse transcription products (e.g., minus-strand strong-stop DNA) or a continuum of DNA species. The endogenous assay is less efficient than the exogenous assay, but because it measures the activity of RT in a relatively native complex, it more closely mimics the conditions of reverse transcription in an infected cell. These assays monitor RT activity in a cell-free setting. Although a limited amount of reverse transcription can take place in the virion under physiologic conditions (382, 626), the overwhelming majority of DNA synthesis does not occur until after entry into the target cell. Thus, the detection of postinfection viral DNA synthesis by polymerase chain reaction (PCR) provides the most reliable method for detecting entry and post-entry steps in the virus replication cycle. As mentioned elsewhere in this chapter, PCR amplification of viral DNA after infection has been used extensively to study defects imposed by mutations in Gag, Env, and accessory proteins.

The mature HIV-1 RT holoenzyme is a heterodimer of 66 and 51 kd subunits. The 51-kd subunit (p51) is derived from the 66-kd (p66) subunit by proteolytic removal of the C-terminal 15-kd RNaseH domain of p66 by PR (see Fig. 5). The structure of HIV-1 RT has been determined by x-ray crystallography in a number of studies. The enzyme was crystallized in several contexts: (a) complexed with the nonnucleoside inhibitor nevirapine (340), (b) bound to a short DNA duplex and the Fab portion of an anti-RT antibody (11, 306, 307), (c) unbound to DNA or inhibitor (165, 532), and, most recently, (d) covalently linked to a complex of primer/template, and dNTP (292). The crystal structure of HIV-1 RT reveals a highly asymmetric folding in which the orientations of the p66 and p51 subunits differ substantially. The p66 subunit can be visualized as a right hand, with the polymerase active site within the palm, and a deep template-binding cleft formed by the palm, fingers, and thumb subdomains (11, 307) (Fig. 23). The polymerase domain is linked to RNaseH by the connection subdomain. The active site, located in the palm, contains three critical Asp residues (110, 185 and 186) in close proximity, and two coordinated Mg^{2+} ions (Fig. 24). Mutation of these Asp residues abolishes RT polymerizing activity. The orientation of the three active-site Asp residues is similar to that observed in other DNA polymerases (e.g., the Klenow fragment of *E. coli* DNA polI) (10). The p51 subunit appears to be rigid and does not form a polymerizing cleft; Asp 110, 185, and 186 of this subunit are buried within the molecule. Approximately 18 base pairs of the primer–template duplex lie in the nucleic acid binding cleft, stretching from the polymerase active site to the RNaseH domain.

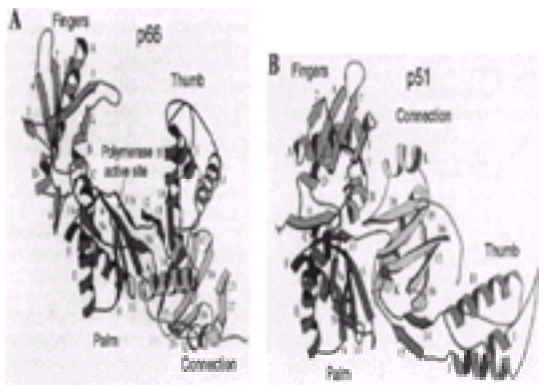

FIG. 23. Ribbon diagrams of the p66 (**A**) and p51 (**B**)RT subunits. Fingers, palm, thumb, and connection domains are shown. (Reprinted from ref. 307, with permission.)

FIG. 24. Active site of a covalently trapped HIV-1 RT catalytic complex. The deoxynucleoside triphosphate (dNTP) pocket is presented, with fingers at the *upper left*, palm in the *center*, and thumb at the *lower right.* The triphosphate of the incoming dNTP is coordinated by residues 65, 72, 113, and 114, and two Mg^{+2} ions. The three active-site Asp residues (110,185 and 186) are shown. A dideoxynucleotide chain terminator at the 3' end of the primer traps the complex prior to attack of the incoming dNTP. (Reprinted from ref. 292, with permission.)

In the RT-primer–template-dNTP structure (292) (see Fig. 24), the presence of a dideoxynucleotide at the 3' end of the primer allows visualization of the catalytic complex trapped just prior to attack on the incoming dNTP. This structure has been particularly informative regarding the orientation of RT with respect to the primer–template, and the location of mutations that confer resistance to RT inhibitors (see later). Comparison with previously obtained structures (see, e.g., ref. 307) suggests a model whereby the fingers close in to trap the template and dNTP prior to nucleophilic attack of the 3'-OH of the primer on the incoming dNTP. After the addition of the incoming dNTP to the growing chain, it has been proposed that the fingers adopt a more open configuration, thereby releasing the pyrophosphate and enabling RT to bind the next dNTP (292). The structure of the HIV-1 RNaseH has also been determined by x-ray crystallography; this domain displays a global folding similar to that of *E. coli* RNase H (127).

The high rate of variation among HIV populations poses one of the fundamental challenges to effectively controlling this pathogen (for reviews, see refs. 412,491, and 509). Variation in a retroviral population is influenced by several factors, including the mutation rate per replication cycle, the replication rate (i.e., number of cycles of virus replication per unit time) and the fixation rate (determined by the selective advantage or disadvantage conferred by a particular mutation) (104,105). High mutation rates are largely a consequence of the error-prone nature of RT, which lacks an exonucleolytic proofreading activity, and frequent template switching during reverse transcription (614). Cell-free error rates for purified RTs have been determined (24,510,530), as have *in vivo* retrovirus mutation rates (147,413,492,493). The cell-free error rate measured for HIV-1 RT is more than 10-fold higher than the mutation rate, suggesting that the *in vitro* reaction lacks factor(s) that increase fidelity (413). The total HIV-1 *in vivo* mutation rate (a composite of substitutions, frameshifts, simple deletions, and deletions with insertions) was measured at 3×10^{-5} per cycle of replication (413). Using similar methods, *in vivo* mutation rates of other retroviruses (e.g., spleen necrosis virus, MuLV, and BLV) were observed to be between 2-fold and 10-fold lower than that of HIV-1 (412).

As discussed earlier, retroviral particles contain two copies of single-stranded RNA. The requirement for template switching during reverse transcription appears to have selected for a low affinity between RT and template (614). As a result, template switches occur frequently during reverse transcription (491). Intramolecular jumps lead to mutations (e.g., deletions, insertions, and duplications), whereas intermolecular jumps generate recombinants.

Two general models have been suggested to explain recombination during reverse transcription. The first, which is operative during minus-strand synthesis, is referred as forced copy-choice (103). The second, which applies during plus-strand synthesis, has been named strand displacement-assimilation (317). The forced copy-choice model proposes that when RT encounters a break in the template RNA it jumps to the copackaged RNA and resumes DNA synthesis. This model has been refined more recently with the suggestion that jumps during minus-strand synthesis can occur in the absence of strand breaks (315). According to the strand displacement-assimilation model, the recombination process begins with the displacement of one fragment of internally initiated plus-strand DNA by another plus-strand transcript. The displaced fragment then binds, at a transient gap, to the complementary region of the minus-strand DNA derived from reverse transcription of the copackaged RNA. This intermediate is then resolved, most likely by cellular enzymes, to generate a recombinant double-stranded DNA copy of the viral genome (317). The impact of high rates of recombination on HIV populations is substantial; as noted earlier, 5% to 10% of sequenced virus isolates have been reported to be intersubtype recombinants (531), and recombinants comprise the predominant strains currently circulating in certain parts of the world. Recombination also provides a mechanism for the rapid generation of multiple-drug-resistant HIV variants. It should be noted that rates of mutation and recombination during reverse transcription can be affected by a variety of viral and cellular factors. Imbalances in the dNTP pools in the infected cell can increase the rate of mutation and template switching (316,421,634), and the fidelity of reverse transcription and frequency of recombination can be influenced by the composition of the large macromolecular complex in which reverse transcription takes place. For example, it has been reported that Vpr increases fidelity (411) and that NC promotes recombination (459).

RT has long been a target in the search for antiviral compounds. A number of RT inhibitors have been developed, including the nucleoside analogs [e.g., 3'-azido-3'-deoxythymidine (AZT or zidovudine), 2'-deoxy-3'-thiacytidine (3TC or lamivudine), 2',3'-dideoxyinosine (ddI or didanosine), and 2',3'-dideoxycytidine (ddC or zalcitabine)] and the nonnucleoside inhibitors (e.g., nevirapine and delavirdine). The nucleoside analogs act as chain terminators, whereas the nonnucleoside compounds inhibit DNA polymerization by binding a small hydrophobic pocket near the RT active site, thereby inducing an allosteric change at the active site (340). Unfortunately, resistance to these compounds develops rapidly in patients, probably reflecting the fact that resistant variants exist even before the initiation of therapy (105). As is the case for mutations that allow escape from PR inhibitors (see earlier), some changes in RT provide drug resistance whereas others appear to be adaptations to resistance-conferring mutations or function to increase levels of drug resistance. Analysis of the location of resistance mutations in the RT structure has provided important insights into the mechanism of resistance and should be useful in designing additional inhibitors that delay the appearance of escape mutants or effectively combat drug-resistant strains.

Integrase

A distinguishing feature of retrovirus replication is the insertion of a DNA copy of the viral genome into the host cell chromosome following reverse transcription. The integrated viral DNA (the provirus) serves as the template for the synthesis of viral RNAs and is maintained as part of the host cell genome for the lifetime of the infected cell. Retroviral

mutants deficient in the ability to integrate generally fail to establish a productive infection.

The integration of viral DNA is catalyzed by integrase, a 32-kd protein generated by PR-mediated cleavage of the C-terminal portion of the HIV-1 Gag-Pol polyprotein (see Fig. 5). The steps in the integration process were originally elucidated in studies using MuLV (59,206), but these findings apply to HIV as well. In all retroviral systems, integration proceeds in the same series of steps (Fig. 25):

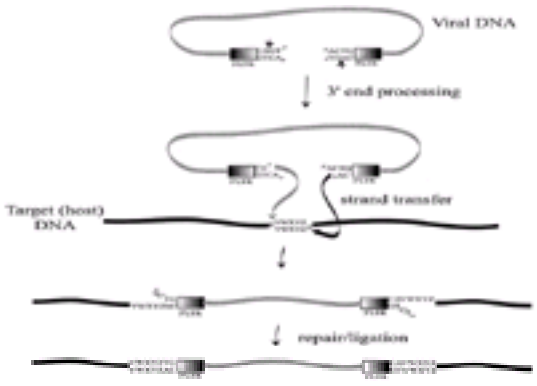

FIG. 25. Schematic depiction of the integration process. Details are provided in the text.

1. IN removes two to three nucleotides from the initially blunt 3' termini of both strands of full-length, linear viral DNA, generating a preintegration substrate with 3'-recessed ends (3'-end processing). This reaction can take place in the cytoplasm of the infected cell.
2. In the nucleus, IN catalyzes a staggered cleavage of the cellular target. Selection of the host DNA target site by IN is essentially sequence independent. The 3' recessed ends of viral DNA are joined to the 5' “overhanging” termini of the cleaved cellular DNA (strand transfer).
3. Cellular repair machinery, perhaps in conjunction with IN, fills the gaps, thereby completing the integration process (59,206,539). The integrated HIV provirus is flanked by a 5-bp direct repeat and terminates with the dinucleotides 5'-TG and CA-3'. The direct repeat results from the duplication of cellular target sequences.

Our understanding of the chemistry of the retroviral integration reaction has been greatly assisted by the development of *in vitro* integration assays. Purified IN, expressed in *E. coli*, can carry out 3'-end processing and strand transfer reactions *in vitro* when combined with short synthetic oligonucleotides that mimic the viral DNA ends and a divalent metal ion (Mg^{2+} or Mn^{2+}) (119,324). Using such systems, IN also catalyzes a reaction, known as disintegration, which in essence is the strand transfer reaction in reverse (93). The predominant product in *in vitro* assays using purified HIV-1 IN is a single end joined to one strand of the target, rather than the more physiologically relevant product in which both ends are integrated into the target. To obtain the integration of both ends with high efficiency requires additional proteins as part of a high-molecular-weight complex (see later), or, at a minimum, the presence of NC (77).

Retroviral IN proteins are composed of three structurally and functionally distinct domains: an N-terminal, zinc-finger-containing domain, a core domain, and a relatively nonconserved C-terminal domain (Fig. 26). Because of its low solubility, it has not yet been possible to crystallize the entire 288-amino-acid HIV-1 IN protein. However, the structure of all three domains has been solved independently by x-ray crystallography or NMR methods. The crystal structure of the core domain of the avian sarcoma virus IN has also been determined (60). The N-terminal domain (residues 1 to 55), whose structure was solved by NMR spectroscopy (68), is composed of four helices with a zinc coordinated by amino acids His-12, His-16, Cys-40, and Cys-43. The structure of the N-terminal domain is reminiscent of helical DNA binding proteins that contain a so-called helix-turn-helix motif; however, in the HIV-1 structure this motif contributes to dimer formation. Initially, poor solubility hampered efforts to solve the structure of the core domain. However, attempts at crystallography were successful (153) when it was observed that a Phe-to-Lys change at IN residue 185 greatly increased solubility without disrupting *in vitro* catalytic activity. Each monomer of the HIV-1 IN core domain (IN residues 50 to 212) is composed of a five-stranded b-sheet flanked by helices; this structure bears striking resemblance to other polynucleotidyl transferases including RNaseH and the bacteriophage MuA transposase (153,525). Three highly conserved residues are found in analogous positions in other polynucleotidyl transferases; in HIV-1 IN these are Asp-64, Asp-116 and Glu-152, the so-called D,D-35-E motif (see Fig. 26). Mutations at these positions block HIV IN function both *in vivo* and *in vitro*. The close proximity of these three amino acids in the crystal structure of both avian sarcoma virus (60) and HIV-1 (153) core domains supports the hypothesis that these residues play a central role in catalysis of the polynucleotidyl transfer reaction that is at the heart of the integration process. The C-terminal domain, whose structure has been solved by NMR methods (158,381), adopts a five-stranded b-barrel folding topology reminiscent of a Src homology 3 (SH3) domain. Recently, the x-ray structures of SIV and Rous sarcoma virus IN protein fragments encompassing both the core and C-terminal domains have been solved (89,683).

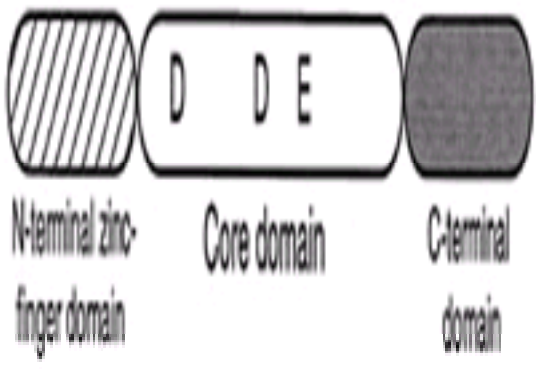

FIG. 26. Schematic representation of an IN monomer. The three structural domains are shown. The catalytic triad of Asp-64, Asp-116, and Glu-152 is represented as D-D-E.

Although it is clear that the core domain of IN plays the central role in catalysis, the functions of the N-terminal and C-terminal domains are less clear. The core domain alone can catalyze the disintegration reaction (66); however, the finding that mutations in the zinc-finger domain disrupt IN tetramerization and catalytic activity suggest a role for this region in stabilizing overall IN structure and promoting the formation of higher-order IN multimers (705). The C-terminal domain is required for both 3'-end processing and integration reactions. Unlike the core domain, which binds DNA only in the presence of a divalent metal ion, the C-terminal domain exhibits strong, and nonspecific, DNA binding even in the absence of metal ion (162). The non-sequence-specific nucleic acid binding properties of the C-terminal domain has led to the suggestion that this region of IN may contribute to the ability of IN to remain associated with the viral genome during reverse transcription (57). It has also been proposed that basic sequences near the junction of the core and C-terminal domains of IN contribute to the nuclear import of the viral preintegration complex (213) (see later).

During reverse transcription and transport of viral DNA to the nucleus, the viral nucleic acid remains associated with a high-molecular-weight PIC composed of both viral and cellular proteins (51,58). Lentiviruses are unique among retroviruses in that the host cell does not have to pass through mitosis for productive infection to occur. The lentiviral PIC must therefore contain determinants that facilitate its transport to, and across, the nuclear membrane. As mentioned earlier, although purified IN can catalyze single-ended integration *in vitro*, such events would be dead-end reactions in an infected cell and would fail to produce an integrated provirus. Additional viral proteins are required for the concerted two-ended reactions *in vitro* that mimic the physiologic integration process. In addition to RT and IN, MA, Vpr, and NC have been reported to be present in HIV-1 PICs, whereas CA is absent (64,214,215). The nonhistone chromosomal protein HMG I(Y) also cofractionates with HIV-1 PICs (171) and the human homolog of the yeast protein SNF5 (referred to as IN interactor 1, or INI1) stimulates HIV-1 IN activity *in vitro* (318). The latter protein, which functions as a transcriptional activator, was postulated to promote integration by targeting viral DNA to transcriptionally active regions of the host chromosome (318). Of particular interest is a host-derived protein that prevents suicidal autointegration events (i.e., reactions in which the DNA ends integrate into internal sites within the same viral DNA molecule). This so-called barrier-to-autointegration (BAF) protein was originally identified by its ability to protect MuLV from autointegration (358) and was subsequently proposed to play a similar role for HIV-1 (85). An additional level of complexity is suggested by footprinting studies demonstrating that the ends of the viral DNA are organized into a large complex composed of both viral and cellular proteins (86,652). These complexes have been termed intasomes to distinguish them from the larger PIC.

The development of *in vitro* assays that faithfully reproduce aspects of the integration reaction provide powerful tools with which to screen for agents that block this critical step in HIV replication. Because PICs catalyze reactions that more faithfully reproduce authentic integration, and because they contain additional viral and cellular proteins, they will prove more useful than purified IN in identifying compounds that exhibit antiintegration activity *in vivo*. Several classes of agents inhibit IN function *in vitro*; these include polyanions, nucleotide analogs, and DNA binding compounds (170). Most of these agents are fairly nonspecific in their mode of action, and many compounds that block activity of purified IN are not inhibitory in assays using purified PICs (172). However, certain compounds, such as the dicaffeoylquinic acids, inhibit integration both *in vitro* and in infected cells and appear to be relatively selective for retroviral IN proteins relative to other DNA modifying enzymes and phosphoryltransferases (709). Recently, diketo acid inhibitors have also been shown to be specific and potent inhibitors of the IN-mediated strand transfer reaction (271).

Env

The HIV Env glycoproteins play a major role in the virus life cycle. They contain the determinants that interact with the CD4 receptor and coreceptor, and they catalyze the fusion reaction between the lipid bilayer of the viral envelope and the host cell plasma membrane. In addition, the HIV Env glycoproteins contain epitopes that elicit immune responses that are important from both diagnostic and vaccine development perspectives.

Biosynthesis and Transport

The HIV Env glycoprotein is synthesized from the singly spliced 4.3-kb Vpu/Env bicistronic mRNA (see Fig. 5, Fig. 13, and Fig. 14); translation occurs on ribosomes associated with the rough endoplasmic reticulum (ER). The 160-kd polyprotein precursor (gp160) is an integral membrane protein that is anchored to cell membranes by a hydrophobic stop-transfer signal in the domain destined to be the mature TM Env glycoprotein, gp41 (Fig. 27). The gp160 is cotranslationally glycosylated, rapidly associates with the host chaperone BiP/GRP78, forms disulfide bonds, and undergoes oligomerization in the ER (154,662). The predominant oligomeric form appears to be a trimer, although dimers and tetramers are also observed. The gp160 is transported to the Golgi, where, like other retroviral envelope precursor proteins, it is proteolytically cleaved by cellular enzymes to the mature SU glycoprotein gp120 and TM glycoprotein gp41

(see Fig. 27). The cellular enzyme responsible for cleavage of retroviral Env precursors following a highly conserved Lys/Arg-X-Lys/Arg-Arg motif is furin or a furin-like protease (262), although other enzymes may also catalyze gp160 processing. Cleavage of gp160 is required for Env-induced fusion activity and virus infectivity (199,427). Subsequent to gp160 cleavage, gp120 and gp41 form a noncovalent association that is critical for transport of the Env complex from the Golgi to the cell surface. The gp120–gp41 interaction is fairly weak, and a substantial amount of gp120 is shed from the surface of Env-expressing cells.

The HIV Env glycoprotein complex, in particular the SU (gp120) domain, is very heavily glycosylated; approximately half the molecular mass of gp160 is composed of oligosaccharide side chains. During transport of Env from its site of synthesis in the ER to the plasma membrane, many of the side chains are modified by the addition of complex sugars. The numerous oligosaccharide side chains form what could be imagined as a sugar cloud obscuring much of gp120 from host immune recognition. As shown in Figure 27, gp120 contains interspersed conserved (C_1 to C_5) and variable (V_1 to V_5) domains. The Cys residues present in the gp120s of different isolates are highly conserved and form disulfide bonds that link the first four variable regions in large loops (364).

Following its arrival at the cell surface, the gp120–gp41 complex is rapidly internalized. Several studies have demonstrated that a Tyr-X-X-Leu sequence in the gp41 cytoplasmic tail (approximately five residues from the membrane-spanning domain) (see Fig. 27) is at least partially responsible for this rapid internalization (351,541). Analogous motifs are also present in the TM Env proteins of HIV-2, SIV, and several other retroviruses. Tyr-based motifs are known to mediate endocytosis of cellular plasma membrane proteins by binding the μ2 chain of clathrin-associated AP-2 complexes (472), and such interactions have been observed with the HIV-1 and SIV gp41 cytoplasmic domains (44,471). It is currently unclear why retroviral Env glycoproteins would evolve a mechanism to promote rapid internalization, although such a strategy could help evade the host immune response and limit Env-induced cytopathicity. Interestingly, it has been reported that $Pr55^{Gag}$ suppresses HIV-1 Env internalization, perhaps by masking the gp41 endocytosis signals (156). By preventing rapid Env internalization in the presence of Gag, Env incorporation into budding particles might be enhanced.

Env Incorporation into Virions

The mechanism by which the Env glycoproteins are incorporated into budding virus particles remains incompletely characterized. Several lines of evidence suggest that HIV-1 Env glycoproteins are actively recruited into virions via direct interactions between Env and MA:

1. Mutations in the HIV-1 MA can block HIV-1 Env incorporation (146,196,197,691); this incorporation defect can be reversed by pseudotyping virions with heterologous Env glycoproteins or by removing the gp41 cytoplasmic tail (196,197,408).
2. HIV-1 Env directs basolateral budding of Gag in polarized epithelial cells (380,481).
3. A direct binding between HIV-1 MA and peptides derived from the gp41 cytoplasmic tail was reportedly detected *in vitro* (117).
4. A single amino acid change in MA reverses an Env-incorporation defect caused by a small deletion in the gp41 cytoplasmic tail (448).

Some evidence, however, supports a more passive mode of Env incorporation. Heterologous Env proteins [e.g., those of MuLV, HTLV, and vesicular stomatitis virus (VSV)] can be incorporated into HIV-1 virions, and some studies observed that most or all of the cytoplasmic tail of HIV-1 can be removed without blocking Env incorporation. A model that is consistent with currently available data would propose that Env incorporation (and the incorporation of multiple cellular membrane proteins) can occur, in some cases, in the absence of an interaction with Gag; however, the incorporation of full-length HIV-1 Env requires a specific interaction between the gp41 cytoplasmic tail and MA. The effect of gp41 cytoplasmic tail truncation was observed to be strongly cell-type dependent (197); recent characterization of this phenomenon revealed that whereas Env complexes lacking the gp41 cytoplasmic tail could be incorporated into virions produced in certain cell lines (e.g., HeLa, MT-4), in most cell lines and cell types, including primary human PBMC and macrophages, such truncations blocked Env incorporation (449). Thus, it is clear that the gp41 cytoplasmic tail plays a crucial role in Env incorporation. The cell-type dependence of this function suggests the involvement of host cell factors.

In addition to the viral Env glycoproteins, HIV-1 virions incorporate substantial levels of a number of cellular surface proteins. These include the histocompatibility leukocyte antigens class I and II (HLA I and II) and the cell adhesion molecules CD44, LFA-I, and ICAM-1 (for review, see ref. 479). The mode of incorporation of these host-derived proteins, and the significance of their incorporation to HIV biology, are not fully understood.

Tissue Tropism

Distinct HIV/SIV isolates display a striking pattern of selective tropism for subsets of CD4+ cells. As noted earlier, many laboratory-adapted isolates (e.g., $HIV\text{-}1_{IIIB}$, $HIV\text{-}1_{LAI}$ or $HIV\text{-}1_{SF\text{-}2}$) readily infect activated human PBLs and T-cell lines but cannot replicate efficiently in primary human monocyte-derived macrophages, which are major *in vivo* HIV/SIV targets in the brain, spinal cord, lung, and lymph nodes. In contrast, the host range of macrophage-tropic (M-tropic) strains of HIV, isolated from asymptomatic individuals, is usually limited to PBLs and cells of the monocyte/macrophage lineage; continuous T-cell lines are usually refractory to infection by these isolates. Over time, the virus present in an infected individual gradually changes and cytopathic isolates capable of inducing syncytia and infecting PBLs and T-cell lines become more prevalent. In fact, the evolution of isolates from primarily M-tropic to T-cell-line (TCL-) tropic may play a major role in disease progression (616). The determinants of M- versus TCL-tropism reside primarily in the V_3 loop of gp120; however, amino acids located in the V_1/V_2 domain may be required for full infectivity of recombinant viruses. Observations indicating that isolates of primate lentiviruses displayed selective tropism for distinct populations of CD4+ cells are consistent with the idea that molecules in addition to CD4 are required for HIV Env-induced fusion and entry;

this hypothesis was confirmed by the discovery of the HIV/SIV coreceptors (see later).

Membrane Fusion

A primary function of viral Env glycoproteins is to promote a membrane fusion reaction between the lipid bilayers of the viral envelope and host cell membranes. This membrane fusion event enables the viral core to gain entry into the host cell cytoplasm. A number of regions in both gp120 and gp41 have been implicated, directly or indirectly, in Env-mediated membrane fusion. Studies of the HA_2 hemagglutinin protein of the orthomyxoviruses and the F protein of the paramyxoviruses indicated that a highly hydrophobic domain at the N-terminus of these proteins, referred to as the fusion peptide, plays a critical role in membrane fusion (657). Mutational analyses demonstrated that an analogous domain was located at the N-terminus of the HIV-1 (200), HIV-2 (198), and SIV (46) TM glycoproteins (see Fig. 27). Nonhydrophobic substitutions within this region of gp41 greatly reduced or blocked syncytium formation and resulted in the production of noninfectious progeny virions.

C-terminal to the gp41 fusion peptide are two amphipathic helical domains (see Fig. 27) which play a central role in membrane fusion. Mutations in the N-terminal helix (referred to as the N-helix), which contains a Leu zipper-like heptad repeat motif, impair infectivity and membrane fusion activity (73,88,152), and peptides derived from these sequences exhibit potent antiviral activity in culture (659). The structure of the ectodomain of HIV-1 and SIV gp41, the two helical motifs in particular, has been the focus of structural analyses in recent years (67,80,611,653). Structures were determined by x-ray crystallography or NMR spectroscopy either for fusion proteins containing the helical domains, a mixture of peptides derived from the N- and C-helices, or in the case of the SIV structure (67), the intact gp41 ectodomain sequence from residue 27 to 149. These studies obtained fundamentally similar trimeric structures, in which the two helical domains pack in an antiparallel fashion to generate a six-helix bundle (Fig. 28). The N-helices form a coiled-coil in the center of the bundle, with the C-helices packing into hydrophobic grooves on the outside. Peptides corresponding to the helical domains presumably inhibit fusion (and virus infectivity) by interacting with their complementary binding partner on gp41 and preventing gp41 itself from adopting the six-helix bundle structure. Binding studies using native HIV-1 Env indicated that gp41 could interact with C-helix-derived peptide only after CD4 binding (211). These results, which extend previous observations of CD4-induced conformational changes in the gp41 ectodomain (552), suggest that the N- and C-helices undergo conformational changes (exposure) following CD4 binding, and that these rearrangements are required for membrane fusion (211). In certain respects, the gp41 ectodomain structure bears a strong resemblance to that of the fusion-competent (low-pH-induced) form of influenza HA_2 (65), although in the latter case the N-helices are outside, not inside, the bundle. Structures for both “resting” and fusion-competent forms of influenza HA_2 have been solved. Upon activation of the resting state by low pH, a dramatic conformational change takes place that causes the fusion peptide to move from the center to the end of the trimer, where it can insert into the target membrane. The similarity between the HIV/SIV gp41 and influenza HA_2 ectodomain structures raises the intriguing possibility (80,611,653) that HIV and SIV Envs trigger membrane fusion by the same “spring-loaded” mechanism proposed for influenza (65). However, as only one structure has been determined for HIV/SIV, strong inferences concerning the rearrangements that ultimately trigger membrane fusion may be somewhat premature. An alternative fusion model that does not require major conformational changes in the gp41 core structure has been proposed (67). In any case, the gp41 ectodomain presents a potential target for drug intervention; indeed, a peptide (referred to as T20) corresponding to the C-helix potently inhibits HIV-1 replication in infected patients (331).

FIG. 28. Trimeric structure of SIV gp41 ectodomain residues 27 to 149. The N-terminal helices are located on the inside of the six-helix bundle; the C-terminal helices pack into hydrophobic grooves on the outside. (Reprinted from ref. 67, with permission.)

Whereas gp41 appears to interact directly with the lipid bilayer to catalyze the membrane fusion reaction, a variety of domains within gp120 are involved in activating Env fusogenicity. The V_3 loop of gp120, which elicits isolate-specific neutralizing antibodies, is an essential player in the membrane fusion reaction. Mutations throughout the V_3 loop of HIV-1 block syncytium formation and virus infectivity without perturbing the processing, transport, and CD4 binding properties of gp120 (201,482). The analogous domain of the HIV-2 Env glycoprotein is also required for fusion and infectivity (198). The importance of V_3 in membrane fusion appears to be a consequence of its role in gp120–coreceptor interactions (see later). In addition to V_3, the V_1/V_2 region participates in some manner in the fusion reaction, as illustrated by the observations that mutations in these variable loops impair fusion (607) and antibodies that bind this region can neutralize virus infectivity (210,431).

CD4 Binding

CD4, the major cell-surface receptor for SIV/HIV, was the first retroviral receptor protein identified (for review, see ref.

48). CD4 is a 55-kd member of the immunoglobulin (Ig) superfamily; it is composed of a highly charged cytoplasmic domain, a single hydrophobic membrane-spanning domain, and four distinct extracellular domains D1 to D4 (397). CD4 normally functions to stabilize the interaction between the T-cell receptor on the surface of T lymphocytes and class II major histocompatibility complex (MHC II) molecules on the surface of antigen-presenting cells. CD4 also recruits $p56^{lck}$ to the T-cell receptor. The high-affinity CD4 binding site for gp120 has been localized to a small segment of the N-terminal extracellular domain, analogous to the second complementarity-determining region (CDR-2) loop of an immunoglobulin light chain variable domain. The cytoplasmic domain of CD4 is not required for its viral receptor function, and mutations that disrupt CD4 internalization do not block HIV entry (25,396). CD4 binding determinants in Env map to the C_3 and C_4 domains of gp120, although a more discontinuous, conformation-dependent domain is clearly involved in high-affinity gp120–CD4 binding. CD4 binding serves not only to promote virion attachment to the target cell but also to induce conformational changes in gp120 and gp41 that are required to activate Env fusogenicity (552,619).

The recent crystallization of a gp120 “core” domain (an unglycosylated gp120 derivative lacking the V_1/V_2 and V_3 loops and the N- and C-termini) complexed with fragments of CD4 and a neutralizing antibody has further refined our understanding of the gp120–CD4 interaction (350,679). The core structure reveals two major domains (referred to as the inner and outer domains) connected by a so-called bridging sheet (Fig. 29). The latter is composed of a four-stranded, antiparallel b-sheet derived from sequences in the V_1/V_2 stem and portions of C_4. Of particular significance is the observation that the CD4 binding site in gp120 (which includes parts of the outer domain and the bridging sheet) is deeply recessed and predicted to be flanked by heavily glycosylated variable regions (350,679). A CD4 residue (Phe-43), shown previously to be critical for gp120 binding, occupies the opening of the CD4-binding cavity while many of the gp120 amino acids important for CD4 binding line the opening of the cavity. A number of the interatomic contacts between gp120 and CD4 are clearly resolved in this structure (350).

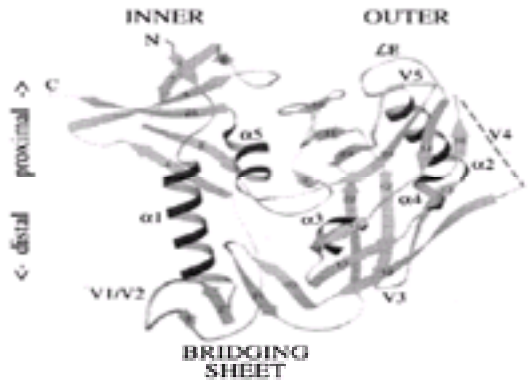

FIG. 29. Ribbon diagram of the gp120 core. In this orientation, the viral membrane would be at the top, the target cell membrane at the bottom. The inner and outer domains are connected by a four-stranded b-bridging sheet. The remnants of variable loops V_1/V_2, V_3, V_4, and V_5 are shown. (Reprinted from ref. 350, with permission.)

The HIV-1 Env glycoprotein binds CD4 not only during the early phase of infection, but also during the transport of gp160 to the cell surface (290). As a consequence of this intracellular association, CD4 is downmodulated from the cell surface, and Env-expressing cells are partially resistant to further infection (47,597). This process, known as superinfection interference, was described many years ago for avian retroviruses (544). In the case of HIV-1, the accessory protein Vpu degrades the CD4 component of gp160–CD4 complexes in the ER, thereby releasing Env for transport to the cell surface (see section on Vpu).

The identification of CD4 as the primary receptor for HIV raised the hope that soluble forms of CD4 (sCD4) might display antiviral activity. Indeed, sCD4 can effectively inhibit syncytium formation and HIV infectivity in tissue culture systems primarily by inducing gp120 shedding (442). Unfortunately, this strong inhibitory effect is generally limited to laboratory-adapted isolates. Primary virus strains are neutralized poorly by sCD4, and sCD4 therapy of HIV-infected individuals has little if any impact on the levels of p24 antigenemia or viremia (122).

Coreceptor Interactions

Soon after the identification of CD4 as the major HIV/SIV receptor, it was recognized that this protein is not sufficient for HIV-induced membrane fusion and virus entry. Mouse cells expressing human CD4 were not infectable (395), whereas CD4+ mouse–human cell hybrids could be induced to fuse upon expression of HIV-1 Env (56,150). Furthermore, as mentioned earlier, different HIV-1 isolates often infected only a subset of CD4+ cells. These observations suggested that, in concert with CD4, secondary receptor(s), or coreceptor(s), expressed on human cells functioned in the membrane fusion process. For more than a decade, the identity of potential HIV coreceptors remained elusive. Then, in a remarkably short period of time, a number of studies demonstrated that members of the G protein–coupled receptor superfamily of seven-transmembrane domain proteins provided the long-sought coreceptor function. By screening a human library for cDNAs that could confer on nonhuman cells the ability to fuse with HIV-1 Env-expressing cells, the a-chemokine receptor CXCR4 (originally designated fusin) was identified as the primary coreceptor for TCL-tropic isolates of HIV-1 (177). A separate line of investigation was stimulated by the observation that several b-chemokines produced by CD8+ T-cells (i.e., RANTES, MIP-1a, and MIP-1b) could suppress infection by M-tropic strains of HIV-1, HIV-2, and SIV (100). This finding simultaneously led a number of investigators to test whether the cell-surface proteins that bound these molecules, the b-chemokine receptors, could function as HIV-1 coreceptors. The results indicated that the b-chemokine receptor CCR-5 could, in fact, render CD4+ nonhuman cells permissive for infection by M-tropic isolates of HIV-1 (8,92,134). In recognition of the importance of coreceptors in determining HIV tropism, a nomenclature scheme was devised based on coreceptor usage; isolates (generally M-tropic) that utilized CCR5 were denoted R5 isolates, strains (generally TCL-tropic) that preferentially used CXCR4 were named X4 viruses, and dual-tropic strains that utilized

both CCR5 and CXCR4 were denoted R5X4 isolates (30).

In the last several years, numerous additional studies have refined our understanding of coreceptor activity and function. Some of the more important findings are described here. (For further information, refer to more detailed reviews in refs. 31 and 143).

1. It is clear that, depending on the virus isolate, multiple sequences in the N-terminal domain and extracellular loops of CCR5 and CXCR4 can influence coreceptor function, and that the gp120–coreceptor interaction is complex.
2. Signaling through the chemokine receptors is not required for their virus coreceptor function (15).
3. Certain isolates of HIV-1, HIV-2, and SIV use coreceptors in a CD4-independent manner (i.e., as primary receptors) (161,419,508). In fact, the designation of the chemokine receptors as coreceptors may be a misnomer; the observation of CD4-independent HIV/SIV infection, together with the finding that feline immunodeficiency viruses utilize the chemokine receptors but not CD4 (661) suggest that the use of chemokine receptors in lentiviral infection may have predated the involvement of CD4.
4. Direct interactions between coreceptor and gp120 have been detected. This interaction is greatly stimulated by, or is dependent on, the presence of CD4 (353,625,676). Association between CD4 and CCR5 in the absence of gp120 has also been reported (680).

Several domains within gp120 directly or indirectly function in Env/coreceptor interactions. Consistent with its influence on HIV-1 tropism, the V_3 loop plays a major role: Single amino acid changes or deletions in V_3 shift or impair coreceptor usage (92,101), V_3 peptides reportedly interact with CXCR4 (547), antibodies to V_3 block gp120–CCR5 binding (625,676), and an HIV-1 variant that escaped the CXCR4 inhibitor AMD3100 contains substitutions within and in close proximity to the V_3 loop (131) . The V_1/V_2 region also helps determine coreceptor usage (537). In addition to the involvement of variable regions in coreceptor interaction, highly conserved portions of gp120 that are exposed upon CD4 binding appear to interact with coreceptor. The binding of antibodies whose epitopes are revealed upon CD4 exposure can block gp120–CCR5 interaction; these epitopes include residues within the gp120 bridging sheet (350,679). By analyzing a number of Env mutants, a highly conserved structure adjacent to V_3 was recognized as playing a major role in CCR5 binding (529).

A generalized scheme for coreceptor utilization is presented in Table 1. TCL-tropic (X4) isolates infect T-cell lines via CXCR4; M-tropic (R5) strains infect macrophages via CCR5. Dual-tropic (R5X4) isolates can utilize either CCR5 or CXCR4 and can therefore infect both T-cell lines and macrophages. Because primary lymphocytes express both CCR5 and CXCR4, they are infectable by X4, R5, and R5X4 isolates. Although this simple model explains many aspects of coreceptor utilization, it fails to account for certain observations. In particular, the details of coreceptor utilization in macrophages remain to be fully elucidated. Some R5 strains do not efficiently infect primary macrophages (90,141), and b-chemokines, which block CCR5, do not always inhibit HIV infection in macrophages (444,556). Furthermore, macrophages have been reported to express relatively high levels of CXCR4 and yet generally (with some exceptions—e.g., see refs. 578 and 688) cannot be efficiently infected with X4 isolates. Several models have been proposed to explain this latter observation, including (a) X4 strains enter macrophages but are blocked at a post-entry step (555), (b) higher-order CD4–CXCR4–gp120 interactions required for membrane fusion do not take place efficiently in macrophages (137,354), and (c) CCR5 out-competes CXCR4 for binding to CD4, thereby rendering CXCR4 nonfunctional as a coreceptor in macrophages (359). Intriguingly, some dual-tropic isolates are reportedly able to use CXCR4 to infect macrophages that lack CCR5 (687); it thus appears that dual-tropism may arise not only from the ability to utilize both CCR5 and CXCR4 but also from the ability to use CXCR4 on macrophages (687).

The observation that CD4 induces conformational changes in gp120 that enhance interaction with coreceptors suggests that events leading up to membrane fusion occur sequentially (Fig. 30). First gp120 interacts with CD4, then a ternary complex forms between coreceptor, CD4, and gp120, and finally conformational changes take place in gp41 that trigger membrane fusion. This step-wise Env activation pathway is also suggested by the finding that binding of antibodies to the putative coreceptor interacting surface of gp120 (350) is enhanced by CD4 (553,619). Thus, interaction of gp120 with CD4 exposes epitopes that are normally hidden in the native Env structure; such epitopes can be constitutively exposed by selecting for HIV-1 variants that replicate in a CD4-independent fashion (286,341).

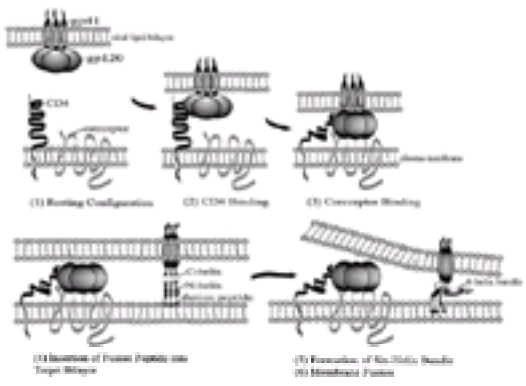

FIG. 30. Schematic representation of the steps leading to membrane fusion. In the resting configuration, the Env glycoprotein complex is in its native state (*1*). CD4 binding (*2*) induces conformation changes in Env that facilitate coreceptor binding (*3*). Following the formation of a ternary gp120/CD4/coreceptor complex, gp41 adopts a hypothetical conformation that allows the fusion peptide to insert into the target lipid bilayer (*4*). The formation of the gp41 six-helix bundle (which involves antiparallel interactions between the gp41 N- and C-helices) (*5*) brings the viral and cellular membranes together and membrane fusion takes place (*6*).

In addition to CXCR4 and CCR5, a number of other chemokine receptors and related “orphans” (receptors whose physiologic ligands are unknown) have been demonstrated to serve as coreceptors for HIV/SIV fusion and infection in culture. These include CCR2b, CCR3, CCR8, APJ, Bonzo (STRL33), BOB (GPR15), and US28 (for review, see ref. 31). The significance of this diverse array of potential coreceptors is currently unclear. Many of the studies that defined the ability of HIV/SIV to utilize these alternative coreceptors employed cell culture systems in which very high levels of coreceptor and CD4 were expressed, and membrane fusion or infectivity could be detected with great sensitivity. Evaluation of coreceptor usage of numerous primary isolates has led some investigators to conclude that CCR5 and CXCR4 are likely to account for most if not all coreceptor activity *in vivo* (702,703). However, the possibility remains that in certain tissues alternative coreceptors may play some role. For example, CCR3 has been implicated in HIV-1 infection of microglial cells (272), and the prevalence of APJ expression in the central nervous system has led to the suggestion that this orphan receptor may perform a role in neuropathogenesis of HIV infection (155).

Certain individuals, despite persistent high-risk behavior, remain HIV-1 uninfected (495). The discovery of CCR5, CXCR4, and related molecules as HIV coreceptors raised the possibility that these individuals might have inherited mutant coreceptor alleles. In some instances, this turned out to be the case. The most clear-cut example of this phenomenon involves a CCR5 allele that is relatively common in Caucasian populations. This mutant allele, referred to as CCR5/D32, contains a 32-base-pair deletion and encodes a truncated protein that is not efficiently expressed at the cell surface and cannot function as an HIV-1 coreceptor (133,295,379,549). Homozygotes for CCR5/D32 are only very rarely infected with HIV-1, highlighting the protective benefits of inheriting this allele. Some limited evidence suggests that CCR5/D32 heterozygosity may confer some protective benefits in slowing disease progression (133,379,549). Since the identification of the CCR5/D32 mutation, other inherited polymorphisms in the CCR5 coding region or its regulatory sequences have been reported to influence HIV-1 transmission or HIV-induced disease progression (420,428). A CCR2 mutation known as CCR2-64I has also been associated with a delay in disease progression, perhaps because of its linkage with the CCR5 gene regulatory region (346,587). It should be emphasized that mutations in genes for currently recognized coreceptors appear to be present in only a minority of multiply exposed, uninfected individuals, suggesting the existence of other mechanisms of protection from HIV infection. One such mechanism associated with altered infectability or disease progression involves the regulation of expression of the chemokines themselves. Analysis of a large number of hemophiliacs exposed to HIV-1-contaminated factor VIII (697) and 100 subjects participating in a Multicenter AIDS Cohort study group (229) revealed that protection correlated with high levels of b-chemokine production. Additionally, a mutation in the regulatory region of the CCR5 ligand RANTES (377), and a single nucleotide polymorphism in the 3' untranslated region of the mRNA encoding the CXCR4 ligand (SDF-1) (669) were reportedly associated with a delay in disease progression.

The identification of HIV coreceptors presents novel opportunities for antiviral therapy. A derivative of RANTES was synthesized by modifying its N-terminus with aminooxypentane. This molecule, known as (AOP)-RANTES does not induce chemotaxis but potently inhibits infection by a variety of HIV-1 isolates (577). Several low-molecular-weight compounds, including the bicyclam AMD3100 (144) and the polyphemusin analog T22 (450), potently block entry via CXCR4. A chemokine agonist encoded by human herpesvirus 8 (Kaposi's sarcoma–associated virus) was reported to inhibit virus entry through CCR3, CCR5, and CXCR4 (337). In addition to chemokine derivatives and low-molecular-weight coreceptor inhibitors, antibodies against coreceptors have displayed antiviral properties (432,677). Some concern about potential undesirable side effects of treatment with anti-coreceptor agents is warranted; although no observable negative consequences of CCR5 deletion are evident among individuals homozygous for the CCR5/D32 mutation, CXCR4 knock-out mice display a range of severe defects including abnormal hematopoiesis, cerebral development, and gastrointestinal tract vascularization (610,713). It is also unclear whether effective therapy would require that multiple coreceptors be targeted simultaneously. Despite these reservations, much effort will undoubtedly continue to be focused on developing coreceptor-based antiviral strategies.

The Accessory Proteins

In addition to the viral structural proteins (Gag and Env), the *pol*-encoded enzymes (PR, RT, and IN), and the regulatory proteins (Rev and Tat), the HIV genome encodes several additional “accessory” proteins: Vif, Vpu, Vpr, Vpx, and Nef (see Fig. 5). Early studies indicated that disruption of the ORFs for these proteins had little or no effect on virus replication in culture; indeed, laboratory-adapted virus isolates often acquire inactivating mutations in several of these genes. However, more recent tissue culture and *in vivo* experiments have revealed a strong requirement for these gene products for efficient virus replication and disease induction. The elucidation of the often-complex functions of these proteins continues to be the focus of active investigation.

Vif

The *vif* (virus infectivity factor) gene is located near the middle of the HIV genome, overlapping with *pol* and *vpr.* The presence of *vif* is a highly conserved feature among lentiviruses; a *vif* product is encoded by all lentiviruses except EIAV (325). Early reports indicated that Vif is not required for efficient virus assembly or release but is necessary for the generation of fully infectious virions (589,600). In subsequent studies, it became apparent that the phenotype of *vif*-defective mutants is strikingly cell-type dependent, and that this phenotype is imposed by the cell in which virus is produced (212). Thus, certain cell lines (e.g., 293T, HeLa, COS, SupT1, CEM-SS, and Jurkat) are “permissive” for *vif*-defective mutants, whereas others, including primary lymphocytes, monocyte-derived macrophages, and some T-cell lines, are “nonpermissive.” This observation suggests that host-cell factors in the virus-producing cell play a role in Vif function. Such a role was highlighted by studies in which the activity of different primate lentiviral Vif proteins was evaluated in cells of both human and nonhuman primate origin (584). The infectivity of HIV-1, HIV-2, and SIV_{agm} could be enhanced by HIV-1 Vif when these viruses were expressed in human cells. In contrast, although SIV_{agm} Vif could modulate infectivity of SIV_{agm} produced in African green monkey cells, it was inactive when expressed in human cells.

The important role of the producer cell in Vif function implies either that Vif-permissive cells express a factor that substitutes for Vif, or that nonpermissive cells contain a factor that suppresses virus replication in the absence of Vif. In

an attempt to distinguish between these possibilities, transient heterokaryons were formed between Vif-permissive (293T) and nonpermissive (HUT78) cell lines (581). The infectivity of virus produced by the resulting heterokaryons was significantly enhanced by the expression of Vif, suggesting that the nonpermissive phenotype is dominant and implying that Vif may suppress a host factor that inhibits virus infectivity. Similar results were obtained by fusing the nonpermissive T-cell line H9 with permissive HeLa cells (394). Intriguingly, the observation that HIV-1 Vif enhances infectivity of MuLV produced in human cells (584) suggests either that Vif suppresses the activity of divergent host factors, or that the putative host factor possesses the ability to inhibit widely divergent (i.e., HIV-1 and MuLV) retroviruses.

The Vif^- defect, while imposed in the producer cell, becomes apparent at an early post-entry step in the virus life cycle. Vif-defective virions, produced from nonpermissive cell types, fail to efficiently reverse-transcribe their genomes following infection (592,646), perhaps as a result of instability of the complex in which reverse transcription takes place (582). Vif-defective virions have also been reported to display morphologic abnormalities evident by EM (45,287) and defects in endogenous RT activity (241). Some investigators reported that Vif mutation causes a defect in Gag processing, such that Vif^- virions contain increased levels of the Gag precursor $Pr55^{Gag}$ (45,576). An effect of Vif on Gag processing has also been observed *in vitro* (347). However, detailed analyses of Vif^- virions obtained from nonpermissive cells failed to reveal any effect of Vif mutation on viral protein composition (50,188,468).

Several groups have detected the presence of small amounts of Vif in virions (70,188,322,378,583). It is currently not clear whether virion incorporation is necessary for the ability of Vif to enhance virus infectivity, or whether its incorporation is nonspecific and not linked to function. Several studies have raised doubts about the relevance of virion incorporation:

1. Vif is incorporated into MuLV particles (70), suggesting that the incorporation of Vif into virions does not require a specific interaction with other virion components.
2. Using cells that express variable levels of Vif, it was observed that the amount of Vif incorporated into virions is influenced by the levels of Vif expressed by the virus-producing cell, and that the resulting variations in the amount of virion-associated Vif have no effect on virus infectivity (583).
3. At least in some cell culture systems, virus preparations may be contaminated with Vif-containing microvesicles, raising the possibility that even the low levels of virion-associated Vif observed in previous studies may represent an overestimate of the amount actually present in virus particles (135).

Vpr

The *vpr* gene, which overlaps with *vif* at its 5' end (see Fig. 5), encodes a 14-kd, 96-amino-acid protein that is incorporated at high levels into virus particles (106,694). The incorporation of Vpr into virions appears to be mediated through an interaction with a $(Leu\text{-}X\text{-}X)_4$ motif near the C-terminus of the $p6^{Gag}$ protein (342,387,494). Vpr contains two predicted helical motifs, one near the N-terminus, the other near the middle of the protein, and a C-terminal Arg-rich domain. Vpr has been predicted to dimerize via residues in the helical domains (563,704). The presence of Vpr is highly conserved among primate lentiviruses. Although Vpr has little influence on HIV replication kinetics in proliferating PBMC or T-cell lines in culture, in nondividing monocyte-derived macrophages its effects are more substantial. Three major functions for Vpr have been proposed: (a) stimulation of gene expression driven by the HIV LTR, (b) transport of the viral preintegration complex to the nucleus during the early stages of the infection process, and (c) arrest of infected cells in the G_2 phase of the cell cycle.

Early studies of HIV-1 Vpr reported that Vpr weakly transactivated expression from the HIV-1 LTR (106). More recent experiments have confirmed this activity and suggest that it may be mediated via interactions between Vpr, Sp1 (554,649), TFIIB (2,334), and TFIID (334). The ability of Vpr to stimulate transcription may be linked to its cell-cycle arrest function (see later).

High levels of virion incorporation suggest a role for Vpr early in the virus life cycle prior to *de novo* gene expression. The observations that deletion of Vpr significantly reduces virus replication in terminally differentiated, nondividing, monocyte-derived macrophages (19), that the HIV-1 preintegration complex contains Vpr (215,274), and that Vpr localizes to the nucleus in HIV-infected or Vpr-expressing cells (388), suggested that Vpr might participate in the nuclear import of the HIV preintegration complex. As discussed previously, this nuclear import capability enables HIV to infect nondividing cells, including monocyte-derived macrophages. Initially, it appeared that MA might play the key role in nuclear import (see section on MA); subsequent studies, however, suggested that MA either has no role or is a relatively minor player in the process compared with Vpr and perhaps IN and a DNA triplex (or flap) structure resulting from lentiviral reverse transcription (see earlier). A number of recent studies have attempted to further define the role Vpr plays in nuclear localization. Mutational analyses have suggested that residues involved in nuclear localization are located at a variety of positions in Vpr, and that the protein lacks a canonical basic-type NLS, such as those found in karyophilic proteins—for example, SV40 large T antigen or nucleoplasmin (for reviews, see refs. 244 and 245). In some cases, Vpr mutations that disrupt nuclear localization may do so by inducing global protein misfolding or instability, complicating the precise mapping of determinants involved in nuclear import. As noted earlier, in the classical basic-type NLS pathway, the importin a (karyopherin a) component of the importin a/b heterodimer binds NLS-containing proteins, whereas the importin b (karyopherin b) component functions in part to mediate docking to the nucleoporins to mediate translocation through the nuclear pore complex (see Fig. 16). This pathway can be blocked by peptides containing the SV40 large T antigen NLS. Other distinct nuclear transport pathways exist, and the process by which cytoplasmic proteins and macromolecular structures destined for the nucleus traffic to the nuclear membrane, associate with components of the nuclear pore complex, and translocate through the nuclear pore is only partially understood. Thus it is perhaps not surprising that no clear consensus exists concerning the mechanism by which Vpr promotes nuclear localization. Divergent observations are in part a result of different experimental systems used to monitor localization, and, perhaps more importantly, due to the fact that some studies assessed the nuclear import of Vpr itself, whereas others followed the fate of a variety of Vpr fusion proteins or HIV-1 PICs containing or lacking Vpr. The diversity of findings is reflected in the results of several recent studies:

1. The observation that Vpr-mediated nuclear localization is not blocked by an SV40 large T antigen NLS suggested that Vpr functions independently of the importin pathway.
2. Based on data using purified PICs either containing or lacking Vpr, it was proposed that Vpr interacts with importin a and increases its affinity for proteins containing basic-type NLSs; it was suggested that Vpr interacts with importin a at a site distinct from that used by either a basic-type NLS or importin b (505,506). This model proposes that Vpr acts to stimulate importin a/b-dependent nuclear import (505,506). Interaction of Vpr with importin a was also reported in a study examining the localization of Vpr itself in both human and yeast cells (640).
3. The analysis of the nuclear transport properties of Vpr fused with heterologous proteins suggested that Vpr interacts directly with nucleoporins (187).
4. The import of Vpr to the nucleus was analyzed using an *in vitro* nuclear transport assay; it was concluded that Vpr contains two independent NLSs that utilize two different nuclear transport pathways, both of which are distinct from the importin a/b pathway (312).

The third major function widely described for Vpr is its ability to arrest cells in the G_2 phase of the cell cycle. Cell-cycle arrest reportedly occurs within hours of infection and is not blocked by antiviral agents, suggesting that it is initiated by the Vpr carried into the cell on virus particles (504). Several studies have indicated that G_2 arrest is linked to inhibition by Vpr of the activity of the $p34^{cdc2}$-cyclin B kinase complex (273,518), perhaps via an interaction with the host protein MOV34 (401). Although the biologic implications of Vpr-induced cell-cycle arrest remain unclear, it has been proposed that increased HIV-LTR-driven transcription in the G_2 phase of the cell cycle could provide a rationale for the evolution of a Vpr cell-cycle arrest function (175,239). This Vpr-induced enhancement of LTR-driven transcription is reportedly mediated by the p300 transcriptional coactivator (175). The nuclear import and cell-cycle arrest functions appear to involve distinct domains in Vpr, as mutations have been reported that disrupt one function but leave the other intact (400). The ability of Vpr to induce cell-cycle arrest not only in primate cells but also in yeast (393) underscores the involvement of highly conserved cellular factors in this phenomenon.

In addition to the properties described earlier, a number of additional functions for Vpr have been described. These include the ability to induce differentiation of rhabdomyosarcoma cells (369), trigger or suppress apoptosis (16,599), interact with the glucocorticoid receptor (519), kill cells by forming ion channels (502), activate HIV gene expression via a transcellular mechanism (370), and reduce mutation rates during reverse transcription (411). The interrelatedness of these diverse functions, and their implications for virus replication and pathogenesis *in vivo*, remain to be characterized.

Vpx

Members of the HIV-2/SIV_{sm}/SIV_{mac} lineage of primate lentiviruses carry an additional gene known as *vpx*. Because of the high degree of relatedness between the *vpr* and *vpx* genes, it was initially proposed that *vpx* was created by a *vpr* gene duplication event after the HIV-2/SIV_{sm}/SIV_{mac} lineage diverged from other primate lentiviruses (623). More recently, it was argued that *vpx* likely arose by acquisition of the SIV_{agm} *vpr* gene by nonhomologous (570) or homologous (624) recombination.

Like Vpr, Vpx is incorporated at high levels in virus particles, also via an interaction with the C-terminus of Gag (276,328,678). Interestingly, in the HIV-2/SIV_{sm}/SIV_{mac} viruses, two of the major functions of HIV-1 Vpr seem to be shared between Vpx and Vpr: Vpx is required for efficient infection of nondividing cells (692), whereas Vpr induces cell cycle arrest but has no role in nuclear targeting (183).

Vpu

A distinguishing feature of the HIV-1 lineage of primate lentiviruses is the presence of the *vpu* gene (see Fig. 4); it is absent from HIV-2 and all SIVs examined to date, with the exception of the closely HIV-1-related SIV_{cpz} (107,298,602). Vpu is a multimeric, 81-amino-acid, integral membrane phosphoprotein containing an N-terminal transmembrane anchor sequence and an approximately 50-amino-acid cytoplasmic domain (402). Three a-helical domains have been predicted: one in the transmembrane sequence (residues 6 to 29), the others in the cytoplasmic domain (residues 32 to 51 and 57 to 72) (675). The sites of phosphorylation have been mapped to two Ser residues at positions 52 and 56 (560). Vpu is translated from a *vpu-env* bicistronic mRNA (567) and is synthesized at intracellular levels comparable to Gag in virus-infected cells. It has not been detected in virus particles. Two principal functions for Vpu have been described: enhancement of virus release and degradation of CD4.

Early studies observed that HIV-1 clones lacking a functional *vpu* gene displayed a pronounced defect in virus particle production (601,602,617). The absence of the Vpu gene product had no discernible effect on the processing or transport of Gag proteins but caused virus particles to be retained at the plasma membrane and in intracellular vesicles (338). The mechanism by which Vpu enhances particle release remains poorly understood; however, a number of observations have been made regarding this property.

1. Enhancement of virus release is independent of Env or CD4 (233,684).
2. Vpu can increase virus production of highly divergent retroviruses (e.g., MuLV, visna, and HIV-2), even though these viruses do not carry a *vpu* gene (247).
3. The N-terminal transmembrane domain appears to be largely responsible for stimulating virus release (558).
4. This function of Vpu does not require phosphorylation (561) but may rely on the ability of Vpu to form cation-selective ion channels (167,559).
5. It was reported that a member of the tetratricopeptide repeat protein family interacts with HIV-1 Gag and suppresses virus release. The ability of Vpu to also interact with this protein and diminish its association with Gag could contribute to the ability of Vpu to enhance virus release (69).

The mechanism by which Vpu induces CD4 degradation has been characterized more completely than its ability to

stimulate virus particle release. Vpu directly binds the cytoplasmic tail of CD4 in the ER; residues in the cytoplasmic domain of Vpu, and Vpu phosphorylation, are critical for this process (49,87,363,561,638,663). The degradation of CD4 by Vpu appears to liberate the Env glycoprotein precursor gp160 from its interaction with CD4, thereby enabling Env to continue its transport to the cell surface (664,665). The presence of Env is not required for CD4 degradation (87,665). Progress has been made recently in elucidating the strategy used by Vpu to induce CD4 degradation: Vpu simultaneously interacts with CD4 and with a protein known as h-bTrCP via WD repeat elements present in the latter protein. The ternary CD4/Vpu/h-bTrCP complexes, which can be detected by coimmunoprecipitation, are then targeted for ubiquitin-mediated proteolysis via interaction between the cellular factor Skp1 and an “F box” motif on the h-bTrCP component of the ternary complex (414). Consistent with this model, inhibition of proteosome activity blocks Vpu-mediated CD4 degradation (205).

Nef

The *nef* gene, present only in primate lentiviruses, overlaps with the 3' LTR and encodes a 27-kd membrane-associated phosphoprotein (see Fig. 5). Membrane binding, mediated by a covalently attached myristic acid moiety and a cluster of N-terminal basic residues (654), is critical for Nef function. Nef is synthesized at high levels from the first mRNA transcript detected after infection. Although Nef was originally labeled a “negative factor” because of reports that it down-regulated virus replication by suppressing transcription from the HIV LTR (3,390), it is now clear that in cultured cells Nef expression either has no effect on virus spread or modestly enhances replication kinetics. The NMR structure of the Nef “core” domain (lacking the N-terminus) has been determined (257,258), and x-ray crystal or NMR structures of the Nef core have been solved for the protein in a complex with Src homology 3 (SH3) domains (12,357) or with the cytoplasmic tail of CD4 (259). In general, the overall folding of Nef is similar to that of helix-turn-helix DNA binding proteins; it contains an unstructured N-terminus, followed by a poly-Pro type II helix (which contains the SH3 binding motif Pro-X-X-Pro), several a-and 3_{10} helices, and a five-stranded antiparallel b-sheet. Residues 150 to 180 form a disordered loop. Interestingly, the binding between Nef and proteins containing SH3 domains (e.g., the Hck Tyr kinase) involves not only the consensus SH3-binding motif (Pro-X-X-Pro) but also discontiguous residues downstream of the poly-Pro type II helix (257).

Several distinct functions for Nef have been widely reported: (a) down-regulation of CD4 and MHC I expression from the cell surface, (b) enhancement of virus infectivity, and (c) modulation of cellular activation pathways. In contrast to the situation in HIV-1-infected cultures, where the role of Nef in virus replication is relatively difficult to measure, Nef appears to be critical for maintaining the high virus loads that are a prerequisite for disease induction *in vivo*. It is currently unclear which of the many activities attributed to Nef in tissue culture are required for its functions *in vivo*.

Like the HIV-1 Env and Vpu proteins, Nef possesses the capacity to down-regulate cell-surface expression of CD4 (226,260). Nef-induced CD4 down-regulation proceeds in a step-wise fashion:

1. Nef interacts with a di-Leu-based motif in the cytoplasmic domain of CD4 (5) and then associates with adapter protein (AP) complexes in clathrin-coated pits to promote rapid CD4 internalization. CD4 endocytosis probably results from a direct interaction between Nef and the µ2 subunit of the AP-2 complex (251,356,500) and perhaps the catalytic subunit of the vacuolar ATPase (386).
2. In the endosome, Nef connects CD4 with the b-subunit of COP I coatomers (b-COP), thereby targeting CD4 for degradation in the lysosome (27,501). Nef may also stimulate CD4 internalization by dissociating CD4 from $p56^{lck}$, which binds the CD4 cytoplasmic tail and normally inhibits CD4 endocytosis (333).

In addition to reducing cell-surface CD4 expression, Nef has been reported to down-regulate MHC I molecule expression (565). In contrast to CD4 down-regulation, which involves a di-Leu-based endocytosis signal in the CD4 cytoplasmic tail, MHC I degradation requires the presence of a Tyr-based motif (356). Cell-surface down-regulation of MHC I by Nef may be accomplished not only by increased rates of protein endocytosis from the cell surface but also as a consequence of reduced trafficking from the trans-Golgi network to the plasma membrane (252,356). Intriguingly, MHC I down-regulation may limit the ability of cytotoxic T lymphocytes (CTLs) to recognize and eliminate virus-infected cells, thus providing HIV and SIV with a mechanism for partially evading at least one component of the host immune response (110). Removing MHC I molecules from the cell surface exposes infected cells to attack by natural killer (NK) cells (689); HIV-1 may avoid this latter problem by not down-regulating those class I molecules (HLA-C and HLA-E) responsible for protection from NK cell lysis (108).

In single-cycle assays, Nef modestly stimulates virus infectivity (95,240,438,594). This function appears to be manifested at an early step in the virus life cycle, since *nef*-deleted mutants fail to efficiently reverse-transcribe their genomes after infection (6,94,564). Mutations in the Pro-X-X-Pro motif, which have no effect on CD4 down-regulation, impair Nef-mediated enhancement of infectivity (240,548), although this phenotype may in part reflect effects of the mutations on protein stability (118). Interestingly, the infectivity defect observed with *nef*$^{-}$ virus can be partially suppressed by pseudotyping with the vesicular stomatitis virus G glycoprotein (VSV-G) (4). VSV-G directs entry via fusion in a low pH endosome following endocytosis, whereas HIV-1 Env mediates entry via direct fusion at the plasma membrane. This observation implies that the role Nef plays early after entry depends on the route of HIV-1 entry. At this time, however, the mechanism by which Nef enhances virus infectivity remains to be elucidated. It is also unclear to what extent this relatively modest effect contributes to the strong requirement for Nef *in vivo*.

Deletion of the *nef* gene has a profound effect in the SIV/rhesus macaque animal model system. Monkeys inoculated with *nef*-deleted virus develop high-level antibody responses but produce no detectable circulating virus (327); these results indicate that Nef plays a critical role in initiating and sustaining SIV infection *in vivo*. Nef has also been proposed to play a major role in pathogenesis in HIV-1–infected humans. This hypothesis stems in part from the findings of some (132,335), but not others (296,297), that virus isolates derived from individuals who progress to disease slowly harbor *nef* mutations. Nef has been shown to be required for HIV-1 replication and pathogenicity in SCID mice containing human fetal thymus and liver implants (310), and transgenic mice expressing Nef in CD4+ T cells and in cells of the monocytic

lineage develop a variety of AIDS-like pathologies (265,266).

A number of studies have suggested that HIV and SIV Nef influence cellular signal transduction pathways and affect the activation state (in either a positive or a negative sense) of Nef-expressing cells (for review, see ref. 416). This area of research remains highly controversial, perhaps owing to differences in the cell culture systems used in various studies, to the inherent complexities of cellular signaling pathways, and to potential differences in the mode of action of SIV versus HIV-1 Nef. As mentioned earlier, HIV and SIV Nef contain a highly conserved consensus binding site for the SH3 domain of Src-family Tyr kinases (Pro-X-X-Pro). Nef has been reported to interact with a Ser/Thr (p21-activated or PAK) kinase and Src-family kinases (e.g., Lyn, Hck, and Lck) and modulate their catalytic activities. As cellular kinases are intimately involved in signaling pathways, such interactions may play a critical role in Nef function. The identification of a naturally occurring mutant SIV *nef* allele has provided some clues into Nef function. A strain of SIV_{mac} known as SIV_{pbj14} induces a rapidly fatal disease in infected macaques characterized by marked T-lymphocyte proliferation, retrafficking of T cells to the gastrointestinal tract, and unremitting diarrhea. In culture, SIV_{pbj14} causes extensive T-lymphocyte activation and displays the unusual property of being able to replicate efficiently in resting PBMC (151). These features are the result of an Arg-to-Tyr mutation that creates a sequence reminiscent of an immunoreceptor tyrosine-based activation motif (ITAM). ITAMs, present in the cytoplasmic tails of T-cell and B-cell receptors, are essential for lymphocyte activation, and the putative ITAM of the SIV_{pbj14} Nef protein appears to activate T-cell signaling (391). Despite the usefulness of the SIV system in understanding Nef function, particularly *in vivo*, caution should be used in extrapolating SIV data to HIV-1 in light of differences observed for SIV versus HIV-1 Nef activities in culture (for example, see ref. 253). In addition to potential direct effects of Nef on stimulating T-cell activation, a recent study demonstrated that Nef expression in macrophages induces the expression and release of the b-chemokines MIP-1a and MIP-1b, which in turn activate resting T lymphocytes (609). Such an effect could help explain the positive effect of Nef on HIV-1 replication *in vivo*.

It has been reported by several groups that Nef is incorporated into virions. Interestingly, as a result of viral PR-mediated cleavage, much of the HIV-1 Nef in virus particles is smaller than the full-length protein detected in infected cells (61,486,655). However, as is the case for Vif, it is not clear what, if any, function is served by virion incorporation, particularly since HIV-1 Nef is incorporated with equivalent efficiency into HIV-1 and MuLV virions (61). The significance of PR-mediated Nef cleavage also remains undefined. In fact, preventing Nef cleavage does not alter its ability to stimulate virus infectivity (437). Recent results suggest that Nef localizes to the viral core (348).

A summary of the major proposed functions of the HIV-1 accessory proteins is presented in Table 3.

Protein	Major proposed functions	Comments
Vif	Enhancement of virus infectivity	Phenotype producer cell-dependent Incorporated at low levels in virions
Vpr	Cell cycle arrest Nuclear import	Incorporated at high levels in virions
Vpx	Nuclear import	Incorporated at high levels in virions Present in HIV-2/SIV_{sm}/SIV_{mac} only
Vpu	Increased particle release CD4 degradation	Not detected in virions Present in HIV-1 only
Nef	CD4 and MHC I down-regulation Enhancement of virus infectivity Increased virus load in vivo Altered cellular activation pathways	Incorporated at low levels in virions

MHC, major histocompatibility complex; HIV, human immunodeficiency virus; SIV, simian immunodeficiency complex.

TABLE 3. *HIV/SIV accessory proteins*

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CHAPTER 60 Pathogenesis and Medical Aspects of HIV-1 Infection

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HISTORY

In June, 1981, the highly unusual occurrence of *Pneumocystis carinii* pneumonia (PCP) in five young homosexual men from Los Angeles was reported to the Centers for Disease Control (CDC) (14). Other unusual cases of diseases indicative of cellular immunodeficiency that occurred in young, previously healthy homosexual men were described soon thereafter (15,205,343,505). Initially, more than 90% of these cases occurred in homosexual or bisexual men, and the term “gay-related immunodeficiency” (GRID) was coined. However, by 1982, hundreds of similar cases of an acquired immunodeficiency syndrome (AIDS) had been reported, not only in homosexual and bisexual men, but also in intravenous drug users, hemophiliacs, blood-transfusion recipients, heterosexual adults from the Caribbean and Central Africa, sex partners of affected patients, and infants born to mothers with the syndrome.

The increase in the incidence of Kaposi's sarcoma (KS) in young gay men after 1979 argued that AIDS was an emerging new syndrome. In addition, sharp increases in the number of requests to the CDC for pentamidine isethionate (a second-line agent for the treatment of PCP) after 1979 further suggested that AIDS was an emerging, new disease entity (501).

An infectious etiology as the cause of AIDS was suggested by geographic clustering of cases, links among cases by sexual contact, mother-to-infant transmission, and transmission by blood transfusion. The depletion in numbers and function of T-helper cells (11) suggested that a lymphotropic agent might be responsible. The prominent immunosuppression that characterized AIDS recalled certain retroviral infections in animals. Human T-cell leukemia virus type 1 (HTLV-I) was known to be tropic for T-helper cells, and was an early candidate in the search for the cause of AIDS (147,179,186). A human T-lymphotropic retrovirus that was immunologically related to HTLV-I was detected in lymph node tissue from a French patient who was at risk for AIDS (32). This virus, later named “lymphadenopathy-associated virus,” or LAV, proved identical or highly related to the virus subsequently isolated at the National Cancer Institute, termed HTLV-III (177,448), and in San Francisco, termed AIDS-associated retrovirus (ARV) (316). These viruses were subsequently cloned and characterized (5,503), and are now known as human immunodeficiency virus type 1 (HIV-1). Molecular and seroepidemiologic studies have consistently demonstrated a near-perfect correlation between infection with HIV-1 and subsequent development of AIDS (194,195 and 196,222). All known HIV-1 sequences are closely related to simian immunodeficiency virus (SIV) sequences harbored by the *Pan troglodytes troglodytes* subspecies of

chimpanzees (182). In fact, zoonotic transmission of retroviruses from chimpanzees to humans is the most plausible explanation for the genesis of the HIV epidemic (182).

Scientific advances in understanding the pathogenesis of HIV infection have proceeded with unprecedented speed. Identification of HIV-1 as the cause of AIDS immediately implicated the viral reverse transcriptase (RT) as a therapeutic target. Azidothymidine (zidovudine), a nucleoside analog RT inhibitor, was shown to delay the onset of AIDS and prolong life in HIV-infected patients with advanced immunodeficiency. Zidovudine was licensed for use in HIV-1 infection in 1987; since that time, 17 additional antiretroviral agents have been approved by the United States Food and Drug Administration.

The discovery of the crystal structure of the HIV-1 protease (393,585) led to the rational design of protease inhibitors, which have revolutionized antiretroviral therapy. The early recognition that the HIV envelope binds CD4 with high affinity and uses this molecule for cell entry (118,287,349) was followed years later by the discovery of coreceptors that are necessary in addition to CD4 for HIV entry and fusion (reviewed in reference 324). Antiretroviral strategies that target this important stage of the viral replicative cycle are currently in clinical trials and appear promising. Many key HIV pathogenesis–related discoveries have elucidated the immunologic mechanisms that regulate HIV replication (158). Manipulation of the immune system as an adjunct to antiretroviral therapy in the long-term suppression of viral replication is in the early stages of development and will likely be an increasingly important tool in efforts to regenerate immune competence.

In spite of the rapid scientific advances that have been made, the brisk pace of the HIV/AIDS epidemic has continued largely unchecked in most parts of the world. Prevention efforts and the availability of antiretroviral therapies have blunted the expansion of the epidemic in the United States and Western Europe; however, expansion of the epidemic continues in sub-Saharan Africa and Southeast Asia, and is now burgeoning in India and in the former Soviet republics. Approximately 300 cases of AIDS had been reported by the end of 1982; by the end of 2000, nearly 22 million individuals had died of AIDS, and 36 million were living with HIV-1 infection worldwide.

INFECTIOUS AGENT

HIV is a member of the *Retroviridae* family, which is characterized by a unique enzyme, RT, which allows the virus to copy its RNA into double-stranded DNA to replicate. Within the *Retroviridae* family, HIV is classified as a lentivirus. The three major coding regions for HIV and all retroviruses encode core (*gag*), polymerase (*pol*), and envelope (*env*) gene products (Fig. 1). The HIV genome additionally encodes several other proteins, initially termed “accessory” proteins (i.e., Tat, Rev, Nef, Vif, Vpr, and Vpu), that may play key roles in the pathogenesis of HIV infection (see Chapter 59); compared with most retroviruses, the HIV-1 genome encodes a large number of proteins (Fig. 2).

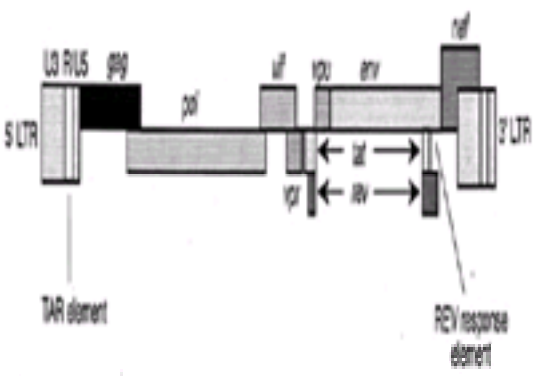

FIG. 1. Genomic organization of human immunodeficiency virus (HIV)-1. Characteristic retroviral elements include the long terminal repeats (LTRs) as well as genes encoding the core proteins (*gag*), reverse transcriptase (*pol*), and the envelope glycoproteins (*env*). Also indicated are genes encoding accessory proteins that are multifunctional and important in the pathogenesis of HIV infection.

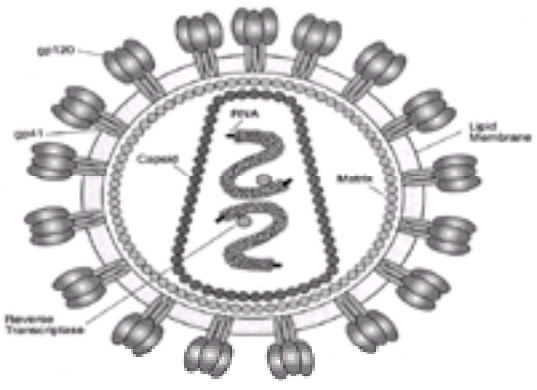

FIG. 2. Organization of a mature human immunodeficiency virus (HIV)-1 virion. Envelope glycoproteins project from the outer lipid membrane. Two copies of the HIV genome are contained within the core.

PROPAGATION AND ASSAY IN CELL CULTURE

A variety of suitable target cells are used for the propagation of HIV-1, including phytohemagglutinin (PHA) or anti-CD3 stimulated primary peripheral blood mononuclear cells (PBMCs), and CD4+ T-cell lines such as CEM, MT-2, HUT78, Jurkat, SupT1, and U937. The kinetics of viral replication vary depending on the input strain of virus and the characteristics of the target cells. Many methods are available to detect virus replication. The most common methods include detection of the viral core p24 antigen in culture supernatants by enzyme immunoassay (EIA) and detection of RT activity in supernatants. Both of these methods detect viral particle production and do not yield information regarding viral infectivity. The RT assay is less sensitive and more labor intensive than the p24 assay; however, the RT assay is considerably less expensive. Assessment of the median tissue culture infectious dose ($TCID_{50}$) is highly labor intensive, but yields quantitative information on infectious virus. Syncytium formation in a cell line such as MT-2 indicates the presence of a syncytium-inducing (SI) strain of HIV; however, this assay is not quantitative and cannot detect non-SI (NSI) strains. The polymerase chain reaction (PCR) is the most sensitive technique available for the detection of HIV. PCR assay for proviral DNA can detect one infected cell per million. Detection of as few as several hundred copies of intracellular HIV RNA can be detected from millions of cells by RT-PCR, and this technique can also detect tens of copies of viral RNA in culture supernatants. Disadvantages of PCR include its expense and labor intensiveness; in addition, because the technique is so sensitive, a great deal of caution must be exercised to avoid contamination.

CYTOPATHIC EFFECTS

The first biologic characteristics ascribed to HIV were its SI and cytopathic effects *in vitro* (Fig. 3) (177, 316,448,503). Multiple mechanisms of cell death appear to be operative after infection of a CD4+ T cell with HIV. A number of different lines of evidence suggest that single-cell killing due to direct infection with HIV is not the predominant mechanism of CD4+ T-cell depletion *in vivo* (see later). In fact, it is uncertain whether HIV has any cytopathic properties *in vivo.* Syncytium formation has been observed only rarely *in vivo* (167,452), and some target cells of HIV infection (i.e., cells of the monocyte/macrophage lineage) appear to be highly resistant to virus-induced cytopathic effects (441).

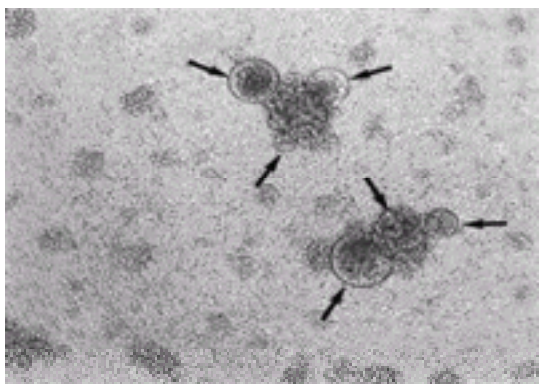

FIG. 3. Typical cytopathic effect of human immunodeficiency virus (HIV)-1 in cell culture; syncytium formation with multinucleated giant cells is shown in a culture of CD4+ T cells.

ACUTE INFECTION

HIV is transmitted either by exposure of the oral, rectal, or vaginal mucosa during sex or breast feeding, or by intravascular inoculation (i.e., through transfusion of contaminated blood products, use of contaminated equipment during injection drug use, or maternal–fetal circulation). Although the earliest events during primary infection may vary depending on the route of exposure, there are no obvious differences in disease manifestations in individuals infected by mucosal versus blood-borne routes. This suggests that the earliest phase of acute infection leads to a common pathophysiologic pathway that is independent of the route of transmission.

Virus that enters directly into the bloodstream is probably cleared from the circulation and is delivered to lymphoid tissue in the spleen, liver, and lungs. Atraumatic application of SIV to tonsils of macaques results in rapid infection of oral mucosa–associated lymphoid tissue, and subsequent dissemination to other lymphoid organs (518). In an animal model of sexual transmission, macaques were exposed to SIV intravaginally; bone marrow–derived dendritic cells (DCs) in the vaginal mucosa were the first cells to contain SIV DNA, which became detectable 2 days after exposure. In subsequent examinations of lymphoid organs, the pattern of appearance and spread of SIV mirrored the course taken by DC on migrating from peripheral tissues to lymphoid organs (516). In addition to serving as direct targets of HIV infection, DCs also are capable of retaining infectious virions on their surface for extended periods (572). Thus their role in the initiation of HIV infection likely includes capturing virions at sites of entry, carrying HIV to the paracortical regions of lymphoid organs, and delivering virus to CD4+ T cells that become activated through their interaction with DCs (Fig. 4). Active viral replication occurs in DC–CD4+ T cell conjugates that are formed *in vitro* (446); similar conjugates of DC and CD4+ T cells containing HIV antigen have been identified *in vivo* in tonsil biopsy specimens from individuals infected with HIV (167), in the peripheral blood in low quantities (572), and in the submucosal tissue after vaginal exposure to SIV (516). Further data from the SIV model suggest that one of the earliest steps of the acute infection process that is necessary for viral dissemination is amplification of infection in macrophages (248).

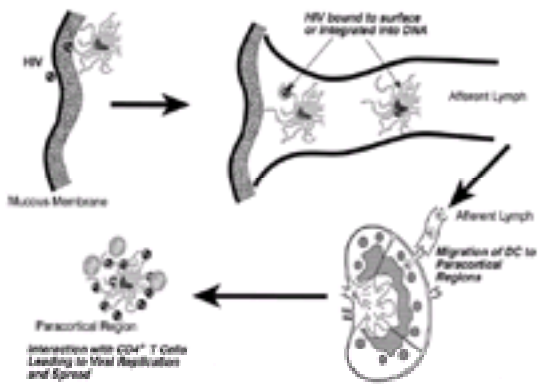

FIG. 4. Role of dendritic cells (DCs) in the initial stages of human immunodeficiency virus (HIV) infection. DCs at the site of HIV exposure transport the virus to paracortical regions of draining lymph nodes, where CD4+ T cells become infected. Reprinted from Weissman D, Fauci AS. Role of dendritic cells in immunopathogenesis of human immunodeficiency virus infection. *Clin Microbiol Rev* 1997;10:358, with permission.

CD4+ T cells and macrophages are key targets of HIV infection. HIV cellular tropism is described in terms of the ability of viral isolates to grow in macrophages or transformed T-cell lines *in vitro.* Isolates that can grow well in macrophages and PBMCs but much less efficiently in T-cell lines are classified as macrophage (M)-tropic, whereas isolates that can grow well in PBMCs and T-cell lines but much less efficiently in macrophages are called T-tropic (185); isolates that can grow well in both macrophages and T-cell lines are referred to as dual tropic. In addition, certain HIV isolates are termed SI, because of their ability to induce giant cell, or syncytium formation in certain T-cell lines *in vitro,* whereas others that lack this ability are referred to as NSI. Considerable but incomplete overlap exists between T-tropism and the SI phenotype and between M-tropism and the NSI phenotype. The viral determinant of cellular tropism maps to the gp120 envelope protein of HIV-1, mostly to the third variable region (V3 loop) (79,262,532,598) that is also the major determinant for coreceptor use (105); most M-tropic strains of HIV use CCR5 as a coreceptor (i.e., R5 strains), whereas most T-tropic strains use CXCR4 (i.e., X4 strains).

Changes in viral phenotype have been observed at different stages of HIV infection. CCR5-using viruses are preferentially transmitted during primary infection and predominate at most stages of infection (612,616). In approximately 50% of HIV-infected individuals, the appearance of CXCR4-using isolates late in the course of disease heralds accelerated $CD4^+$ T-cell decline and clinical disease progression (78,298,538,539). This transition may occur by mutation of only a few amino acid residues, predominantly in the envelope V3 loop (40,79,164,532). Given the high error rate of RT and the rapid kinetics of HIV replication, the surprising failure of such mutants to emerge until late in the course of the disease process indicates a selective advantage of such a mutation during the course of disease progression. The nature of such a selective advantage is currently unknown; however, it may relate to the ability of X4 viruses to gain cellular entry through an expanded repertoire of coreceptor molecules.

The mechanisms responsible for the restriction of R5 viruses during primary infection is the subject of intense investigation. Freshly isolated Langerhans cells (resembling mucosal DCs) express CCR5, but not CXCR4, on their surfaces (604); this finding suggests that infection of DCs may be a necessary first step in acute infection, and that this step is restricted by the expression pattern of HIV coreceptors on DCs. However, HIV-pulsed DCs from individuals lacking a functional CCR5 gene are capable of transmitting infection to CD4+ T cells *in vitro* (140). Thus, although infection of DCs may be dependent on specific HIV coreceptor expression, the ability of DCs to trap virions on their surfaces and infect CD4+ T cells that they encounter is, at least in part, a coreceptor-independent phenomenon.

In animal models of SIV infection, virus can be detected in peripheral lymph nodes within 1 week of infection (73,457,516). Most of the SIV RNA at this time is associated with individual productively infected cells in lymph nodes. This production of virus by individually infected cells in lymphoid tissue precedes and likely is responsible for the peak of antigenemia. Coincident with the appearance of SIV-specific immune responses, a decrease in the frequency of productively infected cells is seen during the second week after infection. By 4 weeks after infection, antigenemia decreases as a result of further elimination of productively infected cells by SIV-specific cell-mediated immune responses and clearance of circulating virions by the formation of immune complexes consisting of virus, antibody, and complement that are trapped within lymphoid germinal centers (73,457).

Cross-sectional studies in patients evaluated soon after acute HIV infection are similar to the findings in the SIV model (Fig. 5). The kinetics of HIV viremia and HIV-specific immune responses in peripheral blood parallel those in the SIV model (45,97,117,301). Preliminary data from a limited number of lymph node biopsies obtained from acutely infected individuals suggest that the SIV model is valid in depicting the early events associated with HIV infection in lymphoid tissue as well (415). In the weeks to months after acute HIV infection, a sharp decline in the frequency of productively infected cells is evident in lymphoid tissue. Germinal center formation within lymphoid follicles becomes pronounced, and viral RNA corresponding to extracellular virions complexed with antibody and complement is detected in the network of follicular dendritic cell (FDC) processes (165,269,419,515). Although the immune system is capable of efficient elimination of a substantial number of productively infected cells in lymphoid tissue, the very earliest interactions between virus and host virtually ensure viral persistence. In this regard, massive numbers of virions trapped within the germinal center FDC network as well as latently infected cells, which harbor proviral DNA but do not express viral proteins (and thus elude the immune response), represent potentially continuous sources of virus for *de novo* infection of CD4+ T lymphocytes that are resident in or migrating through lymphoid tissue (241,261).

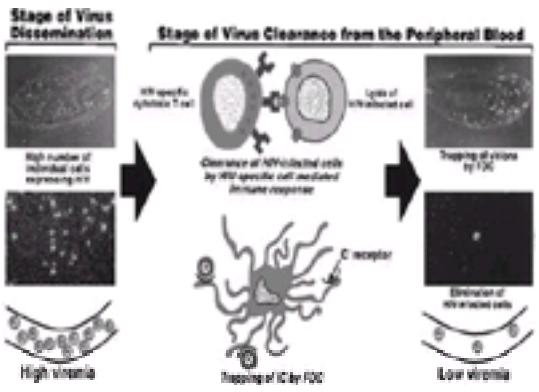

FIG. 5. Cell-mediated and humoral immune mechanisms involved in the down-regulation of viremia after primary human immunodeficiency virus (HIV) infection. Before the emergence of HIV-specific immune responses, large numbers of individual productively infected cells are found in lymph nodes (**left**; low- and high-power photomicrographs of an *in situ* hybridization assay for HIV RNA in a lymph node), associated with high levels of plasma viremia. Emergence of cytotoxic T-cell responses against HIV leads to elimination of a large number of productively infected cells (photomicrograph, **lower right**). In addition, HIV-specific antibodies combine with virions to form immune complexes (*IC*); complement (*C'*) binds to the immune complexes, which are then trapped in the follicular dendritic cell (*FDC*) network of expanding germinal centers by complement receptors (photomicrograph, **upper right**). Adapted from ref. 420, with permission.

VIRAL DYNAMICS

Measures of viral load, including plasma viremia, remain relatively constant in a given patient over a long period (111,432). This steady state is due to an equilibrium between production and clearance of virus. Perturbation of the viral load steady state with highly active antiretroviral therapy allows estimation of the *in vivo* dynamics of HIV infection. Because antiretroviral agents block *de novo* infection of cells, frequent measurement of plasma viremia after administration of these agents yields a decay curve that reflects the clearance rate of virions from plasma as well as the elimination rate of HIV-producing cells. Using different drugs, studies from two groups found the half-life of plasma virus to be approximately 2 days (251,568). In a follow-up study that included more frequent measurements of plasma viremia after drug administration, Perelson et al. (429) estimated the half-life of plasma virus to be 5.8 hours. Rates of virion clearance can also be estimated by acutely removing plasma virions by apheresis and measuring the return of plasma HIV RNA back to baseline. With this approach, the half-life of plasma virus was estimated to be 28 to 110 minutes. Because virus production is proportional to the number of productively infected cells and the number of virions produced per cell, these data imply that the steady-state level of plasma viremia is fueled by rapid and continuous infection of target cells. The average production of HIV virions was estimated to be nearly 10^{10} to 10^{11} virions per day, the average generation time of HIV *in vivo* (i.e., the time from virion release from an infected cell to the time of virus production from a cell infected by the first virion) was approximately 2.6 days, and the half-life of productively infected cells was about 1.6 days (429,453). Although a substantial number of assumptions are built into the mathematical formulae used to derive these estimates, the clear conclusion of these data is that the steady-state level of plasma viremia is dependent on viral replication from a pool of newly infected cells that is rapidly turning over.

VIRAL DISSEMINATION

Like many other invading microorganisms, HIV is transported by antigen-presenting cells to lymphoid tissue. However, in the case of HIV, the host immune system is never able to control and eradicate the infection completely. In an impressive example of subversion of the immune system, HIV uses the activated milieu of lymphoid tissue to its own replicative advantage. Close contact between immune effector cells and high levels of proinflammatory cytokines are characteristic of the lymphoid tissue microenvironment; these conditions favor viral replication in several ways. Activated CD4+ T lymphocytes migrating through lymphoid tissue serve as ideal targets for *de novo* infection with HIV (261,328,339). Activation signals such as those delivered by proinflammatory cytokines, found in abundance within activated lymph nodes, are potent inducers of HIV replication in latently infected cells (53,162,390,527,599) and also are able to increase the pool of activated cells that are susceptible to HIV infection (399,521,523,571). Another example of the ability of HIV to subvert the immune system for its own replicative advantage is the immune hyperactivation induced by the virus itself (408,493). Sequestration of HIV-infected cells within lymphoid tissue due to histopathologic abnormalities and cytokine imbalances (468,579) may further contribute to the high levels of viral replication that occur in lymphoid tissue. Viral replication that occurs in lymphoid tissue before the development of a host immune response is responsible for wide dissemination of virus by lymphatic and hematogenous routes.

Viral dissemination throughout lymphoid tissue is a fundamental pathogenic event during the early phase of HIV infection, and lymphoid tissue remains the most important reservoir of infection throughout the entire course of the disease. In this regard, the frequency of infected cells in lymph nodes exceeds that in peripheral blood by fivefold to 10-fold; differences in levels of viral replication are generally 10- to 100-fold (109,418,419). Up to 25% of CD4+ T lymphocytes present in lymph node germinal centers harbor HIV DNA, further emphasizing the role of lymphoid tissue as a critical reservoir for HIV *in vivo* (142). The continuous state of rapid high-level turnover of plasma viremia derives in large measure from viral replication in lymphoid tissue (67,111,305).

The high concentration of virus in lymphoid tissue is due in part to the normal process of follicular hyperplasia within lymphoid germinal centers after antigenic challenge (41,165,452). The expansion of the FDC network within hyperplastic lymphoid follicles is an efficient mechanism for viral trapping through interactions between antibody and complement-coated virions with the complement receptor molecules C3b and C3d on the surface of FDCs. In addition to this mechanical process, immunologic mechanisms may play a major role in maintaining the viral reservoir in lymphoid

tissue.

During the course of HIV disease progression, there is a shift in the lymphoid histopathologic pattern from follicular hyperplasia to follicular involution (Fig. 6) (419,452). This shift in histopathology is associated with important changes in viral distribution. Disruption of the FDC network is characteristic during this period, leading to reduced trapping of virus in germinal centers and a consequent increase in plasma viremia (419,420). Sequestration of infected cells within lymphoid tissue also becomes less efficient during follicular involution, leading to increased numbers of infected cells in the peripheral blood; as the CD4+ T lymphocyte count decreases to less than 200 cells/μL, there is a tendency for viral load to increase more rapidly in the peripheral blood compartment, leading to equilibration between lymph node and peripheral blood.

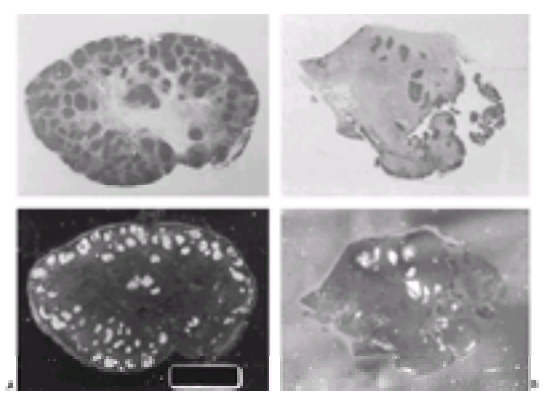

FIG. 6. Changes in lymphoid tissue histopathology and patterns of viral distribution associated with human immunodeficiency virus (HIV) disease progression. In early-stage disease **(A)**, hematoxylin and eosin staining **(top)** reveals florid follicular hyperplasia; *in situ* hybridization for HIV RNA **(bottom)** reveals heavy deposition of HIV in the hyperplastic germinal centers. In intermediate- to late-stage disease **(B)**, loss of normal lymphoid architecture results in follicular involution **(top)**, with consequent loss of HIV trapping capacity **(bottom)**. Reprinted from Cohen O, Pantaleo G, Lam G, Fauci AS. Studies on lymphoid tissue from HIV-infected individuals: implications for the design of therapeutic strategies. *Springer Semin Immunopathol* 1997;18:305, with permission.

Destruction of lymphoid tissue certainly is a major mechanism responsible for the severe immune dysfunction and loss of the ability to inhibit viral replication observed in advanced-stage HIV disease. The ability to maintain an effective immune response to HIV is severely impaired in the absence of intact lymphoid tissue architecture. As a consequence, increased cell-associated viral RNA becomes evident in the paracortical regions of lymph nodes, reflecting increased viral replication. Thus, during progression of HIV disease, there is a reversal in the predominant forms of virus in lymph nodes, with progressive diminution of the extracellular form (i.e., trapped virus) and an increase in cell-associated virus (i.e., cells expressing HIV RNA) (419,420). In the advanced stage of disease, there is almost total dissolution of lymphoid architecture. Follicular involution, fibrosis, frank lymphocyte depletion, and fatty infiltration herald complete loss of functional lymphoid tissue, contributing to the state of immunodeficiency and the dramatically enhanced susceptibility to opportunistic infections.

Disruption of the lymphoid microenvironment during the course of HIV infection remains an enigmatic process with considerable implications for future therapeutic interventions. Productive infection of FDCs by HIV may occur, particularly in the late stages of HIV infection (517); however, the majority of data suggest that productive infection of FDCs is rare during the intermediate stage of disease when dissolution of the FDC network begins (170,486). Direct toxicity of viral gene products and/or factors such as tumor necrosis factor (TNF)-a, nitric oxide, and matrix metalloproteinases may contribute to loss of FDC network integrity (433,567). Several viral gene products can disrupt normal intracellular signaling events (see later), although these effects have been studied largely in CD4+ T cells, and little is known regarding the normal physiology of FDCs and their interactions with HIV proteins. Down-regulation of major histocompatibility complex (MHC) class I expression by viral gene products (254,279,496) may interfere with normal cell–cell interactions in the lymphoid microenvironment. Depletion of CD4+ T cells during the course of HIV disease could also lead to withdrawal of a trophic factor necessary for FDC survival. Of note, some of the HIV-related pathologic changes that occur in lymphoid tissue may be at least partly reversible during potent antiretroviral therapy (614).

Lymphoid tissue is not the exclusive site of HIV infection. Virtually any cell that expresses the CD4 molecule together with an appropriate coreceptor molecule can potentially be infected with HIV. In addition, HIV has been reported to infect *in vitro* a wide range of primary cells and cell lines that may or may not express CD4 or HIV coreceptors (315). These include FDCs, microglial cells, megakaryocytes, eosinophils, CD8+ T cells, B cells, natural killer (NK) cells, thymus and bone marrow stromal cells, astrocytes, oligodendrocytes, renal epithelial cells, cervical cells, trophoblastic cells, rectal and bowel mucosal cells, and parenchymal cells from a variety of organs such as heart, muscle, liver, lung, salivary gland, eye, testis, prostate, and adrenal gland. Infection of at least some of these cell types likely occurs *in vivo.* The consequences of infection of various organs may include pathologic sequelae, such as dilated cardiomyopathy (29) and gastroenteritis (300); in addition, some sites of tissue infection may represent important reservoirs of HIV infection, including not only lymphoid tissue (420), but also the central nervous system (CNS), genital tract, and other sites (495).

IMMUNE RESPONSES

Similar to most pathogens, HIV induces a broad array of host immune responses in an infected individual (Fig. 7). How HIV is able to replicate continuously and cause the inexorable decline in immune system function in the presence of these immune responses is a central question of pathogenesis that has profound implications for the development of effective immunotherapies and vaccines. Some evidence suggests that particular immune responses to HIV may actually

be harmful to the host (20,464,590). A larger body of evidence, however, supports a salutary role for most HIV-specific immune responses. The profound decrease in plasma viremia after acute HIV infection is associated with the appearance of HIV-specific cellular (45,301) and humoral (97) immune responses. The correlation of slow progression with vigorous HIV-specific immune responses also argues strongly in favor of a salutary role for these responses (108).

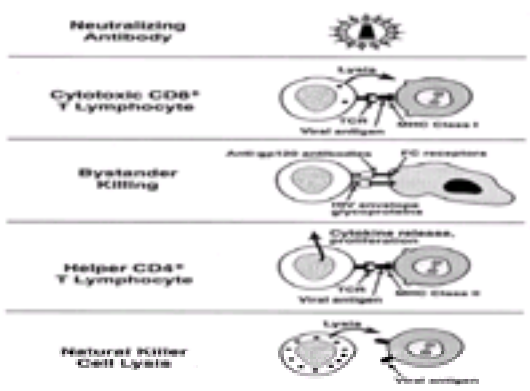

FIG. 7. Immune responses against human immunodeficiency virus (HIV). Neutralizing antibodies bind to virion components and prevent attachment to target cells. CD8+ cytotoxic T lymphocytes recognize viral antigens on the surface of infected cells in the context of major histocompatibility complex (MHC) class I presentation, and are able to lyse these cells directly. Antibody-dependent cell-mediated cytotoxicity (ADCC) results in Fc receptor–mediated elimination of infected cells as well as uninfected cells that are coated with HIV antigen–antibody complexes. CD4+ T cells may recognize viral antigens in the context of MHC class II presentation, resulting in the release of cytokines and cellular proliferation. Natural killer (NK) cells may also directly lyse HIV-infected cells. Adapted from Fauci AS, Lane HC. Human immunodeficiency virus (HIV) disease: AIDS and related disorders. In: Fauci A, Braunwald E, Isselbacher KJ, et al., eds. *Harrison's principles of internal medicine.* 14th ed. New York: McGraw-Hill, 1998:1791, with permission.

Humoral Immune Responses

Antibodies against the viral core protein p24 develop within weeks of acute HIV infection and may play a role in the decline of plasma viremia associated with primary infection. Loss of anti-p24 antibodies is associated with progression of HIV disease (566).

Antibodies that neutralize HIV infectivity may be responsible for at least partial control of viral replication *in vivo* (499,566). eutralizing antibodies may be type specific (i.e., specific for one viral isolate) or group specific (i.e., specific for a broad range of viral isolates). Most type-specific neutralizing antibodies recognize the V3 region of the HIV envelope gp120 protein (206). Neutralizing anti-V3 loop antibodies may prevent conformational changes within gp120 necessary for HIV entry or for cell–cell fusion (168). Group-specific neutralizing antibodies recognize epitopes within the HIV envelope gp41 protein (389), discontinuous conformational epitopes around the CD4 binding site of gp120 (525), or carbohydrate determinants (232). Both type- and group-specific neutralizing antibodies are more efficient in neutralizing laboratory strains of HIV grown in T-cell lines compared with primary isolates grown in PBMCs; this is likely due to differential exposure of the V3 loop and CD4-binding domain epitopes among these isolates (48,529). Some neutralizing antibodies interfere with the interaction between the HIV envelope and CCR5, thereby inhibiting cellular entry of M-tropic strains of HIV (542,591).

Neutralizing antibodies appear to be prognostically relevant to the course of HIV infection (377,434,500). The regularity with which viral variants emerge that resist neutralization suggests that such antibodies are potent impediments to viral replication (3). In nonhuman primate models, neutralizing antibodies are associated with slow rates of disease progression (153) and accelerate clearance of both infectious and noninfectious virions (263); furthermore, passive transfer of SHIV-neutralizing antibodies can protect macaques against a subsequent viral challenge (504).

Some anti-HIV antibodies bind to immunoglobulin G (IgG) Fc receptor–positive cells and sensitize them to mediate antibody-dependent cell-mediated cytotoxicity (ADCC) against HIV-infected or HIV-coated cells (329,403). Most of these antibodies are directed against HIV envelope gp120 or gp41 proteins. $CD16^{+}$ NK cells are important mediators of ADCC (543), and monocytes also may mediate this activity (267). Anti-HIV ADCC antibodies develop soon after primary infection and are detectable throughout the course of HIV disease, with some decrease in titers with the onset of AIDS (323). ADCC also may represent an immunopathogenic immune response that may be responsible for CD4+ T-cell depletion during the course of HIV infection. In this regard, a combination of high levels of plasma viremia with high HIV envelope-specific ADCC activity is correlated with rapid CD4+ T-cell depletion; this correlation may reflect ADCC-mediated “innocent bystander” killing of gp120-coated, but uninfected, CD4+ T cells (508).

Cellular Immune Responses

In HIV-infected individuals, classic MHC class I–restricted, HIV-specific, CD8+ cytotoxic T lymphocyte (CTL) responses have been demonstrated (437,559) against a variety of HIV target proteins including core proteins, RT, envelope proteins, and regulatory proteins. With standard cell-culture methods, a high frequency of HIV-specific CTLs and CTL precursors (up to 1% of peripheral blood T cells) has been observed in asymptomatic HIV-infected patients (252,381); with HLA–peptide tetramers, the frequency of HIV-specific CTLs may be up to an order of magnitude greater than that detected by standard methods (6,401).

A critical role for CTLs in the suppression of viral replication in HIV-infected individuals is suggested by the close correlation between emergence of an HIV-specific CTL response and down-regulation of viremia after acute infection

(45,301,388), the association of vigorous HIV-specific CTL responses with slow progression of HIV disease (289,401,422,461), and the decline in HIV-specific CTL activity with disease progression (252,559). Quantitative studies have revealed an inverse correlation between the frequency of HIV-specific CTLs and levels of plasma viral load (388,401), and between the CTL frequency and the rate of CD4+ T-cell decline (388). A beneficial role for HIV-specific CTLs also is suggested by their presence in the peripheral blood of individuals who are frequently exposed to HIV yet remain uninfected (470,471), and the depletion of HIV-specific CTLs from lymph nodes of patients with advanced HIV disease (536). Finally, studies in nonhuman primates have demonstrated that monkeys subjected to CD8+ T-cell depletion manifest diminished control of plasma viremia during primary SIV infection (487); in addition, CD8+ T-cell depletion during the chronic phase of SIV infection leads to a prompt and profound increase in levels of viral replication (268,345,487).

The quality of HIV-specific CTL response also is an important determinant of the efficacy of these responses in controlling viral replication. The specificity of CTL responses may in part determine their salutary role; in this regard, CTL responses against viral core proteins in particular have been associated with a decreased risk of disease progression (289,401,463). CTL recognition of immunodominant HIV epitopes presented by certain MHC class I alleles may result in potent anti-HIV activity (208) and may in part explain the association of certain MHC class I alleles with slower progression of HIV disease (63,275,276,288). Furthermore, the ability to recruit an HIV-specific CTL response comprising a diverse group of T cell–receptor Vb families that recognize multiple epitopes is associated with better control of viral replication and an improved prognosis compared with a narrow CTL response (238,416).

The loss of HIV-specific CTL activity in patients with progressive disease is likely a result of several factors. Viral proteins such as Tat, Nef, and Vpu can down-regulate cellular expression of MHC class I molecules that are necessary for CTL recognition of infected cells (254,279,496). Increased expression of killer inhibitory receptors also may inhibit CTL activity (131). Another mechanism responsible for the loss of CTL activity is the selective accumulation of CD8+DR $^{+}$ HIV-specific CTLs that lack the interleukin (IL)-2 receptor and are defective in clonogenic potential (421). Finally, the ability of HIV to escape CTL responses by viral mutation or by exhaustion of CTL clones due to high concentration of antigen may explain the loss of CTL-mediated control over viral replication (352,423).

The host CTL response against HIV is constrained by the ability of the MHC class I alleles to bind to various viral epitopes, while the virus is constrained by the degree to which an escape mutation impairs viral fitness (227,271,586). These host–virus dynamics are extraordinarily complex, given the large number of permutations of viral epitopes and MHC class I alleles. Viral mutations within CTL recognition epitopes (i.e., “escape mutants”) are associated with increased levels of viral replication and progression of HIV disease (46,209,293,431). Viral escape mutants may thrive because of the release of CTL control over their replication, and also may inhibit CTL responses against the preescape viral epitope (291,292). A mathematical model of CTL–virus dynamics was provided by Nowak et al. (398), who described disease progression as a result of viral sequence variation that escapes an immunodominant CTL response and shifts the host response toward a weaker epitope. In this scenario, disease progression may be the result of fitness of viral escape mutants outpacing the plasticity of the host CTL response, with slow progression resulting from CTL plasticity overpowering viral escape mutants with limited fitness.

A further mechanism for loss of CTL control over viral replication may be clonal exhaustion. This appears to be a strategy for viral persistence used by other viruses, such as certain strains of lymphocytic choriomeningitis virus, that rapidly and completely mobilize the host CTL response, resulting in CTL exhaustion (i.e., high zone tolerance) (380). CTL exhaustion also may occur to some degree in HIV infection: the disappearance of some CTL clonotypes can be demonstrated in the absence of viral escape mutations that might otherwise explain the phenomenon (352,423).

CD8 Soluble Suppressor Factors

A variety of soluble antiviral factors are elaborated by CD8+ T cells. CD8 antiviral factor (CAF) (560) is noncytolytic, inhibits viral replication at the level of HIV long terminal repeat (LTR) transcription (76), and lacks identity to known cytokines (334). Although CAF activity is non-MHC restricted, its activity is maximal in a syngeneic system (333). CAF activity decreases with disease progression, but remains potent in long-term nonprogressors (31,335).

The combinations of RANTES, MIP-1a, and MIP-1b (the natural ligands for CCR5, a major coreceptor for M-tropic strains of HIV) also are important soluble antiviral factors, and are secreted by CD8+ T cells and other cell types (104,284). These CC-chemokines inhibit viral replication primarily at the level of cell entry. Emerging data support a role for CC-chemokines in protecting against HIV infection and disease progression (171,475,546,601); interestingly, the source of these chemokines does not appear to be exclusively CD8+ T cells, and likely includes CD4+ T cells (171,284,475) and NK cells (404). Macrophage-derived chemokine (MDC) can potently suppress replication of M- as well as T-tropic strains of HIV *in vitro* (412); the role of MDC in controlling HIV replication *in vivo* is uncertain. Finally, IL-16 has been reported to be a soluble antiviral factor (27). IL-16 inhibits HIV transcription and can potently suppress HIV replication when it is expressed in HIV-infected T cells (332,553); furthermore, IL-16 production has been correlated with protection against disease progression in HIV-infected individuals (10,480).

CD4+ T-Cell Responses

HIV proteins contain helper T-cell epitopes that may be presented by MHC class II alleles (68,102). Recognition of these epitopes by CD4+ T cells results in secretion of cytokines and cellular proliferation. These responses may be associated with abortive infection in individuals who are exposed to HIV but who remain uninfected (99), and they decrease with HIV disease progression (68,102).

Studies of other viral infections indicate a central role for T-helper responses in the maintenance of CTL responses. CTLs that persist in the absence of adequate CD4+ T-cell help may be ineffective *in vivo,* as has been demonstrated in the lymphocytic choriomeningitis virus (LCMV) system (605); a similar scenario may occur in HIV infection as well (238).

CD4+ T-helper responses also may be quite important in HIV-specific immunity. In this regard, an inverse correlation has been found between the magnitude of HIV-specific CD4+ T-cell response and levels of plasma viremia (466); these data are consistent with the observed inverse correlation between CTL activity and plasma viremia as well (401). The fate of HIV-specific CD4+ T cells during the course of HIV infection is controversial. Some evidence suggests that these cells are usually depleted in the early stages of disease, and that initiation of potent antiretroviral therapy during acute HIV infection may prevent this loss and lead to more effective control of viral replication (466). Other data suggest that HIV-specific CD4+ T cells are present throughout the course of HIV disease, and that their frequency actually declines during HAART-induced viral suppression (435). Therapeutic strategies currently under consideration include the use of soluble factors that bypass the need for CD4+ T-cell help in maintaining CTL responses.

CYTOKINES AND HIV DISEASE: DYSREGULATION OF CYTOKINE PRODUCTION

Cytokines that are generated during the host immune response to an infection play an important role in determining the outcome of the infectious process; in mice that are susceptible to *Leishmania major* infection, production of the TH2 cytokine IL-4 predominates, whereas the TH1 cytokine interferon (IFN)-g is produced by mice that are resistant to progressive infection (242). Dysregulation of the host cytokine network is characteristic during all phases of HIV infection. Many of the observed alterations in cytokine production contribute to HIV pathogenesis by further stimulating viral replication, suppressing the ability of the immune system to mount an efficient antiviral response, and inducing cytokine-mediated cytopathic effects (108, 150, 152).

HIV infection is associated with increased expression of proinflammatory cytokines; high levels of TNF-a, IL-1b, and IL-6 are secreted by PBMCs and macrophages from HIV-infected subjects, and are found at elevated levels in serum, cerebrospinal fluid, and tissues (reviewed in references 108, 440). High levels of expression of these cytokines, as well as IFN-g (50) and IL-10 (216), are particularly evident in lymphoid tissue, a major site of HIV replication throughout the course of disease (142, 165, 418, 419). Chronically activated CD8+ T cells (266) and HIV-infected macrophages (373) are thought to be major contributors to the elevated cytokine levels observed in HIV-infected subjects.

Another major disruption in the cytokine pattern observed in HIV disease is a progressive loss in the ability to produce immunoregulatory cytokines, such as IL-2 and IL-12 (100, 306). IL-2 and IL-12 are critical for effective cell-mediated immune responses, as they stimulate proliferation and lytic activity of CTLs and NK cells. In addition, IL-12 is essential for stimulating the production of T helper (TH)-1 type cytokines, including IL-2 and IFN-g, that favor the development of cell-mediated immune responses (338, 256). The TH1 limb of cellular immune responses is impaired during the course of HIV infection; this is especially true with regard to defects in IL-2 production and IL-2 receptor expression (98, 121, 336, 362). Although the cause of this TH1 defect in HIV infection is multifactorial, it may derive in part from the ability of the HIV envelope to induce IL-10 production in monocytes, and thereby inhibit IL-2 secretion (30). Some investigators have proposed that a dominance of TH2-like responses (i.e., secretion of IL-4, IL-5, and IL-10) characterizes progression of HIV disease (101, 290). Most studies, however, suggest that cytokine dysregulation during the course of HIV infection is complex and cannot easily be classified in terms of TH1 and TH2 polarity (216, 336, 362).

Effect of Cytokines on HIV Replication

Soluble factors produced by activated PBMCs (162), macrophages (103), and B cells (459) can dramatically up-regulate HIV expression in acutely and chronically infected cells of the lymphocytic and macrophage lineages (Fig. 8). Cytokines that have been reported to up-regulate HIV replication *in vitro* include IL-1b, IL-2, IL-3, IL-6, IL-7, IL-12, IL-15, IL-18, TNF-a, TNF-b, and the colony-stimulating factors (CSF) macrophage (M)-CSF and granulocyte-macrophage (GM)-CSF (reviewed in reference 108; see also 7, 34, 285, 327, 502, and 511). IFN-a, IFN-b, and IL-16 (10, 235) are primarily suppressors of HIV production, whereas other cytokines, such as IL-4 (391, 550), IL-10 (573, 574), IL-13 (391), IFN-g (42, 229), and transforming growth factor (TGF)-b, reduce or enhance viral replication depending on the infected cell type and the culture conditions. The effects of a particular cytokine are often greatly influenced by the activity of other cytokines present in the lymphoid tissue microenvironment (439, 442, 574).

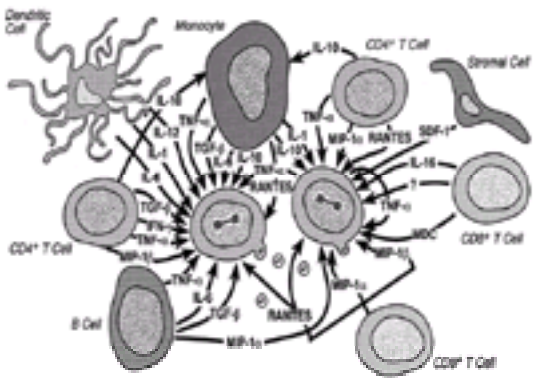

FIG. 8. Cytokine networks that regulate human immunodeficiency virus (HIV) replication. The proinflammatory cytokines tumor necrosis factor (TNF)-a, interleukin (IL)-1b, and IL-6 strongly enhance viral replication. Transforming growth factor (TGF)-b and IL-10 down-regulate viral replication; in the case of IL-10, this occurs at least in part because of down-regulation of the proinflammatory cytokines. The CC-chemokines inhibit R5 strains of HIV, SDF-1 inhibits X4 strains, and MDC-1 inhibits both R5 and X4 strains. Adapted from ref. 152, with permission.

Proinflammatory cytokines, particularly TNF-a, are considered the most potent HIV-inducing cytokines, and their mechanism of action is relatively well understood. Both TNF-a and IL-1b activate the cellular transcription factor nuclear

factor (NF)kB (137,406), a strong inducer of HIV LTR-mediated transcription. IL-6 increases HIV expression primarily by a posttranscriptional mechanism; however, IL-6 can synergize with NFkB-inducing cytokines to enhance HIV transcription (439). The production of HIV by macrophages or PBMCs stimulated with physiologic inducers of proinflammatory cytokine production, such as bacterial endotoxin or IL-2, can be partially or nearly completely abrogated by the addition of antiproinflammatory cytokines (200,573), neutralizing antibodies to the cytokines (285), or receptor antagonists (ra), such as IL-1ra (285). In cultures of HIV-infected macrophages, the viral-suppressive activity of several cytokines, such as IL-10 and TGF-b, is attributable largely to their ability to inhibit the secretion or activity of HIV-inducing proinflammatory cytokines (200,443,573). HIV production by infected T cells is sensitive to both the antiproinflammatory and the antiproliferative activity of such cytokines (202).

Administration of agents that block the action of HIV-inducing proinflammatory cytokines has met with limited therapeutic success. However, the use of cytokine-based therapies aimed at immune reconstitution in HIV disease has expanded over the past several years, particularly with the development of potent antiretroviral therapies that limit the potential for cytokine-mediated increases in viral replication. In this regard, administration of IL-2 to asymptomatic HIV-infected subjects simultaneously receiving effective antiretroviral therapy results in significant and sustained increases in $CD4^+$ T-cell numbers with no concomitant sustained increases in viremia (302). Ongoing studies are continuing to expand the list of cytokines for use as potential immunotherapeutic agents; IL-12, IL-13, IL-15, and IL-16 are all candidates (376,497,615).

Chemokines, or chemoattractant cytokines, are produced by numerous cell types, and may influence HIV replication by binding to chemokine receptors that also serve as HIV coreceptors (Fig. 9) (324). Chemokine production, induced during inflammation, is enhanced by CD40 ligand stimulation (299,350), and by several cytokines including TNF-a, IL-1b, IL-2, and IL-15 (483). Chemokines that bind to CCR5 suppress HIV replication *in vitro* in PBMCs from asymptomatic HIV-infected individuals harboring predominantly R5 strains of HIV (284), but not in PBMCs from individuals with more advanced disease harboring predominantly X4 strains of HIV (265,284). Similarly, HIV isolates obtained longitudinally from individuals with rapid disease progression exhibit reduced sensitivity to inhibition by CCR5 ligands *in vitro* over time (114,265). Ligation of chemokine receptors in different cell types may have different effects on HIV replication; CC-chemokines inhibit replication of R5 strains of HIV in $CD4^+$ T cells, but may actually enhance replication of these strains in monocyte/macrophages (277,485). Unanticipated effects of manipulating the chemokine–chemokine receptor axis have also been observed, including CC-chemokine–mediated up-regulation of CXCR4 expression and enhancement of replication of X4 strains of HIV *in vitro* that is dependent on intracellular signal transduction (135,283).

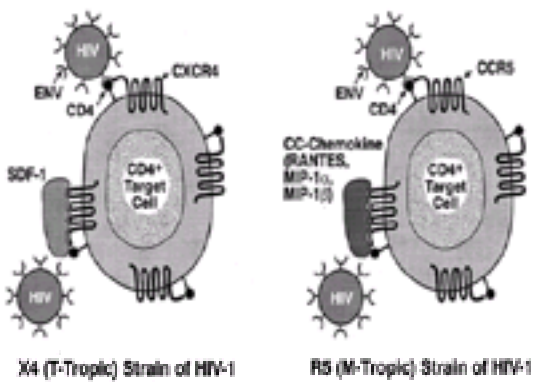

FIG. 9. Model of human immunodeficiency virus (HIV) coreceptor (CCR5 and CXCR4) use and inhibition of HIV entry by coreceptor ligands. Entry of R5 (i.e., predominantly macrophage tropic) strains of HIV is blocked by the CCR5 ligands macrophage inflammatory protein (MIP)-1a, MIP-1b, and RANTES. Entry of X4 (i.e., predominantly T-cell tropic) strains of HIV is blocked by the CXCR4 ligand SDF-1. Adapted from ref. 152, with permission.

Immunologic signals, cytokines, and chemokines influence chemokine receptor expression, which may also exert variable strain-dependent effects on HIV replication and spread (44,283). In this regard, differential regulatory patterns clearly distinguish the major HIV coreceptors, CCR5 and CXCR4 (44,548). CCR5 expression is predominantly seen in previously activated, memory T cells (i.e., $CD26^{high}CD45RO^+$ cells), whereas CXCR4 expression is seen in naive, unactivated cells (i.e., $CD26^{low}CD45RA^+$ cells). Cytokines that are characteristic of TH1-type responses, such as IL-2, IL-12, TNF-a, and IFN-g up-regulate CCR5 expression (44,233,531), whereas TH2-associated cytokines, such as IL-4 and IL-10, tend to up-regulate CXCR4 expression and down-regulate CCR5 expression (176,531,550). Stimulation of CD4+ T cells through CD3 and CD28 protects cells from HIV infection and potently down-regulates HIV replication in infected cells (314,460,465); this effect appears to be due in part to down-regulation of CCR5 (64). Finally, various components of pathogens, including HIV-1 itself, can induce expression of CXCR4 and favor replication of X4 strains of virus that use this coreceptor (258,378,379).

IMMUNE DYSFUNCTION DURING HIV INFECTION

The pathogenesis of HIV disease is a complex, multifactorial process (108,151). A wide array of immune system defects is associated with HIV infection; abnormalities in the function of all limbs of the immune system, including T and B lymphocytes, APCs, NK cells, and neutrophils, have been described.

CD4+ T Cells

CD4+ T-cell dysfunction and depletion are hallmarks of HIV disease. The proximate cause of the susceptibility to opportunistic infections observed with advancing disease is the defects in T-cell number and function that result directly or indirectly from HIV infection. In addition to the decrease in IL-2 production and IL-2 receptor expression, the

percentage of CD4+ T cells expressing CD28 (i.e., the major costimulatory receptor that is necessary for normal activation of T cells) is reduced during HIV infection compared with cells from uninfected individuals (87). $CD28^-$ cells do not respond to activation signals, and express markers of terminal activation, including HLA-DR, CD38, and CD45RO (587). CD4+ T cells from HIV-infected individuals express abnormally low levels of CD40 ligand (47), and this may in part explain the dysregulation of B-cell function that is observed during the course of disease.

A variety of mechanisms, both directly and indirectly related to HIV infection of CD4+ T cells, are likely responsible for the observed defects in T-cell function. Interference with CD4 expression by HIV gp120 (255), Nef (184), and Vpu (581) may impair the ability of the infected CD4+ T cell to interact with appropriate MHC class II molecules. Preferential infection by HIV of CD4+ memory cells and/or the preferential susceptibility of these cells to the cytopathic effects of HIV infection may in part explain the loss of memory responses to soluble antigens and consequent increase in the risk of infection with opportunistic organisms (92,306,490).

The relatively small fraction of T cells that is infected with HIV (i.e., generally <1% even in the late stages of disease) in seropositive subjects at any given time militates against direct infection as the sole mediator of the immunopathogenesis of HIV infection (see later) (418). In addition, CD4+ T-cell dysfunction is evident even in the early stages of HIV infection, before quantitative depletion of this key cellular subset (306). A role for HIV envelope in the immunopathogenesis of HIV disease has been suggested by a number of studies (reviewed in reference 83). The presence of measurable levels of circulating soluble gp120 in HIV-infected subjects (402) and accumulation of high concentrations of virions (both infectious and defective) in lymphoid tissue (165,419,530) underscore the potential for envelope to contribute to T-cell dysfunction in a process distinct from infection of CD4+ T cells.

HIV envelope glycoproteins bind with high affinity to the CD4 molecule and to a number of chemokine coreceptors. Evidence of aberrant intracellular signaling induced by HIV has been available for a number of years (226). Intracellular signals transduced by HIV-1 envelope have been implicated in several immunopathogenic processes, including anergy (409), syncytium formation (106), apoptosis (28,578), and inappropriate cell trafficking (369,575). The molecular mechanisms responsible for these abnormalities include dysregulation of the T cell–receptor phosphoinositide pathway (69), p56lck activation (199,249), phosphorylation of focal adhesion kinase (95), down-regulation of the costimulatory molecules CD40 ligand and CD80 (82), and activation of the MAP kinase and ras signaling pathways (533). Most of these aberrant signals were presumed to result from interactions between HIV envelope glycoproteins and CD4; however, more recently it has become clear that HIV envelope also may transduce intracellular signals through coreceptor molecules such as CCR5 (95,122,575) and CXCR4 (369).

CD4+ T-Cell Depletion

The frequent appearance of X4 strains of HIV late in the course of disease heralds accelerated CD4+ T-cell decline and clinical disease progression (78,298,539). Because the transition from an R5 to an X4 strain of virus may occur by mutation of only a few amino acid residues, predominantly in the envelope V3 loop (79,262,532,598), it is uncertain why such mutants fail to emerge until late in the disease process. The accelerated decline in CD4+ T-cell numbers associated with the emergence of X4 strains of HIV suggests that viral determinants are important with regard to pathogenicity. Because the viral determinant of cellular tropism maps to the gp120 envelope protein of HIV-1 (see earlier, and 105), this observation implicates the viral envelope as a significant mediator of HIV-induced CD4+ T-cell depletion. In an *ex vivo* model system of HIV infection of lymphoid tissue, the viral envelope does indeed appear to be responsible for CD4+ T-cell depletion; although both R5 and X4 strains of virus were capable of depleting CD4+ T cells in this system, R5 viruses depleted only $CD4+CCR5^+$ cells, whereas X4 viruses depleted all CD4+ cells (221). In an SHIV animal model, the viral envelope ectodomains determined the extent of CD4+ T-cell depletion (274). Interestingly, the avidity of the viral envelope for its chemokine coreceptor correlated well with its ability to induce CD4+ T-cell depletion (274) .

Direct Infection as a Cause of CD4+ T-Cell Depletion

The observations that CD4+ T cells are the principal targets of HIV infection *in vivo* and that HIV infection of CD4+ T cells *in vitro* causes cytopathicity led to a reasonable assumption that direct infection of CD4+ T cells *in vivo* results in their depletion. However, quantitative studies of the frequency of HIV-infected cells *in vivo* suggest that single-cell killing by direct infection with HIV may not be the predominant mechanism of CD4+ T-cell depletion (418,489). Although it increases with disease progression, the frequency of HIV-infected peripheral blood CD4+ T cells rarely exceeds 1 in 100, even in patients with AIDS (489). Viral burden and levels of virus expression are far greater in lymphoid tissue compared with peripheral blood (142,418,419); however, these levels even in lymphoid tissue do not appear to be sufficiently high to account for CD4+ T-cell depletion solely by direct mechanisms.

Multiple mechanisms of cell death appear to be operative after infection of a CD4+ T cell with HIV (Table 1). Early events in the viral life cycle, such as accumulation of reverse-transcribed viral DNA in the cytoplasm, may contribute to cell killing in HIV infection (503); however, this is clearly not the sole mechanism responsible for single-cell killing by HIV (38). High levels of viral RNA and aberrant RNA molecules also are present in the cytoplasm of infected cells, and possibly interfere with normal cellular RNA processing (295,513).

Direct mechanisms
• Accumulation of unintegrated viral DNA
• Interference with cellular RNA processing
• Intracellular gp120-CD4 autofusion events
• Loss of plasma membrane integrity due to viral budding
• Elimination of HIV-infected cells by virus-specific immune responses
Indirect mechanisms
• Aberrant intracellular signaling events
• Syncytium formation
• Autoimmunity
• Innocent bystander killing of viral antigen–coated cells
• Apoptosis
• Inhibition of lymphopoiesis

TABLE 1. *Mechanisms of CD4+ T-cell dysfunction and depletion*

The intracellular concentration of envelope gp120 molecules is high during the process of virion assembly in an HIV-infected cell. Several studies have suggested that intracellular gp120 may interact with intracellular CD4 molecules, and that this interaction may induce cell death (133,296). The mechanism of cell death as a consequence of this intracellular gp120–CD4 interaction may be autofusion events that disrupt the integrity of the cell membrane (57). Cell-membrane integrity also may be compromised by the budding of virions from an infected cell (313) and by HIV-induced increases in the concentration of intracellular monovalent cations (558).

HIV-infected cells also may die as a consequence of virus-specific immune responses that occur before the cell succumbs directly to viral infection. Multiple effector mechanisms may be involved in the killing of HIV-infected cells, including CTL responses, ADCC, and NK cell responses.

Indirect Mechanisms of CD4+ T-Cell Depletion

Syncytium formation. HIV envelope glycoprotein molecules are inserted into the host cell membrane during virion assembly, and also are shed by infected cells into the surrounding milieu. Interaction of these HIV envelope glycoprotein molecules with uninfected cells may be an important immunopathogenic mechanism of HIV infection.

The molecular events associated with viral entry that lead to fusion between the viral coat and cell membrane involve the interaction of the HIV envelope glycoprotein, CD4, and a coreceptor molecule. Similar events may occur when an infected cell bearing HIV envelope glycoprotein molecules on its surface encounters an uninfected CD4+ cell with an appropriate coreceptor. Fusion between infected and uninfected cells, resulting in multinucleated giant cells or syncytia, has long been observed *in vitro* (320,512). Other molecules implicated in syncytium formation include LFA-1 (248), CD7 (476), and HLA class I molecules (134). Syncytia have been observed only rarely in tissues obtained from HIV-infected individuals (167,452); thus it is unlikely that syncytium formation is a major pathogenic mechanism of CD4+ T-cell depletion *in vivo.*

Autoimmunity. Autoimmunity may occur during the course of HIV infection as a result of molecular mimicry by viral components. Highly homologous regions exist in the carboxy terminus of the HIV-1 envelope glycoprotein and the amino-terminal domains of different HLA-DR and DQ alleles (197). Sera from a substantial number of HIV-infected individuals react with the shared determinant of gp41 and MHC class II: these sera can inhibit normal antigen-specific proliferative responses and also eliminate class II–bearing cells by ADCC (198). Similar instances of molecular mimicry between HIV-1 envelope constituents and host proteins, which may result in pathogenic autoimmune responses, include the collagen-like region of complement component C1q-A (360), MHC class I heavy chains (215), HLA-DR4 and DR2 alleles (506), variable regions of the T-cell receptor a, b, and g chains (506), Fas (382,602), functional domains of IgG and IgA (506), denatured collagen (213), and a number of nuclear antigens (66). Finally, immune recognition of normal cellular components may be induced by their association with HIV; such phenomena may be responsible for the emergence of CTLs that are specific for autologous CD4+ T cells (212).

Innocent bystander phenomena. Immune responses that target HIV determinants on infected cells also may contribute to elimination of uninfected cells bearing HIV proteins (e.g., gp120) on their surface. Targeting of such “innocent bystander” cells by antibody and cellular immune responses has been described (329,570,606).

HIV-mediated inhibition of hematopoiesis. Failure of normal hematopoiesis is an obvious candidate mechanism to account for depletion of CD4+ T cells during HIV infection. A subset of CD34+ progenitor cells express CD4 and are infectable *in vitro* with HIV-1 (75,163). It is controversial whether CD34+ progenitor cells represent a substantial *in vivo* reservoir for HIV. A number of studies have failed to detect HIV-infected CD34+ progenitor cells in most HIV-infected individuals; however, a large study by Stanley et al. (522) showed that a substantial minority of HIV-infected patients with severe CD4+ T-cell depletion have a reservoir of HIV-infected CD34+ progenitor cells. Recent reports demonstrating expression of HIV coreceptors on CD34+ progenitor cells (62,128,473) suggest that the CD4+ subset of these cells may be infectable with HIV, further substantiating the earlier findings of Stanley et al.

Although the role of direct infection of CD34+ progenitor cells in HIV pathogenesis remains controversial, a large body of evidence suggests that viral proteins and HIV-induced cytokines can impair the survival and clonogenic potential of these cells (130,325,608).

Disruption of the thymic microenvironment (520) and HIV-induced thymocyte depletion also may contribute to the failure of CD4+ T-cell regeneration. Thymic epithelial cells normally secrete IL-6, which can in turn increase HIV replication in infected cells (492). Subpopulations of thymic CD3–CD4–CD8– cells (i.e., “triple negative” cells) are susceptible to infection with HIV *in vitro* (551), and thymic CD3–CD4+CD8– progenitor cells from HIV-infected patients are infected *in*

vivo. Finally, uninfected thymocytes from HIV-infected individuals are primed for apoptotic death, suggesting that indirect mechanisms of defective thymopoiesis are operative as well (528).

Apoptosis. Aberrant intracellular signals transduced by HIV may prime CD4+ T cells for apoptosis, resulting in depletion of these cells during the course of HIV infection (9). Acute infection of T cells with HIV *in vitro* can induce apoptosis (537), and T cells from HIV-infected patients undergo enhanced rates of apoptosis *in vitro* compared with normal T cells, particularly after activation (224,361). Cross-linking of CD4 followed by ligation of the T-cell receptor is sufficient to induce apoptosis, suggesting that uninfected CD4+ T cells can be depleted inappropriately on encountering antigen if CD4 has been cross-linked by gp120 (28).

Fas-dependent and Fas-independent pathways of apoptosis may be triggered by HIV. HIV-mediated Fas-independent apoptosis of CD4+ T cells has been shown *in vitro* by several groups (180); enhanced susceptibility of CD4+ T cells to Fas-dependent apoptosis has been reported as well. Mechanisms that may contribute to this susceptibility include up-regulation of Fas and Fas ligand (24,578); up-regulation and activation of caspase-1 (509), caspase-3 (94), and caspase-8 (33); activation of cyclin-dependent kinases (317); and down-regulation of the antiapoptotic Bcl-2 protein (456). Viral gene products that have been associated with enhanced susceptibility to apoptosis include the viral envelope (28,578), Nef (595,607), Tat (33,578), and Vpu (65).

The susceptibility of uninfected bystander cells to apoptosis is a possible mechanism by which large number of cells may be eliminated during HIV infection. Studies in lymphoid tissue from HIV-infected individuals suggest that the majority of apoptosis occurs in uninfected bystander cells (155,387). Macrophages are efficient mediators of CD4+ T-cell bystander apoptosis. HIV-induced up-regulation of Fas ligand expression on macrophages and enhanced secretion of TNF-a from these cells appear to be responsible in part for this phenomenon (23,24). Another mechanism of bystander apoptosis may involve up-regulation of CD62L on lymphocytes by HIV (564). These HIV-exposed lymphocytes home to lymph nodes, and undergo apoptosis on signaling through homing receptors such as CD62L, CD44, and CD11a (563).

It remains uncertain whether HIV-induced apoptosis plays an important role *in vivo* in CD4+ T-cell depletion. The frequency of apoptotic CD4+ and CD8+ T cells as well as B cells is significantly higher in lymphoid tissue from HIV-infected individuals compared with that from uninfected controls (387,469). The relationship between the frequency of apoptotic cells and the stage of HIV infection remains controversial. Some data indicate a positive correlation between the stage of HIV disease and susceptibility of peripheral blood T cells to apoptosis (207), and another study demonstrated very low frequencies of apoptotic cells in HIV-infected long-term nonprogressors (319). However, other studies found no correlation between apoptosis and stage of disease (363,387). Compelling evidence that apoptosis may play a role in HIV pathogenesis comes from animal models wherein an increased frequency of apoptosis in CD4+ T cells is seen in primates infected with pathogenic strains of SIV, but not in primates infected with nonpathogenic strains of SIV (148).

CD8+ T cells

Dysregulation of CD8+ T-cell numbers and function is evident throughout the course of HIV disease. After an initial decline associated with acute primary infection, CD8+ T-cell counts usually rebound to supranormal levels and may remain elevated for prolonged periods. Increases in CD8+ T cells during all but the late stages of disease may, in part, reflect the expansion of HIV-specific CD8+ CTLs.

During HIV disease progression, CD8+ T cells acquire an abnormal phenotype characterized by the expression of certain activation markers and the absence of expression of the CD25 IL-2 receptor. Alterations in the phenotype of CD8+ T cells in HIV-infected individuals may have prognostic significance. In this regard, individuals whose CD8+ T cells express HLA-DR but not CD38 after seroconversion experience a stabilization of their CD4+ T-cell counts and a less fulminant disease course, whereas individuals whose CD8+ T cells express both HLA-DR and CD38 experience a more aggressive course with rapid CD4+ T-cell depletion and a poorer prognosis (189). CD8+ T cells lacking CD28 expression also are increased in HIV disease (479), possibly reflecting the expansion of the $CD8+CD28^{-}CD57^{+}$ T-cell subset containing *in vivo* activated CTLs (554). The loss of CTL activity with disease progression is not restricted to HIV-specific CTLs; a loss of cytotoxic activity to other common antigens including Epstein–Barr virus (EBV) and *Mycobacterium tuberculosis* also has been observed. In addition to CTL activity, other CD8+ T-cell functions are impaired during HIV disease progression, including loss of noncytolytic non-MHC–restricted CD8+ T cell–derived suppressor activity against HIV (335).

Oligoclonal expansions of CD8+ T cells are characteristic of HIV infection. These expansions are composed largely of HIV-specific CTLs (417,582). The disappearance of CTLs with particular specificity can be due to a number of factors, including mutation of the virus within the CTL recognition epitope. Another cause for the disappearance of some CTLs, however, is clonal exhaustion. This phenomenon, wherein high concentrations of antigen completely mobilize a CTL response, occurs in lymphocyte choriomeningitis virus infection (380); depletion of the CTLs results in persistence of antigen. CTL exhaustion may occur to some extent during HIV infection, where disappearance of some CTLs is observed in the absence of viral escape mutations that would otherwise explain the phenomenon (352,423). Finally, CD8+ T cells may be depleted by apoptosis as innocent bystanders. This phenomenon may be mediated by macrophages in which CXCR4 has been triggered by HIV envelope; membrane-bound TNF-a is up-regulated by HIV in these cells, as is TNF receptor II on CD8+ T cells (245).

Monocytes/Macrophages

Cells of the monocyte/macrophage lineage play key roles in the immunopathogenesis of HIV disease. These cells serve as reservoirs of viral infection and are responsible for a variety of tissue-specific pathologic processes. Dysfunction of these cells contributes to CD4+ T-cell dysfunction, and also to impaired host defense against intracellular pathogens (150,151).

Monocytic cells express CD4 and numerous HIV coreceptors including CCR5, CXCR4, and CCR3 on their surfaces

(77,454,552), and serve as targets of HIV infection. Unlike infection of CD4+ T cells, HIV is relatively noncytopathic for cells of the monocyte/macrophage lineage, and HIV can replicate extensively in these cells in intracellular compartments (187). Chronically infected macrophages may be long-lived, and currently available antiretroviral agents are generally not capable of targeting chronically infected cells; these cells therefore represent a formidable challenge to eradication of HIV (428,495). Circulating monocytes are rarely found to be infected *in vivo* and are difficult to infect *in vitro* (491,549,569); however, infection can readily be demonstrated in tissue macrophages, including resident microglial cells in the brain, pulmonary alveolar macrophages, and mature macrophages derived from blood monocytes *in vitro* (187,294,437). Infection of monocytic precursors in bone marrow may be directly or indirectly responsible for certain of the hematologic abnormalities observed in HIV-infected individuals. Lymphoid tissue macrophages can be prolific producers of HIV in the setting of opportunistic infections, during which the cytokine milieu in the tissue favors a highly productive state of HIV infection (405).

Infection of monocyte/macrophages with HIV or exposure of these cells to viral proteins, including envelope glycoproteins and Tat, leads to a number of functional abnormalities. Impaired accessory cell function (143,597) may result from decreased MHC class II expression, decreased IL-12 secretion, and increased IL-10 secretion (445,597); this HIV-induced dysregulation of antigen presentation may in turn be a significant cause of hyporesponsiveness of CD4+ T cells (143). Defects in ADCC function of monocyte/macrophages, possibly related to low levels of expression of Fc and complement receptors, also have been observed in HIV infection (36,278). Finally, HIV-associated abnormalities in antigen uptake, oxidative burst, and chemotaxis have been described in monocyte/macrophages (383,438,445).

Cells of the monocyte/macrophage lineage are central to the pathogenesis of HIV-induced CNS disease. HIV infection of brain microglial cells, derived from the monocytic lineage, may lead to encephalopathy and neuropathy (51), astrocytosis (395), and cerebral vasculitis (174). Levels of the chemokine monocyte chemotactic protein (MCP)-1 are markedly elevated in the CSF of AIDS patients with HIV encephalitis (96); a major source of these high levels of MCP-1 is likely HIV-infected macrophages (96). MCP-1 in turn recruits and activates monocytes, which elaborate proinflammatory cytokines and thereby enhance HIV replication and induce neuropathologic disease (544). Other mediators of neuropathology that are elaborated by monocyte/macrophages include neurotoxic amines (191), and quinolinic acid (498). These substances may induce neuronal injury by triggering apoptosis (430).

Dendritic Cells

DCs are among the first cells to encounter HIV after mucosal exposure and likely are responsible for transporting the virus to lymphoid organs, thus facilitating infection of CD4+ T cells and viral dissemination. DCs express several different chemokine receptors that can be used as HIV coreceptors for entry (211,472,604). Studies of DCs from HIV-infected individuals have yielded conflicting results; however, productive infection of tissue DCs with HIV appears to be rare (272,351).

It has been reported that infectability of DCs may be dependent on viral subtype (514). This finding could in part explain the differences in the global epidemiology of HIV infection; an HIV subtype that can replicate well in Langerhans cells, likely a major cell type involved in the initiation of viral infection through mucosal contact (516), may be more efficiently transmitted heterosexually. It should be pointed out that these findings are controversial (447), although data do suggest a role for DCs in selecting the strain of HIV that can be transmitted across a mucosal barrier (455).

The degree of DC dysfunction and depletion that occurs during HIV infection remains controversial (56). Emerging data suggest that a subset of DCs expressing CD11c are depleted during the course of HIV infection; interestingly, these cells were not restored during highly active antiretroviral therapy (214). Further studies are needed to clarify the possible role of DC depletion and dysfunction in the pathogenesis of HIV disease.

B Lymphocytes

HIV infection is almost invariably associated with hypergammaglobulinemia and B-lymphocyte hyperactivation. A large component of the immunoglobulin specificity, at least in early-stage disease, is directed against HIV antigens. It has been suggested that a majority of activated B cells produce antibodies directed against HIV during this stage of infection (reviewed in reference 8). In spite of the observed B-cell hyperactivation, the ability of B cells to respond specifically to antigens is reduced. B cells from HIV-infected individuals express abnormally low levels of HLA-DR on their surfaces (188), and also fail to up-regulate CD70 normally after stimulation with activated T cells (587); this latter defect is associated with impaired CD70-dependent immunoglobulin synthesis (587). In patients with advanced-stage HIV infection, B cells fail to proliferate and differentiate in response to ligation of the B-cell antigen receptor and CD40, suggesting a defect in signal transduction (113). Further evidence of HIV-related B-cell dysfunction comes from an *in vitro* lymphoid tissue-culture model of HIV infection. In this model, infection of lymphoid tissue with strains of HIV that use CCR5 as an entry coreceptor results in enhanced antibody responses to recall antigens on challenge; in contrast, HIV strains that use CXCR4 result in profound suppression of these antibody responses (192). This effect appears to be dependent on signal transduction through CXCR4 and may be irreversible (192).

B cells from HIV-infected individuals secrete increased amounts of TNF-a and IL-6, cytokines known to enhance HIV replication (458,459), and express surface-bound TNF-a that can induce the production of HIV from infected CD4+ T cells (330). The secretion of proinflammatory cytokines and the expression of surface-bound TNF-a by B cells in the lymphoid microenvironment may contribute to T-cell activation and HIV replication in these tissues.

HIV gp120 has been observed to bind directly to an immunoglobulin variable chain (VH3) and activate these B cells in much the same manner as a superantigen (37). This antigen-independent polyclonal activation leads in part to the hypergammaglobulinemia and B-lymphocyte hyperactivation of HIV infection. Other portions of HIV, including gp41, directly activate B cells in a non–superantigen-mediated manner (81). Correlates of B-cell dysfunction observed in HIV-infected individuals include an increase in spontaneous EBV transformation *in vitro* and may contribute to the observed increased frequency of EBV-induced lymphomas (374). In addition, the increased susceptibility to bacterial

infections during the course of HIV infection likely results in part from B-cell dysregulation (494).

B cells also may serve as a viral reservoir during the course of HIV infection. In this regard, stimulation of B cells with CD40L and IL-4 up-regulates expression of activation and costimulatory molecules, as well as CD4 and CXCR4. B cells stimulated in this manner are susceptible to infection with dual- and T-tropic strains of HIV (372).

Neutrophils

Neutrophils appear to be hyperactivated during the course of HIV infection, and many functional defects have been observed in these cells (359). Neutrophils from HIV-infected individuals produce more TNF-a and IL-6 in response to lipopolysaccharide (LPS) or *Candida* antigen compared with neutrophils from normal donors (541). These cells also undergo apoptosis at an increased rate compared with those from normal controls (436). When stimulated through Fc receptors or protein kinase C, neutrophils from HIV-infected individuals produced less superoxide compared with controls; this defect could be recapitulated by treatment of normal neutrophils with HIV envelope protein (386).

Dysfunction of neutrophils in HIV-infected individuals has several clinical implications. HIV infection, especially in women, is characterized by an increase in the incidence and severity of *Candida* infections. Although neutrophils from HIV-infected patients readily phagocytize *Candida* organisms, they have a decreased ability to kill the organisms, suggesting a defect in nonoxidative killing (577). This defect may be mediated in part by elevated levels of IL-10 (534).

Natural Killer Cells

Abnormalities of NK cells are observed throughout the course of HIV disease, and these abnormalities increase with disease progression. Most studies report that NK cells are normal in numbers and phenotype in HIV-infected individuals; however, decreases in numbers of the $CD16^+/CD56^+$ subpopulation of NK cells with an associated increase in activation markers has been reported (257). NK cells from HIV-infected individuals are defective in their ability to kill typical NK target cells as well as gp160-expressing cells. The abnormality in NK cell lysis is thought to occur after binding of the NK cell to its target (2). A possible mechanism for defective NK activity includes a lack of cytokines necessary for optimal function. Addition of either IL-2, IL-12, IL-15, or IFN-a to cultures enhances the defective *in vitro* NK cell function of HIV-infected individuals (74,545). Enhanced expression of cytolytic inhibitory receptors during the course of HIV infection also may be responsible for depressed NK cell function (13,175); in addition, selective HIV-mediated down-regulation of HLA-A and -B, but not HLA-C and -E molecules may allow CTL escape and simultaneously prevent NK cell killing of HIV-infected target cells (107).

NK cells are an important source of HIV-inhibitory CC-chemokines in HIV-infected individuals (404). NK cells isolated from HIV-infected individuals produce high constitutive levels of MIP-1a, MIP-1b, and RANTES; high levels of chemokine production also are seen when these cells are stimulated by IL-2, IL-15, or CD16 cross-linking, or during lytic killing (404). Thus NK cells, like CD8+ T cells, may inhibit HIV replication by cell-mediated killing as well as by secretion of soluble HIV-inhibitory factors.

ROLE OF CELLULAR ACTIVATION IN HIV PATHOGENESIS

The result of HIV infection is profound immunodeficiency; however, paradoxically, HIV infection is associated with hyperactivation of the immune system through most of the course of disease (Table 2). HIV subverts the immune system by inducing immune activation and using this milieu to its own replicative advantage (108,151,158).

- Hyperactivation of B cells
- Hypergammaglobulinemia
- Spontaneous lymphocyte proliferation
- Activation of monocytes with secretion of proinflammatory cytokines
- Increased expression of activation markers on T cells
- Follicular hyperplasia of lymphoid tissue
- Elevated circulating levels of neopterin, β_2-microglobulin, acid-labile interferon, soluble tumor necrosis factor receptors, and soluble interleukin 2 receptors
- Autoimmune phenomena

TABLE 2. *Manifestations of an activated immune system in HIV disease*

The replicative cycle of HIV infection is most efficiently achieved in activated cells (53,527,599). Cytokines that induce T-cell activation can further contribute to viral replication by inducing a state of productive infection in latently infected resting T cells (86). Several lines of evidence suggest that HIV replication *in vivo* is dependent on antigen-driven activation of CD4+ T cells. HIV-infected individuals with intercurrent infections experience transient increases in plasma viremia that correlate with the degree of induced immune activation; similar observations have been made in HIV-infected individuals who received immunizations against various pathogens (399,521,523). The amount of viral replication observed after vaccination with influenza vaccine or tetanus toxoid, or during active infection with *Mycobacterium tuberculosis,* correlated inversely with the stage of HIV disease. Individuals with late-stage HIV disease had a moderate increase in viral replication, whereas individuals with early-stage disease had a much greater increase in plasma viremia over baseline, suggesting a correlation between the ability of the immune system to respond to antigen and the magnitude of induction of virus replication. Furthermore, when PBMCs from tetanus toxoid–immunized, HIV-infected individuals were stimulated *in vitro* with tetanus antigen, or when PBMCs from purified protein derivative (PPD)-positive,

HIV-infected individuals were stimulated *in vitro* with PPD or live *M. tuberculosis,* subjects with early-stage disease manifested a much stronger proliferative response to the respective antigens with a larger increase in viral replication *in vitro* than did individuals with advanced-stage disease (201,407,521,523). These studies suggest that the level of viral replication correlates with the level of immune system activation in response to an antigen.

Analysis of viral quasispecies and immune responses within lymphoid tissue from HIV-infected individuals reveals that within individual splenic white pulps, a restricted number of individual antigen-specific immune responses occurs (defined by the analysis of T cell–receptor Vb gene use), and each of the immune responses contains a single or limited number of HIV quasispecies (80). These data support the theory that in the context of individual antigen-specific immune responses, a single quasispecies of HIV, which was present at the initiation of the reaction, spread among the newly activated T cells. Thus it is likely that the continuous daily production of HIV occurs in newly activated CD4+ T cells that are being driven by antigen-specific activation (80,223).

EPIDEMIOLOGY

The Global Pandemic

The global HIV pandemic ranks among the greatest infectious disease scourges in human history. Morbidity and mortality due to HIV infection are staggering; by the beginning of 2001, more than 36 million people worldwide were living with HIV (Fig. 10), and 21.8 million deaths due to HIV infection had occurred since the beginning of the epidemic (547). During 2000 alone, nearly 6 million individuals worldwide acquired HIV infection, and 3 million deaths due to HIV infection occurred; these statistics highlight the continued expansion of the epidemic on a global scale (547).

FIG. 10. Global distribution of the estimated number of individuals living with human immunodeficiency virus (HIV) infection as of December 2000. From ref. 547, with permission.

Phylogenetic analysis of HIV proviral sequences worldwide reveals three major genetic groups of HIVs, the M (major), N (new), and O (outlier) groups. The three major HIV groups are most closely related to SIV sequences (SIVcpz) harbored by chimpanzees of the subspecies *Pan troglodytes troglodytes* (182). The relatively rare O group viruses are concentrated in western Africa in Cameroon, Gabon, and Equatorial Guinea. Group N viruses were isolated in 1998 in two individuals from Cameroon (507). Within the M group, related HIV variants are classified into clades, or subtypes (currently designated A–J) according to their degree of genetic similarity (Fig. 11) (311). At the nucleotide level, viral subtypes differ from each other by about 14% in their *gag* (viral core) coding sequences, and by about 30% in their envelope coding sequences (311). Subtype C viruses are the most common worldwide; they are prevalent in eastern and southern Africa and India, although such viruses also have been isolated in Europe, Russia, China, and Brazil (146). Subtype A viruses are concentrated in central Africa, and also are found in Europe, Russia, east Asia, and South America (146). Subtype B viruses comprise the overwhelming majority of viruses isolated in the United States, likely as a result of a dominant founder effect. Subtype B viruses also are prevalent in Europe, Australia, and South America; variant subtype B viruses have been isolated in Thailand, China, Malaysia, and Japan. Subtype D viruses circulate primarily in central Africa, whereas subtype E viruses are isolated predominantly in the rapidly expanding epidemic in southeast Asia (146). HIV viral subtypes G, H, and J have been isolated in central Africa; however, all three of these subtypes are uncommon.

FIG. 11. Global distribution of human immunodeficiency virus (HIV)-1 subtypes. Subtype B predominates in the United States and Western Europe; however, subtypes C and A are the most prevalent subtypes in the world, particularly in Africa and Asia. From ref. 547, with permission.

The dynamics of the spread of different subtypes of HIV through populations is likely determined by the genetics of the population as well as by viral properties. Some phenotypic properties may be subtype specific. In this regard, the frequency of subtype C HIV viruses that are syncytium inducing and use CXCR4 as a coreceptor is extremely low (427). Additional data suggest that clinical outcomes may vary according to HIV subtype. In one study, infection with subtype A viruses was associated with a more favorable prognosis (273), and in another, infection with subtype C viruses was associated with a poor prognosis (394).

Zoonotic transmission of a retrovirus from chimpanzees to humans in sub-Saharan Africa is the most plausible explanation for the genesis of the HIV epidemic (182). From central, western, and eastern sub-Saharan Africa, the epidemic spread to North America, Latin America, Western Europe, southeast Asia, and southern Africa. Currently, the heaviest burden of the epidemic is concentrated in southern Africa, India, and southeast Asia. Although only 12% of the world's population lives in sub-Saharan Africa, 70% of individuals worldwide who acquired HIV infection in 2000 live there. Of all AIDS deaths since the beginning of the AIDS epidemic, 78% have occurred in sub-Saharan Africa. In the southern African countries of Botswana, Lesotho, Swaziland, and Zimbabwe, 24% to 36% of the population between the ages of 15 and 49 years are HIV infected. The epidemic also is expanding very rapidly in South Africa; 15% of new infections in the entire African continent in 1998 occurred in South Africa. Complex sociologic factors determine the dynamics of the HIV epidemic in different communities. In South Africa, large numbers of male migrant workers are attracted by employment opportunities afforded by valuable natural resources (i.e., gold and diamond mining); this scenario has in turn spawned a large sex-for-money trade that fuels the spread of HIV infection. A great deal of social stigma is associated with HIV infection in South Africa; this further fuels the epidemic, as it hinders open discussion about the disease and its prevention.

Nearly 1% of the adult population in India is infected with HIV. Although this seroprevalence is lower than that in many affected countries, it makes India the country with the largest number of HIV-infected individuals (approximately 4 million). Most HIV infections in India have occurred during the 1990s. Thus an extraordinary burden of HIV-related morbidity and mortality lies ahead in India, as the epidemic there is in its early stages. Alarmingly, among women attending a sexually transmitted disease clinic in Pune, India, more than 90% were married and monogamous; nearly 14% of these women tested positive for HIV (181).

Societal changes are fueling the spread of HIV in eastern Europe and central Asia. Increasing availability and use of illicit drugs, and a burgeoning sex-worker population in the republics of the former Soviet Union have contributed to a 25-fold increase in rates of syphilis in the 1990s (547). Although the absolute number of HIV infections in the region is small, the rate of increase is very rapid. In this regard, the cumulative number of HIV cases reported in the region increased more than five-fold between 1995 and 1997, from 9,111 to 46,573, with Ukraine, Russia, and Belarus accounting for about 90% of all new cases (127). The Yunnan province of China borders the poppy-rich regions of southeast Asia, and 70% of injection-drug users in this province are HIV infected. A similar situation exists in the Xinjiang province, which borders the poppy-growing regions of Pakistan and Afghanistan. Current estimates place the burden of HIV infections at 500,000 in China, and this number appears to be growing rapidly. Molecular epidemiologic studies indicate that nearly 50% of viral isolates originated from injection-drug users in Thailand, and one third of isolates originated from injection-drug users in India.

In the United States, between 600,000 and 800,000 individuals are infected with HIV (547). Although the incidence of new infections declined substantially in the late 1980s as a result of successful prevention efforts, a plateau of approximately 40,000 new HIV infections per year has persisted through the 1990s (467). The availability of HAART in developed countries has led to substantial reductions in the death rate due to AIDS (371,413). Unfortunately, no such benefit has reached the developing world, where AIDS-related mortality continues to exact a devastating toll. The nine African nations with an HIV seroprevalence of greater than 10% will experience a decline in average life expectancy of 17 years by the year 2015; instead of an average life expectancy that would have been on a trajectory to reach 64 years in the absence of HIV, it will decline to only 47 years (Fig. 12) (547). Infant mortality rates are increasing sharply in these countries as well, largely as a direct result of HIV infections. Finally, most developing countries do not have adequate resources to provide for a growing population of AIDS orphans; 20% to 30% of all children younger than 15 years had been orphaned by the year 2000 in nine sub-Saharan countries (540). Many of these orphans, particularly in urban areas, are at high risk for homelessness, starvation, and violent crime.

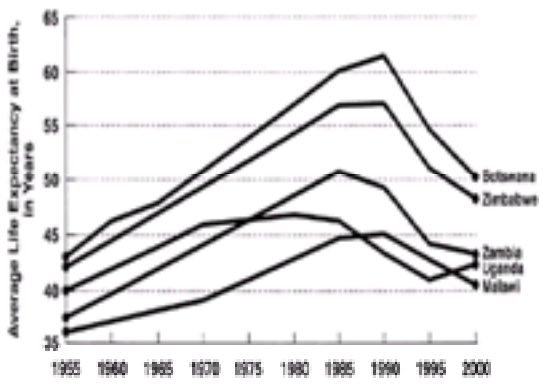

FIG. 12. Projected changes in life expectancy in selected African countries with high human immunodeficiency virus (HIV) prevalence. The extensions in life expectancy realized through improved living conditions and health care from the 1950s through the 1980s are being eroded as a result of HIV-related mortality. From ref. 547, with permission.

Transmission

HIV is transmitted by exposure of the oral, rectal, or vaginal mucosa during sex, by inoculation (i.e., through transfusion of contaminated blood products, use of contaminated equipment during injection-drug use, or maternal–fetal circulation), or by breast feeding. Sexual transmission accounts for more than 90% of HIV infections worldwide. In developing countries, most infections occur as a result of heterosexual contact, with a male-to-female ratio of approximately 1. In the United States and Western Europe, the vast majority of HIV infections that occurred early in the epidemic were associated with homosexual contact. Specifically, unprotected receptive anal intercourse was identified as the strongest risk factor for acquisition of HIV infection in men who reported having sex with men. The demographic characteristics of the epidemic have shifted considerably over the years, with a higher percentage of new infections occurring in injecting drug users and their sex partners. Minority populations are disproportionately affected by HIV; in 2000, AIDS rates per 100,000 population were 84.2 for blacks, 34.6 for Hispanics, and 9.0 for whites (71). Women are also increasingly affected by the HIV epidemic; in the United States, the percentage of AIDS cases occurring in women increased from 7% to 23% between 1985 and 1999 (71). Fewer than 50% of AIDS cases in the United States that occurred in 1999 were in men who have sex with men (71). Behavioral risk factors also have shifted over time. In a cohort of men who have sex with men that was recruited in 1992 through 1994, only 15% of HIV seroconversions that occurred were the result of unprotected anal intercourse; many seroconversions appear to occur after activities previously considered to be of lower risk, including oral sex (555).

Transmission of HIV is dependent on a large number of behavioral and biologic factors. The per-contact probability of male-to-female HIV transmission during vaginal sex is approximately 0.1% to 0.2% (253,410); receptive anal intercourse is associated with a considerably higher (i.e., approximately 0.8%) risk of transmission (555). Not surprisingly, considerable heterogeneity exists in per-contact risk, with some individuals becoming infected after only one or several exposures, and others remaining uninfected despite frequent exposure (18,426,470,555). Some of the many variables that influence the probability of HIV transmission include the genetic background of the potential host (124,322,477), the size and infectiousness of the inoculum, and the local environment in which the exposure occurs. Factors that result in physical disruption of the exposed mucosa, such as the use of vaginal desiccants or the presence of genital ulcer disease, can enhance viral transmission (217,451,519,535).

CLINICAL FEATURES

The clinical features of HIV infection vary depending on the stage of disease. In the acute and early stages of the disease, symptoms resulting from immune hyperactivation predominate; however, with the inexorable depletion of CD4+ T cells that characterizes disease progression, overt cellular immunodeficiency and its many complications develop (Fig. 13). The 1993 CDC classification of HIV infection consists of clinical categories and quantitative CD4+ T-cell categories (Table 3) (70).

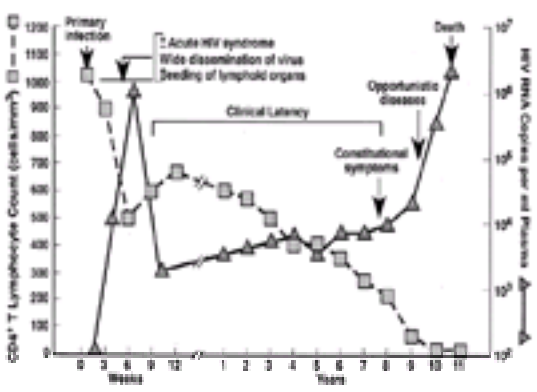

FIG. 13. Natural history of human immunodeficiency virus (HIV) infection. High levels of viremia during acute infection are often HIV associated, with a sharp decline in CD4+ T-cell numbers in the peripheral blood. Emergence of HIV-specific immune responses results in down-regulation of viremia and partial recovery of the CD4+ T-cell count. A long period of clinical latency ensues, during which levels of plasma viremia may remain relatively low, and the CD4+ T-cell count declines slowly. As the CD4+ T-cell count falls below 200 cells/μL, the risk for opportunistic diseases increases. In the late stages of disease, sharp increases in viremia and sharp declines in CD4+ T-cell counts occur. Adapted from Fauci AS, Pantoleo G, Stanley S, et al. Immunopathogenic mechanisms of HIV reproduction. *Ann Intern Med* 1996;124:654.

CD4 cell categories	Clinical categories: A Asymptomatic, lymphadenopathy, or acute infection	B Symptomatic,* not category A or C	C Clinical AIDS†
≥500/μL (≥29%)	A1	B1	C1
200–499/μL (14–28%)	A2	B2	C2
<200/μL (<14%)	A3	B3	C3

Categories A3, B3, and C1–3 are defined as AIDS.

*Examples of these symptoms include bacillary angiomatosis; thrush; vulvovaginal candidiasis that is persistent, frequent, or poorly responsive to therapy; cervical dysplasia or carcinoma in situ; constitutional symptoms such as fever or diarrhea of >1 month duration; oral hairy leukoplakia; multidermatomal or recurrent herpes zoster; immune thrombocytopenic purpura; listeriosis; pelvic inflammatory disease; and peripheral neuropathy.

†Candidiasis of the esophagus or respiratory tract; invasive cervical cancer; extrapulmonary coccidioidomycosis; extrapulmonary cryptococcosis; cryptosporidiosis; extrapyramidal cytomegalovirus infection; herpes simplex with mucocutaneous ulcer >1 month duration, or bronchitis, pneumonitis, or esophagitis; extrapulmonary histoplasmosis; HIV-associated dementia; wasting syndrome; isosporiasis; Kaposi's sarcoma; CNS lymphoma; non-Hodgkin's lymphoma; pulmonary tuberculosis; disseminated *M. tuberculosis*, *M. avium*, or *M. kansasii* infection; nocardiosis; *Pneumocystis carinii* pneumonitis; recurrent bacterial pneumonitis; progressive multifocal leukoencephalopathy; recurrent *Salmonella* septicemia; extrapulmonary toxoplasmosis.

TABLE 3. *AIDS surveillance case definition for adults and adolescents*

The CD4+ T-cell count is an excellent marker of the degree of immunodeficiency and determines the immediate risk of opportunistic infections and other AIDS-related complications (344). The plasma viral load is the most valuable prognosticator of HIV infection that is currently available (Fig. 14). Viral load not only predicts disease progression independent of the CD4+ T-cell count, but also actually predicts the trajectory of the CD4+ T-cell count (356,357). For example, the likelihood of developing AIDS within 3 years, given a plasma HIV RNA level of >30,000 copies/µL is 33% with a CD4+ T-cell count >500 cells/µL, 43% with a CD4+ T-cell count of 351 to 500 cells/µL, 64% with a count of 201 to 350 cells/µL, and 86% with a count of <200 cells/µL (356). Given a CD4+ T-cell count of 351 to 500 cells/mL, the likelihood of developing AIDS within 3 years increases from virtually nil with a plasma viral load of <3,000 copies/mL, to 8% with a viral load of 3,000 to 10,000 copies/mL, to 16% with a viral load of 10,000 to 30,000 copies/mL, and to 43% with a viral load >30,000 copies/mL (356).

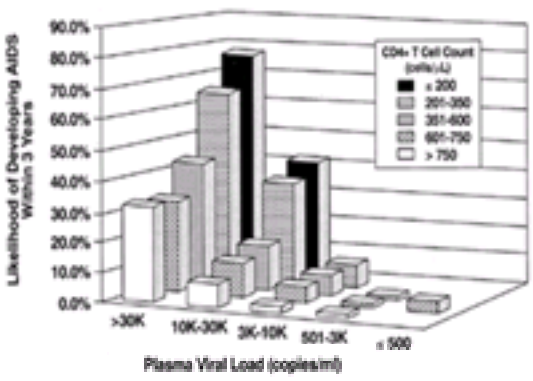

FIG. 14. Likelihood of developing acquired immunodeficiency syndrome (AIDS) within 3 years, given indicated levels of plasma viremia and peripheral blood CD4+ T-cell counts. Adapted from ref. 132, with permission.

Acute Infection

Although more than half of primary HIV infections (and perhaps up to 90%) are accompanied by symptoms, most of these infections are not recognized in the acute setting (481). The differential diagnosis of primary HIV infection is extremely broad, given the variable presentations associated with the syndrome. Fever, rash, pharyngitis, and lymphadenopathy are common during primary HIV infection, as they are in many “flu-like” and “mononucleosis-like” illnesses (397). Myalgias, arthralgias, diarrhea, nausea, vomiting, headache, hepatosplenomegaly, weight loss, thrush, and neurologic symptoms also may occur. Among patients who develop symptoms during primary HIV infection, the mean duration of symptoms is 3 weeks (281).

Plasma HIV RNA levels vary widely in primary HIV infection, from thousands to millions of copies per milliliter (482). After reaching a peak, plasma HIV RNA levels decline by about 6.5% per week over a 4-month period (482). A steep decline of 5.2 cells/µL/week occurs in the peripheral blood CD4+ T-cell count over the 5 months after primary infection (482). The peak level of HIV RNA may have prognostic value (355), and persistently high levels after 4 to 6 months add to the predictive power toward more rapid progression of disease (355,482).

Early-Stage Disease

The natural history of HIV infection includes a long period (i.e., approximately 10 years) of clinical latency between the time of primary infection and the development of symptoms indicative of advanced immunodeficiency. Even during the period of clinical latency, many patients experience fatigue as well as generalized lymphadenopathy. Syndromes indicative of depressed cell-mediated immunity in HIV infection that are not AIDS defining generally begin to appear as the CD4+ T-cell count falls below 500 cells/µL. Examples include oropharyngeal and recurrent vulvovaginal candidiasis, bacillary angiomatosis (usually due to infection with *Bartonella henselae*), recurrent or multidermatomal herpes zoster, listeriosis, infections due to *Rhodococcus equi,* pelvic inflammatory disease, oral hairy leukoplakia associated with EBV, cervical dysplasia (usually associated with human papillomavirus infection), constitutional symptoms such as unexplained fever or diarrhea lasting more than 1 month, idiopathic thrombocytopenic purpura, and peripheral neuropathy (70).

Late-Stage Disease

As the CD4+ T-cell count decreases below the level of approximately 200 cells/µL, the loss of integrity of cell-mediated immune responses allows ubiquitous environmental organisms with limited virulence (e.g., *Pneumocystis carinii* and *Mycobacterium avium*) to become life-threatening pathogens. Conditions indicative of severely depressed cell-mediated immunity due to HIV infection constitute the CDC surveillance case definition of AIDS (see Table 3 and reference 70). HIV-associated diseases indicative of severe impairment of cell-mediated immunity but not included in the current case definition of AIDS include chronic microsporidiosis, gastrointestinal infection with *Cyclospora cayetanensis,* disseminated *Penicillium marneffei* infection (endemic to southeast Asia), cerebral or disseminated *Trypanosoma cruzi* infection (endemic to Latin America), relapsing or chronic visceral leishmaniasis, anal carcinoma, and EBV-positive cases of leiosarcoma, leiomyosarcoma, and Hodgkin's disease (4). Nearly all of these HIV-associated illnesses, including the malignancies, are now known to be caused by infection with or reactivation of opportunistic organisms. In this regard, human papillomaviruses, EBV, and human herpesvirus-8 appear to play causative roles in the AIDS-defining malignancies, cervical cancer, lymphomas, and Kaposi's sarcoma, respectively. The degree to which an infection or reactivation syndrome can be considered opportunistic can be inferred by the CD4+ T-cell counts at which it occurs

(344). Oral candidiasis and tuberculosis tend to occur as the CD4+ T-cell count decreases into the 250 to 500 cells/μL range. Cryptosporidiosis generally does not occur until the CD4+ T-cell count is less than 200 cells/μL. *Pneumocystis carinii pneumonia,* disseminated *Mycobacterium avium* complex infection, cryptococcosis, and toxoplasmosis are indicative of more severe immunodeficiency. At the more extreme end of the opportunistic disease spectrum, cytomegalovirus (CMV) retinitis is usually diagnosed after the CD4+ T-cell count has decreased to less than 50 cells/μL. The steep increase in the risk of developing AIDS-defining illnesses associated with a CD4+ T-cell count of less than 200 cells/μL led to the 1993 revision of the CDC definition of AIDS, which includes a low CD4+ T-cell count (<200 cells/μL) as an AIDS-defining criterion (70).

The incidence of AIDS-defining illnesses in industrialized nations has decreased sharply since 1996. In the United States, the AIDS mortality rate decreased 68% between 1996 and 1999 (Fig. 15) (71,72). These decreases in AIDS-associated mortality can be attributed to a number of factors, including continued progress in prophylaxis against opportunistic infections (Table 4) (17), improved access to health care, and improved antiretroviral therapies (see later).

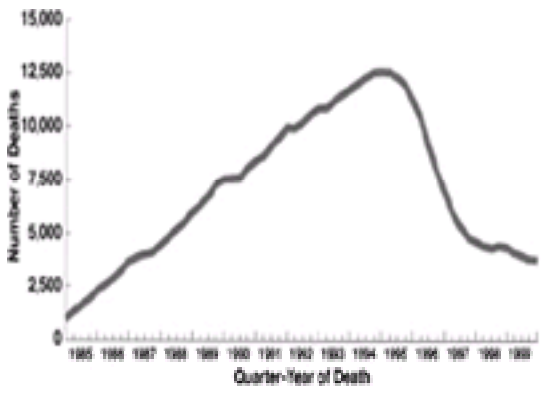

FIG. 15. Numbers of acquired immunodeficiency syndrome (AIDS) deaths in the United States from January 1985 through December 1999. Substantial declines in AIDS-related mortality followed the introduction of potent protease inhibitors in late 1995. From ref. 71, with permission.

Pathogen	Indication	Primary regimen	Alternative
Pneumocystis carinii	CD4+ count <200/μL or thrush	Trimethoprim-sulfamethoxazole	Dapsone; dapsone + pyrimethamine + leucovorin; aerosolized pentamidine; atovaquone
Mycobacterium tuberculosis	Tuberculin skin test reaction ≥5 mm, or prior positive test untreated, or contact with active case	Isoniazid + pyridoxine; rifampin + pyrazinamide	Rifabutin + pyrazinamide; rifampin
Mycobacterium avium complex	CD4+ count <50/μL	Azithromycin; clarithromycin	Rifabutin; rifabutin + azithromycin
Toxoplasma gondii	CD4+ count <100/μL and IgG antibody to *Toxoplasma*	Trimethoprim-sulfamethoxazole	Dapsone + pyrimethamine + leucovorin; atovaquone + pyrimethamine + leucovorin
Varicella zoster virus	Exposure to chickenpox or shingles without historic or serologic evidence of immunity	Varicella zoster immune globulin	—
Streptococcus pneumoniae	All HIV+ patients	Pneumococcal vaccine	—
Hepatitis B virus	All susceptible HIV+ patients (anti-HBc negative)	Hepatitis B vaccine	—
Influenza virus	All HIV+ patients	Influenza vaccine	—
Hepatitis A virus	All anti-HAV negative HIV+ patients with chronic hepatitis C	Hepatitis A vaccine	—

IgG, immunoglobulin G.

TABLE 4. *Prophylaxis recommendations to prevent opportunistic diseases*

Determinants of the Rate of Disease Progression

Myriad host and virologic factors determine the extremely variable rates of disease progression that are observed among HIV-infected individuals. A small percentage of HIV-infected individuals experience no evidence of disease progression over a prolonged period; these long-term nonprogressors provide clues regarding factors associated with disease progression. Although a minority of cases of long-term nonprogressive HIV infection may be associated with attenuated strains of HIV (123,264,286,366), most data suggest that viral attenuation is rare among long-term nonprogressors, and that host factors play a dominant role in determining the state of nonprogression (58,260,365,426).

Host genetic factors strongly influence the rate of disease progression in HIV infection. A number of different mechanisms may be responsible for the observed associations between certain HLA haplotypes and different rates of HIV disease progression (63,275,276,288). The ability of certain HLA molecules to present immunodominant viral epitopes efficiently to generate cell-mediated immune responses may explain an association with slow disease progression. Conversely, other HLA haplotypes may preclude an effective cell-mediated antiviral response and may therefore be associated with more rapid disease progression (63).

Genetic polymorphisms in the HIV coreceptors and their ligands may also significantly affect the course of HIV disease progression. Individuals who are homozygous for a 32-base pair inactivating mutation within the *CCR5* gene (*CCR5-D32*) are afforded a high degree of protection against HIV infection (124,259,477,617), whereas HIV-infected heterozygotes are partially protected against disease progression (110,124,259,364,617). Other mutations that limit the availability of functional coreceptors also are associated with protection against HIV infection and/or partial protection against disease progression. A rare point mutation in the *CCR5* gene (m303) results in a premature stop codon, preventing expression of a functional coreceptor; the *CCR5-D32/m303* genotype confers a high degree of resistance to HIV infection, similar to that afforded by homozygosity for *CCR5-D32* (450). A mutation in the *CCR2* gene involving a conservative amino acid change in a transmembrane domain of the receptor results in a significant delay in disease progression among HIV-infected individuals (510). The mechanism responsible for this observation appears to be related

to the ability of the mutant form of the CCR2 protein to form heterodimers with CXCR4 and CCR5, thus limiting the functional availability of these important HIV coreceptors (354).

Polymorphisms in the *CCR5* promoter have complex associations with HIV disease progression. Two groups have demonstrated that a polymorphism in the *CCR5* promoter is associated with disease acceleration (341,348). Another group has demonstrated that *CCR5* alleles that are partially defined by promoter polymorphisms have variable race-specific effects on disease progression (203,385).

A mutation in the 3' untranslated region of the *SDF-1* gene, *SDF1-3'A,* may be associated with high levels of expression of SDF-1, thereby protecting against disease progression in HIV-infected individuals harboring X4 strains of virus (583). Similarly, a polymorphism in the *RANTES* promoter is associated with enhanced transcription of the *RANTES* gene and retards the rate of HIV disease progression (321).

Genetic polymorphisms in the chemokine–chemokine receptor axis genes represent only one of a number of possible mechanisms responsible for resistance to HIV infection or delayed disease progression. In this regard, down-regulation of coreceptor expression and/or up-regulation of chemokines may recapitulate phenotypes similar to those created by the genetic polymorphisms described earlier. Levels of CCR5 expression exhibit considerable interindividual variability, and these levels correlate with infectability of cells with R5 strains of HIV *in vitro* (592). CCR5 expression occurs predominantly in previously activated, memory T cells (44); treatment of cells with IL-2 slowly up-regulates CCR5 expression (44), whereas stimulation of cells through CD3 and CD28 abrogates CCR5 expression (64). In contrast to CCR5, CXCR4 is expressed on naive, resting T cells (44), and its regulation is reciprocal to that of CCR5 in many respects (44,64). Whether constitutive low-level expression of these coreceptors is associated with a favorable prognosis in HIV infection remains to be determined; in this regard, the more favorable prognosis of *CCR5-D32* heterozygotes should be relevant. A role for high-level expression of HIV coreceptor ligands, such as the CC-chemokines, in protecting against disease progression has been suggested by a number of studies (171,475,480,546,601).

Quantitative as well as qualitative aspects of the host immune response that is mobilized against HIV are important determinants of the rate of disease progression as well. Studies of long-term nonprogressors and exposed-uninfected individuals indicate important roles for CTL, CD8+ T-cell suppressor factors, CD4+ T-helper responses, ADCC, neutralizing antibody responses, and mucosal humoral immune responses in protecting against HIV infection or disease progression (see earlier: Immune Responses).

The morphologic abnormalities of lymphoid tissue associated with HIV disease progression are important determinants of immunodeficiency (420). Despite the long period of HIV infection in long-term nonprogressors, histopathologic examination of lymph node biopsies from these individuals revealed only mild HIV-related abnormalities such as follicular hyperplasia (422). Follicular involution, fibrosis, and lymphocyte depletion, associated with progressive HIV disease, were lacking in lymph nodes from nonprogressors. The degree of follicular hyperplasia, and thus lymphoid tissue activation, seen in nonprogressors is significantly lower compared with that seen in progressors and qualitatively distinct as well, without evidence of large geographic germinal centers extending into the nodal medulla (422). It is likely that preservation of lymphoid architecture in nonprogressors is a reflection of the lower levels of viral replication over time in these individuals. Regardless of the mechanisms responsible for lower levels of viral replication in nonprogressors, preservation of lymphoid tissue architecture is a critical component of the immunocompetence observed. This further highlights the need to understand the mechanisms reponsible for the destruction of lymphoid tissue architecture during progression of HIV disease. If immunorestorative strategies in advanced HIV infection are to be successful, a substrate for the generation of immune responses (i.e., intact lymphoid tissue) must be present, necessitating the prevention or reversal of the histopathologic abnormalities of lymphoid tissue associated with HIV disease progression.

DIAGNOSIS AND LABORATORY MONITORING

Serologic tests for antibodies to HIV form the mainstay of laboratory diagnosis of HIV infection. A solid-phase EIA containing most HIV antigens has a sensitivity of more than 99.5% for the detection of antibodies to HIV. Although the EIA is extremely sensitive, false positives are not uncommon in a number of underlying conditions; therefore, a positive EIA test must always be followed by a confirmatory test. A Western blot assay is the most reliable confirmatory serologic test for the diagnosis of HIV infection. In this assay, HIV antigens are separated by electrophoresis on the basis of their molecular weight and then transferred to a nitrocellulose membrane; after incubation with test serum, antibody reactivity to individual HIV components can be assessed. A negative Western blot demonstrates the absence of any reactivity against HIV components. A positive test demonstrates reactivity to at least two of the following HIV proteins: p24, gp41, gp120, and gp160 (16). Indeterminate tests may be the result of reactivity to antigens that cross-react with HIV determinants; however, indeterminate results also may be a reflection of recently acquired infection and an evolving antibody response. Therefore, clinical follow-up and repeated serologic testing is essential in these cases.

The ideal laboratory marker of HIV disease activity should be easily and reproducibly measurable in all individuals with the disease. Furthermore, it should worsen with progression of disease and improve with positive responses to therapy. Although no ideal marker currently exists, several have been of critical importance in the clinical management of patients, especially with regard to establishing standards of care for initiation of antiretroviral therapy and prophylaxis against opportunistic infections.

Markers of Immune Depletion and Immune Dysregulation

Because CD4+ T cells are the primary targets of HIV infection and because depletion of CD4+ T cells is the immunologic hallmark of HIV disease progression, measurement of CD4+ T cells should be an excellent marker of disease progression. Indeed, studies of the natural history of HIV infection demonstrate that the CD4+ T-cell count is a powerful predictor of the short-term risk of developing an AIDS-defining illness (344). CD4+ T-cell counts frequently increase in response to antiretroviral therapy, and this salutary response has been used as a criterion in the licensing of new

anti-HIV drugs.

Markers of generalized immune activation such as serum levels of b $_2$-microglobulin and neopterin also are valid markers of HIV disease progression. b $_2$-Microglobulin is an 11-kd protein that associates with MHC class I molecules on the surface of most nucleated cells in the body. Serum levels of free b $_2$-microglobulin derive mainly from lymphocytes and are elevated in conditions associated with lymphocyte activation or destruction such as lymphoproliferative syndromes, autoimmune diseases, and viral infections. A direct correlation exists between serum levels of b $_2$-microglobulin and the risk of progression to AIDS (12). Neopterin is a metabolite of guanosine triphosphate, which is produced largely by lymphocytes and by cells of the monocyte/macrophage lineage during tetrahydrobiopterin synthesis. Elevated levels of serum neopterin are seen in patients with autoimmune diseases and viral infections. Like b $_2$-microglobulin, an elevated serum neopterin level is a predictor of progression to AIDS independent of the CD4+ T-cell count (303,358). The combination of a CD4+ T-cell count plus either a b $_2$-microglobulin or neopterin level has more predictive value for disease progression than does any of the three measurements taken individually (149).

The extracellular domains of the receptors for TNF-a are released from the cell membrane by proteolytic cleavage and are detectable in soluble form in serum. Levels of these soluble TNF receptors may reflect levels of TNF-a activity. Insofar as TNF-a has a critical role in up-regulating HIV replication, it is not surprising that levels of soluble TNF receptors serve as a valid surrogate marker of HIV disease progression. In separate studies, levels of soluble TNF receptor type II were found to correlate strongly with plasma viremia and with progression to AIDS (193); however, levels of soluble TNF receptors do not appear to serve as reliable markers of therapeutic efficacy during antiretroviral therapy (169).

A number of other indicators of immune dysregulation correlate with HIV disease progression. The diminished proliferative response of T cells to anti-CD3 antibodies *in vitro* serves as an indicator of immunodeficiency (368), as does diminished delayed-type hypersensitivity response to recall antigens *in vivo* (43). The percentage of peripheral blood CD8+ T cells bearing the activation marker CD38 is positively correlated with disease progression (190). CD8+CD38$^+$ cells mediate cytolytic activity, and increased numbers of these cells in the chronic phase of HIV infection likely reflect a high viral load. Additional markers of HIV disease activity include hematocrit, and serum levels of immunoglobulin A, TNF-b, Fas, and 1,25-vitamin D (237,353,444).

Markers of Viral Load

Before the development of currently available sensitive molecular techniques for the quantitation of HIV RNA molecules, direct culture of HIV from plasma or mononuclear cells was found to correlate with stage of disease (116,250). Changes in levels of culturable plasma viremia further serve as reliable indicators of activity of antiretroviral agents (120); however, these assays are extremely labor intensive and less sensitive than currently available molecular diagnostic tests.

During viral replication, the viral core protein p24 is shed into plasma and can be measured by enzyme-linked immunosorbent assay (ELISA). Measurement of p24 antigenemia is relatively insensitive; however, positivity of a p24 antigen test does herald accelerated decline in CD4+ T-cell counts and the likelihood of progression to AIDS (331). Although a positive test for p24 antigenemia has prognostic value with regard to progression of disease, changes in levels of p24 antigenemia during antiretroviral therapy do not serve as an accurate surrogate for therapeutic efficacy during antiretroviral therapy (126).

The frequency of HIV-infected cells can be quantified by using PCR for detection of HIV-1 DNA, and this measurement has some predictive value with regard to HIV disease progression. Increases of approximately one to two orders of magnitude in the frequency of HIV-infected cells in peripheral blood typically occur among patients who progress to AIDS (489).

Measurement of cell-associated HIV mRNA in peripheral blood, although technically demanding, has been shown by several groups to serve as an independent predictor of disease progression. Particularly impressive is the ability of this marker to predict disease progression in patients with early-stage HIV disease and relatively high CD4+ T-cell counts (476). Determination of the splicing pattern of HIV mRNA in PBMCs can add further predictive value with regard to disease progression. Thus although levels of multiply spliced and unspliced HIV mRNAs increase with disease progression, a disproportionate increase in unspliced HIV mRNA is most predictive of accelerated loss of CD4+ T cells and clinical disease progression (367). Studies of the effect of antiretroviral therapy on the frequency of infected cells in lymphoid tissue, a major *in vivo* reservoir and site of HIV replication, demonstrated that decreases in levels of unspliced HIV mRNA correlated well with decreases in plasma viremia during therapy (111). This finding, combined with the fact that HIV mRNAs are orders of magnitude more abundant in lymph node mononuclear cells compared with peripheral blood mononuclear cells, supports the concept that changes in plasma viremia during antiretroviral therapy are reflective of changes in cell-associated viral mRNA production in lymphoid tissue (111).

The ability to assess the level of viral replication with quantitative assays of HIV RNA in plasma (i.e., plasma viremia) has revolutionized the use of laboratory markers for HIV disease. Measurement of the CD4+ T-cell count yields information regarding the degree of immunodeficiency and short-term risk of opportunistic disease (i.e., a reflection of the damage already sustained by the immune system). In contrast, quantitation of HIV RNA in plasma yields information that predicts the rate and severity of immune deficiency. Technology currently available for reproducible measurement of plasma viremia in clinical specimens includes PCR, nucleic acid sequence–based amplification (NASBA), and branched DNA (b-DNA) assays. PCR and NASBA are both cycle-based target-amplification systems, whereas b-DNA is a single-step signal-amplification system that uses multiple HIV-specific nucleic acid target probes, each of which can be bound to an amplifier molecule containing multiple oligonucleotides. Each oligonucleotide in this array can in turn hybridize to an enzyme-labeled complementary probe, and detection is accomplished by addition of substrate. Each of these assays (PCR, NASBA, and b-DNA) is able to detect as few as approximately 20 to 50 molecules of HIV RNA per milliliter of

plasma, and each has a dynamic range of several orders of magnitude.

The recognition that high levels of HIV replication are sustained throughout the course of infection resulted from a convergence of several lines of data. The enhanced sensitivity of molecular-amplification assays compared with conventional viral detection techniques, and applications of these assays to lymphoid tissue were important developments in this regard (26,142,419,432). The predictive value of plasma viremia as a marker for disease progression was demonstrated in the Multicenter AIDS Cohort Study, in which individuals were stratified by quartiles according to baseline plasma viremia levels. The proportion of patients who progressed to AIDS within 5 years after entry into the study with plasma viremia levels in the lowest through highest quartiles was 8%, 26%, 49%, and 62%, respectively (see Fig. 14) (357). Plasma viremia also was shown to predict progression of disease independent of CD4+ T-cell counts; this measurement was superior to the predictive value of CD4+ T-cell counts (357). Further validation of the use of plasma viremia measurement as a predictor of disease progression was provided by the AIDS Clinical Trials Group, which found that baseline measurements of plasma viremia as well as changes in viremia over time were predictive of disease progression (115,576).

Plasma viral load serves as a valid marker not only for predicting disease progression but also for monitoring therapy in HIV-infected individuals. The link between decreased plasma viremia in individuals receiving therapy and improved clinical outcome was established by the Veteran's Affairs Cooperative Study Group on AIDS (400). Data indicating that decreases in plasma viremia during therapy are reflective of decreases in viral replication in lymphoid tissue further validate the use of plasma viremia as a laboratory marker of a critical aspect of HIV pathogenesis (111).

Levels of plasma viremia become predictive of disease progression after stabilization of viremia (i.e., achievement of a viral load “setpoint”) in the months after primary HIV infection. Mellors et al. (355) found that the level of plasma viremia at 6 months after seroconversion was significantly higher in patients who developed AIDS within approximately 5 years of follow-up compared with levels in those who did not develop AIDS during the same period. A plasma viremia level of >100,000 HIV RNA copies/mL at 6 months after seroconversion was associated with an odds ratio of 10.8 for the development of AIDS. Schacker et al. (482) found that levels of plasma viremia stabilize at a later time than did that reported by Mellors et al. Plasma viremia levels obtained 0 to 6 months after seroconversion did not correlate with subsequent clinical outcome; however, the level of plasma viremia at 7 to 12 months after seroconversion was a strong predictor of CD4+ T-cell depletion during follow-up.

The development of sensitive, quantitative assays for plasma viremia, and the ability of this marker to predict progression of disease and to serve as an indicator of therapeutic efficacy has added an important dimension to decisions regarding initiation and maintenance of antiretroviral therapy in HIV-infected individuals. The variation in plasma viremia by several orders of magnitude in patients with early-stage HIV disease with similar CD4+ T-cell counts allows for the initiation of early therapeutic intervention in those who are most likely to progress. Current recommendations regarding the initiation and maintenance of antiretroviral therapy rely heavily on the two best laboratory markers for HIV disease progression, the CD4+ T-cell count and the level of plasma viremia (59,132).

ANTIRETROVIRAL THERAPY

Recent progress in understanding the pathogenesis of HIV disease and the development of potent antiretroviral agents have resulted in an abundance of treatment options for HIV-infected individuals. The recognition that virus continuously replicates in lymphoid tissue throughout the course of HIV infection, even during the clinically latent stage, strengthened the rationale for early intervention (142,419). Quantitative estimates of the extraordinary rate of viral replication *in vivo* have further strengthened the rationale for early therapy with combination antiretroviral regimens (251,429,568). Combinations of antiretroviral agents that are capable of profoundly suppressing levels of plasma viral load for prolonged periods are referred to as HAART. Significant declines in AIDS-related morbidity and mortality are due in large measure to the wide use of HAART (371,413). However, the optimal use of antiretroviral agents remains a rapidly evolving field, with many obstacles that must be addressed.

Viral Replication Cycle and Antiretroviral Agents

A number of steps in the viral replication cycle can be targeted by agents that are either currently available or under investigation (Fig. 16). Previous disappointment with the use of soluble CD4 as a means of inhibiting viral attachment and entry has yielded to optimism that interference with viral coreceptors may represent an effective therapeutic modality targeted to the earliest stage of the viral life cycle (25). Another promising strategy targets the conformational changes in gp41 that are necessary for fusion of HIV with the target cell membrane (280).

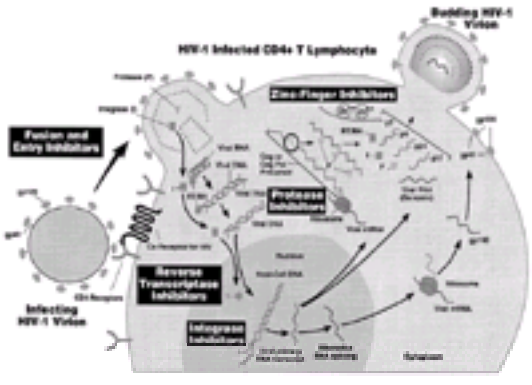

FIG. 16. Targets of antiretroviral therapy. Drugs that are currently licensed for use in human immunodeficiency virus (HIV) infection include reverse transcriptase inhibitors and protease inhibitors. In development are inhibitors of HIV entry and fusion, inhibitors of HIV integrase, and zinc-finger inhibitors.

Inhibitors of viral RT were the first agents to be developed for the treatment of HIV infection; they remain important components of the anti-HIV therapeutic armamentarium. Most RT inhibitors are nucleoside analogs; these agents act as DNA chain terminators by virtue of their structures that lack the 3' hydroxyl moiety necessary for chain elongation. Nonnucleoside RT inhibitors also are used clinically. RT inhibitors as a group have several disadvantages, including their association with multiple neurologic and hematologic side effects, the rapidity with which they select for resistant viral variants when used alone, and their lack of effect on cells already infected with HIV, which no longer require RT to complete the viral replication cycle.

HIV-1 protease cleaves the Gag precursor polyprotein into the p24 and p17 virion components necessary for viral infectivity. Development of HIV-1 protease inhibitors based on the crystal structure of the protease was a triumph for rational and targeted drug design (393,585). The combination of agents that can inhibit *de novo* infection of cells (i.e., RT inhibitors) with agents that lead to the release of defective, noninfectious virions from cells already infected by HIV (i.e., protease inhibitors) results in potent antiviral activity. Protease inhibitors have rapidly become drugs of choice for the treatment of HIV infection in combination with RT inhibitors.

Integration of reverse-transcribed viral DNA (provirus) into the host DNA is facilitated by the viral enzyme integrase. The determination of the crystal structure of integrase (141) has stimulated a search for inhibitors of this enzyme, which we hope will lead to the availability of clinically useful agents.

Targeting the “accessory” proteins of HIV has been hampered by the difficulty in delineating the precise biochemical activities of these proteins. A clinical trial of the Tat antagonist Ro24-7429 failed to demonstrate antiviral activity for this agent (236); however, agents targeting the HIV-1 Rev and Nef proteins are being developed.

Treatment of Primary HIV Infection

Treatment of HIV infection before its wide dissemination to lymphoid tissue and hence before establishment of the viral load setpoint is an approach supported by current understanding of the pathogenesis of early events in HIV infection (270). Unfortunately, the pool of resting, latently infected CD4+ T cells appears to be established during the very earliest stages of acute infection; this process is not abrogated even by initiation of therapy during the symptomatic phase of acute infection (85). Although these data militate strongly against the possibility of eradicating HIV before its wide dissemination during primary infection, the window of opportunity for treatment of primary HIV infection may actually extend to 12 to 18 months after acute infection; this possibility is based on the persistence of high numbers of productively infected cells in lymphoid tissue during this period, despite attainment of the apparent setpoint of plasma viremia (415).

A trial of zidovudine therapy versus placebo given for 6 months after primary HIV infection revealed that those patients assigned to active treatment maintained higher CD4+ T-cell counts, lower levels of plasma viremia, and slower progression to minor clinical end points (e.g., oral candidiasis, herpes zoster, and oral hairy leukoplakia) over a mean follow-up period of 15 months (282). Treatment of patients with recent (i.e., within 90 days) HIV infection with a combination of zidovudine, lamivudine, and a protease inhibitor can suppress plasma viremia to <20 HIV RNA copies/mL for a prolonged period (>2 years) (340); initiation of HAART during acute HIV infection also may prevent the loss of HIV-specific CD4+ T-cell proliferative responses (466). Emerging data suggest that initiation of HAART during acute HIV infection affords significant protection against the development of opportunistic infections and rapid progression to AIDS during a 1.5-year follow-up period (39).

Treatment of Chronic HIV Infection

Early studies of zidovudine monotherapy demonstrated improvements in laboratory markers of HIV disease activity and delayed progression of disease in symptomatic individuals, as well as in asymptomatic individuals with relatively low CD4+ T-cell counts (158,159,556,557,596). Subsequent studies demonstrated that combination therapy with two nucleoside analogs was generally superior to monotherapy (112,129,210,230).

More potent combination antiretroviral drug regimens, such as zidovudine plus lamivudine, which are capable of prolonged suppression of viral replication, became available in 1995 (144). The addition of protease inhibitors to the antiretroviral armamentarium in 1996 resulted in a dramatic enhancement of the ability to suppress viral replication (Fig. 17). The protease inhibitors saquinavir, ritonavir, indinavir, nelfinavir, amprenavir, and lopinavir are all potent antiretrovirals, particularly when used in combination with nucleoside analog RT inhibitors (NRTIs). Such combination therapy is now considered the treatment of choice for HIV infection (132). When used in combination with NRTIs, protease inhibitors typically suppress plasma viral load to undetectable levels in the majority of patients for >48 weeks. In addition, significant reductions in the rate of progression to AIDS or death have been demonstrated for combination regimens including indinavir or ritonavir (55,228).

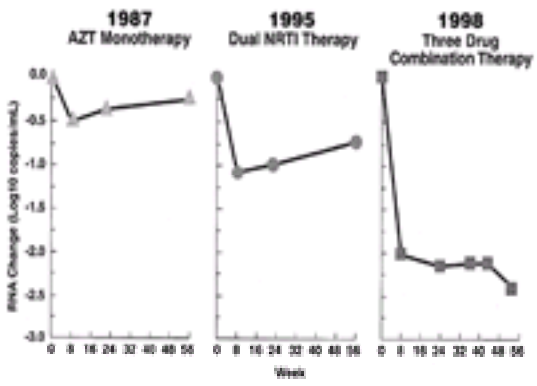

FIG. 17. Representative declines in plasma viral load achieved with zidovudine monotherapy (**left**), dual nucleoside analog reverse transcriptase inhibitor (NRTI) therapy (**middle**), and three-drug combination therapy (**right**).

The decision to initiate antiretroviral therapy should be made jointly by patient and physician. The decision should be driven in part by data regarding the natural history of disease progression at various levels of CD4+ T-cell counts and plasma viral loads (356,357). Treatment is indicated for patients with symptomatic HIV disease regardless of CD4+ T-cell count and viral load (132). Treatment for asymptomatic patients is generally indicated when the CD4+ T-cell count is <350 cells/μL or the plasma viral load is >30,000 copies/mL (132). Some physicians and patients choose to initiate antiretroviral therapy earlier than recommended in official guidelines. Risks and benefits of early initiation of antiretroviral therapy are presented in Table 5; however, unfortunately, no data are yet available regarding the effects of early initiation of HAART on delaying disease progression or survival.

Potential benefits
• Control of viral replication and mutation; reduction of viral burden
• Prevention of progressive immunodeficiency; potential maintenance or reconstruction of a normal immune system
• Delayed progression to AIDS and prolongation of life
• Decreased risk of selection of resistant virus
• Decreased risk of drug toxicity
• Possible decreased risk of viral transmission
Potential risks
• Reduction in quality of life from adverse drug effects and inconvenience of current maximally suppressive regimens
• Earlier development of drug resistance
• Transmission of drug-resistant virus
• Limitation in future choices of antiretroviral agents due to development of resistance
• Unknown long-term toxicity of antiretroviral drugs
• Unknown duration of effectiveness of current antiretroviral therapies

TABLE 5. *Risks and benefits of early initiation of antiretroviral therapy in the asymptomatic HIV-infected patient*

Many treatment options now exist for the HIV-infected patient. A regimen should be selected that will afford a high likelihood of long-term profound suppression of plasma viral load and a significant increase in the CD4+ T-cell count. Many other factors deserve consideration, including the pill burden associated with a particular regimen, dosing frequency, food requirements, convenience, toxicity, and drug-interaction profile. Recommendations regarding specific regimens can be found in Table 6 and in the Department of Health and Human Services/Henry J. Kaiser Foundation Guidelines for the Use of Antiretroviral Agents in HIV-Infected Adults and Adolescents on the worldwide web at http://www.hivatis.org/.

TABLE 6. *Recommended antiretroviral agents for the treatment of established HIV infection*

A change in the antiretroviral regimen may be indicated when a suboptimal reduction in viremia occurs after initiation of therapy, when viremia reappears after suppression to undetectable levels or increases from a nadir of suppression, or when the CD4+ T-cell count declines significantly. When any of these events occurs, it is important to determine whether the underlying cause may be drug intolerance or difficulty with adherence to the drug regimen, suboptimal drug levels due to malabsorption, or drug–drug or drug–food interactions, or development of drug resistance. If drug resistance is suspected, it is appropriate to change the regimen entirely to drugs that have not been used previously and to which cross-resistance is not anticipated. More targeted changes within a drug regimen may be guided by the use of resistance

assays (see later); however, this approach requires further clinical validation. If a regimen is changed because of intolerance or toxicity, it is appropriate to substitute one or more alternative drugs of the same potency and from the same class of agents as the agent suspected to be causing the problem.

Although sustained suppression of viral load is achievable in a high proportion of patients treated with HAART regimens in the clinical trials setting (i.e., nearly 80% at 2 years) (225), only 50% or fewer respond adequately in urban clinic settings (125,326). Predictors of success include lower baseline viral load, higher baseline CD4+ T-cell count, initiation of therapy with multiple drugs simultaneously, and adherence to the drug regimen (125,225,310,326,370,375,524).

Although the use of HAART regimens has greatly improved the prognosis for HIV-infected individuals (371,413), a large number of challenges to the optimal treatment of HIV infection remain to be addressed.

Adherence

Adherence to an antiretroviral regimen is essential to the success of the regimen. The challenges to adherence are considerable. Antiretroviral regimens are complex, consist of multiple agents, and must be taken indefinitely in most cases. Many of the drugs have significant side effects and toxicities (see later). The sheer number of pills required in many regimens (i.e., >20 pills per day) poses a significant challenge to adherence for even the most committed individuals. In addition, many antiretroviral agents, particularly protease inhibitors, have complex dosing requirements in relation to food intake.

Lapses in adherence provide windows of opportunity for virus replication, and enhance the probability of the emergence of drug-resistant virus. In the INCAS drug trial of nevirapine, zidovudine, and didanosine, missed doses of antiretroviral drugs on 28 or more days during the 52-week study significantly predicted a lower likelihood of achieving durable viral suppression (375). In a study of antiretroviral therapy in an urban clinic setting, missed clinic appointments were significantly associated with failure to suppress viral load over a 1-year period (326).

Side Effects and Toxicities

Gastrointestinal disturbances, such as nausea, vomiting, and/or diarrhea, are commonly induced by several of the NRTIs, and by all of the protease inhibitors. Peripheral neuropathy may be caused by most of the NRTIs, and rash and Stevens–Johnson syndrome may occur with use of any of the NNRTIs. Protease inhibitors have a number of drug-specific toxicities, such as the association of nephrolithiasis and indirect hyperbilirubinemia with indinavir; nausea, vomiting, and diarrhea and paresthesias with ritonavir; nausea and diarrhea with saquinavir; and diarrhea with nelfinavir. In addition, several class-specific adverse effects are associated with the use of protease inhibitors. In this regard, a number of metabolic derangements involving lipid metabolism are of serious concern with the use of protease inhibitors. Triglyceride and cholesterol levels may become elevated to dangerously high levels (61,384). Insulin resistance, hyperglycemia, and even frank diabetes mellitus may occur (61,384,562). Additionally, peripheral fat wasting in the setting of central fat accumulation, as well as “buffalo humps” reminiscent of Cushing's disease may occur (61,562). These metabolic derangements may be related to homology between HIV protease and two proteins, cytoplasmic retinoic-acid binding protein type 1 and low-density lipoprotein receptor–related protein that regulate lipid metabolism (60). Finally, a large number of drug interactions may occur with the use of NNRTIs and protease inhibitors, all of which alter cytochrome P-450 metabolism.

Resistance

Failure or loss of suppression of plasma viremia in the setting of good adherence and adequate drug absorption may be due to the emergence of drug-resistant virus. A single nucleotide substitution at position 103 of the reverse transcriptase gene can significantly reduce the activity of the NNRTIs against HIV replication. Similarly, a single nucleotide substitution at position 184 of the reverse transcriptase gene confers resistance to lamivudine. Resistance to some of the NRTIs, such as zidovudine, follows a relatively orderly sequential path whereby accumulation of serial mutations in the reverse transcriptase gene confers increasing levels of resistance to the agent (307); for other drugs in this class, the pathways to resistance are less clear (346).

Resistance to protease inhibitors is a complex phenomenon that has important implications for drug therapy. The HIV protease is a 99-amino-acid protein; that over half of these residues have been reported to be associated with drug resistance is testimony to the genetic plasticity of HIV. Some of these mutations are strongly selected under the pressure of protease inhibitor therapy; however, even when drugs select for unique mutations associated with drug resistance, a great deal of *in vitro* and clinical cross-resistance may still be observed between drugs (484,584). Other mutations are compensatory in nature, and are able to restore partially the replicative fitness that is lost with primary mutations. Distant mutations in the protease cleavage site in the HIV p7/p1 core components create a more optimal substrate for a protease enzyme that is compromised by protease inhibitors (613).

Genotypic and phenotypic resistance assays are now available from commercial sources (247). Genotypic assays generally provide information about the presence of mutations in HIV genes (i.e., reverse transcriptase and protease) that may have been selected for during drug therapy. Phenotypic assays evaluate the replication of virus or of a viral construct containing genes from the patient's virus in the presence of antiretroviral drugs. Interpretation of these assays is fraught with difficulty (247). The tests are insensitive to minor viral variants that may be important *in vivo.* In this regard, viral variants harboring resistance mutations to drugs given in the past may be present at frequencies well below the limits of detectability using these assays. Thus although a positive resistance assay argues strongly that a particular drug will be ineffective, a negative test by no means assures the drug's utility (247).

Preliminary data suggest that resistance testing can be used to optimize the design of antiretroviral regimens. In this regard, one study demonstrated that viral load suppression over a 3- to 6-month period was superior when genotypic resistance assays were used in helping to formulate antiretroviral regimens (138). Another study showed that phenotypic

drug resistance may be detectable before viral load rebound becomes evident during drug treatment (425). These data suggest that drug-resistance assays will likely be an important adjunct in the formulation of antiretroviral regimens in the future.

Persistent Viral Replication

The beginning of the HAART era saw a great deal of optimism that with prolonged suppression of viral load, eradication of HIV infection might be possible. However, Chun et al. (90) reported the presence of unintegrated HIV DNA in lymphocytes from HAART-treated patients who had experienced sustained suppression of plasma viral load for an average of 10 months; because of the short half-life of unintegrated viral DNA, this finding argued strongly that ongoing low-level HIV replication was occurring in spite of undetectable levels of plasma viral load. Support for this same conclusion came from studies showing persistence of HIV transcription in PBMCs from patients with sustained suppression of plasma HIV viral load (173,392). In addition, two other studies demonstrated evolution of proviral DNA in patients receiving HAART with undetectable viral loads, again indicating ongoing viral replication (342,611). Finally, the rapid rebound of plasma viral load that occurs after cessation of HAART after prolonged suppression of plasma viremia also likely indicates ongoing replication during HAART (183,234).

Persistent viral replication in the setting of apparently effective HAART may be due to intermittently suboptimal drug levels (1,526), poor penetration of drugs into various tissue compartments such as genital secretions (145,609), or suboptimal potency of the drug regimen. Clearly, more potent antiretroviral agents that are capable of inhibiting a broader array of HIV target gene products would be welcome additions to the antiretroviral armamentarium. In this regard, inhibitors of the viral entry and fusion process are in clinical trials (280), and inhibitors of the HIV integrase are in development.

Immune Reconstitution

Ultimately, the success of long-term antiretroviral therapy depends not only on suppression of viral replication, but also on at least partial restoration of immune function. Immune competence prevents the onset of HIV-related opportunistic diseases, and also may synergize with antiviral agents to control viral replication. The degree of immune reconstitution that occurs during HAART and the theoretic limits of such reconstitution are matters of intense investigation.

The increases in CD4+ T-cell counts that occur during potent antiretroviral therapy can be modeled to obtain an estimate of CD4+ T-cell turnover at steady state. Initial studies estimated that approximately 2×10^{9} CD4+ T cells turn over each day (251,568). However, the relative contributions of thymic output of new cells, peripheral expansion of preexisting cells, and redistribution between tissue and peripheral blood compartments remain uncertain.

One assay of CD4+ T-cell turnover measures telomeric terminal restriction fragment (TRF) length, a marker of cellular replicative history. After each round of cell division, telomeric sequence is lost, eventually leading to replicative senescence. Data obtained by analysis of telomere lengths do not suggest increased turnover of CD4+ T cells during HIV infection (414,588).

Cell turnover also can be assessed by analysis of incorporation of bromodeoxyuridine *ex vivo* into cellular DNA, quantification of cells expressing Ki67 antigen (a marker associated with cell proliferation), or by analysis of incorporation of infused deuterium into cellular DNA. Studies measuring incorporation of bromodeoxyuridine into lymphocytes *ex vivo* suggest that CD4+ T-cell proliferation is abnormally high in HIV-infected individuals, and is directly proportional to viral load (312). Initiation of HAART resulted in substantial decreases in rates of CD4+ T-cell proliferation measured in this manner (312). In contrast, dichotomous results have been obtained in studies using Ki67 as a marker of cell turnover. In patients with relatively high CD4+ T-cell counts and low levels of viral load, low percentages of CD4+Ki67+ cells are observed, with estimated total body CD4+ T-cell turnover estimated to be normal or moderately increased (161,474). In later stages of HIV disease, in the setting of low CD4+ T-cell counts and high viral load, substantial increases in the percentage of CD4+Ki67+ cells are seen in conjunction with estimated subnormal levels of total CD4+ T-cell production (474). Similar results have been obtained in deuterium infusion studies (243). Thus turnover data obtained by using bromodeoxyuridine suggest that CD4+ T-cell depletion is due not to inadequate T-cell production but to excessive destruction of CD4+ T cells. Turnover data obtained using Ki67 and deuterium suggest that CD4+ T-cell loss is partially compensated for in early-stage disease by a modest increase in production of these cells; however, in late-stage disease, CD4+ T-cell loss appears to result in part from inadequate lymphopoiesis.

Analysis of the phenotype of CD4+ T cells that increase after initiation of HAART reveals that most of the sharp increase in CD4+ T-cell count that is seen in the first weeks of therapy is due to an increase in the memory CD4+ T-cell pool, likely as a result of redistribution from tissue compartments (19,52,411). These findings are consistent with turnover studies using genetically marked cells, in which maintenance of CD4+ T-cell numbers was dependent on peripheral expansion of preexisting mature cells (561).

In many cases of successful HAART, slow increases in CD4+ T-cell numbers follow the sharp increase that occurs within the first weeks of therapy. This later slow increase is largely the result of appearance of naive cells (19,411). Partial normalization of perturbed T-cell receptor Vb repertoires during HAART suggested that some of these cells might be thymically derived cells, and that qualitative as well as quantitative immune reconstitution might be possible (204). Indeed, evidence from several groups suggests the presence of thymic function in HIV-infected adults. In this regard, the radiographic presence of thymic tissue in HIV-infected individuals was associated with higher CD4+ T-cell counts and higher percentages of naive cells compared with those in individuals who did not have detectable thymic tissue (347). Thymic function also can be probed by quantification of cells harboring T cell–receptor rearrangement excision circles (TRECs) that are created during thymocyte development. This type of analysis revealed that HIV-infected individuals have a significantly lower frequency of $TREC^{+}$ cells compared with normal controls, and that this frequency increases significantly during HAART; these increases in $TREC^{+}$ cells correlated with the decrease in viral load and increase in the

number of naive CD4+ T cells (136).

The degree of immune reconstitution that is possible during HAART is unknown. Some data cast doubt on any significant role for the thymus in HAART-related immune reconstitution (240), and one study found that the increased number of thymic emigrants that appear during HAART is insufficient to account for the increased number of naive cells (610). Although some HIV-induced immune damage may be reversible during HAART (614), clearly improvements are more robust in individuals who begin therapy before end-stage disease with near total destruction of the immune system. In this regard, the increase in naive CD4+ T cells during HAART is proportional to their presence at baseline (244). As more is learned about the characteristics of immune reconstitution that occurs during HAART, the fact that *in vitro* responses to antigens associated with opportunistic infections (i.e., cytomegalovirus) are frequently restored during HAART (297,318,462), and that prophylaxis against *Pneumocystis carinii* pneumonia can be safely discontinued as the CD4+ T-cell count increases to >200 cells/μL during HAART (172,488,580) serve as dramatic evidence of the salutary effects of HAART.

Overall, T-cell turnover studies suggest that an important, although not exclusive cause for CD4+ T-cell lymphopenia in HIV infection may be inadequate lymphopoiesis. This is consistent with the known inhibitory effects of HIV infection and HIV proteins on hematopoiesis (see earlier, HIV-Mediated Inhibition of Hematopoiesis). Such a scenario fortifies the rationale for adjuvant immunotherapy in conjunction with HAART that would boost thymic or extrathymic lymphopoiesis and/or peripheral expansion of T cells (139,302).

Latent Reservoirs of HIV Infection

On initiation of HAART, plasma viral load declines in several phases (251,429,568). The first phase of rapid exponential decay reflects the short half-life of free virions and of recently infected virus-producing cells. After the first several weeks of therapy, a slower second phase of viral load decay occurs with a half-life of approximately 14 days. This phase of decay is thought to reflect attrition of longer-lived, chronically infected cells (428). Extrapolation of this decay suggested that treatment of HIV infection with a potent combination drug regimen that completely suppresses viral replication for approximately 3 years may eradicate infection (428). A practical limitation of this estimate is the assumption of complete suppression of viral replication, which is beyond the capability of currently available HAART regimens (90,173,342,392,611).

Another major limitation of the time-to-eradication estimate is the presence of a reservoir of latently infected resting CD4+ memory T cells that persists during HAART. HIV may enter resting CD4+ T cells, and a limited degree of reverse transcription of the viral genome may occur in these cells. This period of preintegration latency may last hours to days; in the absence of an activation signal, the viral nucleic acid loses its capacity to initiate a productive infection. If these cells become activated, however, RT may proceed to completion, followed by nuclear import, and by integration of proviral DNA into cellular DNA (53,527,599). In the majority of CD4+ T cells that become infected with HIV in the *in vitro* setting of a sufficient degree of activation, integration and productive infection occur, followed by cell death. However, a small fraction of productively infected CD4+ T cells may escape cell death and revert to a resting state in which HIV infection is latent (i.e., postintegration latency; Fig. 18). Chun et al. (89,91) estimated the size of this latent reservoir of HIV *in vivo* by examining highly purified resting CD4+ T cells from both blood and lymph nodes of HIV-infected individuals. Using a PCR assay specific for integrated proviral DNA, they estimated that the frequency of latently infected resting CD4+ T cells was approximately 0.05%; interestingly, this frequency was quite similar in the peripheral blood and lymph node compartments, reflecting the fact that resting memory CD4+ T cells may continuously circulate between various lymphoid tissue compartments. Subsequent micro-coculture analysis revealed that approximately one tenth of latently infected resting CD4+ T cells harboring integrated HIV DNA carried replication-competent HIV (91).

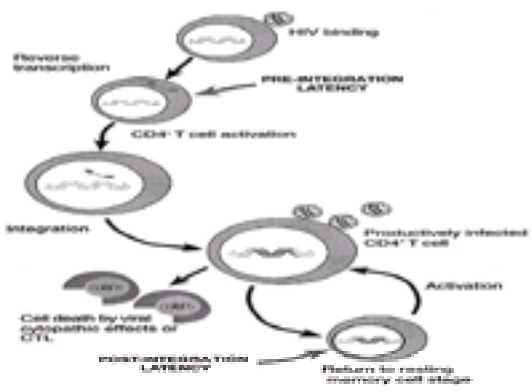

FIG. 18. Human immunodeficiency virus (HIV) entry into the cell may be followed by a state of preintegration latency, productive infection, or postintegration latency. In resting CD4+ T cells, HIV entry may be followed by a limited degree of reverse transcription of the viral genome, resulting in a labile state of preintegration latency. If these cells become activated, reverse transcription may proceed to completion, followed by nuclear import, and by integration of proviral DNA into cellular DNA. Productive infection usually follows integration of HIV DNA, resulting in cell death. However, a small fraction of productively infected CD4+ T cells may escape cell death and revert to a resting state in which HIV infection is latent (i.e., postintegration latency).

Although the size of the pool of latently infected resting CD4+ T cells *in vivo* harboring replication-competent HIV appears to be quite small, it may nevertheless constitute an important obstacle to eradication of HIV infection. Latently infected cells are not subject to elimination by the host immune response because of the absence of expression of viral antigens on the cell surface. Furthermore, integrated proviral DNA cannot be targeted by currently available antiretroviral agents. Therefore the natural half-life of latently infected resting CD4+ T cells may be the rate-limiting step in eradicating HIV

infection. These suppositions are substantiated by several independent studies that attempted to recover replication-competent HIV from cells of patients receiving HAART for prolonged periods. In these studies, virus was recovered from CD4+ T cells in essentially all patients receiving HAART, with levels of plasma viremia that were below the limit of detectability for up to 94 weeks (90,157,589). To estimate the half-life of this pool of latently infected resting memory CD4+ T cells, Finzi et al. (156) performed longitudinal analyses of the number of these cells harboring replication-competent HIV in 20 patients receiving HAART whose plasma viral load remained suppressed to <200 copies/mL over a mean of 14.4 months. The decay of the latent pool of CD4+ T cells was extremely slow, with an estimated half-life of 44 months; given a conservative estimate of the size of the latent pool, this leads to an estimated time-to-eradication of 60 years in patients receiving HAART (156). It is possible, however, that the half-life of the latent pool was overestimated if transient low-level viral replication were reseeding the reservoir over the course of the study. As more potent HAART regimens become available, more accurate assessments of the decay of the reservoir of latently infected cells should be possible.

Strategies aimed at long-term control of HIV infection will need to focus on the pool of latently infected cells that represent a long-lived reservoir capable of “reigniting” viral replication during periods of suboptimal antiretroviral therapy. Antiretroviral regimens that are highly potent and more easily administered should be part of such a successful strategy; however, this approach could be combined with interventions, such as IL-2 therapy, aimed at activating latently infected cells in the setting of HAART as well as enhancing HIV-specific immune responses (86). In this situation, latently infected cells might be driven to a state of productive infection and die; infectious virus released from these cells would be unable to infect new cells under the cover of HAART. Chun et al. (87) found that patients who received IL-2 in addition to HAART had a significantly lower frequency of latently infected CD4+ T cells harboring replication-competent virus compared with patients who received HAART alone. The mechanism of this effect is under investigation; however, unfortunately, even patients who harbored a frequency of latently infected cells that was below the limits of detection experienced prompt viral rebound when HAART was discontinued (93). These data highlight the importance of other reservoirs of smoldering virus replication and/or latently infected cells in the body (88,495).

PREVENTION AND VACCINES

Approaches to HIV prevention must be individualized to the settings in which transmission occurs. In the United States, safer-sex education campaigns within the homosexual community were instrumental in substantially reducing the incidence of new infections in the mid- and late 1980s. Multisector approaches in Uganda and Thailand, including input from governmental and nongovernmental organizations, have resulted in high levels of awareness regarding transmission of HIV; changes in sexual practices, including higher rates of condom use; and commensurate substantial reductions in the incidence of new HIV infections (337,396). Vaginal microbicides that are safe, effective, and acceptable will be welcome additions to prevention efforts in many parts of the world (449).

The most effective weapon in preventing further spread of the HIV epidemic will be a safe and effective vaccine (Table 7). A number of different vaccine approaches might be useful, including those that elicit sterilizing immunity as well as those that may not protect against infection *per se* but protect against development of disease after infection; in addition, others may alter the course of disease in those who had already been infected before vaccination. Vaccine development for many microbes has been aided by the knowledge of the immunologic correlates of protective immunity; however, in the case of HIV infection, correlates of protective immunity are largely unknown (239). Evidence exists supporting salutary roles for neutralizing antibodies, mucosal humoral responses, CD4+ T-helper responses, CD8+ CTL responses, and other immune responses (see earlier); however, none of these responses has been conclusively shown to protect individuals from HIV infection. Therefore HIV vaccine development is a highly empiric endeavor in which stimulation of humoral and cellular immune responses seems to be a reasonable approach. Another major obstacle to developing an effective HIV vaccine is the high degree of genetic heterogeneity of HIV strains within and between clades; protection against one strain by no means guarantees protection against a distantly related strain.

- Peptides (e.g., V3 region of gp120)
- Recombinant proteins (e.g., gp120, gp160, p24)
- Viral vectors (e.g., vaccinia, canarypox, or adenovirus vectors engineered to express HIV proteins)
- Bacterial vectors (e.g., *Salmonella typhi* engineered to express HIV proteins)
- DNA (i.e., plasmid DNA containing HIV genes that are expressed)
- Whole killed virus
- Live-attenuated virus (e.g., viruses harboring mutations in *nef*)

TABLE 7. *Approaches to the development of an HIV-1 vaccine*

Many successful vaccines have been developed that are live-attenuated or killed forms of the relevant microbe. Attenuation of SIV by deletion of portions of the *nef* gene has proven to be an effective vaccine approach in SIV-challenged, immunized monkeys (119,594). However, a number of safety concerns are relevant to further testing in this area. First, *nef*-deleted SIVs do have pathogenic potential in monkeys (21,22). In addition, HIV-infected recipients of transfusions from a single source in Australia were found to be long-term nonprogressors and harbored HIV with deletions in the *nef* gene (308). However, long-term follow-up of this unique cohort has revealed that several of the individuals experienced disease progression characterized by increasing viral load and decreasing CD4+ T-cell counts (309). Finally, another long-term nonprogressor who was infected with a *nef*-defective virus (286) subsequently

experienced HIV disease progression (218).

Neutralizing antibodies are associated with slow rates of SIV disease progression in nonhuman primate models (153), and accelerated clearance of both noninfectious and infectious virions (263). In addition, passive transfer of SHIV-neutralizing antibodies can protect macaques against a subsequent viral challenge (504). More encouraging data regarding a possible role for neutralizing antibodies come from studies by La Casse et al. In these studies, cells expressing HIV envelope were mixed with cells expressing CD4 and a relevant coreceptor and subjected to formaldehyde fixation to capture the transient envelope–CD4–coreceptor structures that arise during HIV binding and fusion. These transient “fusion-competent” epitopes elicited antibodies in a transgenic mouse system and were capable of neutralizing infectivity of 23 of 24 diverse primary HIV isolates from genetic clades A–E (304). Isolation of the fusion-competent epitopes and formulation into a more conventional vaccine is a promising approach.

Viral components, particularly envelope subunits such as gp120, represent the safest vaccine candidates; however, these components tend to be poor immunogens. The only HIV vaccine candidate to enter human efficacy trials to date is a bivalent form of gp120; two monomeric clade B gp120s are being tested in the United States, and a clade B plus clade E formulation is being tested in Thailand (166). It is possible that the immunogenicity of subunit vaccines might be improved by using new adjuvants, using oligomeric as opposed to monomeric envelopes, and by removing some of the envelope carbohydrate that masks HIV from the immune system.

Vaccination with the HIV Tat protein or a detoxified derivative (i.e., Tat toxoid) may prove useful as a preventive and/or therapeutic approach. The rationale for this approach derives from the ability of Tat to be secreted and to act as an immunosuppressant directly and indirectly by inducing interferon-a production from monocyte/macrophages (600). Furthermore, antibodies to Tat correlate with nonprogression of HIV disease (603). Tat toxoid is immunogenic and appears to be safe in HIV-infected and uninfected humans (219,220). Remarkably, immunization of monkeys with Tat protected them from challenge with a pathogenic SHIV (54). Although the mechanism of this protection is not currently known, it is possible that abortive infection occurred, and that neutralization of Tat activity prevented necessary pathogenic events that would otherwise lead to a spreading infection (54,178).

Recombinant microbes that are engineered to express HIV proteins are able to present antigens to the immune system in a physiologic manner. Vaccinia virus vectors have the disadvantage of being potentially pathogenic themselves in immunocompromised hosts. Canarypox vectors and modified vaccinia Ankara (MVA) are nonpathogenic, and are being used to express HIV proteins in clinical trials (231,424). Prime-boost, or combination strategies, in which the vector immunization is followed by or given simultaneous with subunit immunizations, appear promising in their ability to elicit both humoral and cell-mediated immune responses (35,160,618). In addition, cross-clade CTL responses can be elicited by using this approach (154). Recombinant bacterial vectors, such as attenuated strains of *Salmonella typhi,* also are being explored; advantages to this approach include induction of strong mucosal immune responses (593).

Naked DNA, encoding genes of interest, can be readily taken up by mammalian cells. This approach is particularly suited to eliciting cell-mediated immune responses, and shows promise in the search for a malaria vaccine (565). Chimpanzees immunized with an HIV DNA construct encoding *env, rev,* and *gag/*pol developed humoral and cellular HIV-specific immune responses and were protected against a high-titered challenge with a heterologous viral strain (49).

SUMMARY AND CONCLUSIONS

The pathogenesis of HIV infection is a complex, multifactorial process involving viral and host factors. The net degree of virus replication *in vivo* reflects a balance of factors that positively or negatively regulate virus expression; in turn, the amount of virus replication appears to be intimately related to the rate of CD4+ T-cell depletion that occurs, and thus to the rate of disease progression.

HIV is a formidable opportunist of the human immune system, as illustrated by its ability to use activation of the immune system for its own replicative advantage. The virus is able to disarm most components of the host immune response by a variety of direct and indirect mechanisms. A more complete understanding of these mechanisms should aid the rational development of more effective preventive and therapeutic strategies.

Although therapeutic advances for the treatment of HIV infection have been made at a pace that is unparalleled in the study of human viral diseases, a number of sobering obstacles remain in the path of controlling this pandemic. More than 90% of HIV-infected individuals live in the developing world, where expensive combinations of antiretroviral agents are far beyond the capacities of economic and health care infrastructure realities. HIV prevention, including a safe and effective vaccine, remains the best hope in controlling the spread of disease worldwide. Even in the developed world, it is clear that although HAART regimens have vastly improved the prognosis of HIV-infected patients, they are not curative, and their use is fraught with difficulties including numerous toxicities and drug interactions. To truly gain the upper hand against HIV infection, new antiretroviral agents that target different stages of the viral replicative cycle will be needed, as well as immunorestorative strategies that will allow more complete reconstitution of the immune system in the setting of effective antiretroviral therapy.

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CHAPTER 61 Nonhuman Lentiviruses

Ronald C. Desrosiers

HISTORY

Use of the term “slow virus infections” and identification of the first lentivirus is generally credited to Sigurdsson et al. (169,170 and 171) in Iceland. Twenty karakul sheep that were imported from Germany into Iceland in 1933 were responsible for the transmission of a chronic disease and death in massive numbers of Icelandic sheep over the subsequent years. More than 100,000 Icelandic sheep died in the decades that followed. Sigurdsson et al. (169,170 and 171) not only described the disease but also demonstrated that it was due to a transmissible agent. They used the term slow virus infections to refer to this disease and also to what is now known as scrapie (168). The term slow virus infection has subsequently been used to describe a wide variety of nonacute, persisting viral infections. In 1960, Sigurdsson et al. (172) described the cultivation of the transmissible agent in tissue culture, and Gudnadottir and Palsson (70) were able to reproduce the disease with the culture-grown virus. The diseases in the sheep were called *maedi* (Icelandic for dyspnea; i.e., a lung disease resulting in difficulty in breathing) and *visna* (Icelandic for a state of progressive apathy, a “fading away,” resulting from brain disease). Both disease states result from the same virus, now referred to as maedi/visna virus (MVV). MVV and related viruses are called lentiviruses, which is derived from the Latin *lentus* for slow. Approximately 600,000 sheep were slaughtered in Iceland in 1965 to eradicate MVV from the island.

The maedi/visna disease in Icelandic sheep, although the first to be specifically shown to be caused by a defined lentivirus, is probably not, however, the first description of a lentiviral disease in the literature. Vallée and Carré described in 1904 the infectious nature of the disease equine infectious anemia (186), which is now known to be caused by a lentivirus (EIAV). Even for maedi/visna, a chronic, progressive interstitial pneumonia had been described in South African sheep in 1915 and in Montana sheep in 1923 (123). Work with EIAV disease was largely on a parallel track with that of MVV, and the first description of EIAV cultivation appeared in 1961 (104). Only subsequently were EIAV and MVV shown to belong to the same lentivirus subgrouping of retroviruses.

The scenario of disease outbreak in a susceptible population leading to the identification of a new lentivirus has repeated itself dramatically on several occasions in more recent history. In 1964, an emerging infectious disease was first detected in Bali cattle in the Jembrana district of Bali (151,173). Bali cattle are the domesticated form of the wild barteng (*Bos javanicus*). Within 12 months, 26,000 of the 300,000 cattle on the island died of the disease. The cause of the disease was subsequently traced to a bovine lentivirus now called Jembrana disease virus (JDV), a distinct variant of bovine immunodeficiency virus (BIV) (20,96). Bali cattle are particularly sensitive to disease caused by this virus, whereas other species are resistant (174,175,195). Outbreaks of immunodeficiency disease and lymphoma in captive colonies of macaque monkeys (Asian Old World primates) were subsequently traced to the introduction of a simian lentivirus or lentiviruses from African monkeys (11,34,117,120). Simian immunodeficiency viruses (SIVs) naturally infect African nonhuman primates without apparent disease, but Asian macaques appear to harbor no such virus naturally. Even the origins of human immunodeficiency virus (HIV) in people have followed a similar pattern. HIV-2 in people in western Africa quite clearly originated from SIV of sooty mangabey monkeys (22,38,61,78). The natural habitat of sooty mangabey monkeys is the same geographic region in western Africa where HIV-2 is endemic, and SIV from sooty mangabey monkeys is indistinguishable at a genetic level from HIV-2. Sooty mangabey monkeys are naturally infected with SIV at high frequency without apparent disease. More recently, the origins of HIV-1 in central Africa have been traced to the chimpanzee *Pan troglodytes* (29,59). These examples illustrate the importance of studying animal viruses even when they are not apparently associated with any disease.

The earliest descriptions of the isolation of HIV and its association with acquired immunodeficiency syndrome (AIDS) did not appreciate that the virus was a lentivirus (8,150). Only subsequently through more careful examination of electron micrographs and through sequence analysis did it become clear that the virus was a lentivirus (68,128). At the time, study of lentiviruses was a rather obscure discipline with which many scientists were not familiar.

From a historical perspective, MVV (172) and EIAV (104) were discovered, isolated, and characterized long before the discovery of HIV; these viruses have retained use of their original designations. Lentivirus groupings identified after the

discovery of HIV have used a nomenclature similar to that for HIV (i.e., “immunodeficiency virus”). A lentivirus that was originally isolated from a cow with a chronic disease by Van Der Maaten et al. in 1972 (187) received little attention until after the discovery of HIV; the cow lentivirus is now called bovine immunodeficiency virus (BIV) (67). Subsequent to the discovery of HIV, lentiviruses were isolated from monkeys and cats (Table 1). Although discovery of MVV, EIAV, and BIV predates that of HIV, research with HIV has far surpassed that of all other lentiviruses in terms of the level of detailed knowledge. New information on other lentiviruses is thus usually compared with what is known for HIV.

Species	Virus	Year that cultivation was first published
Sheep/goats	Maedi/Visna virus; caprine arthritis encephalitis virus	1960[a]
Horses	Equine infectious anemia virus	1961[b]
Cattle	Bovine immunodeficiency virus; Jembrana disease virus	1972[c]
Humans	Human immunodeficiency virus	1983[d]
Monkeys	Simian immunodeficiency virus	1985[e]
Cats	Feline immunodeficiency virus	1987[f]

[a]Reference 172.
[b]Reference 104.
[c]Reference 187.
[d]Reference 8.
[e]Reference 34.
[f]Reference 142.

TABLE 1. *Known lentiviruses*

INFECTIOUS AGENT

Overview of General Properties

Related lentiviruses have been isolated from sheep, goats, horses, cattle, cats, monkeys, and humans (see Table 1). Genetic analysis of the virus from goats (caprine arthritis–encephalitis virus; CAEV) indicates that it clusters closely with MVV (185) and is thus placed in a single grouping with MVV. Thus, based on host species and the genetic analyses described in more detail later, six discrete evolutionary groupings of lentiviruses are generally recognized (Fig. 1A). It is likely that more remain to be identified. It is important to realize that even within a single grouping, discrete subgroupings based on host species, geography, and genetic distance can be defined. For example, within the SIV grouping, distinctly different subgroupings exist for the SIVs from African green monkeys, sooty mangabey monkeys, Sykes' monkeys, and L'hoesti monkeys (see Fig. 1B). Even among African green monkeys, which inhabit virtually all of sub-Saharan Africa, discrete genetic subgroupings of SIVagm can be defined that correlate with subspecies and natural geographic habitat (Table 2).

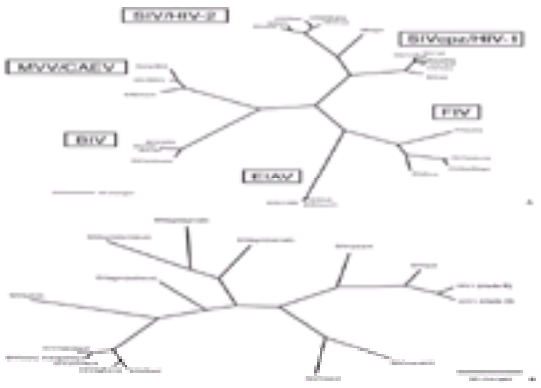

FIG. 1. Phylogeny of the lentiviruses. **A:** Six discrete evolutionary groupings of lentiviruses. Unrooted tree depicts the phylogenetic relationships among the six recognized groups of lentiviruses. The tree is based on a 470-amino-acid alignment of reverse transcriptase sequences from representative members of each group, including the bovine (BIV, Jembrana), equine (EIAV), feline (FIV), ovine/caprine (visna, CAEV) and primate (SIV, HIV) lentiviruses. Maximum parsimony (shown) and neighbor-joining (not shown) analyses give trees of nearly identical topology. Branch lengths are proportional to the number of amino acid replacements. **B:** Phylogeny of primate lentiviruses. Unrooted tree is based on the same amino acid alignment used in **A**. In addition to the viruses shown, SIVs have also been isolated from red-capped mangabeys (SIVrcm) and drill monkeys (SIVdr), but these have not been completely characterized. Courtesy of Welkin Johnson.

Virus designation	Primate lentivirus grouping	Species (common)	Species (formal)	Subspecies isolate	References
HIV-1	HIV-1/SIVcpz	Humans	*Homo sapiens*		6, 134
SIVcpz	HIV-1/SIVcpz	Chimpanzees	*Pan troglodytes*	*Pt. troglodytes*	29, 59, 83, 143
				Pt. schweinfurthii	
SIVsm	SIVmac/SIVsm/HIV-2	Sooty mangabeys	*Cercocebus atys*		57, 78, 104, 130
SIVmac	SIVmac/SIVsm/HIV-2	Macaques	*Macaca mulatta*		11, 34, 98, 133, 135
			M. arctoides		
			M. nemestrina		
			M. fascicularis		
HIV-2	SIVmac/SIVsm/HIV-2	Humans	*Homo sapiens*		35, 60
SIVagm	SIVagm	African green monkeys	*Cercopithecus aethiops*	*C.a. griseoviridis*	3, 4, 35, 54, 55, 77, 90, 92, 131, 139
				C.a. vervet	
				C.a. tantalus	
				C.a. sabeus	
SIVsyk	SIVsyk	Sykes' monkeys	*Cercopithecus mitis*		51, 76
SIVlhoest	SIVlhoest	L'hoest monkeys	*Cercopithecus lhoesti*	*C.l. lhoesti*	74
SIVsun	SIVlhoest	Sun-tailed monkey	*Cercopithecus lhoesti*	*C.l. solatus*	9
SIVmnd	SIVlhoest	Mandrills	*Papio sphinx*		181, 182
SIVrcm	Pending[a]	Red-capped mangabey	*Cercocebus torquatus*	*C.t. torquatus*	64
SIVdrl	Pending[a]	Drill monkey	*Mandrillus leucophaeus*		36

[a]Sufficient sequence information is not presently available to place these isolates relative to the five known groupings of primate lentiviruses.

In addition to the primate lentiviruses listed, serologic surveys for the detection of antibodies to SIV have suggested SIV infection of a variety of other species. These include but are not limited to talapoin monkeys, Delhbrazza monkeys, guenons, Dent's monkeys, Allen's swamp monkeys, and colobus monkeys (118, 139, 177).

TABLE 2. *Detailed listing of primate lentiviruses*[a]

All lentiviruses have a common morphogenesis and morphology that distinguish them from other subgroups of retroviruses (see Chapter 57). Lentiviruses bud from the plasma membrane without a preformed nucleoid, and mature particles typically have a cylindrical or rod-shaped nucleoid (Fig. 2). Classification into the lentivirus subgroup by morphologic criteria alone is entirely consistent with phylogenetic analysis of *pol* gene sequences. Viruses classified as lentiviruses have *pol* gene sequences more closely related to one another than to other retroviruses, and all have the characteristic morphogenesis/morphology. All lentiviruses have a propensity to replicate in macrophages, and all are associated with a chronic disease course in susceptible hosts. The primate lentiviruses have acquired use of CD4 as one of at least two receptors used by virus for entry into cells (Table 3). Lentiviruses from nonprimates do not use CD4 as a receptor for entry. The chronic disease induced by the primate lentiviruses in susceptible hosts has an immunodeficiency component, presumably because of the targeting of $CD4^+$ lymphocytes by use of CD4 as a receptor. All lentiviruses have a number of auxiliary genes (i.e., genes in addition to *gag, pol,* and *env,* not found in other, simpler retroviruses). Major distinguishing properties are summarized in Table 3.

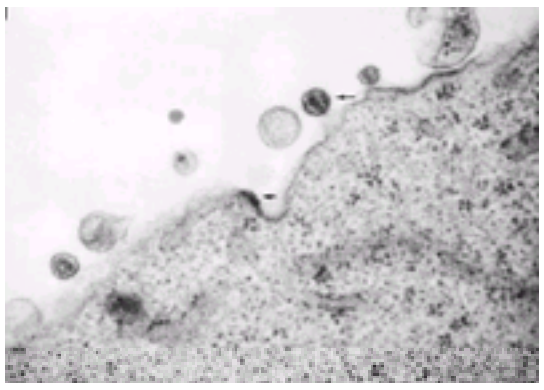

FIG. 2. Lentivirus morphogenesis and morphology. This electron micrograph of a cell infected with simian immunodeficiency virus shows virus budding from the cell in the absence of preformed particles (*lower arrow*) and a mature particle with a cylindrical or rod-shaped nucleoid (*upper arrow*). Photo courtesy of John MacKey.

Property	HIV-1	SIV	MVV, EIAV, BIV, FIV
Morphogenesis/morphology	Lenti	Lenti	Lenti
Macrophage tropism	Yes	Yes	Yes
CD4 lymphocyte tropism	Yes	Yes	No
Use of CD4 as receptor for virus entry	Yes	Yes	No
Use of chemokine receptors as receptor for virus entry	Yes	Yes	Yes
Natural modes of transmission	Sex, blood, vertical	Sex, blood, vertical	Insects, saliva/aerosols, blood, sex, vertical
Genes in addition to *gag, pol, env*	6	5 or 6	3 or more
tat and *rev* activities	Yes	Yes	Yes
dUTPase	No	No	Yes
Persistent viral replication	Yes	Yes	Yes
Chronic, debilitating disease	Yes	Yes	Yes
Immunodeficiency	Yes	Yes	No

TABLE 3. *Properties of lentiviruses*

Genome Organization and Composition

Widespread availability of DNA sequencing capabilities has greatly facilitated our understanding of phylogenetic relationships and the gene products of lentiviruses. Based on sequence analysis, six discrete groupings of lentiviruses are now recognized (see Fig. 1A). It is likely that other groupings remain to be discovered. Extensive diversity exists even within a grouping. For example, among the SIVs, distinct subgroups have been defined from African green monkey, sooty mangabey monkey, L'hoesti monkey, and Sykes' monkey (see Fig. 1B and Table 2). Similar diversity has been observed for FIV in wild and captive cat species (18). Even among four subspecies of African green monkeys (vervet, grivet, tantalus, and sabeus), discrete sub-subgroupings of SIVagm have been defined (see Table 2). These four subspecies naturally inhabit distinct or sometimes partially overlapping habitats that cover almost all of sub-Saharan Africa.

The *pol* gene generally exhibits the greatest degree of sequence conservation. *Pol* gene sequences thus are often used for the comparison of lentiviruses from different groups, subgroups, or sub-subgroups. Sequences from one subgroup of SIV (e.g., SIVsm) will typically exhibit only 55% to 60% identity in *pol* at the amino acid level when compared with sequences from another SIV subgroup (e.g., SIVagm). Diverse members within a subgroup may exhibit as little as 75% to 80% amino acid identity in *pol,* but the number is typically quite higher. When different groups of lentiviruses are compared, for example, MVV with SIV, amino acid identity in *pol* is typically 35% or less. Relatedness in other genes is even less. Nonetheless, these lentiviral sequences are clearly more closely related to one another than to other retroviruses, justifying their classification as lentiviruses on the basis of morphogenesis/morphology and biologic properties.

All known lentiviruses have at least three genes in addition to the standard *gag, pol,* and *env* genes that all replication-competent retroviruses possess (Table 3, Table 4 and Table 5 and Fig. 3). These extra genes likely contribute to a more complicated life cycle for the lentiviruses, which includes persistent viral replication and strategies for immune evasion discussed in more detail later. Genes for *tat* and *rev* are consistently found in all six lentivirus groupings. A *vif* gene is consistently present in five of the six lentivirus groupings; EIAV, however, stands alone in apparently lacking a *vif* gene. Whether another protein may perhaps contribute *vif* function for EIAV remains to be determined. A *nef* gene is found at the 3' end of all primate lentiviruses (see Table 5). None of the more primitive lentiviruses contains a *nef* gene. However, cells infected with EIAV and BIV have been found to make spliced RNAs that predict protein products called Ttm and Tmx that correspond to the carboxy-terminal portion of TM (10,68). Whether these may represent evolutionary precursors to *nef* or perform functions similar to those of *nef* is yet to be determined. All lentiviruses contain an unusually long (>150 amino acids) cytoplasmic domain of TM. In the intergenic region between *vif* and *env,* a number of other genes can be present, particularly in the primate lentiviruses. A *vpr* gene is present in this region in all primate lentiviruses that have been examined. The SIVsm/ SIVmac/HIV-2 subgroup of viruses contains an additional homolog of *vpr* called *vpx,* not found in viruses outside this grouping. HIV-1/SIVcpz contains a gene called *vpu* not found in other lentiviruses.

MVV[a]	EIAV[b]	BIV[c]	FIV[d]
vif	*tat* (S1)	*vif*	*vif*
tat	*rev* (S3)	*tat*	*rev*
rev	*S2*	*rev*	*tat*
	ttm	*tmx*	
		vpw?	
		vpy?	

[a]Reference 185.
[b]Reference 126.
[c]Reference 66.
[d]Reference 140.

TABLE 4. *Auxiliary genes in nonprimate lentiviruses*

	SIVsm/SIVmac/HIV-2	SIVagm	SIVtyk	SIVcpz/HIV-1
vif	+	+	+	+
vpu	-	-	-	+
vpr	+	+	+	+
vpx	+	-	-	-
tat	+	+	+	+
rev	+	+	+	+
nef	+	+	+	+

TABLE 5. *Auxiliary genes of of primate lentiviruses*

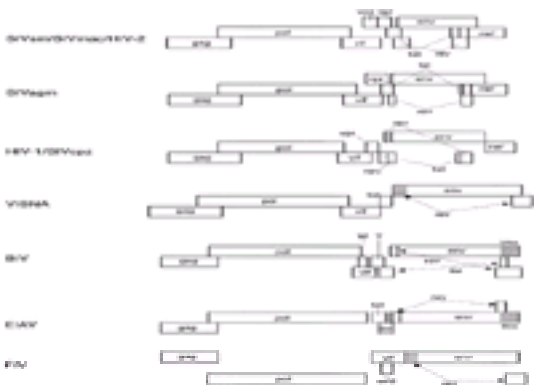

FIG. 3. Genome organizations of representative lentiviruses.

The nonprimate lentiviruses encode a deoxyuridine triphosphatase (dUTPase) activity from a distinct open reading frame located within the *pol* gene (50). Curiously, these dUTPase coding sequences are absent in the primate lentiviruses but present in the type D retroviruses. The explanation for this variable presence of dUTPase is not entirely clear. The type D retroviruses and lentiviruses may possibly have diverged from a common ancestor that possessed such a dUTPase reading frame, and this reading frame may have been lost in the evolution of primate lentiviruses from the more primitive ones. The type D retroviruses and lentiviruses do share a common morphology of the mature particles (i.e., a cylindrical or rod-shaped nucleoid), which also may suggest some commonality. Alternatively, the presence of a dUTPase reading frame in nonprimate lentiviruses and in type D retroviruses could be an example of convergent evolution, with two distinct lineages acquiring these sequences independently by gene capture or by sequence duplication and point mutation.

Another possibility is that one viral lineage may have acquired dUTPase sequences from the other viral lineage by a recombination or capture event (7). The loss of dUTPase in the evolution from nonprimate to primate lentiviruses could relate at least in part to the predominant replication of nonprimate lentiviruses in cells with little potential to divide (macrophages) versus the predominant replication of primate lentiviruses in cells with much greater potential to divide (lymphocytes). Primate lentiviruses incorporate uracil DNA glycosylase into virions through its binding to *vpr* (121). The presence of the dUTPase or uracil DNA glycosylase in virions serves to reduce the mutation frequency in reverse transcription products.

Propagation and Assay in Cell Culture

Primary isolates from all lentivirus groupings can be grown in normal macrophage cultures of the host species. Other types of cells may be more typically used for replicating the individual viruses in routine cell culture. The types of cells that can be used vary with the virus (Table 6) and almost certainly relate to the kinds of receptor that can be used, at least in most cases. Isolates also can be adapted to growth on particular cell substrates. Examples of the latter include growth of FIV on Crandell feline kidney cells and growth of SIVs and HIVs on human tumor T-cell lines (see Table 6).

Lentivirus group	Growth on macrophages	Other cell types	Adaptation to growth
HIV-1	Yes	Stimulated, primary, human CD4+ lymphocytes	Human tumor T-cell lines
SIV	Yes	Stimulated, primary, monkey CD4+ lymphocytes	Human tumor T-cell lines
MVV/CAEV	Yes	Choroid plexus cells; primary synovial cells; endothelial cells	–
FIV	Yes	Primary, stimulated feline lymphocytes	Crandell feline kidney cells
EIAV	Yes	–	Fetal equine kidney; equine dermal
BIV	Yes	–	Bovine, rabbit, canine fibroblasts

TABLE 6. *Cell substrates for growing lentiviruses*

Host Range

The lentiviruses are typically rather restricted in their host range, but both natural and experimental cross-species infections have been documented. SIVs naturally infect a variety of African nonhuman primates, but a single example of natural infection of Asian Old World monkeys has never been documented. The SIVs from sooty mangabey monkeys and African green monkeys, when introduced into macaque monkeys, Asian Old World primates, can infect them and cause an AIDS-like disease (57,75,133). In fact, the SIV of sooty mangabey monkeys was accidentally introduced into macaque monkeys in captive United States colonies on at least one occasion and disseminated unknowingly into other macaques for more than a decade before it was discovered and eliminated (32,120). At least one clear case of laboratory-acquired infection of a human with SIVmac has been documented (100).

Cross-species transmission of SIV to baboons in the wild has been documented. Baboons do not appear to harbor their own SIV naturally. Of 279 baboon sera taken from native habitats in Tanzania and Ethiopia, 277 were negative for the presence of antibodies to SIV. However, two gave strong reactivity to SIVagm antigens (105). One of these two was subsequently shown to harbor SIVagm sequences in stored plasma. The SIVagm sequences clustered with the vervet subtype, consistent with the known cohabitation of vervet monkeys in the same region where the baboon samples were taken (91). An SIVagm variant has similarly been detected in a chacma baboon of southern African (188). Baboons also can be infected experimentally with HIV-2 (116). Evidence for cross-species transmission of SIV from west African green monkeys (*Cercopithecus aethiops sabeus*) to patas monkeys (*Erythrocebus patas*) and to white-crowned mangabeys (*Cercocebus torquatus lumulatus*) has also been presented (12,177). Transmission of SIV or HIV to nonprimates has not been documented.

The origins of both HIV-2 and HIV-1 in humans are now generally believed to be from cross-species transmission. HIV-2 is endemic in western Africa and is only now beginning to be seen detectably in some other regions of the world. The SIV from sooty mangabey monkeys is closely related to HIV-2 and basically cannot be distinguished from HIV-2 on the basis of genetic analysis. The native habitat of the sooty mangabey monkey is the coastal forest regions of western African, the same region where HIV-2 is endemic. Thus strong circumstantial evidence involving both viral sequences and geographic colocalization link the monkey and human lentiviruses in western Africa (23,38,61,78). Similarly, of the four subspecies of chimpanzees, one (*Pan troglodytes troglodytes*) appears to harbor naturally a lentivirus (SIVcpz) that is closely related to group M, N, and O isolates of HIV-1 (29,59). Although more work should be done on the lentiviruses naturally harbored by the four subspecies of chimpanzees, the work to date provides strong support for the introduction of a lentivirus from *P. troglodytes* into the human population on one occasion or more for the origin of HIV-1.

Attempts to transmit HIV-1 experimentally to a variety of animal species have shown a very restricted host range. HIV-1 is infectious for chimpanzees, and a recent chimpanzee-passaged HIV-1 has shown pathogenic potential (137). An early report described HIV-1 infection of pig-tail macaques (*Macaca nemestrina*) (1), but this model has not proven sufficiently robust to date for general use. Attempts to increase the usefulness of this system by serial-passage adaptation of HIV-1 in this macaque species are ongoing.

The more primitive lentiviruses also appear rather restricted in their host range, limited to the same or closely related genera. BIV has been reported to infect New Zealand white rabbits.

Receptor Use

HIV-1 uses a two-receptor system that includes both CD4 and a seven-membrane-spanning chemokine receptor for entry into target cells (see Chapter 59 for details). The SIVs that have been examined to date appear to be similar to HIV in their use of both CD4 and chemokine receptor for entry. Like HIV-1, the SIVsm/SIVmac/HIV-2 and SIVagm groups of viruses appear to use CCR5 as the principal second receptor. However, as with HIV-1, a variety of other chemokine receptors may be used, depending on the virus and the isolate. In addition to CCR5, assorted SIVs have been shown to use CCR2b, CCR3, STRL33 (Bonzo), GPR15 (Bob), and GPR1. The SIVsm/SIVmac/HIV-2 group of viruses in general appears to show less dependence on CD4 for a larger percentage of isolates than does HIV-1 (49). Use of CXCR4 as second receptor also appears to occur much less frequently with SIVsm/SIVmac than with HIV-1. SIV isolates from red-capped mangabeys (*Cercocebus torquatus torquatus*) appear to be unusual in their predominant reliance on CCR2b for use as second receptor (21).

Our understanding of the evolution of receptor-mediated entry among the lentiviruses has been greatly advanced by the discovery that FIV uses CXCR4 as receptor for entry (196). Both primary isolates and laboratory-adapted strains of FIV have been shown to use CXCR4, and the observations have been independently reproduced in several laboratories around the world (147,154). Infection with FIV can be modulated by soluble stromal cell–derived factor, which is believed to be the natural ligand for CXCR4 (81). Little information is currently available on the receptors used by other nonprimate lentiviruses.

The immediate questions regarding receptor use by lentiviruses are obvious ones, but the answers will be instrumental in our understanding of the evolution and logic of receptor use. Do other nonprimate lentiviruses in addition to FIV use seven-membrane-spanning chemokine receptors for entry into host cells? If so, use of chemokine receptors will be a theme common to all lentiviruses. Do the nonprimate lentiviruses also use a two-receptor system for entry into target cells? If so, what is the nature of the other component in the two-receptor system? It is certainly not CD4. In the evolution from the nonprimate to primate lentiviruses, it is possible to envision a switch from a one-receptor system (chemokine receptor) to a two-receptor system (CD4+ chemokine receptor) or a switch from one two-receptor system (X+ chemokine receptor) to another (CD4+ chemokine receptor).

PATHOGENESIS AND PATHOLOGY

Portals of Entry

The primary mode of natural transmission of the nonhuman lentiviruses varies considerably with the virus. EIAV may be the most interesting because it is the only lentivirus for which there is good evidence for vector-borne transmission. During disease episodes, levels of infectious virus in the plasma of horses can exceed 10^4/mL. Transmission via blood can be mediated by inappropriate veterinary practices with needles, scalpels, or by insect vectors. The horse fly appears to be more efficient than mosquitoes, fleas, or other insects for being able to transmit EIAV. Transmission has been documented by following a single horse fly that had taken a blood meal on an infected pony during an acute clinical period (89).

Work on natural modes of infection has demonstrated the capability of both vertical and horizontal transmission for FIV, MVV/CAEV, and EIAV (17). Vertical transmission can occur *in utero,* during parturition, and postnatally via milk. This vertical transmission by these viruses parallels that observed with HIV in humans. Contact transmission also is common, particularly for MVV/CAEV and EIAV when animals are herded closely together in barns or stables. These viruses can be found in semen, lung excretions, and saliva. For cats, bite wounds are believed to be the most important route of transmission (199). FIV infection is much more common in males than in females, consistent with increased fighting and biting among male cats. Cats allowed to roam free in areas with high cat density are at greatest risk of becoming infected.

Information on natural modes of SIV transmission has been harder to come by. One study of wild grivet monkeys in Awash National Park in Ethiopia analyzed SIVagm serologic status with age, sex, and risk (146). Infection was nearly universal in females of reproductive age and nearly absent in younger females. In males, infection was observed in only those that were fully adult. The findings support a predominantly sexual mode of transmission among SIVagm-infected grivets. Maternal–infant transmission of SIV has been documented in captive animals (103).

Experimental infection of laboratory animals has been most commonly achieved by direct needle inoculation. However, mucosal exposure is now being used more frequently, particularly for vaccine studies, as a model for the most common routes of HIV transmission.

Cell and Tissue Tropism

Despite the varied modes of transmission, all lentiviruses are disseminated to an assortment of tissue sites by the blood. Virus can be disseminated as free virus, or more likely by infected blood monocytes or lymphocytes. Differences in the cell tropism of primate versus nonprimate lentiviruses relate largely to receptor use discussed earlier. Infection by the primate lentiviruses SIV and HIV is seen in $CD4^+$ lymphocytes and macrophages, with $CD4^+$ lymphocytes vastly predominating in terms of numbers of infected cells. For the nonprimate lentiviruses, infection of macrophages typically predominates, but infection can be seen in a greater diversity of cell types. Replication in tissue macrophages is a unifying feature of all lentiviruses.

The principal anatomic sites for localization of MVV and CAEV have varied with the study and the strain of virus used. Because many studies of virus localization have used experimental infection, the origin of the infecting strain and the type of cell in which it was grown must be taken into account when interpreting the results of these studies. Various reports have localized MVV principally to lungs, mammary glands, joints, lymph nodes, spleen, and brain. The principal target cell in which MVV and CAEV sequences can be found *in vivo* is quite consistently the tissue macrophage.

However, consistent with the broad range of cell types that can support replication in culture, evidence has been presented for MVV/CAEV replication in other cell types in a less prominent fashion, including epithelial and fibroblast cells in choroid plexus, intestine, and kidneys. An important observation first made with MVV is that although virus may reside in the relatively undifferentiated monocytes in peripheral blood, MVV expression is greatest in the differentiated tissue macro-phages (63,144). This led to the concept of the “Trojan horse” for MVV and other lentiviral infections, whereby undifferential monocytes in the peripheral blood may carry the viral genome without sustaining viral replication until transported to tissues and differentiated into their mature form, the tissue macrophage.

The anemia caused by EIAV is hemolytic and results from the formation of antigen–antibody complexes that can associate with the surface of erythrocytes. The kidney also is affected by this complex formation. A variety of studies, both old and new, have shown the tissue macrophage to be the major cell type producing EIAV (127,138,164). Virus production has been noted in spleen, liver, kidney, lymph nodes, lung, heart, brain, stomach, bone marrow, thymus, adrenals, and intestine. Although EIAV can be adapted to replicate on fibroblasts, virus that comes out of horses during viremic episodes is derived principally from macrophages.

FIV appears to be unusual among the nonprimate lentiviruses in that virus has been more consistently found in a broader range of cell types, particularly lymphocytes, in addition to macrophages. Analysis of tissue and cellular localization of FIV have principally used experimentally infected cats and FIV originally grown in lectin-stimulated peripheral blood mononuclear cell (PBMC) cultures in the presence of interleukin-2. The extent to which cellular and tissue distribution studies represent the situation with natural infection may therefore not be entirely clear. Nonetheless, FIV has been found in a variety of cell types including B cells, $CD8^+$ T cells, $CD4^+$ T cells, macrophages, and cells of the central nervous system (CNS). Macrophages, microglia, and astrocytes, but not neurons, in the brains of cats have been found to be targets of FIV infection (46).

The major sites of pathogenic SIV replication early after infection have been localized to thymus, spleen, gastrointestinal tract, and other lymphoid tissue (107,190). SIV was localized at these early time points primarily within periarteriolar lymphoid sheaths in spleen, paracortex of lymph nodes, and medulla of the thymus. Many early studies have overlooked the fact that the gastrointestinal tract contains most of the lymphoid tissue in the body. SIV infection of rhesus monkeys results in profound and selective depletion of $CD4^+$ T cells in the intestine within days of infection, before any such changes are evident in peripheral lymphoid tissues. The loss of $CD4^+$ T cells in the intestine is coincident with productive infection of large numbers of mononuclear cells at this site. Thus the gastrotintestinal tract appears to be a major target for SIV replication and the major site of $CD4^+$ T cell loss early in SIV infection (190). Within thymus, marked depletion of thymic progenitors has been noted by 21 days; this depletion is followed temporally by increased levels of cell proliferation in the thymus and a marked rebound in thymocyte progenitors (198). The distribution of virus within lymphoid organs varies with the innoculum. SIV also can be found in the central nervous system early after infection. The infected cells in the brain, whether early after infection or late in SIV-induced encephalitis, are primarily cells of monocyte/macrophage lineage (160). These may include perivascular monocyte/macrophages that have migrated from the blood or the resident microglial cells.

Immune Responses and Persistence

Monkeys infected with SIV and animals infected with other lentiviruses typically make strong antibody and cytotoxic T-lymphocyte (CTL) responses to the virus. These immune responses persist at high levels for the lifetime of the infected host, whether infection is by natural or experimental means. Approximately 20% to 30% of macaque monkeys infected with some AIDS-causing strains of SIV develop a more rapid disease course, make few or no antibodies to the virus, and die with AIDS in 3 to 7 months. The deficiency in anti-SIV antibodies in such animals does not appear to be a matter of antigen excess, because B cells from these rapidly progressing monkeys make few or no anti-SIV antibodies (48). The strength of anti-SIV antibody responses also varies with the virulence of the infecting strain (Fig. 4). Deletion mutants of SIV that are progressively more attenuated on the basis of viral load measurements elicit progressively weaker anti-SIV antibody responses (40). CTL responses have been shown to be major histocompatibility complex (MHC) restricted for both SIV and animal lentiviruses. Detailed work on CTL responses has been hindered by limited knowledge of MHC types in these animal species, although a considerable amount of new information is now emerging on MHC alleles in rhesus monkeys and the peptides that they present.

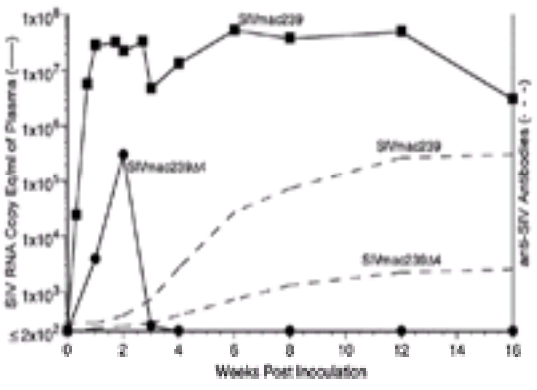

FIG. 4. Attenuation by auxiliary gene deletion lowers viral load and reduces the strength of the antiviral antibody response. SIVmac239D4 has inactivating deletions in *vpr, vpx,* and two in *nef.* From ref. 40, with permission.

The importance of $CD8^+$ lymphocytes in limiting the extent of replication of SIV or SIV/HIV recombinant (SHIV) has been definitively demonstrated by using the approach of CD8 cell depletion (125,161). Extensive or near-total depletion of $CD8^+$ cells was achieved by intravenous administration of large doses of specific anti-CD8 monoclonal antibodies.

Depletion was not permanent because $CD8^+$ lymphocytes returned in the absence of continuous monoclonal antibody. When $CD8^+$ cells were depleted during primary infection, virus replication proceeded unabated after peak loads 10 to 14 days after infection; this contrasts with nondepleted animals, in which immune responses typically result in reductions in viral load beyond 14 days (125,161). Elimination of $CD8^+$ lymphocytes from monkeys during chronic SIV infection resulted in a rapid and marked increase in viremia that was again suppressed coincident with the reappearance of SIV-specific $CD8^+$ T cells (161). Depletion of $CD8^+$ T cells in SHIV-infected animals facilitated the appearance of more highly pathogenic variants (84).

In SIV-induced disease in macaque monkeys with wild-type strains of SIV, viral-specific, proliferative responses of CD4 cells are typically weak or absent. However, infection by attenuated SIV mutants deleted in *nef* results in strong, SIV-specific, CD4 helper cell proliferative responses (62). This situation appears analogous to HIV infection of humans, in which HIV-specific CD4 responses are usually weak or absent in typical progressors but are often very strong in unusual nonprogressors that are controlling their infection (155,156). The picture that emerges with these primate CD4 receptor–using viruses is one of a battle during the early stages of infection. CD4 helper cells try to respond to sites of infection, but these activated CD4 cells at the sites of infection are the ideal targets for replicating virus. In pathogenic infections, the virus wins this early battle and eventually wins the remainder of the war. The status of CD4 helper cell responses in nonpathogenic infection of the natural hosts of SIV (e.g., SIVagm in African green monkeys or SIVsm in sooty mangabeys) has not been investigated.

The lentiviruses persist through chronic active viral replication. The clearest demonstrations of this are with HIV in humans and SIV in macaque monkeys, in which during months and years of chronic infection, millions of viral particles and millions of infected cells are produced and turned over every day (80,193). This does not mean, however, that no cells are infected in a quiescent or latent fashion; some certainly are. The degree of chronic active replication also varies with the strain of virus and the host. The long-term persistence of antibody and CTL responses at high levels is consistent with the prolonged antigen expression of chronic active replication. Even nonpathogenic derivatives of SIV continue to replicate at low levels over prolonged periods, as evidenced by the evolution of sequence changes, persistence of antibody, and other criteria.

EIAV is unusual among the lentiviruses for the episodic nature of the persistent viral replication. Infection of horses with EIAV is associated with recurring episodes of fever, anemia, and thrombocytopenia. These episodic occurrences of clinical disease coincide with bursts of new virus replication, which may be weeks or months apart. Episodes of new virus replication yield EIAV variants that are not neutralized by the horse's own serum; however, the same serum is capable of neutralizing the EIAV variants from earlier episodes (reviewed in ref. 129).

The dilemma of lentiviruses is their ability to replicate persistently in the face of apparently strong immune responses. The levels of anti-HIV antibody and CTLs have been precisely measured and are enormously high. The strategies used by lentiviruses to allow persistent viral replication in the face of these apparently strong immune responses have been recently reviewed (39). These are summarized in Fig. 5 and in points 1 through 5 below.

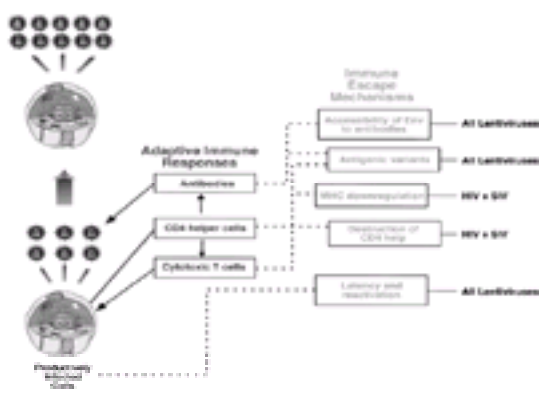

FIG. 5. Human immunodeficiency virus (HIV), simian immunodeficiency virus (SIV), and other lentiviruses use a variety of strategies to avoid both humoral and cellular responses.

1. Differentiation- or activation-dependent expression
   Like all retroviruses, the lentiviruses integrate a proviral DNA copy of their genetic information into the host cell DNA. This proviral DNA may lie dormant for prolonged periods and can be reactivated at a later date when conditions may be more suitable. Triggering events for activation of lentivirus expression can include cellular activation of lymphocytes or macrophages by natural stimuli (antigen, cytokines) and differentiation of monocytes into tissue macrophages, as originally promulgated in the Trojan horse concept.
2. Emergence of antigenic variants
   As RNA viruses, the lentiviruses have an error-prone reverse transcriptase for replicating their genetic information. The emergence of mutant forms of virus (within a single infected individual) that are antigenic variants that escape ongoing neutralizing antibody responses was first described with EIAV, MVV, and CAEV. Subsequent work has provided exquisite detail on the emergence of SIV and HIV variants that escape both neutralizing antibody and CTL responses (14,15 and 16,52).
3. Shielded nature of external surface glycoprotein
   The surface proteins of all lentiviruses are heavily glycosylated, and the external surface glycoproteins of lentiviruses are among the most heavily glycosylated proteins known. The carbohydrate groups help to serve as a barrier that limits the neutralizing antibody response to virus. Strains of SIV lacking specific carbohydrate attachment sites are much more effective targets for antibody-mediated neutralization and are able to elicit considerably higher titers of neutralizing antibodies (19,24,152).

The only lentiviral surface glycoprotein for which three-dimensional data are available is HIV-1 (106). Although the structure lacks loops of variable sequences and lacks carbohydrate, a compilation of antibody mapping and structural and functional data has revealed interesting features that reflect on the replication strategy of HIV. The CD4-binding site is contained within a difficult-to-access pocket, and the variable loops and carbohydrate appear to act largely as a shield to protect regions of the protein that would otherwise make virus sensitive to antibody attack. Although infected people develop high-titer antibody responses reactive with monomeric HIV-1 envelope protein, these antibodies apparently react poorly or not at all to envelope protein as it exists on the surface of virons. These antibodies certainly have very poor neutralizing activites.

4. Destruction of CD4 help
   This immune evasion strategy appears to be applicable only to the primate lentiviruses, which use CD4 as primary receptor. The early destruction of virus-specific CD4 helper cell activity leaves the host without appropriate help for its B-cell and CD8-cell responses.
5. MHC down-regulation
   The primate lentiviruses encode an early gene product, Nef, that down-regulates MHC class I molecules from the surface of cells, making them less-sensitive targets for CTL attack (27,163).

Other RNA viruses with error-prone polymerases, such as poliovirus, seem nowhere near so difficult to neutralize and nowhere near so malleable as HIV. The basis for these differences may lie in the inherent strategies used by persisting versus nonpersisting viruses. The survival of any virus depends on its ability to spread through the population. For a nonpersisting virus such as poliovirus, transmission is limited to the several weeks after the time of primary infection. Resistance to antibodies, once they appear, may not be so important to such a virus. Instead, it is important to replicate maximally for the brief period to maximize the likelihood for transmission. HIV, SIV, and other lentiviruses, in contrast, help ensure their transmission by greatly lengthening the period during which transmission can occur. Thus these viruses may sacrifice inherent replicative capacity in the short term to allow persistent viral replication over the long term.

Virulence

Not all lentiviral infections are uniformly pathogenic. The various forms of SIV are not associated with any disease in their natural hosts. Life-long subclinical infections also have been documented for EIAV and MVV. Various breeds of horses and sheep vary in their general susceptibility to EIAV and MVV, and these may even vary in the degree of susceptibility to specific disease manifestations. For example, the classic CNS form of visna infection originally observed in Iceland is only rarely seen elsewhere. Some strains of SIV are much less pathogenic than others in susceptible macaque monkeys, and some derivatives that have been specifically attenuated by deletion mutations appear to be nonpathogenic.

The diseases associated with lentiviral infections are typically chronic and manifest over a prolonged period. However, there are some prominent exceptions to the classic, prolonged course of lentiviral disease. A particular strain of SIV called SIVsmPbj14 is acutely lethal in rhesus monkeys. Monkeys infected with SIVsmPBj14 typically die within 14 days with very high viral loads, severe gastrointestinal disease, cytokine dysregulation, lymphoproliferative disease, and organ system failure (42,56,58,136). The unusual properties of this strain have been attributed in large part to the unusual positioning of a tyrosine at residue 17 of the nef protein (47). The occurrence of tyrosine at this position of nef creates an immune-receptor tyrosine-based activation motif (ITAM) of the type YXXLX7YXXL that imparts on virus the unusual ability to cause lymphocyte activation and to replicate to high titers in lymphocytes of PBMC cultures without any prior stimulation (47). The BIV variant that is the cause of Jembrana disease in Bali cattle also is acutely pathogenic. About 17% of Bali cattle infected either naturally or experimentally with JDV die with an acute disease within the first few weeks (43,44,195). During acute disease, infectious titers of virus reach 10^{8}/mL of plasma. Jembrana disease has remarkable similarities to the disease induced by the tyrosine- *nef* variant of SIV, SIVsmPbJ. In Jembrana disease, there is marked enlargement of lymph nodes and spleen, which feature proliferating lymphoblastoid cells. Proliferating lymphoid infiltrates are also found in many other tissues. The disease in horses induced by EIAV also is often considered more acute than that occurring with other lentiviruses. The first episode of EIA disease usually occurs 2 to 6 weeks after the initial infection. Subsequent disease cycles are irregular, appearing weeks to months apart, and usually last 3 to 5 days. The frequency and severity of disease episodes usually decline with time. Episodes of clinical disease usually end within the first year after an average of six to eight episodes.

Clinical and Pathologic Features

Good reviews are available on the clinical and pathologic features of nonhuman lentiviral infections (17,94). Some of the most prominent features are highlighted here.

Equine Infectious Anemia Virus

Equines are the only species susceptible to natural or experimental infection with EIAV. Clinical disease is usually divided into acute, subacute, and chronic phases. Acute disease typically results in fever as high as 108°F 1 to 4 weeks after infection. Anemia is not a prominent feature at the outset. Excessive thirst, loss of appetite, weakness, depression, and hemorrhage are seen in the acute phase. The acute disease may result in death. The subacute form is characterized by relapsing fever and recurrence of other signs. Recurrence of episodes may be brought on by hard work or malnutrition. In its chronic form, animals may remain thin despite adequate availability of food, and red cell counts are typically well below normal. Clinical signs in late disease and pathology findings appear to result principally from the hemolytic anemia. Erythrocytes of infected horses are coated with antibodies and complement factor 3, and destruction of red cells is immunologically mediated. Osmotic fragility, shortened half-life, and phagocytosis contribute to the destruction. Bone marrow may also be depressed, but this seems less important than the immune-mediated destruction. Hemorrhage, jaundice, and edema are most commonly found at necropsy. The nature and severity of the lesions will vary with course of disease and duration of the illness.

Maedi/Visna Virus and Caprine Arthritis–Encephalitis Virus

Although maedi (pneumonia) and visna (wasting, depression, paralysis) were once thought to be separate diseases, it is now clear that they are both caused by the same virus. Both systems can be affected in the same animal. Maedi disease in the Netherlands has been called *zwoegerziekte.* Polyarthritis and mastitis also are seen as a result of viral infection. Disease is usually seen only in adult sheep because of the lengthy incubation period, typically 3 to 8 years. The lungs of affected sheep may be 2 to 5 times the normal size, and they exhibit a rubbery loss of elasticity. These abnormalities result from a gross thickening of the alveolar walls from infiltration and proliferation of reticuloendothelial or mesenchymal cells that invade the septa everywhere (Fig. 6A and Fig. 6B). Lymph nodules occur along the bronchi and bronchioles. There is progressive weight loss. Dyspnea is initially apparent only after exercise, but this becomes progressively more severe. Severely dyspneic sheep spend much time lying down. Lesions in the brain consist of demyelination and lymphocytic infiltration (see Fig. 6C and Fig. 6D). Trembling of facial muscles and lips may occur. The onset of clinical signs of visna usually begins with weakness of the hind legs, and this eventually leads to paraplegia. Diseases caused by CAEV in goats are similar to those of MVV in sheep, except that arthritis is usually most prominent, and the pneumonia is usually of lesser severity. Joints are swollen and painful, and this is exacerbated by cold weather. The basic lesion is a proliferative synovitis of joints, tendon sheaths, and bursae.

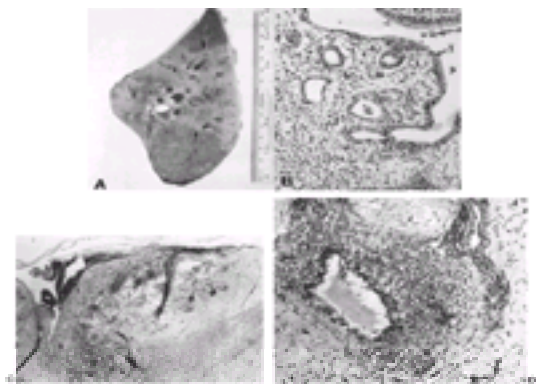

FIG. 6. Pathology of maeid/visna virus (MVV) in sheep. **A, B:** Pneumonia. **C, D:** Encephalitis. **A** and **B** are Fig. 8-69, and **C** and **D** are Fig. 8-68 from ref. 94, with permission.

Feline Immunodeficiency Virus

Primary infection by FIV may lead to low-grade fever, generalized lymphadenopathy, and sometimes diarrhea. During the ensuing months and years, progressing disease is associated with lymphopenia, recurrent fever, lymphadenopathy, anemia, diarrhea, and weight loss of protracted duration. CD4 counts and other cell subsets may be depressed. The final stages of disease are associated with chronic secondary infections, particularly gingivitis, dermatitis, and infections of the upper respiratory tract. Opportunistic infections that have been observed include calcivirus, herpesviruses, toxoplasma, and cryptococcus. Neurologic abnormalities also have been noted, including dementia, twitching tremors, and convulsions. Pathologic lesions primarily reflect those of opportunistic infection.

Simian Immunodeficiency Virus

SIV infection of rhesus monkeys is generally considered the closest model of AIDS in humans. SIV infection of the natural host (e.g., SIVagm in green monkeys or SIVsmm in sooty mangabey monkeys) is not associated with any disease. When SIVsmm was inadvertantly introduced into captive macaque monkey species (Asian Old World primates), AIDS-like disease and lymphomas did ensue (see earlier). AIDS-like disease is generally induced experimentally in macaque monkeys with SIVmac, SIVsmm, or less frequently, SIVagm. As with other lentiviruses, SIV establishes a chronic active infection with a prodromal period of months to years before clinical signs appear. Immunodeficiency is usually but not always associated with marked declines in $CD4^+$ lymphocyte concentrations. Macrophages also are infected. Pathologically, generalized lymphadenopathy is characterized by hyperplasia and is followed by lymphoid depletion in terminal stages (Fig. 7A and Fig. 7B). The gastrointestinal tract, where activated T lymphocytes predominate, appears to be the major site of early viral replication and early loss of $CD4^+$ T cells (see Fig. 7C) (190). However, CXCR4-using viruses may cause a profound loss of T cells in the periphery that is not paralleled in the intestine (72). Marked depletion of progenitor cells occurs in the thymus by 21 days; although a rebound occurs subsequently, thymic dysinvolution is typically seen at terminal stages (see Fig. 7D and Fig. 7E) (198). Nodular lymphocytic infiltrates in a variety of tissues (Fig. 7C), interstitial pneumonia with syncytial cells ("giant cell pneumonia") (Fig. 7H and Fig. 7I), and granulomatous encephalitis (Fig. 7F and Fig. 7G) are variably present. Opportunistic infections are usually present, and these can influence the specific nature of the clinical signs. Common opportunistic infections include *Pneumocystis carinii* pneumonia, generalized CMV, cryptosporidiosis, and *Mycobacterium avium.*

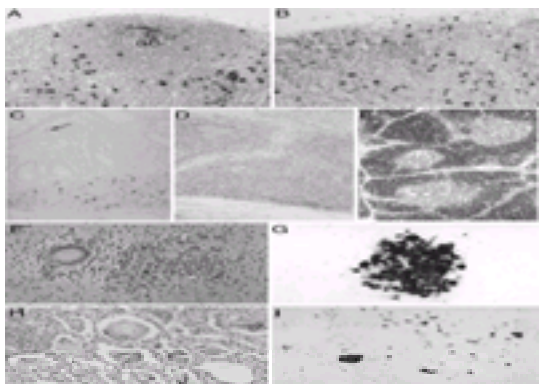

FIG. 7. Acquired immunodeficiency syndrome (AIDS) in monkeys: histopathologic lesions and simian immunodeficiency virus (SIV) localization. **A–C**: *In situ* hybridization for SIV RNA in lymph nodes (**A, B**) and intestine (**C**). In **A**, numerous individual positive cells can be seen in the paracortex of the lymph node of an animal 8 weeks after infection. In addition, diffuse staining of the germinal center (*GC*) of a lymphoid follicle consistent with trapping of virus on follicular dendritic cells can be seen. Note that the lymphoid architecture is relatively intact at this early time point, in contrast to a lymph node from an animal with terminal AIDS in **B**. Although the lymph node in **B** shows severe lymphoid depletion with no evidence of lymphoid follicles, numerous SIV-infected cells can be seen. In **C**, numerous infected cells in intestine can be seen in a submucosal lymphoid nodule with a rare positive cell (*arrow*) in the overlying lamina propria of this animal 2 months after infection. **D, E:** Thymic atrophy. Infection with SIV is associated with profound dysinvolution of the thymus (**D**), as opposed to a normal thymus (**E**) with discrete cortex and medulla. **F, G:** SIV encephalitis. Infection with SIV results in inflammation of the brain in 25% to 50% of the animals that are infected. The brain lesions are characterized by aggregates of mononuclear cells and multinucleated giant cells (**F**). Cells in the lesions contain abundant SIV nucleic acid by *in situ* hybridization (**G**). **H, I:** Giant cell pneumonia. Multinucleated giant cells in this pneumonia (**H**) as well as numerous mononuclear cells contain abundant SIV nucleic acid by *in situ* hybridization (**I**). Figure and legend courtesy of Dr. Andrew Lackner.

Contributions of Individual Genes and Genetic Elements

Because lentiviruses, like other retroviruses, replicate through a proviral DNA intermediate, cloned proviral DNA representing the full-length lentiviral genome can be infectious. Infectious lentiviral DNAs have been used to study the contributions to replication of open reading frames not found in other retroviruses. The unusual, persistent nature of lentiviral replication has raised speculation as to the role of auxiliary genes in the persistent replication and chronic disease of the lentiviruses. Infectious, cloned, lentiviral DNAs also have thus been used to gauge the relative importance and functional role of auxiliary genes in the context of experimental animal infection. The first cloned lentiviral DNA shown to be not only infectious but also pathogenic was one for SIV (97). Infectious clones that are also pathogenic are now available for EIAV, CAEV, and a number of other SIV isolates (28,73,141).

Deoxyuridine Triphosphatase

A dUTPase reading frame is located within the *pol* gene of the nonprimate lentiviruses and the type D retroviruses. dUTPase catalyzes the conversion of dUTP to dUMP and inorganic pyrophosphate (PPi). dUMP is an important precursor for dTTP, used for incorporation into DNA. dUTPase activity in the cell is believed to minimize misincorporation of dUTP into DNA, which can be mutagenic. The effects of eliminating the dUTPase gene (DU) have been studied in EIAV, FIV, CAEV, and visna. In all cases, DU^- virus was still replication competent in cultured cells. Loss of DU in EIAV and FIV had little or no effect on virus replication in permissive fibroblast cell lines that continuously divide. However, loss of DU in these viruses had dramatic effects on virus replication in macrophages (176,192). Macrophages are not a dividing cell and in general have low deoxynucleotide pool sizes. DU^- EIAV exhibited five-fold to 10-fold lower viral loads in plasma at peak compared with the parental virus, and both EIAV and CAEV missing DU were attenuated for pathogenicity (114,183). DU^- FIV and CAEV were found to accumulate increased levels of mutations, particularly G-to-A substitutions (110,183). Visna virus lacking DU also showed decreased viral loads in experimentally infected sheep but still produced neuropathogenic effects on direct intracerebral inoculation (145).

Tat *and* Rev

Tat and *rev* are essential for the replication of SIV and HIV. The *rev* genes of MVV and CAEV have been found to be essential for replication (162,178). *Tat* appears to be essential for replication of BIV and EIAV as well, but in CAEV, where *tat* is only a weak transactivator, *tat* was found to be dispensable for efficient viral replication both in culture and in goats (71). *Tat* is also a weak transactivator in FIV and MVV. Although *rev* is essential for SIV replication, von Gegerfelt et al. (191) have described a rev^- RRE^- strain of SIV that has been rendered replication competent by the addition of a constitutive transport element (CTE). The attenuating effects of loss of *rev* dependence in the rev^- RRE^- CTE^+ SIV are difficult to evaluate because the virus was also nef^-, and no comparison with otherwise isogenic viruses was made. However, based on comparison with published values from other laboratories for nef^- SIV, the loss of *rev* and RRE and the addition of the CTE appeared to have a significant attenuating effect (191). Because antibodies persisted for more than a year without decline, the rev^- RRE^- nef^- CTE^+ SIV appeared to have established a persistent infection but at very low levels.

The tat proteins of lentiviruses can be clearly divided into two groups with respect to whether their transactivating activities are dependent on binding to an RNA sequence element (13,36,130). HIV, SIV, EIAV, and BIV all have long terminal repeats (LTRs) with low basal activity that is strongly induced by their respective tat proteins in a cyclin T1–dependent and RNA sequence–dependent fashion. The LTRs of visna and FIV are strongly active in the absence of any viral proteins, and their tat homologs transactivate via upstream DNA sequence elements. BIV *tat* is unusual in that it is able to bind to the RNA sequence element independent of cyclin T1, but transactivation is nonetheless dependent on the participation of cyclin T1 (13).

S2

An EIAV derivative lacking S2 replicated normally in fetal equine kidney cells, monocyte-derived macrophages, and differentiated macrophages (112). The S2 deletion mutant displayed slightly decreased viral loads and thrombocytopenia-inducing potential when compared with the parental cloned EIAV (111). Infected horses consistently

make antibodies to S2, confirming that S2 is a *bona fide* virus-encoded product.

Vif

Mutations that knocked out the *vif* reading frame of FIV drastically reduced viral replication (165). *Vif*⁻ SIV could be grown significantly only in a *vif*-complementing cell line (40). Thus *vif* is very important for the replication of FIV, SIV, and HIV in most cultured cells. SIVmac239, with a deletion in the *vif* gene, was produced in a *vif*-complementing cell line and inoculated into monkeys (40). Inoculated animals showed no plasma antigenemia, no recoverable virus, and no polymerase chain reaction (PCR)-amplifiable SIV sequences in peripheral blood. However, inoculated monkeys did develop a low-level persisting antibody response. Thus Dvif SIV does appear to be infectious for rhesus monkeys, but it is the most highly attenuated strain yet studied. Little work has been done on the functional activities of nonprimate lentiviral *vif*s, so similarities and differences in their activities compared with HIV-1 *vif* are not known.

Nef

The properties of SIV strains with mutations in *nef* have been extensively studied. SIVmac239 with an in-frame stop at codon 93 of *nef* uniformly reverted to an open *nef* reading frame by 2 weeks after monkey infection (99). Even a form of SIV with a four-amino-acid deletion in *nef* has reverted in about 10% of infected monkeys (194). Thus there is strong selective pressure to maintain an open *nef* reading frame. SIV with deletions in the *nef* gene are markedly attenuated compared with parental virus. Viral loads are typically reduced by 1 to 2 logs at peak 2 weeks after infection and 33–4 logs at set point. Most monkeys infected with SIVmac239Dnef have survived without disease and with undetectable viral loads for as long as they have been studied. Despite the marked attenuation, infection with SIVmac239Dnef is clearly long term and persistent, only at much lower levels. From 10% to 20% of monkeys infected with SIVmac239Dnef have gone on to develop moderate viral loads and to develop AIDS despite the absence of the *nef* gene. Serially passaged Dnef SIV has shown a consistently pathogenic phenotype, and compensatory changes elsewhere in the genome appear to be responsible. *Nef*⁻ SIV replicates similar to wild-type SIV in a variety of standard cell-culture systems, including lectin-stimulated PBMC cultures in IL-2, although conflicting reports have appeared.

The attenuated properties of *nef*⁻ SIV in monkeys appear to be similar to what has been observed in unusual cases of *nef*⁻ HIV-1 infection in humans. One human in the U.S. has been consistently infected with an HIV-1 with large deletions in *nef* since 1983 (101). In Australia, a blood donor with nef-deleted HIV-1 was responsible for infecting eight recipients; the donor and recipients are all slow or nonprogressors with low or undetectable viral loads (37). *Nef*-deleted HIV-1 is clearly an attenuated virus with a similar level of attenuation as Dnef SIVmac. However, as with monkeys infected with SIVmacDnef, some of the humans infected with HIV-1Dnef are showing signs of disease progression (69,109).

The functional properties of SIVmac *nef* appear to parallel those of HIV-1 *nef* in many respects, but there probably are differences in detailed aspects of their function. The amino terminus of SIVmac *nef* consistently overlaps the env reading frame; no such overlap exists for HIV-1. Although there is correspondence in sequence along most of the lengths of SIVmac *nef* and HIV-1 *nef,* SIVmac *nef* is about 40 to 50 amino acids larger because of these extra amino-terminal sequences; these extra sequences appear to have no homologs in HIV-1 *nef.* Most or all of the functional activities that have been reported for HIV-1 *nef* also are seen for SIVmac *nef.* These include CD4 down-regulation, MHC down-regulation, lymphocyte activation, and infectivity enhancement. *Nef* alleles from SIVmac and HIV-2 are able to bind to the zeta chain of the T-cell receptor and activate tyrosine kinase activity (82); HIV-1 *nef* alleles do not do this. Through the use of recombinant viruses, HIV-1 *nef* alleles were shown to be able to substitute for SIVmac *nef* in monkey infections (2). An unusual *nef* allele (Y *nef*; PbJ14 *nef*) has an immunoreceptor tyrosine-based activation motif (ITAM) that results in unusual ability to stimulate lymphocyte activation and to cause an unusually acute disease (47); no analogous HIV-1 *nef* allele has been found.

Vpr *and* Vpx

Two functional activities have been found for *vpr* of HIV-1: facilitation of nuclear import of the preintegration complex in nondividing cells and G_2 cell-cycle arrest (see Chapter 59). HIV-2, SIVmac, and other primate lentiviruses contain two genes related to HIV-1 *vpr*; these have been called *vpx* and *vpr.* The nuclear import function associates with the *vpx* gene, and G_2 cell-cycle arrest associates with the *vpr* gene (53). Two lines of evidence indicate that *vpr* is not a major contributor to the replication of SIVmac in experimentally infected rhesus monkeys. SIVmac239 with a stop signal in the initiating ATG codon of *vpr* was very slow to revert (108). SIVmac239 with *vpr* deleted replicated only slightly less efficiently than did parental virus in rhesus monkeys, and all *vpr*⁻-infected animals have progressed to AIDS (65). The effects of deleting *vpx* from SIVmac239 on levels of replicating virus in rhesus monkeys were more significant, but nonetheless, more than half of the infected animals went on to progress to AIDS (65). A *vpx* deletion of SIVsmPBJ14 was strongly outcompeted when coinoculated with parental virus (79), consistent with these results. Deletion of both *vpr* and *vpx* markedly attenuated the virus, resulting in a virus that was about as attenuated as SIVmac239Dnef (65).

Env

Env is essential for virus replication. The protein products of the *env* gene are responsible for binding receptor(s) on the cell surface and mediating virus entry into cells. The products of *env* are the major or only targets of antibodies that can neutralize infectivity. Determinants of the differences in tropism of related viruses thus often map to the *env* gene. Whereas SIVmac replicates well in stimulated PBMCs from macaques, HIV-1 replicates not at all or only very poorly in macaque cells. Somewhat surprisingly, the block to replication of HIV-1 in macaque cells maps outside of the envelope gene (167). Derivatives of SIVmac in which the envelope gene has been replaced by envelope of HIV-1 are replication competent in macaque cells and capable of infecting macaque monkeys (85,113,119). Such recombinant viruses are called SHIVs. Serial passage of several first-generation SHIVs has resulted in strains that are consistently pathogenic in macaques. A characteristic feature of the first three pathogenic SHIVs that have been derived is that they cause acute,

severe declines in $CD4^+$ lymphocyte concentrations within the first few months of infection.

U3

The U3 regions of the primate lentiviruses are unusually long compared with their counterparts in the nonprimate lentiviruses (86). Most of this unusual length can be accounted for by the overlap of *nef* coding sequences with the LTR. In fact, several lines of evidence suggest that about 300 base pairs (bp) of U3 sequence in SIV and HIV may be nothing other than *nef* coding sequence.

1. In monkeys infected with SIV missing 182 bp in the region that is uniquely *nef,* the SIV progressively and consistently loses about 300 bp of sequence in the region of U3–*nef* overlap (102). The 12 terminal nucleotides of U3 are consistently retained because these are needed for integration. About 50 bp at the C-terminus of *nef* coding sequence, immediately upstream of the nuclear factor kB (NFKB) site, are consistently retained; this region is known to contain a poorly characterized enhancer element (87,122,148).
2. In the human infected with a *nef*-deleted variant of HIV-1, sequences in the U3–*nef* region also were progressively lost over time (101). Again, the U3 terminal nucleotides and 50 bp upstream of the NFKB site were consistently retained.
3. The 300-bp region of *nef*–U3 overlap has been grossly altered in SIVmac239 by 102 point mutations without changing the predicted amino acid sequence of the encoded nef protein (86). This virus replicated like wild-type in rhesus monkeys without evidence of specific reversional events through at least 20 weeks of infection.

These results are most easily explained whereby these 300 bp of U3 sequence serve predominantly or exclusively as *nef* coding sequence. However, it also is possible that this region contains transcriptional control elements that respond to factors induced by *nef* or that in the absence of *nef,* any transcriptional control elements in this region provide less selective advantage than does a shorter genome.

NFKB and Sp1

Sequence elements in U3 for binding NFKB and Sp1 transcription factors have been defined as major enhancer elements of HIV-1, HIV-2, and SIV. SIVmac is still able to replicate well in a variety of cell types, however, in the complete absence of NFKB and Sp1 elements (87). This is due predominantly or exclusively to an enhancer element present within the 50 bp immediately upstream of the NFKB element within sequences that are also *nef* coding sequences (87,122,148). SIVmac239 derivatives with the NFKB sites removed from both LTRs or with all Sp1 sites removed from both LTRs were not detectably attenuated in rhesus monkeys (88). SIVmac239 with NFKB and Sp1 sites removed was detectably attenuated, but not markedly so (88). HIV-1 replication appears more heavily dependent on the presence of the NFKB and Sp1 sites (158), but circumstantial evidence suggests an enhancer element within the 50 bp upstream of the NFKB binding sequence in HIV-1 as well (101). This enhancer element remains poorly characterized.

Multiply-deleted Derivatives

Work in the SIV system has shown that virtually any level of attenuation can be achieved simply by varying the number and locations of deletion mutations (40). Attenuation has been scored by measure of viral load at peak, viral load at set point, strength of the antibody response, and propensity to induce declines in the numbers of $CD4^+$ lymphocytes. Lack of persistence is a phenotype that has never been consistently associated with any mutant strain.

Genetic Resistance

Although individual examples of resistance to disease occur throughout the lentivirus subfamily, the clearest documentations of mechanisms by which it can occur come from studies with humans. In humans, decreased susceptibility to HIV-1 disease is associated with genetic polymorphisms relating to second-receptor use and with MHC diversity (see Chapter 60). In humans, disease progression is quite uniformly related to virus load, with high loads being of poorer prognosis and low loads being of better prognosis.

A very prominent place to look for mechanisms of disease resistance other than in humans is in sooty mangabey monkeys and in African green monkeys. These species are naturally infected with their own SIV without disease, and there is clearly no problem with the SIV that they carry because their SIVs are capable of causing AIDS when transmitted to Asian macaques. Based on experience with HIV-1 in humans, one would expect viral loads in sooty mangabeys and green monkeys to be low. Surprisingly, this is not the case. Many sooty mangabey monkeys harbor SIV RNA loads in plasma in a range (10^5–10^7 copies/mL) in which disease progression uniformly occurs with HIV-1 in humans and SIV in macaque monkeys (95,153). The rates of sequence evolution also are consistent with a high level of continuous virus replication in mangabeys. Data from green monkeys are only beginning to emerge. Thus, the mechanisms of disease resistance in these species remain to be elucidated, but they promise to provide fundamental insights into pathogenesis and immunologic control that have eluded us to date.

A striking example of a genetic polymorphism with analogies to the human situation has been documented in red-capped mangabeys. A 24-bp deletion (D24) was noted in the *CCR5* gene of red-capped mangabeys with an allelic frequency of 86.6% (21). Eleven of 15 red-capped mangabeys were found to be homozygous for this polymorphism. Interestingly, the SIV from red-capped mangabeys does not use *CCR5* as coreceptor but is unusual in its predominant use of CCR2b as coreceptor.

A particular MHC allele in the rhesus monkey has been associated with rapid progression after experimental SIVmac infection. More thorough characterization of MHC types in rhesus monkeys and definition of the SIV peptides that they present may allow testing of how the MHC type of the host and the sequence of the incoming virus influence the extent of

immunologic control that can occur.

DIAGNOSIS

Because lentiviral infections are persistent, antiviral antibodies are present throughout the lifetime of the infected host. Detection of antiviral antibodies is thus the most widely used method for determining the presence of viral infection. A variety of methods are commonly used for antibody detection that vary with the individual virus. These include enzyme-linked immunosorbent assay (ELISA), Western blot, gel diffusion, indirect immunofluorescence, hemagglutination, complement fixation, and neutralization assays. The commonly used Coggins test for the detection of EIAV infection is a gel-diffusion assay for the detection of antiviral antibodies. Attempts are being made to develop simpler ELISAs for EIAV and other nonprimate lentiviruses. ELISAs developed for the detection of antibodies to FIV give a high rate of false positives that is likely due to use of a variety of vaccines containing cellular or serum antigens from production in cell culture. ELISA is most commonly used for the detection of antibodies to SIV; detection by ELISA is usually routine but can be complicated by the history of the monkey, whether the antibodies are to the same or a different type of SIV (i.e., cross-reactive), and in an experimental setting the presence of antibodies at low levels due to attenuation or intervention. Positivity can be confirmed by virus isolation or by identification of viral antigens in plasma or cells. The presence of specific clinical signs and clear demonstration of the presence of antilentiviral antibodies is usually sufficient for a definitive diagnosis.

PREVENTION AND CONTROL

MVV was eradicated from Iceland by a drastic slaughter policy before the availability of diagnostic tests. Test and removal programs have since been and continue to be used as an effective means of control. Test and removal programs may be voluntary or mandated. Buyers of horses have increasingly sought negative test certification for EIAV, and negative test certification is required as a condition for entry into many racetracks, saleyards, and shows. Horses imported into the U.S. and some other countries are required to have a negative test certificate. Testing within a state is not always compulsory, nor is it compulsory for an owner to destroy a positive horse. For FIV, appropriate test and removal programs and certification at the point of sale have been sparingly applied.

The only lentivirus for which a vaccine has been used in the field is EIAV. The vaccine is a live attenuated strain developed by researchers in China by repeated passage in donkey cells (166). The vaccine has been extensively used in China and in Cuba. The vaccine has been administered to millions of horses in China with apparent safety and efficacy. Unfortunately, very little is known by the western world about the properties of this live attenuated vaccine, its genetic composition, or details regarding its performance in the field.

RESEARCH ON VACCINE DEVELOPMENT

One important application of research with nonprimate lentiviruses is in the area of vaccine development. Certainly, development of a safe, effective, affordable vaccine for AIDS is one of the greatest challenges of our times. Most experimental studies have used SIV in rhesus monkeys. Use of SHIV in rhesus monkeys is coming into more frequent use because it allows analysis of HIV-1 env-containing vaccine products in a system in which challenge and meaningful viral load and disease readouts are readily possible. Other studies have used FIV and EIAV.

Expectations were raised when inactivated whole SIV was found to provide protection against pathogenic strains of SIV (41,132). Hope was quickly dashed, however, when it was found that protection occurred only when the vaccine and challenge stocks were grown in human cells (30,31). When the vaccine was grown in human cells and the challenge virus was grown in monkey cells, protection was not observed. Xenoantigens present in virus preparations were able to confer protection when challenge virus was grown in the same xenogeneic cells. MHC class II was found to be at least one of the xenoantigens capable of conferring protection (6). Class II antigens are actually present in greater abundance in virus particles than the virus-encoded envelope proteins (5). The mechanisms by which antibodies to class II present on the surface of virus particles can block viral infection have not been defined, but it could be as simple as steric hindrance. Improved methods for purifying, inactivating, and delivering whole particles justify continued investigation of this vaccine approach (159). Whether the abundance of class II antigens on virus particles has significance for pathogenesis remains to be determined.

Vaccine studies in animal models can provide useful information in several different ways. Head-to-head comparisons of different vaccine approaches can shed light on which approaches perform more effectively, at least under the defined conditions of the experiments. In-depth analyses of individual vaccine approaches also may provide fundamental insights into immunologic control and what is needed for protective immunity. With theses goals in mind, vaccine approaches that have been tried in animal models include a variety of envelope subunit approaches; poxvirus recombinants; DNA; inactivated whole particles; adenovirus recombinants; and prime and boost protocols that use combinations of these approaches. Several themes arising from the studies done to date are worth noting.

1. The particular virus that is used for challenge is one of the single most important determinants of whether an individual vaccine challenge study will be successful. Easy-to-neutralize, nonpathogenic strains of virus have proven relatively easy to protect against. Difficult-to-neutralize pathogenic strains of virus, which could be considered representative of field strains of HIV-1, have proven very difficult to protect against. Homologous cloned virus challenge is less stringent than closely matched uncloned virus challenge, which in turn is much less stringent than challenge by virus with natural, representative levels of sequence divergence.
2. Timing of the challenge is crucial. The vaccine approaches discussed earlier induce transient immune responses; they decay dramatically after the peak of vaccine-induced immune responses, which occurs within the first few weeks after the last vaccine boost. Most challenge experiments have been performed 2 to 4 weeks after the last vaccine boost.
3. It is easier to achieve protection against mucosal challenge than against intravenous challenge. Most early studies used intravenous challenge because of the ability to achieve infection reproducibly in control monkeys with

carefully controlled doses of challenge virus. Many current studies are now using vaginal mucosal or rectal mucosal challenge because these routes represent major modes of HIV transmission around the world.

4. Antibody responses to envelope protein and cellular responses to core *gag–pol* antigens can contribute to protection. Which type of immune response can be most responsible for protection varies with the vaccine approach and with the characteristics of the challenge virus. Inclusion of *gag–pol* and *env* in poxvirus–SIV recombinants results in improved protection in a vaccine/challenge format compared with when either *gag–pol* or *env* was used alone (149).
5. When pathogenic, difficult-to-neutralize strains have been used for challenge, protection has proven very difficult to achieve with the vaccine approaches described. Even using challenge virus identical or highly homologous in sequence and challenge 2 to 4 weeks after the last vaccine boost, the levels of solid long-term protection have seldom reached even 50%.

Live attenuated strains of SIV have performed more impressively as vaccines in experimental monkey studies (33). Although serious hurdles face practical development of live attenuated vaccine approaches for AIDS, continued studies hope to identify critical components of a protective immune response and critical variables that may influence whether a protective immune response is attained. In studies to date, it has been learned that a wide range of attenuation can be achieved by variation of the number and location of deletion mutations (40). Some strains, for example SIVmacD4 (missing *vpr, vpx, nef,* and U3 sequences) and SIVDkRXN (missing NFKB, *vpr, vpx,* and *nef*) are very highly attenuated but still persistent. SIVDvif is so highly attenuated that infection is negative by standard criteria. The ability to achieve protection varies inversely with the degree of protection. Thus more highly attenuated strains are safer but less protective as vaccines (93). Protection is also time dependent; the longer one waits, the better the protection. A protective state can take 6 months or longer to become established. An understanding of protective immunity by live attenuated strains will require an explanation of why it takes so long to develop. Even the live attenuated vaccine strain SIVmac239D3 (missing *vpr, nef,* and U3 sequences) provides only minimal protection against heterologous challenge with SIVsmE660, which contains a level of sequence divergence that one would expect to encounter with a field strain of virus (197). Although this difficulty could conceivably be addressed by using mixtures of sequences in the vaccine strain, the results illustrate the severe difficulties in achieving protection against pathogenic, difficult-to-neutralize strains of SIV. It remains to be seen whether alternate means of attenuation or alternate means of using live attenuated strains (for example, priming with very highly attenuated live virus and subsequent boosting by a different means) can result in improved safety/efficacy profiles.

RESEARCH ON THERAPEUTIC REGIMENS

Antiviral drugs useful against HIV-1 in people have been developed with little or no input from animal models of lentiviral infection. This trend will likely continue. However, there is a growing realization that therapy studies in model systems can provide certain types of valuable information. Cytokines, immunoadjuncts, immune stimulants, immune suppressants, or other highly experimental treatments that could possibly be harmful in the setting of a lentiviral infection can be examined for possible benefit in animal models. Faced with several possible lead compounds, some companies have chosen head-to-head comparisons in an animal model to help select the most promising compound to move forward. Comparisons of experimental regimens during primary infection, analysis of ways to boost virus-specific $CD4^+$ T-helper cell responses during therapy, and analysis of ways to minimize maternal–infant transmission are also areas in which ongoing work in animal models may provide useful information to help guide trials of therapeutic regimens in people.

Some of the antiviral drugs useful against HIV-1 have reduced or marginal activity against SIV. Überla et al. (184) have generated an RT-SHIV in which the reverse transcriptase reading frame of SIVmac239 has been replaced by that of HIV-1. This RT-SHIV is replication competent and capable of inducing disease in monkeys.

PMPA (*R*-9-2-phosphonylmethoxypropyl adenine) is one compound with strong antiviral activity against both HIV-1 and SIV that has been used extensively in monkey studies. Tsai et al. (180) were the first to show that treatment of monkeys with PMPA by 24 hours after SIV inoculation and continuing treatment for 4 weeks prevented the establishment of a persistent infection. Delaying the initiation of therapy to 48 or 72 hours or shortening the duration of treatment significantly reduced efficacy (179). Nonetheless, even when the establishment of persistent infection was not prevented, early treatment significantly reduced viral loads and prolonged AIDS-free survival (179,189).

In a more recent study (115), monkeys were started in PMPA therapy 24 hours after inoculation of SIVsm E660 and kept on drug for 28 days, similar to the studies of Tsai et al. (179) and van Rompay et al. (189). Establishment of a detectable, traditional, persistent infection was prevented, confirming the results of Tsai et al. Interestingly, when three such animals off drug for 6 weeks were subsequently challenged intravenously with SIVsm E660, one animal was completely protected, and the other two showed dramatic reductions in viral load (115). What is most startling and difficult to understand is that protection was achieved in these animals in the absence of detectable SIV-specific antibody or CD4-helper responses (CTLs were not measured) at the time of challenge. Continuation of this line of investigation promises important new insights into immune-mediated control outside the bounds of our current level of understanding.

Hematopoietic stem cell gene therapy is an attractive, futuristic approach for the prevention of HIV-induced disease, but it is clear that many developmental hurdles must be cleared. Research with SIV in monkeys is proving useful toward this goal. Transduction of $CD34^+$ hematopoietic progenitor cells with an anti-tat gene protected T-cell and macrophage progeny from SIV infection (157). Infusion of lymphocytes with antisense tat/rev genes into macaques may have reduced SIV replication on subsequent SIV inoculation (45).

PERSPECTIVE

The worldwide crisis brought on by HIV and AIDS has focused attention on the nonhuman lentiviruses as a source for information that will shed light on the human condition. Lentiviral infections of domesticated animals are economically important in their own right and have been studied historically in this context. The lack of a reliable, usable animal model that uses HIV-1 itself and that recapitulates the features of human infection has resulted in extensive study of nonhuman

lentiviruses as analog models in which susceptible hosts are infected with nonhuman lentiviruses either naturally or experimentally. The most critical issues for future progress include better understanding of pathogenesis; improvements in therapy; improved availability of therapy; and, perhaps most important, development of a safe, effective, affordable vaccine. The most remarkable feature of these viruses, and perhaps most critical for the eventual control of HIV, is their ability to replicate continuously and unrelentingly despite apparently strong immune responses. Further advancement of therapeutic regimens and development of effective vaccines against HIV will have to deal with the strategies of immune evasion used by this group of viruses.

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CHAPTER 62 Other Human and Primate Retroviruses

Maxine Linial and Robin A. Weiss

The preceding five chapters have dealt in some detail with retrovirus replication, with the important human retroviral pathogens, human T-lymphotropic viruses (HTLV-I and HTLV-II) and human immunodeficiency viruses (HIV-1 and HIV-2), and with nonhuman lentiviruses (nomenclature from 54). In this chapter, other retroviruses in humans and in simian species are described. Of perhaps greatest scientific interest are the foamy viruses, which is a group of complex retroviruses that stand distinct from all other retroviruses in their genome organization and mode of replication. Foamy viruses are found in most species of primates studied, although humans zoonotic infections are rare. Foamy viruses can be highly cytopathic in cell culture, but they have not been definitively associated with disease.

The other retroviruses of primates discussed here belong to the *Betaretrovirus* and *Gammaretrovirus* genera. Figure 1 depicts a phylogenetic tree of selected retroviruses, including those discussed in this chapter. Betaretroviruses are B-type and D-type retroviruses that form core particles in the cytoplasm of the host cell before budding from the plasma membrane (see Chapter 57). Simian retroviruses 1 to 5 are D-type viruses that cause immunodeficiency in macaques. The recently discovered human retrovirus 5 (HRV-5) genome is also a distantly related betaretrovirus, as is a major group of noninfectious human endogenous retroviral genomes known as HERV-K. Gammaretroviruses are C-type retroviruses related to murine leukemia virus (MLV). Primate gammaretroviruses include those that can replicate in human cells, such as the infectiously transmitted gibbon ape leukemia virus (GALV), an endogenous baboon gammaretrovirus (79), and the baboon endogenous virus, which is a recombinant genome with a betaretroviral *env* gene (127). There is concern that nonhuman gammaretroviruses might infect people through xenotransplantation of animal tissues and organs; hence, cross-species infection or zoonosis by retroviruses is briefly discussed.

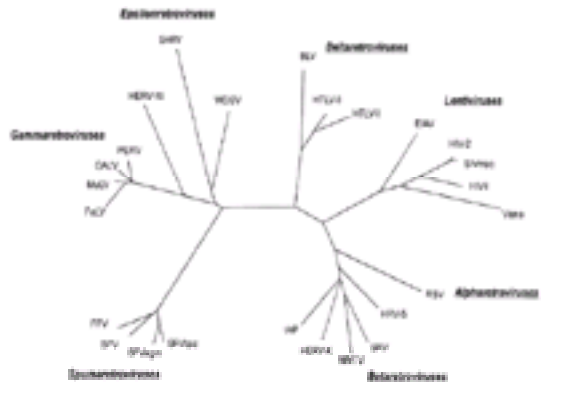

FIG. 1. Phylogenetic relationships of the viruses discussed in this chapter. Alignments were done using the RT domain of Pol. SnRV, snake retrovirus; WDSV, walleye dermal sarcoma virus; BLV, bovine leukemia virus; EIAV, equine infectious anemia virus; RSV, Rous sarcoma virus; IAP, intracisternal A type particles; FeLV, feline leukemia virus; other viruses described in text. (Compilation by D. Griffiths, University College London.)

FOAMY VIRUSES

Description and Prevalence

Despite having been isolated almost 30 years ago, only since the 1990s has there been a large number of reports about foamy virus molecular biology and biology. It was widely assumed for many years that the replication pathway of these complex retroviruses would be very similar to those of the well-characterized gammaretroviruses and lentiviruses. Recent work has, however, belied this idea. The foamy viruses are in the most divergent branch of the retroviral family (the genus *Spumavirus*), and many of the well-accepted paradigms of retroviral replication do not apply to them. In some aspects of their life cycle, the foamy viruses are more similar to the hepadnaviruses, such as human hepatitis B virus (HBV), than to retroviruses.

The highly vacuolating, foamy-appearing cytoplasm of some infected cells and the presence of multinucleated syncytia have given rise to the name of the genus. It is thus surprising that a distinguishing feature of the foamy virus group is a lack of documented pathology in either naturally or accidentally infected hosts. Foamy viruses have been found to be widespread among domestic and feral cats, cattle, and horses and are in most, if not all, primate species other than humans (reviewed in 52). There are also reports of foamy virus detection in sheep, hamsters, and sea lions, but viral isolates from these species are not available for further study. Individual humans have been shown to have acquired foamy viruses as zoonotic infections. Although there is no evidence for viral spread between humans, it is possible that foamy virus may become more prevalent in human populations in the future, especially in populations that have close contact with subhuman primates.

Foamy viruses have been isolated from a number of species. Most research has concentrated on the primate foamy virus, which is the focus of this chapter. The available viral isolates are listed in Table 1. The names given to the primate viruses have resulted in some confusion, especially because cross-species infections occur readily, and similar viruses have been recovered from more than one primate species (115). Additionally, in some cases, the same viruses have been given different names. The phylogenetic relationship of foamy virus to the other retroviral genera is shown in Figure 1. A great deal of work has gone into comparing the genomes of the primate foamy viruses. These viruses appear to have coevolved along with their primate hosts (17). Figure 2 gives the evolutionary relationship among the primate foamy viruses, showing that the sequence similarity among the viruses mirrors that of the host species.

Species	Virus isolates (other designations)	Reference
Feline	FFV (FeFV)	102
Bovine	BFV (BSV)	78
Equine	EFV	125a
Rhesus macaque (*Macaca mulatta*)	SFVmac (SFV-1, SFV-2)	57
African green monkey (*Cercopithecus* species)	SFVagm (SFV-3)	120
Squirrel monkey (*Saimiri sciureus*)	SFVsqu (SFV-4)	58
Galago (*Galago crassicaudatus panganiensis*)	SFVgal (SFV-5)	58
Chimpanzee (*Pan troglodytes* species)	SFVcpz (SFV-6, SFV-7)	103
Spider monkey (*Ateles* species)	SFVspm (SFV-8)	53
Capuchin (*Cebus* species)	SFVcap (SFV-9)	52
Baboon (*Papio cynocephalus*)	SFVbab (SFV-10)	101
Orangutan (*Pongo pygmaeus*)	SFVora (SFV-11)	83
Gorilla (*Gorilla gorilla* species)	SFVgor (SFVgg)	10
Marmoset (*Callithrix jacchus*)	SFVmar	80
Human	SFVcpz(hu) (HFV, HFVhu, CFVhu)	1

Note: The table lists only those foamy viruses whose existence is well documented. In some cases, several different viral types have been given the same isolate number. Infectious clones exist for SFV, FFV, SFV-1, and SFVcpz(hu), which is the prototype for the group and is generally referred to as HFV in the literature. The designations that can be found in the literature are given in parentheses.

TABLE 1. *Foamy virus isolates*

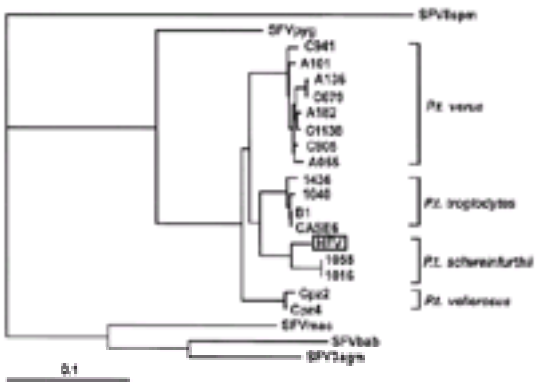

FIG. 2. Phylogenetic relationships of primate foamy viruses. The neighbor-joining method was used to compare integrase sequences from simian foamy virus (SFV), the foamy virus called human foamy virus (HFV) isolated by Achong and associates (1) boxed, and a foamy virus from a human (case 6) infected with a chimpanzee-like SFV. SFV sequences were obtained from chimpanzee subspecies *Pan troglodytes verus* (C941, C679, C1138, A055, A101, A136, A182); *Pan troglodytes troglodytes* (C905, 1040, 1436, B1); *Pan troglodytes schweinfurthii* (1016, 1058); *Pan troglodytes vellerosus* (SFVcpz2, SFVcpz4). SFVpyg, sequence obtained from a pygmy chimpanzee (*Pan paniscus*); other SFVs are described in Table 1. The scale bar represents an evolutionary distance of 0.10 nucleotides per site. (Adapted from ref. 47, with permission).

Origin and Prevalence of Human Foamy Virus

The first description of what was presumably a simian foamy virus in cultures of monkey kidney cells was reported in 1954 (23). However, a primate foamy virus was not isolated until 1971. A virus, with what has proved to be the characteristic foamy virus morphology, was isolated from lymphoblastoid cells released into the supernatant from a human nasopharyngeal carcinoma tissue culture obtained from a Kenyan patient (1). This virus is called human foamy virus (HFV), and is the *Spumavirus*-type species. This so-called HFV was the first foamy virus to be molecularly cloned and sequenced, with an infectious DNA clone derived (31,66,82,100). Later, when a foamy virus was isolated from chimpanzees, it became apparent that the chimpanzee simian foamy virus (SFVcpz) is highly homologous to the laboratory strain of HFV (48). More recently, sequence comparisons of the integrase gene between the HFV isolate of Achong and associates (1), and those of SFVcpz obtained from the four chimpanzee subspecies shows that HFV is most closely related to the foamy virus from *Pantroglodytes schweinfurthii*, which is the species present in Kenya (47) (see Fig. 2). It is therefore possible that the donor from whom the cell culture was derived had acquired foamy virus as a zoonotic infection through chimpanzee contact.

Many papers have been published since the isolation of HFV that purport to demonstrate prevalence of HFV in human populations and to link HFV to different human autoimmune diseases. However, the published data are for the most part based either on a single serologic assay or on polymerase chain reaction (PCR) analysis, rather than both techniques. More recently, two groups have analyzed serum samples taken from large numbers of healthy and diseased individuals for HFV antibodies, including many samples from human populations previously suggested to harbor HFV. In these more recent studies, all samples that were initially positive in one serologic assay were retested with at least one additional assay, or by PCR if DNA was available. Screening of more than 8,000 serum samples revealed no positive cases. Only individuals who acquired foamy virus through zoonotic infection, generally by severe monkey bites, were shown to be infected (3,117). These two studies convincingly demonstrated that HFV is currently not prevalent in the human population. For the purpose of this review, the primate foamy viruses are collectively referred to as PFV. It should be noted that most work has been done with HFV, more correctly called SFVcpz(hu).

Virion Structure

Foamy viruses have a morphology that is distinct from that of the other retroviruses; most noticeably, foamy virus particles have an immature-appearing core and highly prominent surface spikes, composed of the envelope glycoproteins. Virions are seen to bud from both the cell membrane and the endoplasmic reticulum (ER) membrane, where they acquire glycoproteins (Fig. 3A and Fig. 3B). A highly schematized drawing of a foamy virus particle is shown in Figure 4. Very recent structural studies have revealed details of the foamy virus Env protein structure on the surface of released PFV and feline foamy virus (FFV) particles. Electron microscopy of negatively stained particles shows that FV Env proteins are arranged in trimeric complexes, a structural organization considered to be a common feature of retroviral Env surface proteins (see Fig. 3C). The XFV Env trimers are arranged in regular hexameric ringlike strictures, in which two Env glycoprotein trimers are fully integrated into adjacent rings (137).

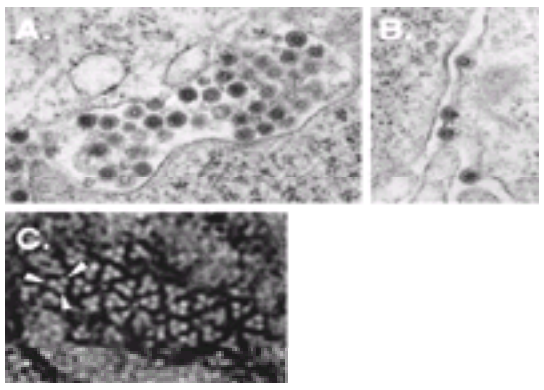

FIG. 3. Electron micrographs of SFVcpz(hu) replicating in human diploid embryonic lung fibroblasts, using negative staining. **A:** Virus budding through the endoplasmic reticulum (ER) membrane. Enveloped particles are seen within the ER. **B:** Virus budding through the plasma membrane. **C:** Electron micrograph of negative-stained SFVcpz(hu). The particles often show a network of trimeric viral spike proteins on the particle surface (*arrowheads*). The 14-nm long glycoprotein complex is predominantly arranged into rings of six subunits. Adjacent hexameric rings always share two completely integrated subunits. (Adapted from ref. 142, with permission.)

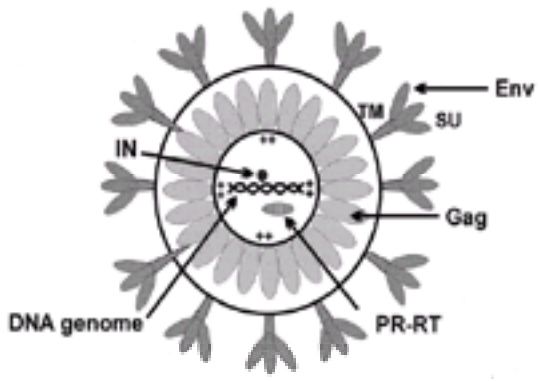

FIG. 4. The foamy virus virion. This highly schematic figure shows the relative locations of the structures and proteins. It does not accurately depict the viral organization. Plus signs (++) indicate the positively charged residues at the carboxyl terminus of Gag in the GR boxes thought to interact with nucleic acid.

As discussed later, efficient cleavage of the Gag precursor protein to the usual retroviral matrix (MA), capsid (CA), and nucleocapsid (NC) proteins does not occur in extracellular, infectious virions. Thus, FV particles appear similar to those of immature gammaretroviruses such as MLV, which are unable to undergo maturation by virtue of mutations in the protease protein. It is likely that the Gag protein is arranged as is the core protein of hepadnaviruses (see Chapter 86), with the amino terminus toward the outside surface of the particle and with the carboxyl terminus, containing positive charged amino acids that are part of the nucleic acid binding domain (143), to the inside of the particle in contact with the genome. However, little is known about other functional domains of FV Gag.

Most extracellular SFVcpz(hu) particles contain RNA; however, a surprisingly large fraction (about 20%) contain apparently full-length double-stranded DNA copies of the RNA (146). Several studies with the reverse transcriptase (RT)

inhibitor 3'-azido-3'-deoxythymidine (AZT) strongly suggest that the infectious genome of SFVcpz(hu) is DNA. In these experiments (88,146), it was shown that treatment of cells with AZT before infection has very little effect on viral infectivity. In contrast, treatment of cells after infection greatly decreased the infectivity of the extracellular particles when assayed on untreated cells, without affecting the amount of virus released. Although the data for SFVcpz(hu) isolate are convincing, similar experiments have not been done using other members of the genus. Additionally, details of the structure of the foamy virus DNA genome have not been reported. Thus, the foamy viruses should be only provisionally considered retroviruses with DNA genomes.

Genome Organization

The general organization of the foamy virus genome is similar to that of the other complex retroviruses, which encode nonstructural proteins as well as Gag, Pol, and Env. The size of the proviral genome is at the upper end of retroviral genomes, about 11.6 kb. As shown in Figure 5A, which diagrams the genome of PFV, the provirus has the requisite retroviral long terminal repeats (LTRs) and a primer binding site, which is complementary to tRNA $_{lys1,2}$. The genome encodes the three characteristic retroviral genes, *gag, pol* and *env,* although as detailed later, the modes of expression and roles of these gene products in viral replication are somewhat unexpected. In addition, there are at least two nonstructural proteins encoded by the primate foamy virus, Tas (previously called Bel-1 for PFV) and Bet. Bet is encoded from both the *tas* and *bel2* open reading frames (ORF). There is no strong evidence for a protein encoded only within the *bel2* ORF, and the *bel3* ORF is not conserved in other primate foamy viruses. The same genomic organization has been found for BFV and FFV, which have also been fully sequenced. These lack the *bel3* ORF. Although the simian, bovine, and feline viruses all encode a Bet protein from the *tas* and *bel2* ORFs, sequence conservation at the amino acid level is low. Thus, although its function may be conserved, it can be provided by highly divergent proteins.

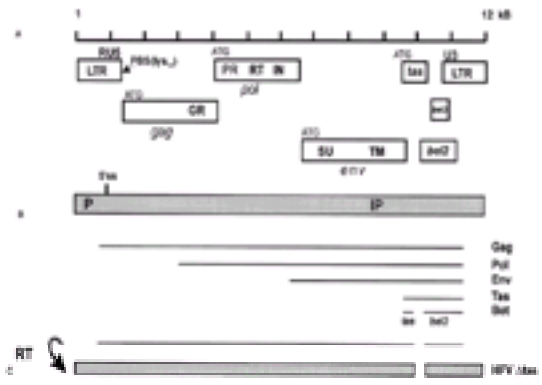

FIG. 5. Foamy virus genome and RNAs. **A:** Organization of the viral genome and coding region of SFVcpz(hu). Open reading frames (ORFs) are shown relative to the proviral structure. Sites of translational initiation are indicated by an ATG initiator codon. ORF names are italicized. *Gag GR* indicates the glycine-arginine boxes, which are the only defined feature of this protein. **B:** Location of the long terminal repeat (LTR) promoter (*P*) and the internal promoter (*IP*) in the provirus. The mRNAs for the five known gene products are indicated below. **C:** Structure of the RNA initiating at the long terminal repeat (LTR) promoter but containing the *tas/bel2* splice, which is thought to lead to the human foamy virus (HFV) D *Tas* genome by reverse transcription. (Adapted from ref. 72, with permission.)

A unique characteristic of the foamy viruses is the presence of a second transcriptional promoter in addition to the normal promoter in the U3 region of the LTR (65). This internal promoter (IP) is in the coding region for the Env protein (see Fig. 5B) and appears to be used for synthesis of the nonstructural protein transcripts, encoding Tas and Bet (64). Bet is synthesized from a transcript in which a portion of *tas* encoding 88 amino acids is fused to the *bel2* ORF. Both the LTR promoter and the IP require the transactivator protein Tas for transcription. As discussed later, these promoters play different roles in viral replication. Additional details about regulation of viral gene expression can be found in recent reviews (72,99).

Another surprising finding about foamy virus gene expression is the method of synthesis of the Pol protein. All viruses in the six other retroviral genera encode Gag-Pol fusion proteins by use of either a suppressor tRNA or ribosomal frame shifting (see Chapter 57). In contrast, the foamy viruses encode Pol from a unique spliced mRNA, using a splice acceptor site at the 3' end of the *gag* ORF (142) (see Fig. 5B). Thus, foamy virus Pol is assembled into virions in a manner different from that of the retroviruses, wherein Pol is assembled by means of the Gag assembly domains in the Gag-Pol polyprotein. The hepadnaviruses also assemble their reverse transcriptase (P) protein into particles independently of the structural proteins (described in detail in Chapter 86). Hepadnaviral P protein binds in *cis* to its own mRNA (which is equivalent to genomic RNA), to initiate the assembly process in conjunction with core protein. Because foamy virus Pol is synthesized from a subgenomic mRNA, a hepadnavirus-like mechanism for Pol assembly can be excluded. Like other retroviruses, the foamy virus Gag protein is encoded from genomic length RNA, and Env protein is encoded from a spliced mRNA. A secreted Env-Bet fusion protein has also been reported in some tissue culture cells, synthesized from an alternatively spliced RNA, which removes a portion of Env (37,71). The function, if any, of this gene product is unknown.

Virion-Associated Proteins

Each of the three virion-associated gene products, Gag, Pol, and Env, has features unique to the foamy virus. Both Gag and Env have features that are shared with human HBV and the other hepadnaviruses. The organization of the viral proteins is shown in Figure 6.

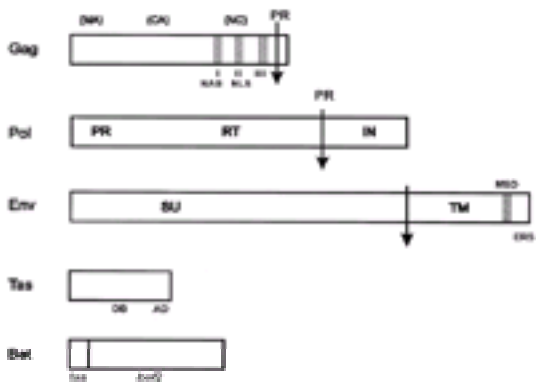

FIG. 6. Viral proteins encoded by SFVcpz(hu). The *arrows* show the most efficient protease cleavage sites. The location of the MA, CA, and NC protein domains in other retroviruses are shown in *parentheses.* The three Gag GR boxes are indicated by *shaded boxes.* The membrane spanning domain (MSD) in *Env* is indicated by a *shaded box*. For *Tas,* the DNA binding domain (*DB*) and activation domain (*AD*) are indicated. NAB, nucleic acid binding; NLS, nuclear localization signal; ERS, endoplasmic reticulum retrieval signal. (Adapted from ref. 72, with permission.)

The foamy virus Gag protein has a calculated molecular weight of 70.6 kd, although this protein migrates on polyacrylamide gels with an apparent molecular weight of 74 to 78 kd. It is phosphorylated on serine residues (24). Unlike other retroviral Gag proteins, it is not efficiently cleaved into matrix, capsid, and nucleocapsid domains in infectious virions. Only one Gag proteolytic cleavage occurs efficiently, in which about 3 kd is removed from the carboxyl terminus. This cleavage occurs about half the time, such that particles contain equal amounts of two large Gag proteins. Gag cleavage is required for production of infectious virus (24). Inefficient processing of the large Gag proteins into smaller components is also seen in infected cells. These secondary cleavages may occur as an early step in infection and may be important for uncoating (95). Foamy virus Gag lacks the two hallmarks of retroviral Gag proteins, a major homology region in capsid, and one or two cystidine-histidine motifs in nucleocapsid. Although it is assumed that the amino terminus of foamy virus Gag has matrix-like functions and that the middle of the protein has capsid-like functions, this has not been directly shown. The carboxyl terminus of PFV Gag has three glycine-arginine–rich regions called *GR boxes.* The first of these binds both RNA and DNA (143), and the second contains a nuclear localization signal. Transient nuclear localization of newly synthesized Gag proteins is a distinct feature of infection of fibroblasts by PFV (111). However, the role of nuclear localization in the viral replication pathway is not clear because a mutant virus lacking a nuclear localization signal (NLS) can still replicate (143). Interestingly, FFV Gag lacks an NLS, and the Gag proteins do not localize to the nucleus (15).

The foamy virus Pol precursor protein, of about 127 kd, encodes the same four enzymatic domains as other retroviruses: protease (PR), RT, RNAase H (RH), and integrase (IN). Unlike, other retroviral Pol proteins, however, cleavage of the protein to PR, RT/RH, and IN moieties does not occur. Instead, only IN is cleaved from the Pol precursor, leaving a PR-RT/RH protein (see Fig. 6), which is a multifunctional enzyme. Active protease is required for viral replication (63). The catalytic domain of the foamy virus RT is not YMDD as in most retroviruses, but YVDD, the same residues as in MLV. Although PFV RT is sensitive to AZT (88,146), it is resistant to most other RT inhibitors developed for HIV (M. Linial and associates, unpublished observations). The IN protein contains the conserved DD35E motif, which is required for integrase function in all retroviruses; mutation of this motif prevents foamy virus replication (25,85). Integrated proviruses contain a 4-nucleotide cellular repeat at each junction (89).

The foamy virus Env precursor protein of about 130 kd is cleaved by a cellular protease into the surface (SU) and transmembrane (TM) moieties, as in other retroviruses. However, there are features unique to the foamy virus *env* gene. Most prominently, extracellular virions are not produced unless Env is present (6,30), reminiscent of hepadnaviruses, rather than retroviruses. The domains in Gag and Env required for budding have not as yet been identified. Foamy virus particles formed by Gag-Gag interactions assemble intracellularly, predominantly in the cytoplasm, probably in a manner similar to that of the betaretroviruses (see Chapter 57). Budding occurs predominantly through intracellular membranes, probably those of the ER, although some particles can be seen budding from the plasma membrane. The foamy virus Env protein contains a dilysine motif at the carboxyl end of the TM protein adjacent to the membrane-spanning domain (see Fig. 6), which has been shown to be a classic ER retrieval signal (39), similar to that of the hepadnaviruses surface protein. The cognate foamy virus envelope glycoproteins are required for foamy virus assembly, release, and infectivity. Unlike with other retroviruses, foamy virus cannot be pseudotyped with other retroviral glycoproteins or the vesicular stomatitis virus G glycoprotein (96). However, MLV can be pseudotyped with a chimeric protein containing the extracellular and transmembrane domains of PFV and the cytoplasmic domain of MLV (70). It is likely that preformed foamy virus capsids must interact with the viral glycoproteins at the ER, or with other cellular membranes, in order for egress from the cell. Mutation of the ER retrieval signal leads to more frequent budding from the plasma membrane but neither increases the amount of extracellular viruses nor decreases the infectivity of the viral particles (38). In general, budding is a highly inefficient process in tissue culture cells; most particles are cell associated rather than released into culture supernatants. Only 1% to 10% of the infectious particles are released from cells, and in order to prepare high-titer stocks of virus, it is necessary to combine free-thawed cell lysates to culture supernatants.

Nonstructural Proteins

Tas is a potent transcriptional transactivator protein with no known nonfoamy virus homologs. It contains a central domain that interacts with DNA, and a carboxyl-terminal activation domain (44). Unlike the transactivator proteins of other complex retroviruses, such as HIV and HTLV, Tas is a sequence-specific DNA-binding protein, which probably requires interaction with cellular DNA binding proteins (43). Tas-binding sites of about 25 nucleotides in length have been mapped to both the LTR and IPs. Interestingly, the binding sites have different primary sequences, and the IP-binding site has a higher affinity for Tas (59). It is likely that early in infection, the IP synthesizes the Tas and Bet transcripts, and after sufficient levels of Tas accumulate, the LTR promoter is used to synthesize Gag, Pol, and Env mRNAs. Such temporal

regulation of transcription is a property more commonly seen in DNA viruses than in RNA viruses and has not been found in any other retrovirus group.

The role of the Bet protein in viral replication is poorly understood. It is not a posttranscriptional transactivator analogous to HIV Rev or HTLV Rex (69). Virus lacking Bet protein can infect tissue culture cells and replicate fairly efficiently (8,69,144). However, conservation of the *bet* ORF from a *tas-bel2*–spliced mRNA in all the diverse foamy viruses suggests that Bet has an important function, perhaps manifested only *in vivo.* There is a suggestion that, at least in fibroblast cultures in which Bet has been overexpressed, Bet can block viral superinfection at an early step (14). Because Bet protein can be taken up by uninfected cells (37), it is possible that Bet could have a physiologic role in modulating infection. It is also interesting that infected cells rapidly accumulate DNA molecules lacking the portion of Tas removed by the Bet splice. This D Tas DNA appears to arise by reverse transcription of a RNA, initiating at the LTR promoter but using the *tas/bel2* splice sites (107) (see Fig. 5**C**). Tas DNA has been proposed to play an important role in establishment of persistent infections by behaving as a defective interfering genome (106). It is not clear that such defective proviruses play a role in *in vivo* infections (27). The *bel3* ORF of PFV has been proposed to encode a superantigen (134), and although there is a report of a *bel3*-encoded protein of 19 kd (135), this report has not been confirmed. Furthermore, this ORF is not conserved in other foamy virus isolates.

Host Range *in Vitro*

The foamy viruses have a broad host range, best characterized for PFV. This virus infects cells derived from all vertebrate species tested, including primates, hamster, mouse, quail, dolphin, and iguana (49). Thus far, no resistant cells have been identified. Infection of primary cells or cell lines often leads to a lytic infection. Productively infected cells, such as human fibroblast cultures, form large multicellular syncytia and are killed within several days. However, little infectious virus leaves the cell, and at least in tissue culture cells, most infectivity is cell associated. A second response to viral infection is seen with some cell lines in which viral infection is not cytopathic but persistent. Cells continue to grow with normal kinetics and continually produce virus, at titers 100-fold or less than the productively infected cells. No syncytium formation of cell death is seen in persistently infected cultures. Again, most of the virus is cell associated. In general, infection of new cells is more easily achieved by cell–cell contact than by free virus (145).

Foamy viruses have been long known to infect a wide variety of cell types, including those of the fibroblastoid, epitheliod, and lymphocytic lineages. PFV can persistently infect a variety of human hematopoietic cell lines, including those of erythroid, T-cell, and myeloid lineages (145). This virus also been shown to infect primary human CD4+ T cells, monocyte-derived macrophages, and microglial cells, whereas CD8+ T cells were less susceptible. Both the CD4+ cells and the microglial cells were rapidly killed by viral infection (87). The observed lack of infection of CD8+ cells is surprising because other work indicates that this lineage is infected in naturally infected primates (131). In addition, it is difficult, if not impossible, to establish T-cell lines from animals that are naturally infected with SFV (29).

Viral Life Cycle

The foamy virus life cycle, based primarily on *in vitro* experiments with SFVcpz(hu), is depicted in Figure 7. Stages of the life cycle that are tentative are marked with a question mark. Virions infect cells through an as yet unidentified receptor(s). It is not clear whether both RNA- and DNA-containing particles can infect cells. Currently, the strength of the evidence suggests that particles with reverse transcription either completed, or ostensibly completed, are the infectious agents (88,146). After adsorption to the cell, the virions are presumably uncoated. During this process, there may be further processing of the large Gag proteins to smaller products (36,95), although the necessity for this, or the function of any of the resultant Gag domains, is not known. Viral DNA is next transported to the nucleus. It is possible that the virion-associated DNA is incomplete and requires some repair using RT, several enzymes, or both. Viral DNA is then integrated into the genome through the action of IN, and provirus serves as a template for transcription and translation using host factors. Viral gene transcription requires synthesis of the Tas protein, which is encoded by the IP. There appears to be temporal regulation of transcription with synthesis of the “early” mRNAs encoding Tas and Bet, preceding that of the “late” RNAs for the virion-associated proteins. Virion assembly is poorly understood, and most takes place in the cytoplasm, although some assembly at intracellular membranes cannot be excluded. Nothing is known about the domains in Gag required for capsid formation. Preformed capsids are transported to intracellular membranes, presumably the ER, where glycoproteins are added. Some glycoprotein addition also occurs at the plasma membrane. Particle egress appears to occur through both the ER and plasma membrane.

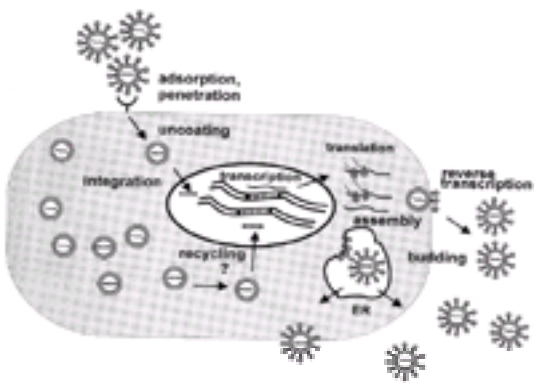

FIG. 7. Foamy virus (FV) life cycle. Viral RNA is indicated by a *wavy line,* viral DNA by a *striped box.* The speculative intracellular recycling pathway is indicated by a *question mark.* Reverse transcription is believed to be a late event for SFVcpz(hu) but has not been shown for other FVs. For SFVcpz(hu), the infectious virion is shown as containing DNA. (Adapted from ref. 72, with permission.)

Foamy virus reverse transcription proceeds presumably using the normal retroviral mechanism (see Chapter 57). Negative-stranded synthesis uses a tRNA primer (in the case of PFV, it is $tRNA_{lys1,2}$). Reverse transcription culminates in production of a cDNA bordered by two LTRs. Reverse transcription appears to occur during viral assembly or shortly thereafter, leading to about 20% of extracellular particles containing DNA (146). The rest of the particles contain RNA, but it is not known how many particles contain dimeric full-length RNA molecules. The RNA in virions is unlikely to be capable of initiating a new round of infection because infectious viral synthesis is sensitive to AZT at a late stage in the life cycle, rather than an early stage (88,146). The temporal regulation of RT/RH activity has not been studied. Perhaps the synthesis of Pol as a free protein instead of a Gag-Pol fusion protein allows RT activity to occur earlier during assembly than with the other retroviruses.

The role of the large numbers of intracellular particles is not known. There is a large reservoir of such particles in persistently infected tissue culture cells. Early in the HBV life cycle, viral nucleocapsids lacking glycoproteins transit back into the nucleus where reverse transcription is completed to produce additional copies of viral covalently closed circular DNA, which is the stable form of the viral genome, akin to the retroviral provirus. It is possible that DNA in these intracellular particles is a source of additional proviruses by a process analogous to this intracellular recycling seen with HBV and other hepadnaviruses. It is attractive to speculate that the NLS in the Gag protein could be involved in such a process. Alternatively, persistently infected cells could provide a reservoir of infected viruses that are budded slowly from cells, or intracellular virus could spread by cell–cell contact.

Overall, the foamy virus life cycle is similar to that of other retroviruses. Notably, reverse transcription is identical to that described for the other six genera of the *Retroviridae* family, and obligate integration (25) is accomplished using an integrase protein with similar features to those of other retroviruses (90). The genomic structure is similar to that of other retroviruses, including the presence of LTRs in proviruses and *gag, pol,* and *env* genes. The LTRs of primary foamy virus isolates range in size from 1.3 to 1.6 kb. However, many of the details of the replication cycle place the foamy virus closer to other RT-encoding viruses (pararetroviruses) with DNA genomes, such as the *Hepadnaviridae* and the less well understood plant *Caulimoviridae* (51), which do not integrate as part of their life cycles.

Viral Transmission and Replication *in Vivo*

Foamy virus infections are extremely widespread in all subhuman primates, including great apes, as well as in cows and cats. Long-term persistent infections ensue, and virus can often be recovered decades after the initial infection. There is no evidence that foamy virus is ever cleared by the host immune system. On the other hand, finding viral expression *in vivo* is extremely difficult, and viral isolation invariably requires cocultivation with highly susceptible cells. Thus, the hallmark of foamy virus infection is a long-term inapparent infection, with a small number of cells that are productively infected and capable of producing new particles.

Although humans are not natural hosts for foamy virus infection, a number of individuals have been identified who have both antibody to a specific SFV and virus that can be recovered by co-cultivation. In each case, transmission can be traced to a severe injury sustained from interaction with primates. In one case, it was shown that an individual was infected with the HFV laboratory strain, perhaps through a laboratory accident (117). In one case of zoonotic transmission, virus highly related to SFVagm was isolated from an animal caretaker who had been bitten repeatedly by African green monkeys, once resulting in a deep injury requiring surgery. Virus could be recovered from primary blood lymphocytes, but not from saliva, from this individual 20 years after transmission; both his wife and daughter were found to be seronegative (114). In recent studies, the results of a screen of individuals with occupational exposures to nonhuman primates were reported (46,47). Of 235 individuals studied, six (about 2.5%) were identified to be infected with various simian foamy viruses. SFVagm, SFVcpz, and SFVbab were all found to be transmitted. All 6 cases were Western blot and PCR positive. Virus could be recovered by co-cultivation of primary blood lymphocytes with canine thymocytes or *Mus dunni* cells from three individuals. No male-to-female sexual transmission of SFV was observed in five spouses who were available for testing (46). In the same study, only one case of simian immunodeficiency virus (SIV) transmission and none of simian T-lymphotropic virus (STLV) transmission was found. SFVbab was also found in humans who had received baboon liver transplants. In a retrospective analysis of tissues from two acquired immunodeficiency patients (AIDS) patients with end-stage hepatic disease who received baboon livers, it was found that baboon cells harboring SFV migrated from the xenografts to peripheral tissues such as lymph node and kidney. In this study, infectious virus could not be isolated (4). This work highlights a potential danger of xenotransplantation using primate tissue (see Cross-Species Retrovirus Infection). It is interesting to note that foamy viruses appear extraordinarily genetically stable compared with the lentiviruses, alpharetroviruses, or gammaretroviruses. For example, comparison of the SFVcpz sequences found in one case where both the recipient and the source chimpanzee were available for samples showed no difference in IN or Gag after at least 15 years of documented infection (47). LTR, IP, ORF-1 (*tas*), and ORF-2 (*bel2/bet*) sequences (see Fig. 5) from one SFVagm-infected person were also compared with those in prototype SFV-3agm. Sequence conservation was seen in all these regions except for ORF-2, which contained premature stop codons (18) that would lead to truncated Bet proteins.

Natural transmission of foamy viruses has been studied in both primates and nonprimate species. In one study, transmission of BFV (previously called bovine syncytial virus, or BSV), which is endemic in domestic cattle, was analyzed. It was found that newborn calves show passive immunity to BFV from ingestion of colostrum but become negative for BFV antibodies by 3 to 5 months of age. No maternal transmission of virus was detected, and even experimental infection of dams with BFV at 5 to 7 months of gestation did not lead to infection of offspring. However, when antibody-negative calves were co-housed with infected adults, by 2 years of age, about 35% of the animals became seropositive within 10 weeks of introduction into the herd, and 85% became seropositive by 2 to 3 years (56). These studies suggest that BFV is transmitted through licking or biting. Similar results were obtained more recently looking at transmission of SFV in baboons. Virtually all adult baboons in a large primate center colony were found to be infected with SFVbab (17). Maternal antibody appears to be protective; most infants have antibodies to SFV, but few have viral sequences. Maternal antibody disappears at about 9 months. Thirteen of 35 young animals from 1.4 to 2.6 years of age were found to be infected with SFV. In contrast, few animals in this age group are infected with sexually transmitted retroviruses. Additionally, SFV antibodies can be detected in juvenile animals, before sexual maturity. These

studies strongly suggest that transmission is not sexual but rather occurs through biting or licking (J. Allan, personal communication).

An important question is how foamy viruses maintain a long-term persistent infection without any disease manifestations, in the presence of neutralizing antibodies (52). Maintenance of persistent infections does not appear to require generation of variant viruses that can escape immune surveillance. Work with SFVagm shows that there is little genetic diversity in the SU portion of Env between isolates from different monkeys or a human animal handler who acquired SFVagm from a monkey bite, although SFVagm isolates could be grouped into four strains. Almost no variation was seen in viruses obtained from a single animal over 13 years (116). In fact, it was found that nonsynonomous mutations were selected against, rather than for, as in the case of lentiviruses. These authors conclude that the absence of viral variation is most likely caused by a low rate of viral replication (116). This conclusion is consistent with results of several studies addressing detection of virus *in vivo,* in both naturally and experimentally infected hosts. One general conclusion is that it is very difficult to detect viral RNA or viral replication *in vivo* using sensitive techniques such as RT-PCR. One possible reason for low viral replication is that replication is suppressed *in vivo* by interferon-g (28). In a recent study of four African green monkeys that had been naturally infected with SFVagm for more than 18 years, viral DNA was easily detected in all organs, including brain, lymph nodes, kidney, and lungs. It was difficult, however, to detect viral RNA, which was found only in the oral mucosa of one of the monkeys (27). This location is consistent with the presence of virus in saliva and the mode of transmission. Whether there are other sites of viral replication, especially earlier in infection, remains to be seen. In another study, lymphocytes, and in particular CD8+ T cells, were shown to be the major reservoir for foamy virus DNA in the peripheral blood, but viral gene expression was not detected (131). Because cell-associated viral particles contain DNA, it is not possible to know whether any of the organs contain integrated proviruses. Also, because at least *in vitro,* infected cells can contain hundreds to thousands of copies of viral DNA, the number of infected cells is hard to determine. Clearly, more studies are required to understand which cells harbor integrated foamy virus proviruses in infected animals and which serve as the major viral reservoir.

Foamy Virus Vectors

The characteristics of foamy virus discussed in this chapter, such as lack of pathogenicity, broad host range, and large genome size, make them attractive agents for development of viral vectors for gene therapy. Recent work that shows that FV DNA can be found in all organs of infected monkeys, including brain (27), provide additional impetus to explore the utility of these agents as vectors. Unfortunately, it is not yet known which cells in brain actually contain foamy virus DNA, and such studies are important for vector development. Unlike lentiviruses, such as human immunodeficiency virus (HIV), foamy viruses cannot replicate with high efficiency in fibroblastoid cells that have been arrested at the G_1/S or G_2 phase of the cell cycle (11). It has been found that foamy virus DNA can enter the nucleus of G_1- or S-phase arrested cells, but no viral replication is observed (108). However, there is evidence that vectors based on SFVcpz(hu) can transfer markers such as alkaline phosphatase to progenitor CD34+ hematopoietic cells more efficiently than MLV-based vectors (50). Thus, integration and expression of the vectors is occurring, and it is possible that foamy virus–based vectors could prove useful for transduction of pluripotent hematopoietic stem cells.

Foamy viruses provide the option of creating replication competent vectors that work with high efficiency in tissue culture cells. Because the *bet* gene is not required for *in vitro* replication, cloning of markers into this region allows introduction of easily selected or assayed marker genes into cells with high efficiency. Such vectors allow infection of all the cells in the culture (112). This could be useful for stable expression of marker genes in cell types that are not killed by foamy virus infection. Vectors that bypass the need for the viral transactivator protein Tas can be made by substitution of the 5' LTR with a strong constitutive promoter (126). Although there are several published vector systems, and a first-generation packaging cell line for SFVmac (140), there is not as yet highly efficient foamy virus packaging cell lines available for foamy virus as there are for MLV and other conventional retroviruses (Chapter 57 includes a detailed discussion of retroviral vectors and packaging cell lines). There have been several reports defining the cis-acting sequences in the foamy virus genome required for transfer of vectors (26,45), but detailed analyses have not yet been done. It is interesting that two regions of the foamy virus genome appear to be required for vector transfer. One is at the 5' end of the viral RNA which does not appear to be involved in RNA packaging (45a). The second is in the *pol* gene and may also be involved in packaging, or perhaps in some posttranscriptional regulation using host factors. Further understanding of the cis-acting elements required for optimal viral replication is important for design of packaging cell lines for nonreplicating vectors.

HUMAN ENDOGENOUS RETROVIRUSES

Most vertebrate species studied contain multiple copies of DNA proviruses integrated in chromosomal DNA, and humans are no exception. Genetic retroelements compose a variety of reverse transcripts, including genuine endogenous retrovirus genomes, human “LINE” elements (retrotransposons lacking LTR sequences but encoding reverse transcriptase), pseudogenes, and “SINE” elements without RT, such as human repetitive *alu* sequences (16). This discussion includes only the HERVs, which are endogenous retroviral sequences representing “fossil” infections and insertion into the germline in the evolutionary past. Primates contain numerous such genomes, including potentially infectious viruses such as baboon endogenous virus, mentioned previously. Many genera of retrovirus have become endogenous in host DNA, although endogenous lentiviruses, spumaviruses, and HTLV-related deltaretroviruses have not been detected; perhaps retroviruses with “complex” genomes containing regulatory and accessory genes do not survive as mendelian elements in host chromosomes.

It is estimated that 0.1% or more of human DNA sequences may be of retroviral origin. With the completion of the human genome sequence, it will become possible to analyze the total distribution, insertion, and expression of HERV elements. Multiple types of HERV exist (Table 2), some as unique copies (e.g., HERV-R) and some as multiple copies (73,74,133). HERVs belong to different retroviral subfamilies and have been classified according to the tRNA thought to associate with the primer binding site; for example, the HERV-K subfamily has tRNA lysine, and the HERV-W subfamily has tRNA tryptophan. HERV genomes proliferated markedly during early Old World primate radiation. Several HERV genera are expressed in the placenta as whole particles (118) or abundant Env protein (86,123), and more selectively in other

human tissues.

Name	Retroviral genus	tRNA primer binding site	Approximate copy number per cell	Reference
HERV-E	Gammaretrovirus	Glutamic acid	50	98
HERV-H (RTVL-H)	?	Histidine	1,000	77
HERV-I	Gammaretrovirus	Isoleucine	10	138
HERV-K	Betaretrovirus	Lysine	100	74
HERV-L	?	Leucine	?	20
HERV-P	Gammaretrovirus	Proline	15	138
HERV-R (ERV-3)	Gammaretrovirus	Arginine	1	128
HERV-W	Gammaretrovirus	Tryptophan	15	12
ERV-9	Gammaretrovirus	Arginine	40	67

Note: Many more HERV genomes that have been partially characterized by *pol* sequences alone are not listed here (138).

TABLE 2. *Human endogenous retroviral genomes*

Ancient proviruses in host DNA tend to be defective, accumulating stop codons and deletions, but some proviruses maintain ORF and functional LTRs. Insertions into new chromosomal sites can be distinguished by comparison with related host species; for example, humans of all ethnic groups possess six HERV-K proviruses not present in chimpanzees (7). HERVs of ancient lineage may maintain an ORF if that retroviral gene has been adopted by its host to perform a function. There is evidence that the HERV Env glycoproteins play a role in the trophoblast development in the human placenta (13,86). HERVs have also been used during primate evolution to modify the expression of cellular genes. An HERV-E insertion 5' to a gene duplication of the pancreatic amylase gene provides a tissue-specific enhancer for salivary gland expression (125), thus allowing humans to enjoy a cereal-rich diet of wheat, corn, or rice. Pleiotrophin expression in the human placenta has been attributed to the insertion of a HERV-E LTR promoter (113), and another placental transcript has a polyadenylation signal provided by a HERV-H LTR (40).

There has been considerable speculation in recent years on the participation of HERV expression in chronic or autoimmune diseases. A HERV-K10 genome was reported to be up-regulated in antigen-presenting cells associated with islets in insulin-dependent diabetes mellitus, and its envelope glycoprotein suggested to have a superantigen effect (19), but these studies could not be confirmed (75). A retroviral genome was detected in multiple sclerosis (94) and designated *multiple sclerosis retrovirus*, but this finding also remains controversial (32). Multiple sclerosis retrovirus is indistinguishable from the newly characterized HERV-W (12,94,130). The most convincing and repeated observation of disease association is of HERV-K sequences in germ cell tumors of the testis, both teratocarcinoma and seminoma (74,109). HERV-K encodes *gag, pol,* and *env* genes and produces both RT activity and retroviral particles. It remains to be determined whether HERV expression plays an etiologic role in the diseases with which HERVs are associated or whether HERV antigens are merely markers of diseased tissues and states (73,121).

Where ORF are maintained in evolution, one might conclude that the HERV element is playing some useful role to the host, but that can be difficult to determine. For example, the evolutionary preservation and high expression of the HERV-3 truncated envelope glycoprotein (128,141) in the syncytiotrophoblast of the human placenta suggested a functional role. However, there is a frequent deletion in HERV-3 in the white population (21). Recently, the HERV-W envelope, which is also highly expressed in the syncytiotrophoblast, was found to be able to induce syncytium formation when transfected into a wide variety of cell types (86) by interacting with the D-type virus receptor (13). This placentally expressed HERV-w *env* gene product may therefore play the role first suggested for HERV-3.

If HERV genomes were highly mobile, one would expect to detect more human genetic diseases caused by insertional disruption of essential genes in the germline, or insertion into somatic cells leading to clonal proliferation and induction of human tumors. However, such events are rare in humans, and where recorded, are more frequently related to retroid LINE or SINE elements rather than HERVs themselves (16). For example, some cases of factor VIII hemophilia in one human lineage are caused by a LINE-1 insertion (61), and Alu insertion into the *NF-1* gene has been correlated with neurofibromatosis (132). Another possible medical concern is the contamination of retroviral vectors to be used in human gene therapy by endogenous retroviral genomes. Although murine packaging cells do incorporate high levels of VL30 murine endogenous retrovirus sequences (110), it is reassuring that human packaging cell lines do not copackage HERV genomes into MLV, and human cells expressing HERV particles do not incorporate and mobilize MLV-based vector genomes (93).

HUMAN RETROVIRUS 5

HRV-5 is a recently identified genome of a retrovirus initially detected by RT-PCR from sucrose gradient concentration of virus-like particles with RT activity (42). However, HRV-5 has not as yet been isolated as replication-competent retrovirus in culture. The HRV-5 genome has not been completely cloned and characterized, but analysis of *gag* and *pol* sequences indicate that it is a betaretrovirus distantly related to SRV-1 to -5 and to murine intracisternal A-particle genomes. Like other betaretroviruses, the protease sequence is in a different reading frame from that of RT and integrase. Southern blot and PCR analysis clearly show that HRV-5 is not endogenous in human DNA. Thus, it appears to represent a novel, infectiously transmitted HRV (42). Its provisional name, HRV-5, denotes that it represents the fifth exogenous HRV, after HTLV-I and -II and HIV-1 and -2.

Although HRV-5 was originally detected in salivary gland biopsies from patients with Sjögren's syndrome, proviral DNA is only rarely detected in such tissue (41). However, a disease association with HRV-5 has become apparent for rheumatoid arthritis (RA) and systemic lupus erythematosus. Some 12% of blood samples from RA patients and 16% of blood samples from lupus patients contain HRV-5 DNA (41). HRV-5 was also detected in about half of synovial biopsies

from arthritic joints, including osteoarthritis and reactive arthritis as well as RA (41). The more frequent detection of HRV-5 by RT-PCR in sucrose gradient fractions of tissue biopsy samples but rarer detection by PCR of proviral DNA indicates that this retrovirus may represent a relatively high prevalence human infection with an exceedingly low viral load in the tissues examined thus far, with less than 1 provirus among 50,000 cells. The higher detection rate in RA and lupus suggests that the virus expression is up-regulated in these conditions, but it would be premature to conclude that it has an etiologic role in disease. Nonetheless, the identification of a novel, presumably infectious retrovirus in humans merits further investigation.

SIMIAN RETROVIRUSES 1 TO 5

The prototype D-type retrovirus is Mason-Pfizer monkey virus (MPMV), also known as SRV-3. SRV strains are associated with a severe, pan-leukopenic immune deficiency in various macaque species (35,104,105). Five serotypes (SRV-1 to -5) are known, of which SRV-1 to -3 have been molecularly cloned and sequenced (104). Although they can be readily distinguished by neutralization in serologic assays, their genomes show 75% sequence similarity. All SRVs use a common cell surface receptor (119), which has recently been identified (97,122). More distantly related D-type retroviruses are endogenous in an Asian leaf monkey (the spectacled langur), in the African yellow baboon, and in the New World squirrel monkey (104,127). These viruses can emerge as infectious retroviruses in culture. The squirrel monkey retrovirus has been detected as a laboratory contaminant of human lymphoblastoid and epithelial cell lines that have a long passage history in various laboratories.

CROSS-SPECIES RETROVIRUS INFECTION

Cross-species infection of retroviruses to new hosts is well known, including potentially infectious endogenous retroviruses with ORFs for all viral genes. The host species harboring the mendelian provirus is often resistant to cell–cell infection and viremia when some host cells become activated to release virions. However, cells of other host species can be experimentally infected, and such retroviruses are called *xenotropic* (16). Sequence analysis of endogenous viruses reveals that some have jumped across broad host phylogenetic taxa. For example, the endogenous viral genome RD114, of the domestic cat, is closely related to baboon endogenous virus but is present only in the tabby species of the genus *Felis* (9). Further, an endogenous genome in the marsupial Koala bear is closely related to GALV (81). GALV itself is possibly a recent cross-species infection from an endogenous virus in Southeast Asian mice such as *Mus caroli.* Certain captive colonies of gibbons are infected with GALV (60), which can cause lymphoid and myeloid leukemia, but its presence in wild gibbons has not been surveyed.

The risk of retroviral zoonosis for human infection is real. HIV-1 probably originated from chimpanzees on at least three separate occasions (33), and it is clear that HIV-2 came from the SIV of sooty mangabey monkeys in West Africa on several occasions (34). Similarly, some strains of HTLV-I closely resemble their simian counterparts in the same geographic area, harbored by baboons and chimpanzees (76,129). As discussed under Foamy Viruses, zoonotic infection also accounts for the few well-documented cases of foamy virus infection in humans by transmission from chimpanzees and monkeys. A replication-competent amphotropic murine gammaretrovirus was derived by recombination from a retroviral vector and caused lymphoma in a macaque recipient of experimental gene therapy (22). Several animal species, such as mice, cats, and pigs, carry endogenous retroviruses capable of infecting human cells in culture. It has been suggested that humans are protected from nonprimate retrovirus infection because human complement (C') inactivates these viruses (136). We now know that C' inactivation is largely mediated through the presence of a_{1-3}-galactose antibodies in human plasma, which bind to a- *gal* xenoantigens on the glycoproteins and glycolipids of enveloped viruses grown in animal cells (124). The mechanism is akin to C'-mediated lysis of ABO-mismatched red blood cells because Old World primates lack the enzyme a_{1-3}-galactosyl transferase and therefore recognize the a- *gal* carbohydrate antigen (e.g., expressed on enteric bacteria) as foreign. It also accounts for hyperacute rejection of xenografts transplanted to primates (5,124). Thus, the generation of transgenic animals expressing human complement-modulating genes (*DAF, CD46,* and *CD59*) could allow enveloped viruses, including retroviruses, which assemble these membrane proteins to transmit more readily to humans in the xenotransplantation setting (133).

There is much debate about the safety of conducting human xenotransplantation. There are several practical, ethical, and safety reasons why simian-to-human xenotransplantation of tissues and organs should not be pursued, including the danger of retrovirus infection (4,5). Pigs are the favored source of tissues, and a number of humans have already been transplanted with fetal porcine brain cells for Parkinson's disease and with pancreatic islet cells for insulin-dependent diabetes mellitus. Extracorporeal perfusion of human circulation through porcine hepatocytes is under trial, and if hyperacute rejection is overcome by using transgenic pigs, kidney and heart xenotransplantation may follow (5,133).

It was therefore timely to demonstrate that porcine endogenous retroviruses (PERV) can infect human cells (92,123,139). Multiple copies of the human tropic PERV strains exist as mendelian proviruses endogenous in all strains of pig examined, and it will not be simple to eliminate them. However, PERVs do not appear to be highly infectious for humans *in vivo,* and there is no evidence of PERV infection in nearly 160 patients after exposure to live porcine cells and tissues (91). It is therefore likely that porcine xenografting to humans will proceed in carefully controlled clinical trials. It is well known that human tumor xenografts in mice can become infected with xenotropic MLV (2). Conversely, graft into host infection by animal retroviruses will need to be closely monitored in human xenotransplantation.

PERSPECTIVES

Less is known about the replication and host interactions of the viruses discussed in this chapter than about the other retroviruses. It has been surprising, given the large numbers of simple replication-competent endogenous retroviruses that have been found in other species, that there have not as yet been any propagated from humans, although some have apparently intact genomes. It is possible that in the future, more cross-species transmission of viruses to humans from primates or other sources may occur, as was the case with HIV. Our current understanding of the role of retroviruses in chronic and autoimmune disease is perfunctory, and this area needs considerable further research and

clarification.

Foamy viral replication has proved to be much more interesting than expected. In some regards, foamy viruses are a link between the *Retroviridae* and *Hepadnaviridae.* There are many open questions about their replication. How is Pol sequestered in particles? What is the role of nuclear localization of Gag? Does an intracellular recycling (retrotransposition) pathway occur? Why is Env required for particle egress from the cell? Does the Bet protein have any role in viral replication? These and other questions should be highly tractable given the ease of work with foamy viruses in tissue culture. An extremely interesting question is why foamy virus are not pathogenic, despite their ability to launch highly cytopathic infections *in vitro.* It appears that they have evolved a lifestyle that permits lifelong maintenance without any gross destruction of tissues, but the role of either viral or cellular factors in this commensal relationship are not known.

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CHAPTER 63 *Polyomaviridae:* The Viruses and Their Replication

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DISCOVERY AND CLASSIFICATION

The *Polyomaviridae* were previously considered a subfamily of the *Papovaviridae,* a name derived from the names of three prototypical members: rabbit papilloma virus (pa), mouse polyomavirus (po), and simian virus 40 (SV40), originally called vacuolating virus (va). Their genomes are single molecules of covalently closed, superhelical double-stranded DNA that are replicated in the nucleus. Thorough investigations since the 1980s indicate that SV40 and mouse polyomavirus differ from the papillomaviruses in having smaller capsids (diameters of 45 nm versus 55 nm), smaller genomes (about 5,000 base pairs versus about 8,000 base pairs), and a different genomic organization. The *Polyomaviridae* are now considered an independent family of viruses. In this chapter, the term *polyoma* is used to refer to mouse polyomavirus, whereas the family of viruses is referred to as *polyomaviruses.*

Thirteen members of the *Polyomaviridae* family have now been identified. All have capsids that are the same size and are constructed from three viral capsid proteins. All have genomes of about 5,000 base pairs and display a similar genomic organization. Many regions of their genomes are highly conserved, demonstrating that the *Polyomaviridae* are descended from a common ancestor. Different family members infect several species of mammals, including humans, other primates, rodents, rabbits, as well as birds. Table 1 lists the members of the *Polyomaviridae* and their natural hosts. Most of these viruses display a narrow host range and do not productively infect other species. However, infection of cultured cells in which these viruses cannot grow productively often leads to the malignant transformation of these cells. Some of these viruses also induce tumor formation in newborn hamsters.

Virus	Host species
Polyomavirus (PyV)	Mouse
Simian virus 40 (SV40)	Rhesus monkey
BK virus (BKV)	Human
JC virus (JCV)	Human
K papovavirus (KPV)	Mouse
Hamster papovavirus (HaPV)	Hamster
Lymphotropic papovavirus (LPV)	African green monkey
Simian agent 12 (SA12)	Baboon
Rabbit kidney vacuolating virus (RKV)	Rabbit
Stump-tailed macaque virus (STMV)	Stump-tailed macaque
Budgerigar fledgling disease virus	Bird

TABLE 1. *The polyomaviruses*

These small DNA tumor viruses, particularly SV40 and polyoma, have been subjects of intensive studies by virologists and molecular biologists since their discovery in 1953 (143). Polyoma was the first family member to be discovered. In his studies on the transmission of murine leukemia, Ludwig Gross noted that extracts from infected animals could be used to transmit leukemia (143). However, some inoculated animals developed salivary gland tumors rather than leukemia. Gross's further studies showed that the two agents could be separated on the basis of differences in sedimentation or filtration (the murine leukemia virus [MLV] was larger) as well as heat inactivation (the agent that induced salivary gland tumors was insensitive to treatment at 65°C, whereas MLV was completely inac tivated). The virus that caused the salivary gland tumors was subsequently named *mouse polyomavirus* because of its ability to cause a variety of different types of tumors in newborn mice (329). Infection of adult mice with polyomavirus does not usually result in tumorigenesis.

SV40 is one of several viruses identified by screening for viruses in the secondary rhesus monkey kidney cell cultures used for production of poliovirus vaccines. Although SV40 does not induce a visible cytopathic effect in rhesus monkey kidney cells, Sweet and Hilleman (338) noted a pronounced cytopathic effect when African green monkey kidney cells were infected with extracts from the rhesus kidney cell cultures. Soon afterward, it was discovered that tumors were induced by injection of SV40 into newborn hamsters (102,133). Many lots of poliovirus vaccine were contaminated with live SV40 virus, raising the concern that this virus, which is oncogenic for newborn hamsters, might also be oncogenic for humans. Fortunately, studies to determine the risk for cancer in those individuals inadvertently inoculated with SV40 during poliovirus vaccination indicate that exposure to the vaccine is not associated with significantly increased rates of ependymomas and other brain cancers, osteosarcomas, or mesotheliomas (244,332). However, SV40 T-antigen sequences have recently been detected in these cancers in the general population, leading to renewed interest in the role of SV40 infection in contributing to the development of some human tumors (reviewed in 39).

Two polyomaviruses of humans have been described. JC virus (JCV) was isolated in 1971 (254) by inoculating human fetal brain cells with extracts of diseased brain tissue from patients with progressive multifocal leukoencephalopathy (PML). BK virus (BKV) was isolated in the same year from the urine of an immunosuppressed renal transplant recipient (123). The genomes of both of these human viruses show closest homology to SV40. Most people worldwide become infected with and acquire antibodies to these viruses during childhood, with no apparent disease manifestations. These viruses are then thought to lie dormant in cells of a subset of infected people. Once extremely rare, the incidence of PML has increased dramatically in recent years as a consequence of the immunosuppression associated with infection by human immunodeficiency virus (HIV). A related simian papovavirus has been isolated from cynomolgus monkeys under immune suppression (363).

Although most polyomaviruses replicate primarily in epithelial or fibroblastic cells, zur Hausen and Gissmann (402) discovered a B-lymphotropic virus antigenically related to SV40 in a B-lymphoblastoid cell line derived from an African green monkey. Because this virus grows only in B-lymphoblastoid cells, it has been named *lymphotropic papovavirus* (LPV). Serologic studies suggest that humans and most other primates harbor viruses antigenically related to LPV. This virus does not induce tumors in newborn hamsters but will transform cultured hamster and mouse cells.

Hamster polyomavirus (HaPV) was isolated originally from a spontaneously occurring hair follicle epithelioma of a Syrian hamster (139). These tumors resemble papillomavirus-induced tumors in being highly keratinized and having virus particles exclusively in the differentiated cell layer. When injected into newborn hamsters, this virus causes leukemias and lymphomas, a tumor spectrum quite distinct in the polyomavirus subfamily. On the basis of nucleic acid sequence homology and genome organization (83,138), this virus is more closely related to polyoma than to SV40 or the human polyomaviruses. Other mammalian polyomaviruses include bovine polyomavirus (BPV) (293), which is sometimes found in fetal calf serum, a rabbit polyomavirus (RKV) (152), a rat polyomavirus (RPV) (370), and Kilham virus, an additional polyomavirus of mice (231).

The first avian polyomavirus (APV) was identified in 1986 (207). Budgerigar fledgling disease virus (BFDV) is classified as a polyomavirus on the basis of nucleic acid homology to other polyomaviruses as well as on morphologic and serologic criteria. It appears to be more similar to SV40 than to mouse polyomavirus because it lacks a middle T antigen. Several additional APV isolates from a variety of avian species have now been characterized; so far, all belong to one genotype and one serotype within the proposed subgenus *Avipolyomavirus* of the family *Papovaviridae* (258).

POLYOMAVIRUSES AS PARADIGMS

The initial impetus for studies of these viruses was their oncogenic potential. However, these viruses are easy to cultivate in tissue culture and to purify, making them suitable model systems for diverse studies in molecular biology. Because of the small size of their genomes, physical maps of their genomes were generated as soon as restriction endonucleases became available in the 1970s, and they were among the first DNA genomes to be completely sequenced (109,271,310,311). Since the 1970s, these viruses have been used to examine many fundamental questions in eukaryotic molecular biology.

Among the major discoveries resulting from work on these viruses are the structure of supercoiled DNA, the identification of eukaryotic origins of DNA replication, the discovery of enhancers and elucidation of the organization of promoters involved in transcriptional regulation, the discovery of alternative splicing, and detailed understanding of the mechanisms of negative and positive regulation of gene expression. Because viral DNA is organized into chromatin, and because replication of viral DNA involves only a single viral gene product and uses almost all of the proteins used for cellular DNA replication, replication of SV40 origin-containing DNA *in vitro* has served as a model for understanding eukaryotic chromosomal DNA replication. Studies to understand the oncogenic potential of these viruses have provided fundamental insights into cell cycle regulation, oncogenes, and tumor suppressor genes. Much of our understanding of the polyomaviruses derives from studies of SV40 and polyoma, and this chapter focuses primarily on these two viruses.

VIRION STRUCTURE

The capsids of the polyomaviruses contain three virus-encoded proteins, VP1, VP2, and VP3, surrounding a single molecule of viral DNA (Fig. 1A) complexed with cellular histones H2A, H2B, H3, and H4 in the form of chromatin (see Fig. 1B). The sizes of the capsid proteins of SV40 and polyoma are shown in Table 2. These proteins are arranged to form a T=7 icosahedral capsid (see Fig. 1C) containing 360 molecules of the major capsid protein, VP1, organized into 72 pentamers. A single VP2 or VP3 molecule associates with each pentamer. The virus particle is 88% protein and 12% DNA and has a sedimentation coefficient of 240S in sucrose density gradients. Because they lack envelopes, the virus particles are resistant to lipid solvents. They are also relatively resistant to heat inactivation. Virions have a density of 1.34 g/mL in cesium chloride (CsCl) equilibrium density gradients, whereas empty capsids have a density of 1.29 g/mL.

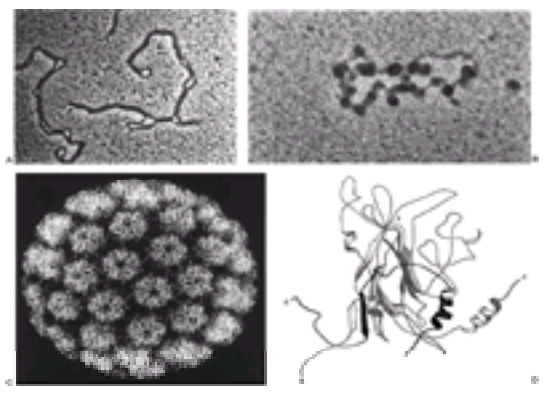

FIG. 1. Viral DNA, minichromosomes, and virions. **A:** Electron micrograph of supercoiled SV40 DNA molecules. **B:** Electron micrograph of an SV40 minichromosome. Note that this particular minichromosome displays a nucleosome-free region. About 20% of minichromosomes have a nucleosome-free region surrounding the regulatory region at the origin of DNA replication. **C:** Computer graphic representation of the structure of the SV40 virion. Note that the shell is made up of 72 pentamers of VP1. Twelve of these lie on icosahedral fivefold axes and are surrounded by five other pentamers. The remaining 60 pentamers, such as the ones near the center of this diagram, are surrounded by six other pentamers. The pentamers are linked together by extended C-terminal arms of VP1 molecules of the subunits. **D:** A ribbon diagram of a VP1 subunit of the mouse polyomavirus virion. *N* refers to the location of residue 15 of the VP1 polypeptide; the first 14 residues are thought to be disordered. *C* is the C-terminus of an invading arm (*darkly shaded*) of another VP1 molecule. At the right of the figure is an *arrow* showing that this VP1 molecule goes off to the right, where it interacts with another VP1 molecule. The interactions of the C-terminal arms of VP1 molecules can be pair-wise with each of two VP1 molecules interacting with the C-terminal arm of the other (as in this figure), or the incoming C-terminal arm of VP1 can come from a third VP1 subunit. (see Chapter 3 for more details on protein–protein interactions in the capsids of the polyomaviruses). *X* is the site of the mouse polyomavirus receptor-binding pocket. (**A** and **B** courtesy of Dr. Jack Griffith, University of North Carolina; **C** and **D** courtesy of Dr. Steven Harrison, Harvard University.)

	SV40		Polyoma		JC virus		BK virus	
	Mol. wt.	Amino acids	Mol. wt.	Amino acids	Mol. Wt.	Amino Acids	Mol. Wt.	Amino Acids
VP1	38,930	362		385	42,666	354	40,126	352
VP2	38,225	352		319	37,966	344	38,345	351
VP3	26,630	234		224	25,745	225	26,778	232

TABLE 2. *Capsid proteins of the polyomaviruses*

Although originally thought to contain 60 hexameric (hexons) and 12 pentameric (pentons) capsomeres, it is now clear that these viruses contain 72 pentameric capsomers, which each contain five molecules of VP1 (209,269). In fact, polyoma VP1 produced in *Escherichia coli* will self-assemble into pentameric capsomeres, and under appropriate conditions, these molecules will further assemble into virus-like empty capsids and other structures (280). The same is true of SV40 VP1 synthesized *in vitro* (126). Icosahedral viruses have both sixfold and fivefold axes of symmetry, and the structure of the polyoma capsids presents a puzzle because these capsids contain capsomeres that have only fivefold axes of symmetry. The high-resolution structure of the SV40 capsid (209,324,383) indicates that the C-terminal arm of VP1 does not form part of the capsomere structure itself but instead makes contacts with neighboring pentamers (see Fig. 1D). It is the flexible geometry of these contacts that appears to allow the pentamers to fit together in such a way that an icosahedral capsid is formed. Calcium ions are required for virion stability (32,52) and are thought to stabilize pentamer–pentamer interactions (209). There are no disulfide bonds within the SV40 pentamer subunits, but disulfide bonds may exist between adjacent pentamers because reducing agents are required to disassemble virus particles.

The sequences of the minor capsid proteins, VP2 and VP3, are overlapping; VP2 contains the entire VP3 sequence at its C-terminus and an additional sequence of about 115 amino acids at its N-terminus. The N-terminus of SV40 and polyoma VP2 is myristoylated (331). The VP2 and VP3 proteins of all polyomaviruses are most highly conserved near their C-termini, and it is this region that interacts with VP1 through its N-terminal region (13,50). The disorder seen for VP2/3 in earlier virion structures reflects the fact that VP2/3 interacts differently with different VP1 molecules within each capsomere, but has an equal probability of interacting most directly with any of the five VP1 molecules (50). Only VP1 molecules are exposed on the outside of the virion. VP1 contains a small groove that interacts with specific carbohydrate residues on the cell surface (325,326 and 327). Oligosaccharide chains that terminate with sialic acid serve as the receptor for mouse polyomavirus (327). Some strains bind only straight-chain oligosaccharides, whereas others bind both straight and branched chains. There are wide variations in the response of mice to polyomavirus infection, ranging from persistent infection to tumorigenesis to death. This can be explained on the basis of the differences in receptor–virion interactions (16,17).

Preparations of polyomaviruses contain three kinds of virus particles. Infectious virus particles contain a single molecule of viral DNA in association with four cellular histones. The DNA and histones are arranged as chromatin, and the histone–viral DNA complex is often referred to as the *viral minichromosome.* This minichromosome has been used as a model system to study the effects of chromatin structure on DNA replication and transcription. Most viral DNA molecules

contain a full complement of nucleosomes, but some lack nucleosomes over viral regulatory sequences. Virion minichromosomes lack histone H1, whereas the form of the minichromosome found within infected cells contains it (364). Virion preparations also contain empty capsids as well as pseudovirions, capsids containing cellular rather than viral DNA. Passage of polyomaviruses at high multiplicities of infection leads to the generation of defective viral genomes containing deletions, duplications, and rearrangements of viral genetic information, often with duplications of the viral origin of DNA replication. If within appropriate size limits, these defective viral genomes can be encapsidated.

GENOME ORGANIZATION

The genomes of all polyomaviruses are divided into early and late regions (Fig. 2). The early region is, by definition, that portion of the genome transcribed and expressed early after the virus enters the cells, and it continues to be expressed at late times after infection, after the onset of viral DNA replication. The late region of the genome is expressed efficiently only after viral DNA replication begins, although low levels of transcription of the late region occur early after infection as well. The first maps of the polyoma and SV40 genomes were divided into 100 map units, with the unique Eco RI site in both the SV40 and polyoma genomes defined as 0/100 map units. Because the complete nucleotide sequences of these genomes have been determined, use of nucleotide positions rather than map units permits more precise description of specific sites within the genome. Nucleotide position 1/5243 for SV40 is the center of a 27–base pair palindrome located at the origin of viral DNA replication (*ORI* in Fig. 3). The numbering system used for SV40 is that of Buchman and colleagues (36). The numbering system used for polyomavirus is that of Griffin and associates (141). For polyoma, nucleotide position 1/5295 corresponds to the center of the Hpa II cleavage site at the junction of the fifth and third largest of eight fragments of polyoma DNA produced by cleavage with Hpa II. This site is also close to the polyoma origin of DNA replication.

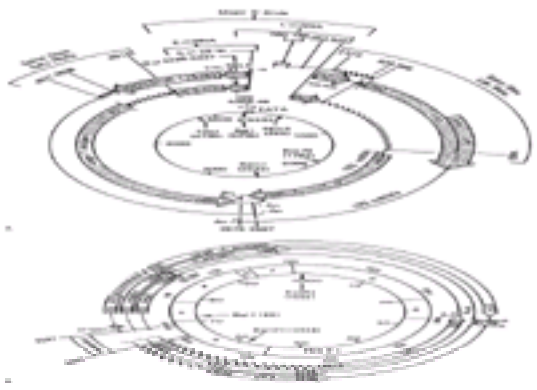

FIG. 2. Genomic organization of SV40 and mouse polyoma virus. **A:** SV40. The origin of replication and transcriptional regulatory region is at the top. The early region extends counterclockwise and the late region clockwise from the top. The regions encoding viral proteins are shaded. Also shown are the nucleotide positions for the 5' and 3' ends of viral mRNAs and the positions of introns. (From ref. 26, with permission). **B:** Mouse polyoma virus. The 0/100 map unit position is at the top and corresponds to the unique Eco RI site. The origin is located counterclockwise about one third the way around the circle. The early region is located in the top half of the figure and the late region in the bottom half. Also shown are the positions where each of the viral gene products are encoded, the nucleotide numbers of the 5' and 3' ends of the viral mRNAs, and the nucleotide positions of the introns. (From ref. 350, with permission.)

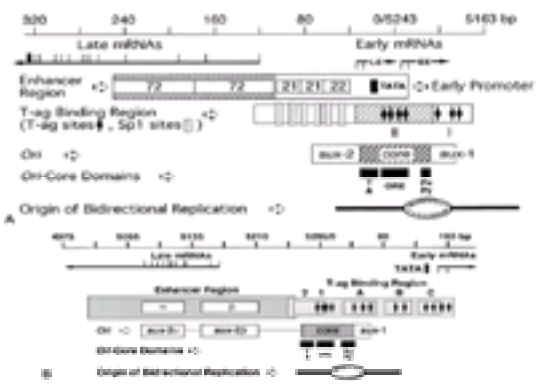

FIG. 3. The regulatory regions of SV40 (**A**) and polyoma (**B**). Shown are the location of the core origin of viral DNA replication and the location of auxiliary sequences (*aux*), which enhance viral DNA replication. Replication of polyoma requires either aux-2a or aux-2b. Within each core origin region are located a TA tract, with a strand bias for Ts and As, the palindromic region that serves as an origin-recognition element (*ORE*), and a region with a purine (*PU*) and pyrimidine (*PY*) strand bias, within which bidirectional DNA replication is initiated. Also shown are sites at which large T antigen binds to viral DNA; pentanucleotides involved directly in binding are indicated by *arrowheads,* which point in the direction of the pentanucleotide, 5'GAGGC3'. Several additional 5'GAGGC3' pentanucleotides are located in the vicinity of the Sp1 sites, to the left of binding site II, but these bind T antigen very weakly and are not thought to play a role in SV40 replication. Sites within this region at which transcription is initiated for production of viral early and late mRNAs are also indicated. The sites marked *EE* for SV40 are those used early after infection; those marked *LE* are used to produce early mRNAs after the onset of viral DNA replication. Additional sites for production of both SV40 and polyoma late mRNAs are located further downstream for both SV40 and Py. For SV40, the locations of the two 72–base pair repeats, which serve as enhancers, and of the 3 nearly perfect 21–base pair GC-rich repeats are also shown. Within these repeats are six sites to which transcription factor Sp1 can bind. (Courtesy of Dr. Mel DePamphilis, National Institutes of Health, Bethesda, MD.)

Two lines of evidence indicate that polyomavirus genomes contain a single unique origin of DNA replication. In one of the earliest uses of restriction endonucleases as tools to address important problems in molecular biology, Danna and Nathans (77) exposed SV40-infected BSC-1 monkey cells to pulses of ^{3}H-thymidine and, at various subsequent times, isolated viral DNA molecules that had completed replication. They then hybridized this labeled DNA to fragments of SV40 DNA generated with restriction endonucleases Hind II + III. In mature viral DNA molecules, label should first appear at sites near the terminus for DNA replication, whereas the origin region should become labeled later because molecules just initiating DNA replication at the time of addition of label require a longer time to be completed. The region of the genome around map position 17 (nucleotide 2622) became labeled first, whereas that near position 67 (nucleotide 0/5243) was labeled last and designated as the origin region.

This location for the origin was also revealed by electron microscopy. Replicating SV40 DNA molecules were cleaved with Eco RI, which cuts SV40 DNA once (nucleotide 1782), and examined by electron microscopy. The unique cleavage site allows mapping of the sites at which replication bubbles are initiated; these studies indicated that replication began one third of the way around the genome from the Eco RI site. By performing similar analyses with viral DNA molecules lacking portions of the early or late region, it was shown that there is no unique site for the termination of DNA replication. Rather, bidirectional replication proceeds away from the origin, with the replication forks meeting at a site about 180 degrees from the initiation site. Detailed mutational analyses to define the origin more precisely are discussed later.

The promoters and enhancers for transcription are located close to the origin of replication (see Fig. 2 and Fig. 3). Together, the promoters, enhancers, and origin are referred to as the *viral regulatory region.* Transcription extends bidirectionally from initiation sites near the origin, with early and late messenger RNAs (mRNAs) being transcribed from opposite strands of the viral genome. Thus, the early region extends from the origin to a site about halfway around the genome. The early region encodes the viral regulatory proteins, the tumor, or T, antigens, so called because they can be detected with antisera derived from animals bearing tumors induced by these viruses or by injection of cells transformed by these viruses. SV40 and the other primate polyomaviruses encode two T antigens—designated large T and small t antigens on the basis of size. Polyoma and closely related viruses encode three T antigens—large T, middle (or medium) T, and small t. All of the T antigens of each virus share N-terminal sequences and contain different C-terminal regions. The mRNAs encoding them are produced by alternative splicing from a common pre-mRNA. Figure 2 shows the common and unique regions of SV40 and polyoma T antigens.

The late regions of these viral genomes encode the three capsid proteins, VP1, VP2, and VP3. As with the early mRNAs, late mRNAs are generated from a common pre-mRNA by alternative splicing. The coding regions of VP2 and VP3 overlap, with VP3 sequences being a subset of VP2 sequences. The coding region for the N-terminus of VP1 overlaps that for the C-termini of VP2 and VP3, with translation of VP1 being from a different reading frame than VP2 and VP3. SV40 and the human polyomaviruses encode a fourth late protein, called the *agnoprotein* (see Table 2). This 62– to 71–amino acid protein is encoded by the leader region of some species of late viral mRNA and accumulates in the perinuclear region during the late phase of the infection cycle (167,253). It was originally named agnoprotein because its function was unknown. It appears to facilitate the localization of the major capsid protein to the nucleus and may also enhance the efficiency with which virus spreads from cell to cell (43,273). Mutants that do not produce the agnoprotein yield fewer progeny virions and produce small plaques (11,238). Pseudorevertants of these mutants map to the major capsid protein, VP1, suggesting a possible interaction between the agnoprotein and VP1. Additional small open reading frames that could encode other proteins are found in other species of late mRNA as well as in some species of early-stranded mRNA present late after infection (186). Thus, many SV40 late mRNA species are bi-cistronic.

VIRAL GENETICS

Genetic analyses have played a central role in our understanding of the biology and replication cycle of the polyomaviruses (reviewed in 351). The first mutants to be isolated were host range mutants of polyoma whose growth was restricted to certain mouse cell lines, and temperature-sensitive (ts) mutants of SV40 and polyoma. The availability of restriction endonucleases led to the construction of a wide variety of deletion and substitution mutants during the 1970s and 1980s. Site-directed mutagenesis and polymerase chain reaction–based approaches have also been used to produce mutants of the polyomaviruses. Today, it is relatively easy to construct any desired point, deletion, or substitution mutant of these viruses.

For SV40, ts mutants were divided into five complementation groups, A, B, C, BC, and D, based on their ability to complement one another to produce plaques at the nonpermissive temperature. We now know that the product of the A gene is large T antigen. VP2 and VP3 are both altered by mutations in gene D, whereas mutants in groups B, BC, and C produce altered VP1. Intracistronic complementation is often seen in genes that encode proteins that assemble into multimers or in multifunctional proteins in which separate domains perform distinct roles. Intracistronic complementation is observed between VP1 mutants in groups B and C but not between those in the BC group and either group B or group C. It has also been observed between mutants of large T antigen that affect the host range of the virus and mutants that affect replicative activities of the protein (352). The ts mutants of polyomavirus have been sorted into similar complementation groups.

By infecting cells at the permissive temperature and shifting them to the nonpermissive temperature at various times after infection, it was shown that the products of genes A and D were required early after infection, whereas the products of genes B and C were required only at late stages of infection. It is not surprising that large T antigen is required early after infection because it is required for viral DNA replication. Studies that suggest a role for VP2 and VP3 in adsorption or uncoating of the virus explain the finding that the D gene product is also required for early events of the infection cycle.

The sites of mutation of representative members of each complementation group were mapped by marker rescue using restriction endonuclease fragments of viral DNA (197). This involved producing partial heteroduplexes containing a circular strand of a particular ts mutant and a linear restriction endonuclease fragment. Successful marker rescue

depends on the wild-type restriction endonuclease fragment containing the information that is mutated in the mutant genome. In this way, the SV40 A gene was mapped to the early region and the SV40 B, C, BC, and D groups to the late region of the genome. Similar mutants of polyoma were mapped to analogous regions of the polyoma genome.

Although these studies were able to identify viral genes and their positions on the physical map of the viral genome, they did not indicate which of the viral gene products were encoded by each gene. Deletion mutants were constructed using restriction endonucleases, and this permitted assignment of some viral proteins to viral genes because it was easy to determine which viral gene product had been shortened by the deletion mutation. Many of these mutants could be propagated only in the presence of wild-type helper virus. Mutants of SV40 with deletions at the unique Eco RI or BamH I sites produce internally deleted or truncated VP1 polypeptides, indicating that the gene for VP1 spans the Eco RI and Bam HI sites (59). This is the region of the genome where marker rescue studies placed mutations in the B, BC, and C complementation groups.

Mutants with deletions at the unique Hae II site affected only VP2 (59). These mutants were able to form very small plaques and hence could be propagated without helper virus. Because these slow-growing mutants could complement tsD mutants at the nonpermissive temperature, they were assigned to a new complementation group E, with VP3 assigned to complementation group D. SV40 mutants with deletions in the early region were also constructed and found to produce large T antigens of reduced size, thus proving that the large T antigen was the product of the A gene rather than a cellular protein induced by SV40 infection (198).

A set of viable mutants with small deletions located randomly around the SV40 genome was produced. Three regions of the viral genome were identified that could accommodate deletions without impairing the plaque-forming ability of the virus (301). These sites are located in the agnogene region, in the region encoding the 3'-untranslated regions of the early and late mRNAs, and in the early region between map positions 54 and 59 (nucleotides 4900 to 4600, approximately).

Normal-sized plaques were produced by the 54/59 region deletion mutants, but these viruses were unable to transform nonpermissive cells as efficiently as wild-type SV40, suggesting that a gene product needed for transformation was encoded in this region. No tsA mutants (affecting large T antigen) had been mapped to this region. Analysis of T antigens encoded by mutants with deletions in the 54/59 region showed that the deletions did not affect large T antigen, although size measurements of large T suggested that most or all of the early region was required to encode it. Studies by a number of investigators subsequently showed that the SV40 early region encodes both small and large T antigens (22,71,256) and that small t antigen was reduced in size by mutations in the 54/59 region. Alternative splicing allows the production of mRNAs that encode these two early gene products; there is not sufficient genetic information in the SV40 early region to encode distinct polypeptides of 84 and 17 kd.

Viable deletion mutants of polyomavirus have also been isolated and characterized. Host range mutants of polyoma were selected that grew poorly or not at all in mouse 3T3 cells but replicated efficiently in primary cells or in cells transformed by retroviruses or by polyoma itself (18). Because these mutants display this host range phenotype and are also defective for transformation, they are called *hrt* mutants. Marker rescue experiments indicated that the hrt mutants map to the analogous region of the polyoma genome as the SV40 54/59 deletion mutants. Complementation is observed between polyoma tsA and hrt mutants to permit cell transformation at a temperature restrictive for tsA mutants. The hrt mutants are known to encode truncated forms of both small and middle T antigens. The role of these proteins in transformation is discussed later.

BIOLOGIC PROPERTIES OF THE POLYOMAVIRUSES

Although SV40 and polyoma are able to infect a wide range of mammalian cell lines and cultures, the response to infection can be productive or nonproductive. In some types of cells, viral DNA replication occurs, followed by the assembly of progeny virions and, ultimately, the death of the cell. This is called a *productive infection,* and cells in which infection is productive are said to be *permissive* for growth of the virus. Monkey cells are permissive for growth of SV40 and mouse cells for growth of polyoma. Some polyomaviruses show an extremely narrow host range for productive infection. For example, JCV replicates only in human fetal glial cells.

Nonproductive infections result when viral DNA replication cannot take place in the infected cell. For viral DNA replication to occur, SV40 or polyoma must produce adequate levels of large T antigen, the only viral protein needed directly for replication, and this protein must interact with cellular replication factors and the viral origin of DNA replication. An inability of SV40 large T antigen to interact productively with host replication factors in rodent cells is the reason that SV40 infection of rodent cells is unproductive. A similar inability of the polyoma large T antigen to interact productively with simian or human host cell factors is responsible for the inability of polyoma to replicate in primate cells.

In nonproductively infected cells, the infection cycle begins normally. The viral early mRNAs are produced, and the viral T antigens can be detected. The early proteins exert a variety of effects on the host cell. Because the polyomaviruses require the host cell DNA replication machinery, they replicate only during S phase, and T antigen stimulates cells to move through the cell cycle into S phase. When expressed in cells that are nonpermissive for viral replication, the viral T antigens also cause cells to acquire the properties of transformed cells. Such cells proliferate in semisolid media or in the absence of a high concentration of fetal calf serum. Usually, these transformed properties are manifested for only a few days. Usually, the viral genome, which does not replicate, is lost from the cells, and they return to normal growth. Such cells are said to have been “abortively” transformed. At a low frequency, the viral DNA becomes integrated into the host cell genome and is subsequently inherited as if it were a cellular gene. Integration appears to occur randomly with respect to sites in both the viral and host cell genomes. If the arrangement of viral DNA sequences after integration permits continued expression of SV40 large T antigen or polyoma middle T antigen, the transformed phenotype is expressed permanently, and the cell is said to have been *transformed* by the virus.

Most studies of transformation by SV40 have been conducted in mouse, rat, and hamster cells, but primate cells can be

transformed by SV40 if replication is prevented by mutation of the viral origin of replication or the viral T antigen gene. The Cos-1 and Cos-7 monkey kidney cell lines express wild-type SV40 large T antigen and contain an integrated copy of SV40 DNA carrying a deletion of sequences within the origin of DNA replication that are essential for DNA replication (135). For polyoma, rat cells are normally used to study viral transformation. Although permissive for replication of polyomavirus, mouse cells can also be transformed by polyoma if the virus carries a mutation affecting large T antigen or its origin of replication or if viral DNA becomes integrated into the mouse cell genome in a way that interrupts the early coding region and prevents continued synthesis of full-length large T antigen. Although multiple regions spanning most of the SV40 large T antigen are required for transformation, the critical protein for transformation by polyoma is the middle T antigen. Although these viral gene products must be present continuously to maintain the transformed phenotype, mutation of cellular genes can eliminate the need for maintenance of the viral transforming region (266,267).

In addition to being able to cause malignant transformation of cultured cells, these viruses can efficiently immortalize primary rodent cells that would normally undergo a limited number of cell doublings. Immortalization is a function of the large T antigens of SV40 and polyoma. Studies of immortalization of primary mouse embryo fibroblasts by mutants of SV40 T antigen suggest that some mutants that can neither immortalize primary cells nor transform established cell lines have the ability to extend the normal lifespan of the primary mouse fibroblasts (64,345). Although the lifespan of human fibroblasts and epithelial cells can also be extended by wild-type SV40 T antigen, their immortalization is a rare event and requires mutations of one or more cellular genes (122,300,362).

THE REPLICATION CYCLES OF SV40 AND POLYOMA

Productive infection of cells by polyomaviruses can be divided into early and late stages. The early stage begins with attachment of virus to cells (Fig. 4) and continues until the beginning of viral DNA replication. Thus, the early stage is marked by adsorption and penetration of the virion and its migration to the nucleus, where the viral genome is uncoated and made available for transcription. During the early phase of infection, the viral early proteins, the T antigens, are produced, and they affect the host cell by stimulating the production of enzymes required for cellular DNA replication, thereby preparing the cell for replication of viral DNA. These viral early proteins also stimulate resting cells to reenter the cell cycle. The late stage of infection extends from the onset of viral DNA replication to the end of the infection cycle and involves the replication of viral DNA, expression of the viral late genes encoding the capsid proteins, assembly of progeny virus particles in the nucleus of the infected cells, and release of virus, possibly at the time of the death of the cell.

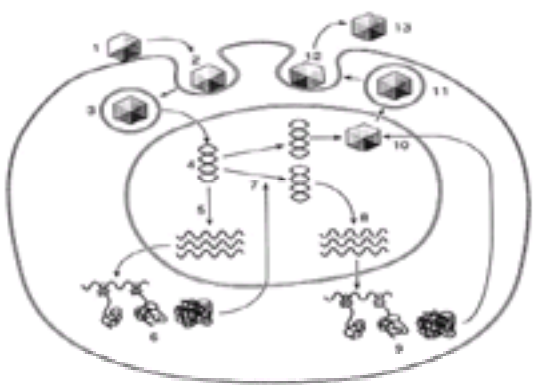

FIG. 4. Replication cycle of polyomaviruses. Steps in the replication cycle are indicated by numbers as follows: *1,* adsorption of virions to the cell surface; *2,* entry by endocytosis; *3,* transport to the cell nucleus (route and mechanism not yet known); *4,* uncoating; *5,* transcription to produce early region mRNAs; *6,* translation to produce early proteins (T antigens); *7,* viral DNA replication; *8,* transcription to produce late region mRNAs; *9,* translation to produce late proteins (capsid proteins); *10,* assembly of progeny virions in the nucleus; *11,* entry of virions into cytoplasmic vesicles (mechanism unknown); *12,* release of virions from the cell by fusion of membrane vesicles with the plasma membrane; *13,* released virion. Virions are most likely also released from cells at cell death when virions have an opportunity to leak out of the nucleus. In nonpermissive cells, the first six steps occur normally, but viral DNA replication cannot occur and subsequent events do not take place.

The time course of infection by SV40 and polyoma depends primarily on two parameters. Infection proceeds more rapidly at higher multiplicities of infection than at lower multiplicities. This most likely reflects the fact that critical levels of the viral early proteins are attained earlier when more viral genomes are available for transcription to produce the viral early mRNAs. The other critical parameter is the growth state of the host cell. At the same multiplicity of infection, viral DNA replication begins sooner, and the production of progeny virions is completed more rapidly when cells are growing exponentially than when cells are confluent and in a G_0 state at the time of infection. Figure 5 illustrates the time course for the expression of polyoma T antigen, induction of cellular enzymes, replication of viral DNA, and production of progeny virions.

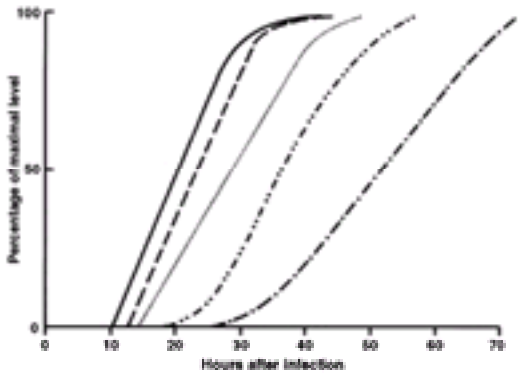

FIG. 5. Idealized time course of central events during productive infection of mouse cells by mouse polyoma virus. Events after infection of monkey cells by simian virus 40 follow a similar course. Parameters shown are expressed as a percentage of maximal level reached. T antigen (——) was measured by percentage of cells showing positive immunofluorescence; the actual rate of accumulation of T antigen may be slower because T-antigen–positive cells continued to synthesize T antigen over a long period. Cellular enzyme production (-----) is expressed as stimulation of enzyme activities compared with activity levels present in uninfected cells. Cellular DNA synthesis (.....), viral DNA and RNA (·····), and infectious virus (-·-·-) are expressed as the percentage of the total final yields of these macromolecules attained at particular times, not the rate of production at any particular time. (From ref. 350, with permission.)

Adsorption and Receptors

Early studies of the initial events of polyomavirus infection were complicated by the fact that virus preparations used contained not only infectious virus particles but also empty capsids and pseudovirions. Less ambiguous results have been obtained using purified virion preparations. Norkin and coworkers found that binding of SV40 virions to LLC-MK2 rhesus monkey kidney cells was inhibited by antibodies to class I major histocompatibility complex (MHC) antigens but not by antibodies to other cell surface proteins (34). Little binding was observed to two human lymphoblastoid cell lines that do not express MHC class I molecules owing to failure to express either b $_2$-microglobulin or HLA class I molecules. Binding occurred when class I expression was restored by transfection of cells with plasmids encoding b $_2$-microglobulin or HLA class I molecules. Different results were obtained by Basak and colleagues, who studied the binding of SV40 virions to polarized monkey kidney cells (Vero C1008) (14). Binding was restricted to the apical surface of these polarized cells, whereas HLA expression was detected at both the apical and basolateral surfaces.

Perhaps class I MHC molecules are required for adsorption of SV40 virions but are not sufficient, with other cell surface molecules also being part of the receptor. In nonpolarized cells, binding sites for SV40 were distributed uniformly over the cell surface. Different subclones of C1008 Vero cells bound different numbers of virions, and the abundance of binding sites was cell cycle regulated. Those cells that bound the greatest number of virions contained about 10 ^{5} receptors per cell.

Competition experiments indicate that SV40 and polyoma receptors are distinct (14). This was also inferred from earlier studies that showed that binding of polyoma was sensitive to treatment of the cells with sialidase, whereas binding of SV40 was not. The polyomavirus receptor is known to contain oligosaccharide chains terminating with sialic acid. Antibodies to VP1 block adsorption of polyoma virions (25). Small and large plaque strains of polyoma produce different VP1 molecules, suggesting that plaque morphology may reflect different efficiencies of interaction of the virus particles with their receptors. When examined by isoelectric focusing (25), polyoma virions are seen to contain six distinct isoforms of VP1 (A to F), which differ in posttranslational modifications. Empty capsids lack one of these species (E) and do not compete with virions for binding to mouse kidney cells, suggesting that species E plays a central role in specific virus adsorption (26). Empty capsids can still bind to guinea pig erythrocytes and are internalized and degraded in lysosomes, rather than being transported to the nucleus for productive infection. Additional evidence for a role for VP1 in virus binding comes from binding studies with capsid-like structures that self-assemble from polyoma VP1 expressed in and purified from bacteria. The fact that the binding of these “capsids” to quiescent cells stimulates modest induction of *c-fos* and *c-myc* (399) suggests that these capsids may be interacting with a growth factor receptor or related protein.

The VP2 polypeptide of polyoma and SV40 is myristoylated at its N-terminus (331), and this modification plays a role in the early events of infection. Deletion mutants of SV40 that produce no VP2 are weakly viable. Plaques formed by these mutants are extremely slow to appear and enlarge slowly, suggesting that these mutants produce a dramatically reduced yield of infectious progeny (59). However, the yield of physical virus particles is nearly normal, suggesting that these progeny are defective in some early event of the infection cycle, perhaps adsorption. Mutation of polyoma VP2 so that it can no longer be myristoylated still permits its incorporation into virions, but infectivity is reduced 15- to 20-fold, and the infection cycle is prolonged by several hours (196,279). Together, these results suggest that the myristic acid moiety of VP2 may interact with cellular membranes, whereas VP1 probably interacts with specific receptor polypeptides. Because VP2 is entirely internal to the virion outer shell, how the N-terminal portion becomes exposed is unknown. There is about a 12.5 Å opening at the top of each pentamer, which may be large enough for the N-terminal portion of VP2 to emerge (50). Weakening of the VP1 contacts in each pentamer could also allow the N-terminal portion of VP2 to be exposed, but no mechanism is known that could explain such a rearrangement. Possibly, initial interactions with the cell (before binding to the true receptor) could trigger such a rearrangement.

Virion Entry and Uncoating

How polyomaviruses gain entry to the nucleus is uncertain. Most particles in a virus preparation do not initiate infection successfully; hence, there is the problem that the particles observed during the early stages of infection are likely to be

primarily those that do not initiate an infectious cycle. After binding to the cell surface, SV40 virions appear to transmit an intracellular signal that in turn facilitates viral entry into caveolae. Class I MHC molecules do not enter cells, indicating that although these molecules mediate binding of virions to cells, they are not required for viral enclosure within the preformed caveolae (2,51). Soon after infection, virus particles can be detected by electron microscopy in small vesicles within the cytoplasm (162,229). These vesicles may ferry virus particles to the nucleus, and the fusion of these vesicles with the nuclear envelope has been observed by electron microscopy (142,250). Virus particles have also been observed within the endoplasmic reticulum (ER) (176); thus, the ER may also provide a pathway to the nuclear envelope.

The nuclear pore complex could serve as an entry portal for virus particles because SV40 virions injected into the cytoplasm enter the nucleus and begin production of SV40 early mRNA (55). This process is blocked by wheat germ agglutinin or by monoclonal antibodies to nuclear pore complex proteins (382), treatments known to block the movement of karyophilic proteins through the nuclear pore complex. Large T antigen is normally not detected until 10 to 12 hours after infection but can be detected within 3 to 4 hours after microinjection of virus particles into the cytoplasm. This time differential may reflect slow steps in penetration and the escape of virions from membrane vesicles into the cytoplasm before entry through the nuclear pore complex. Alternatively, it may mean that the nuclear pore complex is not generally a route of entry into the nucleus for SV40 or polyoma virions, with the pathway to the nucleus involving movement of virus particles within the ER or other membrane-bound structures. Because virus capsid proteins contain nuclear localization signals used to direct the proteins to the nucleus before virion assembly, these same nuclear localization signals may be able to mediate the entry of microinjected virions into the nucleus, even if this route is not normally used.

Most studies indicate that virions are uncoated within the nucleus because intact virions can be detected within the nucleus before expression of the viral T antigen (8,162,219). However, in some studies, partially disassembled virus structures were seen in the cytoplasm and were presumed to be uncoating intermediates (118). Whatever the route of entry, the viral minichromosome is transcribed, replicated, and subsequently encapsidated within the nucleus.

Transcription and Processing of Early Viral Messenger RNAs

Once virions have been uncoated, the viral minichromosome can be used as a template for transcription by RNA polymerase II to produce early viral mRNAs, derived from the early region of the viral genome. Production of mRNAs derived from the late strand of viral DNA is inefficient early after infection. Maximal production of the viral late mRNAs requires both an activity of large T antigen and the onset of viral DNA replication. Viral late mRNAs are discussed later.

For many years, studies on patterns of transcription of polyomavirus genomes were hampered by the very low levels of the viral early mRNAs, which make up only 0.01% to 0.02% of the total cellular RNA synthesized before viral DNA replication. During the initial 10 to 15 hours of an SV40 or polyoma infection, the total production of early mRNA likely represents not more than a few hundred molecules, in comparison to the 300,000 molecules of total mRNA found in a eukaryotic cell (reviewed in 351). From the application of more sophisticated and sensitive mapping techniques, it is now known that transcription initiates within the viral regulatory region and extends at least 180 degrees around the viral genome (188), and may extend further. Functional viral early mRNAs are produced by alternative splicing of this pre-mRNA (22). For SV40, alternative splicing generates two species of early mRNA. Messenger RNAs encoding large T antigen result from excision of an intron that extends from nucleotide 4918 to nucleotide 4571, whereas excision of an intron extending from nucleotide 4638 to the same splice acceptor at 4571 yields mRNAs encoding small t antigen. Thus, the SV40 early pre-mRNA contains two splice donor sites and a common splice acceptor. All of these mRNAs are polyadenylated at the same site at nucleotide 2694. Infected cells may also contain a third alternatively spliced early SV40 mRNA (391).

For polyoma, a similar early pre-mRNA is spliced to generate three polyoma early mRNAs (174,175). These mRNAs share 5' ends located in the viral origin region and 3' ends located at nucleotide 2930, and they differ with respect to sequences excised by splicing. The introns for the mRNAs for the polyoma large, middle, and small T antigens extend from nucleotides 409 to 794, 746 to 808, and 746 to 794, respectively. Thus, there are two splice donors and two splices acceptors. Low levels of polyoma early mRNAs that use a weak polyadenylation site at nucleotide 1525 have also been detected (156,354).

Other polyomaviruses show similar patterns of early transcription. For those that produce two T antigens, including JCV and BKV, two early mRNAs are generated by alternative splicing; for those that produce large, middle, and small T antigens, the three mRNAs are produced by alternative splicing from a common precursor, analogous to the situation with polyoma.

Early Promoters and Enhancers

The sites at which SV40 early mRNAs are initiated have been mapped in three ways—by nuclease S1 protection using end-labeled DNA probes, by primer extension of short labeled oligonucleotides, and by analysis of the nucleotide sequences attached to 5'-capped oligonucleotides produced after enzymatic digestion of purified labeled viral early mRNAs. All three approaches can produce artifactual results, particularly if the RNAs to be analyzed are inabundant (as viral early mRNAs are) or if they have significant secondary structure. Taken together, these experiments suggest that there are multiple clustered start sites for the SV40 early mRNAs, positioned around nucleotides 5237 and 5231 (127,146,348) (see Fig. 3).

Additional species of SV40 early mRNA are also detected that contain 5' ends at nucleotides 34 through 28 (127). Molecules using these upstream starts can be detected relatively early after infection, but these sites become the primary initiation sites after viral DNA replication begins (117). This shift from early-early to late-early start sites requires both T-antigen and viral DNA replication. When all T-antigen–binding sites in the origin region are mutated and a second origin is introduced at another site, the shift from early-early to late-early sites still occurs (37). These 5'-extended early mRNAs have the potential to encode an additional viral polypeptide of 23 amino acids (186) (see Fig. 2), although deletion mutants unable to produce this protein behave like wild-type viruses. Translation from these longer early

mRNAs, in which the initiation codon for the T antigens is not the first one, is inefficient (37).

The promoters that govern the production of the SV40 early mRNAs were among the first to be studied in detail and dissected by exhaustive mutagenesis. The critical features of the SV40 regulatory region are shown in Figure 3. Three sites (I, II, and III) for binding of SV40 large T antigen are located in this region. Upstream from the start sites for transcription is located an AT-rich region that contains a TATA box–like element, TATTTAT. Analysis of deletion and point mutants affecting the SV40 early TATA box and sequences between it and the normal transcriptional start sites demonstrated for the first time that TATA boxes function to direct transcription initiation to a site about 30 nucleotides downstream (65). Detailed point mutagenesis of this region indicates that it actually contains two TATA boxes, which specify initiation at the two clusters, around nucleotides 5237 and 5231 (257).

Upstream from the AT-rich region is a GC-rich cluster that contains two repeats of a 21–base pair sequence and a homologous 22–base pair sequence (often referred to as the *three 21–base pair repeats*). Altogether, this region contains six copies of a GC-rich sequence (CCGCCC) present 40 to 103 nucleotides upstream of the RNA initiation sites. Lying further upstream are two copies of a 72–base pair repeat. A variety of *in vivo* and *in vitro* studies have been performed in many laboratories to determine the importance to viral early transcription of these various regulatory region elements. Together, they indicate that the 21–base pair elements are important promoter elements but are not absolutely essential for early transcription (21,105,117,153). Studies of the SV40 promoter indicate that transcription factor Sp1 binds to these sites *in vitro* and is absolutely required for transcription *in vitro* from the SV40 early promoter (95,96).

The 72–base pair repeat elements function as enhancers for the SV40 early promoter. In fact, it was the study of these repeats that led to the discovery of eukaryotic transcriptional enhancers. These elements act to increase transcription initiation in an orientation-independent mechanism when located either upstream or downstream from transcriptional start sites, with little dependence on distance for their enhancing effects. Many different transcription factor–binding sites are located within the SV40 and polyoma enhancers, but it has not yet been possible to identify which factors actually interact with them *in vivo* (reviewed in 168). The SV40 enhancer is quite strong and functions well in cells of a variety of species and types; it has been widely used as an enhancer to drive expression of heterologous genes. The BKV enhancer consists of three repeats of a 68–base pair sequence, with the middle copy containing an internal 18–base pair deletion, and also includes another sequence element located adjacent to the repeat element closest to the BKV late genes (88). JCV contains two copies of a 98–base pair sequence that functions as an enhancer and that, like SV40 and BKV enhancers, is located on the late side of the origin or replication (183).

The organization of the regulatory region of polyomavirus is similar to that of SV40 (see Fig. 3) but has some important differences. Similar approaches were used to map the major sites of transcription initiation to sequences between nucleotides 147 and 158 (67,173). As in the case of SV40, 5' sites mapping further upstream were detected during the late phase of polyoma infection (67,108,173). In contrast to their production by transcription initiation during SV40 infection, these 5'-extended polyomavirus transcripts appear to arise by processing of long viral pre-mRNAs, which themselves arise by transcription that continues more than once around the complete polyomavirus genome (1). Also in contrast to the late-early SV40 transcripts, these polyoma transcripts are uncapped and primarily nuclear.

The polyomavirus regulatory region contains an enhancer element that has a more complex organization than does the SV40 enhancer. The polyoma enhancer contains a single copy of two different adjacent enhancers, called A and B, or a and b (160), which can function independently and which confer different cell-type specificities. Extensive studies have been conducted of polyomavirus variants selected for efficient growth on either differentiated or undifferentiated embryonal carcinoma cells; these viral variants contain point mutations within the enhancers (76,235,236). Because enhancers contain multiple sites to which transcription factors can bind productively (168), these genetic studies suggest that there has been a selection for mutants that acquire binding sites for transcription factors present in embryonal carcinoma cells.

Regulation of Early Transcription

The early promoters of all polyomaviruses are autoregulated by their large T antigens. This was shown first for SV40 (189,272,344). In cells infected with tsA mutants affecting the large T antigen, early mRNAs are overproduced, and the rate of synthesis of large T antigen is elevated in parallel. A similar result has been obtained for polyoma (57). Autoregulation of viral early transcription has been reproduced *in vitro* and shown to be dependent on T-antigen–binding sites (150,276,277), indicating that large T antigen autoregulates synthesis of its own mRNA. The binding of large T antigen to viral DNA blocks the assembly of a functional transcription complex, thereby repressing early transcription (248). The location of the binding sites that are the target for this repression is different for SV40 and polyoma. The SV40 binding sites I and II (see Fig. 3A) are located downstream of, or overlap, the early mRNA start sites used early after infection, respectively (277), whereas the binding sites critical for autoregulation by polyoma T antigen (see Fig. 3B) are located upstream of the early transcription initiation sites (68,107).

Most studies on autoregulation by large T antigen were performed under conditions in which T-antigen levels were relatively high. Early after infection, when T-antigen levels are much lower, T-antigen–binding sites are unoccupied. Under these conditions, large T antigen stimulates transcription from its own promoter by a mechanism that does not require direct binding of T antigen to viral DNA (375).

Processing of Viral Early Pre-messenger RNAs

Alternative splicing is used to generate multiple species of early mRNA from a single class of mRNA precursor (see Fig. 2). Because of the advantages of working with viral systems, alternative splicing of SV40 pre-mRNA has been extensively analyzed both *in vivo* and *in vitro* as a model system for alternative splicing. The very small size of the intron excised to produce the SV40 small t mRNA (66 nucleotides) helped to define the relationship between minimum intron size and efficiency of excision (119). Splicing to produce large T mRNA uses multiple alternative branch-point sites, whereas splicing to produced small t mRNA uses only the most distal of these sites (251). The use of alternative lariat

branch sites is probably central to alternative splicing in other systems. The ratio of small t to large T mRNA produced varies from cell line to cell line and is 10- to 20-fold higher in human 293 cells than in many other mammalian cell lines (119). Because the ratio of large T to small t splicing *in vitro* in HeLa cell extracts is 100:1 (252), extracts supplemented with fractions from a human 293 cell extract were used to identify and purify a factor from 293 cells that promoted use of the small t intron. This resulted in the discovery of ASF, a protein factor that controls alternative splicing for SV40 (124) and that appears to play an important role in alternative splicing in many cellular gene systems.

For polyoma, the 48-nucleotide intron excised to produce small t mRNA is the smallest known mammalian intron and is below the minimum size thought to be required for excision. Excision of this intron involves two branch-point sites, one only 4 nucleotides upstream of the small t 3' splice acceptor site, and also requires an intact 3' splice site for middle T antigen, located 14 nucleotides downstream of the small t 3' splice acceptor site (125). This indicates that the two 3' splice sites somehow cooperate to permit excision of the tiny small t intron. Such splice-site cooperation could be important for alternative splicing of cellular pre-mRNAs as well.

Synthesis and Functions of the Viral T Antigens

Large T Antigen

The large T antigens of the polyomaviruses are complex multifunctional proteins that have multiple enzymatic activities, interact with several cellular proteins, and perform several different roles during infection. The organization and activities of different large T antigens have been compared and reviewed (260). Considerably more is known about SV40 large T antigen than about the other large T antigens. Although this portion of this chapter focuses on SV40 large T antigen, key differences between SV40 and polyoma are highlighted.

The sizes of the T antigens of several polyomaviridae are listed in Table 2. The functional organization of SV40 and polyoma large T antigens is shown in Figure 6. All of the large T proteins are closely related and contain identical or similar sequences over most of their lengths. Polyoma large T contains a region of 154 amino acids following amino acid 82 with no homology to SV40 large T sequences; the viral DNA encoding this portion of large T is the portion of the polyoma genome that also encodes, in a different reading frame, the unique portion of middle T antigen. Conversely, SV40, JCV, and BKV large T antigen contain about 70 amino acids at their carboxyl termini that have no homology with any sequences in polyoma large T. This carboxyl-terminal domain of large T is involved in host range and adenovirus helper function (see later).

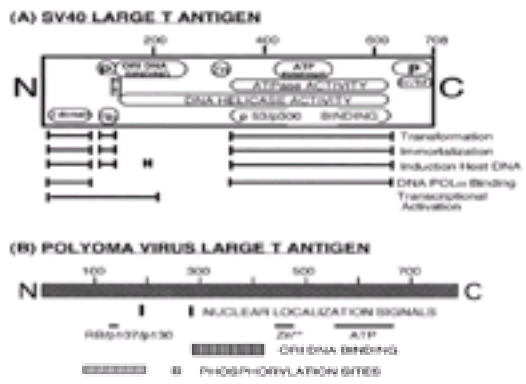

FIG. 6. The domain structures of SV40 large T and mouse polyomavirus large and medium T antigens. **A:** SV40. Shown are the domains of large T antigen required for nuclear localization (*NLS*) and for binding ATP and Zn^{2+} and for binding to the viral origin of DNA replication. The regions of T antigen required for ATPase activity, helicase activity, host range and helper function activity (*hr-hf*), as binding to $p105^{Rb}$/p107/p130 (indicated by *Rb*) and to p53 are shown. The DNA J domain functions like the J domain found in *Escherichia coli* DNA J/hsp40 molecular chaperone proteins and is involved in protein–protein interactions between T antigen and members of the $p105^{Rb}$ family. Also shown are the regions required for transformation of most rodent cell lines, for immortalization of primary mouse fibroblasts, for binding to DNA polymerase a, for induction of host DNA synthesis, and for transcriptional activation of the SV40 late promoter and many simple modular promoters by SV40 large T antigen. **B:** Mouse polyoma virus. Shown are the sequences that serve as nuclear localization signals (*NLS*) and sequences required for binding ATP and Zn^{2+} and for binding to the polyomavirus origin of DNA replication and to $p105^{Rb}$/p107/p130. Sites of phosphorylation are also shown. (**B** courtesy of Dr. Brian Schaffhausen, Tufts University Medical School, Boston, MA.)

The SV40 large T protein is modified posttranslationally in several ways. It contains two clusters of phosphorylation sites (285,343) (see Fig. 6) and is also modified by *O*-glycosylation (165,289), acylation (192), adenylation (30), poly(ADP)-ribosylation (137), and amino-terminal acetylation (237). Little is known about the functions of most of these modifications. However, phosphorylation is known to play a major role in controlling the activities and functions of the protein, and acylation often permits a modified protein to associate with cellular membranes. More than 95% of SV40 large T antigen is located in the nucleus, although a small percentage is found at the plasma membrane. Nuclear T antigen exists both free in the nucleoplasm and associated with chromatin and nuclear matrix (323). It is estimated that lytically infected cells contain between 5×10^5 and 5×10^6 molecules of large T antigen.

Detailed studies of the sequences of SV40 large T antigen required for nuclear localization led to the definition of amino acids 127 to 133 (KKKRKVD) as the first identified nuclear localization signal (170,200). Polyoma large T antigen contains two nuclear localization signals, one at a place analogous to the site of the SV40 nuclear localization signal, the other in a region without homology to SV40 T antigen (275). Phosphorylation sites are located near these nuclear localization signals and may be used to regulate the location of T antigen. For both polyoma and SV40, some of the

phosphorylation sites are substrates for cell cycle–dependent kinases or for isoforms of casein kinase I.

SV40 large T antigen is a DNA-binding protein that interacts specifically with several pentameric sequences (GAGGC) at the SV40 origin of DNA replication. It also binds, but with considerably lower affinity, to both single- and double-stranded DNA in a sequence-independent manner. The organization of these T-antigen–binding sites is shown in Figure 3. Binding of T antigen to site I permits T antigen to autoregulate production of SV40 early mRNAs (91,150,276). Binding at site II plays a central role in initiation of viral DNA replication. Binding site II is the preferred site for binding of SV40 large T antigen in the presence of physiologic levels of adenosine triphosphate (ATP) (27,80), which induces the formation of double hexamers of T antigen bound to site II (225). Individual pentanucleotides within site I are sufficient to direct hexamer formation, whereas certain pairs of these pentanucleotides can direct formation of T-antigen double hexamers (169). These double hexamers are fully able to initiate the structural changes that occur at the origin of replication after T-antigen binding. Because more pentanucleotides than two in site I are required, the others most likely play a role subsequent to the initial formation of double hexamers. In the presence of ATP, binding of polyoma T antigen to the polyoma regulatory region is also stimulated, and binding occurs over an extended portion of the origin (217). Some mutations of phosphorylation sites affect the DNA replication activities of T antigen, and this appears to affect interactions between T antigen hexamers critical for replication (373).

A second activity of large T antigen is DNA helicase activity (78,321). SV40 large T antigen can bind and hydrolyze ATP, and this activity is stimulated by single-stranded DNA (130,350). Helicase activity is crucial for viral DNA replication and requires functional ATPase activity and the ability to bind to SV40 DNA. *In vitro,* T antigen displays helicase activity with substrates lacking specific T-antigen–binding sites. T antigen also possesses RNA helicase activity (284), but the importance of this activity is unknown. There have also been reports that T antigen has (or is tightly associated with) topoisomerase I activity and that T antigen is able to promote strand reannealing and DNA looping (221,224,287). Formation of double hexamers stimulates the helicase activity of T antigen (308,309).

More recently, the N-termini of all known polyomavirus large T antigens have been noted to have sequence homology with the J domain of the DnaJ (Hsp40) family of molecular chaperones (181,261). The T-antigen J domain contains the Hsp70/DnaK family domain histidine-proline-aspartate (HPD) that is conserved within the J domain of all DnaJ homologs (358). T antigen retains its functions when its J domain is replaced by the J domain of two human J-domain proteins (40). *In vitro* studies indicate that this portion of T antigen can stimulate the ATPase activity of hsc70 and also enhance release of an unfolded polypeptide from hsc70 (317). Furthermore, this region has been shown to function as a molecular chaperone mediating phosphorylation of the T-antigen–associated proteins p107 and p130 (333). The domains of T antigen required for these enzymatic activities are shown in Figure 6.

Based on its primary sequence and structural modeling, the DNA-binding domain of large T antigen does not resemble a known DNA-binding domain, and this is confirmed in the solution structure of the DNA-binding domain of SV40 large T antigen, determined using nuclear magnetic resonance spectroscopy (218). Two nearby loops define a continuous surface of T antigen that is altered in the presence of added GAGGC-containing oligonucleotides, suggesting that this surface is the one that directly contacts DNA. The ATP-binding/ATPase domain of large T shows substantial similarity to many known ATP-binding and hydrolyzing proteins. Located between the DNA-binding domain and the ATP binding/ATPase domain is a zinc finger domain important for the overall structure and function of large T antigen (215). Attempts to crystallize T antigen have so far been unsuccessful, perhaps owing to heterogeneity resulting from nonuniform posttranslational modification. High-resolution electron microscopy, image processing, and three-dimensional reconstruction suggest that each T-antigen hexamer at the origin has a propeller-like shape organized around a central channel, similar to what has been seen in other DNA helicases (282).

T antigen forms complexes with several cellular proteins important for DNA replication, including at least two subunits of DNA polymerase a-primase and the single-stranded DNA-binding protein, replication protein A (RPA) (33,63,93,94,161,234,372). T antigen also binds DNA topoisomerase I, and this interaction serves to stimulate initiation of replication at the origin and to prevent the unwinding of nonorigin sites by T antigen (147,303,304 and 305,357).

Complexes are also formed between T antigen and several proteins important for regulation of cell growth. Through conserved sequences located between amino acids 102 and 115, T antigen interacts with the tumor suppressor protein, $p105^{Rb}$, as well as with related cellular proteins, p107 and p130 (82,97,106,149). A sequence very similar to T-antigen amino acids 102 to 115 is found in the adenovirus E1A and the human papillomavirus E7 proteins, which also interact with $p105^{Rb}$ and related proteins. In fact, the adenovirus E1A gene remains functional when its $p105^{Rb}$-binding domain is replaced with the $p105^{Rb}$-binding region of SV40 large T antigen (242). The Dna J domain, in conjunction with an intact $p105^{Rb}$-binding domain, has been shown to perturb p130 and p107 phosphorylation and to target p130 for rapid degradation (334). The mechanism of this effect is thought to require an association between the T-antigen J domain and mammalian hsc70, resulting in activation of the intrinsic ATPase activity associated with hsc70 and alteration of the folding of associated p107 or p130 (81). Small t antigen also contains the J domain and is also able to stimulate the ATPase activity of hsc70 (317).

The tumor suppressor protein p53 forms complexes with SV40 large T antigen (199,213) through portions of a large conformationally sensitive domain located in the carboxyl-terminal half of the protein (190,288,394). Although p53 also interacts with the JCV and BKV T antigens, it does not form complexes with the polyoma large T antigen, and this is one of the most important differences between SV40 and polyoma large T antigens. However, both SV40 (101) and polyoma large T-antigen (249) oncoproteins bind to members of the cap-binding protein (CBP) family of transcriptional adapter proteins (p300 and p400) that contain high histone acetyltransferase activity. The p300 protein, a transcriptional coactivator related to CREB, also binds to the adenovirus E1A protein (374,381), although T antigen and p300 appear to bind to distinct p300 isoforms (5). Furthermore, p300 can also bind directly to and modulate p53 (210), implying the existence of a complex transcriptional regulatory mechanism resulting from the tripartite interaction of large T antigen, p300, and p53.

Small t Antigen

The small t antigens of most polyomaviruses are cysteine-rich proteins of 172 to 195 amino acids (see Table 2) whose amino-terminal 82 amino acids are shared with large T antigen. For those polyomaviruses that encode middle T antigens, almost all of the sequence of small t antigen is contained within the middle T antigen of the same virus. Small t antigen is located in both the nucleus and the cytoplasm (104,398). Studies with mutants of SV40 and polyoma that contain deletions within the region of small t not shared with large T antigen indicate that small t antigen is dispensable for the lytic cycle of these viruses in cultured cells (301,322). SV40 small t antigen promotes G_1 phase progression and increases the efficiency of SV40 transformation of mammalian cells through activation of growth factor–stimulated signaling pathways. Small t antigens (and polyoma middle t antigen) associate with the catalytic (C) and structural (S) subunits of protein phosphatase 2A (PP2A) (255). This interaction displaces the B subunit of PP2A, resulting in inhibition of phosphatase activity (385). Inactivation of PP2A in turn results in the activation of the mitogen-activated protein kinase (314) and stress-activated protein kinase (371) pathways as well as pathways that use nuclear factor-kB (NF-kB), protein kinase C, and phosphatidylinositol-3 kinase (PI-3 kinase) (315). Small t-antigen expression has also been shown to be required to decrease p27 (KIP1) levels during cell cycle reentry of human diploid fibroblasts, perhaps explaining the requirement for small t in cell cycle activation of density-arrested human fibroblasts (263).

In some studies, SV40 small t antigen expressed from plasmids has been shown to be capable of activating transcription from the SV40 late and other viral promoters (23,216). However, when small t was expressed during a viral infection, transcriptional activation was not observed (264), making it uncertain whether small t antigens play a role in regulation of transcription. SV40 small t antigen has been reported to bind zinc ions through its cysteine clusters (361), but the function and importance of this interaction is uncertain because the small t antigens of the bovine and parakeet viruses contain only a single cysteine and would therefore be unable to bind zinc ions. Although the conserved cysteine appears to be required for PP2A binding, interaction with zinc is not required for binding PP2A (226).

There is evidence for yet another t antigen encoded in the SV40 genome, expressed from a third species of SV40 early mRNA that is spliced between nucleotides 4425 and 3679 (391). This alternatively spliced mRNA encodes a 17 kd small t antigen containing the first 131 amino acids of large T antigen and 4 amino acids encoded downstream of the splice junction in an alternate reading frame from that used to produce large T antigen. Whether a similar mRNA and small t antigen is produced by other polyomaviruses is not known.

Middle T Antigen

T antigens of medium size (middle T) are encoded by polyoma and closely related viruses, but not by SV40 or other primate polyomaviruses. The middle T protein is located primarily at the plasma membrane (164,297), where it associates with several cellular proteins important in the signal transduction pathways regulating growth. These include c-src, the cellular homolog of the transforming protein v-src of Rous sarcoma virus (66), and related src family kinase members. This association leads to the activation of the tyrosine kinase activity of c-src. Also complexed with middle T antigen and c-src is PI-3 kinase (4), which in turn leads to activation of the Ser/Thr kinase c-Akt, the cellular homolog of the viral oncogene v-Akt 2A (335). Shc, a two-protein complex that lies upstream of Ras in the signal transduction pathway (41,90) and the A and C subunits of protein phosphatase 2A (255,369), also form complexes with middle T antigen. PP2A binding appears to be required for middle T–src complex formation (134). Mutants of polyomavirus unable to produce middle T antigen are defective for replication, persistence, transformation, and tumor induction in mice (114).

Although formation of complexes with src family kinases and activation of PI-3 kinase signaling pathways are important events for cellular transformation, a role for middle T antigen during lytic infection is not known. Because polyoma large T antigen does not interact with p53, and because the interaction of SV40 large T with p53 is thought to be central to its ability to stimulate cellular S-phase entry and in turn create an optimal environment for viral DNA replication, an interesting possibility is that the polyoma middle T antigen–cellular protein interactions perform a related function for polyoma infection. In this view, middle T antigen plays the role of an activated growth factor receptor through its activation of src family members, PI-3 kinase, and other proteins that mediate signal transduction pathways leading to cell cycle progression and cell division.

Preparation for Viral DNA Replication and Modulation of Cellular Gene Expression

Soon after T antigen is detected within cells, the levels of many cellular enzymes increase (see Fig. 5), and this requires T antigen (reviewed in 351). This probably reflects the ability of T antigen to stimulate expression from a variety of viral and cellular promoters (131,145,274). Diverse mechanisms underlie the ability of T antigen to alter host cell gene expression. For example, when T antigen forms complexes with $p105^{Rb}$ and related proteins, other host cell proteins, including transcription factors of the E2F family, are released and can reach their promoter targets (6,7,48). Many genes involved in growth regulation and cell cycle progression, including *c-fos* and *c-myc*, contain E2F-binding sites.

The regulation of cellular genes is also affected by binding of p53 to T antigen because p53 is a transcription factor (9,184) with both activating (178,390) and repressing activities (132). Binding of p53 to DNA is inhibited by T antigen (10), abrogating the ability of p53 to stimulate gene expression. Among the targets of p53 transcriptional activation is *WAF1/CIP1,* a potent inhibitor of the cell cycle owing to its ability to block the kinase activity of some cyclin dependent kinase (CDK)–cyclin complexes. It is reasonable that *WAF1/CIP1* expression is reduced in the presence of T antigen because this stimulates cell cycle progression by permitting cyclin-dependent kinase–cyclin complexes to function (103,151,380).

Several studies indicate that T antigen can play the role of a TFIID-associated factor (TAF) in transcription initiation by all three RNA polymerases (73,74 and 75,393). T antigen forms a complex with TATA-box binding protein (TBP) and stabilizes the TBP–TFIIA complex on the promoter (74). Direct interactions also occur between T antigen and other cellular transcription factors. Some of these interactions activate, and others repress, transcription of various cellular

genes. T antigen also transactivates transcription by RNA polymerases I and III. Because TBP is involved in transcription by all RNA polymerases, the T antigen–TBP interaction may play a role in activation of multiple classes of genes. In addition, T antigen stimulates the phosphorylation of RNA polymerase I transcription factor, UBF, most likely by recruiting a kinase (392).

T antigen stimulates resting cells to enter the cell cycle and replicate their DNA and, by so doing, creates an optimal environment for viral DNA replication. Multiple distinct subregions of large T are independently able to stimulate cellular DNA replication and probably cooperate to provide maximal stimulation and to activate a broad spectrum of cellular genes (92,316,349). These regions are the Rb-binding domain, the DNA-binding domain, and sequences near the carboxyl terminus of large T (92). Polyoma middle T antigen was also able to stimulate cellular DNA replication. Mutants defective for binding to either SHC or PI-3 kinase were able to stimulate cellular replication, whereas a double mutant (Y250F/Y315F), unable to bind either, was not (245). In some cases, SV40 (226,263) and polyoma small t antigens (292) are required for the support of S-phase induction in concert with the respective T antigens, likely through their ability to stimulate growth factor–signaling pathways. However, in other cases, polyoma small t antigen alone can stimulate cell cycle progression, dependent on its ability to interact with PP2A (245). Because the middle T-antigen double mutant (Y250F/ Y315F) retains PP2A binding, yet cannot stimulate cellular DNA replication, middle T and small t appear to modulate cellular signaling differently when each binds PP2A.

Viral DNA Replication

Replication of viral DNA within infected cells requires a functional origin of DNA replication, large T antigen with its DNA binding and helicase activities intact, and most of cellular proteins involved in replicative DNA synthesis. Viral DNA is in the form of a minichromosome, and its replication occurs in the nucleus during S phase, like cellular DNA replication. By other parameters, including the enzymes used and the geometry of the replication fork, replication of SV40 and polyoma DNA is sufficiently similar to cellular DNA replication that the viral systems have been exploited as powerful tools to understand cellular DNA replication. Excellent *in vitro* systems have been developed (208,330,368,377) that permit accurate and efficient replication of viral DNA using 10 purified cellular proteins, viral DNA containing a functional origin, and purified large T antigen. Viral DNA replication differs from cellular DNA replication in that the SV40 DNA origin can fire multiple times during S phase, whereas cellular DNA replication is tightly controlled to prevent any region of the genome from being replicated more than once in a single cell cycle. This difference reflects the use of the cellular origin-recognition complex (ORC) at cellular origins. T antigen performs ORC's origin-recognition function and is not subject to the cellular mechanism that prevents additional rounds of DNA replication within one cell cycle.

Viral Origin of DNA Replication

The region of DNA required in cis for initiation of viral DNA replication (ORI) has been defined genetically for each of the polyomaviruses by the construction and analysis of detailed sets of deletion and point substitution mutations and by the analysis of evolutionary variants of these viruses, which carry deletions of sequences not required for replication (reviewed in 85 and 86). These studies indicate that both SV40 and polyoma contain a core of sequences absolutely required for viral DNA replication and that auxiliary sequences on either side of the core act to enhance initiation of DNA replication within the viral core (see Fig. 3). For SV40, the core origin of 64 base pairs extends from nucleotides 5209 to 29, whereas the auxiliary sequences extend from nucleotides 5164 to 5208 and 30 to 72.

The major difference between SV40 and polyoma in the organization of their ORI regions is that polyoma replication requires promoter and enhancer sequences on the late side of the ORI core, whereas SV40 replication does not. Critical sequences, shown in Figure 3, include the core, auxiliary sequences on the early side of ORI (aux-1), and enhancer sequences. Replication of DNA-containing polyoma sequences requires core and either aux-2a or aux-2b. The polyoma core covers 66 base pairs. Although quite different in sequence, the auxiliary sequences of polyoma can substitute for those of SV40, and the SV40 72–base pair enhancer can be used in place of the polyoma a and b enhancers. The SV40 21–base pair repeats, which play a role as auxiliary origin elements, cannot substitute for the polyoma enhancer elements. Replication of SV40 in monkey cells and polyomavirus in mouse cells absolutely requires the presence of the appropriate core and large T antigen (19). Interestingly, the SV40 core ORI contains start sites for early mRNA, whereas polyoma early mRNAs are initiated at sites that lie outside of the polyoma core ORI.

The features shared by all polyomaviridae ORI regions are an inverted repeat of 14 base pairs on the early gene side of ORI (see *PU/PY* in Fig. 3), a GC-rich palindrome of 23 to 34 base pairs in the center (see *ORE* in Fig. 3), and an AT sequence of 15 to 20 base pairs on the late side (see *TA* in Fig. 3). That all polyomavirus core origins contain these elements suggests that common structures play a role in binding proteins and in initiation of viral DNA replication. SV40, BKV, and JCV contain a second nearby palindrome of differing size, but it is not required for viral DNA replication and is absent from the polyoma genome.

Initiation of Viral DNA Replication

In the absence of ATP, T antigen binds as a tetramer to binding site II in the origin region and protects about 35 base pairs of DNA from DNAse (278). In the presence of ATP, T antigen undergoes a conformational shift, permitting the assembly of a bi-lobed double hexamer of T antigen at the origin of DNA replication (27,79,80,161,225). Although a single monomer of T antigen can bind to a GAGGC pentanucleotide (see *dark arrowheads* in Fig. 3), clearly not all 12 molecules of T antigen will be bound to a pentanucleotide in the assembled dodecamer–DNA structure. Some of the T antigen subunits can be thought of as playing an allosteric role, modifying the conformation of other subunits to permit proper binding and assembly of the double hexamer (29). Studies of T-antigen mutants indicate that each of the two hexamers interacts with the other. ATP hydrolysis is not required for the assembly of this structure on the DNA.

A variety of physical and chemical analyses indicate that the bound T-antigen double hexamer catalyzes the local unwinding of part of the early palindrome (see *PU/PY* in Fig. 3) and the distortion or untwisting of the TA element (27,299). Once this initial unwinding has occurred, T antigen no longer binds specifically to viral DNA because the ORI

region is no longer double stranded (3). T antigen then associates with RPA, a three-subunit single-stranded DNA-binding protein that keeps unwound regions single stranded, and this permits more extensive unwinding of the DNA using the helicase activity of T antigen (78,378). In its ability to unwind double-stranded DNA from an internal site, T antigen is unusual among DNA helicases (227,321).

The association of the viral ORI, the T-antigen double hexamer, and RPA, is referred to as the *preinitiation complex.* Recruitment of DNA polymerase a-primase into this complex converts it to an initiation complex. It is this step in viral DNA replication that defines the very limited host range for members of the *Polyomaviridae* family. SV40 DNA can be replicated in extracts prepared from murine cells if these are supplemented with DNA polymerase a-primase from monkey cells (247,320). Specific protein contacts form between T antigen, RPA, and DNA polymerase a-primase (62,93,94,121,307), and the nature of these interactions at the viral origin of replication limits productive complex formation with SV40 and polyomavirus T antigens to primate and murine DNA polymerase a-primases, respectively (290). Polymerase a-primase synthesizes short RNA primers, which it then extends into short DNA fragments (38,155,228).

Processive extension of DNA in eukaryotes requires three factors to coordinate their actions. First, DNA polymerase a-primase synthesizes the primed site. Then, replication factor C loads a proliferating cell nuclear antigen (PCNA) clamp onto the primer. Following this, DNA polymerase d assembles with PCNA for processive extension. These proteins each bind the primed site tightly and trade places in a highly coordinated fashion such that the primer terminus is never left free of protein. RPA, the single-stranded DNA-binding protein, forms a common touchpoint for each of these proteins, and they compete with one another for it. Thus, these protein exchanges are driven by competition-based protein switches in which two proteins vie for contact with RPA.

DNA Synthesis at the Replication Fork

After initiation, DNA synthesis moves bidirectionally away from the ORI. Replication fork movement is facilitated by the helicase activity of T antigen, which translocates along the DNA. The cellular replication factor C (RFC) binds the 3' end of elongating DNA strands, displaces polymerase a-primase, and coordinates the switch to DNA polymerase d. RFC, PCNA, and DNA polymerase d can each interact with RFA bound to the growing strand. Multiple subunits of RFC coordinate the assembly of the PCNA sliding clamp and then remain associated with the complex through an RFC–RPA interaction. DNA polymerase d interacts with this complex through its ability to bind to RPA and actually competes with RFC for RPA binding (388). RFC is displaced from the 3' end of the growing strand but remains associated with this complex, which synthesizes both the continuous leading strand (359,360) and the Okazaki fragments on the lagging strand (368). The actions of RNase H, a 5'to 3'; exonuclease (MFI), and DNA ligase I are required for removal of primers and ligation of Okazaki fragments, yielding covalently closed circular form I DNA (367).

Termination of Replication and Separation of Daughter Molecules

Replicative intermediates have been isolated by sucrose gradient centrifugation and analyzed biochemically, electrophoretically, and electron microscopically. Preparations of replicative intermediates are enriched in molecules that are almost completely replicated. Replication moves bidirectionally around the circular chromosome, with both replication forks advancing at about the same rate. These replication intermediates contain three DNA loops—two daughter loops and the unreplicated parental DNA. Molecules that are 85% to 95% complete are enriched in replicative intermediate pools, suggesting that separation of daughter molecules is a slow step in DNA replication (336,337,341). Topoisomerase II is required for this separation (384). There is no specific termination site for viral DNA replication. Deletion analysis indicates that termination can occur within any sequence located about 180 degrees from the origin.

Regulation of Viral DNA Replication by Phosphorylation

The activity of T antigen in viral DNA replication is regulated by phosphorylation of the protein. Both SV40 and polyoma large T antigens carry phosphorylation sites near their DNA-binding domains in the amino-terminal half of each protein. An underphosphorylated population of T antigen isolated from infected cells, or T antigen that is dephosphorylated enzymatically, is more active in DNA binding and viral DNA replication *in vitro* (240). Although phosphorylation of threonine-124 of SV40 T antigen is essential for unwinding of viral origin DNA and replication (239,373), dephosphorylation (240,365,366) or substitution (291) of adjacent serine residues enhances origin unwinding and replication. Interestingly, the phosphorylation site at threonine-124 is a substrate for the cyclin-dependent kinases (233), whereas protein phosphatase 2A is active in dephosphorylation of the nearby serines, whose phosphorylation inhibits viral DNA replication *in vitro* (286). This suggests that the replication activity of T antigen may be regulated during the cell cycle so that T is maximally active for replication during S phase and inactive for replication at earlier stages of the viral infection.

Viral Late Gene Expression

The onset of viral DNA replication brings about a shift in the pattern of viral transcription. The start sites for production of the viral early mRNAs shift to upstream positions (see *LE* in Fig. 3A), and there is a dramatic increase in transcription to produce the viral late mRNAs. Early after infection, late transcription is repressed owing to occupancy of the late promoter by a steroid receptor family heterodimer between RXRa and TRa1, a thyroid hormone receptor (376,400,401).

Infected cells contain up to 200,000 molecules of viral DNA, and more than half of this may be encapsidated into progeny virions. With 360 molecules of VP1 per virus particle, a minimum of 3.6×10^{8} molecules of VP1 must be produced. Assuming a normal loading of ribosomes on viral late mRNA and a normal rate of translation to produce VP1, each molecule of mRNA encoding VP1 could generate 5,000 to 10,000 molecules of VP1 in an infected cell. Thus, more than 30,000 molecules of message encoding VP1 are required. This is several hundred times greater than the abundance of viral early mRNA, making studies of the synthesis and structure of viral late mRNAs relatively easy.

Late transcription of SV40 and polyoma has been studied in the greatest detail. In both systems, two sets of late mRNAs

are produced, originally referred to as 16S and 19S late RNAs based on velocity sedimentation in sucrose gradients. The 16S late mRNAs are considerably more abundant than the 19S late mRNAs. These late mRNAs are all derived by transcription, which begins near the origin and extends around the opposite strand from that transcribed to produce the early mRNAs (see Fig. 2). The start sites for SV40 late mRNAs are heterogeneous and map to many positions (see Fig. 3A) between nucleotides 120 and 482, although the start at nucleotide 325 is the most abundant (128,129,270). All viral late 16S mRNAs contain a leader sequence spliced to a second exon that extends from nucleotide 1464 to a polyadenylation site at nucleotide 2674 (see Fig. 2A). All lack sequences between 527 and 1464. Thus, the 16S late mRNAs differ in the structure of their leader regions, with some containing an additional splice, and a rare group containing a duplication of sequences in the leader region. The 19S late mRNAs are also heterogeneous at both their 5' ends and in the structure of their leader regions. A minor class is unspliced. All of the others contain a leader spliced to sequences extending from nucleotide 558 to the same polyadenylation site used for 16S late mRNAs at nucleotide 2674. All SV40 late mRNAs are derived by processing of precursors that begin at heterogeneous initiation sites and extend most of the way around the viral genome.

Polyomavirus late transcription differs from SV40 in that most viral late pre-mRNAs are extremely large, resulting from transcription completely around the viral genome multiple times (1,206). The mRNAs produced from these giant transcripts contain a single copy of the coding region for late proteins spliced to multiple copies of the leader region by leader-to-leader splicing (172). As with SV40, the start sites for late transcription are heterogeneous (see Fig. 3B); they extend from nucleotides 5075 to 5170, with about 90% of them lying in the region from nucleotides 5077 to 5101 (69,70,110,355). The late promoters of SV40 and polyoma lack TATA boxes, which likely explains the heterogeneity of initiation sites for viral late transcription. Maximal rates of SV40 late transcription depend on sequences within the 21–base pair GC repeats as well as within the 72–base pair enhancers (180,230).

At least three mechanisms are involved in the production of high levels of the viral late mRNAs: amplification of templates for late transcription by viral DNA replication, relief of repression of the viral late promoter, and T-antigen–mediated activation of transcription from the viral late promoter. Studies with origin-defective viral DNA molecules indicate that genome amplification is not required for activation of the late promoter by large T antigen (31,179). The region of large T antigen involved in activation of the late promoter maps to the amino-terminal half of the protein (395). Transcriptional activation of the late promoter by T antigen requires sequences within the 72–base pair enhancer region, including a unique sequence element located at the junction of the two 72–base pair repeats (144). One factor able to bind to these sequences is TEF-1, which is able to form a complex with large T antigen (145,182). Interactions between T antigen and TBP likely also contribute to activation of the late promoter. One mechanism of T-antigen stimulation of transcription involves T antigen functioning as a TAF. In fact, T antigen can complement the ts defect in TAF_{250} in the ts13 cells (73), a baby hamster kidney with a cell cycle defect (340). As a TAF, T antigen acts to stabilize the complex between TBP and TFIIA (74). T antigen appears to be a promiscuous activator, enhancing transcription of both viral and cellular genes in multiple ways. The structure of replicated minichromosomes likely also plays a key role in activation of late transcription (339). Although transcription factors are thought to stimulate DNA replication, complex formation between T antigen and TBP (and perhaps also with other transcription factors) actually inhibits DNA replication *in vitro,* most likely by occupying a binding surface of T antigen required for viral DNA replication (159). SV40 T antigen also interacts with TAF_{110} (232). Overall, T antigen interacts with many cellular proteins as well as with DNA and possibly RNA. This suggests that competition for T-antigen binding can be used to regulate the course of infection.

Synthesis of Late Proteins and Assembly and Release of Progeny Virions

Within the cytoplasm of infected cells, viral 16S late mRNAs are translated to produce VP1, whereas viral 19S late mRNAs are translated to produce VP2 and VP3. Because the sequence of VP3 is contained entirely within that of VP2, VP3 could be derived from VP2 by proteolytic processing. However, deletion mutants lacking the initiation codon for VP2 still produce VP3, indicating that VP3 can be synthesized independently of synthesis of VP2 (59). Because all of the SV40 late mRNAs are polycistronic and contain open reading frames upstream from the capsid protein genes, leaky scanning permits synthesis of capsid proteins from these mRNAs. For some late mRNAs, the upstream ORF encodes the agnoprotein. Studies of translation of SV40 late mRNAs (294,295 and 296) have dramatically increased our understanding of how leaky scanning permits internal AUGs to function efficiently as initiation codons (194,195).

Virion proteins VP1, VP2 and VP3 are synthesized in the cytoplasm of infected cells and transported to the nucleus for assembly into virions. When all three capsid proteins are produced, all three enter the nucleus efficiently. Detailed studies of these proteins indicate that sequences near the amino terminus of VP1 and near the carboxyl termini of VP2 and VP3 are essential for this nuclear accumulation. Within the N-terminus of both SV40 and polyoma VP1 are nuclear localization signals (46,243,379). Sequences required for localization of VP2 and VP3 to the nucleus have also been identified. VP1 is able to cotransport VP2 or VP3, and VP2 or VP3 can cotransport VP1, indicating that these proteins may be imported as a complex as well as separately (13,84,112,163,177). Within the nucleus, these proteins are found in the same ratio as within the viral capsid (212).

Viral chromatin is not required for the assembly of capsids or capsid-like structures. Purified polyoma VP1 self-assembles into pentamers and capsid-like structures (126,280), whereas coexpression of polyoma or SV40 VP1, VP2, and VP3 in insect cells from baculovirus vectors results in the assembly of capsid-like structures containing all three capsid proteins in about the same relative ratio as is found in mature virions (112,283). Sequences within the regulatory region of SV40 have also been shown to facilitate encapsidation (72).

The precise mechanism of encapsidation in polyoma-infected cells remains uncertain. Available data are consistent with two possibilities. Empty capsids can be purified from infected cells and could be precursors to virions. Alternatively, capsomers may condense on viral chromatin to assemble a virus capsid (387). The 75S to 90S chromatin structures containing viral DNA are detected, as are 200S previrions and 240S virions. Although intracellular chromatin contains histone H1, virion chromatin does not, but the precise time or mechanism of removal of histone H1 is not known. Analysis of immunoprecipitates of insect cells expressing one or more polyomavirus capsid proteins indicates that cellular histones associate with capsid proteins and suggest that VP2 or VP3 may be the direct mediator of capsid

protein–histone interactions (112). Polyoma VP1 and SV40 VP2 and VP3 have been shown to bind to DNA in a sequence-independent manner (45,54). The importance of these interactions for virion assembly or stability is uncertain because capsid protein–histone interactions could serve the same roles in assembly or stability as would capsid protein–DNA interactions.

SV40 and other polyomaviruses do not encode a lysozyme that causes cell lysis. It was previously suggested that exit of SV40 virions from infected cells occurs when cells disintegrate or rupture as part of their dying. However, studies of virus-infected epithelial cells lines indicated that SV40 can be released from the cell surface, primarily from the apical surface of polarized epithelial cells and uniformly from the surface of nonpolarized epithelial cells (53). By electron microscopy, SV40 was seen in cytoplasmic smooth membrane vesicles, and release of SV40 was inhibited by monensin, an inhibitor of intracellular vesicular transport. How and where these vesicles form and the fraction of total infectious progeny released in this manner or by leakage from dying cells are not known.

The Host Range and Helper Function of SV40 Large T Antigens

Most monkey cell lines infected by human adenoviruses produce low yields of viral progeny, with the block to productive infection occurring very late in the adenovirus infection cycle (reviewed in 191 and 351). This block can be overcome by the extreme carboxyl terminus of SV40 large T antigen (58,262), which is unrelated to any portions of polyoma large T antigen. Mutants of SV40 whose T antigens lack a normal carboxyl terminus are defective in this helper function and also show a restricted growth range among different African green monkey kidney cell lines (60,61,222,259). Human adenoviruses and host range mutants of SV40 both grow well in some monkey kidney cells lines (e.g., Vero) and poorly in others (e.g., CV-1). This activity of large T antigen is referred to as the *host range and helper function* (hr/hf). It is a distinct and separable activity of large T because a VP1 fusion protein containing the normal carboxyl terminus of large T can provide hr/hf activity (353).

Mutants lacking the hr/hf domain of large T show defects that affect late gene expression. The levels of viral late mRNA and capsid protein are reduced to about one third of their normal levels, but virion production is reduced much more dramatically (319). Agnoprotein is not produced (187,319) because the 5' ends of late viral mRNAs in mutant-infected cells map to normally used minor sites downstream of the agnoprotein initiation codon (319). Provision of agnoprotein in trans permits hr/hf mutants to form plaques but does not increase late mRNA or VP1 production. This suggests that agnoprotein permits more efficient use of the capsid proteins available in mutant-infected cells. This is consistent with studies that suggested a role for the agnoprotein either in transport of VP1 to the nucleus or in assembly of progeny virions (12,43,273).

Evolutionary Variants and Defective Viral Genomes

As with most animal viruses, passage of polyomaviruses at high multiplicities of infection leads to the generation of defective viral genomes and the accumulation of those that have selective growth advantages. These evolutionary variants contain deletions, inversions, and rearrangements, and they often contain multiple origins of DNA replication (140,203). The only sequence retained by all defective viruses is the viral origin region, indicating that it is the only cis-acting sequence required for propagation of the origin-containing DNA. This has led to the development of many eukaryotic expression vectors containing an SV40 origin of replication. These molecules will replicate in monkey kidney cells that provide the SV40 T antigen constitutively (e.g., Cos cells) owing to the integration into the cellular genome of an origin-defective SV40 genome encoding wild-type T antigen.

Those variants with more than one origin replicate more efficiently than wild-type virus and become enriched. Defective viruses require the presence of wild-type virus for their propagation because they often contain no intact viral genes, but nondefective variants have been constructed that contain two functional origins (220). Virus stocks maintained by high multiplicity passage can become contaminated with these evolutionary variants, but this can be prevented by frequent reisolation of wild-type virus through plaque purification.

TRANSFORMATION AND IMMORTALIZATION BY SV40

The polyomaviruses are also known as small DNA tumor viruses. The ability of these viruses to transform established cell lines, to immortalize primary cell cultures, and to induce tumors in animals resulted in the widespread study of these viruses as a window into understanding the molecular basis of oncogenesis and growth regulation. Transformed cells grow readily into tumors when injected into syngeneic or immunocompromised hosts. Transgenic expression of polyomavirus antigens has provided a useful model for studying the development of cell type–specific tumors.

Early studies indicated that genetic information in the viral early region was necessary and sufficient for transformation (reviewed in 116 and 351). One of the first experiments performed after the development of Southern hybridization was an analysis of the patterns of integration of viral DNA in SV40 transformed cells. Although these studies demonstrated that there were no unique or preferred sites of integration in either the viral or host genomes, a functional viral early region were present in all transformed lines analyzed (28,185,281). For SV40, studies using tsA mutants readily demonstrated that T antigen was required for both the initiation and maintenance of the transformed state (35,223,342).

For polyoma, large T antigen is required for transformation (89,99,115), but subsequent studies indicated that T-antigen expression is needed only for initiation but not for the maintenance of the transformed state. The viral DNA in transformed cells is usually integrated to interrupt the early region, blocking continued expression of large T antigen but permitting continued synthesis of both small and middle T antigens (15,201). Studies of polyoma hrt mutants (18), which encode a wild-type large T antigen, demonstrated that other early region gene products besides large T antigen were required for transformation by polyomavirus (100,111). It is now known that a cyclic DNA encoding just middle T antigen is sufficient to transform established cell lines (265,356). The requirement for polyoma large T antigen to initiate transformation after viral infection may reflect modest amplification of the genome by replication. Subsequent integration of the viral DNA often interrupts the large T-antigen–coding region, thereby eliminating the replicative activities of

polyoma large T antigen while leaving the middle T-antigen–coding region intact. Levels of middle T antigen sufficient for transformation may be achieved by transfection, thus eliminating the need for large T antigen. In transformation, polyomavirus middle T antigen activity requires association with the plasma membrane. Here, interaction with many of the same cellular signal transduction proteins mediating growth factor receptor signaling leads to constitutive activation of the cell cycle. Although the interaction between middle T and pp60c-src cannot be essential because middle T antigen can transform cells that contain a disruption of the *c-src* gene, middle T continues to interact with other src family kinases and PI-3 kinase in cells lacking *c-src* (346).

Mutational analyses of transformation by SV40 large T antigen demonstrate that only the amino-terminal portion of large T (amino acids 1 to 121) is required to transform mouse C3H 10T1/2 cells, whereas sequences extending over much of large T antigen are required to transform REF-52 rat embryo fibroblasts and many other established rodent cell lines (318). Transformation by T antigen can be separated from its role in viral DNA replication (136,171) and does not require any of the activities involved in viral DNA replication (e.g., DNA binding, ATP binding, ATPase activity, helicase activity) or host range and helper function. SV40 mutants defective in transformation of REF-52 cells map to three regions of large T antigen: the amino terminus containing the conserved DNA J domain (amino acids 1 to 82), the domain that binds $p105^{Rb}$ and the associated proteins p107 and p130 (amino acids 102 to 114), and the p53/p300 binding domain of the carboxyl half (49,171,396). SV40 small t antigen is clearly required for transformation under many conditions (301,306), but sufficiently high levels of large T antigen (24), growth conditions of the infected cells (298), or contributions to transformation from the cellular genetic background probably account for the lack of a uniform requirement for small t antigen for transformation by SV40. Under circumstances in which SV40 small t antigen is required for transformation, mutants of small t defective for interaction with and inhibition of PP2A are defective for transformation, suggesting that this interaction is central to the effect of small t antigen on cellular growth properties (246).

The requirement for the SV40 large T antigen amino terminus in cellular transformation reflects its DNA J domain activity as a molecular chaperone whereby it disrupts DNA-binding complexes between Rb family proteins and E2F (389). Mutants that express only amino acids 83 to 708 are defective for transformation, as are several deletion, insertion, and point mutations within the first 82 amino acids. Mutants of SV40 that express a T antigen lacking residues 1 to 82 can be complemented for transformation by SV40 small t antigen (241), consistent with the small t-antigen J domain functioning as a molecular chaperone (317). Results of experiments using double knockout mouse embryo fibroblasty (MEFs) (p107–/– and p130–/–) have revealed that the J domain is dispensable for transformation of these cells. This implies that the J domain plays an essential role in SV40 transformation by inactivating p107 and p130 functions (81), even though these proteins do not appear to have tumor suppressor activity.

Binding of T antigen to $p105^{Rb}$, an important tumor suppressor gene product, and the related cellular proteins p107 and p130, contributes to the disruption of the function of these cellular proteins (389). A clue to the mechanism of $p105^{Rb}$ function in cellular growth control suppression is its interaction with the E2F family of transcription factors (6,47). E2F family members bind as heterodimers to sequence-specific sites present in the promoter-enhancer regions of genes known to have important roles in cellular growth control, including *c-myc* and *c-fos* (158). Different E2F family members are thought to associate with different $p105^{Rb}$-related proteins at unique points in the cell cycle (98,205). For example, in G_0 phase, hypophosphorylated $p105^{Rb}$ interacts with E2F, preventing E2F from interacting with target genes (47,157). By binding to the hypophosphorylated form of $p105^{Rb}$, T antigen causes the release of E2F, and this activates E2F-regulated genes, leading to cell cycle progression (48). The related proteins, p107 and p130, complex with different members of the E2F family. The p130 complexes are found predominantly at the G_0–G_1 phase border (56), whereas the p107 complexes are most abundant at the G_1–S phase boundary (98). The concomitant binding of T antigen to p107 and p130 and the amino terminal J domain perturbation of their phosphorylation state result in the release of free and active E2F. Subsequently, genes encoding proteins required for the initiation of DNA synthesis are activated by E2F-dependent mechanisms.

Yet another $p105^{Rb}$, p107, and p130 (“pocket proteins”) mechanism for cell cycle control was identified by the discovery that these proteins, in association with E2Fs, can bind to the cyclins and cdks. The association of pocket protein–E2F complexes with cyclin/cdks appears to be cell cycle regulated and specific for particular cyclin/cdks complexes (42,56,87,204,302). Expression of SV40 large T antigen inhibits the activation of cyclins by cdks, thereby interfering with multiples stages of cell cycle progression (193).

SV40 large T antigen also binds p300 and CBP (5,101) through a common region of T antigen that is required for binding to the tumor suppressor protein p53 (211). The coactivators p300 and CBP form a transcriptional activation complex and can function to promote histone acetylation; binding of p300 and CBP to T antigen disrupts p300-dependent growth control and is likely to contribute to T antigen–induced cell transformation (210). The viral oncoprotein E1A has been shown to repress the normal functions of p300 and CBP by both displacing them from transcriptional coactivating complexes and directly repressing their histone acetyltransferase activity (44). This repression, in turn, leads to p53 deacetylation, thereby disrupting p53 tumor suppressor activities. Whether SV40 large T antigen uses a similar mechanism for direct inhibition of p300 and CBP family histone acetyltransferase activity is not yet known; however, the ability of T antigen to bind directly to p53 (199) is yet another important mechanism through which SV40 can disrupt an important cellular growth checkpoint control protein.

Transgenic mice have been used to study SV40-induced oncogenesis in a broad range of tissues, including mammary gland, salivary gland, pancreas, prostate, liver, lung, kidney, intestine, brain, choroid plexus, lens of the eye, bone, smooth muscle, and cartilage (reviewed in 120). Interestingly, transgenic experiments have suggested that the p300- and p53-binding region may not be required for tumor cell formation in animals. For example, hepatic tumors can be induced by the N-terminal fragment of T antigen (amino acids 1 to 121) encoded by dl1137. This truncated protein retains the ability to bind and inhibit pRb function but cannot bind p53. Surprisingly, these tumors grow significantly faster than those induced by wild-type T antigen (20).

The ability to immortalize primary rodent cells is another property of the large T antigens of polyoma and SV40 that has also been studied in detail. Analysis of the domains of polyoma and SV40 large T antigens required for immortalization

have also been performed, but with conflicting results. Some studies demonstrated that the ability of SV40 large T antigen to bind p53 is required for immortalization of primary mouse cells (347,394), whereas others indicated that the amino-terminal 147 amino acids of large T can immortalize primary mouse or rat cells (312,313). Studies with a ts mutant of SV40 T antigen indicated, however, that T antigen is required continuously to maintain immortalization because cultures shifted to the nonpermissive temperature ceased proliferation (166).

Critical to evaluation of the immortalization potential of these viruses and their individual T antigens are the assays used to measure immortalization. If immortalization of primary cultures requires multiple genetic or epigenetic alterations (e.g., the formation of a complex between SV40 T antigen and p53), mutant viral T antigens may provide some or most of the required functions, and a single additional cellular mutation could result in the full spectrum of genetic changes leading to immortalization. In some assays, no attempts were made to distinguish between extension of normal lifespan, with eventual senescence, and true immortalization. In other assays, populations of cells still proliferating at a time when control cells had senesced were serially subcultured and considered to be immortal if the culture survived for several months. However, the probability is high that cellular mutations will occur that contribute to immortalization of cells expressing mutant T antigens, particularly if mutation of one cellular gene in one cell in the population is sufficient to cause its immortalization and eventual predominance in the culture.

There is solid evidence that mutation of p53 plays a central role in immortalization. Mutation of the p53 gene was seen in all spontaneously immortalized mouse embryo fibroblast cell lines examined (154). It is easy to see that mutation of p53 or its functional inactivation by complex formation with SV40 large T antigen could increase the frequency of cellular mutations because p53 loss of function is known to be associated with gene amplification and genomic instability (214,386). In fact, inactivation of p53 by complex formation with SV40 T antigen is likely to be at least partially responsible for the known genomic instability associated with expression of SV40 T antigen (268,328).

Careful analysis of immortalization by mutant SV40 large T antigens suggests that all three domains required for transformation (J domain, binding region for pRb family proteins, and binding region for p53) are required for immortalization of primary mouse embryo fibroblasts. Substantial lifespan extension was seen in cultures expressing mutant T antigens defective for the amino-terminal transformation function or unable to bind p105 ^{Rb}, but a minority of colonies expressing these mutant T antigens became immortalized after continued subculturing. In contrast, neither lifespan extension nor immortalization was achieved with mutants of T antigen unable to bind p53 and p300. Similar results were shown using amino-terminal (amino acids T1 to T147) or carboxyl-terminal (T251 to T708) fragments of large T antigen: these two regions of T antigen appear to function in the same way as in wild-type T antigen to extend cell lifespan, and together they cooperate to immortalize primary B6MEF cells (345). The fact that immortalization of human cells by SV40 is such an exceedingly rare event may reflect a differential requirement for telomerase activity in human versus rodent cell immortalization because recent evidence demonstrates that introduction of the telomerase catalytic subunit hTRT allows human cells expressing SV40 large T antigen to escape crisis and divide indefinitely (148,397).

Fewer studies have been performed to understand immortalization by polyoma large T antigen, which does not bind p53. An essential role in immortalization for the p105 ^{Rb}-binding activity of polyoma large T antigen has been demonstrated (113,202), and it is likely that additional activities are also required.

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CHAPTER 64 Human Polyomavirus

Eugene O. Major

HISTORY

Isolation of the BK virus (BKV) and JC virus (JCV) human polyomaviruses was first described in companion articles in 1971 (61,122) using laboratory cultivation of BKV in African green monkey kidney cells and JCV in human fetal brain cells. BKV was derived from the urine of a renal allograft recipient, whereas JCV was derived from brain tissue of a Hodgkin's lymphoma patient with the demyelinating disease progressive multifocal leukoencephalopathy (PML). The viruses were named from the initials of these patients and later classified in the family of polyomaviruses based on viral particle size, icosahedral architecture (Fig. 1A), and genome structure of its DNA (see Chapter 63). Recently, the International Committee on Taxonomy of Viruses has recognized that viruses with these and similar characteristics form an independent virus family, *Polyomaviridae,* no longer using the previous larger family designation of *Papovaviridae,* which included the papillomaviruses. Although many genomic variants of both BKV and JCV have been reported since then, there have been no new human polyomaviruses isolated. Before its isolation from urine, BKV was not a suspected pathogen in renal transplant recipients. However, a virus was suspected in the etiology of PML as early as 1958. In the initial description of PML, inclusion bodies were seen in damaged oligodendrocytes as part of the pathology of demyelination (133). In 1969, electron micrographs from PML brain tissue showed a crystalline array of assembled viral particles in the nucleus of the infected oligodendrocyte that were structurally identical to polyoma virions (169), as represented in Figure 1B. Since that time, JCV has consistently been found in brain tissue as well as the cerebrospinal fluid (CSF) of PML patients, which has firmly established JCV as the etiologic agent of PML. Although routinely found in urine of renal allograft recipients and patients with other kidney diseases (10), BKV, as well as JCV, is also found in the urine of normal, healthy individuals. To date, there is no direct correlation between BKV infection and renal or other human diseases. However, BKV infection has been associated with hemorrhagic cystitis in bone marrow transplant recipients, ureteric stenosis in renal transplant recipients that could lead to kidney failure which BK himself demonstrated, and pulmonary infection.

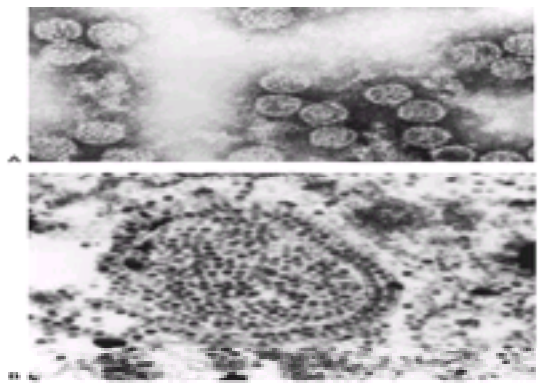

FIG. 1. Electron micrographs of human polyomaviruses. **A:** Negative stain of BK virus in the urine, concentrated by ultracentrifugation. **B:** Transmission electron micrograph of JC virus particles assembled in the nucleus of an infected oligodendrocyte from the brain of a patient with progressive multifocal leukoencephalopathy (×100,000).

Both viruses are associated with diseases affecting immune compromised hosts, suggesting that the human polyomaviruses remain latent in tissues that can be reactivated during times of immune suppression. Because of their presence in urine, BKV and JCV were thought to reside in the kidney as the site of latency (125). More recent work has implicated lymphoid tissues as well as a probable site for latent virus (80). Shortly after their isolation, these viruses were

found to agglutinate erythrocytes similar to the mouse polyomavirus but unlike the simian SV40. This observation helped develop a method to quantitate virus grown in cell cultures for use in seroepidemiologic studies. Such studies demonstrated that nearly 80% of the human population worldwide possessed antibodies to BKV and JCV. Antibodies arise early in life, at juvenile ages, to either or both viruses independently and remain elevated throughout life (65,131). It is still unknown whether the continual presence of antibodies to BKV and JCV results from new or reactivated infection or to what extent circulating antibodies neutralize infection. There is little understanding of the cellular immune response to either virus, mostly because of the immune-suppressed background of individuals in whom these viruses are mostly studied (46,165).

As members of the *Polyomaviridae* family, as shown in Table 1, both viruses were tested for their ability to induce transformation of cells in culture and tumors in experimental animal models. BKV and JCV were able to induce various histotypes of malignant tumors rapidly in rodents depending on route of administration (15). JCV was also shown to cause malignant brain tumors in New World monkeys after long incubation times following intracranial inoculation (102). Considering these results and because of the widespread nature of BKV and JCV infection in the population, there remains a concern about the possible role these viruses may have in initiating oncogenic events in human hosts.

Host	Virus	Characteristics
Human	BK virus (BKV)	Common infection of childhood; persists in kidney epithelium and possibly lymphocytes
	JC virus (JCV)	Common infection of childhood; persists in kidney epithelium, lymphocytes, and bone marrow; produces PML in immunocompromised hosts
Monkey*	Simian virus 40 (SV40)	Natural infection of macaques in Asia; persists in kidney; produces PML-like disease in immunocompromised macaques
	Simian agent 12 (SA 12)	Natural infection of baboons in Africa
	Lymphotropic papovavirus (LPV)	Virus of African green monkey; multiplies in B lymphoblasts
Cattle	Bovine polyoma virus (BPyV)	Common infection of cattle; probably persists in kidney
Rabbit	Rabbit kidney vacuolating virus (RKV)	Infection of cottontail rabbits
Mouse	Mouse polyoma virus	Natural infection of mice in wild; may infect laboratory mouse colonies; persists in kidney
	K virus	Natural infection of mice; infects lung endothelium
Hamster	Hamster papovavirus (HaPV)	Produces cutaneous tumors in hamsters
Athymic rat	Rat polyomavirus	Affects parotid gland
Parakeet	Budgerigar fledgling disease virus (BFDV)	Produces acute fatal illness in fledgling budgerigars

*A virus initially described as originating from squirrel monkeys was subsequently identified as bovine polyomavirus.
PML, progressive multifocal leukoencephalopathy.

TABLE 1. *Host of origin and characteristics of polyomavirus infections*

In the early investigations of BKV and JCV, it was clear that the host range of these viruses reflected the human tissues from their first isolation. BKV multiplies best in kidney epithelial cells of primate origin, whereas JCV multiplies best in glial cells derived from human brain. However, these cell types are not the only cells that support virus multiplication. The current list of cells susceptible to BKV or JCV infection now includes lymphocytes, stromal cells, and numerous cell lines (82). In the three decades since their isolation, experimental emphasis has been on understanding the cellular and molecular mechanisms that control viral host range. Such studies have helped clarify the pathogenesis of virus infection, particularly for JCV and PML, which may lead to the development of new diagnostic tests and therapies against polyomavirus infection.

INFECTIOUS AGENTS

Polyomaviruses are similar in physical virion particle structure and general genome organization. These properties have been discussed in more detail in Chapter 63 and thus are only briefly reviewed here. BKV and JCV particles are about 40 nm icosahedrons, containing a double-stranded, supercoiled DNA of about 5.13 kb. Three virion capsid proteins make up the particle, although the largest capsid protein, VP1, is responsible for virion structure and can self-assemble into icosahedral particles without either VP2 or VP3 (32). The viral genome is organized into coding sequences of about 2.4 kb, which are transcribed before DNA replication, termed *early,* from one strand initiating from the near the DNA replication origin; whereas the *late* coding sequences are transcribed after DNA replication begins from the opposite strand and in the opposite direction (Fig. 2). Both early and late coding regions give rise to multiple proteins from differential splicing events similar to SV40 (see Fig. 2). Early transcripts encode the viral large T and small t proteins, which function similar to the SV40 early proteins (see Chapter 63). There are also several minor transcripts made from the early region sequences that use alternate splicing sites at the carboxyl-terminal end, but no exact functions are known for these proteins (159). Table 2 lists the viral proteins for comparison and the length of their transcripts, mapped to the genomes for both BKV and JCV.

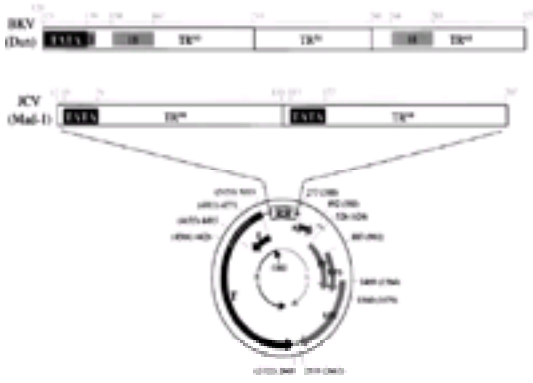

FIG. 2. Graphic representation of the genome organization of the prototype strains of JC virus Mad-1 (55) and BK virus Dunlop (138) human polyomaviruses. Expanded region depicts the regulatory region (*RR*) sequences. Nucleotide numbering has been adopted from references 55 and 138. *Open boxes* represent tandem repeat sequences (TR#) with inclusive base pair length designated by *superscript.* Tandem repeats may include *dark shaded* TATA *boxes* or *light shaded* sequence insertion *boxes* labeled by respective base pair lengths. In the circular genome, the origin of

replication (*ORI*) divides the coding sequences, represented by *broad arrows,* into two expression regions either on the early (*E*) or late (*L*) side of DNA replication. The T and t proteins are expressed E, and the Agno, VP1, VP2, and VP3 proteins are expressed L. *Dots* represent noncoding regions, which include the RR. Numbering of the nucleotides for the proteins surround the genome with JC virus and BK virus numbers in *parentheses*. (Courtesy of Peter Jensen, National Institute of Neurological Disorders and Stroke, National Institutes of Health, Bethesda, MD.)

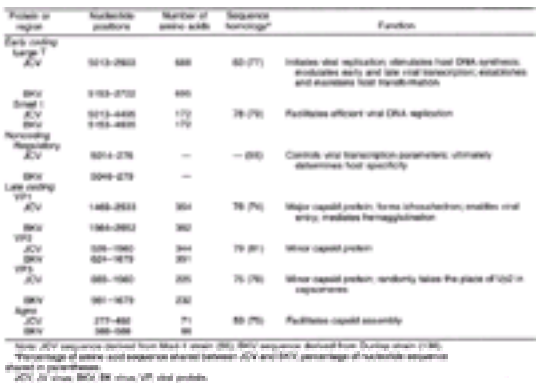

TABLE 2. *Genomic comparison of the human polyomaviruses*

There are, however, significant differences in the genomic organization of the noncoding nucleotide sequences between the human polyomaviruses and the simian polyomavirus, SV40. These sequences function as promoters and enhancers of mRNA transcription and DNA replication and, consequently, make up the regulatory region of the viral genome. Although all polyomaviruses possess noncoding nucleotide sequences located between the early and late coding regions of the viral genome, the arrangement of these sequences differs markedly not only among the primate polyomaviruses but also between BKV and JCV. The prototype strain of the human polyomavirus regulatory region sequences exists as direct tandem repeats, at times occurring in multiple copies as well as demonstrating rearrangements from deletions or insertions of additional regulatory elements. Such *cis* elements may be derived from another structure of the regulatory sequences found frequently in kidney and urine samples. Named the *archetype sequence,* these nucleotides are not arranged in repeats but contain all the elements found in a single repeat along with other sequences, which may function as promoters and enhancers. The regulatory region is widely believed to be the major factor determining host range; therefore, its molecular functions are described in more detail in the discussion of viral biologic properties and pathogenesis. Whether viral genomes with either tandem repeats or archetype arrangements of their regulatory region nucleotides are derived from each other or represent separate strains of BKV or JCV has yet to be firmly established.

Propagation and Assay in Cell Culture

The cell types used for the initial isolation of BKV and JCV have proved the most efficient cell substrates for viral propagation. BKV multiplies well in kidney epithelial cells of primate origin, although progeny virions can be made in human fetal brain cells and, to a lesser extent, in human fibroblasts and lymphocytes (52,128). However, the most productive BKV infection occurs in human embryonic kidney cells (HEK), with more than 70% of epithelial cells yielding virus. The kinetics of infection in HEK cells suggest synthesis of the T and t proteins within 24 hours of infection, but mature, infectious virions cannot be readily detected for several days. During the course of infection, there are marked cytopathic effects typical of polyomavirus infections with rounding of cells, which become translucent in phase microscopy, contain cytoplasmic vacuoles, and detach from cell monolayers in a stringlike appearance (Fig. 3A). BKV forms plaques on HEK monolayers within 2 to 3 weeks. However, HEK cultures do not contain uniform populations of epithelial cells nor are routinely available. Consequently, BKV plaques assays are not usually conducted (137). Because both BKV and JCV agglutinate human type O erythrocytes, hemagglutination remains the routine laboratory assay for quantifying virus.

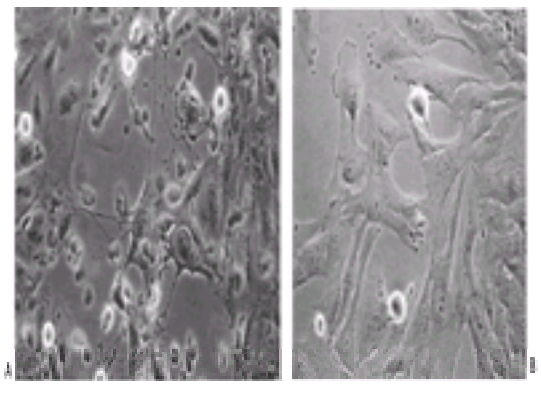

FIG. 3. Cytopathic effects due to polyomavirus lytic infection of susceptible cells in culture. **A:** BK virus infection in human embryonic kidney cells demonstrating cell swelling, pycnotic nuclei, and elongation of cytoplasm before cell lysis. **B:** JC virus infection in human fetal brain cultures, predominantly astrocytes, showing cell enlargement and shrinking of cytoplasmic processes in several cells.

JCV multiplication was originally described in human fetal brain cultures, which are a heterogeneous population of cells, including neural precursors as well as differentiated glial or neuronal cells. Cell type identification in these cultures is usually made through detection of cell type-specific, intermediate filament expression: glial fibrillary acidic protein for astrocytes, neurofilament for neurons, and nestin for progenitors. JCV grows well in astrocytes, which are the predominant cell type in these cultures, and in precursor cells of oligodendrocytes, originally described as spongioblasts, which are not highly represented in these cultures. Therefore, JCV infection occurs predominantly in the glial fibrillary acidic protein–positive cell (11,106) and does not infect neurons. Two glial cell lines established from human fetal brain support JCV multiplication: SVG cells, which were immortalized by insertion of the SV40 T-protein gene; and POJ cells, immortalized by insertion of the JCV T-protein gene (104,109). Although JCV T protein can be detected within 48 hours of infection, the growth of JCV in even highly susceptible cells derived from human fetal brain is slower than BKV. Progeny virions can be detected in several weeks. Typically, JCV T protein is made in about 70% of susceptible cells, but fewer cells actively replicate viral DNA, and even fewer appear able to synthesize capsid proteins and produce infectious progeny. New JCV virions assemble in the nucleus but remain tightly associated in the nucleoplasm, frequently requiring treatment of infected cell cultures with nonionic detergents, such as sodium deoxycholate, to release virus particles (123). Because virions remain in the cell during infection, there is little cell lysis and no easily recognizable cytopathic effects (see Fig. 3B). Because JCV is only gradually released from cells, there is no plaque assay because of the slow kinetics of viral multiplication. Cells that are susceptible to JCV infection do not remain viable for extended periods using agar overlays for plaque assays; 3 to 4 weeks is required for JCV release and cell-to-cell spread of virus. This observation is also true for the progression of the demyelinating plaque lesions seen in the brain in PML patients, which can take months to develop.

Although usually described as strictly neurotropic, JCV can infect many other cell types outside the human nervous system. Table 3 shows the diverse cells types that support JCV multiplication. Most notable are cells of the immune system, discussed under Pathogenesis and Pathology, and tonsillar stromal cells that make up the supporting cellular architecture of tonsil tissue and lymph nodes. JCV can also infect hematopoietic progenitor cells (114), human Schwann's cells (11), and cell lines established from human brain tumors of glial origin (3).

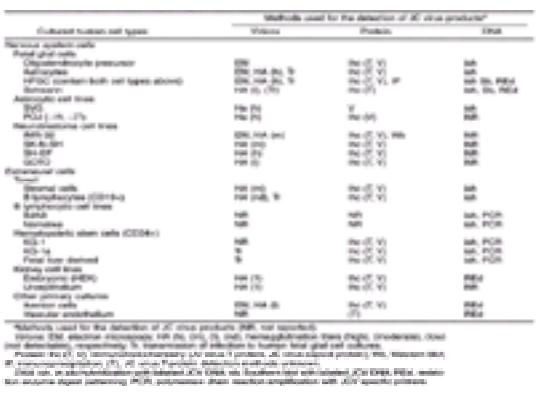

TABLE 3. *Cultured human cell types susceptible to infection with JC virus*

To follow the stages of infection in culture, antibodies to the viral T or capsid proteins are used in immunocytochemistry assays. Both polyclonal and monoclonal antibodies to BKV and JCV have been made, which are cross-reacting or specific to each of the human polyomaviruses (5,87,88,111).

Biologic Characteristics

Typically, DNA-containing viruses cause either permissive or nonpermissive infections, depending on the host cell. For the polyomaviruses, permissive infections occur in the host of origin (see Table 1), whereas nonpermissive infections occur in hosts not related to the species of origin. As described previously for BKV and JCV, cells that are permissive (i.e., all viral genes are expressed) are of primate or human origin. Restriction of viral growth in human cells is cell type specific, controlled at the transcriptional level. For example, virus may attach and enter into human fibroblasts, but early transcripts are not made. Restriction for viral growth in nonpermissive cells is at the level of DNA replication. Cells that allow early transcription of the T protein have DNA polymerases that do not interact with the T protein for initiation of viral DNA replication. Consequently, it is generally thought that cell type specificity is controlled at the transcriptional level, whereas species specificity is controlled at the replication level (50). The viral T proteins of the primate polyomaviruses may substitute for one another but not with the same efficiencies (24,37). This has been demonstrated by construction of hybrid proteins made among BKV, JCV, and SV40, whose T protein substitutes for BKV or JCV T proteins by binding the repeated penta nucleotides near the origin of DNA replication most effectively (30,56,103). Mechanisms of virion uncoating, entry into the nucleus, and assembly have not been studied in detail for the human polyomaviruses but are assumed to be similar to those for SV40. Permissive infection results in progeny virion formation. BKV infection has a higher yield of virus than JCV. Both viruses produce a minority of empty capsid particles along with viral DNA–encapsidated, infectious virions. Both full and empty capsid structures hemagglutinate human erythrocytes. The ratio of hemagglutin titer to infectious particles is about 10^{4} and that to total particles is about 10^{6}, based on plaque assay or TCID 50 doses combined with electron microscopy.

Nonpermissive infection in which only the viral T and t proteins are made commonly results in cell transformation, usually in rodent cells. For example, BKV can readily transform rat, mouse, and hamster fibroblasts, whereas JCV transforms hamster and rat fibroblasts as well as hamster brain cells, but with less efficiency than BKV (54,126,127). The transformed cells can induce tumors in the appropriate animal host and, if explanted, grow in culture and express T protein. Viral DNA is normally integrated in host DNA and can be rescued by polyethylene glycol–mediated fusion with

permissive cells after heterokaryon formation (15,27,35,41,68,107).

BKV and JCV can also initiate clastogenic events in infected cells, causing chromosomal changes during the course of infection. BKV induces high mutation rates in cell lines (155) and chromosomal damage in human fibroblasts expressing T protein (157). JCV-infected cells in culture demonstrate chromosomal abnormalities and damage resembling what is described as “rogue” cells in lymphocytes. Rogue cells show dramatic cytogenetic damage and, probably, do not undergo mitosis. Because of the widespread nature of human infection worldwide with human polyomaviruses and their ability to induce chromosomal damage in cultured cells, a serologic survey was conducted that linked individuals with high antibody titers to JCV, to a lesser extent to BKV, with the presence of such rogue cells (97,117). It is thought that JCV, or possibly BKV as well, may initiate “simple” chromosomal damage, translocations, and unstable multicentric chromosomes, which leads to further DNA damage and ultimately to complex chromosomal abnormalities and cell transformation or cell death (118).

Key Proteins

The human polyomaviruses share a high degree of nucleotide sequence and amino acid protein sequence homology with the primate polyomavirus SV40, as shown in Table 2. Generally, homology exists among all the viral proteins at the amino-terminal end, with divergence not only from SV40 but also between BKV and JCV toward the carboxyl-terminal ends of the proteins. The large T proteins are multifunctional in both lytic and transforming infections, which is required for initiating viral DNA replication (described in detail in Chapter 63) as well as augmenting late gene transcription, binding cell cycle–related proteins, and stimulating cellular gene expression. The small t protein may be dispensable for lytic infection but contributes to transforming infections to establish a fully transformed cell phenotype. The T protein binds the cellular p53 and retinoblastoma proteins in both lytic and transforming infections, which abrogates their cell cycle–regulatory functions. There is also evidence for BKV that inhibition of p53 and pRB protein functions up-regulates transcription factor E2F proteins, which contributes to the release of cells from control of cell cycle pathway or toward apoptosis (73). The JCV T protein also interacts with p53 and pRB cell cycle proteins, which has recently been described in PML brain tissue as well as cultured cells (7,49). JCV T protein may also directly or indirectly interact with cell cycle–dependent kinases, A1 and B1, which disrupts cell cycle movement and induces S-phase synthesis needed for viral multiplication (8).

The viral capsid proteins assemble into naked viral particles 40 nm in diameter according to thermodynamic parameters for icosahedrons. During assembly in lytic infection, replicated viral DNA in its supercoiled form, 5.13 kb, is encapsidated into the particle, which begins to form a crystalline array of virions visualized by electron microscopy. Nonviral DNA, fragmented cellular DNA, may also become encapsidated, which appears limited in size to 5 to 6 kb. The VP1 makes up the major structural protein, is used by the virus for cell attachment, and is the antigen that gives rise to antibody response. Recently, the VP1 of JCV (32,121) and BKV (57) have been cloned into baculovirus vectors and produced in high concentrations in insect cells. VP1 molecules can self-assemble into virus-like particles (VLPs), encapsidate any DNA of appropriate size, and transfer genes into cells in culture. VLPs can be used as a gene delivery vehicle for molecules with therapeutic value (66,121,142). VP1 molecules can also be used as antigens for hemagglutination inhibition or enzyme-liked immunosorbent assays (ELISAs) (71,162).

Infection in Experimental Animals

JCV and BKV infection in experimental animals and in transgenic mice engineered to express the T protein results in tumor formation. The frequency, tumor type, and experimental animal studies have recently been thoroughly reviewed (15). Briefly, BKV induces different types of tumors dependent on the rodent species and route of inoculation. Intracerebral inoculation in hamsters results in ependymomas, neuroblastomas, and pineal gland tumors. Intravenous inoculation causes pancreatic islet cell tumors, fibrosarcomas, and osteosarcomas. Mouse inoculations frequently result in fibrosarcomas, liposarcomas, nephroblastomas, gliomas, and choroid plexus tumors, which are also common in SV40 T-protein–expressing transgenic mice (120). BKV tumor induction shows some viral strain variation with the prototype isolate, BKV Gardner, possessing the greatest oncogenic potential. It is unclear whether trans- or cis-acting factors influence tumor formation frequency, but it is assumed that the regulatory sequence variations have a dominant role (160).

JCV inoculation into hamsters either intracerebrally or subcutaneously also results in multiple tumor types, including medulloblastomas, glioblastomas, and pineocytomas. As reported with BKV, the incidence of tumor formation with JCV showed some viral strain dependence (124). However, unlike BKV or SV40, JCV induces a grade 4 glioblastoma multiforme or malignant astrocytoma upon intracranial inoculation into New World owl and squirrel monkeys. JCV does not induce tumors in Old World macaques or rhesus or African green monkeys. The frequency of tumors is more than 50%, but the time for tumor formation is measured in months (18 to 36 months). Growth of the tumors can be detected by computed tomography (CT) scans and followed by observation of the monkey's abnormal behavior. These tumors progress rapidly, at times metastasizing within the parenchyma of the brain to the noninoculated hemisphere. Tumor cells can occupy nearly one third of the cerebral hemisphere before the animal dies within several months of initial detection of the tumor (79). The astrocytoma cells can be grown in culture, demonstrating the JCV T protein, which binds the monkey p53 protein. In some tumor explants, JCV T protein synthesis gradually diminishes. The cells then quickly enter a senescence period, assumed to be an apoptotic pathway, and die in culture. One owl monkey tumor, however, demonstrated unique properties not only surviving in culture but also in becoming transplantable back into other owl monkeys. JCV DNA in these tumor cells, termed *586,* was both integrated in the cell chromosome and episomal in high copy number. JCV infectious virus was produced continually in culture, resulting in a persistent viral or carrier culture (105). A neuroblastoma cell line has been established that also is a JCV carrier culture (5).

Transgenic mice have also been made that express the JCV T protein. However, it is rare that astrocytomas occur in these animals. There is a hypomyelination that results directly from T-protein disruption of compact myelin formation (143). The T protein affects myelin gene expression by interacting with transcription factors, MEF-1/pura, which may down-regulate myelin basic protein (MBP) synthesis (158). In transgenic mice made using the archetype regulatory region promoters, hypomyelination was not seen. These animals did exhibit primitive neuroectodermal tumors similar in

histology to medulloblastomas with high level of T-protein expression (94). Neural crest–derived tumors in other transgenic mice also showed efficient T-protein synthesis with binding to the p53 and pRB proteins and altered regulation of cyclin-dependent kinases suggesting a mechanism for tumor cell growth (93).

PATHOGENESIS AND PATHOLOGY

Cellular Pathways of Infection and Molecular Regulation of Viral Multiplication

Entry Into Host: Viral Receptors

The simian polyomavirus, SV40, binds the class I major histocompatibility complex (MHC) molecule, which acts to position the viral particle in caveolae for viral entry. However, the efficiency of SV40 infectivity does not completely correlate with the presence or distribution of the class I MHC receptor; consequently, other molecules may act as coreceptors. Antibodies to MHC I do not block human polyomavirus binding to cells, nor does SV40 compete for JCV binding to susceptible cells (100). Because both BKV and JCV agglutinate human type O erythrocytes, as does the mouse polyomavirus, viral attachment was thought to involve sialic acid–containing glycoproteins. In recent studies, treatment of cells with neuraminidase, protease, or phospholipase blocked JCV binding, but only neuraminidase effectively blocked infection. Further competition assays determined that JCV uses an a $_{2–6}$–linked sialic acid residue on glycoproteins for attachment on the cell membrane (101). Little is known about the BKV receptor, although there has been the suggestion that BKV may bind to phospholipids (141). As with many viruses, binding of virus to the cell does not always result in infectivity. There may be multiple binding sites for the polyomaviruses, but only specific molecules function for viral entry, resulting in infection, as suggested by the enzymatic blocking assays. Recently, pseudoreceptors for the mouse polyomavirus were described that selectively blocked binding of several viral strains in various organs, thereby determining spread of virus in the mouse (16). Table 4 lists the currently known cellular receptors for polyomaviruses.

Virus	Receptor	Number/cell	Size (kd)	Tropism	Notes
PyV	N-linked glycoprotein containing α (2–3) linked sialic acid	25,000	1.8×10^{-7} M	Broad	Mutations in VP1 alter sialic acid recognition and tissue tropism
SV40	MHC class 1 proteins, also a possible role for O-linked glycans	90,000	3.75×10^{-9} M	Broad	Only family member that does not interact with sialic acids
LPV	O-linked glycoprotein containing α (2–6) linked sialic acids	1,600	2.9×10^{-7} M	Restricted to B lymphocytes	Presence of a specific receptor correlates with susceptibility to infections
BKV	Glycolipid containing α (2–3) linked sialic acid	Unknown	Unknown	Broad	
JCV	N-linked glycoprotein containing α (2–6)	50,000	Unknown	Oligodendrocytes, astrocytes, and B lymphocytes	Does not share receptor specificity with SV40

PyV, polyomavirus; SV40, simian virus 40; LPV, lymphotropic papovavirus; BKV, BK virus; JCV, JC virus.
Contributed by Walter Atwood, Brown University, Providence, Rhode Island.

TABLE 4. *Polyomavirus receptors*

For the human polyomaviruses, virions are carried to the nucleus, presumably through an endocytotic pathway. Uncoating takes place at the nuclear membrane, although intact virus particles have been described in the nucleus directly after infection. Binding of cellular proteins on the viral regulatory region leads to early transcription and ultimately determines host range or viral tropism (see later).

Site of Primary Infection and Spread in the Host

The widespread nature of both BKV and JCV infection, which occurs at an early age in the human population, strongly suggests a common route for initial infection. Respiratory inhalation had been suggested as a common route of infection, but until recently, there was little evidence for the presence of either JCV or BKV in the respiratory tract. BKV has been reported in lung tissue from one case of interstitial pneumonia (136) in a hematopoietic stem cell transplant recipient; respiratory tract disease and infected tonsil tissue were reported in another case (67). Recently, however, JCV DNA has been identified consistently in human tonsil stromal cells, in the tonsillar tissue itself, and in the B-lymphocyte population contained in the tonsils (115). JCV is also able to infect tonsillar stromal cells productively in culture with an efficiency nearly comparable to human glial cells. BKV has not been shown to infect stromal cells in culture. Because both viruses are not stable under conditions that are consistent with oral ingestion of virus, inhalation of virus or a respiratory pathway for initial infection seems most likely. Whether BKV uses lymphoid tissue for initiation of infection needs further study, but JCV seems able to infect tonsil tissue.

The mechanisms of virus spread throughout the human host remains difficult to ascertain. Using polymerase chain reaction (PCR) analysis, JCV DNA is routinely found in the peripheral circulation in 5% to 40% of normal subjects as well as 70% to 85% of PML patients (164). JCV DNA is also found in a high percentage of patients with immune deficiencies such as allograft recipients treated with immune suppressive drugs, HIV-1 infection, and neoplastic diseases. In the blood, the B lymphocyte appears as the principal cell carrier of virus, but other blood-borne cells, including T cells, may also harbor virus (48). However, if cell tropism is associated with DNA-binding proteins, which function for transcription, only B cells allow viral multiplication (13, 134). There is evidence that BKV can also be found in the peripheral blood from surveys of normal, healthy individuals (R. Frisque, personal communication). Both BKV and JCV have been identified in multiple organs besides kidney and brain (JCV), including heart, spleen, lung, colon, and liver, suggesting a hematogenous route of virus spread (107, 164).

Cell and Tissue Tropism

An early observation of the biologic differences between BKV and JCV was their cell specificity or host range and tissue tropism. BKV could grow in monkey or human epithelial and fibroblast cells in culture with high efficiencies. JCV, on the other hand, was thought to be strictly neurotropic, growing only in glial cells derived from human fetal or adult brain with low viral yields. This pattern of cellular host range was similar whether infection was initiated with virion particles or viral DNA; both BKV and JCV genomic DNA are infectious on transfection into cultured cells (107). Consequently, this observation emphasized that control of host range was at the intracellular, molecular level, which led to investigations on the nature of the viral regulatory region and host factors that recognize these sequences.

Hypervariability of the regulatory region is a typical feature of polyomaviruses, particularly BKV and JCV, which demonstrate multiple arrangements depending on the tissue source of isolation. For example, virus found in the urine contains regulatory region nucleotides, which consistently demonstrate promoter and enhancer sequences in a linear series of 187 base pairs for JCV and 170 base pairs for BKV. JCV isolates from brain or tonsil tissue contain regulatory region variants but consistently demonstrate the promoter and enhancer elements in direct tandem repeats. The prototype strain of JCV, termed Mad-1, named for the location of its isolation Madison, Wisconsin, has direct tandem repeats of 98 nucleotides, including duplication of the TATA sequences. Many other variants of JCV regulatory sequences have been identified in brain, kidney, blood, tonsils, and other tissues that contain multiple forms of this arrangement (reviewed in 82). The linear arrangement found in the urine contains all the elements in the tandem repeat structures plus additional sequences of 23 and 66 base pairs. Figure 4 graphically demonstrates the nucleotide arrangements of these sequences. The linear arrangement of sequences from urine has been termed the *archetype structure* because deletion of the 23 and 66 base pairs and duplication of 25 base pairs, including the TATA sequences as well as 55 and 18 base pair segments, can result in the direct tandem repeats frequently found in tissues outside the kidney (55,167). There have been many isolates of JCV DNA whose regulatory sequences demonstrate variations on either the tandem repeat or archetype arrangement.

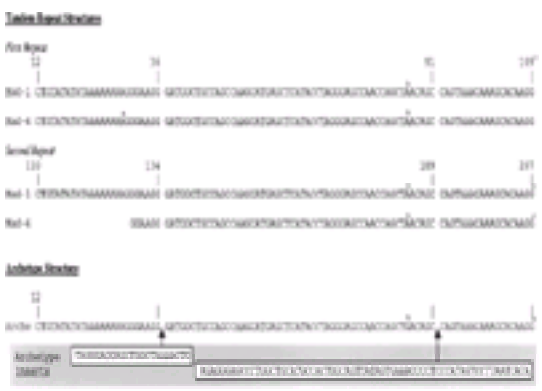

FIG. 4. Nucleotide sequence variations in the regulatory region of JC virus. **a:** This adenine may be deleted in Mad-4 strain. **b:** Variants that have sequence insertions after nucleotides 36 or 91 have a guanine (*G*) at this position. Variants without insertions have an adenine (*A*). **c:** End of the variable portion of the regulatory regions with conserved sequence following GGAAGTGGAAAGCAGCCAAG. . . . **d:** Nucleotide numbering was adopted from Mad-1 (55).

BKV demonstrates a similar phenomenon of archetype sequences found in the urine as well as other repeat structures identified in kidney and other tissues (110). The BKV archetype sequences are composed of blocks of nucleotides directly upstream of the origin of DNA replication and TATA sequences of 68, 39, and 63 nucleotides in length (135). Variations on these nucleotide segments or blocks are found in a number of BKV isolates, which may contain partial duplication of the 68 and 39 segments, the BKV DB strain, minor deletions from the archetype, the BKV AS strain, or triplication of the 68 block of nucleotides, the BKV WT strain (152).

Interestingly, neither BKV nor JCV with archetype regulatory region arrangements are able to grow in cell culture with any degree of efficiency, even though these strains are highly represented in nature. Although it has been suggested that the archetype strains of the human polyomaviruses may be the circulating strain in the human population from which variants are derived by rearrangement of the regulatory sequences, there is no direct evidence that this can occur either by cell culture passage or during the course of active infection in humans (43). It does appear that the archetype sequences cannot be transcribed effectively to produce the viral T protein, but if T protein is already present in the cells, replication of the archetype sequences can occur (72,145). The cell type–specific regulation of JCV, particularly, has been studied in detail because of its limited host range and pathogenicity in humans. Nucleotide sequences that act as transcriptional promoters are principally located in 45 base pairs just upstream from the origin of DNA replication. These sequences include the TATA box and binding sites for transcription factors Sp1, YB1, sup2, pura, NF-1 juxtaposed directly next to AP-1, or c-jun/c-fos (76,129). These sites are all duplicated in the tandem repeat arrangement of JCV, although repeat of the second TATA sequences are routinely not found as seen in the Mad-4 strain, which has greater biologic activity than the Mad-1 strain. The NF-1 proteins exist as a family of proteins expressed in almost all cells but in multiple classes. The NF-1 class D is highly expressed in human brain as well as other cells susceptible to JCV infection, such as stromal cells, and may contribute to the cell-specific regulation of JCV transcription (92,149). Interactions between YB1 and pura also provide cell-specific regulation (34). There is also an NF-kB binding site downstream from the T protein coding sequences that has been shown to be active for late transcription and possible positioning of other DNA-binding proteins, with T protein to initiate efficient transcription (59). Both JCV and BKV contain active AP-1 binding sites, which are necessary for efficient promoter activity (129).

Immune Response

Because of the amino acid homology among the polyomaviruses, many polyclonal or monoclonal antibodies made in rodents to the T or capsid proteins, particularly VP1, are cross-reacting (140). However, there are monoclonal antibodies to the T proteins that discriminate among SV40, BKV, and JCV, which react to epitopes that can be found in the carboxyl-terminal ends of these proteins where amino acid homology is less. Transgenic mice, however, which are tolerant to SV40 T-protein immunization, did make antibody to the amino-terminal end of the JCV T protein upon immunization with JC T protein (153,154). SV40 T-protein transgenic mice can also make antibody to the T protein upon immunization, with the SV40 T protein revealing both major and minor antigenic epitopes in the T-protein molecule. During tumor progression, hamsters develop high-affinity, anti-T antibodies that cross-react with the primate polyomaviruses. Astrocytoma-bearing monkeys develop anti-JCV T antibodies with low affinity and low titer, no doubt owing to the sequestered nature of the tumors in the brain. There are antigenic strain variants of BKV demonstrated by antibody responses raised in rabbits as well as antibodies in the human population (152). Although there are sequence variations in the JCV genome for the VP1 protein, there have not been antigenic strain variants defined as yet by immunologic assays.

BKV inoculation into rabbits also induces antibodies to its own double-stranded DNA as well as eucaryotic double-stranded and single-stranded DNA (131). An anti-DNA antibody response can also be elicited by inoculation with BKV double-stranded DNA, which causes infection.

EPIDEMIOLOGY

Serologic Studies and Amplification of Viral Genome Sequences

Age of Infection and Seroepidemiology

BKV and JCV cause widespread infections throughout the world based on seroepidemiologic studies and identification of viral DNA excreted in the urine. Serologic surveys of populations using hemagglutination inhibition assays for the detection of antibodies indicates that seroconversion takes place early in life, at 5 to 7 years of age (151). Antibodies to either virus remain throughout life but may fluctuate in titer. It is still unclear whether persistence of antibody is the result of reactivation or reinfection. Generally, antibody that is measured by hemagglutination inhibition is immunoglobulin G (IgG), although a number of reports identified an IgM response with onset of clinical symptoms of infection (51). IgM and IgA antibodies have been detected against BKV in bone marrow and renal transplant recipients (47). ELISAs have now been developed using either cloned VP1, viral peptides, or virion particles as antigen (58,71,168). Although there are exceptions, antibody titers show that individuals usually have high titers to one of the human polyomaviruses and low titers to the other. This is an interesting observation because there is such close homology between BKV and JCV in the amino acid sequence of the VP1 molecule against which these antibodies react. Table 5 lists titers of human sera tested for antibodies to BKV and JCV before and after absorption by the heterologous antigen either BKV or JCV. The titers to either virus do not markedly change after absorption with the heterologous virus (71). This observation indicates that antibody made during the course of human infection is selective to unique epitopes on BKV and JCV VP1 capsid proteins, with little or no cross-reaction between viruses. There do not appear to be cross-reacting antibodies to SV40 in human sera (108,146). Whether SV40 independently circulates in the human population as a result of its introduction in contaminated vaccines in the early 1950s or by other means remains a question of considerable concern and debate.

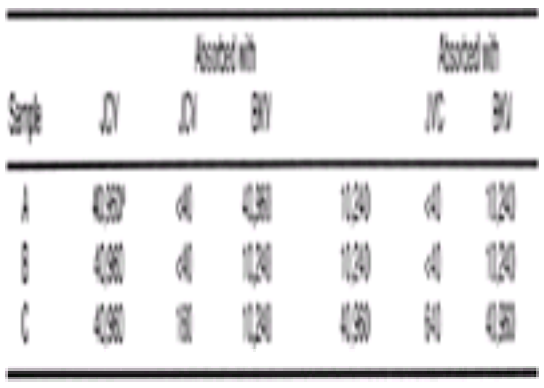

TABLE 5. *Human sera with high antibody titers to either JC virus (JCV) or BK virus (BKV) before and after absorption with either viral antigen*

Spread of Infection

Antibodies to both BKV and JCV are prevalent in populated countries but less prevalent in remote populations. In a recent study analyzing sera from Amerindian tribes from remote regions of South America, a low percentage of samples was positive, demonstrating low titers compared with a collection of Japanese samples of which most were positive with high antibody titers, particularly to JCV (108). It also seemed that introduction of the human polyomaviruses into the Amerindian population occurred at the time of human contact from other countries. However, how the human polyomaviruses are spread among individuals is not clear. Another study collected urine samples from young and older individuals in Japan who would have had contact with American military and their families. The samples were analyzed using PCR to identify JCV DNA and nucleotide sequencing, which ould “type” variants of the DNA; one type was specific to Japan, whereas others were prominent in the United States. The results suggested that none of the JCV DNA types found commonly in the American population were present in any age group in Japan, indicating that virus is not easily

transmitted between populations (84). Other data showed that JCV is readily transmitted from parent to child (95).

New Methods to Track Infection in World Populations

Because urinary excretion of the human polyomaviruses is common, a number of studies have tested urine by PCR for the prevalence of BKV and JCV. As mentioned previously, the nucleotide sequences that compose the regulatory region of both BKV and JCV found in the urine, termed the archetype, rarely contain variants nor tandem repeat sequences, unlike those sequences found frequently in other tissues. In addition to the regulatory region, JCV DNA VP1 sequences from urine samples have also been extensively analyzed. A system for classifying or typing JCV variants has been reported among some groups using nucleotide comparisons and construction of genetic linkages and a phylogenetic tree (83). Genotyping of the JCV VP1 sequences has allowed a geographic distinction of viral variants, particularly from Asia, North America, Europe, and Africa, which has been used to assess migration patterns of human populations of North America Indians (2). Regulatory region sequences have been used as a tool to estimate specific, intercontinental patterns of human population migration (69,148).

In comparison among BKV, JCV, and SV40, it appears that BKV is excreted more frequently than JCV in HIV-1–infected and severely immunosupressed individuals. However, in the population as a whole, JCV is excreted far more commonly—in 40% in the United States and as high as 75% in Japan. The prevalence for BKV is about 15% in the United States and only 2% to 5% in Japan. SV40 has not been found using PCR methods in the urine in the human population.

From the time that JCV was first recognized in lymphoid tissue, it was assumed that JCV or JCV DNA could be identified in the peripheral blood. Many studies have identified JCV DNA using PCR analysis. Reports have also documented BKV in the peripheral circulation, but not with the same frequency (14). There is no consensus yet on the precise percentage of the normal population with JCV in the blood at any time point, but it does appear as a relatively high number. It is clear that at least 5% to 40% of the human population, from predominantly American and European populations with no underlying disease, have JCV in their peripheral blood (48). In the blood, viral DNA is usually associated with lymphocytes, particularly B lymphocytes, with some reports of other cells in the blood or in the plasma (48). Viral mRNA has been reported in blood cells, including B and non-B cells, indicating that some level of infection can take place in these cells. JCV can infect human B lymphocytes as well as human CD34+ hematopoietic cells in culture, but neither T cells nor cells of monocyte origin (31,114).

CLINICAL FEATURES

JC Virus–Induced Demyelination in the Human Central Nervous System

Progressive Multifocal Leukoencephalopathy

PML is a demyelinating disease of immune compromised individuals caused by JCV lytic infection of oligodendrocytes, the myelin-producing cells in the white matter of the human brain. The demyelinated areas form multifocal plaque lesions, which contain enlarged, hyperchromatic oligodendrocytes, macrophages, and bizarre astrocytes, the characteristic features of the histopathology of PML (Fig. 5) described in 1958 (12). Clinically, PML patients demonstrate a typical triad of neurologic symptoms, including muscle weakness and gait disturbance leading to hemiparesis, cognitive abnormalities consistent with a subcortical dementia, and sensory and visual deficits (107). Lesions can be visualized using magnetic resonance imaging (MRI) or CT, most frequently in the cerebral hemispheres in the temporal, frontal, occipital, and parietal regions but also in the posterior fossa in the cerebellum and brain stem (107). Lesions are typically located at the gray matter–white matter junction in areas of highest cerebral blood flow, implicating virus entry by a hematogenous route. Perivascular infiltrates, usually T lymphocytes, have also been described in some PML lesions. In heavily infiltrated regions, few infected oligodendrocytes can be detected. In these lesions, class I MHC has been identified on bizarre astrocytes, whereas class II MHC has been found on macrophages, microglia, and endothelial cells (1). These observations suggest immune clearance of infected cells and JCV antigen presentation in the infected brain in some PML cases. There is a strong correlation between longer-term survival, defined as greater than 12 months, and the levels of CD4 and CD8 cells at the time of diagnosis (17). In these patients, there is also enhancement of lesions on CT scans, suggesting immune reactivity in the brain. Further, intrathecal antibody to JC VP1 protein has been detected in the CSF of PML patients, which again demonstrates both that B cells are present in the PML brain and that an immune response can take place in the brain (162).

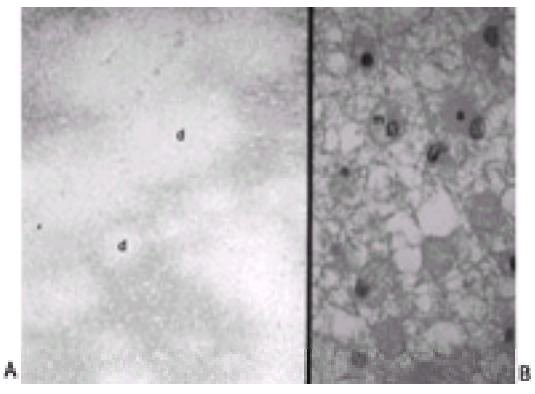

FIG. 5. Histopathology of demyelinated lesion from PML brain tissue. **A:** Luxol fast blue stain of white matter demonstrating demyelinated plaques. **B:** Presence of macrophages (*m*) and astrocytes (*a*) frequently associated with progressive multifocal leukoencephalopathy lesions.

PML can occur at any time during life, even in children (19), although it is usually found in adults with severe immunodeficiencies. Life expectancy ranges from several months to 1 year, depending on the severity of the immune-compromised state, as mentioned previously, and the viral burden in the brain (18). Until the mid-1980s, predisposing immune disorders in PML patients have been lymphoproliferative disorders, neoplastic diseases, granulomatous and inflammatory diseases like sarcoidosis, and allograft transplantation treated with immune-suppressive drugs for graft protection. PML has been recognized more frequently since the mid-1980s and recently in other cases of liver and heart transplantation, Wiskott-Aldrich syndrome in children, Alzheimer's disease, and systemic lupus erythematosus (85). However, the highest number of PML patients occurs in HIV-1–infected patients, reaching as high as 5% to 10% of all acquired immunodeficiency syndrome (AIDS) cases and accounting for nearly nearly 80% of all PML cases. PML has been the presenting AIDS-defining illness in 1% to 3% of HIV-1–infected individuals. Before the AIDS epidemic, PML was considered a rare disease in the United States, with only several hundred reported cases. With the advent of an increased population with severe immune deficiencies, particularly AIDS, the incidence of PML has risen dramatically not only in the United States but also throughout the world.

Pathogenesis

The increased incidence of PML in AIDS patients, particularly, has provided more clinical samples to study virus–cell interactions during the course of disease. Collectively, these cellular events define the stages or pathogenesis of JCV from initial infection, establishment of latency, reactivation, and targeting of glial cells in the brain causing demyelination. The ability of JCV to infect tonsillar stromal cells in culture and the presence of JCV DNA in tonsil tissue have suggested that initial infection can occur in this tissue. The widespread nature of JCV infection in the population also suggests a common route for spread of virus. Infection of tonsil tissue would be consistent with these observations. However, there are no data available as yet on the correlation between the presence of JCV DNA in tonsil tissues and development of antibody. The respiratory tract has also been considered as a potential site. However, there is little evidence for susceptibility of respiratory tract epithelial cells to JCV infection.

JCV causes a subacute or asymptomatic infection that is cleared by immune surveillance or, in some individuals, establishes a latent infection. There is no estimate on the number of individuals who harbor JCV in a latent state. Given the high frequency of urinary excretion of JCV in the population, however, viral latency probably occurs in most infected individuals. Consequently, the kidney has been considered a primary site for establishment of latency. JCV and its DNA have been identified in kidney, in uroepithelial cells, and routinely in urine (see previous discussion). JCV DNA has also been found in lymphoid tissues, such as bone marrow and spleen, particularly in PML patients (80). Viral DNA has also been identified in lymphocytes in the peripheral blood and in B cells isolated from tonsils and PML brain tissues (90,115). It is probable that JCV remains latent in several sites and reactivates at times of immune suppression. Whether JCV DNA integrates into the cellular DNA or remains episomal is not known; nor are there data on the mechanisms for reactivation, although it is assumed that depletion of cell-mediated immunity is a crucial step in release of virus from a latent state.

Because most PML patients have preexisting antibody to JCV, humoral immunity is not considered a factor in viral pathogenesis. It is assumed, however, that an intact cell and humoral immune response to JCV controls infection. Whether virus reactivates from kidney or from lymphoid tissues has been the focus of intense investigations. To address this question, viral nucleotide sequences have been examined from patients with or without PML or AIDS collected from multiple tissues, including kidney, urine, lymphocytes, tonsils, brain, and CSF. As mentioned previously, the regulatory sequences from kidney and urine, the archetype arrangement, do not contain repeats, whereas those sequences from PML brain tissue contain direct tandem repeats (see Fig. 4). Consequently, these sequences have been used as signatures or identifying sequences for tissue origin. One set of data suggests that JCV remains latent in kidney with the archetype arrangement of the regulatory sequences. Viral replication may lead to deletions and rearrangements, resulting in duplication of nucleotide sequences that resemble the tandem repeat arrangement in the brain or the PML type. Virus is carried to the brain through infected cells or through a viremia and initiates a highly productive infection in glial cells. Another set of data suggests that JCV, which resides in a latent state in lymphoid tissues (i.e., bone marrow) and has regulatory sequences in the tandem repeat arrangement and on activation from latency during episodes of immune suppression, is amplified during infection in lymphocytes, which carry the virus to the brain. Examination of the nucleotide sequences from all these tissue compartments provides support for both views. There are many examples of divergence from either principal sequence arrangement—archetype or tandem repeat. There is no clear correlation between strain of virus and the course of the disease or the pathology. There is no animal model as yet available to test this point. However, SV40 latent infection in macaques is associated with a demyelination that pathologically resembles PML (77).

JC Virus and Human Immunodeficiency Virus Type 1 in Acquired Immunodeficiency Syndrome

What mechanisms accounts for the high incidence of PML in AIDS patients is unknown. Whether there is a specific immune control of JCV latency that is deficient in AIDS patients or a direct interaction between the two viruses remains an open question. However, the observation that JCV DNA can be found in lymphocytes in the peripheral blood and brain of PML patients has provided a common host cell for direct interaction between JCV and HIV-1. The JCV T protein can transactivate the HIV-1 LTR in cotransfected cells (64). Conversely, the JCV late promoter is up-regulated through the HIV-1 tat-responsive DNA element in the JCV promoter termed upTAR (36,150). The HIV-1 tat protein complexes with the DNA-binding protein pura at this site (91). Although JCV production in co-infected cells in culture is highly efficient, there has not been identification of co-infected cells *in situ,* either in the brain of PML patients or in lymphocytes.

BK Virus Infection in the Kidney and Other Organs

BKV has been isolated or associated with a limited number of cases of retinitis, nephritis, pneumonia, and encephalitis (74,136,161). Viral DNA or virus was identified in these tissues, suggesting a possible etiological role in these diseases. BKV has also been linked with acute respiratory disease in children in whom an IgM antibody rise was measurable. Viral DNA was found in tonsil tissues in these patients using Southern blot hybridization (67). Virus could not be grown from

tonsil tissue, however, either from transfection of BKV-positive tonsil DNA or from cultivation with human embryonic kidney cells. BKV has been identified in lymphocytes, as mentioned previously, but not in a productive infection. There has been one report of the presence of receptors for BKV on B lymphocytes, which formed rosettes with human erythrocytes (98).

Kidney Infection and Cystitis

BKV has most consistently been identified in kidney diseases and cystitis in renal allograft and bone marrow transplant recipients (6). About 40% of renal allograft recipients shed BKV in the urine (9,10) either transiently or continually over weeks to months. BKV has been implicated in ureteric stenosis and circumstantially associated with kidney failure (25,62). Viral inclusions are present in uroepithelial cells excreted in the urine. Infectious virus can also be isolated from urine of renal or bone marrow transplant recipients. There has been a documented case of BKV transfer from the donor kidney to the recipient patient.

BK Virus in Bone Marrow Transplantation and Hemorrhagic Cystitis

Bone marrow transplant recipients undergo thorough immunosuppressive therapy before transplantation. These patients frequently demonstrate BKV viruria several weeks to several months after transplantation, which ends concurrently with the episodes of cystitis. The frequency of disease is higher in adult allogeneic transplant recipients than in autologous transplant recipients. Both groups have BKV in their peripheral circulation (21). The use of single primed or nested PCR techniques has greatly increased the sensitivity of assays designed to identify BKV in blood and urine of such patient populations. Because of the common occurrence of BKV in the urine in BMT patients compared with BMT patients with cystitis, it has been difficult to identify BKV directly as the cause of cystitis.

In a comprehensive study of children between 7 and 17 years of age followed for nearly 1 year, all the BMT patients who developed hemorrhagic cystitis had antibodies to BKV before transplantation, as did the marrow donors. However, there was a significant increase in hemagglutination inhibition antibodies and presence of IgM antibodies in cystitis patients compared with those with no signs of cystitis, indicating a new or reactivated infection. There was a correlation of the immune status to BKV of the donor and the risk for cystitis in the recipient. Donors of BMT patients without occurrence of cystitis had higher antibody levels compared with donors of BMT patients who did develop hemorrhagic cystitis (22). However, as in the adult patients, there was not a direct correlation between BKV infection and hemorrhagic cystitis.

Polyomaviruses and Human Neoplasia

SV40 in the Human Population

From the mid-1950s to 1963, the simian polyomavirus, SV40, was unknowingly introduced into a large number of individuals receiving contaminated poliovirus vaccines (139). Because SV40 is tumorigenic in rodents and can transform human cells in culture, there has been concern whether SV40 circulates in the human population and consequently is involved in neoplastic diseases. At this time, follow-up studies have concluded that there has not been an increase in neoplasias over three decades in the population that became infected with SV40 compared with matched age, gender, and geographic control populations not exposed to SV40 (140). However, there has been evidence that SV40 DNA and its T protein can be identified in rare human tumors, such as ependymomas, osteosarcomas, mesotheliomas, and choroid plexus papillomas (20,99). A more recent retrospective cohort study of data collected by programs designed to track tumor incidence in specific populations did not find an association between SV40 exposure and increased risk for these rare tumors (146). However, a number of investigations have identified authentic SV40 DNA sequences, T protein, and complexes with p53 and pRb cellular proteins in different human tumors (28,44).

In 1998, the U.S. Food and Drug Administration Office of Vaccine Research and Review convened a meeting of international authorities to discuss the topic of SV40 in the human population and the technical approaches needed to evaluate its presence as an infectious agent and role, if any, in human tumors. A report of this meeting has been published (26) that extensively reviews the available information. As a result of this conference, a study was initiated to standardize the methods for assessing the presence of SV40 in tumor tissues, particularly PCR analysis of tumor tissue, and means of determining antibody in humans to SV40. This study is ongoing.

BK Virus, JC Virus, and Human Tumors

The widespread use of gene amplification methods allows extremely sensitive levels of detection of viral genomes. Levels of detection are now in the range of 1 to 100 copies of viral DNA in 10^4 tumor cells. Consequently, PCR technology has reopened investigations focusing on identification of BKV and JCV DNA sequences in human tumors, particularly in those tumor types that these viruses are known to induce in experimental animals. The role of human polyomavirus infection in initiation of tumors has been thoroughly reviewed recently (15) and is briefly reviewed here.

BK Virus

BKV is highly oncogenic in rodents, as described previously, and can transform human cells in culture if gene expression is limited to the viral T protein. BKV T protein induces chromosomal aberrations, binds the products of tumor suppressor genes, and cooperates with the protein *c-ras* to induce a transformed phenotype (42,156). Many studies have now identified BKV DNA in human tumors, some in the same tumor as SV40 (45,119). Figure 6 shows detection of BKV T protein in a human glioblastoma cell line, which also demonstrates abnormal chromosomal numbers (156). Reports have identified BKV DNA in primary brain tumors, osteogenic sarcomas, and meningiomas. However, BKV DNA has also been described in normal brain, bone, and peripheral blood, so that it is difficult to draw conclusions on the importance of its presence in tumor tissues. However, some of these tumors do not show mutations in *p53* or *RB* genes, as would be expected, and consequently could be targets for BKV T-protein complexes to form, inactivating their normal role in cell cycle regulation. BKV DNA has recently been found in a study of 18 childhood neuroblastomas. Interestingly, p53 in

undifferentiated neuroblastomas becomes sequestered in the cytoplasm. In the tumors in which BKV DNA was found, T protein and p53 were colocalized in the cytoplasm as a complex (53). It is not likely that T-protein binding to p53 contributes to cytoplasmic sequestration because in other tumors, the protein complex does exist in the nucleus. This observation, however, adds to the evidence of possible functions of viral proteins in the phenotype of tumor cells.

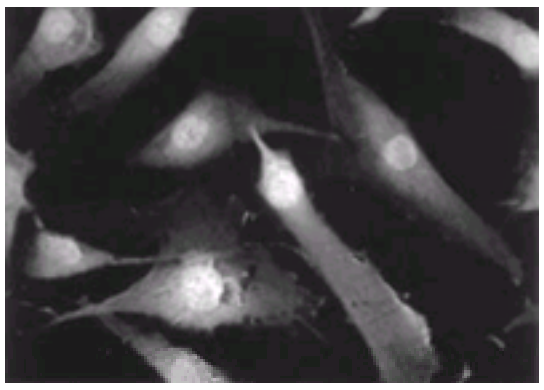

FIG. 6. Photomicrograph of BK virus T protein identified by immunofluorescence in the nucleus of cells derived from a human glioma cell line (111,156). (Courtesy of Mauro Tognon, University of Ferrara, Italy.)

P>*JC Virus*

Because JCV induces glial tumors in nonhuman primates, the major focus of interest in the oncogenic potential for JCV in humans has been the brain. There have been several reports of PML patients with multiple astrocytomas or oligodendroglioma along with evidence of anaplastic astrocytes (29,60). However, this occurrence is not common in PML, nor has there been evidence of JCV in these tumors or in bizarre or anaplastic astrocytes. Pathologic description of astrocytes in PML has suggested a preneoplastic morphology. The lack of viral capsid proteins in these cells is consistent with a transforming, not a lytic, infection. However, JCV replicating DNA has been described in JCV-infected astrocytes in PML, which would not be consistent with tumor formation and describe an abortive infection (4). Recently, an unusual oligoastrocytoma was described containing JCV DNA that expressed the T protein (132) and an xantoastrocytoma that contained JCV DNA by nested PCR detection (23). PML was not a consideration in either of these cases, nor was an immunodeficient condition.

Evidence for the presence of JCV DNA in malignancies beyond the nervous system now extends to leukemias and lymphomas (144) and surprisingly to human colon tumors (96). JCV DNA was found in high copy number in colorectal tumor tissue compared with normal, noninvolved colon tissue from the same patient. In leukemias and colon tumors, malignancies initiate by clastogenic events, chromosomal aberrations, and mutations in tumor suppressor genes, which then become inactivated. JCV infection has been associated with these cellular effects in culture and now in some tumor tissues.

DIAGNOSIS

Clinical Tissue Samples and Biopsy Specimens

JC Virus and Progressive Multifocal Leukoencephalopathy

Clinical history and neuroradiologic evidence of white matter lesions alone cannot confirm a diagnosis of PML. For example, white matter lesions identified in AIDS patients may be caused by HIV-1–associated encephalopathy and not JCV-induced demyelination. JCV DNA or antigen should be demonstrated in the patient in order to make an accurate diagnosis. Shortly after PML was described and JCV was isolated, viral protein was detected in biopsy tissue using antibody to the capsid protein, VP1. The initial antibody used was rabbit polyclonal made to SV40 VP1, which cross-reacts with JCV. Now there are monoclonal antibodies that are specific to each of the polyomaviruses.

As the incidence of PML increased because of AIDS, *in situ* DNA hybridization using probes specific to JCV DNA was frequently used because of its increased specificity and sensitivity. Positive hybridization signals also indicate viral DNA replication indicative of active infection. Use of biotin-labeled DNA probes helped identify not only infected oligodendrocytes but also astrocytes and B lymphocytes in biopsy and autopsy brain tissue (4,85,125). However, the need for brain biopsy complicated the decision whether to proceed for a definitive diagnosis, particularly in children.

Gene-amplification–based assays, usually PCR, have advanced quickly, so that detection of viral genomes in tissue samples is not only highly sensitive but also increasingly more specific. PCR detection of JCV DNA in brain tissue demonstrated that this technique could be reliable and can correlate directly with clinical disease. PCR for JCV DNA was then applied to CSF samples of PML patients, which demonstrated a high degree of correlation with biopsy results (38,75). Estimates of specificity are now in the range of 95% to 98%, and sensitivity rates are as high as 90% (163). Although false-positive results are rarely seen in this assay, false-negative results can occur in about 10% of PML cases. Many laboratories and clinical centers have adopted PCR detection of JCV DNA in CSF samples as the laboratory tool for a confirmation of PML in place of biopsy. Several recent studies have further shown that quantitative measurement of JCV DNA can also be used as a prognostic indicator of the progression of disease. Lower copy numbers or the absence of viral genomes predicts longer-term survival of PML patients (89,166). However, longitudinal studies of serial CSF samples collected from biopsy-confirmed PML patients demonstrated that a single sample may be negative, with viral DNA not detectable below 10 copies/mL, but another CSF sample collected 1 or 2 weeks later might be positive. This suggests that a single PCR-negative CSF sample in a patient in whom PML is strongly suspected warrants collection and

testing of another CSF.

BK Virus

BKV is most closely associated with kidney disease or cystitis in kidney transplant and bone marrow transplant recipients. BKV can be readily detected in urine by PCR methods; hence, viral isolation is not necessary. Viral DNA has also been detected by nucleic acid hybridization assays or viral protein using immunocytochemical techniques that demonstrate intracellular viral infection, predominantly in epithelial cells. It is not common practice to screen clinical samples for BKV at this time. Because the population in general already possess antibody titers to both BKV and JCV, serology has not been useful in assessing the importance of BKV in suspected infections.

TREATMENT

Progressive Multifocal Leukoencephalopathy

Treatment of PML patients has typically used nucleoside analogs in attempts to block JCV DNA replication. Cytarabine (cytosine arabinoside, or ARA-C) has been used most frequently but without consistent results. Several reports described remission of PML symptoms and brain lesions upon intrathecal administration of ARA-C. However, other patients treated with combined intravenous and intrathecal ARA-C did not show remarkable neurologic improvement, although in some cases there was a reduction of viral DNA in the CSF (112,116). With laboratory data showing that ARA-C could specifically block JCV DNA replication without toxicity to human glial cells (78), a multicenter phase II treatment trial of PML patients with AIDS was conducted. Sixty-four PML patients were enrolled in the trial based on brain biopsy examination. The trial was conducted by the AIDS Clinical Trial Group (ACTG 243 protocol) and the Neurology AIDS Research Consortium and represents the largest trial conducted for treatment of PML. ARA-C was administered either intravenously or intrathecally in randomized patients who also received antiretroviral drugs and were followed for several months using clinical improvement as the criterion for drug efficacy. When the trial was ended, there was no evidence of clinical improvement in patients receiving intrathecal or intravenous ARA-C compared with those receiving antiretroviral therapy alone (70). However, there was a reduction or loss of JCV DNA in the CSF in several patients treated with intrathecal ARA-C (166). Consequently, the combined experience of ARA-C in PML patients did not support its therapeutic effectiveness.

With the increased incidence of PML in AIDS patients, there has been the opportunity to evaluate the potential effect of high activity antiretroviral therapy (HAART), which was not available in the ACTG 243 trial. Several studies have now reported a correlation between HAART therapy and a number of parameters in PML patients, including increased survival time up to 1 to 2 years, improvement in clinical status, radiologic features, and, importantly, reduction of HIV-1 load in the blood (39,40,113). In some of these patients, there was also a clearance of JCV DNA in the CSF. These observations suggest that eliminating HIV-1 burden may reduce the multiplication and spread of JCV into the brain either by direct viral interactions or increase in immune reactivity to JCV. In many of these patients, there was a rise in CD4 T cells. However, there is neither cell culture data (78) nor clinical data to suggest that zidovudine (AZT) alone is effective against JCV replication or improvement in PML patients (63).

Several other drugs have been used in PML patients, including cidovofir, a drug mostly used against herpesvirus infections such as cytomegalovirus and interferon-a (81), which showed some evidence of effectiveness in delaying the clinical progression of disease. Cell culture experiments have identified topoisomerase inhibitors, camptothecin and topothecan, that block JCV DNA replication (86). JCV replication requires unwinding of genomic DNA for transcription and replication using topoisomerase, which makes this a target for drug treatment. However, there has been only sporadic use of these drugs in PML patients, with too few results to evaluate their effectiveness.

BK Virus

BKV is a common infection in renal allograft recipients, causing interstitial nephritis, and in bone marrow transplant recipients, associated with prolonged episodes of hemorrhagic cystitis. Both patient groups receive immunosuppressive drugs for graft protection, which may allow reactivation of latent BKV from the kidney. Active BKV infection in kidney transplant recipients at times mimics graft rejection, complicating the use of immune suppressive drugs. In a recent study, antirejection therapy led to an increase in graft loss in most patients, probably owing to increase in viral multiplication (130). Consequently, viral infection leading to interstitial nephritis and graft dysfunction may be sufficient reason to decrease immunosuppressive therapy and closely monitor acute rejection. Vidarabine (adenosine arabinoside, or ARA-A) has been tried in a BMT transplant recipient with a good response (33).

PERSPECTIVES

Since the publication of the last edition of this textbook, there has been intense interest in the human polyomaviruses, demonstrated by a four-fold increase in the rate of related publications. Investigators have contributed rapid advances to understanding the virology and pathogenesis of these agents. The growth of this field is due, in part, to refinements in medical and scientific technologies.

The use of gene amplification methods has allowed routine but highly sensitive detection of viral genomes in human tissues, such as urine, blood, and CSF. Identification and analysis of variant viral DNA sequences isolated by PCR are now being used to track the spread of virus between organs. These data have also aided in the diagnosis of PML as well as in the prognosis of disease severity and progression. New information has also led to reassessments of viral epidemiology on a global scale and even to studies of historical migration patterns of human populations. PCR-based studies have also indicated that both BKV and JCV circulate in the peripheral blood in a large proportion of the normal, healthy population worldwide. The implications of this observation on the public health, as numbers of immune-compromised individuals rise, requires further scrutiny.

Because of unique host range restrictions, viral promoter sequences are now used as tools for studies of cell-specific gene regulation, functional nucleotide rearrangements after replication, and molecular differentiation pathways shared between immune and nervous system cells. Capsid proteins of polyomaviruses, on the other hand, recently have been used to probe for cell receptors. Recombinantly produced capsid proteins promise to bring more readily available antigen for antibody screening and even to generate potential new vectors for gene delivery, employing the self-assembly properties of icosahedral virus-like particles that encapsidate nucleic acids.

In addition to the acute diseases associated with BKV and JCV, both viruses are now implicated in human neoplastic events. New data have now firmly established that these viruses induce chromosomal aberrations with either simple or extensive genetic damage, which could lead to mutations as a first step in oncogenesis. Mechanisms that account for this observation implicate the viral T proteins by interfering with cell cycle–regulatory proteins, p53, pRb, and other cyclins, which remove these proteins from their normal role in directing cells away from mitosis or toward apoptosis. Consequently, more studies are focused on the detection and consequences of the presence of BKV and JCV genomes in human tumor tissues.

Finally, with the ability to monitor these viruses in the population or in individual patients, more extensive clinical trials, particularly for PML, have started. In future studies, new drugs or alternative uses of other drugs, like HAART therapy in AIDS patients with PML, should emerge with potential for treatment. In conjunction with new therapies, hurdles associated with drug delivery must be overcome, particularly to target infected cells in the central nervous system.

Because of the ubiquitous presence of these viruses in the population and their potential for involvement with human diseases, a continued growth in interest and investigations on BKV and JCV is expected.

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CHAPTER 65 Papillomaviruses and Their Replication

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GENERAL DEFINITION AND PROPERTIES

The papillomaviruses are a group of small DNA viruses that induce warts (or papillomas) in a variety of higher vertebrates, including humans. Some papillomaviruses also have malignant potential for animals and people. A number of human papillomaviruses (HPVs) have been implicated as the etologic agents for cervical cancer and other epithelial tumors.

The viral nature of human warts was first indicated more than 90 years ago by Ciuffo, who demonstrated transmission of common warts using cell-free filtrates (48). The first papillomavirus was described in 1933 when Richard Shope recognized the cottontail rabbit papillomavirus (CRPV) as the etiologic agent responsible for cutaneous papillomatosis in the cottontail rabbit (269). This group of viruses remained refractory to standard virologic study for many years because tissue culture systems for the propagation of any of the papillomaviruses in the laboratory did not exist. It was not until the late 1970s, when the first papillomavirus genome was successfully cloned in bacteria, that investigators had reagents that were sufficiently standardized to begin a detailed analysis of the molecular biology of this group of viruses. In addition to standardizing reagents, the molecular cloning of the papillomavirus genomes provided adequate viral genetic material to permit the sequencing of the genomes of a number of papillomaviruses and to initiate a systematic mutational analysis and definition of the genes encoded by this group of viruses. The study of the papillomaviruses was spurred during the 1980s by the development of *in vitro* transformation assays that permitted the analysis of the viral functions involved in the induction of cellular proliferation. The bovine papillomavirus type 1 (BPV-1) initially served as the prototype for studies on various aspects of the molecular biology of the papillomaviruses; therefore, a major focus of this chapter will be on BPV-1. The recognition that specific HPVs are closely linked with cervical cancer, as well as other human tumors, has focused interest on the subgroup of HPVs that are associated with genital tract lesions. Consequently, information on the molecular biology of these HPVs will also be highlighted in this chapter.

CLASSIFICATION

Historically, the papillomaviruses were grouped together with the polyomaviruses to form the papovavirus family. The term papovavirus is derived from the first two letters of the virus first grouped together to form this family of viruses: rabbit papillomavirus, mouse polyomavirus, and simian vacuolating virus (SV40). The properties shared by these viruses include small size, a nonenveloped virion, an icosahedral capsid, a double-stranded circular DNA genome, and the nucleus as the site of viral DNA replication and virion assembly. The papillomavirus particles are about 55 nm in diameter, whereas those of polyomavirus and SV40 are slightly smaller, measuring about 45 nm. Based on this difference in size, the papovavirus family was initially divided into two genera: the polyomaviruses (which include polyomavirus and SV40) and the papillomaviruses. However, comparative molecular biologic studies made possible by the molecular cloning of papillomavirus genomes indicated there were fundamental differences in the genomic organization of the papillomaviruses and the polyomaviruses. Recognition of these differences, as well as others, has led to the papillomaviruses and polyomaviruses now being considered distinct virus families. The genomes of papillomaviruses are larger than are those of polyomaviruses (8,000 base pairs [bp] versus 5,000 bp). Papillomaviruses have more open reading frames (ORFs) than polyomaviruses and encode a different number of structural and nonstructural proteins. The genes of papillomaviruses are all transcribed from the same DNA strand, whereas some polyomavirus genes are transcribed from one strand but others are transcribed from the opposite strand. The two families can also be distinguished by the presence of conserved family-specific epitopes in their major capsid protein (139).

The papillomaviruses are widespread in nature and have been recognized primarily in higher vertebrates. Viruses have been characterized from humans, cattle, rabbits, horses, dogs, sheep, elk, deer, nonhuman primates, the harvest mouse, and the multimammate mouse (*Mastomys natalensis*). Papillomavirus antigens have been identified in a variety of other

mammals, including other nonhuman primates (290). Papillomaviruses have also been described in some avian species, namely the parrot and the chafinch. In general, the papillomaviruses are highly species specific, and there are no examples of a papillomavirus from one species causing a productive infection in a second species. Most papillomaviruses have a specific cellular tropism because squamous epithelial cells are associated with purely squamous epithelial proliferative lesions (warts, papillomas). The lesions can be cutaneous or can involve the mucosal squamous epithelium from the oral pharynx, the esophagus, or the genital tract. Expression of the productive functions necessary for virion replication appears to be limited to the terminally differentiating cells of the squamous epithelium.

There is a distinct group of papillomaviruses, found in ungulates, that induce benign fibropapillomas in which there is a proliferative dermal fibroblastic component in addition to the typical proliferative squamous epithelial component. The best studied papillomavirus of this group is BPV-1. Other papillomaviruses that also induce fibropapillomas include BPV-2, the European elk papillomavirus, the deer papillomavirus, and the reindeer papillomavirus. Interest in this subgroup of papillomaviruses stemmed initially from the fact that, in addition to their ability to induce fibropapillomas in their natural host, they have the ability to induce fibroblastic tumors in other species, such as hamsters (90). Members of this group of papillomaviruses can also readily transform a variety of rodent cells in culture. The capacity of BPV-1 to induce morphologic transformation of tissue culture cells was first demonstrated in the early 1960s (23,25,302). Quantitative transformation focus assays were developed for BPV-1 in the late 1970s, which provided a biologic assay to define and study the viral functions involved in the induction of cell proliferation (79). Although most studies have been carried out with BPV-1, it is notable that the European elk papillomavirus and the deer papillomavirus have also been demonstrated to have these transformation properties.

To date, more than 100 different human papillomaviruses have been characterized, and it is estimated that there may be many additional viral types that have not yet been described (64). Because serologic reagents are not generally available to distinguish each of the known HPV types, they are not referred to as serotypes. The classification of viral types is based on the species of origin and the extent and degree of relatedness of the viral genomes. Hence, the HPV types are often referred to as *genotypes.* For most animal species, only a single papillomavirus type has been described, but this is likely because extensive comparative studies have not yet been carried out for most animal species at a molecular level.

The papillomavirus DNA isolates from one species are classified according to their sequence homology. As noted previously, more than 100 HPV types are now recognized. The initial classification of a specific type was based on the extent of homology of the DNA genomes using liquid hybridization techniques under stringent reassociation conditions (51). Classification of types is now carried out by comparison of the nucleotide sequence of specific regions of the genome. Based on criteria adopted by the Papillomavirus Nomenclature Committee, the nucleotide sequences of the *E6, E7,* and *L1* ORFs of a new type should not exceed 90% of the corresponding sequences of the genomes of known HPV types (63). A closer relationship is considered a subtype or variant of the type with the highest degree of homology.

VIRION STRUCTURE

Papillomaviruses are small, nonenveloped, icosahedral DNA viruses that replicate in the nucleus of squamous epithelial cells. The papillomavirus particles have a sedimentation coefficient (S_{20},W) of 300. The papillomavirus particles are 52 to 55 nm in diameter (Fig. 1). The virion particles consist of a single molecule of double-stranded circular DNA about 8,000 bp in size, contained within a spherical protein coat, or capsid, composed of 72 capsomers. The virus particles have a density in cesium chloride of 1.34 g/mL (54). Fine structural analysis by cryoelectron microscopy on three-dimensional image reconstruction techniques have revealed that the viruses consist of 72 pentameric capsomers arranged on a T=7 surface lattice (10). Like the polyomavirus capsids, the capsomers exist in two states: one capable of making contact with six neighbors, as observed in the 60 hexavalent capsomers, and the other with 5 neighbors in the 12 pentavalent capsomers (Fig. 2).

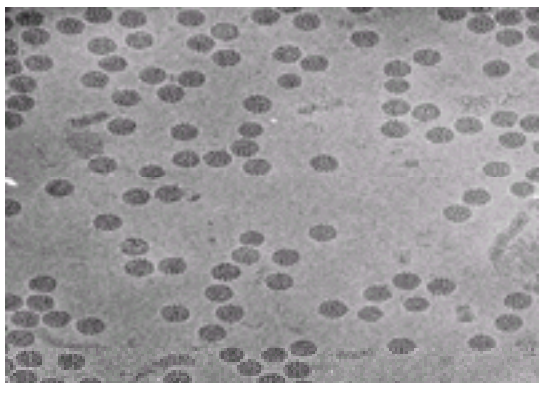

FIG. 1. Electron micrograph of BPV-1 virion particles (55 nm in diameter). (From ref. 10, with permission.)

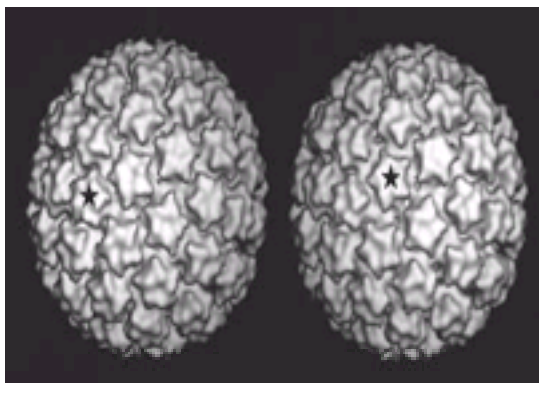

FIG. 2. Surface-shaded displays of papillomavirus three-dimensional image reconstructions of a cryoelectron microscopic analysis of virion particle structures. The individual capsomers exist in either a pentavalent (*starred on left*) or hexavalent (*starred on right*) state. (From ref. 10, with permission.)

The capsid consists of two structural proteins. The major capsid protein (L1) is about 55 kd in size (86,100,222) and represents about 80% of the total viral protein. A minor protein (L2) has a molecular size of about 70 kd. Both of these proteins are virally encoded. In addition, analysis of proteins in the virus particle has shown that the viral DNA is associated with cellular histones to form a chromatin-like complex (86,222). Virus-like particles (VLPs) can be produced from different papillomaviruses by expressing L1 alone or the combination of L1 and L2 using mammalian or nonmammalian expression systems (108,150,243,339). Although not required for assembly, L2 is incorporated into VLPs when coexpressed with L1. The morphology of VLPs containing only L1 appears identical to intact virus particles by cryoelectron microscopy (107). L2 plays a role in the selective encapsidation of papillomavirus DNA in viral capsids and therefore increases the infectivity of papillomavirus virions (338). It colocalizes with the viral E2 transcription and replication factor in PML oncogenic domains (PODs) within the nucleus (60), where it probably fosters virion assembly and perhaps antagonizes some of the activities of E2 (118).

Full papillomavirus particles contain a double-stranded circular DNA viral genome of about 8,000 bp. The guanosine cytosine content of most papillomavirus genomes is about 42%. The DNA constitutes about 12% of the virion by weight, accounting for the density in cesium chloride of about 1.34 g/mL (54).

GENOME STRUCTURE AND ORGANIZATION

The genomes of many of the animal papillomaviruses and human papillomaviruses have been sequenced in their entirety. The genomic organization of each of the papillomaviruses is remarkably similar. The genomic map of BPV-1 DNA is shown in Figure 3. One characteristic of the genomic organization of all of the other papillomaviruses is that all of the ORFs are located on one strand of the viral DNA, indicating that all of the viral genes are located on one strand. Transcriptional studies of the RNAs encoded by the papillomaviruses indicate that only one strand serves as a template for transcription.

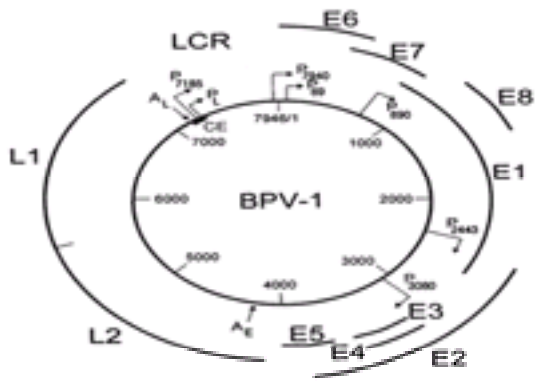

FIG. 3. BPV-1 genomic map. The numbers inside the circle indicate the nucleotide positions. The individual open reading frames of the early (*E*) and late (*L*) regions are depicted as areas outside the double-stranded circular genome. Only one strand is transcribed, and transcription occurs in the clockwise direction. Early promoters are indicated by an arrow labeled P_n, where *n* is the approximate nucleotide position of the RNA initiation site. P_L is the late promoter whose initiation sites map between nucleotides 7214 and 7256. *LCR* designates the long control region, which contains the origin of DNA replication (nucleotides 7911 to 22) (310,328) and the constitutive (*CE*) transcriptional enhancer (nucleotides 7162 to 7275) (312). The early (A_E) and late (A_L) poly(A) sites are located at nucleotides 4203 and 7175, respectively.

The coding strand for each of the papillomaviruses contains about 10 designated translational ORFs that are classified as either early (E) or late (L) ORFs, based on their location in the genome. The early ORFs of the BPV-1 genome are located within the fragment of the BPV-1 genome, which was sufficient for inducing cellular transformation (177). The early region of the viral genome is expressed in nonproductively infected cells and in transformed cells (117). The early region of the papillomavirus genomes encodes viral regulatory proteins, including those viral proteins that are necessary for initiating viral DNA replication. The *L1* and *L2* ORFs encode the viral capsid proteins and are expressed only in productively infected cells (9). The position, size, and function of many of the ORFs are well conserved among the various papillomaviruses that have been sequenced and studied in detail thus far (7,37). The individual ORFs whose functions have been well characterized are described in more detail in the appropriate sections of this chapter.

There is a region in each of the papillomavirus genomes in which there are no ORFs. The region varies slightly in size among the different papillomavirus genomes; in the BPV-1 genome, it is about 1 kb in size. This region has been referred to by several terms, including the *long control region* (LCR), the *upstream regulatory region*, and the *noncoding region.*

VIRUS REPLICATION

As noted earlier, the papillomaviruses are highly species specific, induce squamous epithelial tumors and fibroepithelial

tumors in their natural hosts, and have a specific tropism for squamous epithelial cells. The productive infection of cells by the papillomaviruses can be divided into early and late stages. These stages are linked to the differentiation state of the epithelial cell. Histologically, the lesions induced by papillomaviruses share a number of features. For papillomavirus infections of cutaneous (keratinized) epithelia, there is thickening of the epidermis (acanthosis), usually with some degree of papillomatosis. Keratohyalin granules are often prominent in the granular layer of a keratinized epithelium, and occasionally, basophilic intranuclear inclusions can be detected in the cells of the upper layer of the epidermis. These histologic features reflect the biologic properties of the papillomaviruses; most likely, these morphologic changes are induced by specific viral gene products. The specific tropism of the papillomaviruses for squamous epithelial cells is evidenced by the restriction of the viral replication functions, such as vegetative viral DNA synthesis, the production of viral capsid proteins, and the assembly of virions, to keratinocytes in the process of terminal differentiation. The close link of the papillomavirus life cycle with the differentiation program of the squamous epithelium is depicted in Figure 4.

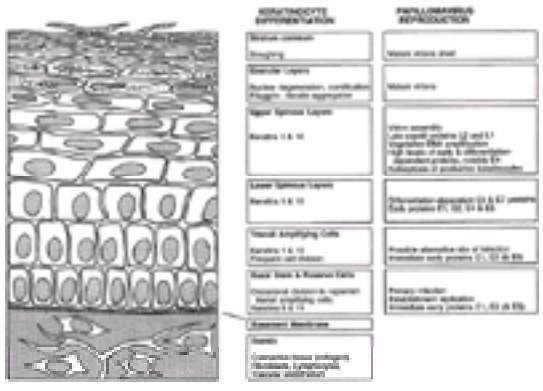

FIG. 4. Differentiation of normal cutaneous squamous epithelium and papillomaviral activities in productively infected benign lesions. The various epithelial strata and the host-differentiation, stage-specific, gene-expression profile are indicated in the *left* and *center* panels. In nonkeratinized squamous epithelia such as cervical or laryngeal, keratins 4 and 13 are expressed in the place of keratins 1 and 10 in the differentiated cells. Although profilaggrin is expressed, there is no granular layer or stratum corneum in nonkeratinized squamous epithelia. The viral activities in the corresponding strata during productive infection shown on the *right* have been determined or inferred from *in situ* hybridization studies. (From ref. 46, with permission.)

Because the basal cell is the only cell in the squamous epithelium capable of dividing, the virus must infect the basal cell to induce a lesion that can persist. By *in situ* hybridization, it has been demonstrated that the viral DNA is indeed present within the basal cells and the parabasal cells of a papilloma (263). Furthermore, using probes to the early gene regions of the papillomaviruses, viral transcripts have been detected in the basal cells of the epidermis (283), and at least some early viral protein is found in basal cells (33).

Late gene expression, synthesis of capsid proteins, vegetative viral DNA synthesis, and assembly of virions occur only in terminally differentiating squamous epithelial cells. The link of viral DNA replication to the state of differentiation of the squamous epithelial cell is illustrated in Figure 5B, which shows an *in situ* hybridization of a BPV-1–induced fibropapilloma. Using a BPV-1 DNA probe, which can hybridize to denatured viral DNA, positive hybridization can readily be detected in the nuclei of cells in the middle and upper layers of the squamous epithelium, but not within the cells of the lower portion of the epithelium or within the fibroblasts in the dermis. This represents a relatively short exposure and is only sensitive enough to detect viral DNA in those cells in which there has been significant amplification of the viral DNA (i.e., vegetative viral DNA replication). Thus, vegetative viral DNA synthesis occurs only in differentiating keratinocytes and is therefore linked to the differentiation program of the squamous epithelial cell. The regulation of expression of the genes encoding the capsid protein is at the level of RNA, as demonstrated in the *in situ* hybridization analysis depicted in Figure 5C, in which a probe complementary to the late viral mRNAs localizes the transcripts to the more terminally differentiated keratinocytes within the wart. Conversely, the major capsid protein (L1) can only be detected in the more superficial cells of the wart (139). The replication cycle for the papillomavirus is depicted in Figure 6 and is discussed in detail below in the appropriate sections.

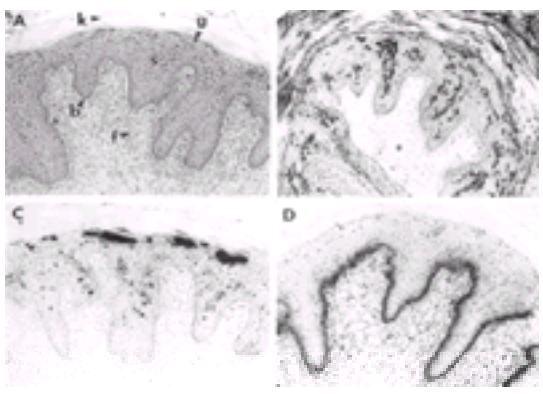

FIG. 5. *In situ* hybridization of a bovine fibropapilloma induced by BPV-1 revealing the linkage of the papillomavirus replication cycle to the squamous epithelial cell differentiation program. **A**: Control section with a sense probe and no denaturation: keratinized horn (*k*), granular layer (*g*), basal layer (*b*), and dermal fibroma (*f*). **B:** Hybridization of a sense probe to a denatured section revealing cells with a high copy number of viral DNA molecules owing to vegetative viral DNA replication. **C:** Hybridization with a probe complementary to the L1 open reading frame indicating late gene expression in the more differentiated epithelial cells of the epithelium. **D:** Hybridization with a probe complementary to a spliced message specific for the E5 transcript revealing maximal expression in the basal cells and some expression in

the dermal fibroblasts. (From ref. 14, with permission.)

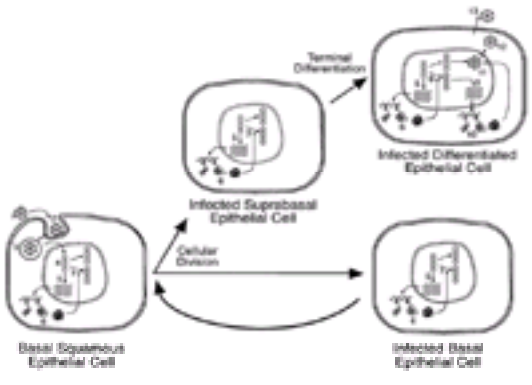

FIG. 6. Replication cycle of a papillomavirus. To establish a wart or papilloma, the virus must infect a basal epithelial cell. Our knowledge is limited about the initial steps in the replication cycle such as attachment (1), uptake (2), endocytosis (3), and transport to the nucleus and uncoating of the viral DNA (4). Early-region transcription (5), translation of the early proteins (6), and steady-state viral DNA replication (7) all occur in the basal cell and in the infected suprabasal epithelial cell. Events in the viral life cycle leading to the production of virion particles occur in the differentiated keratinocyte: vegetative viral DNA replication (8), transcription of the late region (9), production of the capsid proteins L1 and L2 (10), assembly of the virion particles (11), nuclear breakdown (12), and release of virus (13).

Attachment, Entry, and Uncoating

The first step in viral infection is the binding of the virion to the cell. Binding studies with radiolabeled virions have revealed that the papillomaviruses can bind a wide variety of cell types in addition to the normal host cell, the squamous epithelial cell (197,237). Therefore, the specific tropism of these viruses for keratinocytes does not appear to be due to a cell-type–specific receptor. This observation is consistent with studies with the BPV-1, which have shown that this virus can infect and transform a wide variety of cells, including rodent fibroblasts.

The receptors by which papillomaviruses bind and enter cells have not been unequivocally identified. The a_6 integrin was first identified as a candidate receptor for the papillomaviruses based on studies showing that papillomavirus VLPs bound a protein that was identified as a6 and that antibodies to a6 could block binding of VLPs to cells (85). Recent studies have established that the expression of the a6 integrin in a receptor-negative cell line is sufficient to confer papillomavirus binding to the cell, providing functional evidence for the role of a6 as the receptor (189). The a6 integrin partners with a b integrin subunit, either b1 or b4, on the cell surface. The a6b1 integrin is expressed on a wide range of cells, including platelets, lymphocytes, endothelial cells, and so forth, whereas a6b4 is on relatively restricted epithelial cells, mesenchymal cells, and some neuronal cells (189). Papillomaviruses can bind cells expressing either the a6b1 or the a6b4 complex, although the cells with the highest degree of papillomavirus binding match the a6b4 expression profile. The expression of a_6 is widespread, and it is well conserved among species, consistent with the criteria identified for the papillomavirus receptor from the previous binding studies (237). Although evidence suggests that the a6b4 complex is an attractive candidate receptor for papillomaviruses, it is apparently not obligatory because at least some papillomaviruses can infect cells that lack this complex (270). Papillomavirus virions can also bind to heparin and cell surface glycosaminoglycans on human keratinocytes, which may provide an initial binding event that could be followed by receptor binding and internalization (142).

There have been few published studies on the mechanisms by which the papillomavirus enters the cell, gains entry to the nucleus, or uncoats its DNA. It is presumed that these steps proceed similarly to the mechanisms used by the polyomaviruses. In one study using BPV-1, the uptake of the virions was complete by 90 minutes, with most of the capsid antigen localized in the nucleus (337). The virus particles were transported in phagosomes, and their uptake and transport could be inhibited by cytochalasin B and paclitaxel (Taxol), suggesting the possible involvement of microfilaments and microtubules in these processes. Although binding to the plasma membrane and uptake of virions into large cytoplasmic vesicles could be monitored by electron microscopy, no complete virions were observed in the nucleus of infected cells despite a very strong nuclear fluorescent staining for both L1 and L2 proteins (337). This observation suggests that disintegration of the virion occurs in the cytoplasm and that the L1 and L2 proteins may migrate to the nucleus through their nuclear localization signals.

Transcription

The replicative phase of the papillomavirus life cycle has been difficult to study because of its link to the terminal differentiation program of the epithelium. Historically, BPV-1 has served as the prototype for analyzing the papillomavirus transcription program. The studies have been carried out in a variety of systems, including viral RNAs from rodent cells transformed by BPV-1 as well as those from infected wart tissues. Viral transcription studies have been extended to some of the HPVs associated with genital tract lesions, such as HPV-11, HPV-16, HPV-18, and HPV-31, by using HPV-positive clinical lesions, xenograft tissue in nude mice, cervical carcinoma cell lines, and, more recently, organotypic cultures. This section focuses on BPV-1 and on HPV-31, both of which have been extensively analyzed by *in vitro* culture techniques (192).

Viral RNAs and Promoters

Papillomavirus transcription is complex owing to the presence of multiple promoters, to alternate and multiple splice patterns, and to the differential production of messenger RNA (mRNA) species in different cells. Transcription has been most extensively analyzed for BPV-1, for which seven different transcriptional promoters have been identified (1,9,43,281). Their locations are indicated on the circular genomic map of BPV-1 (see Fig. 3). More than 20 different mRNA species have been identified in BPV-1 transformed cells and in the productively infected cells of fibropapillomas (6). The various RNA species and the genes products they might possibly encode are depicted in Figure 7. The known viral RNA species are often present in very low abundance, making it likely that additional RNA species exist. Most RNA species in warts use a polyadenylation site (A_e) at nucleotide 4180, downstream of the early genes. It is the only polyadenylation site used in transformed rodent cells. The mRNAs in transformed cells principally encode viral factors involved in viral plasmid replication, in the regulation of viral transcription, and in cellular transformation. A second set of RNAs, which are expressed in productively infected cells, use the polyadenylation signal (A_l) at nucleotide 7156, positioned downstream of the *L1* and *L2* ORFs. RNAs that use this late polyadenylation site are found only in the terminally differentiating keratinocytes of fibropapillomas and are absent in transformed cells and in the nonproductively infected cells of BPV-1 fibropapillomas. The control of late transcription is complex and involves at least three regulatory mechanisms, as discussed later.

FIG. 7. Transcription map of BPV-1. The genomic map is shown at the *top,* with the *black line* indicating the 69% transforming region containing the long control region (LCR) and the early-region open reading frames (ORFs). The known promoters and poly(A) sites are indicated. The structures of the species A to Q were determined from RNA species in BPV-1 transformed mouse C127 cells by cyclic DNA (cDNA) cloning, electron microscopy, nuclease protection, polymerase chain reaction (PCR), and primer extension. The 5'-most ORF, which is likely to be encoded by the messenger RNA (mRNA) species, is indicated to the *right.* Structures of additional mRNAs that appear to be unique for productively infected BPV-1 fibropapillomas (species R to X) were determined by reverse transcription–PCR and cDNA cloning. Although E2 and E4 are the first significant ORFs for the W and X species, these mRNAs could also encode the L2 capsid protein. (From ref. 6, with permission.)

Six of the transcriptional promoters for BPV-1 are active in transformed cells. These promoters are also active in productively infected fibropapillomas (9). The nomenclature that has been generally adopted for use in the papillomavirus field designates the promoters (P) with a subscript indicating the nucleotide position of the 5' end of the most abundant RNA species expressed from the designated promoter. Thus, for BPV-1, P_{89} is the promoter that has its major transcription start at nucleotide 89. Another promoter, P_{2443}, gives rise to RNA species with 5' ends heterogeneously mapping in the vicinity of nucleotide 2443. The six promoters active in transformed cells that have been mapped to date are P_{89}, P_{890}, P_{2443}, P_{3080}, P_{7185}, and P_{7940} (1,9,43,281,282). One additional promoter, P_{7250}, is referred to as the *major late promoter* (P_L) and is principally active in productively infected keratinocytes, giving rise to the late mRNA species containing the *E4* and *L1* ORFs (9).

A variety of mRNA and promoter mapping studies have also been carried out with the genital tract–associated papillomaviruses, most notably HPV-11, HPV-16, HPV-18, and HPV-31. A transcription map of HPV-31 is shown in Figure 8. As with BPV-1, multiple promoters are involved in generating the various mRNA species for the genital tract HPVs. For HPV-31, P_{97} is the major promoter active in nonterminally differentiated cells. This promoter, which directs the expression of E6 and E7 as well as several other early gene products, is analogous to P_{97} of HPV-16 and P_{105} of HPV-18. Upon differentiation of immortalized keratinocytes harboring episomal HPV-31 DNA, there is activation of the differentiation-dependent late promoter P_{742}, which directs the expression of the late gene products, including E4, L1, and L2, as well as an increase in the level of the E1 mRNA (153,244).

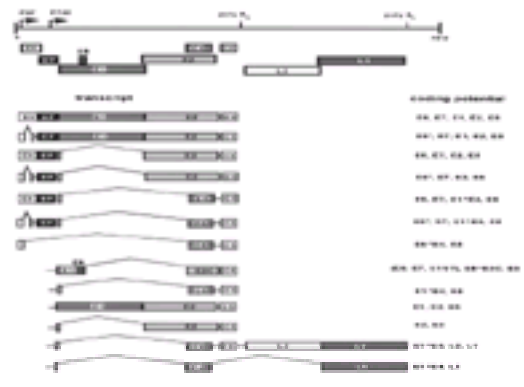

FIG. 8. Transcription map of HPV 31 (134,135,153,213). A linearized version of the genomic map is shown at the *top.*

Transcripts initiated at the early viral promoter, designated P_{97}, are expressed in the nonterminally differentiated cells in the lower portion of the epithelium, whereas those initiating at the late promoter (P_{742}) are expressed upon differentiation in the cells committed to the replication of the progeny virions. (From ref. 289, with permission.)

An important difference in the structures of the E6 and E7 mRNAs and in the manner by which they are expressed distinguishes the high-risk and low-risk HPV types. As discussed in detail in Chapter 66, the HPVs associated with the genital tract lesions can be divided into high-risk (e.g., HPV types 16 and 18) and low-risk (e.g., HPV types 6 and 11) categories based on the risk for malignant progression of the lesions they induce in the cervix. For the high-risk HPVs, such as HPV-16 and HPV-18, a single promoter (P_{97} for HPV-16 and HPV-31, or P_{105} for HPV-18) directs the synthesis of mRNAs with *E6* and *E7* intact or with splices in the *E6* gene (see Fig. 8). The species with *E6* intact could be translated into E6 but not E7 because there is insufficient spacing for translation reinitiation. The mRNAs with the spliced *E6* splice the 5' end of the *E6* ORF (referred to as E6*) to a translation frame with stop codons that provides sufficient spacing for translation reinitiation of the *E7* ORF and are therefore likely to represent the E7 mRNAs. In contrast, the *E6* and *E7* genes of the low-risk HPVs, such as HPV-6 and HPV-11, are expressed from two independent promoters (46).

Regulation of Transcription (cis Elements)

Papillomavirus transcription is tightly regulated by the differentiation state of the infected squamous epithelial cell. This is evident in part through the analysis of the differential expression of viral RNAs in the cells in the different levels of the epithelium in warts (14,47). It is also evident *in vitro* through the analysis of infected keratinocytes using organotypic and suspension tissue culture systems that permit epithelial cell differentiation (72,153,191). Cells transformed by bovine papillomavirus have provided a useful model for examining transcriptional regulation in the nonproductively infected cell, in which transcription is also tightly regulated. The genomes of the papillomaviruses contain multiple cis regulatory elements and encode several transcriptional factors that modulate viral gene expression.

The LCR region of papillomavirus contains enhancer elements that are responsive to cellular factors as well as to virally encoded transcriptional regulatory factors. All of the viral LCRs that have been studied in detail have been found to contain constitutive enhancer elements that have some tissue or cell-type specificity (245). This was first established for HPV-16 and HPV-18 (55,297) and has also been shown for HPV-11 (42) as well as for BPV-1 (312,313). It is thought that these constitutive enhancer elements are essential for the initial expression of the viral genes after virus infection and that they may also be important in the maintenance of viral latency. A number of transcription factor–binding sites have been identified in the LCRs of the various papillomaviruses that have been carefully studied. Included among them are sites that bind AP1, SP1, Oct-1, and YY1, among others (17,36,44,45,123,210,299). The HPV-16 LCR has also recently been shown to contain nuclear matrix attachment sites that may be important for controlling viral gene expression (295).

In addition to the binding sites for cellular transcription factors, the LCR contains binding sites for the virally encoded E2 regulatory proteins and the origin of DNA replication that binds the E1 replication factor, discussed in some detail later.

E2 Regulatory Proteins

The papillomavirus *E2* gene products are important regulators of viral transcription and replication. The *E2* gene product of BPV-1 was first described as a transcriptional activator (278) capable of activating viral transcription through E2-responsive elements located within the viral genome (276). The E2 proteins are relatively well conserved among the papillomaviruses in two domains: a sequence-specific DNA-binding and dimerization domain located in the carboxyl-terminal region of the protein, and a transactivating domain that is located within the amino-terminal half of the protein (99,187). These two domains are separated by an internal hinge region, which is not well conserved in size nor in amino acid composition among different papillomaviruses. The E2 proteins bind the consensus sequence, $ACCN_6GGT$ (2,174), and can regulate transcription from promoters containing E2-binding sites (112,122,275). E2 binds $ACCN_6GGT$ motifs as a dimer; the DNA-binding dimerization domain localizes to the carboxyl terminus of E2 (188).

The E2 proteins have been best studied in the BPV system, where three species have been identified (Fig. 9). The full-length protein (E2TA) can function as a transactivator or a repressor, depending on the location of the E2-binding sites within the enhancer or promoter region (Fig. 10). The two shorter forms of E2, called E2TR and E8/E2, have been described as repressors because they can inhibit the transactivation function of the full-length E2TA (161,162). The shorter E2 proteins contain the DNA-binding and dimerization domains of the C-terminus but lack the transactivation domain. E2TR and E8/E2 can inhibit the transcriptional transactivating function of the full-length polypeptide by competing for its cognate DNA-binding sites and by forming inactive heterodimers with the full-length transactivator protein. The crystal structure of the dimeric DNA-binding domain of BPV-1 E2 revealed a previously unobserved structure for a DNA-binding protein, which is a dimeric antiparallel b-barrel (116). The crystal structure of the N-terminal transactivation domain has also been resolved for HPV-16 and HPV-18 (5,110). The structural studies of the E2 N-terminal domain indicated that it could form a dimer both in the crystal and in solution. Because amino acids that are necessary for transactivation are located at the dimer interface, the dimer structure may be important in the interactions of E2 with viral and cellular transcription factors. The dimer formation may contribute to the stabilization of DNA loops, which may serve to relocate distal DNA-binding transcription factors to the site of human papillomavirus transcription initiation (5).

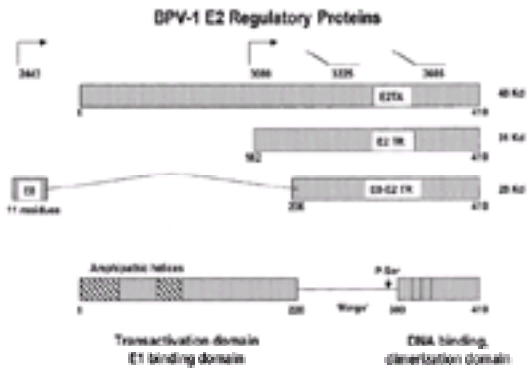

FIG. 9. Structure of the BPV-1 *E2* gene products. The structures of the three known proteins encoded by the BPV-1 *E2* open reading frame are indicated. The 48-kd full-length E2 transactivator can be expressed from an unspliced message from P_{2443} or from a spliced messenger RNA (mRNA) from upstream promoters by using a splice acceptor at nucleotide 2558. The 31-kd and 28-kd forms of the repressor are expressed from P_{3080} and as an E8:E2 fusion by a spliced mRNA as shown, respectively. The transactivation domain consists of a region of about 200 amino acids at the N-terminal region of the full-length E2 protein that is relatively well conserved among papillomaviruses. This region, which is acidic and is predicted to contain amphipathic helices, is only present in the full-length form of E2. The N-terminus of E2 contains the site for binding E1. The 110 C-terminal amino acids are also conserved and make up the DNA-binding and dimerization domain. (Modified from ref. 188, with permission.)

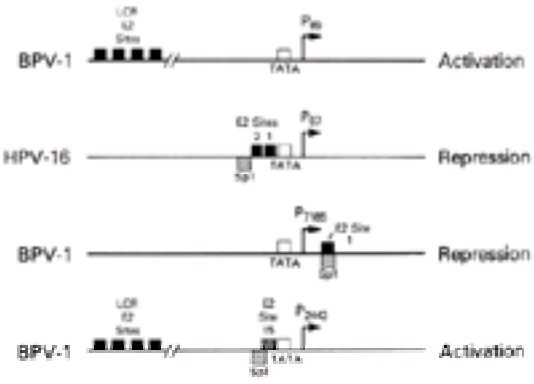

FIG. 10. Papillomavirus E2 transcriptional regulation. Transcriptional activation or repression by E2 depends on the position and proximity of the E2-binding sites in the promoter with respect to the promoter. In this figure, only the E2-binding sites in close proximity to the promoters are indicated. Several E2-binding sites in the BPV-1 long control region (*LCR*) are positioned several hundred base pairs upstream of P_{89}, which is activated by E2 (111,276). The HPV-16 P_{97} promoter (and the analogous P_{105} and P_{97} promoters in HPV-18 and HPV-31, respectively), which directs the synthesis of the E6 and E7 viral oncoproteins, is repressed by E2 through occupancy of E2-binding sites, which overlap an SP1 site and are in close proximity to the TATA box (22,298). It is thought that through displacement of SP1 from the promoter and interference with the formation of the transcription preinitiation complex, E2 may repress transcription. In a similar manner, E2 may repress the BPV-1 P_{7185} promoter by interfering with SP1 binding to a site downstream of the promoter (280,312). The P_{2443} promoter has a low-affinity E2-binding site overlapping an SP1 site, but this does not appear to play an important role in its regulation. The promoter is activated by E2-binding sites located 2.5 kb upstream in the LCR (120,277). (From ref. 188, with permission.)

Mutations in the BPV-1 *E2* ORF have pleiotropic effects on the virus, disrupting transformation, replication, and transcriptional regulation functions. Studies have shown that expression of the early region viral genes is under the control of the viral *E2* gene product through E2-responsive elements located within the viral LCR. The BPV-1 genome contains 17 E2-binding sites (ATTN$_6$GGT), of which 12 are located in the LCR (174). Pairs of the E2-binding sites are sufficient to function as an E2-dependent enhancer (2,113,275). The major E2-dependent enhancer in the LCR, E2RE1, contains four high-affinity E2-binding sites (276) and is located upstream of the P_{89} promoter complex. In addition to activating the P_{89} promoter, E2 can also activate each of the early region promoters (P_{890}, P_{2443}, and P_{3080}) through the E2-dependent enhancer elements in the LCR. Despite this dependence on a common E2-responsive element in the LCR, the various promoters have different sensitivities to the E2 transactivator (293). These differences are most likely due to specific promoter elements in the vicinities of the individual promoters. For instance, there is an SP1 site upstream of the P_{2443} promoter that is necessary for both its basal and E2-transactivated expression (277).

E2 transcriptional regulation has also been well studied for the genital tract–associated HPVs. The binding of E2 to its cognate sites within the LCR of the HPV genomes results in the modulation of viral promoter activity. The *E6* and *E7* transforming genes of HPV-16 and HPV-18 are transcribed from the major early promoter (P_{97} and P_{105}, respectively) contained within the LCR of their respective genomes (272,300). Analyses of promoter activity in human epithelial cells have shown that the P_{97} promoter of HPV-16 and the P_{105} promoter of HPV-18 possess a basal activity that can be repressed by full-length *E2* gene products (22,241,298,300). There are four E2-binding sites within the LCRs of the HPV-16 and HPV-18 genomes. Two E2-binding sites are located immediately adjacent to the TATA box of the P_{97} and P_{105} promoters of HPV-16 and HPV-18, respectively. The basal activity is dependent on the keratinocyte-dependent enhancer contained within the LCR, and E2 repression occurs through binding to the ACCN$_6$GGT sites in close proximity to the TATA box of the early promoter, most probably by interfering with the assembly of the preinitiation transcription complex (22,55,241,291,300). In the context of the full genome, the modulation of HPV gene expression by *E2* is even

more pronounced. In human keratinocyte immortalization assays dependent on the expression of the *E6* and *E7* genes, an intact *E2* gene can decrease the efficiency of HPV-16 immortalization (240). E2 can also suppress the growth of HPV-positive cervical cancer cell lines through transcriptional repression of the viral *E6* and *E7* genes (76,89,136,208,300). These results are consistent with the model that at certain concentrations, E2 can repress the viral P97 promoter through binding to the E2 DNA-binding sites in the proximity of the promoter. In contrast to the repression mediated by E2 on the expression of the high-risk HPVs, in BPV-1, the full-length E2TA stimulates the expression of a number of viral genes, including *E2* itself and *E5* (120,229). This finding has raised the question of whether the transcriptional activation function of E2 is essential for the high-risk HPVs. Indeed, it was recently shown that an HPV-31 genome that carried a mutant *E2* gene, which was defective for transactivation but competent for DNA replication, could still be established as a stable episome and could induce differentiation-dependent late functions (288). Thus, the specific role of E2 as a transcriptional regulator may vary among different papillomavirus types.

E2 is a multifunctional protein. Its functions as a transcriptional activator and repressor are likely mediated by interactions with specific cellular factors, some of which have now been identified (Table 1). In addition to its role as a transcriptional regulator, E2 has critical roles in viral DNA replication and in plasmid maintenance. E2TA, together with the viral E1 protein, is essential for viral DNA replication (41,62,309). E2 may serve as an auxiliary protein in viral DNA replication. This aspect has been best studied in the BPV system, in which E2 was first shown to complex with E1 and to strengthen the affinity of E1 for binding to the origin of DNA replication (195,309). Long-term maintenance of plasmids containing a papillomavirus origin of DNA replication, however, also requires a minichromosome maintenance element, a cis element consisting of multiple E2-binding sites (226). The role of E2TA in plasmid maintenance appears to be due to its role in linking the viral plasmids to the cellular mitotic chromosomes, thereby ensuring that the viral genomes are enclosed within the nuclear envelope for when it reforms during telophase (16,170,271).

Associated cellular proteins	Functional consequences	References
TATA-binding protein (TBP)		233
TFIIB		20, 233
AMF-1/Gps2	Enhance transcriptional activation	30
	Enhance E2 interaction with p300	217
YY1		166

TABLE 1. *Cellular targets and functions of the papillomavirus E2 oncoproteins*

Late Gene Expression

The viral late functions, such as vegetative viral DNA synthesis, capsid protein synthesis, and virion assembly, occur exclusively in differentiated keratinocytes. Transcriptional regulation of the late genes has been most extensively studied for BPV-1, HPV-11, HPV-16, and HPV-31. For each of these viruses, there is a specific promoter, which becomes active only in terminally differentiated keratinocytes. For BPV-1, the late RNAs are transcribed from a wart-specific or late promoter (P_L) at nucleotide 7250 that is located in the 5' end of the LCR (9) (see Fig. 7). Both the *L1* and *L2* genes are expressed from mRNAs transcribed from P_L (14). In addition to the L1 and L2 mRNA species, this promoter also gives rise to other viral mRNAs that are specific for terminally differentiated cells. For instance, one abundant mRNA from P_L gives rise to the message that appears to encode the E4 protein (9), an abundant cytoplasmic protein found predominantly in the differentiated keratinocytes of the wart (73). Thus, although E4 is located in the early region of the viral genomes (see Fig. 3 and Fig. 4), it is a late protein by virtue of the promoter from which it is expressed. Interestingly, the late promoters for the human papillomaviruses that have been analyzed do not map to the LCR. Instead, a differentiation-specific promoter has been identified within the E7 coding region of the HPV-31 genome that gives rise to mRNA species whose 5' ends map to nucleotide 742 (134) (see Fig. 8). The late promoter in HPV-11 also maps to the E7-coding region (46).

Polyadenylation of the BPV-1 L1- and L2-specific mRNAs occurs 3' to the *L1* ORF at nucleotide 7175, a site not used in transformed cells and therefore considered the late poly(A) site (A_L) (9) (see Fig. 7). In the HPVs that have been analyzed, polyadenylation occurs in a similar region of the genome, 3' to the *L1* ORF (see Fig. 8).

Papillomavirus *L1* and *L2* gene expression is also regulated at a posttranscriptional level. There are cis-acting elements that have been described for BPV-1, HPV-16, and HPV-1, which serve important roles in regulating late gene expression for these viruses at a posttranscriptional level. In the 3'-untranslated regions (3'-UTRs) of the late RNAs of each of these viruses, there are negative regulatory elements that can inhibit the generation of stable late mRNAs. In the BPV-1 late 3'-UTR, there is an unused 5' splice element that binds the U1 small nuclear ribonuclear protein and inhibits late polyadenylation, thereby favoring use of the early polyadenylation site (93,94). A negative regulatory element in the HPV-16 3'-UTR contains multiple 5' splice-like sequences, as well as an inhibitory GU-rich region that reduces mRNA stability, that bind to (65,147,154) three different cellular factors (the U2 auxiliary splicing factor 65-kd subunit, the cleavage stimulation factor 64-kd subunit, and the Elav-like HuR protein) (154). In HPV-1, an AU-rich inhibitory region has been identified in the 3' UTR that also binds the Elav-like HuR protein (273). The model emerges that these, and perhaps additional cellular factors, are responsible for the nuclear retention or cytoplasmic instability of the nuclear retention element (NRE)–containing late transcripts. Keratinocyte differentiation would then lead to changes in these cell-encoded factors, thus relieving the inhibition of late mRNA processing.

E4 Proteins

The *E4* ORF of the papillomaviruses is located in the early region (see Fig. 3, Fig. 7, and Fig. 8), yet it is expressed as a late gene with a role in productive infection. It overlaps the *E2* ORF in a different reading frame and therefore encodes a protein with an entirely different amino acid sequence. In general, the *E4* gene is not highly conserved among the papillomaviruses. Transcripts that could encode E4 proteins have been described both for BPV-1 and the HPVs (see Fig. 7 and Fig. 8). A viral transcript formed by splicing a few codons from the beginning of E1 to E4 appears to be the major RNA in HPV-induced lesions for the viruses that have been studied. In papillomas, the E4 proteins are expressed primarily in the differentiating layers of the epithelium, and the E4 proteins are expressed in cells in which vegetative viral DNA replication is ongoing (75). The expression of E4 is not coincident with the expression of the capsid proteins (75), and E4 expression precedes the expression of L1 (56). Although the E4 proteins are expressed at high levels in infected tissues, their precise role in the viral life cycle is unclear. E4 proteins are not found in the virion particles. Mutational analysis of the *E4* gene in BPV-1 showed that E4 was not essential for viral transformation or viral DNA replication (119,204).

In cultured epithelial cells, the E4 proteins are associated with the keratin cytoskeleton (74,236,239). The HPV-16 E4 protein has been shown to induce the collapse of the cytokeratin network (74,236), suggesting that it may function to aid the virus in its egress from the cell. A dramatic collapse of the keratin intermediate filaments has not, however, been observed with cells expressing HPV-1 E4, raising some question as to the generality of this effect (236,239). The available data are compatible with the possibility that E4 may contribute to vegetative DNA replication or to altering the cellular environment in a manner that may favor virus synthesis or perhaps virus release.

Virus Assembly and Release

Little is known about the papillomavirus assembly or release. Virus particles are observed in the granular layer of the epithelium and not at lower levels. The virus is not believed to be cytolytic, and the release of the virion particles does not occur before the cornified layers of a keratinized epithelium (see Fig. 4 and Fig. 5).

VIRAL DNA REPLICATION

The papillomaviruses have three modes of viral DNA replication. The first occurs during the initial infection of a basal keratinocyte by the virus, when there is an amplification of the viral genome to about 50 to 100 copies. Little is known of this process. The next phase is one of genome maintenance, which occurs in dividing basal cells of the lower portion of the epidermis as well as in the dermal fibroblasts in fibropapillomas such as those induced by BPV-1. In these cells, the viral DNA is maintained as a stable multicopy plasmid. The viral genomes replicate an average of once per cell cycle during S phase in synchrony with the host cell chromosome (98) and may be faithfully partitioned to the daughter cells. This type of DNA replication ensures a persistent and latent infection in the stem cells of the epidermis. The third type of DNA replication is vegetative DNA replication, which occurs in the more differentiated epithelial cells of the papilloma. In these cells, which no longer undergo cellular DNA synthesis, one observes a burst of viral DNA synthesis, generating the genomes to be packaged into progeny virions.

Plasmid Maintenance

Plasmid maintenance has been examined in greatest detail in BPV-1 transformed mouse cells, in which the viral genome exists as a stable multicopy plasmid (163). This system has provided a model for studying nonvegetative viral DNA replication, and it has been assumed that viral DNA replication in these mouse cells may be analogous to the stable plasmid replication seen in the dermal fibroblasts of a fibropapilloma and in the basal keratinocytes of a papillomavirus–associated lesion. During the maintenance phase, the viral genome copy number remains relatively constant for many cell generations.

Origin of DNA Replication

The origin of BPV-1 DNA replication was initially localized to the LCR by electron microscopic analysis of replicative intermediates isolated from BPV-1 transformed rodent cells harboring extrachromosomal viral DNA (317). Papillomavirus DNA replication requires the origin of DNA replication in cis and the viral E1 and E2 proteins in trans. *In vivo,* the minimal origin of replication (nucleotides 7911 to 7927) contains an A+T-rich region (ATR), the E1-binding site that includes a region of dyad symmetry (DSR), and an E2-binding site (310) (Fig. 11). The origins of DNA replication have been mapped for a number of the HPVs and contain similar ATR and DSR domains adjacent to E2-binding sites (41,62). Furthermore, in transient replication assays, the viral E1 and E2 proteins can function in trans on replication origins from heterologous papillomavirus genomes (41). Origin-dependent DNA replication of BPV-1 can be achieved *in vitro* with cell extracts containing high levels of E1 alone in the absence of E2 (267,328).

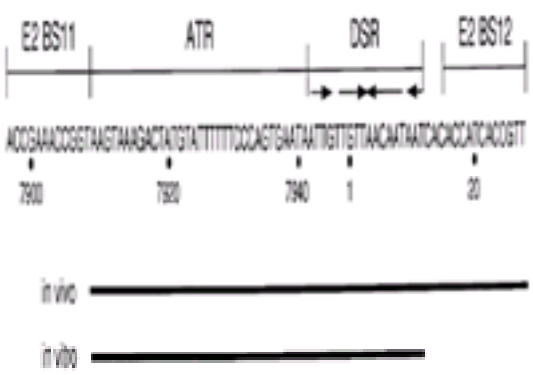

FIG. 11. The BPV-1 origin of DNA replication. The minimal origin for *in vivo* DNA replication (nucleotides 7911 to 7927) requires the A+T-rich region (ATR), the dyad symmetry repeats (DSR), and a binding site for the E2 protein. For *in vitro* DNA replication, there is a requirement only for the ATR and DSR.

E1 Protein

Genetic studies have revealed that stable BPV-1 plasmid replication requires the expression of the viral *E1* and *E2* genes. Initial studies of mutated BPV-1 genomes implicated both E1 and E2 as essential for stable viral DNA replication (70,230) and for transient viral DNA replication (309). However, E1 is the only viral factor that is directly involved in plasmid replication; E2 has auxiliary roles in DNA replication and in plasmid maintenance as discussed below. The *E1* ORF is the largest ORF in the papillomavirus genome and is relatively well conserved among all of the papillomaviruses (Fig. 12). The E1 proteins share structural similarities with portions of the SV40 large tumor antigen (TAg), which is an essential replication protein of that virus. The similarity includes regions of TAg with ATPase, helicase, and nucleotide-binding activities (49,267). The BPV-1 E1 protein is a 68-kd nuclear phosphoprotein that binds specifically to the origin of replication (304,310,324). By itself, E1 binds the origin with weak affinity. However, the binding of E1 to the origin is stabilized through its interaction with E2 and the binding of E2 to its cognate sites adjacent to the origin (195,310).

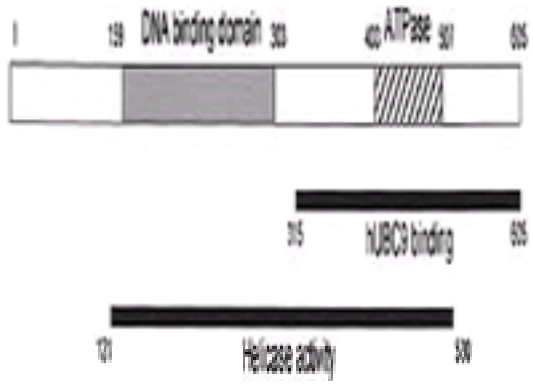

FIG. 12. Schematic representation of the BPV-1 E1 replication protein. The DNA-binding domain of E1 has been mapped to amino acid residues 159 to 303 for BPV-1. The crystal structure of the DNA-binding domain of BPV-1 E1 has recently been determined and shown to resemble (despite an amino acid sequence identity of only 6%) the DNA-binding domain of SV40 tumor antigen (84). The E2-binding domain maps to the C-terminal 266 amino acids.

The papillomavirus E1 protein has DNA-dependent ATPase and DNA helicase activities (28,267,329). E1 is required for both the initiation and elongation of viral DNA synthesis (175). In addition to its interaction with E2, E1 has been shown to bind a number of cellular proteins. E1 interacts with the p180 subunit of the cellular DNA polymerase a-primase and presumably thereby recruits the cellular DNA replication initiation machinery to the viral replication origin (26,215). Several additional host cellular proteins have been found to bind E1, including histone H1 (292), SW1/SNF5 (165), cyclin E/Cdk2 (58,181), Hsp40/Hsp70 (176), and Ubc9 (231,332). Although the physiologic significance of some of these interactions remains to be determined, several appear to be quite interesting. In particular, the efficient cell cycle–regulated replication of papillomavirus genomes is dependent on the association of E1 with the S-phase–specific cyclin E–Cdk2 complex (58). In addition, the interaction of E1 with Ubc9 is required for efficient origin-dependent replication (332). E1 is SUMO-1 modified by Ubc-9, and this modification is required for the intranuclear accumulation of E1 (232).

In addition to the 68-kd protein encoded by the entire gene, the 5' end of BPV-1 E1 encodes a protein with an apparent molecular weight of 23,000 (303). Although the protein has been detected by BPV-1 transformed cells, no function has yet been ascribed to this N-terminal E1 protein (129).

Role of the E2 Proteins

As noted previously, papillomavirus DNA replication also requires the viral E2 protein (309,328). A requirement for E2 in origin-dependent DNA replication has been shown for the HPVs as well as BPV-1 in transient assays. Although not essential for origin-dependent DNA replication *in vitro,* E2 greatly stimulates the ability of E1 to initiate DNA replication (328). E2 interacts with E1 (24,179,195) and greatly enhances the ability of E1 to bind the replication origin (195,264,268). E2 can relieve nucleosome-mediated repression of papillomavirus DNA replication *in vitro* (172). E2 may also stimulate viral DNA replication by recruiting host replication factors to the origin. One of these host factors is RPA (replication protein A), which is a single-stranded DNA-binding protein that can bind to acidic transactivation domains such as the one found in E2 (173). The E1:E2 complex is a precursor to a larger multimeric E1 complex, which after the removal of E2 can distort the replication origin and ultimately unwind the DNA (180) (Fig. 13). E2 serves as an auxiliary factor that fosters the assembly of the preinitiation complex at the origin, but E2 itself plays no intrinsic role in viral DNA replication. A hexameric form of E1 protein is associated with the ATPase and DNA helicase activities intrinsic to its initiator function in DNA replication (88,265).

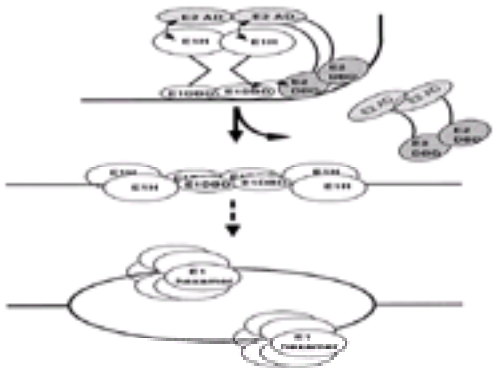

FIG. 13. Proposed pathway for the assembly of an initiation-competent complex at the BPV origin of DNA replication. The E1 initiator binds cooperatively with E2 to the ori, forming a specific E1 $_2$E2$_2$–DNA complex. As a consequence of the interaction between the E1 and E2 DNA-binding domains (*DBDs*), a sharp bend is induced in the ori DNA. The bend promotes the interaction between the E1 helicase domain (*E1H*) and the E2 transactivation domain (*E2AD*). The resulting highly sequence-specific complex serves to recognize the ori. In a reaction requiring ATP hydrolysis, E2 is displaced, and additional E1 molecules are added to the complex, resulting in the formation of a complex in which four molecules of E1 are bound to the ori. This complex can distort the DNA duplex and give rise to partially single-stranded regions. Subsequently, additional molecules are added. In a final step, E1 is assembled onto the exposed single strands, forming a hexameric ringlike structure that constitutes the replicative helicase. (Modified from ref. 84, with permission.)

E2 also has a role in genome maintenance. Plasmids containing the minimal origin of DNA replication are capable of transient replication in cells expressing E1 and E2. With time, however, the plasmids are lost. Long-term, stable plasmid maintenance requires a cis minichromosome maintenance element, which consists of multiple E2-binding sites (226). This, together with the observation that E2TA and the viral genomes are associated with the mitotic chromosomes (137,170,271), suggests that viral E2 protein–mediated attachment of the viral genomes to the host cell chromatin could provide a mechanism for the coupling of viral genome multiplication and partitioning to the host cell cycle during viral latent infection. This association would ensure the localization of the viral genomes within the nuclear envelope when it is reformed during telophase.

Model for Plasmid Replication

In the initial intracellular stages of a papillomavirus infection, the viral genome may undergo amplification for a short period of time. There is then a transition to a maintenance stage in which the plasmids replicate an average of once per cell cycle, in a manner analogous to chromosomal DNA replication. It was initially thought that stable plasmid DNA replication might involve the “marking” of the daughter viral genomes so that they did not return to the replicating pool until the next cell cycle, and that each viral plasmid molecule replicated only once per cell cycle. However, the evidence currently favors an alternative model, in which the plasmid replication is not restricted to once per cell cycle but occurs an average of once per cell cycle and does not rely on plasmid marking (98,202). However, precise mechanisms involved in regulating plasmid maintenance, as well as vegetative DNA replication, have not yet been elucidated.

Vegetative Viral DNA Replication

Vegetative replication of papillomavirus DNA is necessary to generate the genomes to be packaged in virions, a process that normally occurs only in the terminally differentiated epithelial cells of a papilloma. The mechanisms regulating the switch from plasmid maintenance to vegetative viral DNA replication are not known. The switch may involve the presence or absence of controlling cellular factors in differentiating keratinocytes. In addition or alternatively, the relative levels of viral factors such as E1 or E2 (or their modification) may change in terminally differentiating keratinocytes. A high level of BPV-1 DNA amplification occurs in transformed cells after growth arrest, in a process in which both E1 and E2 have been implicated (34,35). There have been few studies that have examined the mode of vegetative viral DNA replication in differentiated cells. One might anticipate that, as with the polyomaviruses, vegetative DNA replication occurs bi-directionally through theta structure intermediates, as it does in the maintenance replication phase. One intriguing study, however, suggests that there may be a switch from a bi-directional mode of replication to what could be a rolling circle mode (87). Additional studies on the mechanism of vegetative replication would appear to be warranted.

VIRAL TRANSFORMATION

BPV-1 Transformation

Certain papillomaviruses are capable of inducing cellular transformation in tissue culture. The most completely studied of the transforming papillomaviruses as been BPV-1. Morphologic transformation in tissue culture was first described for BPV in the early 1960s (23,25,302). In the late 1970s, a focus assay was developed using established cell lines to study BPV-1 transformation (79). In general, investigators have relied on mouse C127 cells and NIH-3T3 cells for these transformation studies, although a variety of other rodent cells, including hamster and rat cells, are susceptible to BPV-1–mediated transformation. Transformation of mouse C127 cells by BPV-1 causes alterations in morphology, loss of contact inhibition, anchorage independence, and tumorigenicity in nude mice (79).

One interesting characteristic of BPV-1 transformed rodent cells is that the viral DNA is maintained as a stable multicopy plasmid (163). Integration of the viral genome is not required for either the initiation or maintenance of the transformed state. However, transformation is dependent on the continued expression of viral DNA, as evidenced by the loss of the transformed phenotype in mouse cells which have been “cured” of the viral DNA by treatment with interferon (308). The extrachromosomal state of the viral genome, however, is not a prerequisite for this transformation because replication

defective viruses are capable of transforming cells (250). In cells in which the extrachromosomal DNA is maintained, it is likely that a high gene dosage resulting from the viral plasmid replication is necessary for efficient transformation by the wild-type genome. Cells harboring BPV-1 mutants, which maintain a low copy number of extrachromosomal DNA, do not have a transformed phenotype (21).

The initial studies with cloned BPV-1 DNA indicated that the entire viral genome was not required for transformation of rodent cells (177). A specific 69% subgenomic fragment was sufficient for inducing cellular transformation; hybrid plasmids containing this 69% fragment could be maintained as stable plasmids within transformed cells. Genetic studies have mapped the BPV-1 transforming genes to the *E5, E6,* and *E7* ORFs, as discussed later. The initial studies establishing that the BPV-1 genome could be maintained as a stable plasmid in rodent cells led to the development of BPV-1–based plasmid vectors for mammalian cells (71,248,249).

BPV-1 has three independent transforming proteins, encoded by the *E6* (258,331), *E7,* and *E5* ORFs (104,259,330). Each of these genes is contained within the 69% transforming fragment, and each is expressed in transformed cells. The *E5* gene is the major transforming gene of BPV-1 in transformed cells. This gene is highly conserved among the group of papillomaviruses that induce fibropapillomas in their natural host and have the capacity to induce fibroblastic tumors in hamsters. The *E5* gene is believed to be responsible for the proliferation of dermal fibroblasts in fibropapillomas.

In addition to the *E5, E6,* and *E7* genes that are directly involved in BPV-1 transformation, the early region also encodes genes that are indirectly involved in transformation. The products of the *E1* and *E2* ORFs that are involved in the transcriptional regulation and replication of the virus regulate the expression of the transforming genes. Initial studies with BPV-1 E2 mutants suggested they were defective for transformation (68,104,230). This defect, however, is not due to the loss of a direct transforming activity of E2 but rather occurs because E2 is necessary for sufficient expression of the E5 viral oncoprotein. Mutations in the *E1* ORF may result in a higher transforming activity as a result of an increase in the transcriptional activity of the mutant viral genome (160,257). BPV-1 E1 can repress the E2 transactivated expression of the virus (246).

BPV-1 E5 Oncoprotein

The BPV-1 *E5* ORF encodes a 44–amino acid protein (262) and is sufficient for the transformation of certain established rodent cells in culture (66,104,230,259). The amino acid sequence of E5 and its predicted structure are shown in Figure 14. Structurally, E5 is composed of two protein domains: a very hydrophobic segment at its amino terminus, which is responsible for localization of E5 in membrane fractions (262), and a carboxyl-terminal hydrophilic domain. The E5 protein is associated with intracellular membranes and is thought to be an integral membrane protein (31). In transformed cells, the E5 protein exists largely as a homodimer and is localized primarily in the Golgi apparatus and in the endoplasmic reticulum (32,33). E5 is not secreted, and the hydrophilic C-terminus extends into the lumen of the Golgi (32). The E5 protein contains two cysteine residues involved in dimer formation that are required for transformation (31,124).

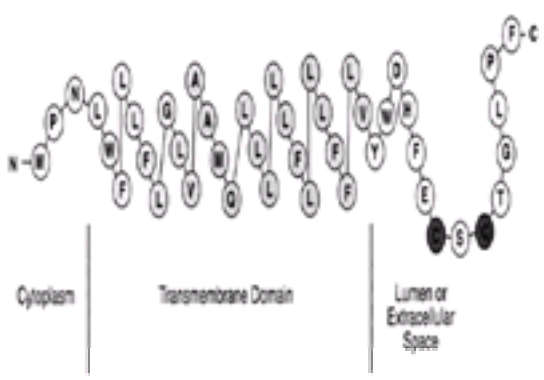

FIG. 14. Amino acid sequence and predicted secondary structure of the BPV-1 E5 oncoprotein. The 27 hydrophobic amino acids composing the first two thirds of the 44–amino acid protein (*shaded lightly*) are predicted to be in a-helical conformation. The 14 carboxyl-terminal residues are predicted to be in a nonhelical configuration. The cysteine residues, which are highly conserved among the E5 proteins of the transforming ungulate papillomaviruses, are *shaded darkly.* These cysteines mediate dimer formation (31,124).

The E5 protein does not possess intrinsic enzymatic activity and functions by altering the activity of cellular membrane proteins involved in proliferation. The first direct evidence that BPV-1 E5 can affect the activity and metabolism of growth factor receptors came from experiments that showed that E5 can cooperate with exogenously introduced epidermal growth factor (EGF) receptor or colony-stimulating factor 1 receptor in the transformation of NIH-3T3 cells (183). Subsequently, it was established that the primary endogenous target for the BPV-1 E5 protein in fibroblasts is the b-receptor for the platelet-derived growth factor (PDGF) (220). There is an increase in the level of tyrosine phosphorylation of the endogenous PDGF receptor in the E5-transformed rodent cells and bovine fibroblasts (218,220), and a prominent form of the tyrosine phosphorylated PDGF receptor in the transformed cells is an intracellular, membrane-associated, premature form of the receptor. Similarly, gene transfer experiments in heterologous cell types demonstrate that BPV-1 E5 transformation can be mediated by the PDGF b-receptor but not by a variety of other growth factor receptors (102,206). E5 activates the receptor and transforms cells in a ligand-independent manner (218,219). The mechanism of E5 activation of the PDGF b-receptor involves complex formation with the receptor, thereby inducing receptor dimerization, trans-phosphorylation of tyrosine residues in the cytoplasmic domain of the receptor, and recruitment of cellular SH2 domain–containing proteins into a signal transduction complex (67). This is depicted in Figure 15, which shows a model suggesting that the individual subunits of the E5 dimer each bind to two different molecules of

the PDGF b-receptor, resulting in receptor dimerization and activation (69). This model is supported by mutational analyses of the E5 protein–PDGF b-receptor interaction, which have identified in E5 the cysteines 37 and 39, glutamine 17, and aspartic acid 33 as essential for receptor binding (124,190,207). Similarly, mutations in the PDGF b-receptor at the transmembrane threonine 513 and juxtamembrane lysine 499 prevent complex formation and transformation (221).

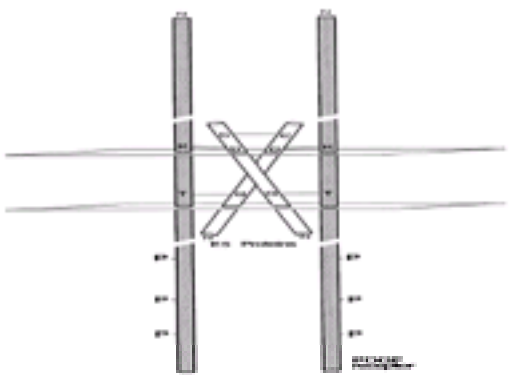

FIG. 15. Schematic model of the E5 protein–platelet-derived growth factor (PDGF) b-receptor complex. The *open rods* represent the E5 dimer, and the *shaded rods* represent two molecules of the PDGF b-receptor. The amino termini of the proteins are designated *N.* The *planes* represent the two faces of the cell membrane. The figure also shows the postulated glutamine-threonine (*Q-T*) hydrogen bond and aspartic acid–lysine (*D-K*) salt bridge linking these two proteins as well as the disulfide bond between the cysteine residues that stabilize the E5 dimer. *P* represents the phosphotyrosines in the cytoplasmic domain of the activated PDGF b-receptor. Not depicted in this figure is the 16-kd transmembrane channel-forming subunit of the vacuolar H^+-ATPase, which also binds E5 (101). (Modified from ref. 67, with permission.)

BPV-1 E5 has also been shown to form a complex with the 16-kd transmembrane channel–forming subunit of the vacuolar H^+-ATPase (101,103), an abundant cellular protein located in the membranes of intracytoplasmic membranes and plasma membranes. Golgi acidification has been found to be impaired in cells transformed by the BPV-1 E5 protein, owing to the inhibition of the vacuolar H^+-ATPase (252). It has not yet been shown directly that Golgi alkalinization plays a role in cellular transformation, but there is a good genetic correlation between the ability of specific E5 mutants to transform cells and Golgi alkalization (252). Because many important growth regulatory proteins, including the PDGF b-receptor, transit through the Golgi apparatus, it is possible that the ability of E5 to perturb the pH of intracellular organelles might affect the activity of some of these proteins and, in so doing, contribute to the transformed phenotype. It is additionally possible that the BPV-1–mediated Golgi alkalinization may affect cellular functions unrelated to transformation that are important in the life cycle of the virus.

BPV-1 E6 and E7 Oncoproteins

The BPV-1 *E6* and *E7* genes also encode proteins with transforming activities. In mouse cells, the full transformed phenotype requires the expression of the *E6* and *E7* genes as well as *E5* (203,250). Furthermore, BPV-1 E6 expressed from a strong heterologous promoter is sufficient for transformation of C127 cells (258). The E6 and E7 proteins are themselves structurally related and are conserved, at least in part, among all of the papillomaviruses. In BPV-1 transformed cells, cyclic DNA (cDNA) has been isolated and characterized, which could encode the full-length E6 protein, the full-length E7 protein, as well as the E6 fusion protein in which the amino-terminal half of E6 could be fused to portions of downstream genes (331) (see Fig. 7). To date, no studies have been done on these potential BPV-1 *E6* spliced genes. The *E6* and *E7* genes of all the papillomaviruses encode proteins with conserved structural motifs. They contain domains of almost identically spaced CYS-X-X-CYS motifs (four in *E6* and two in the carboxyl-terminal portion of *E7*). It has been postulated that the *E6* and *E7* genes may have arisen from duplication events involving a 39-codon core sequence containing one of these motifs (52). The CYS-X-X-CYS motifs found in a number of nucleic acid–binding proteins are characteristic of zinc-binding proteins. The papillomavirus E6 and E7 proteins bind zinc through these cysteine residues (13,105).

The E6 protein of BPV-1 is present at low levels in stably transformed cells. Cell fractionation experiments have localized the BPV-1 E6 protein to the nucleus and to nonnuclear membranes of transformed cells (3). No intrinsic enzymatic activities have been identified for BPV-1 E6, and like the oncogenic HPV E6 proteins, it is believed to function through binding cellular targets. Four different cellular proteins have been found to interact with BPV-1 E6: the ubiquitin protein ligase E6AP (see later), E6BP (also known as ERC55), the g subunit of the AP1 clatherin adaptor complex, and the focal adhesion protein paxillin (38,305,306,311). Unlike the oncogenic HPV E6 proteins (see later), the BPV-1 E6 interaction with E6AP does not result in the binding and degradation of p53. It is not yet known whether the interaction of BPV-1 E6 with E6AP has physiologic consequences for the cell. Similarly, no *in vivo* consequences of the interaction of BPV-1 E6 with either ERC55 or the g subunit of the AP1 on the cell have yet been determined. Analysis of a series of BPV-1 E6 mutants has shown a good correlation between the binding to paxillin and cellular transformation (59,306,311). This binding has also been shown to correlate with the disruption of the cellular actin cytoskeleton, a characteristic of transformed cells (306). E6 binds to charged leucine motifs in paxillin known as LD motifs and, in doing so, competes with the ability of paxillin to bind to vinculin and the focal adhesion kinase (307,311). Nonetheless, a detailed understanding of the mechanisms by which BPV-1 E6 transforms cells remains to be determined.

The product of the *E7* ORF has been detected in BPV-1 transformed cells (138). Although the *E7* gene by itself is not able to induce foci in transformation assays, its integrity is necessary for the fully transformed phenotype as assayed by anchorage independence and by tumorigenicity (203). BPV-1 E6 and E7 are under the negative regulatory control of the viral *E1* and *E2* genes, and in the absence of these gene products, *E6* and *E7* together are potent oncogenes that are

sufficient for the full transformation of C127 cells (314). To date, there have been no functional studies of the BPV-1 E7 protein. Unlike the HPV E7 proteins, BPV-1 E7 does not contain an LXCXE motif for binding the retinoblastoma tumor suppressor gene product (pRB), suggesting that it does not share the property of pRB binding and inactivation with the HPV E7 proteins (see later).

HPV Transformation and Immortalization

Transformation studies with the papillomaviruses have not been limited to BPV-1. Assays have also been developed for the transformation and immortalization functions encoded by those HPVs associated with anogenital cancers in humans. The HPV-16 and HPV-18 genomes are not as efficient as BPV-1 at inducing transformation of established rodent cells; however, alternative assays have been developed. DNA cotransfection with a second dominant selective marker, such as the neomycin resistance gene, and biochemical selection for the transfected cells permit HPV-16 and HPV-18 to transform established rodent cells (333). Assays employing primary rodent cells and primary human fibroblast and keratinocyte cultures have proved even more informative (78,143,185,224,228,261,318). In these assays, the high-risk HPVs, such as HPV-16 and HPV-18, are transformation positive, whereas the low-risk viruses, such as HPV-6 and HPV-11, are not (261,285). These assays have permitted the mapping of the viral genes directly involved in cellular transformation to the *E6* and *E7* ORFs of the high-risk HPV types, which are discussed in detail later.

In established rodent cells, such as the NIH-3T3 cells, the *E7* ORF scores as the major HPV transforming gene (224,296,316,319,334). HPV-16 and HPV-18 by themselves are not able to transform primary rat fibroblasts or baby rat kidney cells (159,224). However, the *E7* gene can cooperate with an activated *ras* oncogene to transform primary rat cells completely (18,174,209,224,285).

The DNA of the high-risk HPVs can also be distinguished from the DNA of the low-risk HPVs by the ability of the high-risk types to immortalize primary cultures of human fibroblasts, human foreskin keratinocytes, and human cervical epithelial cells (78,228,261,318,325). The resulting cell lines are neither anchorage dependent nor tumorigenic in nude mice, but they do display altered growth properties and are resistant in the response to signals for terminal differentiation (78,146,228,261). All of the genital tract HPV types (i.e., HPV-6, HPV-11, HPV-16, and HPV-18) are capable of transiently inducing cellular proliferation. Only the high-risk HPVs, however, are able to extend the life span and give rise to immortalized cell lines that are refractory to differentiation signals (261).

HPV E5 Oncoprotein

As noted previously, the *E5* ORF is highly conserved among those papillomaviruses that induce fibropapillomas and that readily transform rodent fibroblasts in tissue culture, including BPV-1, the deer papillomavirus (DPV), and the European elk papillomavirus (EEPV) (156), suggesting that E5 may play an important role in the tropism for fibroblasts observed *in vivo* for these viruses. Many of the other papillomaviruses that induce purely epithelial papillomas (such as CRPV and the HPVs) also contain *E5* genes with the potential to encode short hydrophobic peptides. The structural similarity of these peptides to the BPV-1 E5 protein has prompted studies of the potential transforming activities of these putative genes. Studies have shown that the HPV-6 *E5* gene can induce some transformation-associated changes of NIH-3T3 cells (39). However, the precise role of HPV-6 E5 in this process is unclear because the HPV-6 DNA is often not retained in transformed mouse cells (196). Transforming activity has also been demonstrated for the HPV-16 *E5* gene. HPV-16 E5 can induce some transformed alterations in mouse fibroblast lines or mouse epidermal keratinocytes (169,171,287), increase the proliferative capacity of human keratinocytes (284), and stimulate cellular DNA synthesis in human keratinocytes in a manner that is potentiated by EGF (287). The biochemical mechanisms by which the *E5* genes of the epitheliotropic papillomaviruses exert their growth stimulatory effects have not yet been fully elaborated. HPV-16 E5 has been localized mainly to the Golgi apparatus, to endosomes, and to some extent to cell membranes (53). HPV-16 E5 has also been associated with the activation of the EGF receptor and with MAP kinase activation (57,287). As with the BPV-1 E5 protein, HPV-16 E5 can bind the 16-kd subunit of the vacuolar ATPase and can inhibit the acidification of endosomes (53,238,286). It should be noted, however, that the *E5* gene is not expressed in most HPV-positive cancers, suggesting that if the *E5* gene does stimulate cell proliferation *in vivo,* it presumably functions in benign papillomas and not in the cancers. It might also participate in the initiation of the carcinogenic process or in some other aspects of the viral–host cell interaction relevant to the pathogenesis of the HPV infection.

HPV E6 Oncoprotein

Although HPV-16 E7 is sufficient for the transformation of established rodent cells such as NIH-3T3 cells and for cooperation with *ras* in the transformation of baby rat kidney cells, the combination of E6 and E7 is required for the efficient immortalization of primary human fibroblasts or keratinocytes (114,198,318). E6 and E7 together can extend the life span of human keratinocytes and lead to the outgrowth of immortalized clones that are resistant to terminal differentiation. This property is dependent on the full-length *E6* gene; mutational analysis has shown that the putative HPV-16 *E6** gene, encoding a truncated form of E6, cannot provide this function (198). Indeed, it appears that the *E6**, if it is actually made *in vivo,* might function in a dominant negative manner to inhibit some of the activities of the full-length E6 oncoprotein of the cancer associated HPV types (227).

The HPV E6 proteins are about 150 amino acids in size and contain four Cys-X-X-Cys motifs, which are believed to be involved in binding zinc (13,105,106). The E6 proteins from the low-risk and high-risk HPVs appear to have similar transcriptional activation properties, as has been shown using “minimal” promoters containing a TATA box element only (266). Because the E6 proteins of the low-risk HPVs have little or no transformation activity in any of the assays tested, this transactivation property of the E6 proteins may not be linked mechanistically to the transforming functions of the E6 proteins of the high-risk HPVs.

The small DNA tumor viruses have evolved mechanisms to complex and functionally inactivate the tumor suppressor gene product p53 (see Chapter 10). Like the SV40 TAg and the Ad5 E1B 55K proteins, the E6 proteins encoded by the high-risk HPVs can complex with p53; however, a functional *in vivo* interaction between the low-risk HPV E6 proteins and

p53 has not been detected (320). Although p53 is not required for normal cellular proliferation, it plays a fundamental role in directing the cellular response to genotoxic and cytotoxic stresses that threaten genomic stability. The function of p53 as a sequence-specific transcriptional activator (92,148) is necessary for its activity in regulating cell growth and in tumor growth suppression. The first downstream target of p53 to be identified was the p21 cyclin-dependent kinase inhibitor, encoded by the *WAF1* or *CIP1* gene (83,109,327). Thus, the p53-mediated suppression of cell growth is due in part to the transcriptional activation of p21 leading to the inhibition of the cyclin-dependent kinases critical for G_1 progression and cell growth. The p53 protein directly regulates the transcription of additional genes involved in the cellular response to genotoxic and cytotoxic stress, apoptosis and cell cycle control, including *Bax, GADD45, IGF-BP3, cyclin G,* and *14-3-3*g (315). The viral oncoproteins SV40 TAg, AdE1B 55K, and the high-risk E6 proteins can each efficiently abrogate the transcriptional transactivation activity of p53 (193,335). Thus, as with SV40 TAg and Ad E1B, HPV E6 has anti-apoptotic activities and can interfere with the negative cell cycle regulatory functions of p53.

Although SV40 TAg, the Ad5 E1B 55-kd protein, and the high-risk HPV E6 proteins can all complex p53, the consequence of these interactions are different with respect to the stability of the p53 protein. In SV40 and adenovirus-transformed cells, levels of p53 are usually quite high, and the half-life of p53 is increased (212,234). In contrast, the levels of p53 in HPV-infected cells are low compared with uninfected primary host cells (128,254). Unlike TAg and the E1B 55-kd protein, which inactivate p53 in part by sequestering it into stable inactive complexes, the E6 proteins of the high-risk HPVs counter p53 by inducing its proteolysis. This was first demonstrated by *in vitro* studies that showed that the high-risk HPV E6 proteins facilitate the rapid degradation of p53 through ubiquitin-dependent proteolysis (256). Under the same conditions, the low-risk HPV E6 proteins, which do not bind detectable amounts of p53, have no detectable effect on p53 stability *in vitro* (256). HPV-16 E6 induces p53 degradation by forming a complex with the cellular ubiquitin–protein ligase E6AP (131,132), which is then able to bind and ubiquitinate p53 (253). E6AP is a component of the ubiquitin-proteasome pathway, which targets proteins for degradation by covalently linking them to multimeric chains of the 76–amino acid protein ubiquitin. In this pathway (shown in Fig. 16), ubiquitin is first activated in an ATP-dependent reaction and then passed from the ubiquitin-activating enzyme (E1) to a ubiquitin-conjugating enzyme (E2). Ubiquitin is then transferred to lysine residues of the target protein with the aid of a ubiquitin–protein ligase (E3). Multi-ubiquitinated proteins are subsequently recognized and degraded by the 26S proteasome (121). Although some classes of E3 proteins, such as the SCF complexes, function as adapters that bring E2 enzymes into complex with their substrates, E6AP belongs to a class of ubiquitin–protein ligases called HECT E3 proteins, which directly transfer ubiquitin to their substrates (255). The catalytic domain of HECT proteins is a conserved 350–amino acid region defined by its *h*omology to the *E*6AP *c*arboxyl *t*erminus (i.e., HECT) (130). The HECT domain binds to specific E2 enzymes (157) and contains an active-site cysteine residue that forms a thiolester bond with ubiquitin (130,255). X-ray crystallography studies have determined that the HECT domain is a bilobed structure, with a larger N-terminal lobe that interacts with the ubiquitin-conjugating enzyme, and a smaller C-terminal lobe containing the catalytic cysteine residue (126). Both E6 and E6-AP are necessary to detect complex formation with p53, and it has not yet been determined whether E6, E6-AP, or both actually contact p53.

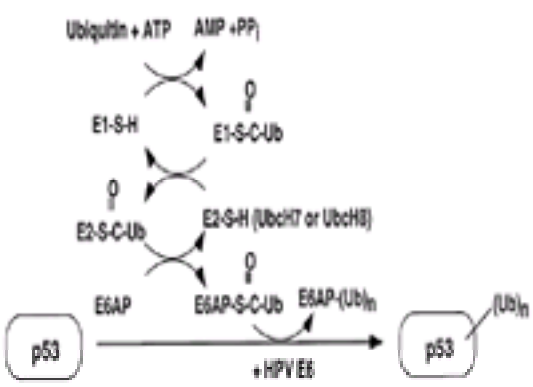

FIG. 16. A ubiquitin thiolester cascade model for the HPV E6–dependent ubiquitination of p53. The E6 protein binds to the cellular protein E6-AP, and the complex together functions as an E3 (ubiquitin protein ligase) in facilitating the ubiquitination of p53 (253). The ubiquitination of a protein involves three cellular activities: E1 (ubiquitin-activating enzyme), E2 (ubiquitin-conjugating enzyme), and E3 (ubiquitin protein ligase). Ubiquitin is activated in an ATP-dependent manner and forms a high-energy thiolester with E1, which can then be transferred to E2 through a thiolester linkage. Ubiquitin can then be transferred to a cysteine within the Hect domain of E6AP, again as a thiolester linkage (255), through the direct binding of E6AP with UbcH7 or UbcH8 (157). In conjunction with HPV-16 E6, E6AP then recognizes p53 and catalyses the formation of an isopeptide bond between the carboxyl-terminal glycine of ubiquitin and a lysine side chain of p53. In catalyzing the ubiquitination of p53, HPV-16 E6 also induces the self-ubiquitination and proteolysis of E6AP (144).

In addition to the *in vitro* evidence that the high-risk HPV E6 proteins accelerate the degradation of p53, E6 also affects the stability of intracellular p53 *in vivo* (11,128,164,254). Levels of p53 in E6 immortalized cells or in HPV-positive cervical carcinoma cells are, on average, twofold to threefold lower compared with primary cells (254). The half-life of p53 is reduced from several hours to 20 minutes in human keratinocytes expressing E6 (128). In uninfected cells, intracellular p53 levels increase significantly in response to DNA damage or genotoxic agents (145). The higher levels of p53 result in either a G_1 growth arrest or apoptosis as part of a cell defense mechanism that allows for either the DNA damage to be repaired before the initiation of a new round of DNA replication or removal of the cell. E6-expressing cells, however, do not manifest a p53-mediated cellular response to DNA damage (149), indicating the ability of E6 to promote the degradation of p53 and prevent the steady level of p53 to rise above a certain threshold level (Fig. 17). Under DNA-damaging conditions, the E6-stimulated degradation of p53 abrogates the negative growth regulatory effect of p53 and as such contributes to genomic instability (321). *In vivo* studies have confirmed the role of E6AP in mediating the E6-dependent ubiquitination of p53 and have established that E6AP in the absence of E6AP is not involved in the

regulation of p53 stability (19,294). Instead, mdm2 functions as the ubiquitin protein ligase for p53 in normal cells (315).

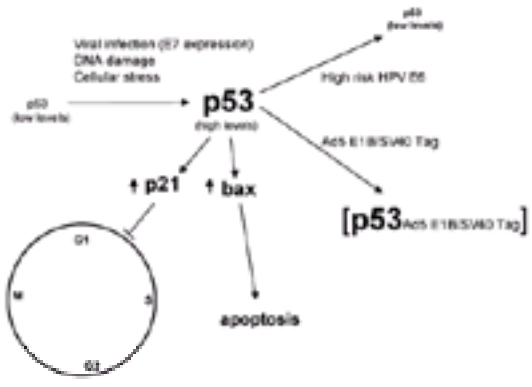

FIG. 17. The level of p53 in primary cells is generally low. DNA damaging agents, viral infection, and expression of E7 increase the level of p53. Elevated levels of p53 can lead to either apoptosis or a cell cycle checkpoint arrest in G_1 phase through the transcriptional activation of *bax* or $p21^{cip1}$. Viral oncoproteins may interfere with this negative growth-regulatory function of p53, either by sequestering p53 into a stable but nonfunctional complex (such as with SV40 TAg or the Ad5 55-kd E1B protein) or by ubiquitination and enhanced proteolysis, as observed with the high-risk HPV E6 proteins.

The HPV-16 E6 protein binds to E6AP within the N-terminal substrate recognition domain, directing E6AP to ubiquitinate p53 (133). Several potential E6-independent substrates of E6AP have now been identified, including the human homolog of the yeast RAD23 protein involved in nucleotide excision repair (HHR23A), the src-family kinase Blk, and the MCM7 subunit of replication licensing factor (155,158,211). In addition, E6 induces self-ubiquitination of E6AP (144). It is conceivable that the redirection of E6AP activity toward p53 by E6 might affect (either enhance or inhibit) the targeting of the normal substrates of E6AP and that such an alteration of E6AP activity could account for some of the transforming activity of E6. Indeed, recent studies suggest that E6 has p53-independent transformation activities. For example, transgenic mice expressing E6 from the epithelial-specific K14 promoter developed epidermal hyperplasia even in a p53-null background (274). Other experiments have shown that an E6 mutant that is unable to bind or degrade p53 can still promote colony formation and growth on soft agar as well as induce cell cycle reentry in cells even when p53 transactivation activity is inhibited (279). Another p53-independent activity of HPV-16 E6 is its ability to activate telomerase, a characteristic of many different types of cancer (152).

A number of additional cellular targets have now been identified for the high-risk E6 proteins in an attempt to define additional p53-independent cellular targets (Table 2). As discussed previously, paxillin, which appears to be a relevant target for the BPV-1 E6 transformation function, can also bind HPV-16 E6, at least *in vitro* (306,311). Interestingly, the high-risk E6 oncoproteins contain a X-(S/T)-X-(V/I/L)-COOH motif at the extreme C-terminus that can mediate the binding of cellular PDZ domain–containing proteins. This motif is unique to high-risk HPV E6 proteins and is not present in the E6 proteins of the low-risk HPV types. The high-risk HPV E6 proteins have been shown to bind a number of cellular PDZ domain–containing proteins, including hDlg (the human homolog of the *Drosophila melanogaster* tumor suppressor disk's large protein), MUPP1 (the multi-PDZ domain protein) and hScrib (the human homolog of *Drosophila scribble*) (151,167,168,201). E6 binding to these PDZ-containing proteins results in their E6AP-mediated ubiquitination and proteolysis (97,201). These PDZ-containing proteins have been shown to be involved in negatively regulating cellular proliferation. At least some of the p53-independent transforming activities of the high-risk E6 oncoproteins may be linked to their ability to bind and degrade some of these PDZ motif–containing proteins. E6 has also been reported to bind the transcriptional coactivator p300/CBP, a target also of adenovirus (Ad) E1A and SV40 large TAg (216,340). The physiologic relevance to the transformation functions of the other E6 cellular targets listed in Table 2 have not yet been elucidated. It is possible that the binding of E6 to some of these targets could contribute to the viral pathogenic functions unrelated to cellular transformation.

Associated cellular proteins	Functional consequences	References
p53	E6AP-dependent ubiquitination and proteolysis	258, 308
E6-associated protein (E6AP)	Ubiquitination and proteolysis of associated proteins	253
	Enhanced auto-ubiquitination of E6AP	144
E6BP (Ert1/	Unknown	38
Paxillin	Disruption of the actin cytoskeleton	306, 311
hDlg, MUPP1, and hScrib	E6AP-dependent ubiquitination and proteolysis	97, 167, 201
IRF-3	Inhibition of interferon-β induction	240
Clathrin adaptor complex AP-1	Unknown	305
Unknown	Activation of cellular telomerase	152
E6TP1	E6-induced degradation	96
Bak	Inhibition of Bak-induced apoptosis	331
CBP/p300	Inhibition of p53 transcriptional activity	216, 340

TABLE 2. *Cellular targets of the papillomavirus E6 oncoproteins*

HPV E7 Oncoprotein

The E7 protein encoded by the high-risk HPVs is a small nuclear protein of about 100 amino acids, has been shown to bind zinc, and is phosphorylated by casein kinase II (CK II) (199). Insight into its mechanism of action came initially from

the recognition that E7 has functional similarities with the Ad 12S E1A product (224). Like Ad E1A, E7 can transactivate the Ad E2 promoter (224), induce DNA synthesis in quiescent cells (251), and cooperate with an activated *ras* oncogene to transform primary rodent cells (185,224).

In addition to these functional similarities, the HPV-16 E7 shares important amino acid sequence similarity with portions of the Ad E1A proteins and the SV40 large TAg (Fig. 18). These conserved regions are critical for the transforming activities in all three viral oncoproteins and have been shown to participate in the binding of a number of important cellular regulatory proteins, including the product of the retinoblastoma tumor suppressor gene *pRB,* and the related pocket proteins, p107 and p130 (61,81,322). Complex formation with pRB involves conserved region 2 of the Ad E1A protein and the corresponding region in the E7 protein and in SV40 large TAg (61,323).

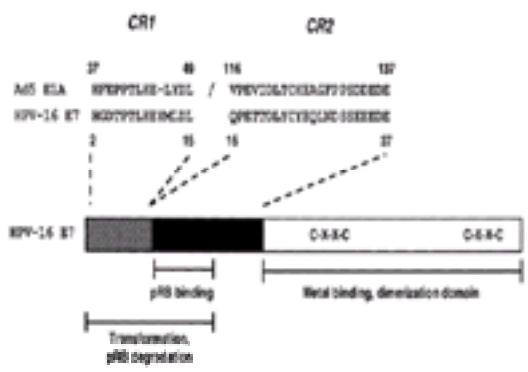

FIG. 18. Amino acid sequence similarity between portions of conserved regions 1 and 2 (*CR1, CR2*) of the Ad5 E1A proteins and the amino-terminal 38 amino acids of HPV-16 E7. CR2 contains the pRB binding site and the casein kinase II (*CKII*) phosphorylation site of HPV-16 E7.

The retinoblastoma protein is a member of a family of cellular proteins that also includes p107 and p130, which are homologous in their binding “pockets” for E7, Ad E1A, and SV40 TAg. The retinoblastoma protein is the most extensively studied member of this family of proteins. Its phosphorylation state is regulated through the cell cycle, being hypophosphorylated in G_0 and G_1 phase and phosphorylated during S, G_2, and M phase. The pRB protein becomes phosphorylated at multiple serine residues by cyclin-dependent kinases at the G_1-to-S phase boundary and remains phosphorylated until late M phase, when it becomes hypophosphorylated again through the action of a specific phosphatase (Fig. 19). The hypophosphorylated form represents the active form with respect to its ability to inhibit cell cycle progression. HPV-16 E7, like SV40 TAg, binds preferentially to the hypophosphorylated form of pRB, consistent with the model that this interaction results in the functional inactivation of pRB and permits progression of the cell into S phase of the cell cycle (50). This property of the viral oncoproteins to complex pRB would appear to account, at least in part, for their ability to induce DNA synthesis and cellular proliferation.

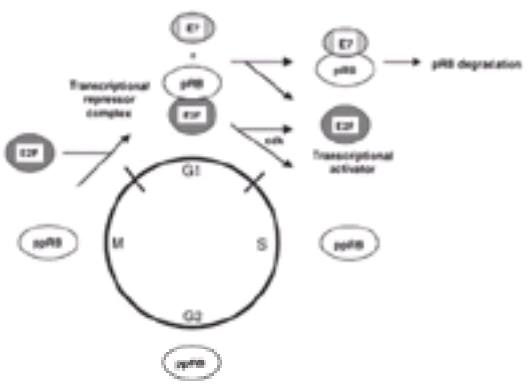

FIG. 19. E7 abrogates the cell cycle regulation mediated by pRB (as well as the related proteins p107 and p130) by complex formation. During the cell cycle, pRB is differentially phosphorylated, and the underphosphorylated form is detected only in the G_0/G_1 phase. This underphosphorylated form is the active form of pRB, acting as a negative regulator of the cell cycle. During the transition to S phase, pRB is phosphorylated by cyclin-dependent kinases (*cdk*), resulting in the inactivation of its cell cycle regulatory functions. Members of the E2F family of cellular transcription factors are preferentially bound to the underphosphorylated form of pRB, and in complex with pRB, they cannot activate transcription. Phosphorylation of pRB or complex formation with E7 results in the release of the E2F factors, allowing them to function as transcriptional activators of cellular genes involved in cellular DNA synthesis and progression into S phase of the cell cycle.

Members of the E2F family of transcription factors are involved in mediating the functions of the pocket proteins (see Chapter 10). The transcriptional activity of E2F is modulated by pRB; when bound to the G_0- and G_1-specific hypophosphorylated form of pRB, E2F functions as a transcriptional repressor. When pRB is phosphorylated by cyclin-dependent kinase complexes near the G_1-to-S phase boundary, the pRB–E2F complex dissociates, and E2F can act as a transcriptional activator (see Fig. 19). The regulated conversion of the transcriptional activity of E2F between repressor and activator contributes to the regulation of G_1-to-S phase progression. The high-risk HPV E7 proteins, like Ad E1A and SV40 TAg, contribute to carcinogenic progression, therefore, at least in part, by disrupting this regulatory

network (205). In the case of the E7–pRB interaction, E7 destabilizes pRB, promoting its proteolysis (27).

The E7 proteins of the HPV high-risk and low-risk types differ in a number of biochemical and biologic properties. The E7 proteins from the low-risk HPV types 6 and 11 bind pRB with about a 10-fold lower efficiency than the E7 proteins of HPV types 16 and 18 (95,200). Furthermore, the E7 proteins of the low-risk HPVs function inefficiently in cellular transformation assays with an activated *ras* oncogene and are phosphorylated by CK II at a lower rate (12). Studies with chimeric E7 proteins containing domains of high-risk and low-risk HPV E7 proteins showed that the difference in transformation efficiency in rodent cells was due to the pRB-binding site (115). Sequence comparison of the pRB-binding sites revealed a single consistent amino acid sequence difference between the high-risk and the low-risk E7 proteins: an aspartic acid residue (Asp 21 in HPV-16 E7) corresponding to a glycine residue in the low-risk E7 sequence (Gly 22 in HPV-6 E7). Substitution of this residue in the respective *E7* genes revealed that this single amino acid residue was the principal determinant responsible for the difference in pRB-binding affinity and in the transforming capacity of the low-risk and the high-risk E7 proteins (115,247).

Mutations in the carboxyl-terminal half of E7 that interfere with E7 function have also generally affected the intracellular stability of the protein (82,223). Nevertheless, several studies have suggested some specific contributions of the carboxyl terminus to E7 functions. E7 can exist as a dimer, and the C-terminal half is important in mediating dimerization (159). The ability of E7 to disrupt the complex between the cellular transcription factor E2F and pRB also involves sequences in the carboxyl terminus of E7, in addition to the pRB-binding site (127,326). Although the ability of E7 to transform rodent cells requires an intact pRB-binding domain, a mutant of HPV-16 E7 that is defective for pRB binding was reported to be competent to cooperate with E6 for the immortalization of primary human genital keratinocytes, indicating that other properties of E7 may also be involved in this function.

Genetic studies indicate that complex formation between E7 and the pocket proteins, including pRB, is not sufficient to account for its immortalization and transforming functions, suggesting that there are likely to be additional cellular targets of E7 that are relevant to cellular transformation (140). Table 3 provides a list of various cellular targets with which E7 has been shown to bind. The physiologic relevance of many of these interactions is unclear. It is possible that some of the interactions may reflect artifacts of the biochemical or yeast two-hybrid approaches used in their identification. It is also possible that some of the interactions may be of physiologic importance to functions of the E7 protein that are not involved in cellular transformation.

Associated cellular proteins	Functional consequences	References
pRB, p107, p130	Disruption of E2F transcription factor complexes	82, 91
	Degradation	27
$p21^{cip1}$	Inactivation of cdk and replication inhibitory activity	91, 141
$p27^{kip1}$	Inactivation of cdk inhibitory activity	336
TBP	↑	184
TAF$_{II}$110	↑	186
AP-1	Activation of c-jun transcriptional activation function	4
MI3] (HDAC)	↑	29
IGFBP-3	Inhibition of IGFBP-3–mediated apoptosis	182
M2 Pyruvate kinase	↑	342
α-Glucosidase	↑	341
hTid-1	↑	260
p48 component of ISGF3	Inhibition of interferon-α stimulation	15
F-actin	↑	225
Forkhead transcription factor MPP2	Activation of MPP2 transcriptional activity	139
Sα subunit of proteasome	↑	20a

TABLE 3. *Cellular targets of the human papillomavirus E7*

Of particular interest are the reports that E7 can interact with cyclin-dependent kinase inhibitors. As with E1A, HPV-16 E7 interacts with and abrogates the inhibitory activity of $p27^{kip1}$ (336). Because $p27^{kip1}$ is involved in mediating the cellular growth inhibition by transforming growth factor-b (TGF-b) in keratinocytes, this activity may contribute to the ability of E7 to override TGF-b–associated growth arrest (225). HPV-16 E7 can also associate with $p21^{cip1}$ and abrogate its inhibition of cyclin dependent kinases as well as its inhibition of PCNA-dependent DNA replication (91,141). The $p21^{cip1}$ is normally induced during keratinocyte differentiation (194), and presumably its inhibition by E7 may be critical to allowing the replication of papillomavirus DNA in differentiated squamous epithelial cells (40).

HPV-16 E7 has also recently been shown to induce abnormal centrosome duplication (77). Although E6 can potentiate this effect, E7 plays a major role in inducing these centrosome-related mitotic disturbances. The mechanism by which E7 affects these centrosome abnormalities are not yet known. Abnormal centrosome duplication rapidly results in genomic instability and aneuploidy, one of the hallmarks of a cancer cell. This activity is therefore likely to be functionally relevant to the contribution of high-risk HPV to malignant progression.

What are the roles of the E6 and E7 oncoproteins in the normal life cycle of an HPV infection? It is likely that they contribute critical functions by providing a cellular environment that fosters the replication of the viral DNA. The viral E1 and E2 proteins are necessary for the initiation of viral DNA replication, but the virus is otherwise totally dependent on host cell factors, including DNA polymerase a, thymidine kinase, PCNA, and so forth, for the replication of its DNA. These are proteins that are normally only expressed in S phase during cellular DNA replication in cycling cells. Vegetative DNA replication for the papillomaviruses, however, occurs only in the more differentiated cells of the epithelium that are no longer cycling (see Fig. 4 and Fig. 5). Thus, the papillomaviruses have evolved a mechanism similar to that of the polyomaviruses and the adenoviruses, to activate the cellular genes necessary for the replication of their own DNA in otherwise quiescent cells. These viruses may do so through the E7 proteins and their ability to release the E2F transcription factors by binding the pocket proteins, including pRB (see Fig. 19). In addition, E7 binds and inhibits the cdk inhibitor $p21^{cip1}$ that is normally induced during keratinocyte differentiation, again presumably for the purpose of permitting viral DNA replication in a differentiated cell. Why, then, does E6 target p53 for proteolysis? The high-risk HPV E7 proteins, when expressed in the absence of E6, result in increased levels of p53, resulting in either a G_1-mediated cell

cycle arrest or apoptosis, depending on the cell type (125,214). The mechanistic link between the pRB and p53 pathways is discussed in Chapter 10. The proposed model, for which there is some evidence, involves the deregulation of pRB by E7, resulting in the induction of E2F-1, which in turn activates p19 ^{arf}, which interferes with the ability of mdm2 to regulate p53 stability. Thus, E7 creates a signal that increases p53 levels. E6, by promoting the degradation of p53, resulting in a lower steady-state level in the cell, counters this activity of E7 and permits the E7-dependent activation of the cellular DNA replication genes required for viral DNA replication.

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CHAPTER 66 Papillomaviruses

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The papillomaviruses (PVs) comprise a group of nonenveloped epitheliotropic DNA viruses that induce benign lesions of the skin (warts) and mucous membranes (condylomas). Some PVs have also been implicated in the development of epithelial malignancies, especially cancer of the uterine cervix and other tumors of the urogenital tract. The recognition that PVs are an important cause of human cancer has heightened their medical importance and stimulated efforts to develop vaccines against these viruses.

HISTORY

Warts were known to the ancient Greeks and Romans. Their infectious nature was recognized, but until the 19th century, genital warts were usually considered to be a form of syphilis or gonorrhea. The viral nature of human warts was demonstrated in the early 1900s when cell-free filtrates from lesions were shown to transmit the disease (50). Although PVs have been identified in a variety of vertebrate species in addition to humans, most PVs are species specific in their host range (reviewed in 161 and 304).

The first animal PV was identified in the 1930s by Richard Shope, who identified and characterized the transmissible nature of papillomas arising in cottontail rabbits (282). The Shope PV, now designated the cottontail rabbit papillomavirus (CRPV), was the first DNA tumor virus identified. Some warts induced by CRPV were shown to undergo malignant progression (256), and for the next two decades, CRPV in the rabbit was an important model for the fundamental study of viral tumorigenesis (157,309). However, the continuing inability to culture CRPV led to its use as a model tumor virus being largely supplanted by the discovery in the late 1950s of the polyomaviruses, which could replicate in cultured cells, could induce morphologic transformation *in vitro,* and were tumorigenic for experimental animals (4) (see Chapter 10).

The advent of molecular cloning in the 1970s initiated an ongoing renaissance in the study of PVs. This technical advance enabled investigators to circumvent partially the inability to culture PVs because the cloning of PV genomes greatly enhanced the ability to study their biologic and biochemical properties. It permitted researchers to determine the function of viral genes, including the multiple PV oncogenes, which fostered a much wider interest in PV research. The bovine papillomavirus type 1 (BPV-1) represented the standard PV for initiating these studies because the virus induced readily identifiable focal transformation of certain established rodent cell lines (21,73). This property meant the genetics of transformation could be studied as well as the biochemical functions of the encoded viral proteins (182). Molecular cloning of PV genomes also led to the recognition that there may be multiple PV genotypes that infect a particular species and that a subset of human papillomaviruses (HPV) was closely associated with human cancers, including cervical cancer (24,62,72,223). The appreciation of their medical importance, combined with improved tools for analyzing PVs, further enhanced the utility of PVs as a model of viral tumorigenesis. Although the study of animal PVs continues to bring new information to the field, the medical importance of human viruses has shifted the emphasis toward the analysis of HPV, especially when it was established that the biochemical properties of some nonstructural viral proteins differed from those of their BPV counterparts (74,263).

INFECTIOUS AGENT

As discussed in detail in the previous chapter, PVs are nonenveloped viruses whose 8-kb closed circular double-stranded DNA genome is surrounded by a 55-nm icosahedral capsid (reviewed in 81 and 301). Most PVs are

classified according to their species of origin and the homology of the viral genomes. There are more than 100 HPV genotypes (types) (62). PVs have been divided into five clades or supergroups, designated A to E (41) (Fig. 1). Animal PVs are found in each of the five supergroups, whereas HPVs are found in three of them. It seems likely that humans were infected more than once with primordial prototypic HPVs because HPVs in the different supergroups are more closely related to animal PVs in the supergroup than to HPVs in the other supergroups. HPVs have infected humans for millennia: at least some genotypes appear to have coevolved with human ethnic groups (123,219). Complete sequence data on many PVs are available (215). Most PV genotypes appear to have arisen through a series of small mutations scattered throughout the genome, rather than by recombination between genomes, because the relative homology between various PVs is often similar regardless of the region of the genome that is compared (41,259).

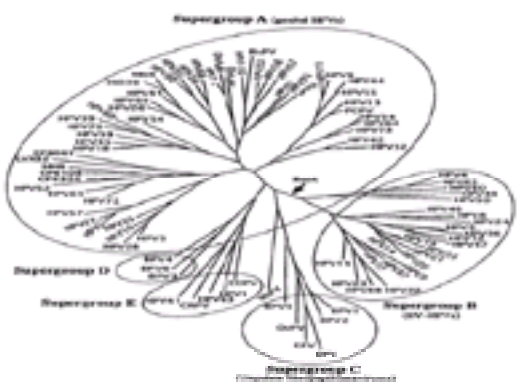

FIG. 1. Phylogenetic tree of human and animal papillomaviruses. The classification is based on the nucleotide sequence comparison of a 291-bp segment of L1. (From ref. 41, with permission.)

Propagation and Assay in Cell Culture

PVs have a similar genetic organization, with all open reading frames (ORFs) present on one strand (Fig. 2). The PV genome contains a main regulatory region, designated the *long control region* (LCR), and coding regions for the nonstructural and structural viral proteins, as described in detail in the previous chapter and reviewed elsewhere (301). Most PVs have six well-described nonstructural viral genes: *E1, E2, E4, E5, E6,* and *E7,* and two ORFs, *E3* and *E8,* whose function is unknown (Table 1). Most viral genes are multifunctional. *E1* has a DNA replicase activity that is required for viral DNA replication. The E2 proteins encode trans-activating and trans-repressor transcription functions and also participate in viral DNA replication. These activities are mediated by E2 binding to specific motifs in the viral genome, particularly in the LCR. *E4* encodes a cytoplasmic phosphoprotein whose biologic function has not been elucidated. PVs have three oncogenes: *E5, E6,* and *E7* (342). The E5 proteins interact with and activate growth factor receptors (192,299). The E5 oncoprotein of BPV-1 preferentially activates the platelet-derived growth factor (PDGF) receptor (68). This property may account for the ability of BPV-1 to induce focal transformation of cultured fibroblasts and contribute to the ability of BPV-1, and related PVs, to induce fibropapillomas, instead of only papillomas. Some E6 proteins induce ubiquitin-dependent degradation of the p53 tumor suppressor protein (261), and many E7 proteins bind and inactivate the underphosphorylated forms of the pRb retinoblastoma suppressor protein (74,113,267). PVs encode two structural viral proteins: L1 and L2. The L1 protein gives rise to the capsid, which is composed of 72 L1 pentamers (12). The immunodominant neutralization epitopes are located on L1. The L2 protein, which is less abundant than L1, interacts with a subset of L1 pentamers (313). It is required for encapsidation of the viral genome (249,339) and also contains neutralization epitopes (38,251).

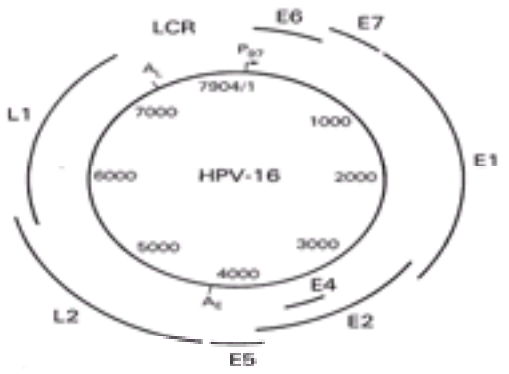

FIG. 2. Genomic map of HPV-16. The genome is a double-stranded circular DNA molecule of 7904 base pairs. Transcription occurs in a clockwise manner; the only transcriptional promoter presently mapped for HPV-16 is designated P_{97}. The open reading frames deduced from the DNA sequence are designed E1 to E7, L1, and L2 and are indicated outside of the circular genome. A_E and A_L represent the early and late polyadenylation sites. The viral long control region (LCR) contains transcriptional and replication regulatory elements.(From ref. 276, with permission.)

ORF	Function
L1	L1 protein, major capsid protein
L2	L2 protein, minor capsid protein
E1	Initiation of viral DNA replication
E2	Transcriptional regulatory protein with an auxillary role in viral DNA replication
E3	No known function
E4	Late protein; disrupts cytokeratins
E5	Membrane-transforming protein; interacts with growth factor receptors
E6	Transforming protein of HPVs; targets degradation of p53
E7	Transforming protein of HPVs; binds to the retinoblastoma protein
E8	No known function

ORF, open reading frame; HPV, human papillomavirus.

TABLE 1. *Functions assigned to the papillomavirus open reading frames*

PVs have proved difficult to propagate *in vitro* because these viruses replicate in stratified squamous epithelium, which is not mimicked in monolayer cultures. Therefore, most clinical identifications of PV infection rely on a technique that identifies the viral DNA, such as polymerase chain reaction (PCR) or a molecular hybridization technique, rather than on virus isolation.

Although the species specificity of PVs has also limited the utility of animals for the study of HPVs, one approach for PV propagation has been to expose initially the primary cultured epithelial cells to virus and then to place the cells under the renal capsule of nude mice (124). This immunologically protected site can support the growth of heterologous cells and foster formation of a multilayer epithelium that resembles a stratified squamous epithelium. The xenograft approach has led to the successful propagation of several HPVs, although it is not suited to routine virus isolation. The technique is difficult to use for large-scale virus production or for the molecular or biochemical analysis of virus replication.

It has been shown that the complete virus life cycle can be recapitulated in cultured cells by using a “raft” culture system composed of stratified squamous epithelial cells placed at an air–water interface (205). This technique is more complex than growing cells in monolayers, and complete replication occurs in a small minority of the cells. However, it can be used to analyze aspects of PV biology, genetics, and biochemistry (195) (Fig. 3). Late gene expression can also be studied by suspending HPV containing epithelial cells in semisolid medium (257).

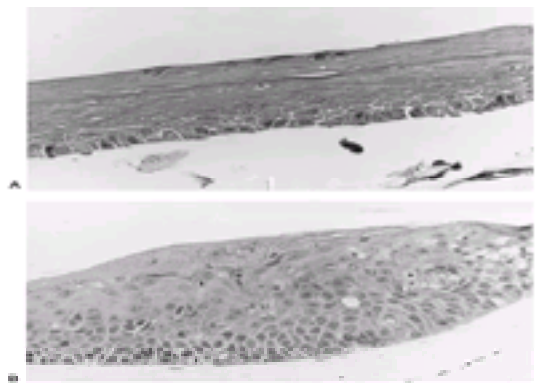

FIG. 3. Raft cultures showing normal keratinocytes (**A**) and keratinotcytes transfected with the full-length HPV-16 genome (**B**). The normal keratinocytes stratify and differentiate with an increase in the cytoplasm-to-nucleas ratio in cells of the upper half of the epithelium and a prominent granular layer. In keratinocytes transfected with HPV-16, while stratification takes place, differentiation is abnormal. There is hyperplasia of cells in the parabasal layer, and mitotic figures are observed in the upper half of the epithelium. No granular layer is seen, and the cytoplasm-nucleas ratio does not change throughout the stratified epthelium. This morphology is similar to that of cervical intraepithelial neoplasia (CIN). (Courtesy of D. McCance, University of Rochester. From ref. 276, with permission.).

Many aspects of PV replication can be studied with monolayer cultures. The most frequently used source of infectious virus is BPV-1 obtained from cattle carrying experimentally produced lesions, which have a high density of infectious virions. The availability of cloned plasmid DNAs for many PVs, including HPVs, makes it possible to use transfection, or infection with heterologous viral vectors, as another approach to study aspects of viral replication.

PV infection of monolayer cultures, whether epithelial cells or fibroblasts, leads to expression of the nonstructural proteins, except for E4, and to episomal viral DNA replication (116). It is therefore possible to use monolayers to analyze these nonstructural viral genes and viral DNA replication (reviewed in 47,235, and 301). The principal restriction to virus replication in cell monolayers is the lack of expression of the structural viral proteins. Infectious virus can be generated in monolayers if the genes encoding the two structural viral proteins are provided to PV-infected monolayer cells through other vectors, such as defective Semliki Forest virus (SFV) vectors (249). This type of system permits dissection, in monolayers, of the requirements for the production of infectious virus.

Biologic Characteristics

Important parameters of PVs include their species specificity, their epitheliotropism, their ability to stimulate abnormal cell growth and establish chronic infection, and the capacity of particular types to infect preferentially particular regions of the

body. The molecular basis for many of these fundamental properties is incompletely understood.

The receptor for PVs has not been clearly identified. Biochemical evidence that the receptor might be a $_6$,b$_4$ integrin (199), although plausible, has not been supported by genetic studies (283). Biochemical and genetic analyses suggest that all PVs may use the same receptor, that the receptor is evolutionarily conserved, and that it is expressed on many cells in addition to epithelial cells (210,250). Indeed, mouse fibroblasts can be readily infected with BPV-1 or with a BPV-1 genome pseudotyped by the HPV-16 structural proteins, and virus-like particles composed of HPV-16 L1 can interfere with BPV-1 infectivity (249).

These results imply that the species specificity of PVs probably lies in their responding to yet to be determined species-specific host regulatory proteins rather than to a restriction at the level of virus adsorption and penetration. Conversely, the epitheliotropism of PVs is likely to result from intracellular restrictions in nonepithelial cells. Consistent with this hypothesis, the LCRs of epitheliotropic PVs bind epithelial-specific factors that regulate viral gene expression (258).

The animal PVs that cause fibropapillomas, such as BPV-1, represent an exception to the strict epithelial tropism of most PVs (161). The viral genomes from this class of PV nonproductively transform the dermal fibroblasts underlying the papillomatous portion of the lesion, in addition to inducing papillomatous changes analogous to those that occur with other PVs. The efficient transformation of the fibroblasts by these PVs probably occurs because their genomes are adapted to be regulated appropriately by factors present in fibroblasts (258) and because their oncogenes are also adapted to transform fibroblasts (68). As noted earlier, such adaptation appears to have occurred with the BPV-1 *E5* oncogene, which in cultured fibroblasts accounts for most of the transforming activity of the BPV-1 genome. BPV-1 *E5* induces a potent activation of PDGF receptors, which are found on fibroblasts but not on epithelial cells, whereas HPV E5 oncoproteins appear to transform cells through a partial activation of epidermal growth factor (EGF) receptors (299) and have weak activity in fibroblasts.

The correlations that have been made between the various HPV types and their clinical manifestations have disclosed preferential sites of infection for many types. Most HPVs can be broadly classified into one of three groups: cutaneous types, EV types, and genital-mucosal types. The cutaneous types cause nongenital skin warts in otherwise normal individuals. The EV types of HPV cause nongenital skin lesions primarily in individuals with epidermodysplasia verruciformis (i.e., EV), a rare condition characterized by a susceptibility to widespread chronic infection by this class of HPVs, or in immunosuppressed individuals. However, inapparent infection with EV HPVs is quite common (25). The genital-mucosal types may infect the genital skin, genital mucosa, or nongenital mucosa. The genital-mucosal types are often further subdivided into high-risk and low-risk types, depending on whether or not they are found regularly in cervical cancer.

Despite evidence that the cutaneous types and the genital-mucosal types tend to infect nonoverlapping areas of the body, there is no molecular insight into what accounts for these distinct profiles of infectious sites. Because, as noted previously, the initial steps in PV infection appear to be through conserved mechanisms that are neither region specific nor species specific, it seems likely that putative intracellular regional-specific regulatory factors would account for the regional restriction of the HPV types.

The ability to induce papillomas, which is the hallmark of PVs, derives from their capacity to alter the normal growth characteristics of the infected epithelium. As described in detail in the previous chapter, *in vitro* studies have examined the biologic and biochemical characteristics of the viral oncogenes and their encoded protein products in considerable detail. These studies imply that three oncoproteins, E5, E6, and E7, perturb cell growth by nonoverlapping, but cooperative, mechanisms. The papillomas themselves appear to arise by means of increased cell proliferation combined with a prolonged retention of the superficial epithelial layers (296).

Papillomavirus Infection of Experimental Animals

The species-specific nature of PVs has thus far prevented adaptation of HPV infection to experimental animals. Despite these limitations, several useful animal models have been described. Animal PVs are found in a variety of mammals and in birds (161,217,304), and transgenic rodent models that use HPV genes have also been developed.

Wild cottontail rabbits (*Sylvilagus floridanus*) represent a natural host for CRPV (33,157,282). Experimental studies of CRPV can be carried out in the natural host, but difficulties in maintaining cottontail rabbits under typical cage conditions have led to most CRPV studies being carried out with domestic rabbits (*Oryctolagus cuniculus*). Cottontail and domestic rabbits represent closely related species, which probably accounts for the ability of CRPV to infect domestic rabbits. CRPV infection of cottontail rabbits induces papillomas, which can progress to cutaneous squamous cell carcinomas, although most lesions remain benign papillomas that will eventually regress. CRPV can also readily induce papillomas in domestic rabbits, and progression to cutaneous cancers occurs more frequently. Another difference is that although papillomas in cottontails usually contain large amounts of infectious CRPV, the lesions in domestic rabbits contain little or no infectious virus.

The canine oral papillomavirus (COPV) causes oral mucosal lesions (217). The papillomas it induces usually regress 1 to 2 months after infection, which makes this model well suited to studying aspects of host defense mechanisms against PV infection.

BPV-1 is the prototype of a group of animal PVs, found in ungulates, that cause fibropapillomas (161). The ability of these PVs to induce nonproductive transformation of dermal fibroblasts leads to their having a wider host range than other PVs, although the increased host range is limited to fibroblasts. Benign dermal tumors, known as *equine sarcoid tumors,* appear to arise after accidental dermal infection of horses with BPV-1. An experimental counterpart is the ability to induce nonneuronal tumors in hamsters inoculated intracerebrally with BPV-1.

BPV-4 can induce oral mucosal lesions, as well as esophageal papillomas, in cattle (37). When cattle are fed bracken fern that contains chemical carcinogens, it can lead to esophageal cancers. One interesting feature of these cancers is that although the BPV-4 infection plays a role in their induction, the tumors themselves do not contain detectable BPV-4 DNA. This represents a rare instance of virally induced tumors that arise by means of a “hit-and-run” mechanism.

A rhesus papillomavirus (RhPV) has been described that appears to be sexually transmitted between monkeys and to be associated with the development of cervical cancer (224). RhPV and HPV-16 are highly homologous, which suggests this animal model might have many similarities to human cervical infection with HPV. However, infectious RhPV has not yet been isolated or propagated, severely limiting experimental analysis of this potentially useful model.

Although no PV has been identified that is infectious for laboratory mice or rats, several useful transgenic mouse models that express a subset of viral genes have been developed. In one model, mice transgenic for BPV-1 develop dermal fibromas, which can progress to fibrosarcomas (286). In another system, when the early region of HPV-16 was placed under control of the keratin 14 (K14) promoter and female mice were treated with estrogen, cervical and vaginal tumors developed in a high proportion of the animals (8). A fivefold lower dose of estrogen resulted in tumors that were localized to the transformation zone of the cervix (77). Neither dose of estrogen was associated with a change in viral gene expression, suggesting that estrogen was stimulating tumor formation, in cooperation with the HPV genes, by altering the expression of one or more cellular genes.

Other mouse transgenic systems have examined the activities of high-risk *E6* and *E7* genes because these are the two viral genes that are preferentially retained and expressed in cervical cancer (270). In one, the expression of *E6* and *E7* from high-risk HPV-16 was directed to the eye by placing each gene under control of the lens crystalline promoter (229). The E7 transgenic mice displayed increased cell proliferation and increased apoptosis, and the E7 activities depended on the Rb-binding site in E7. The E6 mice had impaired lens fiber denucleation and a decrease in the apoptotic-like DNA degradation normally seen in the developing lens. Cooperation between E6 and E7 was seen, in that tumors developed in the lens of E6 × E7 double-transgenic mice but did not develop in E6- or E7-only mice. When the transgenic mice were placed on a p53 null background, it was determined that the E7-dependent apoptosis and the E6 inhibition of apoptosis were each mediated by processes that were partially p53 dependent and partially p53 independent (230).

The *E6* and *E7* genes from high-risk HPV-16, or HPV-18, when placed under control of the K14 promoter, lead to the development of benign or malignant cutaneous tumors (102, 160). Transgenic mice that express HPV-16 E6 or E7 alone, under control of the K14 promoter, were constructed, and cooperation between E6 and E7 was demonstrated for tumor formation, in that E6 × E7 double-transgenic mice developed benign and malignant cutaneous tumors much faster than lesions developed in E6- or E7-only mice (292). The role of E6 and E7 in the tumorigenic process has also been analyzed by testing the ability of the mice expressing only E6 or E7 to function in a standard cutaneous carcinogenesis protocol, which consists of initiation, promotion, and progression. In the K14 transgenic model, E7 functioned primarily as a promoter, leading to the formation of benign papillomas in skin that had been initiated with a chemical carcinogen. By contrast, E6 was found to foster progression from benign papillomas to malignant tumors. These distinct roles of E6 and E7 in tumorigenesis provide at least a partial explanation for the cooperation noted previously in the E6 × E7 double-transgenic mice.

PATHOGENESIS AND PATHOLOGY

Papillomas

Papillomas are benign epithelial lesions that arise following a variable latency period of weeks to months. The papillomas may develop on virtually any stratified squamous epithelium and may therefore involve the skin or mucous membranes. They are usually called *warts* when they are found on the skin and *condylomas* on the genitalia. Lesions may remain for months to years and then regress spontaneously. To establish this chronic infection, it is believed that the virus must infect those epithelial cells that possess long-term proliferative capacity. Minor trauma to the upper layers of the epithelium is probably required at the time of inoculation because the proliferative cells are located in the basal cell layer of the epithelium. Such trauma occurs frequently during intercourse, which may account for the high rate of genital HPV transmission following sexual exposure. Based on X-chromosome–linked marker studies, nongenital skin warts have been reported to be clonal in origin (213), but it remains unclear whether genital warts are similar in this regard (36).

The lower layers of the epidermis harbor low numbers of viral DNA genomes and transcribe low levels of viral RNA (81, 298). Vegetative viral DNA replication, high-level expression of the viral proteins, and virus assembly all occur in the upper epidermal layers, which are undergoing terminal differentiation. Virus assembly occurs in the nucleus, where virions often remain until after the cells are desquamated into the environment.

Papillomas are characterized by a thickened epidermis (acanthosis) with elongated rete pegs (papillomatosis), often with hyperkeratosis and parakeratosis. Cells in the upper layers of the epidermis may have abnormal nuclei (koilocytosis), which are eccentric, pyknotic, and surrounded with a halo. The increase in basal cell mitoses is usually slight, and the basement membrane is intact. The dermal capillary vessels may be prominent and thrombosed.

Although these lesions arise as a direct result of viral infection, the mechanisms by which the virus induces these pathologic changes are poorly understood. Koilocytic atypia was described first in cervical dysplasia and is diagnostic of PV infection (154, 200). However, most of the abnormal nuclei are not associated with viral particles or viral protein, and the mechanisms underlying these cytopathic changes are unknown. As noted earlier, the E5, E6, and E7 proteins of PVs can directly stimulate abnormal cell growth. However, with the possible exception of E5 (35, 194), these genes are expressed at very low levels in infected basal cells (298). It seems possible that viral gene expression in suprabasal cells may stimulate the release of cytokines that in turn stimulate growth of the basal cells. Part of the increased epithelial thickening arises secondary to abnormal retention of cells in the upper epidermis (296). The mechanisms that underlie

this process are not known.

PVs are resistant to desiccation (247). They can remain infectious in the environment for extended periods, and transmission through fomites may occur relatively commonly for nongenital warts (95). However, genital warts, because of their location, are generally transmitted by person-to-person contact. Because of the apparent need for PVs to infect basal cells at the time of inoculation, maceration of the skin may predispose to cutaneous infection. An individual's own PV can represent potentially important sources of infectious virus for transmission to uninfected areas. Lesions involving apposing areas of skin or mucous membrane, as occurs frequently, probably develop by auto-inoculation.

Dysplasias and Carcinomas

A subset of PVs is clearly implicated in the development of malignancy in humans and animals (33,37,342) (Table 2). In humans, these HPVs include those found in cervical cancer and anal cancer (especially HPV-16, HPV-18, HPV-31, and HPV-45) as well as those found in cutaneous squamous cell cancers associated with EV (especially HPV-5 and HPV-8). HPVs are also present in some cases of vulval and vaginal cancer, penile cancer, and oral cancer, especially tonsillar cancer. There is some evidence associating HPV infection with some esophageal cancers, and sensitive HPV DNA detection techniques have identified a variety of HPV types in nonmelanoma skin cancers (NMSCs; basal cell and squamous cell tumors) arising in long-term renal transplant recipients as well as in nonimmunosuppressed individuals. In animals, CRPV and BPV-4 have been shown most clearly to have oncogenic potential.

Species	Cancer	Predominant viral type	Cofactors
Humans	Skin carcinomas in EV patients	HPV-5, -8	Sunlight, genetic defect
	Lower genital tract cancers	HPV-16, -18, -45, -31	Not known
	Malignant progression of respiratory papillomas	HPV-6, -11	X-irradiation
Cattle	Alimentary tract carcinoma	BPV-4	Bracken fern
	Eye and skin carcinoma	Not characterized	Sunlight
Sheep	Skin carcinoma	Not characterized	Sunlight
Cottontail rabbit	Skin carcinoma	Cottontail rabbit papillomavirus (CRPV)	Methylcholanthrene and coal tar (experimentally)

EV, epidermodysplasia verruciformis.

TABLE 2. *Naturally occurring cancers associated with papillomaviruses*

The cancers attributable to PV infection do not occur until many years after the initial infection, which typically induces either benign papillomas or mild dysplasia. Persistent infection with high-risk types, especially with high viral loads, places women at considerable risk for progression to high-grade dysplasia and more severe pathologies, with continuing infection appearing to be necessary for the development of malignancy (119,122,174,325,333). Most infections, even with oncogenic HPV types, have a benign outcome. Lesions that progress to invasive cancer may begin as papillomas or as lesions with low-grade dysplasia and then become more dysplastic over time. However, malignant progression is not an inevitable outcome. Lesions at any stage before the development of invasive cancer may fail to progress or may regress, although the likelihood of regression is inversely related to the degree of dysplasia.

The long interval between the initial infection and the development of cancer implies that other factors in addition to HPV infection play crucial roles in malignant progression. Immune status represents one important host parameter, with impaired cellular immune function being associated with a greater risk for persistent infection and progression to high grades of dysplasia (266). Women who are long-term renal transplant allograft recipients are at increased risk for HPV and for progression to cervical cancer (6,9,284). Women who are infected with human immunodeficiency virus (HIV) are also at increased risk for these outcomes, as discussed in greater detail in the section on HIV and HPV infection (228,315).

Exogenous exposure of PV-induced lesions to co-carcinogens represents another mechanism that can contribute to malignant progression. This phenomenon probably operates in the malignant tumors that arise in EV. Most of the cutaneous cancers in EV occur on sun-exposed skin, which implies that ultraviolet light is serving as a co-carcinogen (187,222). In rabbits with CRPV-induced lesions, malignant tumors develop more frequently, and with a shorter latency period, if the lesions are exposed to dimethylbenzanthracene, methylcholanthrene, or tar (252). In the BPV-4 model of esophageal cancer, the presence of carcinogens in the bracken fern that the cattle eat contributes to the malignant progression (37). Some epidemiologic studies of cervical HPV infection suggest that smoking may be a risk factor for malignant progression, which raises the possibility that chemicals in cigarette smoke could be functioning as co-carcinogens, promoters, or immunosuppressants (58,92,149,240).

As suggested previously, viral genotype also plays an important role in progression. This aspect is clearly seen in EV and cervical cancers. In EV, many different HPV types may cause chronic benign papillomas, but about 90% of the malignant tumors arise in lesions that contain HPV-5 or HPV-8 (187,222). The critical molecular differences that account for their greater oncogenicity has not been determined. In cervical infection, a variety of HPV types can infect the cervix, but only some of these types are consistently associated with cervical cancer (23,181). Based on the differences in the frequency with which these types are found in cancer, compared with their distribution in nonprogressed lesions, the HPV types found regularly in cervical cancers may be designated as high risk or intermediate risk; low risk applies to those HPVs that infect the cervix but do not appear to be associated with malignant potential in the cervix. Important molecular differences have been identified between high-risk or intermediate-risk and low-risk types. These include the much greater efficiency with which high-risk HPVs inactivate the tumor suppressor proteins p53 and pRB as well as other cellular proteins that may negatively regulate cell growth, in contrast to the low-risk HPVs (74,263). There are also

probably quantitative differences in oncogenic potential between high-risk and intermediate-risk types (181) and even within a single high-risk type (40,180,331,332,335), but the molecular basis for these differences has not been clearly delineated.

Although viral DNA remains exclusively episomal in benign papillomas, integration of a partially deleted viral genome is present in most cervical cancers (270). Integration occurs most often in conjunction with the development of high-grade dysplasia, which is also associated with an increase in viral gene expression in basal cells (298). The E6 and E7 viral oncogenes are preferentially retained and expressed in severe dysplasias as well as in frankly malignant lesions. The viral DNA integration occurs in transcribed areas of the host DNA, is often associated with increased expression of E6 and E7, and results in selective growth advantage (129,130). *In vitro* studies of cervical cancer cell lines make it likely that the E6 and E7 oncoproteins contribute directly to the abnormal growth characteristics of dysplasias and carcinomas. The consistent loss of expression of viral genes other than E6 and E7 implies that the process of progression has selected against them. Expression of these other genes may be less compatible with E6 and E7 activities in lower levels of the epidermis and may render the cells more susceptible to immune recognition.

In contrast to the continued expression of E6 and E7 in cervical cancer, viral gene expression may not be obligatory for all cancers that are associated with HPV. In at least some human tumors that contain HPV DNA, viral gene expression appears to have been selectively suppressed (99). As noted earlier, the development of esophageal cancer in cattle by BPV-4 and bracken fern is associated with complete loss of the viral genome in the malignant tumor (37). This hit-and-run phenomenon suggests that the virus is required for tumor induction but that its continued presence is selected against in progressed lesions. The evidence for the involvement of HPV in human esophageal cancers is somewhat controversial (289,305). However, the failure of some investigators to find viral DNA in these cancers could represent a phenomenon analogous to the situation in cattle.

Immunity

The virus life cycle, which occurs in maturing keratinocytes and results in the release of virions through desquamating cells, appears to have evolved to minimize the exposure of the immune system to viral antigens. However, many aspects of papillomavirus biology point to the importance of the immune response in determining the course of infection (32,87,188,297,311). T-cell immune responses appear to be most important after the host has been infected, whereas humoral immunity may help prevent the spread of infection to new sites within the host and reduce the likelihood of reinfection. Protection appears to be largely type specific (310), which may provide at least a partial explanation for the large number of HPV types. However, the apparent antigenic stability within a given HPV type may suggest that each type is currently subjected to relatively little pressure for immune selection.

When multiple warts are present, it is not uncommon for removal of one wart to be followed by the regression of others, suggesting an immunologic response triggered by release of antigen from the treated wart. The spontaneous regression of flat cutaneous warts, in which many lesions may regress simultaneously, or of condylomas or cervical dysplasia, is associated with a mononuclear cell infiltrate consistent with a cellular immune response (52,127). Cutaneous warts occur more frequently in patients with tumors associated with depressed T-cell function, such as leukemias and lymphomas, whereas their frequency is not increased in patients with tumors associated with impaired humoral immunity, such as multiple myeloma (207). Patients with other conditions that affect T-cell immunity, such as pregnancy, immunosuppressive chemotherapy, or HIV infection, are at increased risk for HPV infection that persists as well as for cervical cancer (6,9,84,90,228,315). Even benign HPV infections in immunosuppressed patients are notoriously difficult to treat, and reduction of the immunosuppression may be associated with the spontaneous improvement of the HPV-induced lesions.

While these observations indicate that the immune system, especially T cells, can influence the outcome of HPV infection, protective immunity is incompletely understood, and the viral determinants that induce the immune responses responsible for regression remain uncertain. It seems likely that several viral antigens may possess this capacity, given the genetic heterogeneity of human immune responses and the fact that HPV infection is self-limited in most immunocompetent individuals. Animal papillomavirus models support this possibility, with experimental evidence that cellular immune responses to E1, E2, E6, E7, and L2 may each contribute to the regression of lesions (38,163,178,271). Indeed, experimental CRPV infection of outbred rabbits appears to be prevented in a higher proportion of rabbits when they are immunized with a “cocktail” consisting of genes encoding several nonstructural viral proteins rather than with only one of the viral genes (110). In women, T-cell responses to HPV-16 E6 and E7 occur more frequently in patients with a history of regressed lesions than in those with persistent lesions (133,216,314). However, it is difficult to know whether these particular antigenic responses helped to mediate the regression or are simply markers for regression.

Regression of infection and resistance to reinfection appear to be type-specific phenomena. For example, infection with more than two HPV types occurs relatively frequently, and in these situations, the duration of infection by one type does not appear to be influenced by the duration of infection by the other HPV type (310). Conversely, resistance to reinfection by one HPV type does not appear to confer resistance to reinfection by other types. These results are consistent with the finding that most T-cell and B-cell epitopes in HPV proteins are type specific.

Prevention against reinfection probably depends on both B-cell and T-cell immunity. The ability of T-cell responses to various viral proteins to protect against experimental infection in animal models was noted previously. In addition, L1 induces the immunodominant neutralizing antibodies, which are conformationally dependent and type specific (87). As discussed in greater detail in the section on prevention, neutralizing antibodies are able to protect against experimental challenge with the homologous virus in animal PV models, although the antibodies do not induce regression of established infection (31,145,306). Most women infected with HPV-16 develop low titers of type-specific serum and vaginal antibodies directed against conformational L1 epitopes, as measured in HPV-16 enzyme-linked immunosorbent assays (ELISAs) that use virus-like particles (VLPs) with L1 as the target antigen (39,65). Similar results have been found for VLP-based ELISAs for other HPV types.

There is evidence that certain HLA polymorphisms may predispose individuals toward or against the development of cervical dysplasia and cervical cancer, some with type specificity, presumably because they influence the likelihood of persistent infection (reviewed in 32 and 297). The issue is complicated because various studies have implicated different haplotypes, perhaps because of confounding ethnic factors or choice of control group. The most consistent associations have involved class II alleles, particularly DQ3 and DQB1*03, and two DR-DQ haplotypes, which have been reported to be associated with an increased risk in different populations. A negative association with DRB1*13 has also been reported in some populations. In addition, a naturally occurring HPV-16 E6 variant that affects a putative HLA-B7–restricted cytotoxic T-lymphocyte epitope has been found in the cancers of HLA-B7–positive women, raising the possibility that this mutation might render the virus less immunogenic for these women (76). The plausibility of class II alleles serving an important role in the immune response to HPV infection is strengthened by data obtained with CRPV infection of rabbits. In this animal PV system, both susceptibility and resistance to persistent infection and progression to carcinoma have been linked to class II alleles (32). In addition, infection with two closely related CRPV strains, which differ primarily in their E6 and E7 amino acids, has identified some class II alleles associated with regression against one strain and persistence in the other (259). These observations support the notion that an allele-specific restriction in the binding and presentation of CRPV antigens plays a critical role in determining the outcome of infection.

Patients who develop one HPV-associated tumor are at increased risk for a second HPV-associated tumor (89,92,242,302). In renal transplant recipients, this association is almost certain to depend on their immunosuppression. By extension, it is possible that nonimmunosuppressed individuals who develop additional HPV-associated tumors may have an impaired immune response to the HPV infection. Although a genetic component is plausible, cigarette smoking, which may be carcinogenic or immunosuppressive, has been found to be an associated risk factor.

Asymptomatic Infection and Latency

PVs have the ability to establish clinical latency, although it remains unclear to what degree these situations represent true latency versus persistence in association with very low levels of virus replication. The presence of viral DNA has been documented in the larynx during disease-free periods in patients with laryngeal papillomas (191,295). In genital lesions, the presence of viral DNA in clinically normal tissue surrounding lesions may be associated with a greater likelihood for recurrence (26,83). In the cervix, infection is more readily detectable in pregnant women or in women who have been on long-term immunosuppressive therapy (6,9,84). It is likely that many of these infections represent reactivation of latent disease rather than being secondary to new exogenous exposure. Scarification of domestic rabbits with CRPV DNA represents an experimental model of latent papillomavirus infection (338).

The advent of highly sensitive techniques, such as nested PCR, has also shown that HPV infection can be completely inapparent (10,27). EV-specific types have been isolated regularly from plucked eyebrows of normal individuals. Similar techniques have also identified the DNA of various HPV types in clinically normal skin and mucosa. Their presence in normal tissue means that when sensitive techniques are used to find HPV DNA in a lesion, additional evidence should be sought to determine whether the virus has contributed to the development of the lesion. EV-specific types, or antibody responses to them, have also been found in patients with psoriasis and other conditions associated with rapid epidermal cell growth (79,80,319). The relationship of HPV infection to these disease processes is unclear.

There is also evidence for asymptomatic perinatal HPV transmission, although its relative frequency and potential biologic significance remain conjectural. Pregnancy is associated with a high proportion of cervical samples being HPV positive, which places infants at risk for exposure to genital HPV types during the perinatal period (84). Epidemiologic data support the conclusion that most childhood cases of recurrent respiratory papillomatosis (RRP) arise after intrapartum transmission of HPV from mother to child, as discussed in greater detail in the section on RRP (137). Many cases of childhood RRP occur in children older than 1 year of age, which implies that there has been a period of latent HPV infection at least in these cases. Such data, and the occasional identification of HPV DNA by Southern blotting in neonatal foreskins (253), indicate that perinatal HPV transmission occurs. However, discrepant results have been reported regarding the frequency of such transmission (66,151,241,244,318). Some groups reported that when sensitive nested PCR techniques were employed, up to half of children younger than 11 years of age harbored asymptomatic HPV-16 DNA in their buccal mucosa; other groups found that samples from children, taken from buccal mucosa or the anal region, yielded positive results in fewer than 5% of samples. The reported differences in frequency do not appear to result only from differences in sensitivity of sampling techniques. The presumed high carrier rate of HPV-16 has suggested the possibility to some investigators that genital HPV infection may frequently arise by auto-infection or that many HPV-16 carriers would be immunologically tolerant to this infection. These scenarios appear unlikely to be a common occurrence, in view of the overwhelming epidemiologic evidence that most genital HPV infections in adults are transmitted sexually and the recent evidence, discussed in more detail in the section on prevention, that an HPV-16 L1 virus-like particle vaccine has thus far been found to be immunogenic in all subjects who have received the vaccine (111).

CLINICAL FEATURES AND EPIDEMIOLOGY

Nongenital Skin Warts

These benign papillomas occur most commonly on the hands and feet, although they can arise in almost any location (34,127,128). They occur frequently in older children and young adults, with some surveys of schoolchildren indicating a prevalence of about 10% (164). Most lesions are present for several months and regress spontaneously within 2 years (204), although some may persist indefinitely.

Multiple lesions are most common, and they may be distributed symmetrically or be unilateral. Common warts are scaly, rough, spiny papules or nodules. They often occur as single or grouped papules on the hands and fingers. Flat warts are slightly elevated flat-topped papules that have minimal scale and are found most frequently on the face, arms, or lower extremities. Filiform warts occur most frequently on the face or neck. Plantar warts (on the plantar surface of the foot) and

palmar warts (on the palms) may be thick, endophytic, and hyperkeratotic lesions. Mosaic warts result from the coalescence of plantar or palmar warts into large plaques.

Although not absolute, there is a reasonably good correlation between the clinical lesion and the HPV type (Table 3). HPV-1, HPV-2, and HPV-4 are usually associated with common warts and plantar warts, including mosaic warts, whereas HPV-3, HPV-10, HPV-28, and HPV-41 are found in flat warts. Butchers and meat handlers may have an unusually high prevalence of hand warts. There is no evidence that these warts are caused by animal PVs. Instead, HPV-7 is found in most lesions. It is believed that the combination of maceration and trauma predispose these workers to the development of clinical lesions when they are exposed to this HPV type.

Clinical type	Location and characteristics	Associated HPV (s)[a]
Deep plantar wart	Bottom surface of feet; generally single	HPV-1
Common wart	Mostly on hands; generally multiple	HPV-2, -4
Mosaic wart (superficial spreading wart)	Feet and hands; resistant to treatment	HPV-2
Flat wart	Arms, face, around knees; multiple	HPV-3, -10, -28, -41
Reddish-brown (macular) plaques of EV	Potential for malignancy in light-exposed areas	HPV-5, -8, -9, -12, -14, -15, -17, -19, -20, -21, -22, -23, -24, -25, -36, -37, -38, -47, -49
Butcher's warts	Common warts on hands of butchers and meat handlers	HPV-7

[a] HPV-27, -29, -37, -38, -46, -48, -49, and -49 are also recovered from skin warts.
EV, epidermodysplasia verruciformis.

TABLE 3. *Association of HPV types to morphology and location of skin warts*

Regression of lesions is believed to be mediated immunologically. When most lesions regress spontaneously, there may be a mild mononuclear cell infiltrate, although regression of flat warts may be associated with an erythematous reaction around the lesions in association with an intense inflammatory infiltrate. The low incidence of warts in older people may imply that immune mechanisms have rendered them resistant to infection.

Epidermodysplasia Verruciformis

EV is a rare disorder in which affected individuals have a unique susceptibility to cutaneous HPV infection (187,222). The warts usually develop in childhood, become widespread, do not tend to regress, and in some instances may progress to squamous cell cancers.

There are two predominant types of lesions. Some have the appearance of flat warts, whereas others are flat, scaly, red-brown macules that resemble lesions of pityriasis rosea or tinea versicolor. Both types often occur in the same patient. The flat warts are caused by the same HPV types that induce flat warts in the general population, usually HPV-3 or HPV-10. The pityriasis-like lesions are associated with EV-specific HPV types, most frequently HPV-5 and HPV-8. EV patients are frequently infected with several HPV types.

In about half of affected patients, EV occurs as an inherited disorder, and many EV families have a history of parental consanguinity. Inheritance appears to have an autosomal recessive pattern in most affected families, although one family with apparent X-linked recessive inheritance has been reported (7). Cases with autosomal recessive inheritance are genetically heterogeneous because the condition in different families has been mapped to two distinct chromosomal loci (243). EV patients do not ordinarily display an increased susceptibility to clinical infection with other microbial agents, including the mucosal HPV types or those HPV types associated with common warts. Because many normal individuals have been found to harbor EV-specific HPV types in normal skin, EV patients are unusual in that these HPV types produce clinically apparent lesions. The lesions in EV patients are resistant to usual treatment modalities. These features make it likely that EV patients suffer from an inadequate immune response to infection by the subset of HPV types to which they are susceptible. Consistent with this possibility, global defects in cellular immune function, such as inability to be sensitized to dinitrochlorobenzene (DNCB) and impaired mitogenic responses of cultured lymphocytes to phytohemagglutinin, can be demonstrated in most, but not all, patients with EV. In addition, acquired EV has been described in association with HIV infection (17), and clinical lesions associated with EV-specific HPV types have been described in other immunosuppressed individuals (172).

About one third of EV patients develop skin cancers in association with their lesions. Most of the malignant tumors remain local, but regional and distant metastases may occur. The risk for malignant progression is limited to the pityriasis-like lesions, which are the lesions that contain the EV types. HPV-5 and HPV-8 appear to be the most oncogenic because about 90% of the skin cancers contain one of these two types. Another important feature of the skin cancers is that they usually develop on sun-exposed areas. It therefore appears that carcinoma develops in EV by a combination of infection by an oncogenic EV HPV type plus the co-carcinogenic effects of ultraviolet light. Some EV patients may develop more than one skin cancer. However, cancer progression is a relatively rare event for EV lesions because even in these individuals, most lesions remain benign. Because the tumors do not undergo normal differentiation, the tumors do not contain detectable virions. However, multiple copies of viral DNA are found in primary tumors and in their metastases.

Nonmelanoma Skin Cancer

NMSCs, which are extremely common, are generally subdivided into basal cell carcinomas and squamous cell carcinomas (239). They generally arise on exposed areas of skin; exposure to sunlight is a predominant risk factor; and tumors associated with sun exposure are usually locally invasive but only rarely metastasize. The skin of

immunosuppressed individuals is at high risk for warts as well as premalignant lesions and NMSC, especially squamous cell, in exposed areas (reviewed in 146).

The known oncogenic potential of at least some HPVs and the consistent finding of certain HPV types in squamous cell carcinoma associated with EV make HPV infection an attractive etiologic agent for at least some NMSCs in individuals who do not have EV. Consistent with this possibility, sensitive detection techniques, such as nested PCR, have led to HPV DNA being identified in a substantial proportion of NMSCs in immunocompetent and immunosuppressed patients (60,63,278). When these sensitive techniques are used, about 30% to 40% of NMSCs from immunocompetent patients may be HPV positive, with the rate being about twice as high in tumors from immunosuppressed patients. However, the fraction of these tumors that should be attributed to HPV remains unclear.

The current evidence favoring a pathogenetic role for HPV is stronger in immunosuppressed individuals than in immunocompetent individuals (61,146,233). Several factors contribute to the uncertainty. Predominant HPV types have not been as clearly identified in NMSC in these populations, in contrast to squamous cell carcinoma in EV or to cervical cancer. One heterogeneous range of HPV types appears to be associated with NMSC in immunocompetent patients, and another in immunosuppressed patients. This observation differs from what is seen in cervical cancer, in which the HPV types detected appear to be relatively independent of the immune status of the patient. The NMSCs that arise in immunosuppressed patients are preferentially associated with EV-specific types, but precisely which ones has not reached consensus. In immunocompetent patients, less sensitive detection techniques, such as Southern blotting or type-specific PCR, have identified HPV DNA in only a small minority of NMSCs, although these techniques are adequate to detect HPV DNA in most cases of squamous cell carcinoma in EV or in cervical cancer. One large study that employed these less sensitive techniques identified HPV DNA in less than 3% of the tumors from immunocompetent patients (141). However, these techniques have identified HPV DNA in 30% to 45% of NMSCs in immunosuppressed patients (reviewed in 233). The positive HPV results that require the use of extremely sensitive detection techniques imply that these tumors contain low levels of HPV DNA, probably much fewer than one copy per cell. These positive results also need to be compared with those obtained when noninvolved skin is examined. Using highly sensitive techniques, one study reported HPV DNA in 90% of NMSC lesions from immunosuppressed patients, compared with 35% positive in samples from nonlesional skin (61,63). A recent report of basal cell carcinoma in immunocompetent patients examined paired samples and found that tumor tissue was not more likely to be HPV DNA positive than was normal uninvolved skin (320).

Infection of the Genital Tract

HPV infection of the anogenital tract represents the most important medical burden from infection with this group of agents (Table 4). Infection affects the genital skin and mucosa, including the vaginal tract, cervix, and anal canal. Most of these infections are sexually transmitted, which means that their prevalence is usually correlated with measures of sexual promiscuity, such as number of lifetime sexual partners, recent change in sexual partners, and a history of other sexually transmitted infections.

Clinical association	Viral type(s)
Genital tract	
Subclinical infection	All genital HPVs
Exophytic condyloma (any site)	HPV-6, -11
Flat condyloma (especially cervix)	HPV-6, -11, -16, -18, -31, others
Bowenoid papulosis	HPV-16
Giant condyloma (Buschke-Lowenstein tumor)	HPV-6, -11
Cervical cancer	
Strong association	HPV-16, -18, -31, -45
Moderate association	HPV-33, -35, -39, -51, -52, -56, -58, -59, -68
Weak or no association	HPV-6, -11, -26, -42, -43, -44, -53, -54, -55, -62, -66
Vulvar cancer	HPV-16
Penile cancer	HPV-16
Respiratory papillomas	HPV-6, -11
Conjunctival papillomas	HPV-6, -11
Oral cavity	
Focal epithelial hyperplasia	HPV-13, -32
Infection with genital tract HPVs	HPV-6, -11, -16
Lesions on lip	HPV-2

TABLE 4. *Major clinical association of genital tract and other mucosal HPVs*

Infection of the anogenital tract with HPV is extremely common and has a wide range of clinical manifestations and outcomes, varying from asymptomatic and self-limited to persistent and associated with malignant progression. Viral factors and host factors each contribute to determining the outcome.

Anogenital Warts

In contrast to nongenital warts, these lesions are most prevalent among young sexually active adults, and their frequency usually parallels that of other sexually transmitted conditions. One study reported that condylomas developed in about two thirds of the sexual partners of individuals with condylomas (221). The mean incubation period was 2.8 months, with a range of 3 weeks to 8 months. It is possible that viral transmission might have been delayed in some cases with longer incubation periods. Penile lesions occur commonly in sexual contacts of women with cervical intraepithelial neoplasia (14).

The increase in sexual promiscuity that began in the mid-1960s was associated with a substantial increase in the incidence of condylomas. Between 1966 and 1981, the number of visits to private physicians increased sixfold, from about 170,000 to almost 1 million (1). The number of office visits in 1981 for condylomas was about three times higher than that for genital herpes. Similar findings were made in a population-based study in Minnesota, where the incidence of condylomas between the early 1950s and the mid-1970s increased from 13 per 100,000 to 106 per 100,000 population (49).

Condylomas are usually exophytic lesions that are frequently multiple (220). They may arise anywhere on the external genitalia and may be found simultaneously in multiple sites. In males, they occur most commonly on the penis and anus, and in females on the perineum and anus. The anus may develop multiple lesions that coalesce to surround the anal canal. Most condylomas are usually self-limited, regressing spontaneously or after local treatment, but some lesions may persist for years. As with other HPV infections, genital warts when they arise in patients with impaired cellular immunity may be extremely refractory to treatment. They may also increase in size and number during pregnancy and regress after delivery. This sequence of events may also be a reflection of the immunosuppression associated with pregnancy.

Most genital warts are caused by HPV-6 or HPV-11, which are closely related to each other, with HPV-6 being responsible for most of them (104). Other HPV types, including HPV-16, may also be found in these lesions. Bowenoid papulosis is a related entity (105). The lesions usually consist of multiple small papules, which histologically resemble Bowen's disease or squamous cell carcinoma *in situ.* Most of these lesions contain HPV-16, but the rate of transition to frank malignancy appears to be much lower for the external genitalia than for the cervix. Genital warts in children may be sexually transmitted, secondary to sexual abuse (51,218). However, the genital lesions in children may commonly result from virus inoculation at birth or from incidental spread from cutaneous warts. In contrast to anogenital lesions in adults, a significant proportion of genital warts in children contain HPV types that are usually isolated from nongenital warts, perhaps because, compared with adults, the genital skin in children is more susceptible to infection with these “nongenital” HPV types.

Cervical Cancer

Cervical cancer is the third most common cancer among women worldwide, with about 400,000 newly diagnosed cases each year (231,237). Despite its worldwide distribution, the frequency of cervical cancer varies considerably, being about 10 times more common in some countries than in others (2,126,266). Cervical cancer is the most common cancer of women in most developing countries, where it may account for as many as one fourth of female cancers. It occurs less frequently in developed countries. In the United States, there are about 15,000 newly diagnosed cases annually, and about one third of these women die from malignant disease. The incidence of cervical cancer in the United States varies considerably between ethnic groups, with the rate among black women being about twice that of white women.

Most cancers occur in the transformation zone of the cervix, where the columnar cells of the endocervix form a junction with the stratified squamous epithelium of the exocervix (156,327). About 85% of cervical cancers are squamous cell cancers. Most of the other cases are adenocarcinomas, with a small number being small cell neuroendocrine tumors.

Lesions that are destined to become malignant squamous cell carcinomas typically undergo a series of dysplastic changes over a time span of many years (156,245,327). The severity of the lesion is determined by the degree to which the squamous epithelium is replaced by basaloid cells, with the entire thickness being replaced in the most severe dysplasias. In the traditional classification of cervical intraepithelial dysplasia (CIN), grades I,II , and III correspond, respectively, to mild dysplasia, moderate dysplasia, and severe dysplasia or carcinoma *in situ* (Fig. 4). In the more recently described Bethesda system (158), these abnormalities have been classified as low-grade and high-grade squamous intraepithelial lesions (SIL), with low grade corresponding to mild dysplasia and high grade encompassing the more advanced lesions (Fig. 5). There is also a designation ASCUS (for *a*typical *s*quamous *c*ytology of *u*ndetermined *s*ignificance) for equivocal lesions.

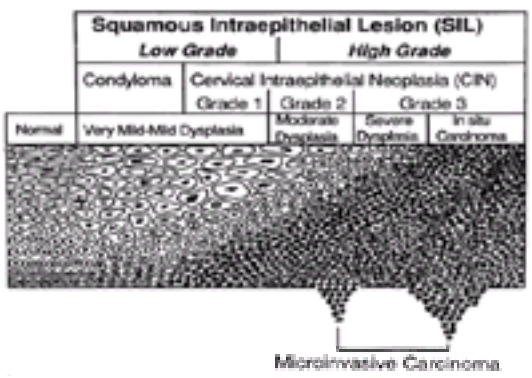

FIG. 4. Cervical squamous carcinoma precursors. Schematic representation of cervical cancer precursors and the different terminologies that have been used to refer to them. The risk for microinvasion from different states of squamous intraepithelial lesions (cervical intraepithelial neoplasia) is arbitrarily represented and is not necessarily proportional to that illustrated in this scheme. (From ref. 327, with permission.)

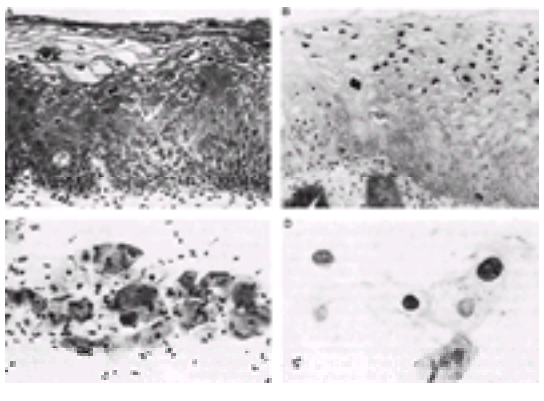

FIG. 5. Low-grade squamous intraepithelial lesions of the cervix. **A:** Histologic section showing koilocytes with vacuolated cytoplasm and parakeratosis (H&E, ×234). **B:** The darkly stained nuclei contain papillomavirus capsid antigen. [Immunoperoxidase stained with genus-specific antiserum (H&E, ×234)]. **C:** Cervical smear. The squamous epithelial cells are rounded. They occur in clumps and display koilocytic changes (Papanicolaou stain; H&E ×375). **D:** Cervical smear. The darkly stained nuclei contain papilloamavirus capsid antigen. (Immunoperoxidase staind with genus-specific antiserum; H&E, ×750). (Courtesy of P. Gupta, University of Pennsylvania. From ref. 276, with permission.)

Most dysplasias do not progress and, in fact, resolve spontaneously, with the likelihood of resolution decreasing with the severity of the dysplasia (Fig. 6). However, the more severe dysplasias generally arise from less dysplastic lesions after several years (132,174), although some high-grade dysplasia may develop rapidly without passing through a low-grade stage (155). The cervical dysplasias have their counterpart in the exfoliated cells present in the Papanicolaou (Pap)smear, by the presence of basaloid cells and koilocytosis. Because of the long interval between the development of cervical dysplasia and the development of invasive cancer, Pap smear screening programs can identify most premalignant lesions. Appropriate follow-up of women with these abnormalities, together with appropriate treatment, can thereby prevent the development of most cases of cervical cancer.

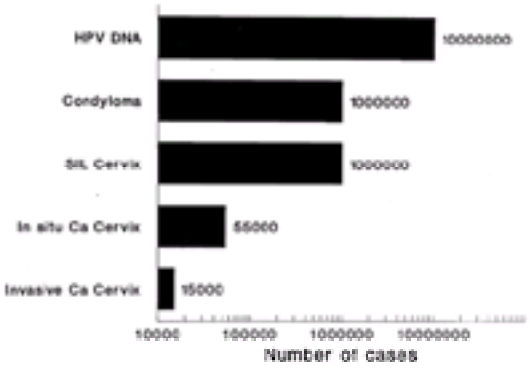

FIG. 6. Estimates of prevalence of genital tract HPV infections and of HPV-associated diseases, in U.S. women. Estimates of number of invasive and *in situ* carcinoma cases are from the American Cancer Society. Other estimates are crude and derived from many sources. (From ref. 276, with permission).

Cervical cancer has been recognized for decades to behave as a sexually transmitted disease, long before sexually transmitted HPV infection was implicated in its pathogenesis. Many studies have concluded that this cancer does not occur in virgins, is most common among prostitutes, and is correlated with number of lifetime sexual partners. Indeed, most of the wide differences in the incidence of cervical cancer in different populations can be accounted for by the sexual practices of the men and women in these populations and by the availability of effective Pap smear screening programs (Table 5).

Number of lifetime sexual partners				
Females	Males	Health care	Cervical cancer incidence	Example
0	–	Variable	0	Nuns, virgins
Few	Few	Variable	Very low	Religious groups, e.g., Amish, Mormons
Few	Many	Poor	Very high	Latin America
Many	Many	Poor	Very high	Some countries in Africa
Many	Many	Good	Low	U.S., Europe

Adapted from ref. 153, with permission.

TABLE 5. *Relationship of risk factors to cervical cancer incidence*

Human Papillomavirus and Cervical Cancer

In the mid-1970s, Meisels and his colleagues recognized, on morphologic grounds, that papillomavirus infection of the cervix occurred frequently, often with the histologic characteristics of mild CIN (200). These observations coincided with the proposal by zur Hausen that HPV infection might be the putative sexually transmitted agent responsible for cervical cancer (341). The CRPV system had provided experimental evidence that animal PVs could induce malignant tumors, whereas the development of skin cancer in EV patients had demonstrated that some HPVs might have malignant potential. As noted earlier, another known feature of EV was that only a subset of the HPVs isolated from EV patients were found in the skin cancers, implying that HPV types might vary in their oncogenicity. The identification in the early 1980s of HPV-16 and HPV-18 by zur Hausen and his colleagues (24,72) provided the field with HPV types that could be shown to be present in most cervical cancers and could, by lower stringency hybridization techniques, identify HPV DNA

in an even greater proportion of these tumors. Subsequent studies, carried out by many investigators, have sought to understand the natural history of HPV infection of the genital tract, determine the biologic properties of different HPV types, elucidate the role of the virus in the pathogenesis of cervical disease, and identify nonviral factors that may influence the outcome of HPV infection.

More than 25 HPV types can infect the genital tract and be associated with cervical dysplasia in some infected women, with no single HPV type accounting for most infections. However, only a subset of the genital-mucosal HPV types are found regularly in cervical cancers. Furthermore, the likelihood of finding certain genital types in more severe dysplasias and in the cancers can be shown to increase rapidly with some types, to remain approximately unchanged with other types, and to decrease with others (Fig. 7). These differences, in association with progression to cervical cancer, have led some types to be designated as high risk (such HPV-16, HPV-18, and HPV-45), intermediate-risk (such as HPV-31, HPV-33, HPV-35, HPV-51, and HPV-52), and low-risk (such as HPV-6, HPV-11, HPV-42, HPV-43, and HPV-44) (23,126,181). This classification has considerable utility but must not be overinterpreted. The classification for a particular HPV type is based on the assumption that the relative frequency of exposure and infection has remained constant over many years, which may not be warranted in some instances. Another issue is that even low-risk HPV types may occasionally be associated with cervical cancer (100). HPV-6 and HPV-11 isolated from cervical cancers may have alterations in their genome that could have increased their oncogenic potential, or an HPV type might be more oncogenic in an immunocompromised host. It should also be recognized that this classification may not apply to HPV infection in all sites. For example, the Buschke-Lowenstein tumor, which is a low-grade squamous cell carcinoma of the external genitalia, is usually associated with the low-risk HPV-6 or HPV-11 (106,193). Laryngeal cancer complicating recurrent respiratory papillomatosis also involves these low-risk types (136).

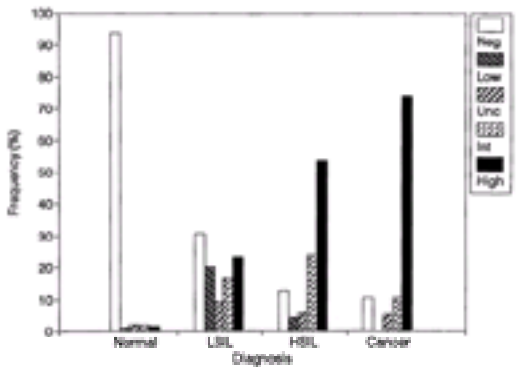

FIG. 7. Patterns of HPV type distribution within four diagnostic categories: Neg, negative; Low, low-risk HPVs (HPV-6, HPV-11, HPV-42, HPV-43, HPV-44); Unc, unclassified HPVs; Int, intermediate-risk HPVs (HPV-31, HPV-33, HPV-35, HPV-51, HPV-52); High, high-risk HPVs (HPV-16, HPV-18, HPV-45, HPV-56); LSIL, low-grade squamous intraepithelial lesion; HSIL, high-grade squamous intraepithelial lesion. (From ref. 181, with permission.

Nonmalignant and Premalignant Cervical Infection

Cervical HPV infection is extremely common among young sexually active women (2,126,266). The estimated prevalence of infection varies with the age of the population and depends on the sensitivity of the assay used to detect HPV DNA in clinical isolates. The peak prevalence is seen in women 15 to 25 years of age, which may be 25% to 40%, and then declines with increasing age (Fig. 8). The decreased rate of HPV infection with age probably results from the self-limited nature of most infections, decreased HPV exposure, and resistance to reinfection. An increase in HPV DNA positivity in women older than 60 years of age has been identified in some populations (119). It is not clear whether to attribute this phenomenon to a cohort effect, a decrease in immune surveillance, or some other explanation.

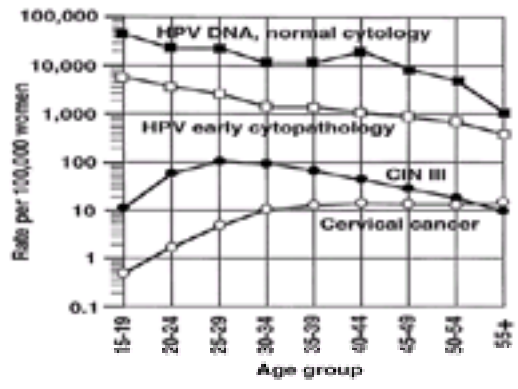

FIG. 8. Age-specific rates of cervical HPV infection and associated abnormalities. HPV infection with normal cytology is much more common than HPV infection associated with abnormal cervical cytology. The prevalence of HPV infection decreases with age. Peak incidence of cervical intraepithelial neoplasia type III (CIN III) develops in women who are older than those with the highest rates of HPV infection. Peak incidence of cervical cancer develops in women who are older than those with the highest rates of CIN III. HPV DNA with normal cytology and early cytopathology are point-prevalence data, whereas the CIN III and cervical cancer data are incidence rates. The data are from several sources in the United States. (Adapted from ref. 266, with permission.)

Although most women with HPV infection of the genital tract do not have any detectable cytologic abnormalities, the epidemiology of genital HPV infection appears to account for the epidemiology of cervical neoplasia, including the dysplasias that precede cervical carcinoma. Some analyses published in the 1980s failed to show a strong association between HPV infection and cervical neoplasia. These conclusions were the consequence of false-negative and false-positive HPV DNA results obtained with incompletely validated assays. More recent analyses, based on validated assays, have consistently shown that HPV infection represents a major risk factor for cervical neoplasia (2,126,266).

In multivariate analyses, HPV infection accounts for at least 80% to 90% of the attributable risk for the development of cervical dysplasia. In one cross-sectional study, HPV prevalence in patients with SIL was 81%, compared with 18% in cytologically normal specimens (264). When five cytologists examined the same set of “atypical” Pap smears, the HPV DNA prevalence was 100% for those specimens classified by all of them as abnormal, compared with 21% for those smears that they all classified as normal (280). A key epidemiologic question is whether HPV infection precedes the development of cytologic abnormalities. In this context, cohort studies of cytologically normal women have shown that HPV infection frequently precedes the development of cytologic abnormalities and that women infected with HPV-16 or HPV-18 are at highest risk for dysplastic changes. In a cohort of women with negative cytology at recruitment, the cumulative incidence of SIL at 2 years was 28% in the HPV-positive women, compared with 3% in the HPV-negative women (155). In another study, cytologically normal women who were HPV DNA positive at recruitment were 3.8 and 12.7 times more likely to develop low-grade and high-grade SIL, respectively, compared with HPV DNA–negative controls (174).

Human Papillomavirus in Invasive Cervical Cancer

A worldwide study of almost 1,000 cervical cancers, from paraffin-embedded sections, indicated that more than 90% contain HPV DNA (23). The results confirmed and extended observations made in more restricted studies of cervical cancer (2,126). Although more than 20 HPV types were found in the tumors, four types (HPV-16, HPV-18, HPV-31, and HPV-45) accounted for close to 80% of the HPV-positive cancers. Conversely, low-risk HPVs were found in only two of these cancers. In almost all areas of the world, HPV-16 appears to be the most common type found in the cancers. However, there does appear to be some regional variation in the specific proportion of HPV types. A follow-up evaluation of the cancers in the international study that were initially thought to be HPV-negative indicated either that they were false-negative results or that the DNA in the specimens was too degraded for the negative results to be deemed reliable (317). The conclusion from this study was that at least 99.9% of cervical cancers contain HPV DNA.

Most studies have focused on the more common squamous cell cancers. However, most adenocarcinomas, adenosquamous carcinomas, and carcinomas with neuroendocrine differentiation also contain HPV DNA. One apparent difference is that most studies have found HPV-18 to be the predominant type in adenocarcinomas and neuroendocrine carcinomas, in contrast to HPV-16 being most common in the squamous cell cancers. Some evidence has suggested that HPV-18 infection may progress more rapidly and carry a poorer prognosis than HPV-16.

A Mechanistic Role for HPV in Cervical Neoplasia

Epidemiologic data have clearly established that cervical HPV infection precedes the development of cytologic abnormalities, that strong previous risk factors associated with cervical dysplasia and cervical cancer tend to be eliminated when controlled for the presence of HPV, that HPV infection can account for most cervical dysplasias, and that HPV DNA is found in virtually all cases of cervical cancer. Odds ratios of 10 to more than 200 have been found for HPV infection as a risk factor for these clinical abnormalities, with the highest odds ratios seen for infection with HPV-16 and HPV-18. These odds ratios are the highest for a human carcinogen. In addition, results of a validated serologic assay that can identify currently and previously infected individuals also implicate HPV-16–related viruses in the development of cervical cancer (171).

Laboratory-based studies of HPVs also strongly support the inference that HPVs are etiologically involved in cervical cancer. The viral DNA is present in all cells of the primary tumors, as well as in metastases, and is transcriptionally active. In mildly dysplastic lesions, each of the viral ORFs appears to be expressed, mainly in the upper levels of the epithelium (298). In high-grade dysplasias, by contrast, a more restricted number of ORFs are expressed, primarily *E6* and *E7,* and their expression is now found in the basal, proliferating layer of the epithelium. More progressed lesions are associated with a higher expression of these genes. High-grade dysplasia and progression to carcinoma is associated with integration of the viral genome, with deletion of large segments of the viral genome and with transcription of sequences downstream from the integrated LCR (11,270) (Fig. 9). In this integrated form, the *E6* and *E7* ORFs remain intact in the integrated viral DNA, from which they can be transcribed. Disruption of the viral *E1* and *E2* genes as well as of downstream viral sequences may permit higher levels of *E6* and *E7* transcription (64,130,254), whose RNA is stabilized following fusion to downstream cellular sequences. Viral DNA integration occurs in almost all tumors and tumor-derived cell lines that harbor HPV-18, and in most with HPV-16, although the DNA remains episomal in some HPV-16 tumors and cell lines (57). Most cell lines derived from cervical cancer contain HPV DNA, either HPV-16 or HPV-18 (270). They continue to express E6 and E7, which appear to be necessary for their continued viability and their ability to proliferate (64,316). However, some tumor-derived cell lines are negative for HPV DNA (262,324), raising the possibility that some primary cervical cancers may be HPV DNA-negative.

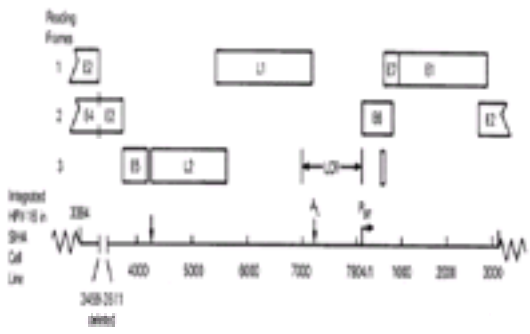

FIG. 9. Structure of the single copy of HPV-16 DNA integrated into the SiHa cell line derived from a cervical carcinoma. The *jagged line* represents human chromosomal sequences; the nucleotide numbers pertain to the integrated HPV-16 genome. The *open boxes* indicate the early and late open reading frames (ORFs). Integration has occurred in the *E2* ORF, and a portion of the *E2* ORF has been deleted. (From ref. 11, with permission.)

Experimental studies with HPV genomes and individual viral genes emphasize the greater biologic activities of the high-risk HPVs compared with the low-risk types, especially the critical role of E6 and E7. In organotypic raft cultures, high-risk HPV can induce changes that resemble dysplastic lesions (195). Furthermore, the high-risk HPV genomes, such as HPV-16 and HPV-18, are capable of immortalizing human keratinocytes, in contrast to the low-risk HPV genomes, although cooperating cellular changes are probably also necessary (342). In experimental results that parallel the observations in the cervical cancers and cell lines derived from them, efficient immortalization requires cooperation between high-risk E6 and high-risk E7 (114,211). Substitution of either low-risk E6 or low-risk E7 severely reduces the efficiency of immortalization.

Thus, the *E6* and *E7* genes from high-risk HPVs collaborate to interfere with normal growth and cell cycle checkpoint controls, and with the regulation of chromosomal stability (94). Mutational and biochemical analyses of the proteins encoded by *E6* and *E7* have provided insight into some of the complex mechanisms by which these multifunctional genes act. As noted previously, the high-risk E6 proteins inactivate p53, whereas the high-risk E7 proteins bind and inactivate pRb. HPV-16 and HPV-18 positive cell lines derived from cervical and anal cancers harbor wild-type versions of pRb and p53, whereas HPV-negative tumor-derived cell lines have mutant Rb and p53 (55,56,262,324). These results imply that E6 and E7 generally induce substantial functional inactivation of p53 and pRb *in vivo* and support the conclusion that inactivation of pRb and p53 make an important contribution to the biologic activities of these proteins. In at least some instances, however, there may be some residual p53 activity, as evidenced by HPV-positive tumors with mutant p53 in metastases (54). The p53 protein is a transcription factor that, by means of its ability to regulate a series of target genes, orchestrates a pleiotropic cellular response to various genotoxic and cytotoxic stresses, to preserve genomic integrity and normal cell growth (173). Abrogation of p53 activity by E6 therefore disrupts a critical inhibitor of genomic instability and of abnormal cell growth (190). E6 interferes with p53 function primarily by inducing the ubiquitin-dependent degradation of p53, a function that is mediated by the ubiquitin ligase E6-AP (261,263). However, E6 can also interfere more directly with p53 activity by other mechanisms. These include the ability of E6 to inhibit the interaction of p53 with its cognate DNA-binding sites in transcriptional targets and the ability of E6 to bind to p300/CPB, which is a coactivator of p53 (168,340). One important biochemical consequence of the interaction between E7 and pRb is the release of the transcription factor E2F1, which, in the absence of E7, is bound to the underphosphorylated pRb that inhibits growth (43,225). The release of E2F1 leads to its activation, which probably makes an important contribution to the pRb-dependent biologic activity of E7 (202,208).

At least some effects of E6 and E7 are p53 independent and pRb independent, respectively (190,343). Mutants with impaired biologic activity *in vitro* have been described for E6 proteins that still induce degradation of p53 and for E7 that still bind pRb (13,85,179). Several cellular proteins in addition to p53 and pRb have been reported to interact with E6 or E7, as described in the previous chapter, although their precise role in tumorigenesis is still unclear. For example, E6 can interact with and inhibit the protein encoded by the discs large gene (DLG), and with interferon regulatory factor 3, resulting in the inhibition of its transcriptional activity (148,169,255). In addition to its interaction with Rb, E7 has been reported to bind and interfere with at least two additional cell cycle inhibitors, p21CIP1 and p27KIP1, and to activate cyclins A and E (93,131,198,336,343).

One important HPV-dependent effect relevant to cancer is the activation of telomerase (147,150). In human keratinocytes, this activity is induced primarily by E6, by a mechanism that is largely independent of p53. Efficient immortalization of keratinocytes by E6 and E7 probably depends on the ability of E6 to inactivate p53 and to activate telomerase, although keratinocyte immortalization can be induced by E7 plus either a mutant p53 or the catalytic component of telomerase. In cervical lesions, telomerase activation is associated with progression to severe dysplasia and cervical cancer, and cell lines derived from cervical cancers are uniformly positive for telomerase (139,214,291).

There is also evidence that high-risk HPV can induce abnormal centrosome duplication, which can result in genomic instability and aneuploidy (69). The deregulation of this mitotic event appears to depend on E6 and E7, with the latter protein being most responsible for the effect.

It has also been found that the oncogenic potential of some intratypic variants may be greater than that of others (40,180,331,332,335). This phenomenon has been best documented for HPV-16, but it also appears to apply to HPV-18 and perhaps to other HPV types. Although HPV types have a worldwide distribution, certain variants are more frequently identified in certain regions. By this criterion, HPV-16 can be divided between those that are considered to be European variants (also referred to as *prototype-like* because a European variant was the first HPV-16 identified) and those that are more distantly related (designated *non–prototype-like*). Several studies have now found that non–prototype-like variants

are associated with a greater likelihood of persistent infection and with high-grade cervical dysplasia and cervical cancer as well as with anal carcinoma *in situ.* It should be recognized, however, that prototype-like HPV-16 variants still account for most cases of severe dysplasias and cancer associated with HPV-16. It is unclear whether the differences in oncogenic potential are attributable primarily to variation in the LCR (134), in the biologic activity of one or more viral proteins, or in the immune response they induce (334).

In summary, the evidence linking HPVs to cervical cancer meets all the criteria for an etiologic relationship. The association of HPVs with cervical cancer is very strong, consistent, and specific; it holds for cervical cancers throughout the world; cohort studies show that HPV infection precedes dysplasias and malignancy; and important aspects of the disease can be reproduced in experimental models. Thus, the evidence for biologic plausibility is as strong as for any human carcinogen.

Cellular Events in Cervical Cancer

Although infection with high-risk HPV may be necessary for the development of cervical cancer, or other cancers attributable to HPV infection, it is clearly not sufficient. Cancer must arise only after other factors have collaborated with the infection. Some changes may be virus specific, as discussed previously. For example, viral integration into the host DNA leads to decreased expression of some viral genes, increased expression of E6 and E7, selective growth advantage, and progression to high-grade dysplasia and cancer (57,129,130,270).

Other changes associated with progression include genetic alteration of cellular genes. Cervical cancers are aneuploid, and as discussed previously, the *E6* gene probably plays an important role in fostering genomic instability, although *E7* may also contribute to this abnormality. Tumors typically have several cytogenetic alterations, which implies loss or gain of function of several cellular genes. In most instances, the specific gene that has been affected by a given cytogenetic alteration has not been identified. Although most of these cytogenetic changes are probably not a direct consequence of viral DNA integration, cellular genes can be directly affected by the viral integration. For example, viral DNA integration near *Myc* and *JunB* has been associated with increased expression of these protooncogenes (46,71).

Consistent with the multistep nature of tumorigenesis, cervical cancers may show additional cytogenetic alterations when compared with adjacent dysplastic lesions (143). Certain cytogenetic changes have been found in a relatively high proportion of tumors (reviewed in 166). Those found in one fourth to one half of tumors include loss of heterozygosity in chromosome regions 3p14, 4p16, 4q21 to 4q35, 6p21 to 6p22, 11p15, 11q23, 17p13.3, and 18q12 to 18q22. Loss of heterozygosity in 3p has been identified in cervical dysplasias adjacent to cancers that also have this alteration (108). This observation implies that inactivation of a putative tumor suppressor gene in this region may occur as an early event that could predispose to further progression. The *FHIT* (fragile histidine triad) gene, which is a putative tumor suppressor gene, is a candidate for the 3p locus. It is located at 3p14.2, and its expression is inversely correlated with the severity of dysplasia or cancer (20). However, reintroduction of the *FHIT* gene into tumor cell lines did not alter their biologic properties (329). In contrast to the multiple loci with loss of heterozygosity, a gain of chromosome 3q24 to 3q28 has been reported to be present in almost all cervical cancers but to be absent from most cases of severe dysplasia (120). PIK3CA, which is located in 3q26.3 and is involved in the PI3 kinase–AKT signaling pathway, has been proposed as a candidate oncogene for this amplified locus (184). Amplification or overexpression of other oncogenes may occur, including *c-Myc, EGFR,* and *Her-2/Neu,* and may be associated with a poorer prognosis (142,206,236,246). A possible difference in risk for cervical cancer was recently proposed for individuals whose p53 contains Arg or Pro at residue 72, which are common allelic variants of p53 (reviewed in 190). This proposal was based on the experimental finding that the Pro-72 allele was more resistant to E6-dependent degradation than the Arg-72 allele, raising the possibility that individuals with the Arg-72 might be at increased risk for cervical cancer. However, an initial report that suggested such an increase in patients with cervical cancer has not been confirmed in several subsequent studies.

Insights into the collaboration between viral oncogenes and cellular genes in tumorigenic progression have also been obtained with cervical cancer–derived cell lines, by inducing their reversion and retransformation. Studies by Stanbridge indicated that the tumorigenic phenotype was recessive by showing that it could be suppressed by fusion of HeLa cells, a cervical adenocarcinoma–derived cell line that contain HPV-18, with normal human fibroblasts or keratinocytes (294). Reversion could be reproduced by microcell transfer of only chromosome 11 into the HeLa line or the SeHa line, which are HPV-16 positive (152). Conversely, loss of the short arm of chromosome 11 in fibroblasts was associated with their being more efficiently transformed by HPV-16 than wild-type fibroblasts (287). Taken together, the results are consistent with the existence of a tumor suppressor gene on the short arm of chromosome 11. It is unknown whether this gene and the 11p15 locus identified as a frequent site for loss of heterozygosity in cervical cancers are the same or distinct. Regardless, suppression of HeLa cell tumorigenicity in nude mice is associated with a decrease in the level of E6 and E7 expression *in vivo* (although not *in vitro*), in which tumor necrosis factor-a (TNF-a) probably plays an important role. Suppression can be abrogated by ectopic expression of the *c-fos* oncogene, which increases AP-1 transcription activity and renders the cells resistant to the inhibitory effects of TNF-a (293).

There is also evidence that cervical cancers may have undergone changes that help them to evade the immune system (reviewed in 32,87,188,297, and 311). The most frequent changes involve the down-regulation of HLA class I alleles, which are normally expressed on keratinocytes, in contrast to class II (53,297). Consistent with a role in progression, down-regulation of one or more class I alleles occurs in most tumors, is reported to be greater in metastases compared with the primary tumor, HLA-B7 positive patients whose tumors down-regulate this allele have a poorer prognosis, and selective loss of HLA-B7/40 allelic products is associated with lymph node metastases. Several mechanisms may account for the down-regulation of class I expression (28,53,153). Some affect class I alleles directly by mutation or deletion, whereas others affect the class I processing system, primarily by down-regulation of the TapI transporter, which is required for class I processing. The HLA region is located on the short arm of chromosome 6, at 6p21.3, which is within the 6p21 to 6p22 region that, as noted previously, has been reported to be a frequent site of cytogenetic abnormalities in cervical cancer. It is therefore likely that many of the cytogenetic rearrangements in this region reflect alterations in class I alleles. In addition to these class I changes, most primary tumors aberrantly express HLA class II molecules, perhaps in response to inflammatory cytokines produced by infiltrating lymphocytes (32,53,297). Class II expression is not a feature

of metastatic lesions.

Human Immunodeficiency Virus

The immunosuppression resulting from HIV infection is associated with an increased susceptibility to infection by many other microbial agents. The risk for genital HPV infection may be particularly high for HIV-infected individuals because infection by both viruses is often through sexual transmission, and it is well known that exposure to one sexually transmitted agent places an individual at higher risk for exposure to other sexually transmitted agents. Thus, individuals at high risk for sexual exposure to HIV would also tend to be at high risk for exposure to genital HPV infection. On the other hand, the lesions of uncomplicated cases of genital HPV infection are not usually associated with erosion or ulceration and thus would not be expected to provide an efficient portal of entry for HIV, in contrast to the predisposition to the acquisition of HIV infection associated with genital ulceration.

It has been well documented that cervical HPV infection is detected more frequently in HIV-positive women than in those who are HIV negative, the infection is even more likely to persist, it is more likely to result in cytologic abnormalities, and there is much greater risk for high-grade dysplasia (228,303,328). In a prospective study of women in New York at high risk of HPV infection, cervical swabs taken at the initial examination detected HPV DNA about twice as frequently in HIV-positive women compared with HIV-negative women (56% versus 31%), and persistent infection was six times as frequent in the HIV-positive women (24% versus 4%) (303). Low-grade dysplasia (CIN I) was about three times as frequent in HIV-positive women (13% versus 4%), whereas high-grade was seven times more frequent (7% versus 1%) (328). Low CD4 counts in HIV-positive women represent an independent risk factor.

These features of HPV biology in HIV-positive women make it likely that HIV-positive women would suffer from a high rate of cervical cancer. Indeed, retrospective studies from New York reported that an unexpectedly high proportion of cervical cancers diagnosed in women under 50 were HIV-positive (186), leading the CDC in 1993 to designate cervical cancer as an AIDS case-defining illness. Analysis of large cohorts has documented that HIV infection places women at increased risk of developing cervical cancer (90,272). In a report from the AIDS-Cancer Match Registry Study in the United States, HIV infection was associated with a relative risk of about 5 for women of any age (90).

HIV infection appears to represent a risk factor for other malignancies associated with HPV infection, including cancers of the vulva, penis, and anus (90). The relative risk for developing these cancers was higher for individuals who were under 30 years of age than for older individuals. As discussed below, the relative risk for anal cancer is especially high for HIV infected male homosexuals.

Other Cofactors for Cervical Cancer

Many additional factors have been examined for their possible influence on the incidence of cervical cancer and its precursor lesions. When controlled for the presence of HPV infection, the factors of smoking, oral contraceptives, pregnancy, parity, nutrition, micronutrients, and sexually transmitted diseases have been found in some studies to be associated with increased risk (15,58,97,149,212,266). In most instances, it is not clear how the cofactor may contribute to dysplasia and carcinogenesis. Possibilities include increasing the risk for establishing nfection, decreasing local or systemic immunity, stimulating the growth of HPV-infected tissue, and inducing mutations in infected tissue. It may be relevant that higher levels of a tobacco-specific carcinogen have been identified in the cervical mucus of cigarette smokers (240). The possible relationship between cervical cancer and certain HLA class II alleles was also noted previously (32,297). It is not surprising, therefore, that some evidence exists for an hereditary contribution to the risk for cervical cancer (117).

Although those factors may make some contribution to the development of cervical cancer, they are unlikely to explain the wide variation in cervical cancer incidence in different populations throughout the world. In this context, the incidence of cervical cancer in an area depends primarily on the relative endemnicity of HPV infection and the effectiveness of cervical cancer screening programs (126).

Anal Cancer

Anal cancer appears to share important similarities with cervical cancer, as well as some differences. The disease is somewhat more frequent in women than in men, with about 1,900 female cases and 1,400 male cases per year in the United States (162). Anal HPV infection appears to be sexually transmitted in most instances, and a history of receptive anal intercourse in women and of homosexual activity in men is associated with an increased risk for this cancer (91). However, such a history is often lacking, which makes it likely that some anal infections have spread from other genital areas. Consistent with this interpretation, simultaneous cervical and anal infection, often with the same HPV type, may be relatively common. The rate of anal infection by HPV appears to be similar to that of cervical infection, although anal HPV infection has been studied less systematically than cervical infection (323). As with cervical cancer, high-risk HPVs have been found in most anal cancers, with an even greater preponderance of HPV-16 than in cervical cancer, and most anal cancers arise in the transition zone between columnar and squamous epithelium. In one large study from Scandinavia, 83% of HPV-positive tumors contained HPV-16; 93% of tumors from women were HPV positive, whereas 69% of the male tumors were HPV positive (91). It would therefore appear that most of these cancers in the women were attributable to HPV infection, whereas some proportion of the anal cancers in men might not be associated with HPV infection.

One important difference from cervical cancer is that the risk for anal cancer in the general population appears to be much lower than for cervical cancer, although it is not known what may account for these differences in rates of progression. In countries with effective cervical cancer screening programs, comparisons between the incidence of cervical cancer and anal cancer underestimate the difference in risk for progression because these programs prevent many potential cases of cervical cancer. However, in developing countries where there is almost no impact of Pap smear screening on the incidence of cervical cancer, the incidence of anal cancer in women is less than one tenth that of

cervical cancer (231).

The risk for anal cancer among HIV-positive individuals is much greater than in the general population, with especially high rates for HIV-positive male homosexuals. According to the AIDS–Cancer Match Registry Study in the United States, HIV infection was associated with a relative risk, for invasive cancer, of 6.8 for women and 37.9 for men (90). The difference in risk between women and men was attributable to the group of HIV-positive homosexual men, whose relative risk was 59.5, compared with a relative risk of 5.9 for HIV-positive male intravenous drug users. A very high proportion of men and women with HIV infection also have anal HPV infection. Studies from San Francisco report anal HPV infection in 93% of HIV-positive male homosexuals (versus 61% of HIV-negative homosexual males) (226), compared with 76% of HIV-positive women (227). It is therefore unlikely that the increased risk in male homosexuals for anal cancer results primarily from a higher incidence of anal HPV infection. Instead, it appears reasonable to postulate that an additional feature specific to receptive anal intercourse, such as trauma, may collaborate with HIV and HPV infection (90). Consistent with this interpretation, at least some of the increased risk in male homosexuals for anal cancer appears to have predated the HIV era (201). The high risk for anal cancer among homosexual and bisexual men who are HIV positive has led some authors to advise routine screening of this population for anal squamous epithelial lesions (98).

Human Papillomavirus in Other Genital Sites

Genital HPV may also routinely infect other genital areas that contain stratified squamous epithelium. The risks associated with HPV infection at these sites appears to be similar to those of cervical infection (126). The penises of male partners of women with cervical dysplasia are frequently infected with HPV, often with the same HPV type as their partner (14,322). HPV DNA, usually HPV-16, is found in a subset of cancers of the vulva, vagina, and penis. Giant condyloma acuminata, also called the *Buschke-Lowenstein tumor,* is a low-grade locally invasive squamous cell carcinoma that involves the external genitalia, most frequently the penis. It is associated with low-grade HPV types, such as 6 and 11 (106,193). HPV may also be present in a high proportion of urethral tumors (321).

In squamous cell carcinoma of the vulva, most of those invasive tumors designated morphologically as being warty or basaloid arise in younger women and contain HPV DNA, especially HPV-16 (126). Smoking and infection with herpes simplex virus type 2 have been identified as possible cofactors for vulval cancer. In contrast to basaloid vulval cancers, HPV DNA is found in only a minority of keratinizing squamous cell carcinomas of the vulva, which is associated with older women and with mutation of p53 (170).

Human Papillomavirus in the Aerodigestive Tract

RRP (laryngeal papillomas), which is caused primarily by HPV-6 and HPV-11, is the best characterized HPV-associated disease in the aerodigestive tract (5,137,209). The oral cavity may be infected with any of the genital-mucosal types (238,308). Infection by HPV-13 and HPV-32, which are responsible for focal epithelial hyperplasia, may be limited primarily to the oral cavity. HPV infection has also been implicated in cancer of the oral pharynx (96,203,269,289) and may also play a role in some cases of esophageal cancer (305).

Recurrent Respiratory Papillomatosis

RRP is a rare condition whose papillomas may severely compromise the airway, particularly in young children (5,137,209). Most lesions are caused by low-risk genital HPV types, especially types 6 and 11. Lesions frequently recur after surgical treatment.

Hoarseness or change of voice is the usual presenting symptom in RRP. The vocal cords of the larynx is the site most commonly affected, but papillomas may arise at other sites, such as the trachea, lungs, nose, and oral cavity, even without laryngeal involvement. Lesions may be single or multiple. Papillomas may be life-threatening if their bulk severely restricts the passage of air to or from the lungs. This complication may arise more frequently in infants because of the small size of their airway. Symptomatic papillomas are generally treated surgically. The treated lesions frequently recur, and surgery may lead to an increase in the number of lesions. The recurrent nature of the disease means that periodic surgical treatment may be required. Although most patients do not need to be treated more than once a year, some papillomas grow very rapidly, requiring surgical intervention as frequently as every 2 weeks. It is important to avoid the necessity of performing a tracheostomy in patients with RRP because papillomas often grow along the tissues involved in the tracheostomy, resulting in severe morbidity.

Epidemiologic studies suggest there may be at least two modes by which RRP develops (138,277). One primarily affects young children and the other is first diagnosed in older individuals. There is a steep rate of rise in the incidence of RRP during the first 5 years of life, with about half of cases developing in children by the time they are 5 years old (Fig. 10). RRP may occur at any age, but the risk for developing it decreases after the age of 5 years. In younger children, a history of genital warts in the mother is a risk factor for RRP. Depending on the study, 30% to 60% of mothers whose children develop juvenile RRP have a history of genital warts, compared with less than 5% of mothers of unaffected children. Children born by cesarean section are at much lower risk for RRP, and HPV transmission, than those born by vaginal delivery (277). Taken together, these observations suggest that most cases of RRP that arise in early childhood are attributable to intrapartum transmission of HPV from the mother to the child. *In utero* transmission probably also occurs, as suggested by occasional cases of neonatal RRP, but this mode of transmission appears to be rare. In addition to a history of genital warts and vaginal delivery, other risk factors in the mother associated with juvenile onset of RRP include young age of pregnancy and first pregnancy. It appears reasonable to speculate that these risk factors may represent markers for the likelihood of high levels of HPV being present in the genital tract at the time of delivery. A history of oral-genital sex may be a risk factor in adult RRP.

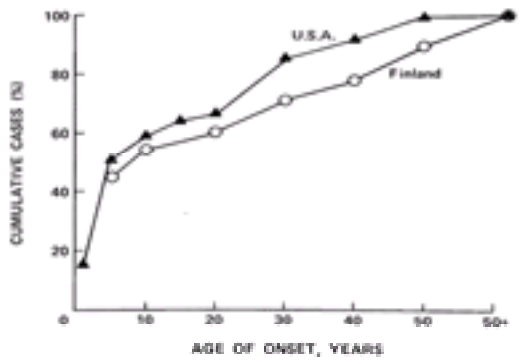

FIG. 10. Cumulative percentage of cases of respiratory papillomatosis in the United States and Finland, by age of onset. (From ref. 209, with permission.)

Infection with HPV-6 and HPV-11 accounts for most cases of RRP. Although the relationship between childhood RRP and genital HPV infection in the mother is well established, the incidence of RRP is much lower than that of adult genital infection with HPV-6 and HPV-11, which suggests that other factors also contribute to determining whether an exposed neonate will develop symptomatic RRP. One possible factor might be the HPV type to which the infant is exposed because HPV-6 accounts for most cases of genital condylomas, whereas HPV-11 is found in most cases of RRP (137,209). Given the presumed single exposure to HPV during vaginal delivery, the long and variable latent period of up to 5 years for childhood RRP remains to be explained. One possible explanation could be that viral latency is established in a proportion of exposed neonates before they develop symptomatic infection. If that were the case, activation of the latent HPV after a variable interval might account for the first clinical episode developing almost any time during childhood. The plausibility for this scenario is supported by two observations. First, it is known that the larynxes of patients with RRP who are in remission can harbor latent HPV DNA, which can subsequently be reactivated (5,295). Second, persistent asymptomatic carriage of HPV DNA in the oral cavity may occur in some children, although, as discussed previously in the section on latency, its frequency and significance remain controversial.

RRP is associated with a low risk for progression to severe dysplasia and even more rarely to cancer. The risk for such progression is increased if the papillomas have been subjected to x-irradiation, which was a common treatment of RRP in the 1940s. In one report, the average time interval between papilloma onset and diagnosis of cancer was 10 years in the irradiated cases, compared with 30 years in the nonirradiated cases (136). In contrast to cervical cancer, HPV-6 and HPV-11 in RRP are clearly associated with severe dysplasia and cancer, although high-risk types, such as HPV-16, may sometimes be found in laryngeal cancer.

Nasal Papillomas

Nasal papillomas are uncommon and may be present by themselves or with papillomas elsewhere in the respiratory tract. They are classified as *squamous* or *fungiform papillomas* (which closely resemble laryngeal papillomas clinically and histologically), *inverted papillomas* (in which the mucosa extends inward into the underlying stroma), and *cylindrical cell papillomas.* Most lesions are associated with HPV-6, HPV-11, and HPV-57, although some have been found to harbor HPV-16 (274,330). Carcinomas develop rarely and may be associated with HPV, including type 57.

Human Papillomavirus in the Oral Cavity

HPV infections of the oral cavity occur commonly (reviewed in 238 and 308). They may be asymptomatic or associated with single or multiple lesions in any part of the oral cavity. Genital-mucosal HPV types, especially HPV-6, HPV-11, and HPV-16, have frequently recovered from oral tissue. HPV is usually identified in at least half of papillomatous lesions in the oral cavity lesions, but some lesions appear to be HPV negative. Papillary lesions are traditionally classified as follows: (a) oral squamous cell papilloma, which are mostly solitary tumors and are the most frequent benign epithelial tumors of the oral cavity: (b) oral condyloma acuminatum, which are often multiple and present a cauliflower-like appearance: and (c) oral verruca vulgaris, which show marked keratinization. The designations *condyloma acuminatum* and *verruca vulgaris* implied that the infections were derived, respectively, from genital and common warts. Indeed, when verruca vulgaris involves the vermilion border of the lip, HPV-2, which is a nongenital cutaneous type that is a frequent cause of skin warts, has usually been found. However, when the lesion is present in the oral mucosa, HPV-6, HPV-11, and HPV-57 have been the most prevalent types. HPV-6 and HPV-11 appear to be responsible for most oral condylomas. Results in oral squamous cell papillomas have been less consistent, with HPV-6, HPV-11, and HPV-16 reported to be present in a variable proportion of the lesions. The association of HPV with malignant tumors of this region is discussed in the next section.

Focal epithelial hyperplasia (FEH) is a well-defined clinical entity that occurs only in the oral mucosa (238,308). Its distribution is worldwide, but it is most prevalent in the indigenous populations of Central and South America and of Alaska and Greenland. In Greenland Eskimos, the prevalence in different localities varied between 7% and 36%. In contrast, the prevalence among white residents in the same localities was less than 1 in 300. Cases of FEH in whites have been reported from many European countries (323). Clinically, FEH is characterized by discrete, multiple, elevated nodules on the oral mucosa. The disease may affect children or adults and in areas of high prevalence my affect several members within a single family. The lesions may be self-limited or persist for years without dissemination. They do not ordinarily undergo malignant transformation. Most are attributed to HPV-13 or HPV-32, two types that appear predominantly to infect the oral cavity (118).

Conjunctival Papilloma

Conjunctival papillomas are rare and occur at any age. HPV-6 and HPV-11 are associated with nearly all childhood conjunctival papillomas and with a proportion of the adult tumors (197).

Cancers of the Aerodigestive Tract and Other Regions

A consistent association of HPVs with the rare cancers that arise in respiratory papillomas, discussed previously, and in oropharyngeal and tonsillar carcinomas has been established (96,203,269,289). Cigarette smoking and alcohol consumption are two well-known risk factors for oral cancer. Although HPV does not appear to play a role in most cancers in this region, a subset of these cancers do appear to be attributable to HPV infection. Most of these HPV-associated cancers are located in the oral pharynx, which includes the tonsils, tonsillar fossa, base of the tongue, and soft palette. HPV-positive tumors have a characteristic basaloid morphology and are less likely to harbor mutations of p53 or pRb. They may also have improved disease-specific survival. Genital-oral sex may be a risk factor for these tumors, and the risk of HPV infection and cigarette smoking may be more than additive. HPV-16 is by far the most frequent HPV type isolated, accounting for 90% of the HPV-positive tumors in one study.

The evidence for the role of HPVs in respiratory cancers unassociated with RRP has been inconsistent (289). HPVs have been recovered from a small proportion of squamous carcinomas of the larynx (29). However, a firm association between specific HPV types and a specific respiratory cancer has not been established.

The role of HPV in esophageal cancer is uncertain (42,165,305). HPVs have been identified in a variable proportion of these cancers, ranging from 0% to 70%. HPV may be detected in esophageal tumors more frequently in regions with a high incidence of this cancer, such as China and South Africa. HPV-16, as well as other types, have been detected. Results from serologic studies, using antibodies to HPV-16 capsid antigen, have also given conflicting results, with some suggesting a strong association and others not (65,67,159). Because BPV-4–induced esophageal cancers of cattle may be negative for viral DNA (37), a hit-and-run phenomenon for HPV might be given some consideration in this tumor.

HPVs have been recovered from a minority of conjunctival and lacrimal sac carcinomas (185,196,312). These tumors are more frequent in HIV-infected individuals. HPV may also be found in a small proportion of bladder cancers (285). The suggestion that HPV might be implicated in cancers of the ovary, endometrium, colon, and prostate has not been confirmed (18,167,275,300).

DIAGNOSIS AND TREATMENT

Human Papillomavirus Diagnosis

Diagnosis of an HPV type in a tissue requires nucleic acid hybridization studies. Election microscopic examination for viral particles and immunologic detection of viral capsid antigens (with a broadly cross-reactive, genus-specific antiserum) are of limited value because viral particles and capsid antigens are not uniformly present, even in histologically diagnosed warts, and are seldom found in high-grade intraepithelial neoplasias and invasive cancers. Antisera are generally not available for identification of individual HPV types in clinical samples.

Hybridization Assays

A variety of hybridization assays have been employed for detection of HPVs (62,101,107,273). Nick-translated or random-primed DNA probes and *in vitro*-transcribed RNA probes are prepared from reference DNA clones. The probes can be labeled with radioactive or nonradioactive compounds. Southern hybridization of DNAs extracted from fresh or fresh-frozen tissues has served until recently as the gold standard for HPV detection because the sizes of the hybridizing bands provide an internal positive control for the specificity of the annealing reaction. In addition, the information allows identification of HPV subtypes and can provide evidence suggestive of integration, which occurs in many HPV-associated cancers. Also, Southern hybridization under low-stringency conditions allows identification of related viral types. However, the technique is labor intensive, and it may result in interlaboratory discrepancies when used as the only criterion for the presence of HPV DNA. Dot-blot and slot-blot hybridization techniques avoid the gel electrophoresis and transfer steps of Southern hybridization. These tests are suitable for large-scale studies, but faint true-positive signals are difficult to distinguish from the background because the size of the hybridizing fragment is not known. The Southern and dot-blot hybridizations can detect 10^5 to 10^6 copies of the HPV genome. In reverse-blot hybridization, cellular DNA is labeled and is used as a probe to hybridize at a high effective temperature with a panel of reference HPV DNAs that have been electrophoresed, denatured, and transferred onto a membrane. The test is somewhat less sensitive than PCR but allows screening of an HPV in a tissue against a large number of known HPVs in a single test.

In tissue *in situ* hybridization tests, cells or tissue sections on slides are hybridized using radioactive or nonradioactive probes (109,298). This is the only test in which the signal is seen in topographic relation to the pathologic lesion. The demonstration that the viral genome is in the affected cells can be an important step in investigating the role of HPVs in the etiology of cancers. By using exon-specific probes, it is possible to correlate the pathology of a lesion with the expression of specific viral genes.

HPV diagnosis based on amplification of segments of the viral genome by PCR combines sensitivity and specificity, when properly performed. Emerging data implicating viral load as an important correlate of risk for progression imply that quantitative or less sensitive assays might have utility in some clinical settings (132,333). The reverse hybridization procedure is very specific, can be used as a semiquantitative assay, and appears to detect most clinically relevant infections (265). Several different strategies for PCR amplifications for detection of genital-mucosal HPVs have been reported (100a,101,288,290). In a PCR method that has been widely used in the United States, consensus, degenerate primers targeted to amplify a segment of the L1 region of HPVs and capable of amplifying a large number of HPV types are employed; the PCR products are identified by type-specific oligonucleotide probes of several HPV types (264). The method is highly sensitive and highly specific, requires a small amount of the specimen, and is capable of amplifying a

large number of HPV types in a single amplification reaction. A wide range of nongenital cutaneous or EV-associated HPVs can be detected by a nested PCR approach (25,60,63,112). PCR-based methods can detect 10 to 100 copies of the viral genome, as compared with the conventional methods, which can detect 10^5 to 10^6 copies of the viral genome.

Utility of Diagnosis

The recognition that HPVs are etiologically associated with cervical cancer raises the possibility that HPV diagnosis might be useful, as an adjunct to Pap smear or even as a replacement for it, in screening for cervical cancer and in the clinical management of women who have abnormal cervical cytology. A significant proportion of women with invasive cancer give a history of negative Pap smears before onset of disease (59). It has been suggested that the presence of HPV DNA in the cervix would help identify the 20% to 30% of women with cervical disease who have false-negative results of Pap smear screening. These considerations need to be addressed in the context of the resources available to the population being evaluated, to evolving technology of Pap smear screening (119,125,279), and to the apparent utility of self-collection of cervical samples (100a, 325,326). Because the major goal of such testing is to prevent cervical cancer, it is possible that tests for nonviral molecular abnormalities might eventually have utility (177,260).

There may be specific settings in which the inclusion HPV determination may aid in the management of patients, but it does not appear at this time that an HPV diagnosis would be a useful adjunct to the general screening of a population that also undergoes high-quality Pap smear screening. However, the high rate of cervical HPV infection, especially in younger women, means that the routine use of HPV diagnosis would reveal, in the United States, millions of young women who have HPV DNA in the genital tract, normal cervical cytology, and little risk for cervical cancer. In the absence of an antiviral agent that is effective against HPV, there is little that can be done to eliminate these infections, and the perception of a woman that she is infected with a “cancer virus” could create needless anxiety and subject her to unnecessary treatment. On the other hand, it may possibly be useful to include HPV diagnosis with Pap smears in older women because HPV DNA prevalence is low and cervical cytology less reliable in this group (175). In older women, the presence of HPV DNA has a higher predictive value for cervical disease than in younger women. HPV diagnosis may also help clarify the interpretation of equivocal cytology and help with its management (189,219a). However, in younger women with low-grade cervical SIL in high-resource settings, HPV diagnosis does not appear to enhance clinical management (3). On the other hand, HPV diagnosis may be more useful in settings that lack high-quality Pap smear screening (265,326). It is also possible that tests that determine HPV type and measure viral load might have improved clinical utility (132,333).

Clinical Diagnosis and Treatment

Clinically, the diagnosis of a viral wart is usually made by the clinical appearance but can also be suggested by histologic examination. At times, other dermatologic conditions may be confused with warts (34). Depending on the location of the lesions, these conditions may include molluscum contagiosum (caused by a poxvirus), plantar corns, skin tags on the neck and in the axillae, keratoses, lichen planus, condylomata lata (secondary syphilis), and other diverse conditions. Virologic diagnosis was described previously.

Most warts regress spontaneously, although some may persist indefinitely. Depending on the location and extent of the infection, patients may seek treatment for cosmetic reasons, pain, discomfort, disability, hoarseness of voice, and so forth. The response of nongenital warts to treatment may be better in children and is also better for single warts and for warts of short duration. Pregnant women, children with recurrent laryngeal papilloma, immunodeficient patients, and patients with EV pose difficult problems for therapy.

There is no specific antiviral therapy for HPV lesions (234). Most treatments are aimed at destroying the lesional tissue while causing little long-term damage to the surrounding normal tissue. There is no treatment that will cure all warts, which has led to the wide range of therapies. Effectiveness of therapy is difficult to assess because of spontaneous regression on the one hand and recurrence on the other. At least partial regression can be obtained with many therapies, but even complete clearance that is then followed by recurrence is usually of limited clinical value. Traditional therapies include topical application of caustic agents (e.g., salicylic acid, podophyllin), cryotherapy, inhibitors of DNA synthesis (5-fluorouracil), and surgical therapy or laser treatment. Immunotherapy with autogenous vaccines, as well as sensitization to DNCB followed by application of DNCB to lesions, has been tried, with limited success.

In genital warts, topical application of imiquimod, which is an immunomodulating agent that can induce interferon and other cytokines, is more effective than placebo (19,75,307). Complete clearance of lesions in 50% to 70% of patients has been reported, compared with placebo group clearance of 3% to 11%. Lesions recurred in 3% to 19% of patients with complete clearance.

Intralesional and parenteral interferon therapy has been shown to be more successful than placebo for refractory genital warts (78,88,268). In two trials, repeated intralesional administration of interferon-a resulted in cure rates of 62% and 36%, as compared with cure rates of 21% and 17%, respectively, in the placebo recipients. Interferons have antiviral, antiproliferative, and immunomodulating effects. Parenteral administration was associated with even better responses.

Interferon therapy of recurrent respiratory papillomatosis has been less successful than that of genital warts. In one randomized national study, children with severe disease were treated either with surgery plus parenterally administered interferon-a or with surgery alone (135). Complete response was achieved in 3 of 28 patients who received surgery plus interferon, but in none of 28 patients who received only surgery. In another large study, children receiving surgery plus parenterally administered human leukocyte interferon (for 1 year) were compared with children receiving surgery alone (115). In the first 6 months of the treatment, the papilloma growth rate in the interferon group was significantly lower than that in the control group; however, in the second 6 months, this difference diminished and was no longer significant. No complete responses were seen.

Depending on the setting, treatment of cervical dysplasia may be surgical, with cryotherapy, by loop electrosurgical

excision repair, or by laser. Most successfully treated cases became negative for HPV DNA, whereas incompletely treated cases may remain positive (22).

PREVENTION

Interruption of Transmission

As noted previously, the epidemiology of various forms of HPV infection may differ drastically. Therefore, approaches to control genital HPV infection would be distinct from those to control nongenital infections. Pap smear screening can prevent most cervical cancers, which represent the most serious public health consequence of HPV infection. However, current efforts to prevent HPV infection have not been particularly successful.

Epidemiologic findings, discussed earlier, suggest that most cases of laryngeal papillomas that arise in infants and young children have been transmitted from exposure to the mother's HPV during vaginal delivery (137,277). This hypothesis raises the possibility that the use of cesarean section for the delivery of infants of mothers with known genital HPV infection would reduce the risk for HPV exposure. However, the incidence of genital HPV infection is high, whereas that of laryngeal papillomas is low, which means that many cesarean sections would be needed to prevent each case of laryngeal papillomas. These considerations suggest that the morbidity and mortality risk to the mother associated with cesarean section may, in most situations, be greater than the risk of laryngeal papillomas to the baby, which argues against the widespread use of this procedure to prevent laryngeal papillomas. However, because the risk for papillomas is greater in younger women and in first pregnancies, there may be a subset of HPV-positive women for whom the cost-benefit analysis might favor cesarean section (277).

Public health efforts that can prevent other sexually transmitted infections should, in principle, also be effective in preventing genital HPV infection. Indeed, decreased sexual promiscuity on the part of both sexual partners should reduce the likelihood of exposure to genital HPVs. It would also seem reasonable to expect that condoms would be able to reduce the incidence of genital HPV infection. However, such protection has been shown for transmission of external genital warts to men but has not yet been clearly established for internal or external genital lesions in women (121), perhaps because epidemiologic studies have not focused specifically on this issue. In the absence of specific treatment that would eradicate the infection, investigation of sexual partners of infected individuals would not be expected to have much impact on HPV transmission, although it may help identify infected individuals who otherwise might not realize that they are infected. Although likely, it is not known definitively whether eradication of visible lesions lowers the transmission rate of genital HPV infection.

Vaccine Prospects

The important public health consequences of genital HPV infection make it highly desirable to develop an effective vaccine against those HPV infections associated with cancer, especially cervical cancer. Natural history studies support the conclusion that protective immune responses occur regularly in most cervical infections (65,310). These responses appear to confer type-specific protection, although the critical viral determinants have not been established. Because the same HPV types are also implicated in most other genital cancers and in oral cancers associated with HPV infection, these less common malignancies might also be prevented by a vaccine targeted to the HPVs implicated in cervical cancer. Experimental vaccine studies in animal papillomavirus models have identified several possible vaccine targets, including L1, L2, E1, E2, E6, and E7 (38,163,178,271). Intradermal injection of a vaccine composed of formalin-inactivated COPV virions was shown to protect against COPV-induced oral lesions under field conditions (16). This result demonstrates that systemic immunization can induce protective immunity against a mucosal PV infection under natural conditions. The L1 neutralization epitopes were present in the vaccine, but the viral determinants that conferred protection were not definitively identified.

Most studies have focused on prophylactic vaccine strategies, although immune reactivity against certain viral proteins may have some therapeutic potential (reviewed in 30,70,86, and 183). The chronicity of HPV infection, combined with the fact that serious pathologic sequelae arise after many years of infection, provides a highly desirable setting for developing an HPV vaccine with activity against established infection. The continuing challenge of this goal is that there is no precedent for an effective therapeutic vaccine. In addition, from a public health point of view, protective immunity must be of long duration. The neutralizing antibodies induced by most prophylactic vaccines represent the traditional cornerstone of preventive activity, in part because these antibodies can persist for years after vaccination. The activity of therapeutic vaccines would probably depend primarily on their induced cellular immune reactivity, whose duration remains unknown. However, the approach itself is feasible. For example, HPV-16 E7 is immunogenic, and immunization with E7 peptide can induce protection in mouse studies against challenge with a tumorigenic cell line that expresses E7 (45,82). The immunogenicity of E7 can also be enhanced by various manipulations, such as by redirecting the protein to a class II pathway (176), increasing its rate of class I presentation by a mutation that increases the degradation rate of the protein (281), or fusing it to heat shock protein 70 (44).

Several prophylactic vaccine studies in animal papillomavirus models have emphasized the potential of L1 as an immunogen because it contains the immunodominant neutralization epitopes. When expressed at high levels, L1 can self-assemble into VLPs, which are empty capsids that closely resemble those of authentic virions morphologically and immunologically (144). As with authentic virions, systemic immunization of animals with L1 VLPs has been found to induce high titers of neutralizing antibodies that are conformationally dependent and type-specific. In cutaneous (CRPV) and oral mucosal (COPV and BPV-4) PV models, systemic immunization with L1 VLPs can confer 95% to 100% protection against experimental high-dose challenge with the homologous virus (31,48,145,306). Protection is type-specific and can be passively transferred with immunoglobulin G, implying that most of the protective effects are mediated by the neutralizing antibodies. An interesting feature of papillomavirus VLPs is that adjuvant is not required for eliciting protective immunity. An L2 neutralization epitope that is broadly cross-reactive against genital-mucosal HPVs has been identified, but its immunogenicity is lower than the type-specific L1 neutralization epitopes (140,248).

Evidence that cellular immune reactivity against certain nonstructural viral proteins can induce some protection in animal models has also led investigators to incorporate peptides from these proteins into VLPs by fusing the peptides to L1 or L2, to form “chimeric” VLPs. When L2 is coexpressed with L1, it coassembles with L1 to form L1 and L2 VLPs. The L1-to-L2 ratio in these VLPs is close to the 30:1 that is found in virions isolated from papillomas. Polypeptides several hundred amino acids in length can be incorporated into VLPs by fusing them to L2. Alternatively, shorter peptides, up to about 60 amino acids in length, can be fused to a truncated C-terminus of L1 without eliminating the ability of L1 to self-assemble into VLPs. Although fewer amino acids can be incorporated than with L2 fusions, L1 fusions have the advantage of being present in greater abundance in VLPs. Protection against a tumorigenic mouse cell line transformed with HPV-16 E7 has been shown for chimeric VLPs composed of a C-terminally truncated L1 fused to an E7 peptide and for chimeric VLPs composed of full-length L1 and of L2 fused to full-length E7 (103,232).

Early-phase human clinical trials have been carried out for L1 VLPs and for some vaccines that contain nonstructural viral proteins. The vaccine studies in humans have confirmed that the human immune responses are similar to those seen in animals (111,337). It remains to be determined what approaches may be effective prophylactically or therapeutically.

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CHAPTER 67 The Viruses and Their Replication

Thomas E. Shenk

Adenoviruses were first isolated and characterized as distinct viral agents by two groups who were searching for the etiologic agents of acute respiratory infections. In 1953, Rowe and colleagues (333) observed the spontaneous degeneration of primary cell cultures derived from human adenoids. The pathogenic changes proved to result from the replication of previously unidentified viruses present in the adenoid tissues. In 1954, Hilleman and Werner (172) were studying an epidemic of respiratory disease in army recruits, and they isolated agents from respiratory secretions that induced cytopathic changes in cultures of human cells. The viruses discovered by the two groups were soon shown to be related (188), and, in 1956, the agents were named *adenoviruses,* after the original tissue (adenoid) in which the prototype viral strain was discovered (99). Epidemiologic studies confirmed that adenoviruses are the cause of acute febrile respiratory disease among military recruits (90,122). It soon became clear, however, that adenoviruses are not the etiologic agents of the common cold; they are responsible for only a small portion of acute respiratory morbidity in the general population and for about 5% to 10% of respiratory illness in children. Besides respiratory disease, adenoviruses cause epidemic conjunctivitis (196), and they have been associated with a variety of additional clinical syndromes—perhaps most notably, infantile gastroenteritis (450). Today, more than 100 members of the adenovirus group have been identified that infect a wide range of mammalian and avian hosts. All of these viruses contain a linear, double-stranded DNA genome encapsidated in an icosahedral protein shell.

In 1962, Trentin and colleagues (398) made a seminal discovery: human adenovirus type 12 induces malignant tumors after inoculation into newborn hamsters. This was the first time that a human virus was shown to sponsor oncogenesis. No epidemiologic evidence has been reported linking adenoviruses with malignant disease in humans; extensive searches have generally failed to find adenovirus nucleic acids in human tumors (144,254). Nevertheless, the ability to induce tumors in animals and to transform cultured cells has established adenovirus as an important model system for probing the mysteries of oncogenesis.

As the interest in adenoviruses as tumor viruses intensified, their virtues as an experimental system became evident. The prototype human adenoviruses are easily propagated to produce high-titer stocks, and they initiate synchronous infections of established cell lines. Further, the viral genome is readily manipulated, facilitating the study of adenovirus gene functions by mutational analysis. Studies of adenovirus-infected cells have made numerous contributions to our understanding of viral and cellular gene expression and regulation, DNA replication, cell cycle control, and cellular growth regulation.

Perhaps the signal contribution to modern biology of the adenovirus system has been to host the discovery of messenger RNA (mRNA) splicing. Studies on the biogenesis of viral mRNA first demonstrated that many mRNAs are produced from a large nuclear transcript (17,421), and subsequent analysis of the structure of adenovirus mRNAs revealed the existence of introns (30,65). Today, the utility of adenovirus as a vector for gene therapy is the subject of intense exploration. This chapter overviews the structure of the adenovirus particle, the adenovirus replication cycle in human cells, its ability to oncogenically transform cells, and its interactions with host cells and host organisms. Details of adenovirus transformation are also considered in Chapter 10, and a discussion of the pathogenesis, clinical syndromes, epidemiology, techniques for diagnosis, modes of treatment, and utility of adenoviruses as vectors follows in Chapter 68.

CLASSIFICATION

The adenoviruses constitute the *Adenoviridae* family of viruses, which is divided into two genera, *Mastadenovirus* and *Aviadenovirus* (297). Whereas the *Aviadenovirus* genus is limited to viruses of birds, the *Mastadenovirus* genus includes human, simian, murine, bovine, equine, porcine, ovine, canine, and opossum viruses. Although there is antigenic cross-reactivity among members within each genera owing to conserved epitopes located on the hexon protein of the virion (296), there is no known antigen common to all adenoviruses.

Forty-nine human adenovirus serotypes (Table 1) have been distinguished on the basis of their resistance to

neutralization by antisera to other known adenovirus serotypes. Type-specific neutralization results predominantly from antibody binding to epitopes on the virion hexon protein and the terminal knob portion of the fiber protein (296). Hypervariable regions have been identified on the hexon that make up serotype-specific loops on the surface of the protein (75). The various serotypes are classified into six subgroups (see Table 1) based on their ability to agglutinate red blood cells (330). The central shaft of the viral fiber protein is responsible for binding to erythrocytes, and the hemagglutination reaction of an adenovirus is inhibited by antisera specific for viruses of the same type but not by antisera to viruses of different types. A variety of additional classification schemes have been explored, including subgroupings based on oncogenicity in rodents, relatedness of tumor antigens, electrophoretic mobility of virion proteins, or genome homologies. The various schemes produce reasonably concordant groupings (see Table 1), suggesting that the widely used classification based on hemagglutination is a reasonable standard.

Subgroup	Hemagglutination group	Serotypes	Oncogenic potential		Percentage of G+C in DNA
			Tumors in animals	Transformation in tissue culture	
A	IV (little or no agglutination)	12, 18, 31	High	+	48–49
B	I (complete agglutination of monkey erythrocytes)	3, 7, 11, 14, 16, 21, 34, 35	Moderate	+	50–52
C	III (partial agglutination of rat erythrocytes)	1, 2, 5, 6	Low or none	+	57–59
D	II (complete agglutination of rat erythrocytes)	8, 9, 10, 13, 15, 17, 19, 20, 23–30, 32, 33, 36–39, 42–48	Low or none (mammary tumors)	+	57–61
E	III	4	Low or none	+	57–59
F	III	40,41	Unknown		

Modified from refs. 23 and 240, with permission.

TABLE 1. *Classification schemes for human adenoviruses (mastadenovirus H)*

VIRION STRUCTURE

Adenoviruses are icosahedral particles measuring 70 to 100 nm in diameter (177) (Fig. 1). The particles (virions) contain DNA (13% of mass), protein (87% of mass), no membrane or lipid, and trace amounts of carbohydrate because the virion fiber protein is modified by addition of glucosamine (143,191). Virions consist of a protein shell surrounding a DNA-containing core. The protein shell (capsid) is composed of 252 subunits (capsomeres), of which 240 are hexons and 12 are pentons (125). As suggested by their names, penton and hexon subunits are surrounded by five and six neighbors, respectively. Each penton contains a base, which forms part of the surface of the capsid, and a projecting fiber whose length varies among different serotypes (295).

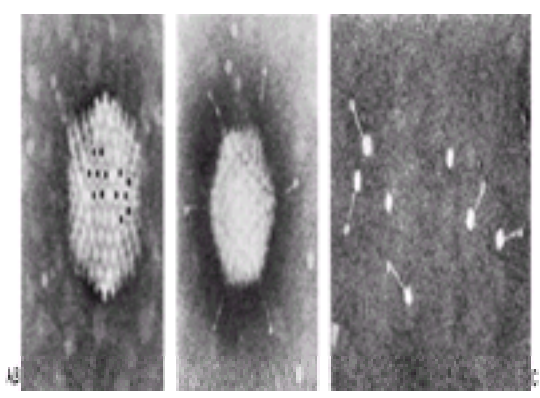

FIG. 1. Adenovirus type 5. **A:** The virion is an icosahedron. One of the 240 hexon capsomeres surrounded by 6 hexons and one of the 12 penton capsomeres surrounded by 5 hexons are marked (*dots*). **B:** Six of the 12 fibers that are present on each virus particle are shown projecting from penton capsomeres located at the vertices of the icosahedral capsid. **C:** Free penton capsomeres containing penton base and fiber (×285,000). (From ref. 401, with permission.)

Virion Polypeptides and DNA

Most of the structural studies of adenoviruses have focused on the closely related adenoviruses type 2 and 5 (Ad2 and Ad5). Electrophoretic analyses of purified virions disrupted with sodium dodecylsulfate was employed initially to identify structural polypeptides (191,407). Comparison of electrophoretic results with genomic open reading frames (ORFs) suggests there are probably 11 virion proteins. These proteins are numbered by convention (255), with no polypeptide I because the moiety originally designated I proved to be a mixture of aggregated molecules.

The outer shell or capsid of the virion is composed of seven known polypeptides. Polypeptide II (967 amino acids) is the most abundant virion constituent. The hexon protein is composed of three tightly associated molecules of polypeptide II (179), and this trimeric protein is referred to as the *hexon capsomere.* Polypeptides VI (217 amino acids), VIII (134 amino acids), and IX (139 amino acids) are associated with the hexon protein after various isolation procedures (107). All three polypeptides likely stabilize the hexon capsomere lattice, and polypeptides VI and VIII probably bridge between the capsid and core components of the virion. Five copies of polypeptide III (571 amino acids) associate to form the penton base protein (407), which is found at each vertex of the icosahedral particle. Polypeptide IIIa (566 amino acids) is associated with hexon units that surround the penton after pyridine dissociation of virions, and it appears to link adjacent facets of the capsid and bridge between hexons and polypeptide VII of the core (106). Polypeptide IV (582 amino acids)

forms the trimeric fiber protein (407), which projects from the penton base at each vertex of the icosahedron. The combination of penton base plus fiber is called the *penton capsomere.* All human adenoviruses examined to date encode a single fiber protein with the exception of Ad40 and Ad41, which encode two fiber proteins and incorporate both polypeptides into their virions (205). Because the fiber interacts with a cellular receptor protein, the incorporation of two fiber proteins might extend the range of cell types to which these viruses can bind. Hexon and penton capsomeres are the major components on the surface of the virion, and their constituents, polypeptides II, III, and IV, contain tyrosine residues that are exposed on the virion surface and can be labeled by iodination of intact particles (106).

The core of the virion contains four known proteins and the viral genome. Polypeptides V (368 amino acids), VII (174 amino acids), and mu (19 amino acids) are basic, arginine-rich proteins (181,335) that contact the viral DNA (6,56). The function of the mu protein is unknown (181). Polypeptide VII is the major core protein, and it serves as a histone-like center around which the viral DNA is wrapped (56,272). Polypeptide V can bind to a penton base (106), and it might bridge between the core and capsid, positioning one relative to the other. The fourth protein in the core is the *terminal protein* (671 amino acids), which is covalently attached to the ends of the viral DNA. It was first identified indirectly by its ability to mediate circularization of the viral DNA through a protease-sensitive, noncovalent interaction (327). The circularizing agent was shown to be a 55-kd protein covalently attached to each 5' end of the viral DNA (324), and the protein was subsequently visualized on viral DNA by avidin-biotin labeling (110a). The linkage between DNA and protein is a phosphodiester bond formed between the b-hydroxyl group of a serine (residue 562 of terminal protein) and the 5' hydroxyl of the terminal deoxycytosine residue (83,360). The protein is not evident in electrophoretic analyses of virion proteins because it is present in only two copies per particle. The terminal protein serves as a primer for DNA replication (49,52,236,382), and it mediates attachment of the viral genome to the nuclear matrix (36,115,342).

Ad2 DNA was the first adenovirus genome to be completely sequenced (326), and it includes a total of 35,937 base pairs (bp). Ad5 (67), Ad12 (365), murine adenovirus (268), canine adenovirus (279), and avian adenovirus genomes (62,312) have also been sequenced. The DNAs of different adenoviruses have inverted terminal repeat sequences ranging in size from about 40 to 160 bp (12,119,396). The inverted repeats enable single strands of viral DNA to circularize by base-pairing of their terminal sequences, and the resulting base-paired panhandles are thought to be important for replication of the viral DNA.

Capsid and Core Three-Dimensional Structure

X-ray crystallography, electron microscopy, and combinations of the two methods have been used to generate a fairly refined picture of the adenovirus capsid (reviewed in 372). The x-ray structure of the major capsid protein, the hexon, has been determined to a resolution of 2.9 Å (13,325). The hexon protein, which is also referred to as the hexon capsomere, is a trimer composed of three interwoven hexon polypeptides. As described first for the picornaviruses, a b-structure stabilizes the association of the three subunits into a larger structure consisting of two domains: a triangular top facing the outside of the capsid and a hexagonal base with a central cavity. Group-of-nine hexons (“ninemers”), which can be isolated from each triangular face of the virion by treatment with 10% pyridine (313), provided insight to how interactions between hexons are stabilized. The structure of these ninemers was examined by subtracting an array of nine projected x-ray–determined hexons from scanning transmission electron microscopic images (116). The resulting two-dimensional difference image revealed density from a minor protein component extending along the hexon–hexon interfaces. Biochemical analysis had previously identified this component as polypeptide IX (407), a minor capsid constituent involved in stabilization of the capsid (69). Its carboxy-terminal domain is exposed on the surface of the capsid (3).

The three-dimensional structure of the complete adenovirus particle was determined to 21 Å resolution by image reconstruction from cryoelectron micrographs (63,371,372). A density map of the virion was generated from multiple images of the particle in different orientations. The reconstruction process relied on the known icosahedral symmetry of the virion to align the individual images. This work provided the first detailed visualization of the vertex proteins, including the penton base and its protruding fiber; it confirmed the earlier placement of protein IX; and it located minor capsid polypeptides at the edges of triangular facets, bridging hexons in adjacent facets. The three-dimensional structure of the virion has been refined by subtracting 240 copies of the crystallographic hexon from the cryoelectron microscopic image reconstruction (372). The difference map revealed more precisely the positions of several capsid proteins (Fig. 2). A less abundant stabilizing protein is located at each specialized position in the hexon assemblage. The penton complex fills the large gaps at the vertices, polypeptide IX stabilizes hexon–hexon contacts within a facet, polypeptide IIIa joins hexons of adjacent facets, and polypeptide VI anchors the ring of peripentonal hexons on the inside surface of the capsid and connects the capsid to the core. The structure of the particle has been further refined by employing constrained maximum entropy tomography (359).

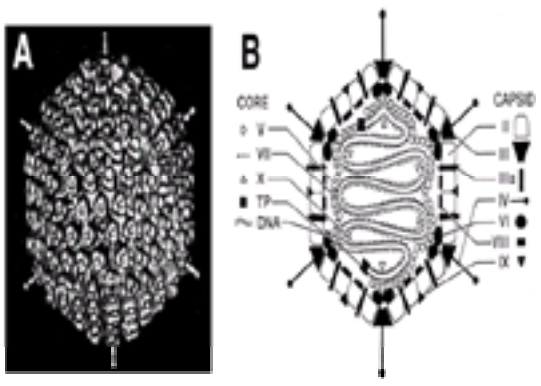

FIG. 2. Models of the adenovirus virion. **A:** Three-dimensional image reconstruction of the intact adenovirus particle viewed along an icosahedral threefold axis. **B:** Stylized section of the adenovirus particle based on current understanding of its polypeptide components and DNA. No real section of the icosahedral virion would contain all components. Virion constituents are designated by their polypeptide numbers with the exception of the terminal protein

(*TP*). A from ref. 371, with permission; B from ref. 370, with permission.)

The fiber protein that projects from each vertex of the particle is composed of three copies of polypeptide IV (334,407). The amino-terminal 40 residues of each subunit are embedded in the penton base (84,423). A central extended shaft is composed of repeating motifs about 15 amino acids in length, and the number of repeat units differs among adenovirus serotypes (145). The carboxy-terminal 180-residue segment of each subunit composing the fiber protein contributes to formation of a terminal bulb (84). The structure of the terminal bulb has been determined at 1.7 Å resolution (442), and a three-dimensional model of the fiber shaft has been proposed (375), predicting that its constituent polypeptide chains form a left-handed, triple helical structure composed of short b-strands interspersed with extended loops that follow the overall helical path. This model fits reasonably well with the cryoelectron microscopic results (372) and mutational studies (173).

The adenovirus core is composed of the linear, double-stranded DNA and four virus-coded proteins. Unlike cellular chromatin, which yields discrete mononucleosomal DNA fragments of about 146 bp on digestion with micrococcal nuclease, the adenovirus core yields a heterogeneous population of DNA fragments ranging from about 50 to 300 bp in length (74,272,410,411). These fragments are associated with polypeptide VII (272,410). Although the reason for the variability in DNA length is not understood, it nevertheless seems clear that adenovirus chromatin is organized into unit particles composed of DNA and polypeptide VII (272,410). The notion that adenovirus chromatin is arranged in a repeating unit is consistent with electron microscopic visualization of particles within the adenovirus core (41,411) and with x-ray scattering patterns obtained with cores (85). Digestion of cores with limiting amounts of nuclease fails to produce a ladder-like series of DNA fragments on electrophoresis, as would be predicted for an oligomeric structure, and this might be due to the fragile and unstable nature of the core after release from virions (411).

The higher-level organization of the core remains obscure. Psoralen cross-linking of viral DNA within partially disassembled virions indicates that the linear molecule is organized into eight supercoiled domains (440), in agreement with earlier electron microscopic studies suggesting that the core is organized into 8 to 12 domains (41). It is intriguing to note that the eight-domain organization places most of the viral transcription units into independent supercoiled domains (440).

GENOME ORGANIZATION

All human adenovirus genomes that have been examined to date have the same general organization; that is, the genes encoding specific functions are located at the same position on the viral chromosome. Avian adenoviruses differ in that they lack several of the early genes identified in the human viruses and contain ORFs of unrelated sequence in their place (62,312). The linear genome contains two origins for DNA replication. The two origins are identical, and one is present in each terminal repeat. The genome also includes a cis-acting packaging sequence (135,155,166,168) that must be located within several hundred base pairs of an end of the chromosome to direct the interaction of the viral DNA with its encapsidating proteins (165).

The viral chromosome carries five early transcription units (E1A, E1B, E2, E3, and E4), two delayed early units (IX and IVa2), and one late unit (major late) that is processed to generate five families of late mRNAs (L1 to L5), all of which are transcribed by RNA polymerase II (reviewed in 308) (Fig. 3). Families of mRNAs were initially localized on the adenovirus map by hybridization to restriction enzyme–generated fragments of the viral DNA (351), and transcription units were defined by ultraviolet light–mapping experiments (32,439). The chromosome also carries one or two (depending on the serotype) virus-associated (VA) genes transcribed by RNA polymerase III. By convention, the map is drawn with the E1A gene at the left end. Both strands of the viral DNA are transcribed with the so-called rightward reading strand on the conventional map coding for the E1A, E1B, IX, major late, VA RNA, and E3 units and the leftward reading strand coding the E4, E2, and IVa2 units.

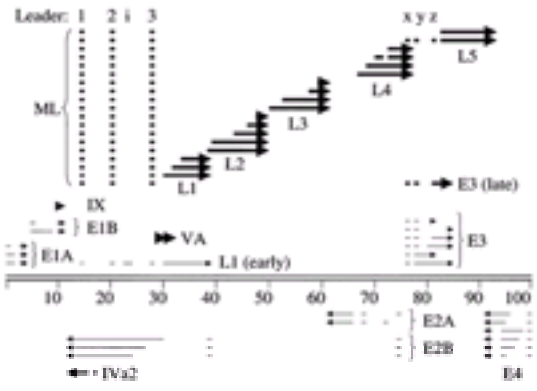

FIG. 3. Transcription map of adenovirus type 2. The early mRNAs are diagrammed with their exons represented by *thin arrows,* late mRNAs are drawn with *heavy arrows,* and delayed early mRNAs (*1Va2* and *IX*) are designated with *arrows of intermediate thickness.* Most late mRNAs originate at 16.3 map units and contain the tripartite leader whose components are labeled *1, 2,* and *3.* Some of the late mRNAs also contain leader segments *i, y,* or *z* polypeptide. Introns are indicated by gaps in the lines comprising the *arrows.*

Little is known about the functional and evolutionary considerations that have led to the current organization of the transcription units on the viral chromosome. Their arrangement might serve a timing function. Viral cores have an

organized structure (41,440), and they are converted from a more compact to a more open structure as the early phase of infection progresses (41,440). Perhaps RNA polymerase initially interacts with promoters exposed at the ends of the chromosome, and transcription of the terminal units drives further opening of the core structure. This proposal predicts that the terminal E1A and E4 transcription units would be the first to be expressed, and this is the case. Thus, the location of a transcription unit on the chromosome could influence the order in which it is expressed relative to other units during the early phase of the infectious cycle.

The activation of transcription units can also be influenced by their location relative to other units on the chromosome. Insertion of a strong transcriptional termination sequence between the E1A and E1B units inhibits activation of the E1B unit, suggesting that transcriptional read-through from E1A to E1B contributes to the activation of E1B (108). One might also imagine that transcription units embedded within the major late unit would be influenced in cis by its very active expression late after infection. However, different viral molecules could serve as templates for early and late transcription units, avoiding complications that might arise from overlapping transcription units. Further, initiation is rate limiting; hence, even if opposing and overlapping units were transcribed from the same template, transcription complexes would seldom meet on the chromosome.

Each of the adenovirus genes transcribed by RNA polymerase II gives rise to multiple mRNAs that are differentiated by alternative splicing and in some cases by the use of different poly(A) sites. The structures of mRNAs were defined by a variety of procedures, including electron microscopic heteroduplex analysis (64,66) and S1 nuclease mapping (32); 5' ends were localized by primer extension analysis (464). As mentioned earlier, the analysis of adenovirus mRNA structure led to the discovery of splicing (30,65) (Fig. 4).

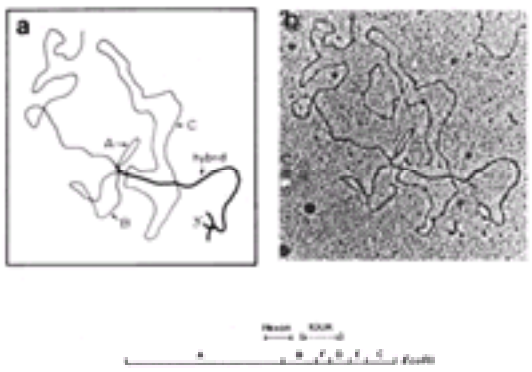

FIG. 4. Detection of adenovirus hexon transcripts processed by splicing near their 5' ends. When hexon mRNA was hybridized to viral DNA, it formed a duplex structure with three short DNA loops (labeled *A, B,* and *C*), which correspond to introns removed from the mRNA by splicing. **A:** Diagram of duplex molecules. **B:** Electron micrograph of the RNA:DNA hybrid. The position of the hexon and L4 100-kd protein coding regions are displayed on the EcoR1 cleavage map of adenovirus type 2 (*bottom*). (From ref. 30, with permission.)

Some of the protein products generated from the same transcription unit are partially related in their sequence (e.g., the two major polypeptides encoded by the E1A unit); others have no sequence in common (e.g., the two major E1B-coded polypeptides). Unfortunately, no consistent terminology has been adopted for naming viral proteins: the E1A proteins are named for the sedimentation coefficient of the mRNAs that encode them; E1B and E3 proteins are designated by their molecular mass; E2 proteins are named for their functions; E4 proteins are named for ORFs; and the structural proteins encoded by the late transcription unit are termed II to IX. The various historical names of viral polypeptides are used in this chapter, generally preceded by the name of the transcription unit or family of late mRNAs that encodes them (e.g., E1A 13S, E4orf6, L5-IV).

Many of the individual adenovirus transcription units encode a series of polypeptides with related functions. As discussed later, the E1A unit encodes two proteins that activate transcription and induce the host cell to enter the S phase of the cell cycle; E1B encodes two proteins that block apoptosis; E2 encodes three proteins that function directly in DNA replication; E3 encodes products that modulate the response of the host to infection; and the late family of mRNAs is concerned with the production and assembly of capsid components. Only the E4 unit encodes an apparently disparate set of functions. E4 products mediate transcriptional regulation and mRNA transport and modulate DNA replication and apoptosis. One might speculate that an ancestral adenovirus encoding fewer gene products evolved to generate a modern virus in which many of the ancestral genes have given rise to groups of more specialized products that remain functionally related. The grouping might also be driven in part by the advantage of using a single transcriptional control element to regulate the expression of multiple polypeptides that are needed simultaneously to execute a function such as DNA replication. Further, it might be useful to group closely the coding regions for products that interact physically or functionally to reduce the frequency with which they can be separated by recombination. Otherwise, recombinant variants, which could be defective because they encode products unable to function well together, might be generated at high frequency.

GENETIC SYSTEM AND RECOMBINATION

Adenovirus mutants have proved invaluable as reagents for the study of viral physiologic processes. Most genetic studies have used Ad2 or Ad5. Initially, physical and chemical mutagens were employed to generate variants that could be recognized on the basis of their growth phenotype. Subsequently, mutants have been produced by the targeted manipulation of viral DNA. The ability to propagate mutant viruses is currently the only limitation to their production. Many variants have proved viable, often growing more poorly than their wild-type parent, but growing sufficiently well for the production of useful virus stocks. Other mutants have been selected for conditional growth. These include

temperature-sensitive as well as host range variants. Host range mutants have played an especially important role in adenovirus genetics. E1A and E1B mutants can be propagated in 293 cells, a human embryonic kidney cell line that contains and expresses the Ad5 E1A and E1B genes (136); the physiologic consequences of the mutations can then be analyzed in standard laboratory host cells such as HeLa cells. Similarly, complementing cell lines have been developed for propagation of viruses with mutations in the E2, E4, and L5 genes (5,212,221,415,425).

Recombination between adenoviruses contributes to the evolution and diversity of viral serotypes. It is well established that recombination occurs among adenoviruses of the same subgroup. Homologous recombination occurs with high efficiency during growth in co-infected cultured cells (127,437), and comparison of field strains with disparate neutralization (hexon) and hemagglutination (fiber) serology (170) indicates that such exchanges also occur in nature. Recombination between viruses of different subgroups appears to have given rise to Ad4, the sole member of subgroup E adenoviruses. Ad4 exhibits sequence similarity in its E1A (0 to 5 map units) and E2 regions (62 to 66 map units) to group B adenoviruses (209,392), and its hexon gene (51 to 61 map units) is immunologically related to group B viruses (298), whereas the Ad4 fiber gene sequence (146) and immunologic characteristics (296) are most similar to group C viruses. Thus, the simplest model for the origination of Ad4 posits a single recombinatorial crossover event somewhere between the E2 coding region of a group B virus and the fiber coding region of a group C virus (146).

Efficient adenovirus recombination requires viral DNA replication (453), but the virus does not appear to encode any gene products that function specifically to facilitate recombination (103). Rather, it appears that single strands of DNA produced during the viral replication process, which can pair with each other or invade duplex DNAs, are the driving force behind recombination (111).

Although adenoviruses have never unambiguously been shown to integrate into the chromosomes of permissive host cells, integration does occur during the process of transformation. No specific motifs have been identified in the cellular DNA sequences at adenovirus integration sites.

REPLICATIVE CYCLE

Studies of the human adenovirus replication cycle have focused primarily on the closely related Ad2 and Ad5 viruses. These serotypes have been favored because they are easily grown in the laboratory, and an extensive collection of Ad2 and Ad5 mutant viruses have been developed. When other human serotypes have been studied, their growth strategies have proved similar to the paradigm established for the prototypes. Most studies of adenovirus growth have been performed by infection of HeLa or KB cells at fairly high multiplicities of infection (more than 10 plaque-forming units per cell). High multiplicities of infection have been used so that all cells in the culture are synchronously infected, allowing the ordered series of biochemical events during the infectious cycle to be observed in a time-wise fashion. HeLa and KB cells have been favored as hosts because they are easily propagated in large quantities, and the viruses grow in them rapidly and to high yield. These tumor cells support more rapid viral growth than human diploid fibroblasts, in which the replication cycle is substantially prolonged.

The replication cycle is divided by convention into two phases that are separated by the onset of viral DNA replication (Fig. 5). Early events commence as soon as the infecting virus interacts with the host cell. These include adsorption, penetration, movement of the viral DNA to the nucleus, and expression of an early set of genes. Early viral gene products mediate further viral gene expression and DNA replication, induce cell cycle progression, block apoptosis, and antagonize a variety of host antiviral measures. In HeLa cells infected at a multiplicity of 10 plaque-forming units per cell, the early phase lasts for 5 to 6 hours, after which viral DNA replication is first detected. Concomitant with the onset of viral DNA replication, the late phase of the cycle begins with expression of late viral genes and assembly of progeny virions. The infectious cycle is completed after 20 to 24 hours in HeLa cells. At the end of the cycle, about 10^4 progeny virus particles per cell have been produced, along with the synthesis of a substantial excess of virion proteins and DNA that are not assembled into virions (141). Cells infected at high multiplicity seldom divide; hence, at the completion of the replication cycle, the DNA and protein content of the infected cell has increased by a factor of about two.

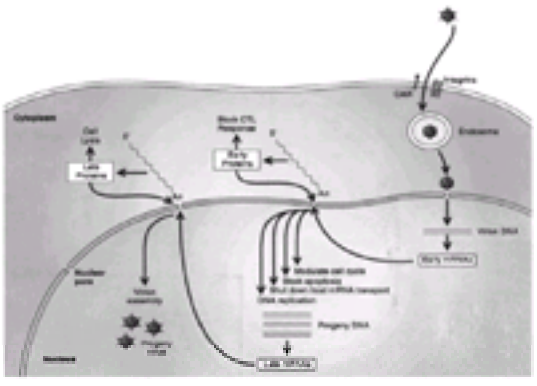

FIG. 5. Cartoon of the adenovirus life cycle.

Although *early* and *late* are convenient terms for description of events that occur during the replication cycle, the functional distinction between early and late events is often blurred. Early genes continue to be expressed at late times after infection, and the promoter controlling expression of the major late transcription unit directs a low level of transcription early after infection. The viral genes encoding proteins IVa2 and IX begin to be expressed at an intermediate time (308) and thus form a *delayed-early* category.

Adsorption and Entry

Attachment of Ad2 to cells is mediated by its fiber protein (246). The distal, carboxy-terminal domain of the fiber protein terminates in a knob that binds to the cellular receptor (84). The CAR (coxsackievirus and adenovirus receptor) protein, which belongs to the immunoglobulin superfamily, is an adenovirus receptor (29,395). It serves as a high-affinity receptor for human adenoviruses from subgroups A, C, D, E, and F, but not subgroup B (329). The class I major histocompatibility complex (MHC) a_2 domain has also been reported to serve as a receptor for Ad5 (174).

The observation that Ad2 binds to some cells but does not efficiently enter them (357) raised the possibility that a second protein–protein recognition event might be needed for internalization, and a second interaction has been identified. The penton base protein binds to members of a family of heterodimeric cell surface receptors, termed *integrins* (436). The a_vb_3- and a_vb_5-integrins and perhaps other integrin family members interact with the viral protein. The interaction occurs through an arg-gly-asp (RGD) sequence present in each of the five polypeptide III molecules that compose the penton base, the same sequence motif present in a number of extracellular adhesion molecules that bind to integrins. RGD-containing peptides can block internalization of Ad2 (436), and Ad5 variants with mutations that alter the RGD sequence in polypeptide III exhibit a delay in the onset of viral DNA synthesis under certain conditions, consistent with a reduced efficiency of internalization (20). The structure of a_vb_5-integrin bound to virions has been determined at 21 Å resolution by cryoelectron microscopy (63).

It seems likely that the fiber–receptor interaction serves to bring the virion to the cell surface, facilitating the critical integrin–receptor interaction. Consistent with this view, insertion of an RGD motif into the Ad5 hexon partially overcomes the need for a fiber–receptor interaction (414).

The tropism of adenoviruses is influenced by the availability of cellular receptors for fiber and penton. Airway epithelial cells lack both receptors (128,455). This deficiency might reflect evolutionary pressure to block a port of entry for respiratory adenoviruses, and it has frustrated efforts to use adenovirus as a vector to introduce therapeutic genes to the lungs.

After adsorption, Ad2–receptor complexes diffuse into coated pits, and they are internalized by receptor-mediated endocytosis (54,55,110,409). The process is triggered by the penton–integrin interaction because purified penton but not fiber protein is rapidly internalized by cultured cells (436). The process requires dynamin (419), a protein that specifically regulates clathrin-mediated endocytosis; and it requires activation of the lipid kinase phosphotidylinositol-3-OH kinase (230), which induces reorganization of actin filaments. The internalization of adsorbed virus is remarkably efficient (140). Eighty to 85% of virus that binds to the surface of a susceptible cell is internalized, and penetration occurs rapidly, with half of the adsorbed virus moving to endosomes within 10 minutes. About 90% of the virus within endosomes successfully moves to the cytosol with a half time of about 5 minutes. The movement to the cytosol is triggered by the acidic pH of the endosome (349,379), and the penton base is believed to play an essential role in the process. The rapidity of the movement to the cytosol suggests that the virus escapes from the endosomal unit, known as the *early endosome,* before formation of a lysosome (270). The endosome appears to be substantially disrupted because DNA–protein complexes that enter the endosome together with virus particles—but not physically attached to virions—also escape to the cytosol with high efficiency (451). Virus particles are transported across the cytoplasm to the nucleus by a process that involves microtubules (76,378). *In vitro* studies have shown that virions can attach to microtubules through the hexon, and some drugs that interact with microtubules can inhibit adenovirus infection (76). About 40 minutes after penetration, virus particles are seen at nuclear pore complexes by electron microscopy. After 120 minutes, about 40% of internalized particles have released their DNA free from hexon proteins, although it is not known what portion of this released DNA is localized to the nucleus (140). Inhibitors of nuclear import block the accumulation of adenovirus DNA in the nucleus, indicating that it enters through the nuclear pore (138).

During the internalization process, there is a sequential disassembly of the virion (140). The disassembly occurs by selective dissociation and proteolytic degradation of virion constituents. First, the proteins at the vertices of the particle are lost. Polypeptide IV that trimerizes to form the fiber, polypeptide IIIa located near the peripentonal hexons linking adjacent facets of the capsid, and polypeptide III that forms the pentameric penton base are substantially lost by 15 minutes after penetration of the cell. Polypeptides IV and IIIa dissociate somewhat more rapidly than III, and this could reflect a need to free the penton base for a role in escape from the endosome. Polypeptide VIII dissociates from the particle as the penton capsomeres are lost, and, shortly after entry of the particle to the cytosol, polypeptide VI is degraded by the virus-coded protease (139). Both polypeptides VI and VIII bridge from the DNA core to the capsid, and their loss prepares the particle for release of its DNA. Somewhat later, polypeptide IX, which stabilizes hexon facets, exits from the infecting particle; finally, the DNA-containing core is freed from hexons. Thus, the virion, which is very stable outside the cell, is dismantled by an ordered elimination of structural proteins on entry to the cell so that it can deliver its DNA to the nucleus.

The events that signal disassembly are mostly unknown. Acidification induces exit of the infecting particle from the endosome to the cytosol, but disassembly of the vertex constituents can occur in the absence of acidification (140). It seems likely that the signal for the initial dissociation of the fiber from the penton is a determining event that initiates a cascade in which each sequential disassembly event is driven by changes in the structure of the virion that result from previous events.

A DNA–protein VII complex reaches the nucleus (138). The viral DNA has been reported to convert into a structure that can be digested with a low concentration of DNase I to generate DNA fragments that form a ladder pattern similar to that of cellular chromatin on electrophoresis (384), suggesting that cellular histone proteins might replace polypeptide VII, the major core constituent, before the infecting DNA is transcribed. However, using sensitive assays, others have been unable to detect nucleosomal particles composed of viral DNA and cellular histones (410). It is possible that the viral genome is expressed as a chromosomal structure containing protein VII rather than cellular histones. When the viral DNA reaches the nucleus, it associates with the nuclear matrix through its terminal protein (36,115,342). DNAs from

mutant Ad5 variants with lesions in their terminal protein genes fail to become tightly associated with the nuclear matrix, and they also fail to be efficiently transcribed (342). This correlation between nuclear matrix association and activation of transcription suggests the two events are functionally interrelated. Apparently, the terminal protein, which arrives with the infecting genome, is the first viral gene product that functions within the nucleus to initiate the program of viral gene expression.

Activation of Early Viral Genes

There are three main goals of early adenovirus gene expression. The first is to induce the host cell to enter the S phase of the cell cycle, providing an optimal environment for viral replication. As discussed later (see Interactions with the Host), E1A, E1B, and E4 gene products play roles in this process. The second is to set up viral systems that protect the infected cell from various antiviral defenses of the host organism. The E3 and VA RNA genes contribute to these defenses, and these are also discussed later (see Interactions with the Host). The third is to synthesize viral gene products needed for viral DNA replication. All three of these goals depend on the transcriptional activation of the viral genome, and the principal activating proteins of adenovirus are encoded by the E1A gene.

E1A is the first transcription unit to be expressed after the viral chromosome reaches the nucleus. Transcription of the E1A unit is controlled by a constitutively active promoter, and it encodes two mRNAs during the early phase of infection (Fig. 6). Three additional E1A mRNA species accumulate later in the infectious cycle (369), but no distinct function has been described for their products. The two early mRNAs contain identical 5' and 3' ends, differ internally owing to differential splicing, and encode proteins that are identical except for an additional 46 amino acid segment that is present in the larger polypeptide. The two polypeptides are commonly referred to as the 12S and 13S E1A proteins. The primary E1A translation products undergo extensive phosphorylation that is mediated, at least in part, by cyclin-dependent kinases (256) and mitogen-activated protein kinase (430).

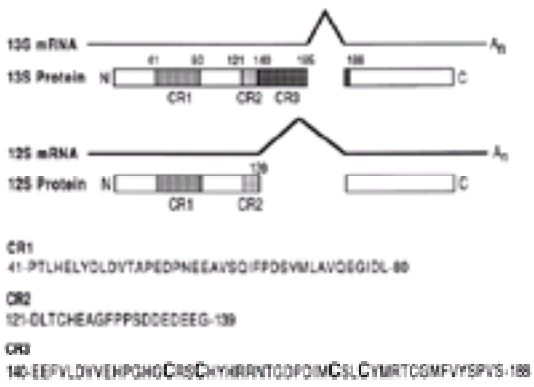

FIG. 6. Diagram of E1A mRNAs, the polypeptides they encode, and the amino acid sequence of conserved domains. 13S and 12S mRNA exons are represented (*lines*), as are introns (*carets*) and poly(A) sequences (A_n) The polypeptides encoded by the 13S and 12S mRNAs are designated (*rectangles*). Domains in the proteins that are conserved among adenovirus serotypes are identified as conserved regions 1 to 3 (CR1, CR2, CR3), and the amino acids at the boundaries of conserved regions are indicated (*above rectangles*). The amino acid sequences of the conserved domains are displayed at the bottom of the figure, and the cysteines composing the zinc finger motif in CR3 are marked (*enlarged bold letters*).

When the amino acid sequences of the E1A proteins encoded by a variety of human serotypes are compared, the E1A products prove to be constructed of three conserved regions (CR1, CR2, and CR3) (see Fig. 6) separated by less highly conserved domains (278,290,408). The E1A proteins do not exhibit sequence-specific DNA binding; rather, they bind to cellular proteins and modulate their function. The three conserved regions in the E1A proteins mark domains that play major roles in protein–protein interactions.

The ability of E1A proteins to activate expression of the other adenovirus transcription units was discovered when viral mutants were examined that carried either a single base-pair substitution (31) or a deletion (198,353) within the E1A coding region. These mutant viruses, which could be grown in transformed human cells expressing the E1A and E1B genes (293 cells) (136), failed to accumulate early viral mRNAs with normal kinetics when grown in HeLa cells that were unable to complement the defect. Compared to wild-type virus, they accumulated reduced quantities of early mRNAs only after a long delay. Subsequent analysis demonstrated that E1A proteins acted to increase the rate of transcription (289). Since the E1A proteins can activate other genes in trans, they are often referred to as *trans-activators.*

Initially, attempts to identify a promoter element that is responsive to the E1A proteins proved frustrating. In a number of studies, it was possible to mutate any specific DNA element without blocking the ability of E1A to activate test promoters, provided that basal transcription was not eliminated. The reason for the promiscuous activation is now clear. The E1A proteins activate transcription by binding to a variety of cellular transcription factors and regulatory proteins (Table 2). The E1A proteins can interact directly with auxiliary factors that mediate basal transcription, with activating proteins that bind to upstream promoter and enhancer elements, and with regulatory subunits that influence the activity of DNA-binding factors. All three conserved domains of E1A mediate protein–protein interactions that activate transcription.

E1A-binding protein	Binds to		Other proteins in complex
	12S E1A	13S E1A	
pRB	+	+	Unknown
p107	+	+	Cyclin A, cdk2/cyclin E
p130	+	+	Cyclin A, cdk2/cyclin E
p300	+	+	Unknown
TBP	−	+	Unknown
hTAF(II)135	−	+	Unknown
SUR-2	−	+	Unknown
Dr1	+	+	Unknown
ATF-2	−	+	Unknown
YY1	+	+	Unknown
Sp1	?	+	Unknown
MAZ	?	+	Unknown
STAT1	+	+	Unknown

Note: This listing contains only those cellular proteins that have been shown to bind directly to E1A proteins, and it is limited to binding partners for which there is evidence that the interaction is biologically relevant.

pRB, retinoblastoma susceptibility protein; cdk2, cyclin-dependent kinase 2; TSP, TATA-binding protein; TAFs, TBP-associated factors.

TABLE 2. *Cellular E1A binding partners*

E1A proteins activate transcription through the TATA motif (142,441), an element found 25 to 30 bp upstream of transcriptional initiation sites in many viral and cellular genes. The activation is believed to result in part from the ability of the 13S E1A protein to bind directly to the TATA-binding protein (TBP) (175,226), which is the DNA-binding subunit of the auxiliary transcription factor IID (TFIID), the first auxiliary transcription factor to interact with TATA-containing promoters. The interaction between TBP and E1A is mediated by the 13S-specific CR3 domain of the E1A protein (226), and this helps to explain why the 13S E1A protein activates transcription through TATA motifs more efficiently than the 12S protein. Part of the mechanism by which the 13S E1A protein activates transcription as a result of binding to TBP appears to involve the cellular tumor suppressor protein, p53, which can bind to TFIID (244,350) and repress transcription (350). The two proteins bind to overlapping domains on TBP, and the E1A protein can displace p53 from TBP and relieve p53-mediated repression (176). The 12S E1A protein can also activate through a TATA motif (219), but more weakly than the 13S protein. This activation event involves Dr1, a factor that can bind to TBP and inhibit transcription (190). The 12S and 13S proteins bind directly to Dr1, preventing it from associating with TBP (218). Thus, both 12S and 13S proteins can activate transcription through TATA motifs by binding to cellular factors and relieving transcriptional repression. E1A also has been reported to bind to several TAF subunits of TFIID (121,265) and to the rap30 subunit of TFIIF (242).

Recently, the 13S-specific CR3 domain of E1A has been shown to bind to the human SUR-2 protein (38). This interaction is very likely to be functionally significant because point mutations in CR3 that block transcriptional activation also block the interaction with SUR-2. SUR-2 is a constituent of a large protein complex known as *Srb/Mediator* or *TRAP.* The complex contains about 30 polypeptides and functions as a transcriptional coactivator or corepressor. The herpesvirus VP16 and p53 activation domains also interact with this complex, but through a different subunit (38,192).

In addition to interactions with the basal transcriptional machinery, E1A proteins can activate transcription through the binding sites for factors that bind well upstream of the basal promoter. The best-studied activation event occurs through E2F-binding sites. This transcription factor is named for the first promoter where it was found to bind, the adenovirus E2 promoter (214). E2F forms a complex with the cellular retinoblastoma tumor suppressor protein, pRB, and pRB inhibits transcriptional activation by E2F. The 12S and 13S E1A proteins activate transcription through E2F-binding sites (215,445,456), and both E1A proteins bind to pRB (435) through CR1 and CR2 (96,189), dissociating it from E2F (18,19,21). The strong correlation between the dissociation of the E2F–pRB complex and transcriptional activation by E1A proteins argues that E1A activates transcription by dissociating pRB from E2F. The activation of E2F by E1A not only affects the expression of viral genes but also influences the expression of cellular genes, dramatically affecting cell cycle progression (see Activation of the Host Cell).

E1A activates transcription by binding directly to several additional cellular factors. All of the early adenovirus promoters, with the exception of the E1B promoter, contain binding sites for the ATF (also termed *CREB*) family of transcription factors. E1A activates transcription through ATF-binding sites (243), presumably in part because the 13S E1A protein can bind to ATF-2 (1,239,243). The interaction occurs between the DNA binding domain of ATF-2 and CR3 of E1A. Different subdomains of CR3 mediate E1A binding to ATF-2 and TBP, raising the possibility that E1A functions as an adapter, bridging between ATF-2 bound at an upstream site and TBP (226). The SUR-2–containing complex, discussed previously, might also participate in the bridging. Such a bridging function might stabilize the initiation complex at the promoter.

E1A proteins can also activate through YY1 recognition sites (354). YY1 represses transcription when bound upstream of a transcriptional initiation site. The E1A proteins bind to YY1 through an amino-terminal sequence of E1A, and, in the case of the 13S E1A protein, the interaction is stabilized by CR3 (229). This binding correlates with the relief of repression mediated by YY1. The murine *c-fos* promoter provides a specific example of YY1-mediated repression that is relieved by E1A (462). This promoter contains both YY1- and ATF/CREB-binding sites, and YY1 binds to ATF/CREB, inhibiting its ability to activate transcription. E1A binds to YY1, blocking its interaction with ATF/CREB and relieving the repression. YY1 also interacts with p300 (see Activation of the Host Cell), and E1A potentially influences the activity of the YY1–p300 complex through its ability to bind to p300 (224).

E1A proteins have been reported to both activate and repress through the transcription factor AP1. These activities involve an amino-terminal domain and the CR1 domain of E1A (101,299). E1A can repress the action of AP1 through its consensus binding sites in the collagenase and stromelysin genes (299) and enhance the ability of AP1 to activate through the ATF sites (to which AP1 can bind) of adenovirus early genes (100,157). The AP1 transcription factor is a dimeric molecule that is generally composed of *c-fos* and *c-jun* family members, although *jun* family members can also heterodimerize with members of the ATF family (27). E1A repression appears to result from a block to DNA binding by *c-fos/c-jun* heterodimers (151), and activation by E1A through AP1 occurs, at least in part, by an indirect mechanism. E1A proteins cooperate with cyclic adenosine monophosphate (cAMP) to induce the level of AP1, which activates

transcription of viral genes (284). The E1A-mediated induction of AP1 activity results from transcriptional activation of the *c-fos* and *junB* genes through their TATA motifs (211), and the *c-jun* gene has been shown to be induced through an ATF-like element in its promoter, which probably responds to a *c-jun*/ATF-2 heterodimer (402). Hence, E1A can activate transcription indirectly through AP1-binding sites by inducing AP1 activity, and E1A can activate through ATF-binding sites either directly by binding to ATF-2 or indirectly by inducing AP1 activity. A second adenovirus protein contributes to the induction of AP1 activity. The E1B 19-kd protein activates the *c-jun* N-terminal kinase (JNK) (347), which strongly induces *c-jun*–dependent transcription.

Expression of the adenovirus VA RNA genes is induced by the E1A proteins (358,452). These genes are transcribed by RNA polymerase III, and it appears that the amount of the 110-kd subunit of transcription factor IIIC2a is increased by the E1A proteins (358). RNA polymerase III–sponsored transcription is repressed by pRB (434); hence, E1A might act through pRB to induce the synthesis of the 110-kd subunit.

Besides the E1A proteins, two additional early gene products have been shown to activate adenovirus promoters. The E4orf6/7 polypeptide binds to the cellular transcription factor E2F, with two molecules of the E4 protein apparently bridging between a pair of E2F molecules and causing them to bind cooperatively to the pair of E2F-binding sites within the E2 promoter (156,187,318). The E2 DNA-binding protein can activate a variety of early adenovirus promoters as well as the major late promoter (53), but the mechanism by which it stimulates transcription is unclear.

Individual adenovirus early promoters are activated by multiple mechanisms. For example, the E2 promoter can be activated by E1A through its TATA motif, ATF site, or E2F sites. In addition, the E4orf6/7 protein contributes to activation at the E2F sites, and the E2 promoter can also be induced by the E2 DNA-binding protein. Perhaps the activation pathways function with different efficiencies within various cell types, and these apparently redundant activation mechanisms have evolved to ensure that early viral promoters will be efficiently activated in a variety of different cell types as the virus spreads within its infected host.

Generally, adenovirus early genes remain active throughout the viral replication cycle, although the rate at which they are transcribed slowly declines. In part, the decline results from cell death. However, there are three known down-regulatory events, and in each case, it appears that a viral protein, which accumulates in response to an activation event, subsequently acts to inhibit continued transcriptional stimulation mediated by one or more early promoter elements. First, the E1A proteins can repress the activity of a variety of known enhancers, including the enhancer residing upstream of the E1A gene itself. This down-regulatory function correlates with the ability of the E1A protein to bind to a cellular protein, p300 (368), and the consequences of this protein–protein interaction are considered later (see Activation of the Host Cell). Second, whereas the E2 DNA-binding protein activates some promoters (53), it inhibits transcription from the E4 promoter (293) by an unknown mechanism. Third, the induction of AP1 activity by E1A and cAMP is transient (284). It is antagonized by the E4orf4 polypeptide, which accumulates in response to the E1A-mediated activation of the E4 gene (283). Mutant viruses unable to produce E4orf4 protein are viable but are more cytopathic than the wild-type virus, a counterintuitive result because expression of E4orf4 in the absence of infection induces apoptosis (222,356). E4orf4 binds to protein phosphatase 2A, and phosphatase activity is essential for the down-regulatory event (211).

Once early mRNAs have been synthesized, they are translated on polysomes together with cellular mRNAs. Initially, they do not appear to enjoy a competitive advantage, but as the infection enters the late phase, cellular mRNAs are excluded from polysomes (see Activation of Late Gene Expression and Host Cell Shutoff).

Activation of the Host Cell

Adenovirus infection has long been known to induce quiescent cells to enter the S phase of the cell cycle, creating an environment optimally conducive to viral replication. Modulation of the cell cycle is primarily a function of the E1A proteins, and the modulation is mediated by their CR1, CR2, and nonconserved amino-terminal domains. The key to understanding how E1A proteins manipulate cell cycle regulation came from the observation that a set of cellular proteins can be co-immunoprecipitated with the E1A proteins (158,446). The most abundant coprecipitating cellular polypeptides have molecular weights of about 33, 60, 80, 90, 105, 107, 130, 300, and 400 kd (Fig. 7); several of these polypeptides have been shown to interact directly with E1A proteins (see Table 2). The 105-kd moiety was the first to be identified (435). It is the retinoblastoma tumor suppressor protein, pRB, which, as discussed earlier (see Activation of Early Viral Genes), regulates the ability of the E2F cellular transcription factor to activate transcription. The human papillomavirus E7 protein and the large T antigens of papovaviruses also bind to pRB, underscoring its importance as a target of oncoproteins encoded by DNA tumor viruses. Although E2F sites are present in the adenovirus E1A and E2 promoters and contribute to their activation, neither papovaviruses nor papillomaviruses have E2F binding sites in their genome. Therefore, tumor virus oncoproteins must target pRB for reasons other than to influence E2F function at their own promoters.

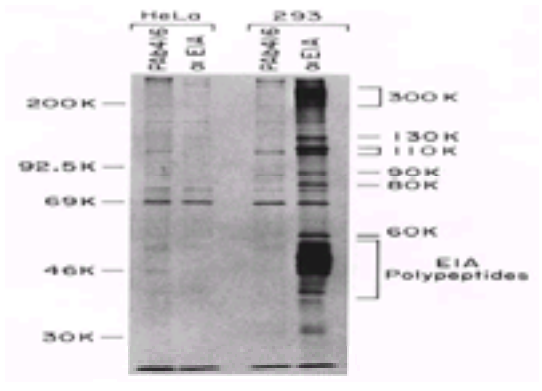

FIG. 7. Co-immunoprecipitation of cellular polypeptides with E1A proteins. Radioactively labeled polypeptides were

immunoprecipitated from lysates of HeLa or 293 cells with a monoclonal antibody specific for SV40 T antigen (PAb416) or a mixture of antibodies specific for E1A proteins (aE1A), and immune complexes were analyzed by electrophoresis. The size (kd) of marker proteins is indicated (*left*), and the size of polypeptides coimmunoprecipitated with E1A proteins, as well as the location of E1A polypeptides, is indicated (*right of autoradiogram*). (From ref. 158, with permission.)

Overexpression of pRB inhibits cell cycle progression, causing cells to arrest in middle to late G_1 phase, and growth arrest correlates with the ability of pRB to bind to E2F and block E2F-dependent transcriptional activation. pRB activity is regulated by cyclin-dependent kinases. Hyperphosphorylation of pRB inhibits the formation of the pRB–E2F complex, and it reverses the ability of pRB to arrest cell growth. These observations are consistent with the fact that hyperphosphorylated pRB and free E2F accumulate as growing cells reach late G_1 and enter S phase. The strong correlation between the ability of pRB to bind E2F and to block progression from G_1 to S phase supports the conclusion that pRB regulates cell cycle progression through its interactions with E2F. pRB inhibits E2F function: it likely masks the E2F activation domain, and it binds to histone deacetylase. Deacetylation of histones inhibits transcription, consistent with the observation that the E2F–pRB–deacetylase complex actively inhibits the transcription of E2F-responsive genes. Free E2F activates a series of genes important for S phase and cell growth that contain E2F-binding sites in their promoters (e.g., dihydrofolate reductase, DNA polymerase a, *cdc2,* cyclin E, and *c-myc.* Indeed, ectopic expression of E2F induces quiescent cells to enter the S phase of the cell cycle.

The CR2 domain is primarily responsible for the interaction of E1A proteins with pRB, and the amino-terminal portion of the CR1 domain plays an auxiliary role, stabilizing the interaction (96,189). CR2 contains the sequence Leu-X-Cys-X-Glu (where X is any amino acid), which is present in other viral and cellular proteins that bind to pRB. The E1A-binding domain on pRB is often referred to as the *pocket,* and it is composed of two essential sequences, termed A and B, separated by a spacer region (184,199). E2F also binds to the pocket domain; hence, E1A binding competes with E2F for access to the pRB pocket. As a result, E2F can be liberated from pRB by E1A (18,19,21), and the free E2F can then activate cellular genes that facilitate cell cycle progression. This accomplishes what appears to be a common goal of DNA tumor viruses: to move the infected cell into the S phase, creating an environment favorable for efficient viral DNA replication. Mutant E1A proteins that fail to bind pRB are unable to activate transcription through E2F or modulate cellular growth.

Two additional cellular pRB family members have been identified that interact with E2F—p107 and p130—and E1A can bind to them (see Fig. 7), dissociating E2F. As is the case for pRB, the interaction is mediated by the E1A CR2 domain, and E1A–p107 binding is stabilized by interactions within the CR1 domain (418). The proteins p107 and p130 associate with cyclins and cyclin-dependent kinases. E1A can be found in complexes that include kinase activities (169,210), and the phosphorylation state of p107 and p130 in these complexes is substantially elevated (22). Whether these complexes represent nonfunctional byproducts of the release of E2F or whether the complexes that include E1A, p107 or p130, cyclin and cyclin-dependent kinase (p60 and p33) (see Fig. 7) perform an active, E1A-modulated function is unknown. Not only are there multiple pRB family members present in E2F complexes, but there are also multiple E2Fs. The E2F transcription factor is a heterodimer composed of one of six known E2F family members and one of three DP family members. In summary, the E1A proteins disrupt a series of complexes that contain different pRB family members and multiple E2F subunits, forcing quiescent cells to begin DNA synthesis.

In addition to disrupting pRB–E2F complexes, E1A blocks the action of a cyclin-dependent kinase inhibitory protein. Cyclin-dependent kinases target pRB family members and regulate their activities. Hyperphosphorylated pRB does not bind E2F, allowing the transcription factor to stimulate S-phase entry. Cyclin-dependent kinases are themselves regulated by several mechanisms, including association with cyclin-dependent kinase inhibitory proteins, such as $p27_{kip1}$. Overexpression of $p27_{kip1}$ blocks cell cycle progression in the G_1 compartment. E1A antagonizes the inhibitory activity of $p27_{kip1}$ (4,257), preventing G_1 arrest and favoring progression to the S phase.

The E1A proteins facilitate cell cycle progression through a second pathway by binding to the closely related p300 and CBP proteins (see Fig. 7). Their binding site on E1A includes the poorly conserved amino-terminus and the carboxy-terminal half of CR1 (418). E1A mutants lacking CR2 and unable to bind pRB family members can nevertheless stimulate cellular DNA synthesis, provided their p300/CBP-binding site remains intact. Thus, the E1A proteins contain two independent domains, either of which can stimulate cells to progress from G_1 to S phase (183,240,459). The p300/CBP proteins serve as coactivaters for a number of transcription factors, including CREB, STATs, and nuclear receptors. The coactivaters are brought to the promoter through their interaction with DNA-binding factors. The p300/CBP proteins exhibit intrinsic histone acetylase activity, and they interact with a factor termed *P/CAF* that is also a histone acetyltransferase. Acetylation of histones is believed to weaken their interaction with DNA, producing a chromatin structure that is more conducive to transcription. Thus, p300/CBP is thought to function, at least in part, by modifying chromatin structure. When E1A binds to p300 or CBP, it simultaneously displaces P/CAF and inhibits the intrinsic acetyltransferase activity of the coactivator (48,154,307,444). The inhibition of p300/CBP acetyltransferase activity can explain the earlier observation that E1A inhibits the function of some enhancer elements (328,368,418).

S-phase entry is facilitated by the ability of E1A to inhibit p300/CBP function. The p53 tumor suppressor protein is one target of this activity. The *p53* gene is the most commonly mutated gene in human tumors, and the loss of p53 function or its alteration by mutation can contribute to tumor progression. Consistent with its frequent loss in tumor cells, high-level expression of p53 promotes cell cycle arrest or apoptosis. The p53 protein is a DNA-binding transcription factor, and p300/CBP serves as a coactivator for p53. By inhibiting p300/CBP activity, E1A proteins antagonize p53 function (238,362). Although inhibition of p53 will relieve a block to cell cycle progression, it does not by itself stimulate progression into S phase. It is likely that there are unidentified genes whose expression is modulated by the interaction of E1A with p300/CBP, inducing the cell to synthesize DNA. Alternatively, the domain on E1A that mediates its interaction with p300/CBP might interact with another unidentified target. Indeed, this domain binds to p400, the largest protein known to interact with E1A. Although p400 and p300/CBP are related in their amino acid sequences (22), the function of

p400 is unknown.

Two separate regions of the E1A proteins can induce cells to move from G_1 to S phase through two independent mechanisms: binding to pRB family members and binding to p300/CBP. Presumably, E1A proteins employ two mechanisms because together they function more effectively than either alone or because there may be cells in which one pathway works more efficiently than the other. Further, although the E1A domains mediating binding to either pRB family members or p300/CBP can induce DNA synthesis in quiescent cells, both regions are required to pass the G_2- to M-phase checkpoint and progress to mitosis (182,183,417,459). This could be an important function in animal hosts, in which infections are likely to proceed much more slowly than in cultured cells. Without the ability to divide, cells that pass completely through S phase would be stuck in G_2; if the cells can divide, they can continue cycling and return to S phase, during which viral replication is most efficient. Possibly, the simultaneous inhibition of both the pRB family and p300/CBP by E1A binding somehow enables the infected cell to pass through the G_2- to M-phase checkpoint. Alternatively, E1A proteins might alter the function of these regulatory proteins rather than simply inhibit them. E1A has been reported to bind simultaneously to a pRB family member and p300/CBP (22), and this new complex might influence passage through mitosis. It is also possible that E1A proteins bind to additional, as yet unidentified, proteins to antagonize this checkpoint.

The level and activity of p53 are induced in response to a variety of stresses, including the presence of E1A proteins (79,248,337). E1A activates p53 through a pathway that includes the p19 ^{ARF} tumor suppressor protein (78). This protein can associate with p53 to alter its level and activity. E1A induces p19 ^{ARF} through the same domains required for the induction of p53, and E1A fails to induce p53 in cells lacking p19 ^{ARF}. Although E1A inhibits the p53 transcriptional coactivator p300/CBP, p53 nevertheless acts to limit its oncogenic potential. As noted previously, high-level expression of p53 promotes cell cycle arrest at the G_1- to S-phase boundary or apoptosis. Although the transcriptional activation function of p53 mediates its block to cell cycle progression and apoptosis, p53 can induce apoptosis through a second pathway that does not depend on its ability to stimulate transcription. This helps to explain why adenoviruses have evolved additional mechanisms to counter p53 function.

The Ad5 E1B 55-kd protein binds to p53 within infected cells (339), and it can block transcriptional activation by p53. Analysis of mutant E1B 55-kd proteins has revealed a strict correlation between the ability of E1B to block p53-mediated transcriptional activation and its ability to cooperate with E1A in the oncogenic transformation of cells (447). The E1A and E1B proteins of adenovirus are oncoproteins (see Oncogenesis), and they cooperate to transform cultured cells. Oncogenic transformation is a manifestation of the ability of these proteins to interfere with the normal function of tumor suppressor proteins. Thus, the correlation between a block to transcriptional activation and transforming activity of the viral protein suggests that the E1B 55-kd protein antagonizes the ability of p53 to block cell cycle progression and induce apoptosis by inhibiting its activation function. The E1B 55-kd protein binds to the amino-terminal, acidic transcriptional activation domain of p53 (200), and this suggests that the viral protein might simply mask the activation domain. Steric hindrance might contribute to the ability of the E1B 55-kd protein to block activation by p53, but it is not the entire mechanism. The E1B 55-kd protein can inhibit transcription if it is artificially anchored to a promoter, indicating that it can actively repress transcription (448). Not only does the interaction of the E1B 55-kd protein with p53 serve to bring the viral protein to the promoter, but also the E1B 55-kd protein increases the affinity of p53 for its DNA-binding site (261). In conjunction with an as yet unidentified corepressor (261), the E1B 55-kd protein not only blocks the activation function of p53 but also actively represses transcription. Thus, like E1A, the E1B 55-kd protein binds to a tumor suppressor protein, antagonizes its normal activity, and helps to deregulate cell cycle progression.

In contrast to E1A, expression of the E1B 55-kd protein alone is not sufficient to stimulate quiescent cells to enter the S phase of the cell cycle. This is consistent with the observation that deletion of both p53 alleles does not directly lead to the loss of regulated cell division (93). Nevertheless, p53 is targeted by a variety of tumor virus oncoproteins: SV40 large T antigen binds to p53, blocks its DNA-binding activity, and interferes with its ability to activate transcription; and human papillomavirus type 16 E6 protein complexes with p53 and cooperates with cellular proteins to promote its degradation. Presumably, then, the E1B 55-kd protein collaborates with E1A to activate quiescent cells more effectively. Further, because E1A causes p53 to accumulate, E1B must contribute to the activation of quiescent cells by preventing the implementation of a cell cycle block or apoptosis by elevated levels of p53.

The E1B 55-kd protein also enables the virus to overcome a restriction to its replication imposed by the cell cycle (131) independently of the presence of wild-type p53 (132). Mutant viruses unable to express the E1B 55-kd protein fail to produce progeny efficiently unless cells are infected in the S phase of the cell cycle. The mechanism by which the E1B 55-kd protein mediates this intriguing function is uncertain. Mutant viruses that fail to express E4orf6 protein exhibit a similar cell cycle phenotype as E1B mutants (133). A complex of the E1B and E4 proteins controls the transport of viral and host mRNAs from nucleus to cyto plasm (see Activation of Late Gene Expression and Host Cell Shutoff), but this function does not appear to underlie the effect of the E1B 55-kd and E4orf6 proteins on the cell cycle restriction.

It is noteworthy that the Ad12 homolog of the Ad5 E1B 55-kd protein can inhibit p53-mediated transcriptional activation and cooperate with E1A to transform cells (447), even though it shows no indication of p53 binding (137,458). Also, the Ad5 E1B protein can sequester a substantial portion of the cell's p53 in a discrete cytoplasmic structure outside of the nucleus (457), whereas the Ad12 protein cannot (458). This has been taken as further evidence that the Ad12 protein does not bind to p53. The Ad12 E1B protein might bind weakly to p53, and the resulting complex might not be sufficiently stable to be detected by the assays that have been employed. The p53 protein is stabilized in Ad12 transformed cells, and the stabilization is dependent on expression of the E1B protein (403). Stabilization is often observed for mutant p53, but the p53 present in the Ad12 transformed cells is wild type. Further, although the Ad5 E1B protein inhibited transformation by *myc* plus *ras,* the Ad12 E1B protein, like mutant p53, enhanced the number of foci produced by *myc* plus *ras.* If there is no direct interaction between the Ad12 E1B protein and p53, these observations suggest that the Ad12 protein somehow causes wild-type p53 to be modified, perhaps by association with another cellular protein, so that it behaves in some respects like mutant p53.

In addition to the E1B 55-kd protein, the E4orf6 protein can bind to p53 and block its transcriptional activation function (92). Like the E1B 55-kd protein, E4orf6 inhibits the interaction of p53 with the TAF31 subunit of TFIID; however, unlike

the E1B 55-kd protein, which binds to the amino-terminal activation domain of p53, E4orf6 binds within its carboxy-terminal region. The E1B and E4 proteins cooperate to shorten markedly the half life of p53 (276,287,331,366). Given its ability to antagonize p53, it is not surprising that E4orf6 can cooperate with E1A in cell transformation assays (see Oncogenesis).

E4or6, but not the E1B 55-kd protein, can bind to p73 and inhibit its function (171,367). The p73 protein is a member of the p53 family that is able to activate transcription through p53 response elements. Thus, E4orf6 antagonizes the function of both p53 and p73, blocking their potential inhibitory effects on viral replication.

Inhibition of Apoptosis

E1A proteins trigger apoptosis through both p53-dependent and p53-independent mechanisms (386). The p53-independent apoptosis is an indirect effect of E1A. It results from the E1A-mediated expression of the E4orf4 protein (222,356). E4orf4 might induce apoptosis as a consequence of its ability to bind to protein phosphatase 2A and inhibit its activity (211). This phosphatase targets Bcl-2 and caspase 3, which are known to regulate and mediate apoptosis, respectively. Apoptosis is not observed when E4orf4 functions in the context of an infected cell.

As has been found for other viruses, adenoviruses encode proteins (E1B 55-kd, E1B 19-kd, and E4orf6) that block apoptosis. As discussed previously, the E1B 55-kd and E4orf6 proteins bind to p53 and inhibit its function, blocking p53-dependent apoptosis. The E1B 19-kd protein is a member of the Bcl-2 family of proteins and can block p53-dependent or p53-independent apoptosis (316). In this respect, it is similar to Bcl-2, which resides in the outer mitochondrial membrane and inhibits the translocation of cytochrome c from mitochondria to the cytoplasm. When cytochrome c enters the cytoplasm, it facilitates activation of caspase-9, provoking an apoptotic response.

Adenovirus mutants that fail to produce a functional E1B 19-kd protein induce extensive degradation of host cell and viral DNA, enhanced cytopathic effect, and reduced virus yield when grown in cultured cells (310,380,433). Presumably, the inhibitory effects of premature cell death on viral propagation would be even more severe in natural infections, in which the viral growth cycle is slower than in cultured HeLa cells. Thus, apoptosis is an example of a cellular response to infection that has the potential to inhibit viral growth and block its spread within the infected organism.

Adenoviruses encode additional proteins (E3 gp19-kd, E3 14.7-kd, E3 14.5-kd, and E3 10.4-kd) that block the induction of apoptosis by cytotoxic T lymphocytes (CTLs) and macrophages, and their mode of action is discussed later (see Viral Antagonists of Cytotoxic T Lymphocytes and Tumor Necrosis Factor-a).

Viral DNA Replication

As E2 gene products accumulate, the stage is set for viral DNA replication. Ad2 or Ad5 DNA replication begins between 5 to 8 hours after infection of HeLa cells at a multiplicity of 10 plaque-forming units per cell, and it continues until the host cell dies.

The inverted terminal repeats of the viral chromosome serve as replication origins. *In vivo* studies support a model in which adenovirus DNA replication takes place in two stages (223) (Fig. 8). First, synthesis is initiated at either terminus of the linear DNA and proceeds in a continuous fashion to the other end of the genome. Only one of the two DNA strands serves as a template for the synthesis; thus, the products of the replication are a duplex consisting of a daughter and parental strand plus a displaced single strand of DNA. In the second stage of the replication process, a complement to the displaced single strand is synthesized. The single-stranded template circularizes through annealing of its self-complementary termini, and the resulting duplex “panhandle” has the same structure as the termini of the duplex viral genome. This structure allows it to be recognized by the same initiation machinery that operates in the first stage of replication, and complementary strand synthesis generates a second completed duplex consisting of one parental and one daughter strand. Adenovirus DNA was the first eukaryotic template to be replicated *in vitro* (50), and increasingly, defined cell-free systems have allowed the analysis of replication in considerable detail.

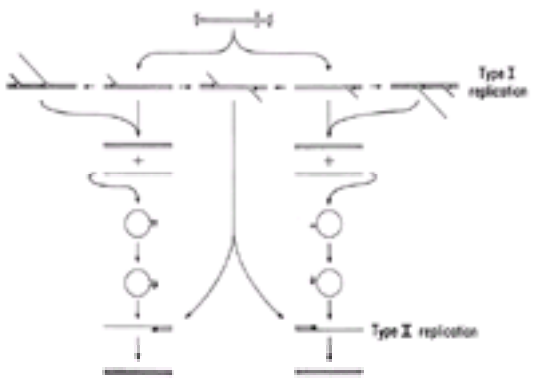

FIG. 8. A model for adenovirus DNA replication. Replication begins at either end of the duplex adenovirus DNA molecule, displacing one of the parental strands and subsequently replicating the displaced strand. Type I replication refers to replication on duplex templates, and type II replication refers to replication initiated on a single-stranded molecule. (From ref. 223, with permission.)

The cis-Acting sequences composing the replication origins are located within the inverted terminal repeats of the viral chromosome. Three functional domains have been defined within the terminal 51 bp of the repeats. Domain A consists of the first 18 bp of the viral DNA, and it functions as a minimal origin of replication. This domain is required for replication

but supports only limited replication on its own. The sequence between base pairs 9 to 18 (5'ATAATATACC-3') is conserved among different human adenovirus serotypes, and a complex of two viral proteins binds here (59,281,385): the preterminal protein (pTP) and the DNA polymerase. The E2-coded terminal protein (TP) is synthesized as an 80-kd polypeptide (pTP) that is active in initiation of DNA replication (49,373) and, as discussed previously, is found covalently attached to the 5' ends of the viral chromosome. It is subsequently processed by proteolysis during assembly of virions to generate a 55-kd fragment (TP) that is covalently attached to the genome (51), but it appears that the entire protein with cleaved peptide bonds remains associated with the genomic termini (342). The E2-coded polymerase is a 140-kd protein with biochemical properties distinct from other known DNA polymerases (102,109). It contains both 5'-to-3' polymerase activity and 3'-to-5' exonuclease activity, which probably serves a proofreading function during polymerization (109). The pTP and the polymerase form a heterodimeric complex in solution (102,235,374,385) and thus would be expected to bind to the origin as a unit.

Domain B consists of base pairs 19 to 39, and domain C includes base pairs 40 to 51. These two elements are not absolutely required for adenovirus DNA replication, but they substantially enhance the efficiency of the initiation reaction. Cellular factors bind to these elements: nuclear factor I (NFI) binds as a dimer in domain B, and nuclear factor III (NFIII) binds in domain C. NFI interacts with the DNA polymerase, stabilizing the pTP–polymerase complex at the origin (37,59,282). The binding of NFI at domain B appears to be stimulated by the third viral protein that functions in DNA replication, the E2-coded single-stranded DNA-binding protein (68,376). NFIII binds to the pTP and helps to stabilize the pTP–polymerase complex at the origin (406). NFIII binding induces DNA bending, and this might also contribute to its function (413). NFI and NFIII are also transcription factors. NFI is a member of the CTF family of transcriptional activators (197), and NFIII is also known as Oct 1, a member of the family of proteins that contain POU DNA-binding domains (302,315).

There is flexibility in the requirement for the B and C domains in the replication origin. A mutant Ad5 lacking domain C grows well, with sequences adjacent to the inverted terminal repeat compensating for the lack of an NFIII-binding site (162). Ad4 1acks domain B, the binding site for NFI, and its replication appears to require only domain A (159,163). Possibly, the Ad4 pTP–polymerase complex has an intrinsically higher affinity for its binding site in the minimal origin than is the case for the complex encoded by other adenoviruses, and this could bypass the need for the stabilizing function of NFI.

A cloned adenovirus terminal sequence will support the initiation of replication if it is cleaved to produce an end near the normal terminus (382). However, a natural template with a molecule of TP attached at its 5' end is replicated at much higher efficiency. The activity of the cloned template can be substantially increased if it is treated with a 5'-to-3' exonuclease. Thus, the production of a single-stranded 3'-terminus can compensate for the absence of an attached molecule at the 5'-terminus, and this suggests that TP bound to the template DNA normally helps to open the terminal duplex. The pTP–polymerase complex can bind in a sequence-specific fashion to the double-stranded form of the A domain of the replication origin (385), and it also can bind to single-stranded DNA (59,204). A sequential interaction of the pTP–polymerase complex, first with the duplex terminus and subsequently with a single strand, could help to unwind the terminal duplex DNA.

The pTP interacts with the nuclear matrix (36,115,342) and serves as a primer for DNA replication. Within the nuclear matrix, pTP appears to contact CAD, a multifunctional pyrimidine biosynthesis enzyme (9). This interaction might anchor adenovirus replication complexes in the vicinity of useful cellular factors.

As a primer, pTP preserves the integrity of the viral chromosome's terminal sequence during multiple rounds of DNA replication. The priming reaction begins with the formation of an ester bond between the b-OH of a serine residue in pTP and the a-phosphoryl group of deoxycytidine monophosphate (dCMP), the first residue at the 5' end of the DNA chain (49,236). The pTP–dCMP interaction requires the presence of polymerase and is template dependent (52,236,382), suggesting that it occurs after the pTP–polymerase complex is properly positioned on the template. The 3'-OH group of the pTP–dCMP complex then serves to prime synthesis of the nascent strand by the DNA polymerase. The study of mutant templates in a reconstituted replication system has revealed that pTP–dCMP first primes the synthesis of a pTP–CAT initiation intermediate by copying the second three mononucleotides of the template, and then the newly synthesized complex jumps back to base pair at the terminus (207). This jumping mechanism succeeds because the sequence starts with a repeated trimer: 3'-GTAGTA. After the synthesis of the first three or four nucleotides of the new DNA strand, the polymerase separates from pTP (206). The dissociation coincides with a change in its sensitivity to inhibitors and in its K_m for deoxynucleoside triphosphates, as the polymerase becomes a more efficient elongation enzyme.

Chain elongation requires two virus E2-coded proteins, the polymerase and single-stranded DNA-binding protein, and a cellular protein, nuclear factor II (NFII). The virus-coded DNA-binding protein is a 59-kd phosphoprotein that migrates in SDS-polyacrylamide gels with an apparent molecular weight of 72 kd. It binds tightly and cooperatively in a sequence-independent fashion to single-stranded DNA (404). The role of the DNA-binding protein in DNA replication was first revealed by analysis of an adenovirus temperature-sensitive variant carrying a mutation within the E2 gene; its ability to replicate DNA is exquisitely temperature dependent (405). The three-dimensional structure of the DNA-binding protein has been determined (400), and it contains a terminal extension that hooks onto an adjacent DNA-binding protein monomer. This drives the formation of long, multimeric protein chains bound to single-stranded DNA. If the carboxy-terminal extension is removed from the DNA-binding protein, strand displacement on a double-stranded template no longer occurs (81). This indicates that the polymerization of the DNA-binding protein on single-stranded DNA drives strand separation, consistent with the lack of a requirement for a DNA helicase to unwind the double-stranded template in reconstituted adenovirus DNA replication reactions. In the presence of the DNA-binding protein, the polymerase is highly processive (109,241) and can travel the entire length of the chromosome after it has separated from pTP. NFII copurifies with a cellular DNA topoisomerase activity (285), and mammalian topoisomerase I will substitute for it in an *in vitro* replication reaction. NFII does not significantly enhance the synthesis of nascent chains up to 9,000 nucleotides in length; hence, it must be needed to overcome a DNA structural problem that arises only after extensive replication.

In summary, a set of proteins has been identified that mediates the initiation of adenovirus DNA replication (pTP,

polymerase, NFI, and NFIII) and chain elongation (polymerase, DNA-binding protein, and NFII). These polypeptides, together with a template containing an adenovirus replication origin, are sufficient to reconstitute the complete viral DNA replication reaction *in vitro.* The adenovirus E4 gene encodes one or more products required for efficient DNA replication (39,153,426), but their role in the process is almost certainly indirect (266).

Activation of Late Gene Expression and Host Cell Shutoff

Adenovirus late genes begin to be expressed efficiently at the onset of viral DNA replication. The adenovirus late coding regions are organized into a single large transcription unit whose primary transcript is about 29,000 nucleotides in length (105,291). This transcript is processed by differential poly(A) site utilization and splicing to generate multiple distinct mRNAs (see Fig. 3). These mRNAs have been grouped into five families, termed L1 to L5, based on the use of common poly(A) addition sites (66,291,463). Expression of this large family of late mRNAs is controlled by the major late promoter. This promoter exhibits a low level of activity early after infection, and it becomes several hundred-fold more active on a per DNA molecule basis at late times (352). It is strongly activated by the E1A proteins through its TATA motif and Sp1/MAZ binding sites (E1A binds to the Sp1 and MAZ transcription factors) (306), but this does not account for the delayed kinetics of the activation event. There appear to be at least two distinct components that contribute to the delayed activation of the major late promoter: a cis-acting change in the viral chromosome, and induction of at least one virus-coded transacting factor.

The time-dependent cis-acting modification of the adenovirus chromosome was revealed by the sequential infection of cells with two closely related adenovirus strains whose late products could be distinguished (389). Although the first virus to reach the nucleus was actively expressing its late products, the second viral chromosome did not express late gene products until it had completed the early phase and had begun DNA replication. Thus, gene products from the first virus did not act in trans to initiate late expression from the second virus.

There are several possible explanations for this observation. It might be necessary to alter the constituents of the viral chromatin during the process of DNA replication to activate the major late promoter. Unreplicated viral chromatin appears to be associated with a different set of proteins than replicated chromatin (56,82). Viral DNA might remain associated with virus-coded core proteins until cellular histones bind during replication. Such an exchange could be required to activate the major late promoter. However, this proposal seems unlikely. Adenovirus DNA does not appear to be associated with histones (410), and papovaviruses exhibit a DNA replication–coupled early-to-late switch even though they package their DNA in cellular histones. Perhaps the replication process allows transcription factors to gain access to the promoter. Histones or histone-like proteins are displaced and then reassemble on DNA during replication, and this could provide an opportunity for transcription factors to compete for binding to the DNA. Alternatively, a newly arriving viral core might simply require a period of time to decondense and become available to the transcription machinery. Chromosomal domains could become accessible to the transcriptional machinery in a defined order, and the promoter responsible for most late gene expression might become accessible only after a delay. The major late promoter is active at a low level early after infection, but this might reflect early activation by a relatively minor subset of the total infecting population of viral chromosomes. It is also possible that the viral chromosome might establish a compartmentalized environment in the nucleus, and it might take a period of time to recruit cellular factors to its defined environment that are needed for late gene expression. Discrete viral centers at which replication and transcription occur have been observed in the infected nucleus by electron microscopy (262,280).

Whatever the underlying mechanism, *in vivo* footprint analysis has revealed that a transcription factor, termed *USF* or *MLTF* (45,273,341), binds to its upstream site in the major late promoter after the onset of DNA replication but not before (397). This factor activates transcription when it is bound to its recognition sequence in the major late promoter. However, a point mutation that blocks binding of USF/MLTF does not prevent the normal activation of the major late promoter (319,320). Hence, if it normally plays an important role in the late activation process, there must be redundant activators that can work in its absence, or USF/MLTF might be brought to the promoter through a protein–protein interaction in the absence of its binding site. Nevertheless, the demonstration that a factor gains access to the major late promoter only after the onset of DNA replication suggests that the time-dependent cis-acting modification to viral chromatin likely involves changes that permit access of transcription factors to the template.

The second component that contributes to activation of the major late promoter is a virus-coded transcription factor. In addition to the upstream region with USF/MLTF-, CAAT-, Sp1/MAZ-, and TATA-binding motifs, the region between +85 and +120, downstream of the initiation site, contributes to activation of the promoter (227,259). This downstream domain contains binding sites for at least two factors that can cooperate with the upstream USF/MLTF element to activate transcription (274,275). One of these factors is coded by the adenovirus *IVa2* gene (399). The *IVa2* gene has been classified as a delayed early gene because it is activated somewhat later than the standard early genes. Its promoter also contains important downstream elements (43,201), and these elements include the binding site for a factor that represses *IVa2* transcription (58). The mechanism by which repression is relieved is not yet known, but it seems likely that an early gene product plays a role. One can build a model in which a cascade of events leads to activation of the major late promoter: early transcription is activated; an early gene product relieves repression of the IVa2 promoter; and the IVa2 protein binds downstream of the major late start site and contributes to its induction. This model is attractive because the sequential nature of these events can serve as a timer, and this clock, together with the requirement for DNA replication, delays the synthesis of the late mRNAs until their products are needed for virus assembly. The IVa2 protein might work in concert with the L1 52/55-kd protein to activate the major late promoter because the two proteins have been reported to interact (149).

Adenovirus encodes a second delayed early gene product, protein IX. It activates transcription in transfection assays but does not exhibit specificity for the major late promoter (250).

Accumulation of late mRNA not only requires activation of the major late promoter but also depends on the alleviation of premature termination by RNA polymerase as it traverses the 29,000-bp late unit. Premature termination occurs at two positions within the unit. The first termination occurs close to the start site. During the late phase of infection, 80% of the RNA chains initiated at the major late promoter terminate before elongation has proceeded 500 nucleotides

(104,114,348). This termination primarily mitigates against late mRNA accumulation because prematurely terminated transcripts with 3' ends in this region are not detected early after infection (114). The second termination event occurs early after infection when molecules of polymerase fail to transcribe beyond the end of the L2 coding region (292); later, the polymerase transcribes through all of the late coding regions to the end of the genome (113). The molecular basis for the termination at early but not late times remains a mystery. Among other possibilities, it could result from cis-acting changes in the structure of adenovirus chromatin.

When DNA replication begins and all the late mRNAs are synthesized, the cytoplasmic accumulation of cellular mRNAs is blocked (26). Synthesis and processing of cellular transcripts continue, but the mRNAs fail to accumulate in the cytoplasm, suggesting that their transport is blocked. The block to cellular mRNA accumulation is mediated by the E1B 55-kd polypeptide (15,311) and by the E4 34-kd polypeptide (153,426), which exist in a complex (338). The same E1B and E4 proteins are required for efficient cytoplasmic accumulation of viral mRNAs late after infection (16,153,311,426). Thus, a complex that includes two viral proteins inhibits accumulation of cellular mRNAs and facilitates cytoplasmic accumulation of viral mRNAs. Analysis of late viral mRNA metabolism in cells infected with a mutant virus unable to synthesize the E1B 55-kd protein indicated that a block occurred immediately before movement of the mRNA from the nucleus to the cytoplasm (228). As discussed previously, the E1B 55-kd protein also binds to p53, but there is no evidence that p53 plays a role in the mRNA transport function mediated by the complex formed between the E1B and E4 proteins.

How does the E1B–E4 complex move mRNAs from the site of synthesis? The E4orf6 protein shuttles between the nucleus and cytoplasm (91,134). E4orf6 contains a nuclear export sequence, a motif first identified in the human immunodeficiency virus (HIV) rev protein, that would be expected to mediate its interaction with the cellular nucleocytoplasmic shuttling apparatus. How does the E1B–E4 complex interact with RNAs to be transported? The E1B 55-kd protein has been reported to exhibit intrinsic RNA-binding activity (178), and the viral protein interacts with a cellular protein termed *E1B-AP5* (117). E1B-AP5 is a member of the heterogeneous nuclear ribonucleoprotein family of RNA-binding proteins, it contains an RNA-binding motif, and its overexpression overcomes the block to host cell mRNA cytoplasmic accumulation in adenovirus-infected cells. A complex of the E1B and E4 proteins with their cellular partners transports newly synthesized viral mRNAs to the cytoplasm.

The discrimination between host and viral mRNAs for transport to the cytoplasm within virus-infected cells is not based on the identity of individual mRNAs. Cellular genes expressed from recombinant viral chromosomes accumulate in the cytoplasm late after infection as if they were viral mRNAs (120,167). Further, mRNAs are transported to the cytoplasm when encoded by cellular genes, such as the *hsp70* gene, that are transcriptionally induced within infected cells (277,443). Immunoelectron microscopy revealed that the E1B–E4 protein complex is localized within and surrounding intranuclear inclusions believed to represent viral transcription and replication centers (304). Together, these observations suggest a model in which the E1B–E4 complex relocalizes to viral transcription centers a cellular factor required for transport of mRNAs (304). This proposal can explain the simultaneous inhibition of host and activation of viral mRNA transport because a transport factor would be moved from the many sites of host transcription and processing to the viral centers.

In addition to their facilitated transport from the nucleus, viral mRNAs are preferentially translated when they reach the cytoplasm late after infection (461). During this period, when viral mRNAs constitute about 20% of the total cytoplasmic pool (381), they are translated to the exclusion of host mRNAs (14,449). Host mRNAs are not degraded; if they are extracted from the infected cell, they can be translated with normal efficiency in a cell-free extract (387). The translational block is not dependent on the inhibition of host cell mRNA accumulation in the cytoplasm. In contrast to most host mRNAs, b-tubulin mRNA continues to accumulate in the cytoplasm, but it is not translated (277). Also, 2-aminopurine can prevent the inhibition of translation without relieving the block to accumulation of host mRNAs (185).

Several regulatory components cooperate to facilitate selective translation of viral mRNAs late after infection. The first involves the cellular protein kinase R (PKR), which is activated by double-stranded RNA that accumulates within adenovirus-infected cells (260,301). After activation, it can phosphorylate the eukaryotic initiation factor-2a (eIF-2a), inactivating the initiation factor and blocking translation. Translation of host cell mRNAs is not inhibited after infection of cells deficient in PKR activity (185,300), suggesting that the kinase, and presumably inactivation of eIF-2a, are key components of the block to host cell translation. The adenovirus-coded VA RNAs (see Viral Antagonists of Interferon-a and -b) inhibit activation of the cellular PKR. These small RNAs have been shown to copurify with viral mRNAs (263); as a result, they might protect viral but not cellular protein synthesis, providing a functional compartmentalization.

Inactivation of eIF-4F also contributes to selective translation in adenovirus-infected cells (186). This initiation factor binds to the cap of mRNAs and facilitates scanning of the 40S ribosome from the cap to the AUG through its intrinsic helicase activity. eIF-4F is normally activated by phosphorylation, and it becomes substantially inactivated by dephosphorylation late after adenovirus infection when cellular mRNAs are not translated. The five families of mRNAs encoded by the adenovirus major late transcription unit all contain the same 200-nucleotide 5'-noncoding region, which has been termed the *tripartite leader sequence* (30). This 5'-noncoding region is important for translation of mRNAs late but not early after infection (33,245). Tripartite leader–containing adenovirus mRNAs continue to be translated late after infection because the 40S ribosome can scan from cap to AUG by a process termed *ribosome jumping* (454), without the need for a helicase as eIF-4F activity becomes limiting. In contrast, most cellular mRNAs are no longer translated in the absence of eIF-4F because they require the helicase to permit scanning through the more extensive secondary structure at their 5' ends.

Finally, there is a selective activation of late viral protein synthesis by the 100-kd protein encoded by the L4 family of late mRNAs (164). A mutant virus expressing defective L4 100-kd protein fails to translate its late mRNAs efficiently, but it is nevertheless able to block host cell translation. The L4 100-kd protein can bind to mRNA (2), suggesting it may function at the polysome to facilitate translation.

Virus Assembly and Release from the Cell

The replication of viral DNA, coupled with the production of large quantities of the adenovirus structural polypeptides, sets the stage for virus assembly. Trimeric hexon capsomeres are rapidly assembled from monomers after their synthesis in the cytoplasm (180). Assembly of the hexon requires the participation of a second late viral protein (the L4 100-kd protein), the same protein that stimulates late viral translation. Biochemical experiments indicate that a multimeric complex of the L4 100-kd protein binds to hexon monomers (47), and genetic analyses have demonstrated that mutations in the L4 100-kd protein can block assembly of the hexon capsomere (237,303). Apparently, the L4-coded protein acts as a scaffold to facilitate assembly of trimers, but the mechanism underlying the process is unknown. Penton capsomeres consisting of a pentameric penton base and trimeric fiber assemble somewhat more slowly in the cytoplasm (180). Pulse-chase experiments indicate that the penton base and fiber assemble independently and subsequently join to form a complete penton capsomere (180,412). After their production, hexon and penton capsomeres accumulate in the nucleus, where assembly of the virion occurs.

Mutations within a variety of viral genes can interfere with the assembly process. As would be expected, alterations in structural polypeptides that comprise the hexon or penton base can prevent accumulation of mature virions as well as subassemblies, such as empty capsids (87,97,336). Alterations in the polypeptide forming the fiber, polypeptide IIIa, or in the L1 52/55-kd protein can prevent assembly of virions and lead to the accumulation of incomplete capsid-like particles (57,86,87,97,336). Finally, a mutant virus with a defective L3-coded protease accumulates noninfectious virion-like particles with a set of unprocessed polypeptides (420).

Studies of mutant viruses, combined with analysis of the kinetics with which polypeptides are incorporated into capsids and mature virions, have provided a rough outline of the adenovirus assembly process. Assembly appears to begin with the formation of an empty capsid (309,377). Subsequently, a viral DNA molecule enters the capsid. The DNA–capsid recognition event is mediated by the packaging sequence, a cis-acting DNA element that is centered about 260 bp from the left end of the viral chromosome (135,155,165,390). Several cellular proteins have been reported to bind at the packaging sequence (343), but their role in the interaction between DNA and capsid is unclear. The packaging element can function only when it is positioned near an end of the viral chromosome, raising the possibility that proteins interacting at the terminal replication origin might contribute to its function. This could explain the ability of mutations in the virus-coded DNA polymerase to interfere with assembly (87). No empty capsids accumulate in cells infected with a mutant adenovirus containing a partially defective packaging sequence, even though all of the known constituents of the capsid accumulate (160,166). This suggests that capsid assembly does not occur spontaneously in the absence of an interacting DNA; rather, it is probably initiated in association with a viral DNA, an association mediated by the viral packaging sequence. Encapsidation of the chromosome is polar, beginning with the left end of the viral DNA (390), as one might anticipate given the location of the packaging sequence. The mechanism by which the viral DNA enters the capsid is controversial (87,160,422). Some experiments favor the possibility that viral DNA replication and encapsidation are coupled; that is, that the viral DNA enters the capsid as it is replicated. Other work suggests that ongoing viral DNA replication is not required and that replication and encapsidation occur in separate nuclear compartments. The L1 52/55-kd protein, which is composed of two differentially phosphorylated forms of a 48-kd precursor polypeptide, facilitates the encapsidation process (148,160,161). The L3-coded proteinase, a cysteine proteinase that requires DNA and a fragment of another viral polypeptide as cofactors (89,258,391,424), functions late in the assembly process. It cleaves at least four virion constituents, generating the mature VI, VII, and VIII and terminal protein moieties. These cleavages stabilize the particle and render it infectious.

There appear to be at least two viral systems that facilitate the escape and spread of progeny virus. The first involves disruption of intermediate filaments, which are components of the cytoskeleton. Vimentin is cleaved rapidly after infection, reportedly both by a reaction initiated by the adsorption process that does not require viral gene expression (24) and in response to the E1B 19-kd protein (431,432). As a result, the extended vimentin system collapses into the perinuclear region (80,460). Late in the infectious cycle, the L3 proteinase cleaves the cellular cytokeratin K18 (61). The cleavage event occurs at amino acid 74 of the cytokeratin, creating a “headless” protein that is not able to polymerize and form filaments; rather, it accumulates in cytoplasmic clumps. A normal intermediate filament system helps to maintain the mechanical integrity of cells, and perturbations to the network would be expected to make the infected cell more susceptible to lysis. The second system that facilitates the release of progeny virions involves the E3 11.6-kd protein. This protein, which is also referred to as the *adenovirus death protein,* kills cells as it accumulates during the late stage of infection (394). The mechanism by which it kills is not known.

INTERACTIONS WITH THE HOST

Adenovirus can maintain a long-term association with its human host, persisting for years after an initial infection. Adenovirus DNA has been identified by *in situ* hybridization in 1 in 10^4 to 10^6 peripheral lymphocytes of healthy individuals (123), and lymphocytes could be the source of virus in the original isolations of adenoviruses from adenoid tissue. Thus, adenovirus probably persists in lymphocytes. The virus encodes three gene products that should facilitate persistence by antagonizing antiviral responses of the host: E1A proteins and VA RNAs, which inhibit the cellular response to interferon-a and -b; and E3-coded products that protect infected cells from killing by CTLs and tumor necrosis factor (TNF). It is intriguing to note that these protective viral products are expressed from the three adenovirus genes most likely to be expressed in lymphocytes in the absence of active viral replication. The E1A promoter and the polymerase III–transcribed VA RNAs are constitutively expressed in all cells that have been tested, and the E3 promoter is unique among adenovirus control elements in that it contains several binding sites for the lymphoid-specific NFkB transcription factor, facilitating constitutive expression in lymphoid cells (438). Additionally, it appears that these protective viral genes might be further induced in response to the production of interleukin-6 (IL-6), a cytokine that is induced at the site of adenovirus infection in experimental animals (124), as a host antiviral measure. Transcription of the E2 gene is strongly activated by IL-6 through the cellular transcription factor NF–IL-6 (364); the resulting E2-coded DNA-binding protein, in turn, can activate the E1A and E3 promoters (53). Thus, the production of a cytokine by the host

as a protective response likely induces a viral countermeasure.

Viral Antagonists of Interferon-a and -b

The adenovirus E1A protein and virus-associated (VA) RNAs both afford protection from interferon-a and -b. E1A proteins inhibit the antiviral affect of interferon (7) by blocking the activation of interferon response genes (150,321). At least part of this effect results from the ability of E1A to bind to STAT1 (247) and its coactivator p300/CPB (see Activation of the Host Cell) and inhibit their activity. STAT1 is a transcription factor that mediates activation of interferon-responsive genes.

The small, abundant VA RNAs were named when their viral origin was still uncertain (322). Different adenovirus serotypes have been reported to encode one or two VA RNAs; Ad2 and Ad5 encode two species, termed VA RNA_I and VA RNA_{II}. The RNAs are each about 160 nucleotides in length, are GC rich, adopt stable secondary structures that are important for their function, and are transcribed by RNA polymerase III (112,147,427). VA RNA synthesis begins during the early phase of the infectious cycle and dramatically accelerates during the late phase. VA RNA_I accumulates to about 10^8 molecules per infected HeLa cell, roughly the abundance of ribosomes, and VA RNA_{II} reaches about 10^7 molecules per cell.

The first hint to the function of VA RNAs came from analysis of a mutant Ad5 virus in which the VA RNA_I gene was deleted (387). The virus grew poorly, and its defect was traced to inefficient protein synthesis during the late phase of infection. Additional work identified a defect in polypeptide chain initiation (323,345) resulting from phosphorylation of the eukaryotic initiation factor-2 (eIF-2) (323,344).

The eIF-2, which includes three subunits (a, b, and g), is central to one of the best-studied pathways of translational control. Early in the initiation process, eIF-2 binds to guanosine triphosphate (GTP) and the initiator transfer RNA (tRNA) to form a ternary complex, which then interacts with the 40S ribosomal subunit. Subsequent steps in the initiation process involving additional factors result in the binding of mRNA and the 60S ribosomal subunit. As the round of initiation is completed, eIF-2 leaves the ribosome, lacking the initiator tRNA and complexed with guanosine diphosphate (GDP) instead of GTP. For eIF-2–GDP to participate in another round of initiation, its GDP must be replaced with GTP in a reaction catalyzed by eIF-2B (also termed *guanosine nucleotide exchange factor*). The exchange reaction is inhibited by phosphorylation of the a subunit of eIF-2. The eIF-2a(P) forms a tight complex with eIF-2B, preventing it from cycling and catalyzing the exchange reaction. As a result, initiation is brought to a halt when about one third to one half of eIF-2 is phosphorylated, trapping all available eIF-2B. The degree of eIF-2a phosphorylation is greatly increased during the late phase of infection with the VA RNA_I–negative mutant (301), and protein synthesis in extracts of these infected cells can be restored to normal levels by addition of eIF-2 or eIF-2B (323,344). Further, the mutant phenotype is abrogated in cells that contain mutant forms of the eIF-2a subunit (77) or lack the eIF-2a kinase (301,344). Thus, the reduction in late translation results from the phosphorylation and inactivation of eIF-2.

Phosphorylation of eIF-2 is mediated by PKR. Synthesis of an inactive form of PKR is induced by interferon, and the latent enzyme is activated by double-stranded RNA. PKR is activated by autophosphorylation, which appears to require that two molecules of the enzyme interact with a molecule of double-stranded RNA (213). Activated PKR can phosphorylate the eIF-2a subunit; this leads to sequestration of eIF-2B and the cessation of translation. VA RNA_I can bind to PKR (203,269) and block its activation (208,300). The current structural model for VA RNA_I (Fig. 9A) suggests that it consists of two extended duplex domains bracketing a central domain that is composed of several stem-loop structures. The central domain includes two complementary tetranucleotides that are conserved in VA RNAs encoded by a variety of adenovirus serotypes (251,253), and mutational analysis indicates that the conserved tetranucleotide pair is essential for VA RNA_I structure and function (252).

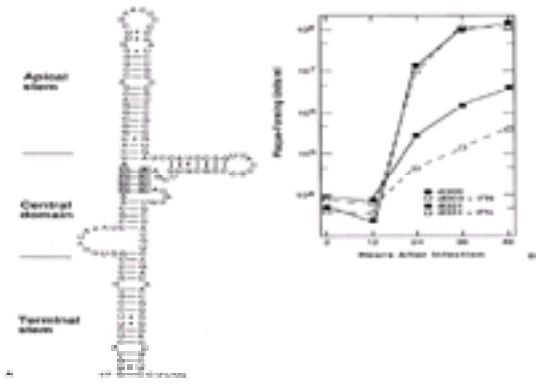

FIG. 9. Adenovirus VA RNA structure and function. **A:** Current model for the secondary structure of Ad2 VA RNA_1. The molecule is divided into three domains by convention: apical stem, central domain, and terminal stem. The two tetranucleotides in the central domain that are conserved among different adenovirus serotypes are identified (*shading*). **B:** VA RNA_1 antagonizes the antiviral effects of a-interferon. *Cell*s were infected with a virus that contained a wild-type VA RNA_1 gene (*d1309, circles*) or a mutant with a deletion blocking expression of VA RNA_1 (*d1331, squares*), and either treated (*open symbols*) or not treated (*solid symbols*) with a-interferon. The adenovirus with the wild-type VA RNA_1 gene was resistant to a-interferon, whereas the mutant virus was inhibited. (**A** modified from ref. 252, with permission; **B** from ref. 208, with permission.)

In an adenovirus-infected cell, PKR is probably activated by double-stranded RNA produced as a result of the symmetric

transcription of the viral chromosome (260,301). Activation of endogenous latent kinase is likely responsible for the inhibition of translation observed in cells infected with VA RNA $_{I}$–negative mutants (264). The level of latent PKR is induced by a factor of 5 to 10 in response to interferon. Thus, one might anticipate that VA RNA $_{I}$ antagonizes the antiviral effect of interferon by blocking activation of PKR, and this has proved to be the case (208). Whereas interferon-a inhibits the growth of VA RNA$_{I}$–negative virus, it has no effect on wild-type Ad5 (see Fig. 9B). In summary, VA RNA$_{I}$ blocks activation of latent PKR, and this function would seem critical for optimal viral growth in an infected host capable of responding to viral infection by the production of interferon.

In contrast to VA RNA$_{I}$, VA RNA$_{II}$ exhibits limited ability to block PKR activation. VA RNA $_{II}$ binds to RNA helicase A and NF90, a component of the nuclear factor of activated T cells (234). It is not yet understood how these interactions might promote viral replication.

Viral Antagonists of Cytotoxic T Lymphocytes and Tumor Necrosis Factor-a

Upon intranasal inoculation of cotton rats (*Sigmodon hispidus*) or mice with Ad5, viral gene expression is detected in the epithelial cells of the bronchi and bronchioles of the lung and nasal mucosa. The pulmonary histopathology is similar to that observed in humans (126), and disease proceeds in two stages (314). The earlier stage consists of a mild-to-moderate damage to bronchiolar epithelial cells and a diffuse cellular infiltration of peribronchiolar and alveolar regions. At this time, TNF-a, IL-1, and IL-6 appear in infected lung tissue (124). The later stage of the disease consists almost entirely of an infiltration of lymphocytes. A normal early phase ensues, but little late response is observed when athymic nude mice are infected (124), consistent with the interpretation that the late phase results from infiltration of virus-specific CTLs. Thus, the two stages of pathogenesis appear to result from an initial nonspecific host response to viral infection that includes synthesis of cytokines followed by an immunologically specific CTL response. Adenovirus E3-coded proteins, which are not needed for efficient viral growth in cultured cells, combat at least a portion of these host antiviral measures, inhibiting cytolysis of infected cells by CTLs or TNF-a.

Recognition and lysis of a virus-infected cell by CTLs requires that viral peptide antigens be displayed on the infected cell's surface in a complex with a class I MHC antigen. Ad2- or Ad5-infected cells expressing the E3-coded 19-kd glycoprotein (E3 gp19-kd) are considerably less sensitive to CTL-mediated lysis than cells infected with mutant viruses unable to express the E3 protein (8,42). E3gp 19-kd is a transmembrane protein of 142 amino acids, after removal of its amino-terminal signal sequence. It resides in the membrane of the endoplasmic reticulum, where it is held through the action of a retention signal that has been mapped to its C-terminal domain (305). The luminal domain of E3gp 19-kd binds directly to the peptide-binding domain of class I MHC antigens (42,220), and this interaction retains class I antigen in the endoplasmic reticulum through the di-lysine retention signal in the E3 protein. E3 gp19-kd also binds to TAP (28) and prevents it from transferring peptides processed in the cytosol to class I antigen in the endoplasmic reticulum. A reduced level of class I antigen on the cell surface and a block to TAP-mediated loading of class I antigen should protect against premature lysis of the infected cell by CTLs. Such protection can be inferred from experiments with cotton rats (126), but not mice (363). Pulmonary infection of cotton rats with a mutant virus, unable to express E3gp 19-kd, induced a markedly enhanced late phase inflammatory response (126). Thus, the E3 protein apparently ameliorates the late-phase response in cotton rats by protecting against lysis by CTLs.

CTLs carry the Fas ligand on their cell surface and induce apoptosis when the Fas ligand interacts with the Fas receptor (CD95) on a target cell. Products of the E3 region block Fas-mediated apoptosis. The membrane-bound E3 14.4-kd–E3 10.4-kd protein complex mediates internalization and degradation of the Fas receptor (98,355,393). Further, the E3 14.7-kd protein interacts with FLICE, a caspase acting downstream of the Fas receptor, and inhibits Fas ligand-induced apoptosis (60).

TNF-a is a cytokine that is secreted by activated macrophages and lymphocytes. It exerts cytotoxic or cytostatic effects on many tumor cells, and it can induce apoptosis and the subsequent lysis of cells infected with some viruses. Whereas uninfected cells or cells infected with wild-type adenovirus are not affected, cells infected with mutant adenoviruses lacking the E3 coding region are lysed by TNF-a (129). The CR1 domain of the E1A proteins is responsible for inducing susceptibility to TNF-a (95). This is the same E1A domain that has been reported to antagonize the activation of interferon-responsive genes, and the mapping does not precisely correlate with the binding requirements for any known E1A target. Either the E3 14.7-kd protein (129) or the E3 14.5-kd–E3 10.4-kd protein complex can prevent cytolysis by TNF-a (130). Both inhibit the activation of cytosolic phospholipase A2, which acts downstream of the TNF-a receptor (88,216,465). As noted previously, the E3 14.7-kd protein interacts with FLICE (caspase 8) (60). FLICE acts downstream of both the FAS and TNF receptors, and this interaction likely accounts for part of the mechanism by which the E3 14.7-kd protein blocks the induction of apoptosis by both receptors. The E3 14.7-kd protein also binds to a set of proteins termed FIP-1, -2, and -3 (231,232 and 233). When overexpressed, FIP-3 appears to activate a cell death pathway and to block an NFkB-mediated survival pathway (231). The interaction of the E3 14.7-kd protein with FIP-3 presumably blocks both of these pro-apoptotic activities.

In addition to its effect on the Fas receptor and the response to TNF-a, the E3 14.5-kd–E3 10.4-kd protein complex reduces the level of epidermal growth factor receptor on the surface of infected cells by inducing its internalization (44,393). It is not yet clear whether the internalization stimulates an epidermal growth factor–like response or abrogates function of the receptor.

ONCOGENESIS

All human adenoviruses that have been tested are able to transform cultured rodent cells to an oncogenic phenotype. The transformants undergo morphologic changes, and they tend to grow as dense, multilayered colonies. They can display various phenotypic hallmarks of oncogenic transformation, including growth in reduced serum, anchorage-independent growth, and growth to higher densities than normal. Only a subset of adenovirus serotypes can directly induce the formation of tumors within rats and hamsters (see Table 1). Group A adenoviruses (e.g., Ad12, Ad18) are highly oncogenic, producing tumors in most animals within 4 months; group B viruses (e.g., Ad3, Ad7) are weakly

oncogenic, inducing tumors within 4 to 18 months; at least one group D virus (Ad9) is oncogenic, efficiently inducing mammary tumors within 3 to 5 months; and group C and E viruses are not known to be tumorigenic.

Adenoviruses transform rodent cells much more efficiently than human cells, and the oncogenic adenoviruses induce tumors in rodents, whereas extensive screens have failed to correlate the presence of adenoviruses with human cancer. Initially, it seemed that the more efficient oncogenic transformation of rodent than human cells might result from the fact that rodent cells are generally less permissive for growth of human adenoviruses, and, as a result, they could more readily survive an infection and yield transformants. However, cloned E1A and E1B genes exhibit the same host cell preferences for transformation as does whole virus, arguing that differences in permissivity to viral growth are not a primary determining factor in transformation efficiency. There is a fundamental difference in the biology of rodent and human somatic cells that likely influences their response to adenovirus oncoproteins. Murine cells express telomerase and have longer telomeres than their human counterparts, which lack telomerase activity. It has recently been shown that the catalytic subunit of telomerase can cooperate with SV40 T antigen plus an oncogenic H-ras protein to transform efficiently human fibroblasts to a tumorigenic phenotype (152). T antigen plus oncogenic H-ras do not transform human cells in the absence of telomerase. This experiment suggests that the lack of telomerase expression in somatic cells is, at least in part, responsible for the block to adenovirus oncogenicity in humans. It is also possible that the immune response to adenovirus transformants (see Tumorigenesis) is fundamentally different in rodents and humans.

Transformation

Three lines of evidence initially demonstrated that the E1A and E1B genes mediate transformation. First, virus-transformed cells always contain and express these genes; second, transfection of cultured cells with cloned E1A and E1B genes leads to transformation; and, third, mutant viruses with alterations to these genes can be defective for transformation. As discussed previously, the E1A and E1B proteins manipulate cellular growth regulation. In a lytically infected human cell, E1A proteins induce quiescent cells to enter the S phase of the cell cycle, and E1B proteins prevent p53-mediated G_1 phase arrest and block the onset of apoptosis, creating an environment optimally conducive to viral replication. In the context of a rodent cell, these same events lead to oncogenic transformation.

One can construct a model for how the E1A and E1B 55-kd oncoproteins deregulate the G_0- to G_1- to S-phase cell cycle checkpoint through their interactions with pRB family members, p300 and p53. However, although E1A proteins have been shown to drive cells past the G_2- to M-phase checkpoint into mitosis, the mechanism by which this checkpoint is antagonized remains a mystery. Mutations in the E1A proteins that interfere with the binding of p300 or pRB family members can block the ability of E1A proteins to induce mitosis (182). Perhaps these cellular proteins, either in their normal complexes or as a result of their interaction with E1A proteins, influence both the G_0- to G_1- to S-phase and the G_2- to M-phase cell cycle checkpoints. Alternatively, the E1A proteins interact with additional, as yet unidentified proteins that control the G_2- to M-phase checkpoint.

Either the E4orf6 (276,286) or the E4orf3 protein (288) can substitute for E1B and cooperate with E1A to transform cells. Further, both of these proteins can enhance the tumorigenic potential of cells transformed with the E1A plus E1B oncogenes. E4orf6 inhibits p53 function and cooperates with the E1B 55-kd protein to reduce the half life of p53 (see Activation of the Host Cell). The ability to neutralize p53 probably accounts for the oncogenic activity of E4orf6. E4orf3 and E1A localize to and cause a morphologic rearrangement of POD/ND10 structures (46,94). The function of these intranuclear structures and their possible relationship to the oncogenic activity of E4orf3 are unknown.

Tumorigenesis

Rat cells transformed by the highly oncogenic Ad12 generally produce tumors in newborn syngeneic rats, whereas most cells transformed by the nononcogenic adenoviruses, such as Ad2 or Ad5, are not tumorigenic. However, some Ad2-transformed rat cell lines can induce tumors in immunosuppressed rats (118), suggesting that cells transformed by nononcogenic adenoviruses are rejected by the host's immune system. Newborn hamsters, which have not yet developed a thymus-dependent immune response, are also susceptible to tumor production by Ad2-transformed cells. Although thymectomized hamsters remain susceptible, normal animals become resistant to tumor challenge at the age of about 3 weeks when their thymic response has developed (71), suggesting that the thymus-dependent cellular immune system plays a key role in the rejection.

The thymus-dependent CTL response leads to lysis of transformed cells. As mentioned earlier, CTLs target tumor cells by recognizing foreign antigens displayed on their cell surface in the context of class I MHC antigens. In the case of Ad5 transformed cells, the E1A protein itself serves as a cell surface target (25), and several specific E1A-encoded epitopes have been identified that serve as targets of class I–restricted CTLs (202,332).

The Ad12 E1A proteins inhibit the CTL response that blocks tumor formation by cells transformed with nononcogenic adenoviruses (35). Ad12-transformed cells contain reduced quantities of class I MHC antigen on their surface (346), and, after introduction of a class I gene MHC that cannot be down-regulated by the Ad12 E1A proteins, Ad12 transformants are unable to induce tumors (383). Thus, the Ad12 E1A proteins repress expression of the class I antigen, and this prevents recognition by CTLs. The repression occurs at the level of transcription, and it appears that the activity of at least two factors that interact with the class I gene control region are altered (217,267,294).

Not all Ad12 transformants have proved resistant to CTL lysis (271), and some Ad12 transformed lines have become more tumorigenic (i.e., fewer cells can induce a tumor) when they were modified to express higher levels of class I antigen (361). Thus, although Ad12 E1A proteins reduce class I MHC levels in some transformed cells, facilitating escape from class I–restricted CTL-mediated destruction, the correlation is not reliable. This indicates that there are additional immune mechanisms that govern adenovirus tumor formation.

Many Ad2 transformed cells are markedly less efficient than Ad12 transformants in the formation of tumors in athymic

nude mice that lack class I–restricted CTLs. Nude mice possess natural killer (NK) cells, a class of immunologically nonspecific cytotoxic lymphocytes. Ad2 transformants are more susceptible to NK cell–mediated lysis than their Ad12 counterparts (70,317). Susceptibility to NK killing is governed by the E1A gene (72,73,340); addition of the Ad5 E1A gene to a highly tumorigenic sarcoma cell line induced susceptibility to lysis by NK cells, blocking its ability to form tumors (416).

Non-E1A–mediated mechanisms can also influence tumorigenesis. The E3 19-kd glycoprotein can influence class I MHC levels (see Viral Antagonists of Cytotoxic T Lymphocytes and Tumor Necrosis Factor-a). Thus, the E3 gene might also contribute to the differential CTL sensitivity of Ad12 versus Ad2 or Ad5 transformants. Although the E3 gene is often present in Ad12 transformants, it is generally not present in Ad2 or Ad5 transformed cells. Although there are exceptions, most rodent cells transformed with Ad2 or Ad5 contain only portions of the viral genome; only the E1A and E1B genes are consistently present. In a similar vein, additional E3-coded proteins, if present, might afford protection from TNF-a. The notion that additional genes play auxiliary roles in tumorigenesis is consistent with the observation that substitution of the Ad12 E1A or E1B genes into the corresponding Ad5 domain did not equip the resulting hybrid viruses to induce tumors in newborn rats or hamsters (34,340).

In contrast to group A adenoviruses such as Ad12, which induces tumors at the site of injection, Ad9, a group D virus, induces mammary tumors, irrespective of the site of injection. Subcutaneous injection of newborn Wistar-Furth rats produces mammary tumors in females but not males (11). The tumors are predominantly benign fibroadenomas, but phyllodes-like tumors and malignant sarcomas are also produced; all of the tumors are estrogen dependent (10,193). When hybrid viruses were constructed between Ad9 and a closely related group D virus that fails to induce mammary tumors, the Ad9 E4 gene was found to be required for mammary tumorigenesis (194). Remarkably, the E1A and E1B genes are dispensable for tumor induction (388). The Ad9 E4orf1 protein is responsible for Ad9 tumorigenesis. It is able to transform cultured cells efficiently to an oncogenic phenotype (195), and E4orf1 from group A and C adenoviruses can also transform cells if overexpressed (428). The 12.5-kd E4orf1 protein is localized predominantly to the cytoplasm (429), and it binds to a PDZ domain–containing protein, hD1g/SAP97 (225), a human homolog of the *Drosophila* discs large tumor suppressor protein. The carboxy-terminal domain of E4orf1 that is required for oncogenesis is also required for hD1g/SAP97 binding, suggesting that E4orf1 might act through this protein to mediate its oncogenic activity.

PERSPECTIVES

Much is known about the adenovirus growth cycle; indeed, the advances in our understanding during the 5 years since I first wrote this chapter are remarkable. An adenovirus host cell receptor has been identified, the protective (from the virus's point of view) roles of E3 proteins have been clarified, and the oncogenic potential of E4 proteins has been discovered. Yet, much remains a mystery, including the structure of the viral core both within the virion and as viral DNA is transcribed, the mechanism by which viral DNA is encapsidated, and the basis for host restrictions and tissue tropisms in infected organisms. There is no doubt that these and many other questions will be resolved in the near future.

Adenoviruses have brought much more to the table than a system of viral replication; they have also contributed to a molecular understanding of many fundamental cellular processes. For example, studies of adenoviruses have yielded important insights to the complex mechanisms by which cells express and process mRNAs and control their growth. Early adenovirus gene products have helped to identify many important cell cycle regulatory proteins, and they continue to serve as probes with which to manipulate these cellular factors and reveal their normal functions.

Adenoviruses no longer serve simply as systems for study. As discussed in Chapter 68, they are now being developed as vectors for gene therapy. Indeed, adenovirus vectors have been created that carry no viral genes, and it is becoming possible to modify the tropism of vectors by directing the virus to proteins on the cell surface other than its natural receptor. If the virus can be tamed so that it delivers and expresses therapeutic genes without attendant pathology, we will undoubtedly find ourselves “sleeping with the enemy” in the near future.

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CHAPTER 68 Adenoviruses

Marshall S. Horwitz

HISTORY

During attempts to establish tissue culture lines from tonsils and adenoidal tissue surgically removed from children, Rowe and colleagues (224) recognized that a transmissible agent was causing degeneration of the epithelial-like cells. Thus, adenoviruses (Ads) were first cultured and reported as distinct viral agents in 1953. Similar viral agents were isolated from febrile military personnel with a variety of respiratory illnesses (114,124). It was soon realized that there were multiple serotypes of these new agents and that they were antigenically related by a group-specific complement fixation antigen. These viruses were first called *adenoid degeneration* (AD), *adenoid-pharyngeal conjunctival* (APC), and *acute respiratory disease* (ARD) agents. The current nomenclature of adenoviruses was subsequently adopted in 1956 (59).

Epidemiologic studies initiated by the Commission of Acute Respiratory Diseases of the U.S. Armed Forces confirmed that the adenoviruses were the cause of a large number of acute febrile respiratory syndromes among military recruits (37,50). In retrospect, acute respiratory disease syndromes caused by adenoviruses had been defined for perhaps 100 years before their association with this group of viruses (50). In addition, adenoviruses were isolated from outbreaks of epidemic keratoconjunctivitis (EKC), which had been described previously as a distinct clinical entity by German clinicians in the 1920s (118,133). As the number of adenovirus serotypes increased to the current 51 human types, additional clinical syndromes such as acute hemorrhagic cystitis, hepatitis, gastroenteritis, and myocarditis were associated with these agents (46,47,112,198,226). In 1962, it was demonstrated that Ad12 could cause tumors in rodents; this was the first description of a pathogenic human virus that could induce malignant tumors in animals (251). The oncogenic potential of the adenoviruses propelled this group of agents into the forefront of molecular biologic research, which has produced a detailed understanding of many aspects of both the lytic and transforming cycles. However, at this time, the adenoviruses have not been associated convincingly with any malignancies in humans (93). The clinical manifestations and epidemiology of this large family of human viruses are quite varied and will be discussed in this chapter.

The molecular biology of the adenoviruses has been extensively studied both during the lytic cycle in cells of human origin and during transformation in rodent cells (see Chapter 67). Molecular biology has contributed greatly to our understanding of intracellular events that could explain some of the pathologic lesions observed during infection (209,235,253). In addition, the tools of molecular biology are being increasingly applied to the diagnosis of adenovirus infections. Clinical isolates and various viral variants that occur under natural conditions have been analyzed by restriction endonuclease digestions of the genome as well as by DNA sequencing (71,104). Molecular footprints can (a) trace epidemiologic associations between clinical specimens, (b) assist in the characterization of new isolates, and (c) aid in the evaluation of variations in the structure of previously described serotypes. Polymerase chain reaction (PCR) to amplify viral DNA and nucleic acid hybridization can be rapidly applied to tissues and excretions from patients so that inactivated viral specimens that are no longer viable for growth in tissue culture can still be characterized. This has been most important for the characterization of adenoviruses in formalinized tissue fixed for histology and in the recent association of adenovirus infection with myocarditis (158,166,254). Adenovirus vectors are being used extensively for the delivery of cloned DNA into cells or into animals, including humans. There is an intensive effort by many investigators to introduce genes for therapy of various genetic diseases as well as malignancies and vascular occlusive disease using

adenovirus vectors (76,167,179,219,228,247). In parallel efforts, genes that code for epitopes that might induce immunity to various infectious diseases are being introduced with the help of adenovirus vectors (32; reviewed in 160).

INFECTIOUS AGENT

Propagation and Assay in Cell Culture

Growth of adenoviruses from patient material is best achieved in cells of human origin (11). Primary human embryonic kidney (HEK) cells are probably the best host for the replication of the entire range of human adenoviruses (153). However, HEK cells are expensive and may be contaminated with the adeno-associated virus (AAV). The A549 cell line, which was derived from a human lung carcinoma, is also a good host for most of the human adenoviruses, but some of the ocular strains may not grow well on these cells. The continuous epithelial lines, such as HEP-2, HeLa, and KB, are also highly sensitive but are difficult to maintain for the long period of time (28 days) that may be required to isolate some of the ocular adenovirus strains, such as Ad8 and Ad19 (91). However, adenovirus detection in epithelial cells has been greatly accelerated by centrifugation of viral specimens directly onto the tissue culture cells (HEP-2), using the shell vial technique. This approach has yielded positive cultures in 1 to 2 days for most of those eventually positive by conventional culture techniques (60,151). After growing for 1 to 2 days, the tissue culture cells were tested with monoclonal antibodies against the group-reactive epitope on the hexon. Human fibroblast lines are easier to maintain for long periods of time without media changes, but they are less sensitive than primary HEK cells or continuous epithelial lines.

The 293 cell line appears to be a good host for some strains of the two enteric adenoviruses, EAd40 and EAd41 (244). The sensitivity of this transformed line appears to offer the clinical laboratory a convenient host system for the growth of fastidious adenoviruses that are pathogenic in the intestinal tract (230,244). The 293 cell, which is a primary HEK transformed to a continuous line by Ad5 (88), retains the E1A and E1B regions of the adenovirus genome covalently linked to the host DNA (see Chapter 67). Infectious adenovirus cannot be produced from the endogenous sequences alone. The mechanism of facilitation of the growth of the EAd40 in 293 cells appears to be a function of the E1B-55 kd protein (169).

Primary monkey cell lines have been used to isolate human adenoviruses. However, there is a large amount of accumulated evidence that most adenoviruses grow poorly in these cells unless co-infected with the papovavirus, simian virus 40 (SV40) (10). The block in adenovirus replication is late in the viral replicative cycle after the synthesis of viral DNA, and the helper function of SV40 is supplied by the carboxyl-terminal part of the T-antigen polypeptide (10,63,283). Intramolecular hybrids between adenoviruses and SV40 have formed under conditions in which SV40 was an endogenous infection of the monkey kidney cells used for adenovirus isolation (222). Rhesus or other simian primary kidney cells are more easily maintained, require less feeding, and are less expensive to purchase. Therefore, primary monkey kidney cells are in widespread clinical use to isolate adenoviruses, even though such practices are not optimum.

Biologic Characteristics

Adenovirus cytopathogenic effect (CPE) consists of rounding and grapelike clustering of the swollen infected cells (144) (Fig. 1). Initial CPE may start at the periphery of the monolayer before generalizing. However, some of the group B adenoviruses do not cause cell enlargement or clustering. Adenoviruses increase glycolysis in continuous cell lines and thereby induce the cells to produce large quantities of acid (64). In some laboratories, the color changes of indicators caused by the lowered pH in the medium have replaced microscopic examination for CPE for some adenovirus culture work, such as viral neutralization tests. Rapid cytopathology can be induced within several hours of inoculating concentrated crude virus preparations and is not related to viral replication. The rounding and detaching of cells in such cases is caused by the penton base component of the free viral penton capsomere that is made in far greater quantities than needed for viral assembly; however, these cytopathic effects do not result in cell death. The penton base–induced cell detachment domain has been mapped to the integrin-binding RGD sequence (6).

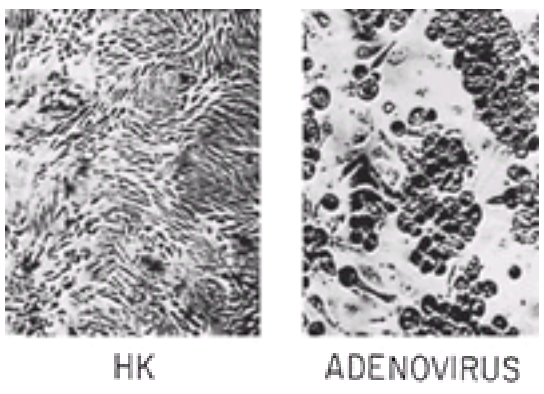

FIG. 1. Cytopathic effect induced by an adenovirus in human kidney (*HK*) tissue culture cells. Control monolayer (HK). Adenovirus-infected culture showing rounding, ballooning, and clustering of cells. (From ref. 119, with permission.)

Adenoviruses assemble in the nucleus of infected cells, and the nuclear morphologic changes can be used for diagnostic purposes. However, the viral proteins are synthesized in the cytoplasm, where some of them accumulate, and they can also be recognized by immunologic techniques. The nuclear changes after Ad1, Ad2, and Ad5 (group C) infection include overall enlargement and intranuclear inclusions that initially are Feulgen negative and eosinophilic but become Feulgen positive and basophilic as the infection progresses (27) (Fig. 2A and Fig. B). The Feulgen-positive inclusions contain adenovirions by electron microscopy (EM) (see Fig. 2C), and it is the DNA within the viruses that contributes to the

Feulgen stain. In addition, there are paracrystalline aggregates that contain viral protein without nucleic acid (180). The group B viruses also cause nuclear inclusions, and infection results in rearrangement of basophilic nuclear material (see Fig. 2).

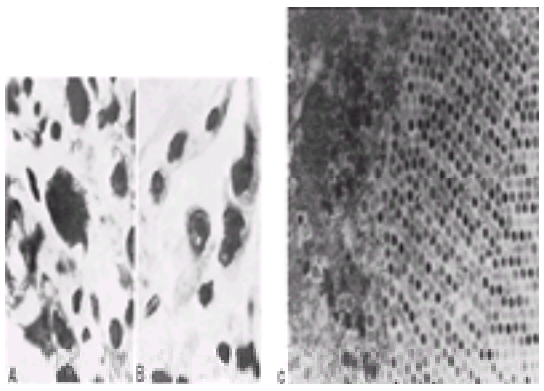

FIG. 2. Adenovirus cytopathology in human tissue. **A:** Lung tissue from a fatal case of adenovirus pneumonia showing in the center of the field a desquamated alveolar lining cell with a basophilic inclusion body filling the entire nucleus and obliterating the nuclear membrane (smudge cell). **B:** There is another desquamated cell with less-advanced cytopathology manifested by a smaller nuclear inclusion body, highlighted by a clear halo, contained within the nuclear membrane (×2800). **C:** The nuclear inclusion is full of virions, of which there may be as many as 10,000 particles per cell (EM, ×237,200). (From refs. 142 and 190, with permission.)

Although these light-microscopic cytologic changes are not absolutely pathognomonic of adenovirus infection, they are useful for the histopathologic diagnosis of biopsies, especially of lung tissue, and as an aid to tissue culture differentiation of adenoviruses from other agents. Suggestive adenovirus isolates based on the pattern of cellular degeneration must be further characterized by adenovirus-specific serologic or PCR assays for definitive identification, as described later.

Description of Key Antigens

The adenovirus proteins that provide the clinically useful antigens are the three coat proteins: hexon, penton base, and fiber. The hexon and fiber contain most of the epitopes recognized by neutralizing antibodies, but there are epitopes on penton base that are also recognized by some neutralizing antibodies (reviewed in 206a). In experimental infections in humans using replication-defective adenovirus vectors, it has been shown that the antibody responses to fiber and penton base ntigens have a synergistic effect in neutralization assays (77). The differences in neutralization of viral infectivity in tissue culture assays are the basis for the classification of distinct serotypes. The neutralization properties of polyclonal antibodies are often concordant with the inhibition of adenovirus-induced hemagglutination (HA) of selected red blood cells. However, the HA functions are the property of the fiber protein, which usually must be linked to the penton base for complete HA to occur. Because of recombination within subgroups in clinical isolates, it is not uncommon to isolate a virus that demonstrates discordant reactions in the neutralization and HA inhibition reactions. The functions of these proteins in viral entry (fiber) and in transport through the endosome membrane (penton base) and across the cytoplasm into the nucleus (hexon) are described in Chapter 67.

The cell protein receptor named CAR (coxsackie-adenovirus receptor), which is a receptor shared between these two groups of unrelated viruses and is the target of all subgroups of adenovirus fibers except for Group B, has recently been isolated (19,217). The class I major histocompatibility complex (MHC) molecule has also been proposed as a second receptor for Ad5 (119) but has not been confirmed by other groups (44,172). One of the group D adenoviruses, Ad37, appears to bind to sialic acid rather than to a protein at the cell surface (4). The penton base may be more important for the entry of some adenoviruses into hematopoietic cells (193).

There are group- and type-specific epitopes on both the hexon and fiber polypeptides. Type-specific domains have been mapped to unique sequences in loop 1 (amino acids 281 to 292) of the hexon and in loop 2 (amino acids 441 to 455) by generating neutralizing antibodies to peptides from each of these regions (248). Loops 1 and 2 had previously been shown to be on the surface of the virion by crystallography. Neutralizing antibodies to these epitopes have been found in human sera, but they do not inhibit attachment or internalization of the virus. These observations suggest that the antihexon antibody inhibits viral replication after these early steps are completed. Differences in the HA properties of rhesus and vervet erythrocytes for two important subtypes of Ad11 (Ad11p and Ad11a) have been related to nucleotide sequence differences in the shaft and knob region of the fiber. These viruses have some differences in tissue tropism in that Ad11p can be persistent in the urinary tract and Ad11a causes acute respiratory tract infections. These properties of the whole virus may be related to the changes in fiber polypeptides that are otherwise identical for 92.3% of their amino acids (173).

In addition to the type-specific neutralizing antibodies, there are group-reactive antibodies that react with domains of the hexon from all human serotypes (197). These interactions were classically measured by the complement fixation (CF) test and were useful in identifying an agent as an adenovirus. Subsequent serologic techniques, such as immunofluorescent (IF) antibody and enzyme-linked immunosorbent assay (ELISA) determinations, were also capable of detecting group reactivity shared by most of the human adenoviruses. Sequencing studies completed on adenoviruses from groups A, B, and C have confirmed that there are common sequences within the hexon protein coding domain that are extensively shared (110).

Infection of Experimental Animals

Human adenoviruses are species specific in their replication cycle, as are most adenoviruses of other mammals or avian species. There are some crosses of infectivity between closely related species, but even simian species are poor hosts for the replication of human adenoviruses. The replication of human adenoviruses in monkey cells can be achieved, but it requires co-infection with SV40 or the use of an adenovirus host range mutant (Ad5hr404, which contains a point mutation in the DNA-binding protein) for efficient replication (148). Human group C adenoviruses can replicate in the lung of cotton rats, and the pathogenesis of adenovirus pneumonia has been studied in these animals (209). Observations from this model have been reviewed in Chapter 67. An ocular model of infection with either Ad5 or Ad8 has been described in cotton rats, and the clinical manifestations, including subepithelial corneal opacities, were similar to epidemic keratoconjunctivitis (252). The animals shed virus, developed specific antibodies to the infecting virus, and even were able to spread the infection to control cotton rats. A similar ocular model was reported in New Zealand rabbits after topical or intrastromal inoculations of Ad5 (86). The virus appeared to replicate, and most of the animals developed a humoral immune response. There were findings of blepharitis, conjunctivitis, iritis, corneal edema, and subepithelial corneal infiltrates that were consistent with immune-mediated clinical disease. Other animals have demonstrated limited replication of single adenovirus serotypes but have served as neither animal reservoirs for disease nor useful models for *in vivo* studies with adenoviruses.

Adenovirus infections in a cotton rat pneumonia model, in which the E3 effects on pulmonary inflammation were studied, have been described in Chapter 67. Although human adenoviruses fail to produce virus in most other species, the virus does adsorb, penetrate, and make early proteins in a variety of cells of other animals. For example, mouse cells have been shown to support the synthesis of a variety of early adenovirus proteins, and mice have been used in pathogenesis studies, although little or no progeny virus is produced (55,85). Pulmonary pathology has been reported from the instillation of very high doses of Ad5 intranasally into mice, and the production of interleukin-1 (IL-1), IL-6, and tumor necrosis factor-a (TNF-a) has been observed in this nonreplicating pathogenesis model (85,235).

Isolated adenovirus early region 3 (E3) proteins, such as the gp19 kd, which inhibits the transport of the class I MHC to the cell surface, or the 14.7 kd, which inhibits some of the functions of TNF-a, have been studied for their potential to alter virulence of vaccinia virus in mice (94,253). The gene for Ad 14.7 kd, which was cloned into vaccinia virus together with the *TNF*-a gene (VV/TNF/14.7K), increases the virulence of the vaccinia virus carrying the TNF gene alone (VV/TNF) by reversing the antiviral effects of TNF-a. These results were obtained both in immunocompetent and in SCID mice in a pneumonia model, with death as well as inflammation as an end point (253a). The advantages of these systems are that the model includes a replicating virus, and the only permutation was the insertion of a single adenovirus gene. Other models for studying the role of single or groups of adenovirus genes in pathogenesis in mice have been developed by inserting the Ad E3 region from human Ad2 into transgenic mice (57). The Ad E3 genes placed under the control of the rat insulin promoter for targeted expression in murine pancreatic islet cells can prevent autoimmune diabetes in a virus-induced (lymphocytic choriomeningitis virus) murine model of type I diabetes or in nonobese diabetic (NOD) mice (58,261).

Human adenoviruses inoculated into a variety of rodent species cause tumors. Ad12, Ad18, and Ad31 are highly oncogenic in hamsters, and much has been learned about the mechanism of action of oncogenes from these viral models. The adenovirus genes responsible for these tumors are the *E1A* and *E1B* genes (see Chapter 67). Ad9 causes fibroadenomas and mammary sarcomas in rats, and an early region 4 gene seems to be important in this model (132). However, there is no evidence that adenoviruses are etiologically involved in human malignancies, but they are found as opportunistic infections in patients immunosuppressed by either their malignancies or chemotherapy (67).

PATHOGENESIS AND PATHOLOGY

From the interactions of adenoviruses and cells described in Chapter 67, it is possible to reconstruct some of the events that contribute to the pathologic lesions seen during the course of adenovirus infection. During the lytic cycle of many of the adenovirus serotypes, transport of host messenger RNAs (mRNAs) from the nucleus to the cytoplasm is inhibited by the action of the Ad E1B-55 kd and E4-34 kd protein complex (206,276). The inhibition of cell DNA and protein synthesis, subsequent to the interruption of the normal processing of host mRNAs, is incompatible with cell survival. The infected cell degenerates in specific ways that help the pathologist diagnose adenovirus infection on biopsy or autopsy tissue (190). The respiratory epithelial cells that are affected during adenovirus pneumonitis have enlarged nuclei containing amphophilic or basophilic inclusion bodies surrounded by thin rims of cytoplasm. Some of these cells are referred to as *smudge cells* (see Fig. 2A) and contrast with those infected cells with nuclear inclusions but intact nuclear membranes (see Fig. 2B). On occasion, these nuclear inclusions have been mistakenly identified as those of cytomegalovirus (155). However, there are no syncytia or multinucleated giant cells as found with herpesvirus infections. As in tissue culture, the epithelial cells are the primary target for adenovirus cytopathology *in vivo* (27).

One of the adenovirus structural proteins, the penton base, made in much larger excesses than needed for the assembly of the adenovirus, has been shown to bind to the a_vb_3- and a_vb_5-integrins and to cause cells to detach rapidly from the surface of monolayer tissue culture dishes (268). This effect can be demonstrated even in cells that are not directly infected with adenoviruses; it has been postulated that this protein might be involved in pathogenesis. It had initially been thought that the penton cell-rounding effect led to cell death, but it now appears that the cells are viable upon removal of the viral protein (194). Although the importance of penton in human disease has not been determined, it has been found in the blood of several fatal cases of adenovirus pneumonia (154a).

Adenovirus-induced changes at the level of single cells result in considerable organ toxicity during serious tissue invasion by adenoviruses. Necrotizing bronchitis, bronchiolitis, and interstitial pneumonia, as well as fibrin and hyaline membranes within the alveoli, characterize adenovirus pulmonary syndromes (52). Comparable lesions occur within the conjunctiva, in which exudative and mononuclear infiltrates are found. However, the lesions occur beneath the

epithelium, so that ulceration or neovascularization of the cornea usually does not occur (134,176).

Proliferative responses similar to transformation have not been recognized in acute adenovirus infections in humans. However, lymphatic tissue is often hypertrophied, and active germinal centers are found. For example, the appendices of children undergoing surgery to correct bowel obstruction found in intussusception may be associated with enlarged mesenteric lymph nodes and adenovirus isolation from stool specimens (279,280). These lymphocyte changes are often in close proximity to areas of desquamated epithelial cells in which typical viral inclusions may be found. The inclusions contain viral particles in crystalline arrays that are visualized by EM. Some of the lymphocytes are probably CD8 cytotoxic T lymphocytes (CTLs) that recognize proteolytic peptide products of viral proteins that have not been further elucidated for the human CTL response. However, in the mouse, the E1A and E1B viral polypeptide sequences have been recognized in the context of the syngeneic MHC in the plasma membrane (16,72,142,154). The potential modulation of this response by a product of the E3 region is discussed in Chapter 67.

Entry into the Host

Adenoviruses enter susceptible hosts either by the mouth, the nasopharynx, or the ocular conjunctiva. As described previously, recent experiments have defined the receptors for all the subgroups except for subgroup B as the CAR receptor (19,20,217,247a). Entry of a single group D serotype (Ad37) appears to be an exception that requires sialic acid rather than the CAR protein (4). The entry of adenoviruses into a variety of hematopoietic cells may involve the penton base rather than the fiber (193). The lower serotypes, Ad1, Ad2, Ad5, and Ad6, are ubiquitous, particularly in young children, and may be shed for months, especially in the stool, which is probably responsible for the endemic spread of these agents by the fecal-oral route to new pools of susceptible infants and children (73). An epidemic form of adenovirus keratoconjunctivitis has been spread in swimming pools (75) from contaminated water and in medical settings (135) from adenovirus-infected ophthalmologic instruments, especially tonometers (183). A third pattern of spread was unique in the military setting, occurring during the early period after induction into service. Ad4 and Ad7 caused acute respiratory distress (ARD), including pneumonia, and were the result of airborne inoculation of the respiratory tract (52,81).

Site of Primary Replication

It is clear from the original findings of adenoviruses in tonsils and adenoids that these tissues of the oropharynx were a major initial site of replication for the entering adenoviruses. The initial replication most likely occurs in the nonciliated respiratory epithelium, although some limited replication and persistence can also occur within lymphocytes (1; reviewed in 159). These conclusions are based not on careful observations of cells *in situ* but on the cells in which adenovirus replicates in tissue culture. Ciliated respiratory epithelium of the lower airway is difficult to infect with adenoviruses through the apical surfaces, which do not contain the CAR receptor; however, disruption of the integrity of cell–cell contact allows basolateral infection of such polarized epithelial cells using the CAR receptor (205,265).

Spread of Virus and Tissue Tropism in the Host

Most of the manifestations of adenovirus infection are locally in the eyes and pharynx, but contiguous extension into the lungs results in some cases. Even in the nonreplicating mouse model of human adenovirus infection, the virus can cause inflammation in the lungs, which is strong evidence for direct extension into these target organs (85). However, adenoviruses have been cultured from the blood during fatal respiratory disease caused by these agents, suggesting viremic spread in some situations (35). The successful use of oral, live, microencapsulated adenovirus vaccines by the military to prevent ARD suggests that if the respiratory tract can be physically bypassed by Ad4 and Ad7, intestinal replication of the virus causes an immunizing rather than a virulent infection (39,40). These vaccine viruses have not been intentionally attenuated by serial tissue culture passage and are believed to retain some of their initial virulence. Although most adenoviruses replicate in the intestine without causing gastroenteritis, Ad40 and Ad41 are responsible for intestinal disease. Adenovirus disease in the urinary bladder, primarily by group B adenoviruses in immunocompetent hosts, suggests that the virus probably is viremic at some stage in order to reach this organ. There is no evidence for ascending infection for these group B serotypes, which are less commonly found in the intestine and are more common in young males than in females. The route of infection of the liver, especially in immunosuppressed liver transplant recipients, is unknown, but some patients might be infected by latent adenoviruses that are present in cells such as lymphocytes in the transplant.

Immune Response

Various aspects of the immune response to this family of viruses have been described in other sections, and mechanisms that the virus uses to evade the immune response have been reviewed recently (121,163,272). Early cytokine responses (IL-1, IL-6, and TNF-a) have been described in the murine pneumonia model after nonpermissive infections with human adenoviruses (85). A biphasic mononuclear cell response in the cotton rat suggested an early appearance of monocytes and NK cells followed by specific T and B lymphocytes. The cell infiltrate was changed from a mononuclear to a polymorphonuclear leukocyte response by deletion of the adenovirus E3 anti-TNF genes (*14.7 kd, 10.4 kd/14.5 kd*). Deletion of the anti-MHC Adgp19 protein increased the intensity of the inflammatory response in the cotton rat but not in the mouse (84,235). These observations suggest that immunopathology plays a role in the host response to adenovirus infection, and this process is ameliorated by the E3 inhibitors of the host inflammatory response (83,209). However, testing the effects of the isolated adenovirus anti-TNF *14.7 kd* gene or the Ad *gp19 kd* anti-MHC gene in other animal model systems suggests that the *14.7 kd* gene can increase viral virulence in a pneumonia model and that the *gp19 kd* gene may not affect virulence in a similar model (41,94,253). Whether these adenovirus genes would affect viral persistence has not been tested. Thus, it is not clear at this point whether the cell-damaging effects of adenovirus infection or the host immune responses to the pathogen are responsible for the tissue pathology and the clinical manifestations. Despite the down-regulation of class I MHC by the Ad *gp19 kd* protein, the CTL response (in the mouse) is induced usually to a peptide from the E1A region of the virus. There is evidence that both the CD8 and CD4 responses of human cells to adenovirus infections are not serotype specific because there is cross-reactivity to targets infected by adenoviruses of different groups (70,179,234). It is important to understand these issues of pathogenesis and the immune

responses because adenoviruses are being used as vectors to deliver foreign genes into human hosts. Although most of these adenovirus vectors have been enfeebled by deletion of the E1A and E1B regions and therefore fail to grow efficiently in human cells (see Adenoviruses as Vectors for Vaccination and Gene Therapy), it is still essential to determine whether the E3 anti-MHC and anti-TNF genes should be deleted or retained in a form that would be expressed in the vectors for gene therapy.

Neutralizing and nonneutralizing antibodies are discussed in the sections, Description of Key Antigens and Viral Serology. After infection, most patients develop both group- and type-specific antibodies to the infecting adenovirus strain. Group-specific antibodies are not neutralizing of viral infectivity but are useful in measuring patient response when infection with an adenovirus is suspected but no isolate was obtained. In this case, acute and convalescent sera taken several weeks apart may demonstrate rising antibodies to a group-specific adenovirus antibody such as that measured by CF, IF, or ELISA, using a viral-infected tissue culture extract or purified hexon from a single adenovirus type. Type-specific antibodies are measured by either the neutralization test or inhibition of HA, as described in the section, Viral Serology. It is clear that patients may continue to shed adenoviruses intermittently, especially in their stools, for many months after a successful humoral immune response has occurred (73). Neutralizing antibodies may be protective against disease manifestation in the previously infected host or against reinfection with the same serotype, but they do not eliminate the carrier state.

Virulence

There is active research into the genes that can alter virulence, as discussed for the E1, E3, and E4 genes (see Chapter 67). However, it is unclear why certain serotypes characteristically cause disease in some organs and not in others. The gastroenteritis-inducing capacity of Ad40 and Ad41 is unexplained, as is the tropism of type B adenoviruses for the urinary tract or the types (Ad8, Ad19, and Ad37) that are particularly virulent in the conjunctiva. Tissue culture experiments or the existing animal models do not explain the mechanisms of such tissue tropism or organ-specific pathogenicity. Because all but the group B adenoviruses can attach to the CAR receptor, it is unlikely that this receptor can explain differences in the *in vivo* tropism of the various serotypes. Even the mechanism of attenuation of the oral vaccine strains used to prevent ARD in military personnel may depend more on physical formulation (i.e., enteric coating) of a live virus that bypasses the oropharnx and first exposes the intestinal epithelium than on mutation of virulence genes.

Persistence and Latency

Although low levels of replication-competent virus remain in tonsils, adenoids, and intestine of infected hosts, and shedding can occur for months to years (128,191,256), little is known about the mechanism of latency for adenoviruses in humans or animals. Adenovirus DNA has been detected in adult human lymphocytes, and the number of such positive samples appeared to increase with increasing age, as if latency were the end result of previous adenovirus infection (120). However, another group using PCR techniques failed to find evidence of adenovirus persistence in the lymphocytes of their study group, although they were able to isolate an Epstein-Barr–positive lymphocyte line from one patient who persistently produced adenoviruses (68,69). Carrier cultures of adenovirus-infected tissue culture cells in which the virus waxed and waned upon continued passage of the cells have been established (9). However, molecular mechanisms to maintain latency or the genes required for such a state are not known.

EPIDEMIOLOGY

Adenovirus infections occur worldwide in humans as well as in a variety of animals. With few exceptions, the human adenovirus serotypes are generally not pathogenic to animals, and animal adenoviruses are only pathogenic within the species of origin (246). However, asymptomatic infection across these species barriers has been documented by antibody determinations. Simian species occasionally have been shown to have antibodies to human Ad12 (141). Antibodies to bovine, simian, and canine adenoviruses have been detected in human sera evaluated by neutralization tests. The transmission of adenovirus infection and disease varies from sporadic to epidemic. The pattern often correlates well with the viral serotype and the age (children or adults) of the susceptible population. Because many of the adenovirus-related diseases are not clinically pathognomonic of the etiologic agent, the epidemiology of adenovirus infection is crucially dependent on the techniques used to prove the identity of the infectious agent. Therefore, the incidence and clinical spectrum of sporadic adenovirus disease were only elucidated by prospective monitoring of families, orphanages, and children's homes (260). A virus watch study was undertaken in New York and Seattle (73,74). Respiratory and fecal samples, as well as histories of disease, were collected twice a month in these latter studies. Much of the adenovirus epidemiology in civilian populations was learned from these studies, including the important information that there was a high incidence of recurrent shedding, especially of the lower serotypes, in fecal specimens. In addition, the large number of completely asymptomatic adenovirus infections was documented in such studies. Fecal-oral transmission accounts for most infections in young children. Initial spread may occur by the respiratory route, but the prolonged carriage in the intestine makes the feces a more common source during both the acute illness and intermittent recurrences of shedding (73). The epidemiologic importance of the long latency in tonsil tissue is not known. Adenoviruses probably account for 3% of the infections in civilian populations and for about 7% if only febrile illnesses are calculated (73). The corresponding figures for young children are about 5% and 10%, respectively.

Serologic surveys have furnished some estimates of the prevalence of adenovirus infections in various populations. Antibodies to Ad1, Ad2, and Ad5 are most common and are present in 40% to 60% of children (28,124,136). The incidence of antibodies to Ad3, Ad4, and Ad7 is low at the same ages. These antibody results probably explain why adults are uncommonly infected with Ad1, Ad2, and Ad5 but are more susceptible to infections with Ad3, Ad4, and Ad7. During the surveillance for the virus watch studies, it was documented that only about 75% of the adenovirus isolates were accompanied by an antibody response, as measured by the CF test (73). Some of the serologic nonresponders may be related to the insensitivity of the CF assay or the failure of some children to produce such antibodies.

The epidemic forms of adenovirus disease were studied in different ways from the sporadic endemic occurrences. The

epidemics of acute respiratory disease were well known during World War II, and this awareness preceded the isolation and the characterization of the first adenovirus by about one decade. This ARD, which occurred almost exclusively in recently assembled military recruits, was most common in winter. It did not occur in seasoned personnel in close contact with the recruits and was later identified as an Ad4 or Ad7 infection in most outbreaks (113,115,223). This disease rarely occurred in similarly congregated college students, suggesting that additional factors, such as more crowded sleeping conditions or the fatigue associated with basic training, contributed. In support of these cofactors has been the observation that ARD-causing adenoviruses did not spread to civilian personnel in contact with the military. In recruits congregated during the summer months, ARD often did not occur until the onset of colder weather in the fall. Influenza A could be distinguished because it affected both experienced and new recruits. Adenovirus-induced ARD often affected 80% of the recruits, with 20% to 40% hospitalized. The duration of infectivity was rather short; virus was not demonstrable after 4 days of illness (223). Controlled studies of routes of infectivity for the ARD-causing adenoviruses have demonstrated that aerosolized virus inhaled into the lungs of volunteers produced the disease, whereas application to the mouth, the nasal mucosa, or the intestine in enteric-coated capsules failed to produce the lower respiratory disease (39). ARD outbreaks were effectively controlled by vaccination of recruits in the first few days of military service; however, recent interruption in the supplies of adenovirus vaccine has resulted in the reappearance of the epidemic form of ARD. This is discussed further in the section on vaccines later.

The epidemiology of pharyngoconjunctival fever and keratoconjunctivitis, both of which may occur in epidemic proportions, is described later with the individual disease entities. Infection resulting in several of the adenovirus syndromes can be acquired in hospitals and can be spread as nosocomial infections. The isolation of the fastidious enteric types of adenovirus from a group of hospitalized infants on a pediatric ward demonstrates that this type may be spread within the hospital environment (277). Other adenovirus serotypes have probably been spread as nosocomial infections (12,30,260).

CLINICAL FEATURES

Adenoviruses can commonly infect and replicate at various sites of the respiratory tract as well as in the eye and gastrointestinal tract. Less frequently, adenoviruses can infect the urinary bladder and liver. On occasion, these viruses may also cause disease in other organs, such as the pancreas (196), myocardium, or central nervous system, which may be involved in meningoencephalitis complicating an adenovirus infection of the respiratory tract (146). Although there are at least 51 distinct human adenovirus serotypes, most human disease is associated with only one third of these types. Many adenovirus infections are subclinical and result in antibody formation that is probably protective against exogenous reintroduction of the same adenovirus serotype. However, the virus itself may be grown, especially from the gastrointestinal tract (73) and respiratory tract (61), for months after the initial infection and immune response. The more common illnesses associated with various adenoviruses are described in Table 1 and in the following paragraphs.

Disease	Individuals most at risk	Principal serotypes
Acute febrile pharyngitis	Infants, young children	1-3, 5-7
Pharyngoconjunctival fever	School-aged children	3, 7, 14
Acute respiratory disease	Military recruits	3, 4, 7, 14, 21
Pneumonia	Infants, young children	1-3, 7
Pneumonia	Military recruits	4, 7
Epidemic keratoconjunctivitis	Any age group	8, 11, 19, 37
Pertussis-like syndrome	Infants, young children	5
Acute hemorrhagic cystitis	Young children	11, 21
Gastroenteritis	Infants, young children	40, 41
Meningoencephalitis	Children and immunocompromised hosts	7, 12, 32
Hepatitis	Infants and children with liver transplants	1, 2, 5
Myocarditis	Children	?
Persistence:	Bone marrow transplant recipients; patients with acquired immunodeficiency or other immunosuppression syndromes	
In urinary tract		34, 35
In colon		40-49

Modified from refs. 109 and 160, with permission.

TABLE 1. *Adenovirus infections associated with disease or persistence*

Respiratory Diseases

Endemic Adenovirus Infections of Young Children

About 5% of ARD cases in children younger than 5 years of age are due to an adenovirus (28). The usual symptoms include nasal congestion, coryza, and cough. Other patients may have an exudative tonsillitis that may be clinically indistinguishable from disease caused by the group A streptococcus (82,100). The respiratory symptoms are often accompanied by systemic manifestations, such as generalized malaise, fever, chills, myalgia, and headache. The common serotypes are Ad1, Ad2, Ad5, and Ad6 (group C or HA group III) and occasionally Ad3 (group B or HA group I), which are endemic in most populations. Sporadic cases may be indistinguishable from other viral respiratory infections, such as influenza, parainfluenza, and respiratory syncytial virus. If conjunctivitis accompanies the signs and symptoms already described, the disease is designated as *pharyngoconjunctival fever* (12). The conjunctivitis is usually follicular, unilateral or bilateral, and characteristically mild. The adenovirus serotype most commonly involved is Ad3, but Ad7 and Ad14 within the same HA group have been isolated from such patients (107,124,202). In more severe cases, pneumonia may occur. Adenoviruses are probably responsible for about 10% of the pneumonias of childhood (164). Most patients recover from these lower respiratory infections, but some epidemics of Ad7 have resulted in considerable mortality (189). Sequelae in those who recover may include bronchiectasis that can clinically manifest years after the primary infection (156,233).

Acute Respiratory Disease of Military Recruits

In many respects, ARD is similar to the description furnished earlier of the respiratory infection of children. The syndrome

is caused by Ad4, Ad7, and occasionally Ad3. ARD is a syndrome that frequently occurs under the special conditions of fatigue and crowding created soon after the induction of young military recruits (107,175,178). It is less common among healthy adults. Some cases have had a fatal outcome from the pneumonitis that may accompany and complicate the other, milder respiratory symptoms. Further aspects of this disease are discussed in the section entitled Epidemiology.

Pertussis-like Syndrome

The association of adenovirus infection with a pertussis-like syndrome has been noted, leading to some speculation that adenoviruses rather than *Bordetella pertussis* caused many of the cases of clinical whooping cough (241). Claims were made that even the lymphocytosis of pertussis might be related to the adenovirus infection (35). This point of view was given some credence by the isolation of an Ad5 from multiple organs of a patient with severe whooping cough with lymphocytosis that ended fatally (35). Later data from a controlled study of 134 children with a pertussis-like illness and 101 healthy controls reported the common association of adenoviruses with whooping cough symptoms (192). However, there was no evidence that the adenoviruses alone were responsible for the syndrome. The large number of adenovirus isolates may be due to conditions favorable for reactivation of latent viruses from tonsillar tissue during concurrent *B. pertussis* infection (195).

Infections of the Eye

An acute follicular conjunctivitis may occur as part of a respiratory-pharyngeal syndrome or as a separate entity (18,188). Both bulbar and palpebral conjunctival involvement may occur and affect both eyes. The disease is often accompanied by significant preauricular lymphadenopathy (12). Complete recovery without sequelae is the most common result of this rather mild illness. The incubation period is usually 6 to 9 days, but it was as short as 2 days in experimental infections in volunteers (14,140). Epidemiologically, these infections can occur sporadically or cause disease in large groups of contacts. Family members may be affected. When the source is a swimming pool or small lake, large numbers of children and young adults may develop symptoms. Swimming pool conjunctivitis is probably most commonly due to adenoviruses (75). Although the virus is isolated from the conjunctiva of affected individuals, it has not been isolated from water samples from putatively infected sources. The common-source water-borne outbreaks usually occur in summer and are caused by Ad3 and Ad7; however, other types, such as Ad1, Ad2, Ad4, Ad6, Ad9, Ad10, Ad11, Ad15, Ad16, Ad17, Ad20, and Ad22 (groups B, C, D, and E and HA groups I, II, and III), have been associated with this syndrome (18,188).

In contrast to the milder form of ocular disease described previously and limited to the conjunctiva, EKC is a highly contagious and more serious disease. The clinical entity was first described before the isolation of adenoviruses and occurred among German workers in the late 19th century (118,133). It was subsequently observed in shipyard workers in Hawaii and in the continental United States. “Shipyard eye” was probably transmitted in the medical facilities that cared for chemical and physical trauma to the eyes of the workers. After an 8- to 10-day incubation period, a follicular conjunctivitis with edema of the eyelids, pain, lacrimation, and photophobia began. Corneal subepithelial infiltrates often followed the initial conjunctival involvements (45). The disease is often unilateral with preauricular lymph node hypertrophy. Occasionally, other lymph nodes were involved, and constitutional symptoms occurred, especially in children. However, most often the disease was limited to the involved eye and its draining lymph nodes. Corneal opacities in some cases lasted for several years and, uncommonly, would remain for longer periods. In some patients, the involvement slowly progressed to a hemorrhagic conjunctivitis that should be distinguished from the rapidly evolving acute hemorrhagic conjunctivitis associated with enterovirus 70 (45). Ad8 was the original cause of EKC. Between 1973 and 1976, Ad19 was common (51,96,258). Ad37 was isolated in 1976 and has been the predominant cause of EKC since 1977 (147). Rarely, other types caused a similar syndrome. In addition to viral inoculation during an ophthalmologic procedure such as tonometry, EKC has occurred as a late summer and fall epidemic in certain parts of Japan, Taiwan, and Vietnam. EKC has accompanied endemic trachoma in Saudi Arabia (15). Ad8 antibody is uncommon in American schoolchildren, whereas about half the Japanese or Taiwanese children have such antibodies (245).

Acute Hemorrhagic Cystitis

Acute hemorrhagic cystitis, an illness occurring almost exclusively in boys and associated with Ad11, is characterized by gross hematuria (198). Its significance lies in the potential confusion with other, more serious diseases of the kidney (such as glomerulonephritis). This self-limited disease is usually not accompanied by fever or hypertension, and tests of renal excretory and concentrating functions have been essentially normal. Ad21, like Ad11, is a group B HA type I and can also cause hemorrhagic cystitis (185). In Japan, when acute hemorrhagic cystitis occurs in a boy between the ages of 6 and 15 years, an adenovirus isolation from urine or a rise in neutralizing antibody occurs in about 70% of patients (198). In comparable studies of hemorrhagic cystitis in the United States, only 20% of cases can be linked to an acute adenovirus infection, and for 60% of the total, the etiology remains unexplained (185).

Patients have developed Ad11 acute hemorrhagic cystitis after renal transplantation and its accompanying immunosuppression (99,149). In some patients, it appears that the infection was introduced with the transplanted kidney. Some of the causes of hematuria in immunosuppressed patients include cytotoxic drugs used to treat the underlying disease, but even in such patients, the prevalence of adenovirus isolates from the urine is increased. Various adenovirus serotypes have occasionally been isolated from urine during acute systemic adenovirus infections. Two other group B serotypes, Ad34 and Ad35, were isolated first from renal transplant recipients. Ad34 was isolated from urine, whereas type 35 was isolated from kidney and lung tissue at autopsy (109,190). Although neither was accompanied by the symptoms of hemorrhagic cystitis, the Ad35 clearly contributed to the patient's demise from pneumonia.

Meningoencephalitis

It is rare to isolate any of the adenoviruses from either the cerebrospinal fluid (CSF) or the brain. However, several reports have directly demonstrated adenoviruses in CSF (Ad3, Ad5, Ad6, Ad7, Ad7A, and Ad12). One patient with malignant lymphoma, immunosuppressed by chemotherapy, had an Ad32 isolated from brain at autopsy (33,146). There are other cases of meningoencephalitis in which viral isolation from extraneural sites or antibody titer increases have

been used to make a diagnosis, especially associated with epidemic Ad7 pneumonia in children (232). A case of sudden unilateral deafness was associated with an Ad3 infection of the nasopharynx (129).

Gastrointestinal Diseases

Diarrhea

The relationship between adenoviruses and diarrhea has had a long and complicated history but has been clarified considerably in recent years (177,269,273,277). Because many adenoviruses replicate efficiently in the intestine and are excreted in the stool, it was assumed that they would be strong candidates for causing diarrhea (54,66). However, most earlier epidemiologic studies generally found as many adenovirus isolates in the stools of controls as in those with diarrhea (211). The failure to correlate adenovirus growth from stool with clinical illness was a good example that adenoviruses should not be designated as the cause of a whole spectrum of medical illnesses just because they can be cultured from the stool of an individual with a disease. Normal children can clearly shed adenoviruses in stool and often develop antibodies to the particular type grown (73,116). These subclinical infections probably result in lifelong immunity. That young children with systemic adenovirus infections can develop diarrhea as an accompanying problem is well known but probably no more common than after any other systemic infection.

The whole issue of adenoviruses as a cause of infantile gastroenteritis was reexamined based on the observation that initially “noncultivatable” adenoviruses were seen on EM examination of stool smears of affected children (66). Serologic detection methods such as ELISA for enteric adenovirus antigens have been developed to supplement the EM observations (270). Alternate tissue culture host–cell systems, such as the HEK-293 line, have resulted in the growth of some of the “noncultivatable” viruses (47,243,270). Two different enteric adenoviruses, EAd40 and EAd41, have been associated with diarrhea (47,80,270). Epidemiologic studies to assess the importance of these agents have been completed for several population groups. A report of 14 enteric adenovirus–related cases of diarrhea in 27 hospitalized patients studied during a 12-week period suggested that these viruses may be an important cause of acute gastrointestinal disease in hospitalized young children and may be nosocomially transmitted; this report also suggested that respiratory symptoms may be a prominent part of the clinical manifestations (277). The incidence of adenovirus-related gastroenteritis differs considerably in the various studies and locations reported by many authors. In general, it is not as prevalent as rotavirus diarrhea, occurs most often in children younger than 4 years of age, and is not easily distinguished on clinical grounds from rotavirus infection (215). In Bangladesh, it was responsible for 2.8% of the cases of diarrhea in 4,409 specimens but reached 12.3% in some months as diagnosed by a monoclonal antibody specific for Ad40 and Ad41 (131). In day care centers in Houston, weekly stool specimen collections from children 6 to 24 months of age were used to demonstrate that 38% of 249 children present during 10 separate outbreaks associated with enteric adenoviruses had this agent in their stool specimens. However, 46% of these samples came from patients who were asymptomatic, demonstrating that even during epidemics of enteric adenoviruses, many infected children do not develop gastroenteritis (257). In another study of children in day care reported from the Centers for Disease Control and Prevention in Atlanta, the prevalence of all adenoviruses in stool was 8%, and EAd40 and EAd41 were 2% in both 565 patients with diarrhea and 129 controls (157). In the latter study, astroviruses were more common in children with diarrhea (4%) than in controls (1%). Thus, it is clear that the role of adenoviruses as a cause of diarrhea can vary in different clinical settings. In some population groups, 50% of the children at the age of 4 years have antibodies to the enteric adenoviruses (230).

Intussusception

Another intestinal syndrome, intussusception, has been linked in some patients to adenovirus infection (13). The telescoping bowel characteristic of intussusception may be caused by mesenteric adenitis acting as a lead point to the mechanical obstruction. Adenoviruses (group C, HA group III, Ad1, Ad2, Ad5, and Ad6) have been isolated from both stool cultures and the involved lymph nodes removed at surgery (34,207). However, there is no evidence that EAd40 and EAd41 are involved in this syndrome (22). An alternative explanation, that some hyperirritability of the small intestine might be caused by adenovirus infection and lead to the intussusception, has been proposed (280). However, many patients with intussusception have no evidence of adenovirus infection, and the disease is probably multifactorial. Adenovirus inclusions have been seen in some appendices removed at surgery (279).

Celiac Disease

Structural homology between the Ad12 E1B protein and A-gliadin, a major component of a-gliadin known to activate celiac isease, was noted. In addition, most patients with celiac disease had evidence of prior Ad12 infection, in contrast to matched controls. These observations suggest that a viral protein product of an adenovirus found in the intestine may play a role in the pathogenesis of celiac disease, perhaps by inducing cross-reacting antibodies to a-gliadin (139). In two recent studies to determine whether there was persistent Ad12 infection in the intestinal mucosa of patients with celiac disease, PCR was used. Four of 18 patients had detectable adenovirus DNA, as did 2 of 24 controls, leading the authors to conclude that Ad12 persistence is not a major element in celiac disease (162). Similar conclusions were reached by others but do not preclude that prior Ad12 infection might have been involved in the evolution of celiac disease (259).

Myocarditis

Adenovirus DNA has been detected by PCR in most myocardial biopsies primarily from children with acute myocarditis in two studies (158,166). Although there was clinical and electrocardiogram evidence of myocarditis, most of the adenovirus-positive biopsies did not show classic inflammation, in contrast to those that appeared to be associated with enterovirus infection.

Diseases Associated with Immunocompromised Patients

Although the adenoviruses are not nearly as prevalent as the various herpesviruses during immunosuppression,

adenoviruses have been isolated from immunocompromised hosts and have contributed to their morbidity and mortality. Fifteen immunocompromised patients seen between 1967 and 1978 with either an adenovirus isolate (14 patients) or typical adenovirus histology on postmortem (1 patient) have been reviewed (281). Twelve patients had pneumonia, 8 of 11 patients had hepatic abnormalities, and 9 patients died of either respiratory or hepatic failure. Only 3 patients had evidence of other concurrent infections. The adenovirus serotypes were Ad1, Ad2, Ad5, and Ad6 in children and Ad4 in the adults. These same ubiquitous serotypes were also prevalent in patients with other, less serious illnesses in normal age-matched populations. These data would imply that immunocompromised patients may be no more commonly infected with adenoviruses than normal hosts but that the outcome may be more serious and even result in fatalities. This general conclusion was reached by other investigators reporting an individual case of Ad11 pneumonia in an agammaglobulinemic patient (231), an Ad2 fatal hepatic and bronchial necrosis in a child with thymic alymphoplasia (271), and an Ad35 fatal disseminated infection in a renal transplant recipient (237).

The problem of adenovirus hepatitis in 5 of 224 pediatric hepatic transplant recipients has been demonstrated (29). The serotypes involved were common types, such as Ad1, Ad2, and Ad5. The clinical manifestations were often the direct result of infection of the transplanted liver, and viral intranuclear inclusions were readily identified. The disease was acquired *de novo* or by reactivation of latent virus from an endogenous source. Forty-nine of 484 pediatric liver transplant recipients had an adenovirus isolated from either liver, lung, or gastrointestinal tract. Twenty children had invasive adenovirus infection, which culminated in the death of 9 patients (174). Some immunocompromised patients recovered from their adenovirus infections after discontinuation of immunosuppressive drugs, but the reported cases clearly emphasize the poor outcomes of some of these acute infections. No specific antiviral agents are effective against such adenoviral diseases.

Twelve percent of patients with acquired immunodeficiency syndrome (AIDS) have been reported to have group B adenoviruses in their urine (48,122). Restriction endonuclease analyses of viral DNA of 24 urinary isolates have shown that all were related to Ad34 and Ad35. However, some of the AIDS urine isolates had the Ad7 hemagglutinin by serologic classification, suggesting recombination between the Ad35 and Ad7 group B (HA group I) agents. In addition to the HAs of Ad7 and Ad35, HAs from Ad3 and Ad11 have been found in other Ad35-like isolates from AIDS patients (122). Occasionally, Ad35 has also caused fatalities from pneumonia or hepatitis, as observed in 3 pediatric patients (152).

Adenoviruses were observed by EM in colonic tissue of 5 of 67 homosexual men infected with human immunodeficiency virus type 1 (HIV-1) and studied because of persistent diarrhea. However, it is not clear whether group D adenoviruses are associated with the chronic diarrhea that is common in these immunocompromised patients. The tissue biopsy samples had foci of mucosal necrosis, chronic inflammatory cells, and amphophilic nuclear inclusions observed by light microscopy. Thus, adenoviruses, primarily newer isolates of group D, are present in the colonic lesions of some immunocompromised patients and may cause diarrhea but do not appear to be the etiology of the large number of intestinal dysfunctions that affect AIDS patients (112,130).

After allogenic bone marrow transplantation, adenovirus isolation rates as high as 20.9% and disease in 6.5% of the patients have been reported (67). Most of these isolates were group B agents, serologically typed as Ad35 by viral neutralization assays, but were typed as Ad11, Ad34, and Ad35 by DNA analysis (67) (see Identification and Classification of Viral Isolates). More than half of these patients with adenovirus diseases succumbed to their infections. Although these rates of isolation and disease are higher than previously reported (229), the trend to more high-risk bone marrow transplantations may have contributed to the increased incidence of adenovirus infection and disease.

Latency

Adenoviruses were discovered because of their propensity for latency in adenoidal tissue (1), and there are several patterns of latent infections. The persistence or reisolation of adenovirus in normal human hosts has been documented for 24 months after initial infection (73,116). The potential role of lymphocytes (especially B cells) has been suggested in the maintenance of latency by several groups of investigators who have found low levels of adenovirus DNA persistent in peripheral blood lymphocytes (1). Confirmation of true latency in tonsillar tissue has been reported in a study demonstrating adenovirus nucleic acid sequences in this tissue but no infectious virus, even after repeated passage (195). Latent adenoviruses have been postulated to be important in the pathogenesis of chronic obstructive pulmonary disease (COPD) that occurs in cigarette smokers (168). Adenovirus DNA sequences from the E1A region were found by PCR more often in resected pulmonary nodules from patients with COPD than patients without airway obstruction. However, the detection of adenovirus DNA sequences from the E3 region was not different in the two groups studied. The viral E1A DNA was found in the epithelial cells, suggesting that it might be involved in COPD. In another study in children, persistent infection with adenoviruses was associated with asthma as well as with chronic airway obstruction (161). The significance of these observations, which were reviewed recently (159), awaits further study.

Persistent or latent adenoviruses could explain the clinical illnesses in immunocompromised hosts who might be reinfected from an endogenous source at the time of immunosuppression. However, in some renal transplant recipients, the infection appears to be exogenously acquired in the donor organ (99). Another possible outcome of infection would be the stable integration of E1A and E1B sequences of the adenovirus DNA in covalent linkage with the host chromosome. The Ad E1 region sequences have been shown to be sufficient for transformation of rodent cells by various adenovirus serotypes. In model systems in animals, it has been clearly demonstrated that some adenoviruses, especially Ad12, Ad18, and Ad31, are highly oncogenic. However, most attempts to associate adenovirus infection with the etiology of human tumors have been unsuccessful. In the adenovirus oncogenesis model, infectious virus is no longer present, but a defined segment of viral DNA is integrated into the host chromosome, from which it is actively transcribed and translated into viral polypeptides. No viral macromolecules (DNA, RNA, or proteins) suggesting an adenovirus infection have been found in most studies of the etiology of human malignancies (92,93). However, one group of investigators has found adenoviral nucleic acid in neurogenic tumors (125). These latter results await confirmation by others.

DIAGNOSIS

Differential Diagnosis

Many adenovirus infections of the respiratory tract are difficult to distinguish from other respiratory viruses and even some bacterial infections. In children younger than 3 years of age, small discrete white spots of exudate can appear on the tonsils and are consistent with adenovirus infection. They have to be distinguished from streptococcal infections and the manifestations of the Epstein-Barr virus mononucleosis syndrome or even some cases of herpes stomatitis, which in older children or young adults might start as exudate on the faucial tonsils. The most characteristic manifestation of adenovirus infection is the conjunctivitis as part of the keratoconjunctivitis syndrome, which usually begins unilaterally, causes moderate enlargement of the preauricular lymph node, and may subsequently involve the contralateral eye. The adenovirus-induced upper respiratory syndromes or pneumonias are impossible to distinguish clinically from other viral infections of these organs and must employ laboratory diagnosis for clarification of the etiologic agent. Hemorrhagic manifestations of the urinary tract can be caused by bacterial infections as well as by adenovirus infections. In patients immunosuppressed with cyclophosphamide for bone marrow transplantation, manifestations of hemorrhagic cystitis can be due to drug toxicity in the bladder soon after transplantation. However, the isolation of adenoviruses later in the course as a cause of the cystitis was reported in 13% of cases (150). Adenovirus as a cause of hemorrhagic cystitis is more common in Japan than in the United States, for reasons that have not been explained. The gastroenteritis that is manifest during Ad40 and Ad41 infection may be nosocomial and should be sought in hospital-related outbreaks in young children, when a bacterial etiology has not been identified.

Laboratory Diagnosis

Collection and Preparation of Specimens

Collection of specimens from affected sites early in the illness is necessary to optimize viral isolation and to detect adenovirus antigens or nucleic acid directly in clinical samples. The duration of excretion of adenoviruses at the time of acute infection is about 1 to 3 days from throat in adults with the common cold; 3 to 5 days from nose, throat, stool, and eye from patients with pharyngoconjunctival fever; 2 weeks from eye cultures from keratoconjunctivitis; 3 to 6 weeks from throat or stool of children with respiratory or generalized illnesses; and 2 to 12 months or longer from urine, stool, throat, or organ biopsies of immunocompromised patients (110). However, after the acute period, adenoviruses may be latent in some tissues, such as the tonsils, or may be reisolated intermittently from throat or stool cultures for periods of months to years after primary infection in some patients (73,116). Adenoviruses obtained from throat swabs, nasal washes, conjunctival swabs or scrapings, and anal swabs are generally stable agents and withstand freezing and storage at –70°C, if immediate inoculation is not possible (144). Because some of the urines may be toxic to the tissue culture lines employed, it is best to remove the input inoculum after 2 to 4 hours of incubation. This time allows the virus to be absorbed before the cultures are re-fed with fresh medium. When cultures of blood mononuclear cells are desired, heparinized blood is fractionated on Ficoll-Hypaque gradients, and the washed cells are cultured in RPMI medium with fetal calf serum (144). Portions of these cells should also be co-cultivated on such cells as HEK cells, which are susceptible to adenovirus infection.

Viral Serology

Agents growing in tissue culture and demonstrating typical adenovirus cytopathology should be further processed to identify the isolate definitively. Serologic confirmation is useful in this characterization and can be separated into two categories. The IF and CF tests, using tissue culture material as antigens and reference antibodies, measure group characteristics of the adenoviruses and are therefore usually shared by all human isolates (79,144). The hemagglutination inhibition (HAI) and serum neutralization (SN) tests measure type-specific parameters that can distinguish members of each group (144).

The adenovirus CF antigen is a property of the soluble hexon capsomere, which is made in great excess over that assembled into the virion. Although the hexon carries multiple antigenic epitopes, the alpha measured in the CF test is common to all human adenoviruses (204,246). In many laboratories, the IF or ELISA recognition of adenovirus isolates is considerably easier than CF characterization. Thus, a single reference antibody can be used for initial characterization of a clinical isolate as an adenovirus. Type-specific monoclonal antibodies that could be used in the IF test as well as in ELISA and latex agglutination procedures are currently used for differentiating EAd40 and EAd41 from other adenoviruses in the feces (105). Specific serotyping includes the use of monoclonal antibodies to type-specific epitopes on hexon or fiber.

In current practice for specific adenovirus serotyping, the HA properties of a virus isolate are determined, followed by serologic tests to inhibit HA or to neutralize the virus with type-specific antibodies. Agents preliminarily characterized as adenoviruses are tested for their ability to hemagglutinate either rhesus or rat cells (218). Further HA testing using human cells, as well as cells from various other animal species, can provide supplemental information (108,147,160). Classification of adenoviruses by HA of rat or rhesus cells is shown in Table 1 of Chapter 67. In addition to group B (HA group I), which completely hemagglutinates rhesus cells, and group D (HA group II), which does the same with rat cells, there are the group III agents, which only partially hemagglutinate rat cells. Once an adenovirus can be placed in an HA group, the various type-specific antibodies within that group are added to find the one that will inhibit the HA reaction. Preparation of the sera includes absorptions with kaolin, inactivated filtrates of *Pseudomonas* species, or heparin-MnCl2 to remove nonspecific inhibitors (144).

Various cross-reactions among serotypes commonly occur (e.g., among Ad7, Ad11, and Ad14 within subgroup I). In addition, a number of recombinants occur between adenoviruses within a subgroup (111). These recombinations appear both naturally in patients and artificially in tissue culture dually infected as described in Chapter 67. Recombination of patient-derived material complicates the typing procedure. The SN and HAI results may be discordant as a result of recombination of two adenovirus genomes between the hexon and fiber genes (Fig. 3; see Chapter 67). When the results of the SN test differ from those for the HAI test, they are reported as SN/HAI. Because the HA is a property exclusively of

the fiber, the HAI test is able to measure antigenicity from only 5% of the genome. Adenovirus neutralization is a property of both the hexon and the fiber, which together represent 15% of the genome. Because adenoviruses spread slowly from one cell to another, it takes considerable time to reach an end point in a neutralization test. To speed up the SN, many laboratories have adopted various modifications that rely on the kinetics of neutralization rather than on a stable end point. Various intersecting pools of antibody mixtures have been devised to decrease the number of individual neutralization reactions that must be used for virus identification.

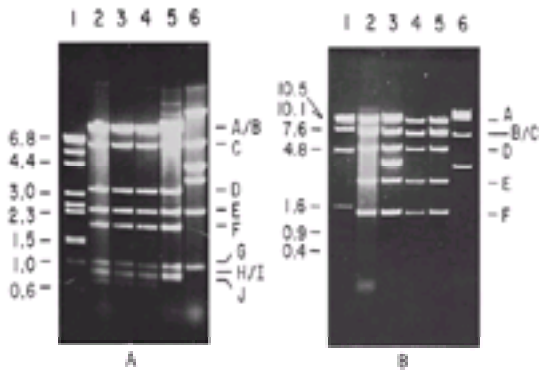

FIG. 3. Characterization of clinical adenovirus isolates by restriction endonucleases. **A:** Smal restriction endonuclease was used to digest and compare six adenovirus DNAs that were electrophoresed in 1% agarose and stained with ethidium bromide. *Lane 1:* Prototype Ad2. *Lane 2:* Prototype Ad35. *Lanes 3–6:* Clinical isolates. Lanes 3 and 4 are identical to Ad35, but lane 5 is missing one restriction cut, causing fragment J to remain fused to fragment A. Lane 6 is another clinical isolate unrelated to types 2 or 35. **B:** The same DNAs as those in **A** were digested with Hpal restriction endonuclease. Note that there is a single-band difference between lanes 3 and 2 or 4, all of which were identical following Smal digestion. (From ref. 48, with permission.)

Viral DNA

Other properties of the virus can be exploited both for identification of an isolate as an adenovirus and for subgroup typing. Characterization of the viral DNA by both hybridization and restriction endonuclease digestion patterns has been used for clinical isolates (48,147,242,262). In contrast to the limited regions of the genome analyzed by either HAI or SN tests, restriction endonucleases recognize and cut the DNA at specific sequences found throughout the genome. Viral footprints characteristic of the DNA digestion patterns for the various serotypes have been used for virus identification and classification. Modified Hirt extracts of infected cells separate the viral from the host DNA in a rather simple purification scheme before the restriction endonuclease step. Characterization of patient isolates by these techniques is shown in Figure 3 (48). For finding small differences in DNAs, multiple enzymes may be necessary. Other investigators have found it unnecessary to separate host from viral DNA and have obtained similar results (242). These procedures can be made even more sensitive by Southern blotting of the DNA restriction digests, followed by DNA–DNA hybridization (242). This procedure, which used small numbers of cells and adenovirus-specific radioactive-DNA probes, may be positive for viral DNA before any evidence of viral growth by CPE. The specific viral radioactive-DNA probes can easily detect the viral DNA restriction endonuclease bands among the contaminating host DNA. However, this procedure is tedious for routine clinical use and clearly must be reserved for special situations.

Currently, PCR assays are being used for diagnosis of adenovirus infections in various tissue specimens and body fluids. For reactivity with most human adenoviruses, PCR primers have been chosen from the hexon coding region or from the VAI and II (110,274) regions, for which there is extensive homology among serotypes. For type-specific probes, sequences from the E1B region have been used to identify the two enteric types (3). The sensitivity and specificity when compared with viral culture or the presence of hexon antigen was greater than 90% in one study (110). Such an approach would be useful late in an illness, when little viable virus was present. Modifications of these approaches are also suitable for fixed tissue specimens prepared for microscopic pathologic examination (254). However, some investigators have found that the hexon primers result in many positive PCR specimens from stool samples of normal patients, such as 50% of symptom-free children with a mean age of 21 months or 18% of normal young adults tested (3). These latter results may be detecting latent viruses from a variety of adenovirus serotypes because the PCR probes specific for the E1B gene of enteric adenoviruses were negative in the normal patients.

Direct Microscopy of Clinical Specimens

Exfoliated Cells

Rapid diagnosis by direct examination of the cytopathology of exfoliated cells has not been generally successful because IF techniques have detected only one third of the nasopharyngeal secretions from which adenoviruses subsequently grew in tissue culture (78). However, other authors have reported that the IF technique to detect adenovirus-infected cells is as sensitive as tissue culture growth (170). The IF technique has not been useful in early diagnosis of conjunctival adenovirus lesions but may be more useful in studies of exfoliated bladder cells from patients with acute hemorrhagic cystitis (17). Latex agglutination tests for detecting enteric adenovirus proteins in stool are available. They depend on specific adenovirus antibody to agglutinate latex beads in the presence of adenovirus proteins. Although evaluation of their sensitivity and specificity has indicated that they are useful in diagnosing enteric adenovirus infection, they are less sensitive than culture, they may give positive results with nonenteric adenoviruses, and a significant number of positive reactions of stool extract with the latex controls can invalidate the test (90).

Electron Microscopy

EM of clinical specimens has been used in special situations to identify viral agents rapidly and specifically. The discovery of the “noncultivatable” adenoviruses was made by EM examinations of stool extracts (66). The procedures used were developed to detect other fecal agents, such as rotavirus and Norwalk agent, that grew poorly in tissue culture (25,65,143). Because the EM morphology of the adenoviruses is unique, it was not as critical to use immune EM to differentiate the viral agents from other nonviral components of stool. On specimens from other body sites, immune EM for rapid diagnosis of respiratory infections was successful in detecting 19 of 25 throat swabs from which adenoviruses were grown (56). Although less sensitive than tissue culture growth, the EM technique was rapid. However, such approaches are not generally used to diagnose adenovirus infection of the respiratory tract.

Antibody Response to Adenovirus Infection

Patient antibody responses to infection or epidemiologic surveys have generally used CF, HAI, SN, and ELISA tests as described for serologic identification of adenovirus isolates. Because the CF antibody response is a group reactivity, single CF antigens detect responses to many adenovirus serotypes. However, adenovirus CF serologic responses may vary with the virus used as a source of the antigen, and some adenovirus infections may not be detected by this technique (225).

Although the HAI and SN antibody responses are more specific for a single infecting serotype, these tests may detect heterologous responses within a subgroup, especially when adults are being studied. Children have more specific serotype responses, perhaps related to their limited experience with the adenovirus family. Other serologic tests (e.g., Ouchterlony agar gel diffusion, radial gel diffusion, and HA enhancement) have been used by various investigators (89,197). Each has an advantage, such as economy of reagents, ease of performance, or high sensitivity for group-reactive antigens, but none has found general use in clinical laboratories.

PREVENTION AND TREATMENT

Therapy

Specific antiviral therapy for adenovirus ocular infections has been attempted. The keratoconjunctivitis has been treated during the acute inflammatory phase, and the effect of therapy on both the acute symptoms and chronic corneal sequelae was evaluated. Iododeoxyuridine and adenine arabinoside, which both inhibit the replication of the DNA-containing herpes family of viruses, were unsuccessful in the treatment of adenovirus-induced epidemic keratoconjunctivitis (53,267). Neither the acute phase nor the delayed appearance of corneal opacities has been benefited by these topical antiviral agents. Two nucleoside analogs, (S)-9-(3-hydroxy-2- phosphonylmethoxypropyl) adenine (HPMPA) and 2'-nor-cyclic guanosine monophosphate (cidofovir), have been reported to be potent, nontoxic inhibitors of adenovirus replication in human embryonic fibroblast cultures (5). Cidofovir, the cytidine analog of HPMPA, is also effective in tissue culture against Ad1, Ad5, and Ad8 (87). This drug, which is used to treat cytomegalovirus disease, has shown some efficacy in the rabbit ocular model of Ad5 infection (86). There is also a case report of a single patient with adenovirus disease complicating stem-cell transplantation treated successfully with cidofovir (216). Intravenous ribavirin has been used to treat severe local or systemic adenovirus infections in immunosuppressed patients. The reports, usually of single patients, have had mixed results (31,137,165,186). In a refractory case of adenovirus hemorrhagic cystitis after bone marrow transplantation (186), the hematuria and urinary symptomatology resolved; however, in two other similar cases, the patients succumbed to the adenoviral infection. Controlled trials must be done for both cidofovir and ribavirin for the efficacy of either drug to be determined. The use of antivirals for the adenovirus-induced respiratory syndromes, including pneumonia in immunocompetent patients, have not been reported. When these diseases occur sporadically, they are difficult to distinguish from similar illness caused by a multitude of respiratory RNA viruses. In the military, where epidemics of adenovirus pneumonia are easier to diagnose, attempts at control have focused on prevention by vaccination.

Vaccine

Effective oral enteric-coated vaccines exist to prevent disease by Ad4 and Ad7 (249,250). Although these preparations have been in use in the military, they are not licensed for administration to civilians. Adenovirus vaccines are therefore not used in susceptible children. The vaccine serotypes are packaged together in enteric capsules so that they bypass the respiratory epithelium and only replicate once they reach the intestine (40). The enteric infection is asymptomatic, and it results in good neutralizing antibody responses. Circulating antibody is a reliable standard of immunity after administration of adenovirus vaccines.

Previous attempts to immunize military recruits were beset by many problems that are illustrative of the biology of adenoviruses as well as of SV40 with which they interact (106,221,222). Early batches of adenovirus vaccines were grown in primary monkey kidney cells before the discovery of SV40. After SV40 was discovered, it was soon appreciated that this virus was also present in the adenovirus vaccine strains. In attempts to rid the adenovirus vaccine strains of SV40, the virus mixtures were passed in the presence of SV40 antibody. The elimination of replication-competent SV40 was achieved by this step, and SV40 viral (V) antigens were no longer detected in the adenovirus vaccine. However, in subsequent passage of the adenoviruses, other SV40 antigens were again detected. The then newly discovered SV40 tumor (T) antigen (an early nonstructural SV40 antigen) was demonstrated in the adenovirus-infected cells. The SV40 T antigen arose from a subgenomic piece of SV40 DNA that had been stably integrated into the adenovirus DNA and was actually needed as a helper gene for adenovirus growth in monkey kidney cells. This was the first demonstration of an adenovirus–SV40 hybrid virus. Because of the deletion of adenovirus sequences to accommodate the SV40 insertion, the hybrid virus was lacking the coding capacity for some essential adenovirus polypeptides, and it was therefore defective. The adenovirus–SV40 recombinants required the helper functions provided by the whole, intact adenovirus genomes packaged separately into other particles. Many different adenovirus–SV40 hybrid viruses have been isolated

subsequently and include nondefective recombinants (145). These latter adenovirus–SV40 hybrid viruses were discovered to have SV40 inserts and a compensatory adenovirus deletion entirely within a region of the adenovirus genome (79 to 85 map units) that is not essential for adenovirus growth in tissue culture.

While the adenovirus–SV40 recombinant viruses were being studied, it was realized that Ad12 was highly oncogenic in newborn hamsters (251). Subsequent testing of all adenoviruses known at that time revealed that Ad7 also induced tumors in rodents, but the incidence and rapidity of tumor induction were less than for Ad12. Considerable apprehension about the use of Ad7 in vaccines was subsequently allayed when more experience with isolates from humans was obtained. The failure to show an association between human tumors and infection by any adenovirus allowed the vaccine program for the military to proceed (250). Now the vaccine strains are grown in human embryonic fibroblasts and appear to be safe and effective. Initially, Ad4 was used as a vaccine strain. After successful control of this strain, Ad7 appeared and was, in turn, controlled by including it in the enteric-coated oral vaccine preparations. Further changes in strain types, anticipated to fill the gap created by successful vaccination with Ad4 and Ad7, have not materialized.

Recently with the supply of adenovirus vaccines for the military interrupted by production problems, outbreaks of ARD have recurred in military installations (7,81,103). The only manufacturer of adenovirus vaccines has stopped producing the virus. Because of limited amount of vaccine, the adenovirus immunization program was initially reduced, especially for new recruits entering the military in the summer months, when ARD was characteristically uncommon. However, several large outbreaks have occurred at Fort Jackson, South Carolina, with hospitalization rates reaching 11.6% per week and 1,000 total cases. The last outbreak was due to Ad4, which is a serotype that was successfully controlled by the standard vaccine.

Use of adenovirus vaccines has not been extended generally to young children; however, Ad4 attenuated vaccine strains were given to a small number of children during a clinical trial (184). The infection spread to household contacts and resulted in a few clinically manifest illnesses. Similar vaccine administration to adults commonly spread to sexual partners but infrequently to other adults housed together (238). This pattern of infection suggests that vaccine strains spread by contact, in contrast to the airborne or droplet routes that appear to explain many of the observations collected in studies of natural infections among military recruits. The common adenovirus types, Ad1, Ad2, and Ad5, which infect children, have not been attenuated for vaccine use (227). These strains would be expected to spread and therefore cause disease in close contacts of vaccinees. The lack of attenuation and the lingering apprehension about the oncogenic potential of the adenoviruses have interdicted their general use as routine vaccines of childhood.

Adenoviruses as Vectors for Vaccination and Gene Therapy

Adenoviruses as vectors for the delivery of foreign DNAs are being used extensively as tools of modern molecular biology. There have been many hundreds of papers published using adenovirus vectors in each of the past several years. There are at least four general goals in these approaches. The first is to deliver DNA effectively to cells in culture. Because some cells are hard to transfect but most are susceptible to adenovirus infection, the delivery of functional genes cloned into various adenovirus constructs is widely employed. The second goal is to deliver a functioning gene to a cell *in vivo* to correct a congenital absence or mutation in an essential enzyme, a critical transporter or structural protein. Adenoviruses have been used to deliver the complementary DNAs (cDNAs) for many enzymes, including ornithine transcarbamylase, blood clotting factors VIII and IX, and bilirubin UDP glucuronosyl transferase (126,127) to hepatocytes in rodents and primates. Delivery of the cystic fibrosis transporter (219) and the dystrophin cDNAs (210) using adenovirus vectors has been studied in numerous laboratories. Some of these studies have involved human subjects, but there are many obstacles to overcome before these approaches are useful in humans. The third use of adenovirus vectors is to deliver genes to control cell growth and apoptosis. For the treatment of cancer, there are numerous approaches to introduce cytotoxic and pro-apoptotic genes using adenovirus vectors or to make the virus itself selectively toxic to malignant cells (23,42,76,101). There are also considerable efforts being made to control the growth of blood vessels and their endothelial lining cells. In some uses of the latter approach, growth factors are included in adenovirus vectors to promote the growth of new vessels to replace occluded ones. In other cases, adenoviruses are being used to prevent the overgrowth of the arterial wall during the healing phase after angioplasty is performed to open blocked cardiac arteries (228,247). A fourth use of adenovirus vectors is to deliver DNA that can code for an epitope or antigen that would be useful as an immunizing agent against another infectious agent or tumor cells. Both the humoral and cell-mediated immune responses can be stimulated by these approaches. Some of the protein targets that have been inserted into adenoviruses include the hepatitis B surface or core antigens (181); HIV-1 env, gag, or p24 proteins (49); glycoproteins, such as the gB from herpes simplex virus (171) or the gP from rabies virus (208); and the respiratory syncytial virus F protein (36). This is only a short list of the many antigenic proteins that have been cloned into adenovirus vectors. In general, the protein-coding regions inserted into adenovirus vectors express much larger amounts of product than the native virus from which they were derived. In the trials to induce tumor immunity, genes for various cytokines and putative tumor antigens have been inserted into adenovirus vectors in an attempt to increase host immunity against malignant cells (2,203,240,266).

The advantages of using adenoviruses as vectors are that virus replication does not require that the recipient host cell is dividing, as for most retrovirus vectors, except for lentiviruses. Adenoviruses can be designed to enter mammalian cells and express proteins but can be otherwise defective for production of infectious progeny virus. These viruses can be grown to high titers. The disadvantages are that adenoviruses are highly immunogenic, inducing both humoral and cell-mediated immune responses (43), and do not efficiently transduce some cell types. The immune responses appear to decrease the length of time that the transgene is expressed after the initial vector administration as well as interfere with the effective readministration of the vector. Many studies have attempted to understand the components of the immune response that limit adenovirus-based expression vectors; however, the practical results for more effective gene therapy still require immunosuppression of the host to lengthen adenovirus expression. To address the difficulties of transducing some cells or to increase the specificity of adenovirus delivery to selected cell types, some investigators have changed the adenovirus fiber to increase the efficiency of attachment to specific cell types (236,268).

Adenoviruses are expressed primarily as episomal DNA because they integrate their DNA into the host chromosomes with very low efficiency. Although the absence of integration is an advantage when considering the deleterious effects

that integration could have on antioncogenes, such adenovirus vector-delivered DNAs are lost from the cells and are not conducive to long-term expression of the foreign gene. Recently, by combining the advantages of the adenovirus system with the integrative properties of the rep protein of adenoassociated virus, it has been possible to achieve integration of adenoviruses into chromosome 19 (214).

Adenovirus vectors can be prepared to be either replication competent or conditionally defective in a variety of genes required for a productive infection. From the experience with adenovirus–SV40 recombinants, it was learned that the entire adenovirus E3 region could be deleted without a major change in viral growth in tissue-cultured cells (Fig. 4; see Chapter 67). This region can be substituted with foreign DNA and the adenovirus grown in any cell line permissive for wild-type adenovirus infection. E3-substituted adenoviruses have been used primarily for the insertion of genes to produce proteins for immunization. Some of the immunogens have been described earlier. Various promoters have been used to maximize expression, but it has often been observed that strong upstream promoters, such as the adenovirus major late promoter with its tripartite leader, are the origins of the mRNAs from which the E3-inserted genes are transcribed. For antigens that do not elicit high enough titers on a single administration, booster immunizations have been possible by duplicate cloning of the desired gene into two or more distinct adenovirus serotypes (123). Because the initial immunization produces antibodies against the adenovirus surface proteins that contain neutralizing epitopes, it is usually not possible to readminister the same serotype and obtain sufficient replication for adequate reexpression of the cloned DNA insert. The second serotype, to which the host is not immune, thus serves as a booster for augmenting antibody titers to the foreign DNA contained in common between the two adenovirus serotypes.

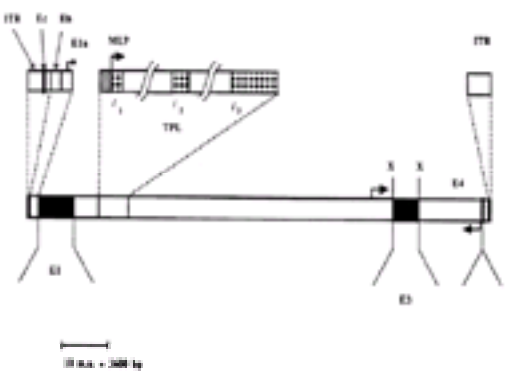

FIG. 4. Foreign genes can be inserted and expressed in the three areas (E1, E3, or near E4) as well as behind the major late promoter. The E1 or E3 genes may be deleted, whereas insertions at the right end are placed between the inverted terminal repeat (*ITR*) and region E4. The direction of transcription from the promoters is shown (*arrows*). Ec, sequence required for encapsidation of adenovirus DNA; Eh, enhancer; MLP, major late promoter; l1, l2, l3, parts of the tripartite leader (TPL) separated by introns; X, XbaI restriction exonuclease sites. (From ref. 95, with permission.)

In contrast to adenoviruses constructed for use as vectors for immunization where viral replication is desired to increase the amount of the immunizing epitopes, viruses engineered as tools for gene therapy are generally designed to be defective for replication. Most of the inserts of foreign DNA for the latter vectors replace the deleted E1A and E1B regions (see Fig. 4). The constructs are made by a variety of protocols but include plasmids into which the foreign DNA is inserted, flanked by adenovirus sequences (21,117). These sequences serve as control regions that contain the DNA packaging sequences, the left-hand origin of DNA replication at the physical end of the DNA, as well as sites for recombination with the rest of the viral DNA to reconstitute conditionally defective adenovirions. These virions grow only in cells such as the 293 cell line, which has stably expressed E1A and E1B sequences that complement the defect in the reconstituted virus. Although the viral replication cycle is aborted without producing much progeny virus, these recombinant adenoviruses are fully competent to enter human cells without the E1 region and transcribe the inserted sequences. Numerous inserts have been made using this protocol. Although the viral vectors have E1 deletions and are therefore defective for efficient growth, the E1-transactivating functions for other adenovirus promoter-enhancers can be supplied in trans by cellular genes under some special conditions (187,200). There was also low-level contamination with E1-containing viruses that were reconstituted by recombination with the endogenous E1 gene from the 293 complementing cell line used to grow the viral stocks. This problem has been solved by reconstructing new cells with a more limited E1 region that prevents the reconstitution of wild-type adenoviruses by recombination between the integrate E1 genes from the cell and the plasmids containing various other components of the adenovirus DNA (62).

The accumulated knowledge about adenovirus molecular biology during 46 years of research on these agents has been employed to make the adenovirus vectors more defective. The goal was to eliminate the leakiness of expression of other viral genes, especially those that result in immunogenic coat proteins expressed from late genes in constructs deleted of only E1 and E3 (275). Thus, the first-generation adenovirus vectors have been further altered by substituting a temperature-sensitive DNA-binding protein gene into the vector or making selective deletions of the adenovirus E4 open reading frames (ORFs) (212). The temperature-sensitive mutation made it necessary to grow the vector at lower temperatures, whereas the E4 ORF deletions required insertion of these viral genes into the complementing 293 cell line. Further vector permutations have produced “gutless” vectors, which contain only the necessary cis-acting elements, the two origins of DNA replication at each of the two physical ends of the virus, and the packaging sequences from within the first 500 base pairs of the left-hand end (182,201). The gutless vectors require a complementing helper virus, which is a temperature-sensitive adenovirus that provides all the required early and late gene products, including the structural proteins, for the assembly of progeny virus particles. Because the amounts of genomic DNA are different between the helper and gutless adenoviruses, they have different buoyant densities in the cesium chloride density gradients used to separate these two viral constructs. It was assumed that the more adenovirus genes eliminated from the vector construct, the less immunogenic and toxic it might become. These assumptions have not yet been validated sufficiently. For example, replication-incompetent adenoviruses treated with psoralen and ultraviolet light to inactivate all transcription retain the ability to induce inflammation through class I MHC and chemokine-dependent mechanisms (26,138). The

coding capacity for some of the first-generation adenovirus vectors limited the foreign gene insert to 8.3 kb before the packaging limit of 105% of the normal genome was reached. The gutless vectors could presumably contain inserts of more than 30 kb but were not efficiently assembled unless more than 75% of the original genome size was provided in the construct.

There are other ways in which adenoviruses are being used to facilitate the insertion of foreign DNAs into cells. The mechanisms of viral attachment, processing through the endosomes, and eventual delivery of DNA to the nucleus are being used to co-internalize foreign DNA external to the adenovirus particle. In a series of studies, it has been shown that DNA complexed with polylysine can be joined to adenovirus particles by several linking methods and co-internalized (38,263). The complex enters cells presumably by attachment of the fiber to its putative receptor, but there have been modifications of these techniques to include ligands-like transferrin to facilitate entry into cells with the transferrin receptor (264). Recently, it has been demonstrated that it is unnecessary to link the virus and the external DNA for efficient entry of the DNA into cells in culture (278). Although these methods have proved efficient for the insertion of foreign DNA into a variety of tissue-cultured cells, *in vivo* delivery has lagged behind.

Some of the safety and efficacy issues in the use of live adenoviruses, including vectors in humans, are worth summarizing. More than 10 million people have been immunized safely with live Ad4 and Ad7 vaccines. There are numerous phase I and II human trials in which adenovirus vectors are currently being used; most are attempts to treat various types of cancer by direct intralesional injections. In one example, there was an attempt to engineer an adenovirus, which would be selectively toxic to malignant cells. A head and neck tumor trial is using a virus called Onyx-015, which was originally described as dl-1520 and was defective in the synthesis of E1B-55 kd (24,98,101). Because the E1B-55 kd protein binds to and inactivates the function of the *p53* antioncogene, it was postulated that the Onyx-015 virus would grow and be selectively toxic to the many malignancies that are deficient in *p53* function. In normal cells with functional *p53,* the adenovirus vector would presumably not replicate. Although there are many exceptions to this paradigm of selective toxicity of Onyx-015 to *p53*-deficient cells, the virus is being studied using intralesional injection into a variety of human malignancies (97,98,102,220,239). Additional viral genes have been manipulated in attempts to make adenovirus vectors that would be selectively toxic to tumor cells. Overexpression of either the E3-11.6 kd adenovirus death protein or the E4orf4 proteins, which also induce cell death, is being exploited in attempts to selectively kill tumor cells. Other studies being done in a variety of clinical trials are using the *p53* gene inserted in adenovirus vectors in an attempt to restore the antioncogene effects of this cellular gene on malignant cells. Although preliminary evidence of efficacy has been reported at various symposia at which individual patient results were discussed, definitive results from these studies on larger numbers of patients are not yet available. In none of these studies in humans is the inserted “foreign” gene of interest deleted to establish an appropriate control virus group to study the effect of the adenovirus infection itself.

In the area of gene delivery to replace a congenitally absent gene of importance, a phase I trial of the ornithine transcarbamylase (*OTC*) gene delivered to the liver in an adenovirus vector deleted of the E1 and E4 regions has recently been completed. The experiment was done to test for the short-term expression of *OTC* because it was anticipated that the immune response would eliminate any long-term expression of the enzyme. The trial consisted of an escalation of viral dose to determine effective delivery of a functioning gene to the liver as well as to monitor toxicity. Although most patients tolerated the right intrahepatic artery injection with only minor toxicity, one subject at the highest dose had a fatal outcome 4 days after the gene therapy. Analysis of the data for gene expression in the liver both in the fatal case and in the others was disappointing in that only small amounts of gene expression could be detected in human hepatocytes. This contrasts with the exuberant amount of adenovirus gene expression in the liver of mice injected at doses that do not cause mortality (213,282). Whether the difference of expression correlates with the amount of mRNA for the adenovirus receptor, CAR, which is high in mouse liver but low in human, is not known (19,20). Although there was modest hepatocyte expression of *OTC* in nonhuman primates at viral doses that were compatible with survival, it remains a challenge to deliver adenoviruses safely to human liver in doses that would not be toxic systemically (8,199). The issue of adenovirus being a tumor virus in animals seems less of a problem because of its long safety record as a vaccine for young adults, the failure to find adenoviral DNA in human tumors, and the fact that the E1-transforming region has been removed from most adenovirus vectors. Clearly, this is an active field of research, and we can anticipate considerable progress in the near future.

PERSPECTIVES

An amazing wealth of information about cell and molecular biology has come directly from studying adenovirus replication and pathogenesis for the past half century. Some of the seminal observations in cell biology were made before the ease of cloning opened up the possibilities of studying cell processes directly without the aid of viruses. Some of these discoveries have been described in both Chapter 67 and this chapter. Future studies should lead to better use of adenoviruses as vectors for the delivery of therapeutic proteins to humans with a great variety of illnesses. Although the problems of specific and efficient delivery to selected organs and cells with a minimum of toxicity remain significant, numerous investigators are working to modify the virus in rational ways. These studies have been facilitated by recent discoveries about receptors and coreceptors for the binding and entry of adenoviruses.

Control of the immune system to foster long-term gene expression has also been a difficult task. However, much has been learned about the nature of the host immune responses to adenoviruses and those components of the immune system that limit virus expression. The large number of immunoregulatory genes that adenoviruses possess offers strong clues as to their importance both in virus replication and survival through quiescent stages, such as latency. The possibility of using the viral immunoregulatory genes out of the context of the virus to control immune and cytokine responses remains a tantalizing possibility for application in the areas of autoimmunity and transplantation. Thus, adenoviruses not only point the way to study what is important in the immune system but also provide investigators with useful new proteins to serve as reagents to understand these immunologic processes. We all hope that, with respect to therapeutics for humans, the best is yet to come from understanding the viral immunoregulatory genes and their cellular targets.

At the same time that many novel discoveries are being made using adenoviruses and their isolated genes, the supply of

live attenuated adenovirus used for many years as a vaccine in the military is being jeopardized. While we shed many old techniques and reagents in the process of acquiring new ones, it would be unfortunate to lose the protection of a very effective vaccine formulated in the days before cloning and genetic manipulations, which have become so common today.

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CHAPTER 69 *Parvoviridae*: The Viruses and Their Replication

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The parvoviruses are among the smallest of the DNA animal viruses. The virion has a diameter of 18 to 26 nm and is composed entirely of protein and DNA. The family *Parvoviridae* (21) contains two subfamilies: the *Parvovirinae*, which infect vertebrates, and the *Densovirinae*, which infect insects. Each of the subfamilies contains three genera (Table 1). Because the host range of the *Densovirinae* lies outside the scope of this volume, the replication of these viruses is not reviewed. The interested reader is referred to several recent articles (14,29,151). The *Parvovirinae* have a wide distribution among warm-blooded animals, ranging from domestic fowl to humans (see Table 1). Dependoviruses, also known as adeno-associated viruses (AAV), are unique among animal viruses in that, except under special conditions, they require co-infection with an unrelated helper virus, either an adenovirus (Ad) or a herpesvirus, for productive infection in cell culture (11,38). In spite of this distinction, there are many similarities between AAV and the members of the *Parvovirus* and *Erythrovirus* genera in genome structure, organization, and expression. It is the intent of this chapter to compare and contrast the replication of these three genera at the molecular and biologic levels.

TABLE 1. *Parvoviridae*

THE VIRION

The parvovirus virion has a relatively simple structure composed of a mixture of VP1, VP2, and VP3, which encapsidates a linear, single-strand DNA molecule. The particle has icosahedral symmetry (277). The crystal structures of canine parvovirus (CPV), feline panleukopenia parvovirus (FPV), minute virus of mice (MVM), human parvovirus B19, and wax moth densovirus have been determined and their basic capsid organizations are similar (2,3,277, and refs. therein). The 60 protein subunits that make up the capsid have a common structure, arranged with T=1 icosahedral symmetry. The main structural motif is an eight-stranded, antiparallel b-barrel, which also has been found in most other viral capsid structures. The b-barrel motif contains only approximately one third of the amino acid composition of VP2, the major structural protein in most parvoviruses that comprises about 90% of the capsid. The remaining two thirds is present as large loops connecting the strands of the b-barrel. The loops form much of the capsid surface, onto which a number of biologic features, such as host species and tissue tropism, receptor binding, and antigenic properties have been structurally and genetically mapped. The insertions between the bD and bE strands of the b-barrel form an antiparallel b-ribbon, which together with similar insertions in four other fivefold related polypeptides form a cylinder about the fivefold icosahedral axes, with an opening to the exterior surface of the capsid in CPV, FPV, and MVM, but not in B19. VP2 is thought to be externalized for maturation cleavage to VP3 via this opening in the latter viruses. Other characteristic features of the parvoviral capsid (Fig. 1) include spikelike protrusions at the icosahedral threefold axes, a

15-Å canyon-like depression about the fivefold axes, and a dimple-like depression at the icosahedral twofold axes. Antigenic regions have been mapped to the threefold protrusions (2), and the twofold depression has been implicated in the attachment of host cell factors (3).

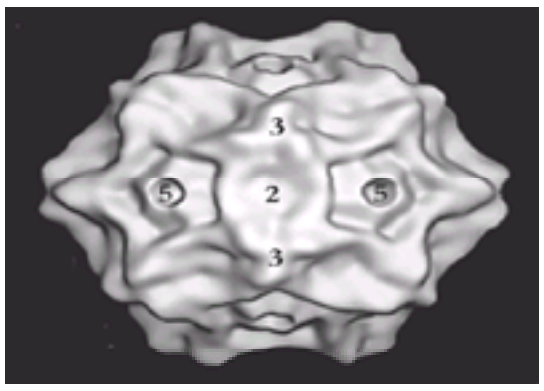

FIG. 1. *Parvovirus* structure. Cryoelectron micrograph of canine parvovirus showing some of the surface features around the two-, three-, and fivefold axes of symmetry.

The particle has a molecular weight (MW) of 5.5 to 6.2 × 10^6 daltons. Approximately 50% of the mass is protein, and the remainder is DNA. Because of the relatively high DNA-to-protein ratio, the buoyant density of the intact virion in cesium chloride (CsCl) is 1.39 to 1.42 g/cm^3 (21). The heavy buoyant density in CsCl permits the ready separation of AAV from helper Ad in co-infections. Both heavier and lighter forms of the virion occur (22,84). The latter are particles containing DNA molecules with significant deletions, and these particles can function as defective interfering (DI) particles. The exact role of the heavier-than-normal particles in infections is unknown. The encapsidated DNA molecules are indistinguishable from those of normal-density particles and so, presumably, some of the coat protein molecules are missing. Whether the missing proteins constitute a specific set is unknown. Finally, the sedimentation coefficient of the virion in neutral sucrose gradients is 110 to 122 (300).

Possibly as a consequence of its structural simplicity, the virion is extremely resistant to inactivation. It is stable between pH 3 and 9 and at 56°C for 60 min. The virus can be inactivated by formalin, b-propriolactone, hydroxylamine, and oxidizing agents (21).

The Genome

The genome is a linear, single-stranded polydeoxynucleotide chain (80,237). Several parvovirus DNAs have been completely sequenced (see Table 1) and the genome size is typically about 5 kilobases (kb). MVM DNA (the prototype autonomous parvovirus) contains 5,084 bases; AAV2 DNA (the prototype dependovirus) contains 4,679 bases (10,264). In general, autonomous parvoviruses encapsidate primarily strands of one polarity, the strand that is complementary to mRNA (i.e., the minus strand), whereas AAV encapsidates strands of both polarities with equal frequency (80,237). However, this distinction is not absolute. In the case of bovine parvovirus (BPV), only approximately 20% to 30% of the encapsidated strands are of the same polarity as mRNA, and in some hosts, LuIII (another autonomous parvovirus) encapsidates equal numbers of strands of both polarities (17).

All autonomous parvovirus genomes have palindromic sequences at both the 5' and the 3' termini of the virion (antimessenger) strand (Fig. 2). The palindromic sequence at the 3' end of the virion strand (also commonly referred to as the left end) of most murine autonomous parvovirus DNAs is approximately 115 bases long (8). In the human parvovirus B19, this sequence is more than 300 nucleotides in length (86). Because of its palindromic nature, the 3' end can fold back on itself to form a hairpin structure stabilized by hydrogen bonding between self-complementary sequences. This hairpin structure (see Fig. 2) is believed to be shaped like either a Y or a T, but its exact three-dimensional structure is not known. There is also a conserved GAA/GA mismatch bubble in the stem of the hairpin.

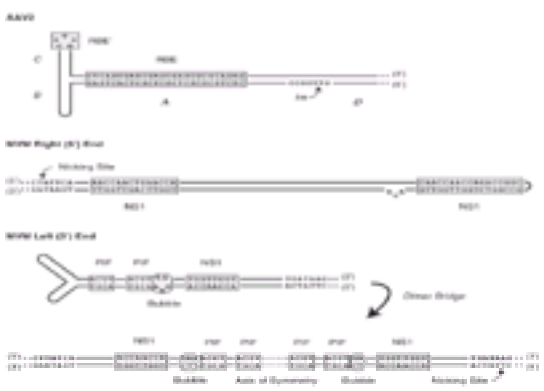

FIG. 2. Comparison of the 3' and 5' terminal palindromic structures of MVM DNA with the AAV2 terminal repeat, which is identical at both ends. Also shown is the 3' dimer bridge that is formed in a dimer replicative intermediate during MVM DNA replication. In each case, the key sequence elements are indicated that are required for AAV Rep nicking at the *trs*, or MVM NS1 nicking at the 5' (right end) hairpin or in the 3' (left end) dimer bridge. The hairpin sequences are shown in their most stable secondary structure. In the AAV hairpin, B and C by convention are the small internal palindromes flanked by the A palindrome that forms the stem of the first 125 bases. The D sequence comprises the remaining 20 bases of the TR that are inboard of the *trs*. See text for details.

The 5' end (or right end) of autonomous parvoviruses is also palindromic and can form a hairpin, but the sequence is completely unrelated to that at the 3' end of the virion strand (see Fig. 2). An exception is the 5' end of the human B19 genome, in which the two ends are identical (86). The MVM 5' palindrome is 207 bases long (10). Like the 3' terminal sequence, the 5' terminal sequence of MVM is not a perfect palindrome (see Fig. 2); again there are short, self-contained internal palindromic sequences (not shown) and an AGA bubble. The 5' terminal sequence is also not unique. Two sequences that represent an inversion of the terminal segment of the genome are found with equal frequency and are called flip and flop. The two orientations are a consequence of the fact that an inversion of the 5' sequence occurs during DNA replication (see later). Because the 5' hairpin is not perfectly symmetric, the two forms can be distinguished by restriction analysis.

In contrast to the sequence at the 5' end of the virion strand, which can exist in either of two orientations, the 3' terminal sequence is usually unique; only one orientation is found. (The relevance of this organization to the process of DNA replication is discussed later.) The exception is BPV, in which about 10% of the 3' end of the genome is, like its 5' end, inverted (255).

Unlike autonomous parvovirus DNA, the AAV genome has an inverted terminal repetition of 145 nucleotides (181). The first 125 bases form a palindromic sequence that, when folded to maximize base-pairing, forms a T-shaped structure almost identical to that formed by the 3' terminal sequence of the virion strand of autonomous parvoviruses (see Fig. 2). As with the sequence in autonomous parvovirus DNA, the palindromic terminal sequence in AAV DNA may be characterized more accurately as two internal palindromes (nucleotides 42 to 84) flanked by a more extensive palindrome (nucleotides 1 to 41 and 85 to 125). In the folded configuration, only seven of 125 bases remain unpaired. Six are required to allow the internal palindromes to fold over, and the seventh separates the two internal palindromes. Both ends of AAV exist in two orientations, flip and flop, which are essentially inversions of the terminal 125 bases. In this respect, the AAV terminal repeats (TRs) are similar to the autonomous parvovirus 5' (right end) palindromic sequence. Again, the two orientations can be detected because the two internal palindromes are not identical. In the flip orientation, the B palindrome (see Fig. 2) is closer to the end of the genome than the C palindrome, whereas in the flop orientation, the C palindrome is closer to the end. Furthermore, there is an additional heterogeneity in the extreme terminal bases. The canonical 5' terminal sequence in AAV2 DNA is 5' TTG. However, 50% of the molecules isolated from virions lack the first T, and a further 15% lack both Ts.

Because mature virion DNA is the end product of DNA replication, any model proposed must be able to account for the nucleotide sequence arrangements found in virion DNA. The terminal sequence organization has offered valuable clues to the mechanism of DNA replication. Thus, the similarities and differences between the terminal sequence arrangements of autonomous parvovirus DNA and those of AAV DNA have led to similar models of DNA replication for the two genera that differ in several key steps (see later).

There are additional similarities in the internal organization of the genomes of the vertebrate viruses (Fig. 3). All have two large open reading frames (ORFs) that do not overlap (10,228,264). One extends approximately from map position (mp) 5 to 40, whereas the second extends approximately from mp 50 to 90. As is discussed later, the ORF in the right half of the genome codes for all the caspsid proteins, and that in the left half codes for nonstructural proteins.

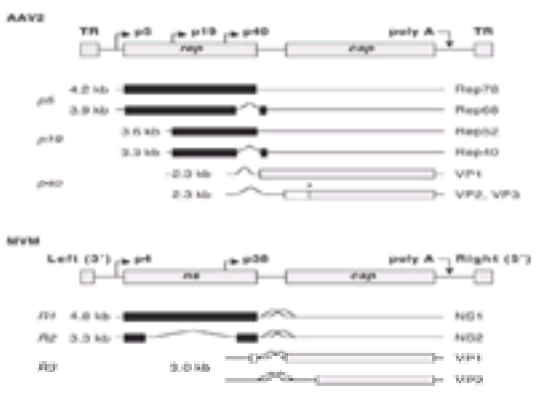

FIG. 3. Transcriptional maps of MVM, the prototype autonomous parvovirus, and AAV2, the prototype dependovirus. Terminal palindromes and ORFs are represented by *boxes.* The approximate positions of the promoters and polyadenylation sites in each genome are indicated by *arrows.* AAV has three promoters, at map positions 5, 19, and 40; MVM has two promoters, at mps 4 and 38. All AAV and MVM transcripts are co-terminal at a polyadenylation signal near the right end of the genome. Below each genome are the major mRNAs synthesized by each virus along with (on the left) their common names and their sizes in kilobases. At the right of each message is (or are) the viral protein(s) translated from each mRNA.

Coat Proteins

Both autonomous parvovirus and AAV virions contain three capsid proteins [with the exception of Aleutian disease virus (ADV), B19, and simian parvovirus, which contain only two coat proteins] (21). For different species of autonomous parvoviruses, the proteins have approximate MWs of 80,000 to 86,000 (VP1), 64,000 to 75,000 (VP2), and 60,000 to 62,000 (VP3). Those of lapine parvovirus are significantly larger: 96,000 (VP1), 85,000 (VP2), and 75,000 (VP3). Except for ADV, VP3 is the major coat protein, representing 80% to 90% of the total mass. Some virus preparations are lacking in VP3, and its abundance appears to depend on the time during infection when the virions are isolated. LuIII

preparations, for example, may contain only VP3 in detectable quantities. All AAV preparations characterized to date have three coat proteins: VP1, 87,000; VP2, 73,000; and VP3, 62,000 (143). In neither genus is there any evidence for glycosylation of any of the coat proteins; however, all parvovirus proteins appear to be phosphorylated. The N terminus is blocked in the case of the AAV VP3 protein (19,143).

In addition to the fact that all three parvovirus coat proteins appear to be coded for by overlapping in-frame DNA sequences, a further complexity has been reported. In the cases of both AAV1 and AAV2, the coat proteins VP1 and VP3 can be further subdivided by polyacrylamide gel electrophoresis into several subspecies (191). The molecular basis for the difference in mobility is unknown.

PARVOVIRUS INFECTION

Host Range

Parvoviruses replicate in the nuclei of infected cells. Of all the DNA viruses, they seem to be among the most dependent on cellular function. The autonomous parvoviruses require the cell to go through S phase to replicate (269). Unlike the polyomaviruses and Ad, the parvoviruses do not have the ability to stimulate or turn on host DNA synthesis in resting cells. The autonomous parvoviruses simply wait for S phase in the host they infect. AAV relies on a helper virus, adeno- or herpesvirus, to induce S phase or its equivalent (11,38) (Fig. 4). (The nature of the helper factors is described later.) There are also cell lines that can be made semipermissive for AAV infection, without helper co-infection, by various chemical or physical treatments, described in the section on helper functions. The autonomous parvoviruses are relatively species specific, although some hamster viruses were first isolated as apparent contaminants from human tumor cells grown in the laboratory, and MVM will grow in both mouse and human transformed cell lines (73). In the case of MVM, tissue specificity has been observed for differentiated cells (262). The prototype strain MVM(p) replicates in murine fibroblasts but not in T lymphocytes. A second strain has been discovered, MVM(i), that displays the opposite phenotype.

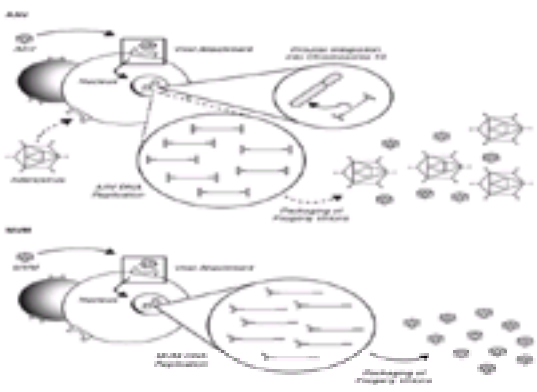

FIG. 4. Top: Replication of adeno-associated virus (AAV). Under nonpermissive conditions, AAV integrates into the q arm of human chromosome 19, where it remains silent until challenged by a helper virus (e.g., an adenovirus). This leads to rescue of the integrated virus from the chromosome and induction of the lytic cycle. Under permissive conditions (i.e., in the presence of a helper virus, such as adenovirus), AAV replicates, resulting in host cell lysis. **Bottom:** Replication of the autonomous *Parvoviridae.* After adsorption of the virus at the cell membrane of a variety of host cells, the virus enters and is transported to the nucleus. During S phase, the cellular replication machinery is recruited for viral replication, leading to virus production and cell lysis.

Genetic analysis of parvoviruses has been greatly facilitated by the discovery that clones of complete parvovirus genomes in bacterial plasmids are infectious when transfected into mammalian cells. This fact, together with the complete sequence of several prototype strains, notably MVM and AAV2, has made it possible to construct and propagate any mutant in a bacterial plasmid vector, and then to transfect the intact plasmid clone into cell culture. If the cells are permissive or (in the case of AAV) infected by helper virus, the parvoviral genes are expressed, the inserted genome is rescued from the plasmid and replicated, and infectious virions are produced. In this way, it has been possible to correlate specific phenotypes with specific mutations at fixed points along the genome, and thus it has been possible to develop meaningful genetic maps (125,244). The infectivity of parvovirus plasmids has also been the key to their development as mammalian transduction vectors (126).

Tissue Specificity, Viral Entry, and Cryptic Infection

Although dividing cells are required for the replication of autonomous parvoviruses, not all dividing cells are susceptible to, or permissive for, viral infection. In the case of MVM, susceptible cells have large numbers of surface receptors (10^5 or more). The nature of these receptors has not been defined; however, the receptors appear to contain sialic acid, as pretreatment of cells with neuraminidase prevents viral attachment (73). The surface receptors are missing from B lymphocytes. Additionally, MVM(p) is restricted in T lymphocytes at the level of gene expression even though the virus can enter the cell; in contrast, the related variant MVM(i) is permissive in T cells (262). The host range restriction is caused by several mutations in the capsid (13). In other cells, transcription can occur but DNA replication is inhibited. The loss of permissiveness seen at the level of cell culture is reflected in the general resistance of older animals to infection in spite of the presence of rapidly dividing tissues.

The narrow host range of B19 virus (primarily erythrocyte precursors) is partly the result of the distribution of its receptor. The human B19 virus receptor is globoside, the blood group P antigen, which is found on only a few cell types (35). Some of the narrow host-range specificity can also be attributed to tissue-specific gene expression (162). Both

nonstructural (NS) and capsid genes have been implicated in the porcine parvovirus host range (281). In the case of MVM, NS2 has been specifically implicated in tissue specificity (198).

Parvoviruses frequently cause semipermissive (sometimes called restrictive) infections, which can imply a low level of viral multiplication in the intact host (73). At the level of cell culture, this seems best illustrated by the establishment of carrier cultures in which viral multiplication also occurs at a low level. The situation is the result of a low fraction of cells that are permissive, and it is plastic in that viral variants frequently arise that can infect the majority of cells in the culture (236). The reciprocal situation has also been observed, in which resistant cells arise from clones of susceptible cells. The frequency of semipermissive infection raises the question of whether the autonomous parvoviruses can cause latent infections by integration of the viral genome into cellular DNA as a provirus, as is seen with AAV. To date, no evidence for integration of autonomous parvoviral DNA during viral replication can be detected, nor has integration been observed during abortive infections.

Several receptors used by AAV2 for viral entry have been identified. These include heparin sulfate proteoglycan (267), the fibroblast growth factor receptor 1 (222), and the integrin $a_v b_5$ (266). Uptake of AAV by susceptible cells occurs rapidly (half-life, 10 min) through a standard receptor-mediated endocytosis from clathrin-coated pits (16). When the virus escapes from endosomes is not clear, but within 30 minutes the virus accumulates perinuclearly and within 2 hours virus particles are detected within the nucleus. It is still not clear whether uncoating occurs in the nucleus or at the nuclear pore.

DEPENDOVIRUSES

Overview

One of the outstanding features of AAV replication in cell culture has been the requirement for co-infection of the cell by an unrelated helper virus (see Fig. 4). Either an Ad or a herpesvirus can supply complete helper functions for fully permissive AAV infection (11,38). In the absence of helper virus, AAV can establish a latent infection in cell culture that involves integration of the viral genome into a unique site on human chromosome 19 (48,155,248). Superinfection of cell lines carrying a provirus with Ad or herpes will rescue the integrated genome and initiate a fully productive infection (48,169,190). In light of this, it has been assumed that AAV uses latency in chromosome 19 as a strategy to survive in the absence of a helper.

The situation during natural infections is not as clear. *In vivo* (i.e., in human infections), AAV has typically been found as a contaminant of Ad isolates (24). It is generally assumed, therefore, that natural AAV infections occur via the respiratory or gastrointestinal route as is the case for Ad. However, as yet, it is not clear what tissue or organ is a preferred site of latency in humans. Efforts by several groups to demonstrate latency in human lung samples have failed to detect AAV. AAV has been recovered from a small percentage (1% to 5%) of hematopoietic cells and from the genital tract of female patients suspected of herpes infections (106,117). One report (273) suggested that AAV was present in some first-trimester abortion material, but this has not been confirmed (106). However, AAV has been found at a surprisingly high frequency (20%) in muscle biopsies (Xiao, personal communication, 2000). Recently, Dutheil et al. (97) demonstrated that the chromosome 19 integration site that is preferred by AAV is linked to the slow skeletal muscle–specific gene troponin T1 (TNNT1), and the cardiac troponin I gene (TNNI3). Several proviral cell lines established *in vitro* were shown to contain rearrangements of the TNNT1 gene due to integration. Additionally, Samulski and his colleagues (Samulski, personal communication, 2000) have demonstrated that recombinant AAV preferentially transduces slow skeletal muscle fibers in rodent models. Together, these observations have led to the hypothesis that the natural site of AAV latency in humans might be skeletal muscle. Skeletal muscle is known to be resistant to Ad and herpes infection when these viruses are administered intravascularly, thus muscle could be a reservoir that is protected from rescue by AAV helper viruses. Because muscle fibers are multinucleated, integration of AAV and the possible disruption of the TNNT1 locus would produce minimal effects on the host. However, as yet, no one has demonstrated that AAV is integrated into the chromosome 19 target in human samples.

As discussed later, the role of the Ad helper functions seems in part to be the induction of the appropriate cellular milieu required for AAV DNA replication. The notion that AAV replication is possible once the appropriate cellular genes have been induced suggests that the appropriate cellular milieu could be established in the absence of helper virus. Indeed, several groups have reported that pretreatment of transformed or tumor cell lines with a variety of genotoxic agents can render the cells semipermissive for AAV replication in the absence of helper virus co-infection (307, and refs. therein). Agents that have been successfully used include ultraviolet (UV) irradiation, cycloheximide, hydroxyurea, aphidicolin, topoisomerase inhibitors, and several chemical carcinogens. Recently, Hermonat and colleagues (personal communication, 2000) have demonstrated that keratinocyte cultures can support AAV replication, albeit poorly, in the absence of a helper virus. In this system, basal cells are nonpermissive for replication but become semipermissive as the cells differentiate. The value of these semipermissive systems is that they may help identify cellular functions that are normally induced by the helper virus.

Helper Functions

Adenoviruses, herpes simplex virus (HSV) types I and II, cytomegalovirus, and pseudorabies virus all serve as complete helpers for AAV replication (11,38,197). The AAV host range is identical to the normal host range for the helper virus. Genetic analysis of helper functions has been most extensive for Ad. Many of the identified Ad early functions serve as helper functions for AAV replication, but no Ad late functions have been found to be necessary. Four Ad proteins have been shown to be required for complete helper function: the early region IA (EIA) transactivator protein, the EIB 55-kd protein, the E4 34-kd protein, and the E2A DNA-binding protein. Additionally, synthesis of the Ad virus-associated (VA) RNAs is required. Ad EIA function is required for the other Ad early regions to be transcribed (197). Similarly, an EIA function is required for AAV transcripts to be detected by Northern blotting (140,170,232). Two EIA proteins with overlapping sequences of 289 and 243 amino acids, respectively, have been identified. The former can both activate and inhibit gene expression in trans, whereas the latter primarily inhibits gene expression. The 289-amino-acid EIA protein is

responsible for transactivation of AAV gene expression (46). EIA, in concert with other Ad early genes, also induces a variety of changes in cellular gene expression. The most important may be that EIA induces cells to enter S phase and to synthesize cellular DNA replication proteins that are likely to be needed for AAV DNA replication (197).

E4 was originally identified as encoding a helper function specifically required for AAV DNA replication. The E4 product involved is a 34-kd protein that is the product of the E4 ORF 6 gene and it can form a complex with the 55-kd EIB protein during a productive Ad infection (135). Both the 55-kd and the 34-kd proteins have been shown to regulate the expression of AAV genes, possibly at the level of transport of mRNA to the cytoplasm (246). In a general sense, EIB has also been identified as being required for AAV DNA replication to occur, but it does not seem to have a consistent effect on AAV transcript accumulation (140, 170, 232, 246). More recently, studies of transduction mediated by recombinant AAVs (rAAVs) that carry marker genes have suggested that the E4 ORF 6 protein enhances the conversion of single-stranded input viral genomes to a duplex form by promoting second-strand synthesis (101, 102). Grifman et al. (115) have shown that the ORF 6 gene product promotes degradation of cyclin A and inhibits the kinase activity of cdc2. The net effect of these changes would be to stop the cell cycle in late S or G2. Because cellular DNA replication enzymes are induced during this phase of the cell cycle, this could in part explain the effect of ORF 6 on AAV DNA replication.

E2A encodes a 72-kd single-strand DNA binding protein (DBP) that is required for Ad DNA synthesis but that does not appear to be required for AAV DNA replication (39), although particle formation is greatly inhibited by certain E2A gene mutations (141). DBP stimulates transcription from AAV promoters (39, 45) and may be involved in mRNA transport and stability. In spite of the absence of genetic evidence that Ad DBP is required for AAV DNA replication, recent *in vitro* studies of AAV DNA replication using cell-free extracts suggest that Ad DBP is preferentially used for AAV DNA replication in place of the human single-strand DBP, replication protein A (RPA) (290). In contrast, the E2B region, which produces the Ad terminal protein, and the Ad DNA polymerase, both of which are directly involved in the process of Ad DNA replication, is not required for AAV replication. Finally, Ad VAI RNA has been reported to facilitate the initiation of AAV protein synthesis (139) by preventing the interferon-induced host-cell shutoff of translation.

Two detailed studies on the HSV-1 genes required for productive AAV infection have been reported. Unfortunately, the conclusions were somewhat different. The first study (193) identified the following HSV proteins as providing helper functions for AAV: ICP4 transactivator, DNA polymerase, ICP8 single-strand DBP, the origin-binding protein, and two of the three subunits of the helicase–primase complex (UL5, UL8). The second study (294) identified ICP8 and all three components of the helicase–primase complex as helper functions. It should be noted that the latter study used a heterologous promoter; normally, ICP4 function is required for expression of HSV replication genes. The former study found that maximal AAV replication required all the genes identified but that only ICP4 was essential for AAV replication per se. It is not clear why with Ad, most of the viral DNA replication genes are not required, but with herpes, most of the helper functions are replication enzymes.

Genetic Map

The AAV genetic map (see Fig. 3) has been derived primarily from studies of AAV2 (125, 190, 247, 252, 274) but is highly conserved among all of the AAV serotypes. There is a large ORF (cap) on the right side of the genome (mp 50 to 90), which encodes the three coat proteins of the virus. Frameshift and deletion mutants within this region do not block DNA replication, but the accumulation of progeny single strands is inhibited, presumably because this requires encapsidation (126, 274). Mutations in the N-terminal region of the cap ORF, which affect VP1 exclusively, package DNA but produce virus particles that have lower infectivity. There is also a large ORF in the left half of the genome (mp 5 to 40), which has been called the rep region because any frameshift mutation or significant deletion within the region blocks DNA replication (126, 274). At least four proteins have been detected (Rep78, Rep68, Rep52, and Rep40), which correspond to the four mRNAs that have been mapped to this region (see Fig. 3) (192). It is possible to selectively eliminate Rep78 and 68 (from the p5 transcripts), and this type of mutation completely blocks all AAV-directed transcript accumulation that can be detected by Northern analysis, and it completely eliminates DNA replication (126, 168).

The larger Rep proteins play a critical regulatory role in every phase of the AAV life cycle. Under nonpermissive conditions (no helper virus) Rep68/78 negatively regulates AAV gene expression and DNA replication and is required for site-specific integration in the host cell genome to establish a latent infection (148, 164, 253, 275). In the presence of helper, Rep68/78 is a transactivator of AAV gene expression (168) and is essential for DNA replication and rescue of the viral genome from the integrated state (125). Mutants that are defective for the synthesis of the two smaller Rep proteins (Rep52 and 40 from the p19 promoter) have been made by changing the initiator AUG for these proteins to GGG (47). The resulting mutant is able to replicate DNA, but no mature single-stranded DNA is encapsidated. The phenotype is thus similar to a coat protein mutant. Other smaller ORFs exist near the middle of the genome, but the production of proteins corresponding to these regions is less clear.

Mutations in either the rep or cap ORFs can be complemented in trans. In contrast, the inverted TRs of 145 bases are required in cis for both DNA replication and transcription (18, 252). In addition to these functions, the TR is required for encapsidation (190), integration of the genome during the establishment of a latent infection (309), and rescue of the genome from the integrated state (247). All of these functions are detailed later.

Transcription

In a productive infection, six polyadenylated, capped RNAs are detectable by Northern blotting (see Fig. 3) (171). In decreasing size, the mRNAs have apparent lengths of 4.2 kb, 3.9 kb, 3.6 kb, 3.3 kb, 2.6 kb, and 2.3 kb. These are synthesized from three conventional RNA polymerase II promoters at mp 5, 19, and 40 (114, 182). All of the RNAs are 3' co-terminal and are polyadenylated downstream from an AATAAA signal at mp 96 (264). The two largest transcripts (4.2 and 3.9 kb) are synthesized from the promoter at mp 5 (p5), the next two (3.6 and 3.3 kb) are from the promoter at mp 19 (p19), and the two smallest RNAs (2.6 and 2.3 kb) are from the promoter at mp 40 (p40). There is an intron at mp 42 to 46, and there are both spliced and unspliced mRNAs produced from all three promoters, hence the three pairs of

transcripts. The smallest RNA (2.3 kb) is the major species and is produced from the p40 promoter. It is used to translate all three capsid proteins. The 2.3-kb species actually consists of two alternatively spliced mRNAs (196). The major spliced species directs the synthesis of the major capsid protein, VP3, from a conventional AUG codon, and the minor capsid protein, VP2, from an upstream in-frame ACG codon. The minor spliced 2.3-kb message includes an upstream AUG codon that allows the translation of the entire cap ORF to produce the minor capsid protein VP1. No clear role has been established for the unspliced 2.6-kb message. The p5 and p19 promoters generate two mRNA species each (spliced and unspliced), which code for the four AAV nonstructural proteins, Rep78 and 68 from p5, and Rep52 and 40 from p19 (182,192). In the case of the p5 and p19 RNAs, the unspliced species is the major one, but the reverse is true of the p40 RNAs. During the course of infection, the unspliced p5 transcript, and the Rep78 protein coded by it, appear first (192). This is followed shortly by the synthesis of the p19 and p40 transcripts and the proteins they encode. Additionally, the spliced p5 and p19 RNA species accumulate later in infection than the unspliced species. This is apparently the result of a change in splicing that is caused by either Ad infection or Rep78 expression (276).

Several groups have mapped the regulatory signals that control AAV transcription (Fig. 5). Chang et al. (46) mapped two regulatory sequences in the p5 promoter. The first corresponded to a sequence in the Ad major late promoter activated by EIA (Ad MLP). The second was a sequence that binds the cellular transcription factor YYI. Two YYI sites were found, one upstream of the TATA box and one overlapping the p5 message start site. McCarty et al. (188) subsequently mapped a Rep binding element (RBE) between the TATA box and the downstream YYI site. There are four additional RBEs, two in each of the two TRs. Together, these elements seem to be responsible for controlling AAV p5 transcription (164,212). Regulatory sequences upstream of the p19 and p40 promoters have also been identified (see Fig. 5). In the p19 promoter, the proximal TATA box, two Sp1 sites, and a novel sequence that binds an as yet unidentified cellular factor, cellular AAV activating protein (cAAP), were found to be sufficient by mutation analysis for p19 transcription (214). In the p40 promoter, an Sp1 site and the TATA box were essential, and ATF and AP1 sites also may play a role (213). The regulation of AAV transcription is complex. This is apparently because following infection, the virus can choose one of two paths for persistence: It can either establish a latent infection or proceed through a productive viral infection. The switch for these two paths appears to be controlled primarily by the Ad EIA gene and the AAV *rep* gene, as discussed later.

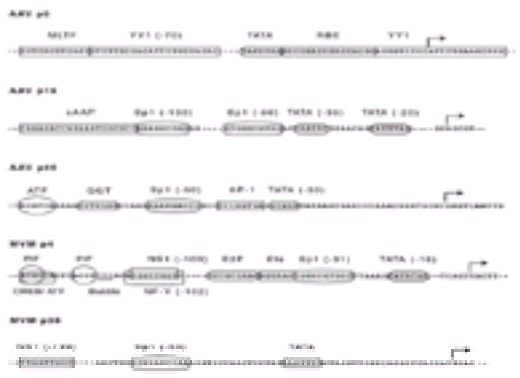

FIG. 5. Approximate position of promoter elements within the AAV2 and MVM promoters. *Arrow* indicates the start of the major mRNA from each promoter. See text for details.

In the absence of helper virus, only a small amount of Rep78 is synthesized from the p5 promoter. This is sufficient to bind to the p5 RBE and completely repress further p5 transcription (164,212). Mutation of the p5 RBE can lead to higher p5 expression and a virus that is partially permissive for DNA replication in some cell lines (288). Repression is tight, so that generally it is not possible to detect p5 transcripts or Rep protein. However, it is possible to detect *rep* gene function biologically because Rep inhibits expression from a number of heterologous promoters (122,128,134,167). Although the domain of Rep78/68 that binds to the RBE has been mapped to the region of Rep78 that is not present in Rep52 and 40 (210), the p19 Rep proteins are also capable of repressing the p5 promoter (163). Furthermore, although there is no strong RBE within the p19 promoter, Rep52 is capable of repressing p19 transcription (134,163). This suggests that binding to the RBE at p5 is not the sole mechanism by which Rep proteins can repress transcription. Indeed, studies of the papilloma virus transcription control region and the human immunodeficiency virus (HIV) long terminal repeat (LTR) suggest that Rep can repress transcription from these heterologous promoters, which have no RBE, by interacting with a variety of transcription factors (134). Although there is active repression of p5 and p19 promoter activity by the Rep proteins, repression of p40 has been difficult to demonstrate. Yet there is little accumulation of p40 transcripts or capsid protein in the absence of Ad infection (169,186).

In the presence of Ad co-infection, a cascade of transcriptional regulation seems to rapidly induce the synthesis of all AAV genes. First, the Ad EIA transactivator protein induces transcription from the p5 promoter and synthesis of Rep78/68. The mechanism of EIA transactivation of p5 is not clear, but it may involve interaction with YYI, which can function as a transactivator or a repressor (254). Additionally, Rep may interact with the TATA binding protein (TBP) or Rep may be displaced from the p5 RBE by YY1, thereby derepressing the p5 promoter (127,164). Rep78/68 in turn now behaves like a transactivator protein rather than a repressor and induces transcription from the p5 and p19 promoters (168,212,293). To accomplish this, Rep apparently needs to be bound to the RBE that is present either in the TR or at the p5 promoter, as the absence of both elements reduces transactivation of p19 and p40. Curiously, mutations in the p5 RBE continue to derepress p5 transcription (212), suggesting that Rep bound to the p5 RBE continues to repress p5 even while it transactivates p19 and p40. The absence of the RBE within the TR, however, leads to lower p5 activity, suggesting that Rep bound to the TR transactivates the p5 promoter as well as the p19 and p40 promoters (18,212). Finally, transactivation of the p19 and p40 promoters and repression of p5 appears to be coordinately regulated by the p5 RBE and Rep protein (186,212,213 and 214). Deletion of the p5 RBE element (and the TR) simultaneously increases p5 transcription and decreases p19 and p40 transcription. The elements in p5, p19, and p40 required for this coordination have been partially mapped (see Fig. 5). p19 Transcription requires the p5 or TR RBE, as well as the –50

p19 Sp1 site and the cAAP site upstream of p19 (214). Transactivation of p40 requires the same elements required for p19 transactivation and the proximal –50 Sp1 site upstream of the p40 start (213). How this is accomplished is not clear, but Rep has been shown to bind to Sp1 (214), TBP (127), and PC4 (292), a general transactivation protein for pol II transcription. Presumably, some or all of these Rep interactions are involved.

Several other regulatory circuits have been uncovered: (a) Rep52/40, which represses p5 in the absence of Ad, derepresses p5 in the presence of Ad, possibly by interacting with the larger Rep proteins (212). (b) Overexpression of Rep78/68 reduces the level of capsid protein by a posttranscriptional mechanism, possibly by reducing translation of the capsid message (276). (c) At least one cis-active negative regulatory sequence has been mapped to a region between mp 28 and 37 (168). Deletion of the sequence leads to higher levels of the upstream transcripts, and insertion of the sequence downstream of the p40 promoter reduces p40 transcript levels. Whether this cis-active negative regulatory sequence inhibits transcript elongation or adversely affects transcript stability is not known.

The net effect of these positive and negative feedback loops appears to be the maintenance of a constant ratio of Rep78/68, Rep52/40, and the three capsid proteins during productive infection. Why it is critical to maintain this ratio so tightly is not entirely clear. One possibility is that overexpression of capsid proteins might prematurely deplete the pool of replicative intermediates required to sustain a productive infection. On the other hand, overexpression of the Rep proteins might increase the replicative pool of DNA at the expense of packaging.

DNA Replication

AAV DNA replication occurs via a single-strand displacement mechanism (Fig. 6) (120,265). No evidence has been found for RNA primers or for the equivalent of Okazaki fragments (the presence of which would be indicative of lagging strand synthesis). Because all known DNA polymerases have a requirement for a primer with an available 3' OH, in addition to a template, linear DNA genomes have had to evolve specialized terminal sequences to allow them to maintain the 5' terminal sequences intact when the primer structure is resolved during replication. The basic model for parvovirus DNA replication (often called the rolling hairpin model) was first described by Straus et al. (265) and Tattersall and Ward (272). As noted earlier, the two ends of AAV are identical in sequence and palindromic. The palindromic inverted TR at the 3' end of either strand can form a hairpin to serve as a primer to initiate synthesis of the complementary strand (181). This produces a linear duplex molecule in which the original 3' end is covalently closed in the hairpin configuration, also called a monomer turnaround form (see Fig. 6, mT). The hairpin is nicked at a site near the end of the terminal palindrome (nucleotide 124 of the AAV sequence), and this produces a new 3'-hydroxyl primer that allows repair synthesis of the hairpin. This process is called terminal resolution (or strand transfer) and the site-specific and strand-specific nicking site is called the terminal resolution site (trs). The net result of terminal resolution is the first complete duplex molecule in which both ends are extended, the monomer extended form (see Fig. 6, mE). Either end can then denature and reanneal to form a double hairpinned structure, or rabbit ears (a process sometimes called reinitiation), and the newly hairpinned 3' end initiates leading-strand displacement synthesis to generate a single-stranded genome (which is packaged) and a duplex genome covalently closed at one end. The process of terminal resolution and reinitiation is then repeated (see Fig. 6, gray box). Each time the cycle is completed, a new single-stranded progeny strand (see Fig. 6, ss) is generated, and the strand transfer process that occurs during terminal resolution inverts the TR. If a molecule covalently closed at one end is not resolved before completion of strand displacement from the other end, then a dimer molecule can be formed in which two genomes are present in an inverted orientation (head to head or tail to tail) with a single TR at their junction (see Fig. 6, dimer turnaround or dT).

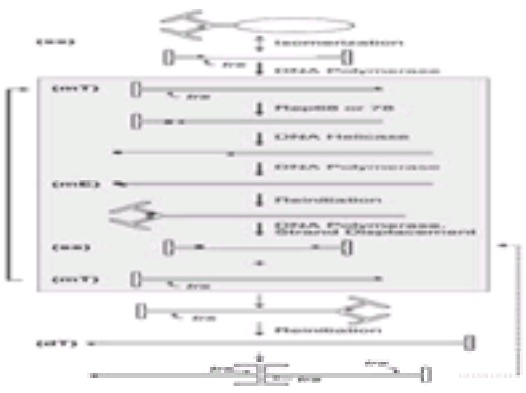

FIG. 6. Model for AAV DNA replication. *Gray box* highlights the reactions essential for generating single-stranded progeny DNA. mT, monomer turnaround RF; dt, dimer turnaround RF; ss, single-stranded viral DNA; mE, monomer extended RF. See text for details.

A large amount of evidence now supports this mechanism. All of the predicted replicative intermediates have been found *in vivo* (201,265) and can also be generated with *in vitro* replication systems (201). These include monomers and dimers, with both ends extended or with one end covalently closed (201,265). Two orientations of the TR (flip and flop) are seen in replicative intermediates and in packaged DNA as predicted (180,244). Biochemical analysis of the Rep protein carried out by several groups *in vitro* also supports this mechanism. This has shown that Rep78/68 protein carries out the site-specific nicking reaction necessary for terminal resolution and has a DNA-DNA (and DNA-RNA) helicase activity and an ATPase activity, that is essential for nicking and probably for strand displacement (138,260,302).

The outstanding feature of the TR is the T- or Y-shaped structure that is formed when the terminal 125 bases are folded on themselves to optimize base-pairing (181). This structure is quite stable and appears to exist predominantly as B form DNA. Recent thermal melting and UV spectra analysis suggests a DH_{cal} of 614 kcal mol^{-1} (226). Thus, the T-shaped structure is likely to need the active intervention of a DNA helicase to unwind during terminal resolution and to re-form

during the reinitiation step following resolution. Another issue is that the nicking site (*trs*) is present in both the hairpinned and linear extended forms of the TR, as well as in the linear junction between two genomes within a dimer molecule. Thus, some method for discriminating between these three forms must exist that allows nicking preferentially on the hairpinned substrate and the dimer, rather than on the linear extended TR, in which nicking appears to be minimal (50,188).

Mutational analyses of both the Rep coding sequence and the TR, as well as the development of a number of *in vitro* biochemical assays, have led to a good preliminary understanding of the terminal resolution reaction (see Fig. 2). The TR contains at least three specific sequences that direct the Rep-mediated *trs* endonuclease reaction. A 20- to 22-bp linear sequence, the RBE (also called RRS or RBS), within the stem of the T structure is sufficient to bind a Rep78 or 68 complex that consists of two to six Rep molecules (50,188,241,259,295). Most of the RBE sequence consists of a tandem $(GAGC)_4$ repeat. As few as two GAGC repeats are sufficient to bind Rep, and considerable heterogeneity in the RBE is tolerated (51,188,303). One of the functions of the RBE is apparently to assemble the Rep complex in a particular orientation that is subsequently required for *trs* endonuclease activity. Substrates that contain inversions of the RBE, for example, will bind Rep but are poorly nicked (32). The enzyme also makes contact with a GTTTC motif that is present in the small internal palindrome that is furthest away from the *trs*. This sequence motif, called the RBE' (or RRS'), is present in both the flip and the flop orientations in the same position with respect to the *trs* (see Fig. 2) (32,241). Contacts with this motif as well as with several other bases within the small internal palindromes apparently account for the difference in binding affinity between the hairpinned and linear forms of the TR, which has been estimated to be as much as 100-fold (189,259), and the fact that the linear form of the TR is nicked with a lower efficiency than the hairpin (32,50,188,259,304). DNase protection and chemical interference assays suggest that the enzyme complex bound to the RBE in the absence of ATP does not actually make contact with the *trs* site (137,241).

Once bound to the TR, Rep carries out two sequential reactions. First, the DNA helicase activity of Rep unwinds the stem of the TR in a reaction that requires ATP hydrolysis and generates a single-stranded *trs* site (31,83,259); this explains why the *trs* endonuclease reaction is ATP dependent (138). A highly conserved inverted repeat sequence that flanks the nick site is probably used to form a cruciform structure, which brings the *trs* closer to the Rep complex that is still bound to the RBE (31,32). Evidence that Rep does not let go of the RBE prior to nicking comes from mutants in which the spacing between the RBE and the *trs* is increased; insertion of even three bases significantly reduces nicking activity (32). In the second step of the reaction, the endonuclease active site carries out a transesterification reaction, in which the tyrosine at amino acid position 156 of Rep is covalently joined to the 5' phosphate end of the nick at the *trs* (83,138,257). This reaction is ATP independent. Y156 is part of a rolling circle replication (RCR) protein motif that is common to all parvovirus NS proteins and is similar to motifs found in many enzymes of bacterial and eukaryotic origin that nick the origin of plasmids that undergo rolling circle DNA replication (136).

The *trs* is also a specific sequence recognized by Rep. It consists of a seven-base sequence (3'CCGGT/TG5') that is recognized only on the correct strand (31). Nicking occurs between the two Ts (138), and mutation of any of the seven bases on the nicked strand reduces nicking (31). Changes in the sequence of the complementary strand, however, have no effect on nicking. Thus, the AAV origin for DNA replication is a tripartite sequence that consists of the RBE, the RBE', and the *trs*.

Several groups have clearly demonstrated that the transesterification reaction does not require ATP. The best evidence is that Rep will nick a variety of substrates in an ATP-independent reaction, provided that the RBE is duplex but the *trs* is single stranded (259). Generally, these substrates lose some specificity in the nicking site. However, recently Brister and Muzyczka (31) have constructed what appears to be the actual intermediate for nicking by deleting the *trs* sequences on the complementary strand, thus artificially extruding the *trs* into a stem-loop structure that contains the *trs* in a single-stranded loop. This substrate is cleaved in an ATP-independent fashion only at the correct nicking site; moreover, the substrate is nicked more efficiently than the conventional hairpin. Most recently, Rep also has been shown to nick a completely single-stranded substrate that contains only one strand of the RBE and the *trs* (257).

Additional evidence for the two-step *trs* endonuclease reaction comes from mutagenic analysis of the Rep coding sequence. Rep contains two domains that can be functionally separated. The C-terminal end that is present in Rep52 and 40 contains the Rep-associated DNA helicase activity (256). Rep52, like Rep78 and 68, has full DNA helicase activity when tested on primed single-stranded DNA substrates and behaves like a conventional 3' to 5' DNA helicase (256,316). In contrast, C-terminal truncations of Rep78, which retain the N-terminal sequences that are not present in Rep52, have no helicase or ATPase activity, but they are capable of binding to the RBE and they nick substrates that contain a single-stranded *trs*—that is, substrates in which the DNA helicase activity is not necessary to generate a single-stranded *trs* (83). Thus, the N-terminus of Rep78/68 contains most of the amino acid residues that are required for specific binding to the RBE and nicking the *trs*.

Unlike conventional RCR proteins, there does not appear to be an unlinking step for the Rep tyrosine phosphate bond with DNA. The Rep protein continues to be joined to the 5' end of the nick through subsequent steps of DNA replication, and it apparently remains attached during packaging of the progeny strand (96,161,219,300). Mature, highly purified virus particles, however, do not appear to have significant quantities of Rep protein (161,317); thus, some mechanism exists for hydrolyzing the tyrosine phosphate linkage that has not yet been discovered.

Once the terminal hairpin is nicked, it is believed that the Rep helicase unwinds the hairpin and a cellular DNA polymerase copies the TR to form an extended terminal sequence. Evidence for this comes primarily from *in vitro* reactions in which Rep is the only helicase available (138). The possibility that a cellular helicase or a viral-encoded helicase (in the case of herpesvirus) participates in the reaction has not been excluded. Rep helicase is then most likely responsible for unwinding and refolding the duplex end to form the double-hairpin TR that is needed for further DNA synthesis. Evidence for this comes from *in vitro* assays in which cell-free extracts are used (289). By themselves, the extracts replicate linear AAV templates poorly. When purified Rep is added, these extracts become competent for AAV DNA replication. This suggests that Rep78/68 can induce the formation of the double-hairpin substrate required to initiate strand displacement synthesis. That Rep is capable of initiating the double-hairpin intermediate is also supported by characterization of its DNA helicase activity using defined substrates. Simple linear oligonucleotides appear to be poor

substrates for Rep helicase, but oligonucleotides that contain an RBE can be unwound efficiently (316). Thus, the extended duplex TR, which contains two RBEs, is an efficient substrate for Rep helicase activity.

Both uninfected and Ad infected cell-free extracts have been used to replicate AAV DNA *in vitro* in a Rep and AAV ori-dependent manner. Typically, these extracts are supplemented with Rep78 or 68 purified from baculovirus, vaccinia, or bacterial clones, and study of these *in vitro* systems has provided additional information about AAV DNA replication. In general, Ad-infected extracts are significantly more active for *in vitro* AAV DNA synthesis (131,202,289). The block in DNA synthesis when uninfected extracts are used is not in the terminal resolution reaction but rather in the elongation reaction during strand displacement synthesis (202,289). Ward et al. (290) have presented evidence that the Ad-encoded DBP increases the processivity of AAV replication *in vivo*, allowing more efficient strand elongation. In spite of the fact that deletions of DBP are apparently viable for AAV DNA replication *in vivo* (39), DBP is preferentially used in place of the host single-strand binding complex, RPA, in the *in vitro* reaction. However, adding Ad DBP to uninfected extracts does not raise the level of DNA replication to the same level seen with an Ad-infected extract, suggesting that there are additional factors in an Ad-infected extract that are needed.

Ni et al. (201) have also examined AAV DNA replication in uninfected extracts. This group found conditions for stressing cells that apparently produced cell extracts that were permissive in the absence of Ad infection. Such conditions might be a model for the semipermissive *in vivo* AAV replication that has been demonstrated by several groups. As expected, Ni et al. (201) found that AAV DNA replication did not depend on DNA polymerase alpha–primase but was dependent on the presence of RPA, proliferating cell nuclear antigen (PCNA), and replication factor C (RFC). PCNA is a processivity factor that is essential for DNA polymerase delta and epsilon, both of which are believed to be involved in replication of cellular DNA. Epsilon was originally identified as a DNA repair enzyme. Both polymerases are essentially leading-strand DNA synthesis enzymes, and either would be appropriate for AAV DNA replication. RFC is a 3' primer recognition enzyme that assembles PCNA at a 3' hydroxyl. Reconstitution of the *in vitro* reaction with one or both purified polymerases, however, did not restore AAV DNA replication, suggesting again that there is an additional unidentified cellular factor necessary for AAV DNA replication.

At least two other cellular factors have been implicated in AAV DNA replication. The first is high mobility group protein 1 (HMG1), a protein that binds cruciform DNA (65). This protein has been shown to bind AAV hairpins and Rep. HMG1 stimulates Rep nicking of the TR, Rep ATPase activity and Rep repression of the p5 promoter. It is, therefore, likely to be directly involved in DNA replication. The second factor is a protein that binds specifically to one strand of the D sequence within the AAV TR (see Fig. 2), hence the name single-stranded D binding protein (ssD-BP) (183,223). This protein exists in two phosphorylation states. When bound by the phosphorylated form, the input single-stranded AAV genome is unable to synthesize the second strand to form the first replicative intermediate. The protein, which appears to be the 52-kd FK506 binding protein, FKBP52, binds to the 10 bases of D sequences (see Fig. 2) proximal to the *trs* and prevents DNA replication. When the nonphosphorylated form of the protein binds, second-strand synthesis proceeds normally. Phosphorylation of the protein appears to be controlled by the epidermal growth factor receptor protein (EGF-R) tyrosine kinase and correlates well with the ability of cells to be transduced by recombinant AAV vectors in the absence of helper virus. This is because the input vector single-stranded DNA must synthesize the second strand to produce a substrate for RNA polymerase II transcription. The role of this protein in productive infections (i.e., when helper virus is present) has not been reported.

Three types of substrates have been used for *in vitro* DNA replication. The first is a linear AAV duplex molecule, which is covalently cross-linked at both ends by the hairpinned form of the TR, a substrate called NE (no end) DNA. In this assay, Rep 68/78 protein is required, as is an extract from cells infected by Ad (202). The major product is linear duplex AAV DNA containing one newly synthesized strand and extended ends. Extracts of uninfected HeLa cells do not support replication, even if supplemented with Rep68. A second assay has been described (49,131,132,291) in which the substrate is either linear duplex AAV DNA or the same DNA contained in a bacterial plasmid vector. Again, Rep68/78 is required and an extract from uninfected HeLa cells does support replication. When the template is the plasmid construct, replication leads to rescue of the AAV insert from pBR322 and, in some instances, pBR322 gets replicated in addition to AAV. Whether the vector sequences are preferentially replicated as opposed to the AAV sequences appears to depend on the presence of Ad DBP.

An important observation is that rescue of the AAV insert from bacterial plasmids *in vitro* can be separated from the initiation of DNA replication in terms of template sequence requirement. Deletion of the terminal 55 bases from both TRs in the plasmid construct prevents rescue of the AAV insert but allows the entire construct to be replicated. The implications of this are that ori function does not require the entire TR and that a hairpin per se is not required for DNA replication (131). The minimal requirements for this reaction are an RBE and a *trs*. Replication in this case appears to be similar to the *in vitro* replication seen with plasmids that contain a copy of the chromosome 19 integration sequence (280). These plasmids have been shown to undergo a rolling circle type of replication in the presence of Rep and cell-free extracts, and a nick at the *trs*-like site within chromosome 19 apparently initiates replication. In both cases, nicking by Rep appears to occur because of the presence of an RBE and a nearby *trs* without an RBE' or significant secondary structure. *In vitro* assays using such substrates have demonstrated that Rep can both bind and nick substrates that do not have an RBE' or the cross-arms of the AAV hairpin (50,188). This reaction proceeds 20- to 100-fold less efficiently than nicking of the complete hairpin and may be an important aspect of the mechanism for AAV integration or rescue from human chromosome 19. There is evidence as well that rescue of AAV does not require Rep or DNA replication. One group identified a cellular enzyme, endo R, that recognizes potential triple-stranded DNA in the TR and excises AAV from recombinant plasmids (113). Another group has shown that a plasmid containing a single TR with two D sequences, analogous to an internal TR junction from a dimer replicative form (RF) molecule (see Fig. 6), can be converted rapidly to linear molecules when transfected into cells in the absence of Rep expression (306).

Single-stranded AAV DNA can form circles that are hydrogen bonded through the 20-bp D sequence (see Fig. 6). This type of structure is believed to be responsible for the ability of AAV TR deletions to be repaired to the wild-type sequence (247). As described earlier, the cloned duplex form of the genome is infectious when transfected into Ad-infected cells. Clones with deletions of up to 113 bases at one end are still infectious, and the resulting progeny contain genomes with wild-type termini at both ends (247). The repair process is thought to involve (a) base-pairing between the remaining

complementary bases (32 base pairs in this case) and (b) use of the shorter 3'-terminal strand as a primer for repair synthesis, using the intact 5' TR strand as the template. Mutants with deletions that extend beyond the bounds of the TR cannot be rescued. Thus, not only does the TR help to resolve the problem inherent to linear genomes of maintaining the integrity of the 5'-terminal sequences during the process of DNA replication, but it also confers the ability to repair terminal deletions of the type described earlier.

Deletion of an 11-base symmetrical sequence (at nucleotides 50 to 60) from both TRs, thus effectively removing one of the cross-arms of the T, abolishes the ability of a cloned genome to be rescued and replicated (247). Substitution of an alternative 8- or 12-base symmetrical sequence restores viability to the cloned genome (172). No differences can be detected in either the rate or the extent of DNA replication that occurs after viral infection under permissive conditions. Substitution of an asymmetric 12-base sequence does not significantly restore viability (28). This observation has been interpreted to mean that, at least in the region of the cross-arms, the conformation of the TR, when folded on itself, takes precedence over the actual sequence in terms of rescue and replication of the genome from the cloned state in a plasmid. However, it is now known that the sequence deleted in these constructs was the RBE' motif that makes specific contacts with Rep. The RBE' has a specific three-dimensional location with respect to the RBE and *trs* in the hairpinned TR and this, in addition to the sequence of the RBE', may play a role in TR recognition by Rep. Thus far, it has been difficult to separate the role of the hairpin secondary structure from the RBE' sequence for Rep binding, helicase, and nicking activity. Recent experiments in which the RBE' sequence has been mutated without changing the overall structure of the TR suggest that the RBE' contacts are important primarily for initiating DNA helicase activity on the hairpin and are essentially dispensable for the transesterification reaction (32).

The viability of the symmetrical substitutions offers the possibility of testing whether or not the ability of the TRs to perfectly base-pair and form a single-stranded circle is a requirement for normal AAV DNA replication. Constructs in which a viable TR substitution is inserted in one end and not the other are found to be capable of being packaged. Under some conditions, the chimeras were able to undergo many rounds of replication and encapsidation without the occurrence of gene conversion (27). Similarly, packaged genomes derived from wild-type AAV are equally divided between those whose TRs at both ends are in the same orientation (flip or flop) and those with TRs in the opposite orientation (180). Thus, although the termini definitely can interact during the process of replication, TR interaction or gene conversion does not appear to be essential for replication.

As with many DNA viruses, AAV DNA replication generates defective genomes. These structures contain internal deletions flanked by TRs. In some cases, the defective genomes contained inverted duplications of sequences from either end of the genome (84,121).

Protein Synthesis

Four nonstructural proteins, Rep78, 68, 52, and 40, have been identified immunologically and purified from bacterial or eukaryotic expression vectors. They have been named according to their apparent MWs on sodium dodecyl sulfate (SDS) acrylamide gels, but their actual MWs are 72, 62, 46, and 34 kd, respectively. The amounts of the individual proteins correspond reasonably well to the relative accumulations of the individual transcripts. The two larger species are predominantly nuclear proteins, whereas the smaller ones, Rep52 and 40, are also found in the cytoplasm (192).

Various functions ascribed to the Rep proteins have been mapped along the primary structure. As noted earlier, Rep52 (amino acids 225 to 621 of the rep ORF) retains full helicase activity (256). The region unique to Rep78/68 plus a few amino acids shared with Rep52/40 (amino acids 3 to 251) are sufficient for nicking a single-stranded *trs* substrate (83), and a slightly smaller N-terminal region is sufficient for RBE binding (210). Efficient TR binding, however, requires sequences within the C-terminal region as well (187). A number of specific amino acids involved in TR binding have been mapped (82,110,187,279). The N-terminal region contains several motifs common to RCR proteins (amino acids 9 to 14, 89 to 94, and 146 to 156) (136) that are believed to be involved in nicking activity, and mutations in these motifs are defective in both *trs* endonuclease activity and integration at chromosome 19 (279). This includes the tyrosine residue at amino acid 156 that makes a covalent linkage with the *trs* site (83,257). C-terminal deletions (amino acids 490 to 621) appear to retain most Rep functions *in vivo* and *in vitro* (134,154). This includes the region of Rep52 and 78 that is spliced out in Rep40 and 68 (amino acids 529 to 621). However, repression of the p5 promoter and a heterologous promoter, the HPV18 upstream regulatory region, was most pronounced with Rep78, followed by Rep68 and Rep52. Rep40 repression was undetectable (134). Transactivation of the p40 promoter appears to be exclusively caused by either Rep78 or 68, with Rep68 having a more pronounced effect (293). A zinc-binding domain in the C-terminal region that is spliced out in Rep68 and 40 has been mutated; the mutant Rep78 protein no longer binds Zn *in vitro*, but the mutation does not change the ability of Rep78 to inhibit transcription from AAV or heterologous promoters *in vivo* (134). Rep is phosphorylated primarily on threonine residues, but these have not been mapped (62). As yet there is no clear evidence that Rep phosphorylation has an effect on any of its biochemical activities. A canonical ATP binding site is located at amino acids 334 to 349. Mutations in this region are defective for ATPase and helicase activity, DNA replication, and transactivation and repression of the p5 promoter *in vivo*; however, they retain some ability to repress the p19 and heterologous promoters. Curiously, virtually all mutants that are defective for helicase or DNA replication activity are also defective for transactivation; this has led to the suggestion that the helicase activity may be involved in transactivation (187). Mutants in other putative helicase motifs conserved among parvovirus nonstructural proteins eliminated helicase activity (187,284). A divalent cation binding site, apparently the one used for DNA helicase activity, has been mapped at amino acid 421 (110). Another divalent cation binding site in the N-terminus that is used for nuclease activity (presumably one of the conserved RCR motifs discussed earlier) remains to be definitively mapped. A nuclear localization region has been mapped to amino acids 483 to 519 (313). Rep 68/78 inhibits oncogenesis induced by a variety of carcinogens; mutants involving amino acids 25 to 56, 73 to 74, 164 to 165, and 257 to 346 had a significantly reduced capacity to inhibit EIA + *ras* transformation (312). Rep interacts with a variety of cellular proteins, but most of the interaction domains have not been completely mapped. Most of the N-terminal region (amino acids 1 to 174) is dispensable for interaction with PC4 (a transcription activator), as well as for inhibition of herpes virus replication (154,292). Rep–Rep interaction domains have been mapped to several specific regions (82,83,96,258), and Rep–capsid protein interaction has been mapped to amino acids 322 to 482 (96).

As mentioned earlier, all of the coat proteins, VP1 to VP3, are translated from two alternatively spliced p40 mRNAs (196). The most abundant of these messages is used to translate VP2 from a nonconventional ACG codon, and VP3 from a downstream AUG. VP1 is translated from the low-abundance spliced p40 message. All three proteins share the same ORF. Amino acid numbers below begin with amino acid 1 of VP1; VP2 begins at amino acid 138, and VP3 at 203. Why the first AUG in the predominant mRNA should be so far downstream is uncertain. However, evidence has been presented that *rep* activity can inhibit gene expression from the p40 promoter at the level of protein translation when a vector with a reporter gene under control of the p40 promoter is co-transfected with a plasmid containing *rep* into Ad-infected cells (275). Thus, it is possible that the long leader sequence in the major p40 spliced RNA from which VP2 and VP3 are translated serves to protect against this type of inhibition.

Expression of the structural proteins in insect cells leads to spontaneous particle self-assembly. VP2 was specifically required for self-assembly; either VP1 or VP3 could be omitted, but all three were required for infectivity (239). VP3, however, lacks a nuclear localization signal (NLS) (299); if a heterologous NLS is fused to VP3, it also becomes capable of self-assembly into empty particles (133). At least one NLS has been mapped to the N-terminus of VP2, amino acids 167 to 172 (133), but there appear to be redundant NLS sequences (305). As mentioned earlier, AAV2 uses heparin sulfate proteoglycans as a primary cell surface receptor (267). The heparin sulfate binding region of the AAV2 capsid protein has been mapped to two clusters, amino acids 509 to 522 and 561 to 591 (305). Several substitution and insertion mutants have been characterized in a preliminary way (305, and refs. therein). In general, insertions into putative loop regions are on the surface of the capsid and at least partially viable. The N-terminus of VP2 and a portion of the N-terminus of VP1 are also on the capsid surface. The N-terminus of VP3 and the C-terminus are internal to the capsid and essential for capsid structural integrity. One mutant (R432A) makes only empty capsids. Nonviable mutants tend to map to alpha-helix or beta-sheet regions, which are known to be necessary for capsid integrity.

Latent Infection

In the absence of a helper virus co-infection, the AAV genome is often integrated into cellular DNA to establish a latent infection that can be activated by a subsequent helper virus infection. AAV latent infection was discovered by Hoggan et al. (130) in the course of a federal project to screen primary cell lots intended for vaccine production. Although there was no immunologic evidence for AAV, upon challenge by Ad infection, 20% of the lots of African green monkey kidney cells and 1% to 2% of the lots of human embryonic kidney cells tested produced AAV. Thus, AAV latent infection *in vivo* appeared to be common. Although most human transmission of AAV appears to be horizontal, evidence for vertical germline transmission of avian AAV has been reported in chickens (23). Human cells in culture can be latently infected simply by infecting with a high multiplicity of AAV (multiplicity of infection of 10 to 1,000) in the absence of helper virus (126,169). The frequency of positive clones (estimated either by isolation of single-cell colonies or by selection with recombinant vectors carrying a *rep* gene and a selectable marker) suggests that up to 10% of the infected cells become latently infected (126,169). These figures exceed those seen with other DNA animal viruses and approach the efficiency of lambda bacteriophage lysogeny and rescue.

Characterization of AAV DNA in latently infected clones shows that the viral genome is in most cases integrated into cellular DNA (48,169). The junction with cellular sequences is usually within the TR, but the site of integration within the viral TR is not unique. Recombination junctions occur throughout the TR sequences and the p5 promoter region (155,309), and portions of the TR are deleted. The viral DNA in most latently infected cell lines is integrated as a tandem (head-to-tail) array of several genome equivalents (48,169,190). Inverted (tail-to-tail or head-to-head) arrays have also been seen but occur at a much lower frequency (155). The presence of TR deletions raises the question of how the AAV provirus is rescued. Presumably this occurs by excision of an internal tandemly repeated genome that is intact. Indeed, the junctions between tandem repeats appear to be intact, consist of a single TR flanked by two D sequences, and correlate with rescue (94,190,306).

It is not known whether deletions of the TR occur during integration or during subsequent passage of the cells. In one clone of latently infected cells that was followed for over 100 passages, the restriction patterns obtained, using enzymes that cut in the unique sequences, were unchanged, but significant changes were noted using a restriction enzyme that cuts exclusively in the TR (48). This raises the possibility that the TR is an unstable sequence. This notion was supported by the observation that after 100 passages, free copies of the AAV genome were present, even though all of the detectable sequences had been integrated into high-MW DNA in earlier passages.

Initially, it was concluded that integration was random with respect to cellular sequences because restriction analysis found that junction fragments of viral and cellular DNA differed in size in every independently derived clone carrying a provirus. It is now clear that integration occurs at a specific site on human chromosome 19q13.3-qter (157,158,248). Integration is by nonhomologous recombination, although there are four to five base homologies at the site of recombination, and integration is often associated with rearrangements and inversions of both viral DNA and cellular sequences (156,309). Although integration is site specific, it is not specific at the individual nucleotide level but occurs within a region of several hundred nucleotides (156,248,309). Additionally, only about 70% of the integration events appear to occur on chromosome 19.

The preintegration site has been isolated as an 8-kb fragment, of which the first 4 kb have been sequenced (156). The sequenced region, called AAVS1, contains all of the known sites of recombination. The average base composition is 82% GC in the first 900 bases, which are near the recombination sites. Toward the 3' end of the AAVS1 sequence, there is a tandem repeat of 10 copies of a 35-mer minisatellite, which occurs at 60 places in the human genome, all of which are on 19q. There are also a large number of direct repeats in which there is an 11/12 base match. The first 900 bases fulfill the criteria for a CpG island, which could serve as a promoter for expression of the ORF found in the middle of the sequenced region. As noted earlier, Dutheil et al. (97) have recently identified two of the coding regions within this region. A gene proximal to the integration site codes for the slow skeletal muscle troponin T gene, TNNT1, previously mapped to 19q13.4. This gene is located approximately 15 kb to the telomere side of AAVS1, and integration into AAVS1

has been shown to cause rearrangements of TNNT1 in some proviral clones.

The most interesting feature of the preintegration site is that it contains an RBE and an abbreviated version of the *trs*. The sequences within the AAVS1 site necessary for site-specific recombination have been identified by the use of an extrachromosomal Epstein-Barr virus shuttle vector carrying the preintegration sequence. When human cells carrying the shuttle vector are infected with wild-type AAV virions, the viral genome preferentially integrates into the chromosome 19 target site. The critical region has been mapped to a 500-base fragment, which contains the RBE and a *trs*-like element (111). It was possible to dissociate the RBE from the sequences at which recombination occur in the latently infected cell lines. Thus, it appears as though the critical factor is a recognition site, and the actual recombination takes place several hundred bases distal to the recognition site. Additionally, any higher-order chromatin structure specific to chromosome 19q does not appear to be essential for site-specific integration. Site-specific mutagenesis has been used to further define the critical elements. Mutation of either the RBE or the putative *trs* site dramatically reduces site-specific integration (176), and these two elements appear to be the only essential elements required for integration.

Several groups have shown that Rep expression is essential for site-specific integration. Recombinant AAV (rAAV) vectors carrying a selectable marker flanked by two TRs, but deleted for the *rep* gene, do not integrate into chromosome 19 (148), whereas those that contain the *rep* gene integrate site specifically. Plasmids carrying an rAAV genome, when co-transfected with a Rep-expressing plasmid (or Rep protein) into human cells, will excise the rAAV genome and integrate it into chromosome 19 (253). The same occurs when human cells are infected with herpes, Ad, or baculovirus vectors carrying an rAAV genome, provided Rep is expressed (225, and references therein). Most recently, site-specific integration has been demonstrated in nonhuman cells. Mice do not have the AAVS1 preintegration sequence. However, when cells from transgenic mice carrying the AAVS1 locus are infected with rep^+ AAV, integration occurs in the transgenic S1 locus (233).

Biochemical evidence also supports a role for Rep in site-specific integration. Weitzman et al. (295) have demonstrated that Rep68 can bind to a plasmid containing a fragment of the preintegration site and can also serve as a bridge between the AAV TR and the chromosome 19 RBE. Thus, *in vitro*, Rep can bring the chromosome 19 target site and the AAV genome together through Rep-Rep contacts. As noted earlier, Urcelay et al. (280) have shown that plasmids containing the AAVS1 site can be nicked by Rep, which in turn initiates DNA replication. Recently, Smith and Kotin (257) have shown that Rep can both nick a *trs* and ligate the nicked DNA to another sequence in a second transesterification reaction *in vitro*. Dyall et al. (99) have also provided evidence for nicking and joining *in vitro*.

Integration in the absence of Rep generates proviral structures in cell culture that are essentially the same as those seen in the presence of Rep, with the exception that the site of integration is not chromosome 19 (93,190,240,309). (This includes the formation of head-to-tail repeats, deletions within the TR, rearrangements of cellular DNA, and microhomologies at junctions between cellular and viral DNA.) The efficiency of Rep-minus integration is variable and seems to depend on the cell line under study, but it can be as high as 80% (190). This suggests that cellular enzymes are largely responsible for recombination, and Rep is essential only for site specificity. The only AAV related sequence that is necessary for integration seems to be the TR, which is recognized by the cellular machinery as a recombination or amplification signal (309).

No clear consensus model has emerged thus far for integration. The key elements to explain are the tandem (head-to-tail) repeats, the high rescue frequency implying that at least one intact copy is always integrated, the role of Rep, the ability of Rep to rearrange the host target site even in the absence of an input AAV genome, and the lack of site specificity at the sequence level for either viral or host DNA. The existence of head-to-tail repeats prompted some to suggest that the integration intermediate is a circular AAV genome that undergoes RCR (169,190). Both AAV and chromosome 19 could be nicked at their respective *trs*s, exposing a TR end for illegitimate recombination to a random cellular sequence in the S1 site. This would explain the frequent occurrence of tandem repeats with intact internal TRs. Giraud et al. (112) have suggested that copy-choice replication mechanisms during RCR could account for the rearrangements and inversions that have been found in some proviruses. Dyall et al. (98) have suggested a deletion substitution method of recombination.

The need for *trs* nicking on the input genome apparently has been eliminated by Young and Samulski (314), who demonstrated that site-specific integration occurs even when plasmids carrying AAV with mutant *trs*s are transfected into human cells. The mutant *trs* is defective for Rep-mediated nicking or DNA replication, but it integrates into chromosome 19 at about the same frequency as wild-type DNA.

Evidence for the formation of circular intermediates comes from recent studies of rAAV transduction in cell culture and muscle tissue (94). These experiments are done in the absence of Rep expression but, as noted earlier, this produces similar proviral structures in cell culture. Persistent transduction appeared to correlate with the formation of circular rAAV intermediates, which could be recovered by subsequent transfection into bacteria. Previous studies had shown that the expression of the Ad E4 ORF 6 gene product or a variety of genotoxic agents would increase transduction or reduce the time required for expression of a transgene in cell culture and *in vivo* (101,102). This was interpreted to mean that a variety of agents could promote second-strand synthesis, which was required for transcription. More recent experiments suggest that either linear duplex or circular duplex intermediates are formed, depending on the type of stimulus—for example, UV light versus ORF 6 gene expression (249). The significance of this is not clear.

Perhaps the most surprising development in rAAV integration has been the realization that recombinant AAV genomes persist *in vivo* for extended periods of time (up to a year) as episomes. These are converted slowly from linear or circular monomer genomes to high-MW concatemers, and this may or may not be accompanied by integration (1,105,148,184,282). In addition, it is now clear that two different rAAV genomes will recombine to form circular or linear concatemers. Several groups have shown that this occurs frequently and can be used to reconstruct split genes that are infected into cells in two different vectors (95,268,308).

AAV as a Vector

AAV has received a significant amount of interest as a potential vector for gene therapy (41,126,245). There are a number of characteristics that contribute to the potential utility of the virus for this purpose. Recombinant AAV vectors can efficiently transduce a variety of nondividing, terminally differentiated tissues *in vivo*. These include cells of the eye, central nervous system, muscle, liver, and lung. Significantly, the vector genome persists for extended periods (up to 2 years in muscle, brain, and eye of rodents) with no apparent decrease in expression. In part, this is because essentially no pathogenicity or inflammatory response is generated by *in vivo* injections of vector. The cytotoxic T-lymphocyte (CTL) response also appears to be lower than that seen with most other viral vectors (20,52,129). This may be due in part to the absence of genes (other than the transgene) in the vector, or to the observation that AAV does not efficiently infect antigen-presenting cells (145). Long-lived expression is due also to the fact that foreign promoters appear to behave as expected in the context of an AAV vector. Tissue-specific and inducible promoters are expressed only in the appropriate cell types or in the presence of inducer and do not appear to be shut off by mechanisms that affect other types of vectors. Although the AAV TR is itself a TATA-less promoter (104), its basal level of expression is low and detectable only in some cell types. Expression from an rAAV vector typically takes 3 to 6 weeks to reach a maximum level (149,261). The slow onset is generally believed to result from the slow synthesis of the second strand (101,102).

Ex vivo gene therapy has also been investigated, particularly in hematopoietic cells. One group has successfully transplanted ablated mice with transduced hematopoietic stem cells and achieved long-term expression in recipients. However, it is clear that the efficiency of transduction is variable, and that it depends on several factors that have so far been poorly defined but may include the genotype of the donor cells (218), the phosphorylation state of ssD-BP (221), the ability of virus to traffic to the nucleus, or the presence of the appropriate cell surface receptors (222,266,267).

Permanent transduction in cell culture was first shown by Hermonat and Muzyczka (126). The vectors used in these studies deleted the capsid gene and substituted foreign DNA. This type of vector retains the *rep* gene and can integrate with high frequency into chromosome 19. The second type of vector, which simply puts an AAV TR on both ends of the foreign DNA (190), has received the most attention because it contains more room for the transgene, about 4.5 kb. This type of vector was the first to be used *in vivo* (103), and it is currently being used in two human trials for the treatment of cystic fibrosis and factor IX deficiency. Preliminary results from both trials have been encouraging (147,283).

The rAAV vector system has suffered from several disadvantages, some of which have now been addressed or eliminated. The small packaging size has been a problem for some transgene–promoter combinations. Two groups have recently showed that this problem can be overcome by the use of split genes that are cloned into two separate vectors (95,268,308). These are expressed *in vivo* from head-to-tail concatemers of the vector with the use of appropriate splice signals. Another problem has been the lack of effective methods for purification of rAAV virus, and this also has been addressed. There are now several new methods for virus production and purification that produce virus with low particle-to-infectivity ratios and low wild-type AAV and helper contamination (59,116,317). Scalable production methods have also been developed (59,63,108). Several problems remain, however. The slow onset of transgene expression eliminates rAAV as a vector for acute clinical applications. It is still not clear from biodistribution experiments whether rAAV permanently transduces germline cells *in vivo*. Finally, because rAAV integrates into some primary cells, the potential long-term risk from insertional mutagenesis needs to be assessed.

Inhibition of Helper Virus Replication and Heterologous Gene Expression

In addition to AAV gene expression and replication being affected by Ad or herpes co-infection, the course of Ad infection is also significantly affected by AAV co-infection (40,42,43). There is also evidence that Rep can directly or indirectly interfere with replication of other viral genomes, specifically SV40, papilloma, and herpes, as well as with replication of cellular DNA (123,154,166,310,311). In the case of Ad, the net effect is enhanced AAV gene expression and replication, with greatly reduced Ad gene expression and replication. These effects depend on the ratio of the multiplicities of infection of the two viruses and the temporal order of addition. AAV is effectively autoinhibitory because, if the ratio of AAV to Ad becomes too great, the inhibition of Ad gene expression is sufficient that it no longer provides the helper functions needed for AAV replication. By the same token, if AAV infection is delayed until Ad DNA synthesis has begun, AAV replication occurs but that of Ad is not inhibited. The inhibition appears to require *rep* gene expression because DI particles of AAV, which have very large internal deletions but intact TRs, cannot inhibit Ad replication (84). Whether the inhibition of Ad includes direct effects on DNA replication or simply a lack of early regulatory proteins is not known. This type of inhibition is not usually seen with herpesvirus co-infections (15), probably because the herpesvirus infection is so rapid, but it is seen when the *rep* gene is overexpressed (154).

Initially, infection by AAV in the absence of helper virus was not noted to affect the cell phenotype. More detailed studies of latently infected cells have indicated several phenotypic changes. These include decreased plating efficiency, evidence of cell cycle arrest, and increased sensitivity to a variety of genotoxic agents [e.g., UV, chemical carcinogens (286,287)]. Latent infection can also inhibit DNA amplification after exposure to genotoxic agents (298). AAV infection of the human leukemic cell line HL60 and immortalized keratinocyte lines led to reduced cell growth and reduced levels of detectable differentiation-associated antigens, plus altered expression of c- *myc*, c-*myb*, and c-*fos* (153). In the case of c-*myc* and c-*fos*, this appears to result from Rep repression of the cellular promoters (124). This may also account for the observation of Yang et al. (311), who have shown that the *rep* gene is cytostatic. Cells carrying an inducible *rep* gene were arrested for growth when the *rep* gene was induced and returned to normal growth in the absence of inducer. Even low constitutive levels of *rep* expression increased cell doubling time.

AAV has also been reported to inhibit permanent cellular transformation by selectable markers under the control of the SV40 early promoter, the HSV type 11 thymidine kinase promoter, and the inducible murine metallothionein promoter. The level of inhibition was greater than 95% and depended on an intact *rep* gene. Under the conditions employed, the cells were not killed by *rep* gene expression, nor was the ability of plasmid DNA to integrate into cellular DNA decreased. Thus, it appeared as though the inhibition was caused by repression of gene expression. This notion was supported by

the observation that in co-transfections, the AAV *rep* gene was able to inhibit chloramphenical acetyl transferase (CAT) expression under control of the SV40 early promoter. These types of inhibitory effects have been observed in both murine and human cells (167,276), but the extent of inhibition was very dependent on the specific cells used.

Inhibition of Adenovirus, Herpesvirus, and Papillomavirus Oncogenicity

Oncogenic serotypes of Ad induce tumors in newborn Syrian hamsters. Co-infection of AAV with oncogenic Ad reduces the frequency of tumors and lengthens the induction time of those that do occur (152,185). AAV co-infection also inhibits the ability of Ad to transform cells. Ad-transformed hamster cells are also oncogenic in the newborn, and this oncogenicity was reduced by infection of the cells with AAV, although the growth rate in culture was unaltered (209). The primary effect appeared to be on the level of E1B expression in the transformed cells. de la Maza and Carter (85) reported that DI particles of AAV and DI genomes could inhibit Ad induction of tumors in hamsters. Because some of the preparations of defective particles contained only the termini of the AAV genome, it was suggested that the termini may be sufficient for inhibiting Ad oncogenicity. AAV has also been reported to inhibit the oncogenicity of HSV-II-transformed cells after infection (81). Not only was the incidence of tumor induction decreased after cells were injected into hamsters, but the induction period was lengthened. Possibly the most interesting result was that even in those animals that did develop tumors there was no evidence of metastasis. AAV has also been shown to inhibit transformation by bovine papilloma virus and human papilloma virus 16 (HPV16), and this inhibition is attributable to Rep (123,285). Rep-mediated inhibition appears to involve binding of Rep to an HPV16 promoter and direct interaction between Rep and the HPV E7 oncoprotein (128). Similarly, Rep inhibits cell transformation by the combined action of the E1A and human *ras* oncogenes (150).

The reports of AAV inhibition of oncogenicity have raised the question of whether AAV might inhibit oncogenicity in humans. More than 90% of adults are seropositive for AAV, suggesting that many carry AAV as a latent infection. According to the model hypothesized, latent virus might be activated by oncogenic virus infection and, once activated, inhibit oncogenesis. Several retrospective epidemiologic studies have been done, studying patients suffering from cervical carcinoma. Interestingly, in each study the patients were markedly deficient in antibodies to AAV compared to a group of matched controls (263, and references therein). These results raise the unusual possibility that a viral infection might, for once, be beneficial to the host.

AUTONOMOUS PARVOVIRUSES

Genetic Map

Like that of AAV, the autonomous parvovirus genome contains two large ORFs (see Fig. 3). The first covers much of the left half of the genome (approximately mps 6 to 42) and encodes two NS proteins, NS1 and NS2, from alternately spliced mRNAs that are initiated at mp 4 (see Fig. 3). Mutations within NS1 block viral replication and gene expression (229,230). Only NS1 is absolutely required for DNA replication. NS2-specific mutants are defective for capsid synthesis, gene expression, and DNA replication in murine cells but show variable phenotypes in human cells (36,68,174,198,199). The second large ORF occupies much of the right half of the genome (mp 45 to 90) and encodes the coat proteins (228,255). Up to three coat proteins have been detected in the virion. In the case of MVM, the smallest capsid protein, VP3, is generated in the intact capsid by proteolytic cleavage of VP2 (271). The amino acid sequences of VP1 and VP2 are identical except for additional amino acids at the N terminus of VP1, and they are synthesized from two alternatively spliced messages initiated at mp 38 (see Fig. 3). The VP1 message contains a second (smaller) ORF at the 5' end of the major right-hand ORF (165,194). Mutants altered in either the NS or coat protein ORFs can be complemented in trans. However, the palindromic sequences at both termini are required in cis for DNA replication and packaging.

Transcription

For most autonomous parvoviruses, three major size classes of polyadenylated transcripts have been identified (see Fig. 3). Two of these (R1 and R2) are initiated downstream of a promoter (TATA box) at mp 4 and extend to a point near the right end of the genome near mps 95 to 96, where a polyadenylation signal (AATAAA) is located. Both of these transcripts have an intron removed between mps 44 and 46, and one has a large intron removed between mps 10 and 39 (144,194,217). These two transcripts are translated to yield NS proteins NS1 and NS2, respectively (72,228). The other major size class (R3) is initiated downstream from a promoter at mp 38 and alternatively spliced between mps 44 and 46 to produce the coat proteins VP1 and VP2 (165,194). The region between mps 44 and 46 is unusual in that two pairs of donor and acceptor splice sites occur in a very small region. In all, three of the four possible splicing combinations that could occur in this region are observed in each of the three major mRNA size classes, R1, R2, and R3 (see Fig. 3), to produce a total of nine mRNA species that are made at detectable steady-state levels (144,194). Unspliced mRNA species are also a major portion of nuclear RNA found in infected cells. In the case of MVM, excision of the large intron and the ratio of the R1 and R2 messages depend on sequences within the small intron (mps 44 to 46). The role of the small intron in this process has been hypothesized to involve initial entry of the spliceasome (315). Improvement of the polypyrimidine tract of the large intron relieves the requirement for the small intron and makes prior excision of the small intron unnecessary for large intron excision. As an added factor, any mutations that insert stop codons in either exon will block splicing (200).

Significant variations of this general scheme occur. All transcripts of the autonomous human parvovirus B19 have been reported to initiate from the promoter at mp 6 (25,90,211). Potential internal polyadenylation signals (AATAAA) exist near the middle of several parvovirus genomes, and these sequences are used for polyadenylation in the case of ADV and B19 virus (6,211). An additional NS protein, NS3, has been identified in ADV, which is coded by an alternatively spliced left-side ORF transcript (58).

Both MVM NS1 and H1 NS1 (a closely related parovirus) are necessary for transactivation of their respective p38 promoters (92,230). In the absence of functional NS1, p38 mRNA is difficult to detect by most RNA detection methods. The transactivation domain in NS1 has been mapped to the C-terminal 129 amino acid residues (173). Three sequences

are essential for transactivation. The first is the tar element (transactivation response element, sensitive to NS1) or an alternative NS1 binding site–containing sequence (178,179,231). NS1 binds to the tar region in an ATP-dependent manner (53). However, because there are many NS1 binding sequences upstream of the p38 promoter (more than 100), mutations of the NS1 binding site within the tar region have not always shown a decrease in p38 transcription, presumably because of redundant NS1 binding sequences. Two other sequences are essential for transactivation, an SP1 site and the TATA box (5,179) (see Fig. 5). NS1 can bind to Sp1, and the general transcription proteins TBP and TFIIA in the absence of DNA (159,179), and, presumably, these interactions are involved in transactivation. However, the mechanism of transactivation is not yet clear.

Activation of p4 transcription by NS1 has been seen in LuIII, B19, and MVM (91,119), and it depends in part on sequences within the upstream terminal palindrome, presumably because they contain NS1 binding sites (see Fig. 5). Transactivation of p4 by NS1 essentially creates a positive feedback loop for p4 transcription, thereby creating a switch that commits the virus to lytic infection.

Several other elements necessary for p4 transcription have also been mapped in MVM (4,87,88,107,118,215). A TATA box and an Sp1 site at –29 and –45 bases relative to the p4 mRNA start (see Fig. 5) are essential for promoter activity (4). Additionally, because MVM requires cellular proteins that are expressed in S phase for productive infection, some mechanism must exist for sensing when S phase begins, because unregulated expression of NS1 is cytotoxic (235). This is apparently supplied by an E2F site (see Fig. 5) at –60 relative to the message start (87,88). Mutation of this site abolishes NS1 induction in S phase and produces a replication-negative phenotype that can be rescued only if NS1 is supplied in trans. The E2F transcription factor consists of a heterodimeric factor composed of E2F and DP family members. Their transcriptional activity is controlled during the cell cycle by binding to members of the pocket protein family (pRb, p130, and p107). Free E2F heterodimers are active for transactivation and are released at the G1–S transition as a result of phosphorylation of the pocket proteins by cell-cycle-regulated cyclin-dependent kinases. A second transcription factor family also is involved in sensing and responding to the cell cycle. Two cyclic AMP response elements (see Fig. 5, CREs), which are present within the left palindrome (at –130 and –160), and which bind the ATF/CREB family of transcription factors, activate the p4 promoter in growing and serum-starved cells but repress the p4 promoter in contact-inhibited cells (87,215). Promoter silencing in contact-inhibited cells is apparently mediated by the cyclin-dependent kinase inhibitor (CKI) p27, as overexpression of p27 reduces p4 transcription in growing cells. Thus, control of NS1 expression by the E2F and CREB families of transcription factors effectively couples MVM p4 transcription (and DNA replication) to the cell cycle.

Two other transcription factors participate in p4 control, NF-Y and Ets (see Fig. 5). NF-Y binds to an atypical site, CCAAC, which is present in the left TR (–100 upstream of p4) as well as at the internal dimer junction of MVM. Its mutation reduces p4 transcription approximately twofold (118). The Ets transcription factor binding site is located at approximately–50, just upstream of the Sp1 site, and it also increases p4 transcription (about threefold) (107). The Ets site appears to cooperate with the nearby Sp1 site in activating p4. Interestingly, both the Ets site and the CRE sites increase transcription from the p4 promoter in a *ras*-transformed cell (107,215). The increased NS1 levels in *ras*-transformed cells may account in part for the well established sensitivity of some tumor cells to MVM killing (235).

Negative regulation of p38 has also been observed (109,160), which can be caused by a cellular repressor. Evidence for regulation by RNA processing and protein stability has also been found (251). Another form of regulation that apparently occurs involves attenuation of transcription of the p4 promoter via an RNA polymerase pause site (*att*) between nucleotides 259 and 383. The *att* site contains a sequence that can form a cruciform in the DNA template (216, and refs. therein). Temporal regulation of gene expression has been reported for MVM (61), which is consistent with transactivation of the p38 transcript by the NS1 protein.

Regulation of gene expression is also a major factor in tissue specificity. The parvovirus HI NS2 gene regulates gene expression in a manner dependent on cell type; it does so by virtue of sequences in the 3' untranslated region (175). As noted earlier, a different type of host-range mutant is observed for MVM. Wild-type MVM(p) productively infects murine fibroblasts but not T lymphocytes. A mutant MVM(i) has the reverse phenotype. Both strains are replicated for one round after transfection. The genetic difference is in the middle of the capsid gene and represents several amino changes on the surface of the virion. However, simple binding to the cells is not affected. Thus, the physiologic lesion is somewhere between entry and uncoating (13).

DNA Replication

Like AAV, autonomous parvovirus DNA replication takes place in the nucleus. The location of replication, combined with the fact that the cell is required to go through S phase for replication to occur (227,269,301), suggests a very close relationship between viral and cellular DNA replication. Sequence analysis of the termini of virion and replicative forms of MVM DNA suggests a modified rolling hairpin model for autonomous parvovirus DNA replication (7,272). However, there are some important differences between the model for autonomous parvoviruses and the mechanism described for AAV. Like AAV, the autonomous parvovirus genome has palindromic ends. Unlike AAV, the two ends are not identical in size or primary sequence (9,228). Furthermore, unlike in AAV, only one of the two strands (the minus or noncoding strand) is packaged in most rodent autonomous parvoviruses. This places significant constraints on the model of autonomous parvovirus DNA replication (Fig. 7) as compared with the AAV model described earlier (see Fig. 6). The following is currently the consensus model for rodent autonomous parvovirus DNA replication, and it is drawn primarily from work done with MVM. However, there are still some significant steps that are not clearly supported with experimental evidence, so the model should be considered one example of how MVM might replicate its DNA to generate the species of replicative intermediates and packaged viral DNA that are seen in cell culture.

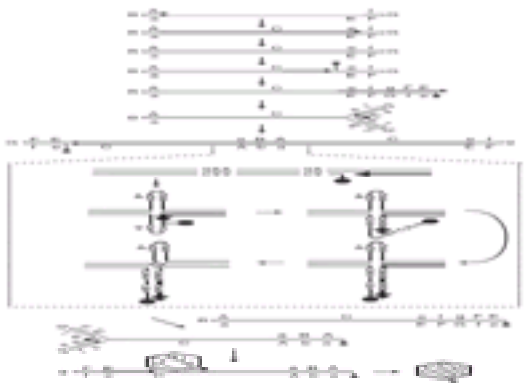

FIG. 7. Model for MVM DNA replication. See text for details. ABa, 3' terminal palindrome of virion strand; FGf, 5' terminal palindrome of virion strand; e, 18- to 26-nucleotide sequence present in replicative intermediates but not in DNA of nuclease-treated virions; V, virion strand; V^{par}, parental virion strand; V^{prog}, progeny virion strand; C, complementary strand; *heavy arrows*, new DNA synthesis; *solid circle*, site of nicks and NS1 covalent attachment in right end and dimer bridge. (The model was developed from the discussion in ref. 78, with permission.)

It is assumed that the 3' terminus (by convention the left end) of the virion strand folds back on itself to serve as the primer to initiate DNA synthesis of the complementary strand (see Fig. 7). When DNA synthesis reaches the hairpinned 5' end, it is believed that a ligation occurs to produce a linear molecule in which both ends are covalently joined—that is, there are no free 5' or 3' ends. Such molecules have been found *in vivo* (69). (They can be thought of as single-stranded dimer circles that have collapsed via base pairing to duplex linear molecules.) The 5' end (by convention the right end) is then nicked at a site that is inboard of the original 5' end of the parental molecule by NS1, which forms a covalent linkage with the 5' end of the nick. This generates a 3' OH that is used to repair the right end to a duplex extended form (see Fig. 7, steps 4 and 5). In the process, the right end undergoes a strand-transfer event, which inverts its sequence and results in the two orientations seen at the right end, flip and flop. Subsequently, the newly repaired extended right end forms a double hairpinned intermediate in which the 3' strand at the right end of the genome serves as a primer for further DNA synthesis (see Fig. 7, steps 6 and 7). Synthesis proceeds through the left end to form a duplex dimer molecule in which the left end (the original 3' end) is now in the form of a linear junction between two copies of the MVM genome that are inverted with respect to each other. The viral right ends, which flank the dimer, can continue to undergo terminal resolution and strand transfer, generating flip and flop orientations. The junction in the middle of the dimer is often called the left-left junction, the dimer bridge, or the 3' junction. Unlike the right end that exists in two orientations (flip and flop), the left end is found in only one orientation, called flip. Thus, a mechanism exists to maintain only one orientation of the left end and to generate only one strand of the viral genome for packaging. This is accomplished by asymmetric nicking at the internal left-left junction in a process called junction resolution (see Fig. 7, steps 8 to 12), which generates 3' termini in only one orientation (7,78). Thus, one of the key replicative species in MVM DNA replication appears to be an obligatory dimer intermediate containing a left end–left end junction. The products of bridge resolution have been shown *in vitro* (71) to be a monomer intermediate that is covalently joined at the left end and a monomer intermediate in which both ends are extended (see Fig. 7, step 13). The latter can undergo DNA replication from the right end (the 5' end) of MVM to generate the minus strand of the virus with the correct orientation at its 3' end (and either orientation at its 5' end) for packaging into mature virions. (This process could be repeated multiple times.) The former intermediate can also undergo DNA replication from its right end to regenerate a dimer intermediate with a left end–left end bridge.

The details of this model have not been completely settled but several lines of evidence support the keys steps outlined. Concatemers of dimer and tetramer length, as well as molecules covalently closed at both ends, have been detected in parvovirus-infected cells (69,270) as predicted by the model. Additionally, the expected bias toward left-left bridges has been seen *in vivo* (278). Biochemical characterization of NS1 also supports the model. In addition to having endonuclease activity, NS1, like the AAV Rep protein, is a DNA helicase and an ATPase (57,297). Whenever NS1 nicks, it forms a covalent linkage with the 5' end of the cut (12,74). Linkage is through a tyrosine residue at amino acid 210 of NS1 (204), which is part of the RCR motif (136). NS1 cuts the 5' (right end) at a position some 18 bases upstream of the sequence that is found in viral DNA (7), and it remains attached to the DNA during packaging. Mature MVM virus particles initially contain NS1 still attached to the DNA on the outside of the viral capsid (75). The protein is subsequently removed along with some of the 5'-end DNA, by nucleases in the culture medium or during cell entry. Recovery of the 18 bases missing from the right end presumably occurs when NS1 nicks the initial RF form (12,69), which is made *in vivo* and is covalently closed at both ends.

In vitro DNA replication studies using viral single-stranded DNA (or duplex RF) and cell-free crude extracts supplemented with purified NS1, have also confirmed key elements of the model (12,296). First, there is an apparent preference for formation of the linear duplex covalently closed at both ends from viral DNA, which is apparently the first RF intermediate made (12). Second, the left end is relatively insensitive to NS1 resolution when it is in the hairpin configuration (12); NS1 cuts the left end only when it is in the linear dimer bridge form. This is important because it accounts for why only one orientation of the left end (flip) is seen. Third, NS1 helicase activity is apparently responsible for unwinding and refolding the linear extended right end into the double hairpin that is used for initiation of DNA replication at several steps in the model (296). Unwinding is ATP dependent and NS1 mutants that have mutations in the canonical ATP binding site are inactive for the refolding reaction. Additionally, the TR isomerization occurs more efficiently at the right extended TR than at the left end in the extended configuration (12). This means that initiation of strand-displacement DNA synthesis is initiated preferentially at right ends. Coupled with the absence of left-end hairpin resolution, this would explain the preferential formation of dimer molecules with left-end bridges and the predominant synthesis of only minus strands.

Resolution of left-left and right-right end junctions also has been demonstrated *in vitro* and *in vivo* using plasmids that contain these junctions. In both cases nicking requires MVM NS1 protein and NS1 becomes covalently attached to the 5' end of the nick (70,71,76,77,177). Resolution of the left-left dimer bridge *in vitro* and *in vivo* is asymmetric as predicted, producing predominantly extended forms from one arm of the bridge and predominantly hairpinned (also called

turn-around) forms from the other arm (71,76). Precisely how one of the products winds up having a covalently closed end is still not clear. The model illustrated in Figure 7 is just one possible mechanism. Cleavage of the right-right junctions, on the other hand, produces predominantly extended structures in both the flip and flop orientations with covalently associated copies of NS1 (70,76). NS1 also cuts the right end hairpin conformation, which results primarily in extended forms both *in vivo* and *in vitro* (12), again as predicted by the model.

An implication of the model for MVM DNA replication is that NS1 must be able to cut two dissimilar sequences in the right-end hairpin and the left-left dimer bridge. This has been confirmed *in vitro* and the sequences required for nicking at the right and left ends have been identified. The left end in the hairpin configuration contains a bubble in the stem (see Fig. 2), in which the inboard arm contains a GAA sequence and the outboard arm contains a GA doublet directly opposite the GAA. When these are replicated to form the dimer bridge, one arm of the duplex bridge contains the doublet GA/TC (usually called the TC arm) and the other contains the triplet. NS1 nicks only the arm with the doublet TC sequence *in vitro* when the substrate is a plasmid containing the MVM dimer bridge and the reaction is supplemented with a crude cell-free extract (see Fig. 2) (77). Purified NS1 binds the sequence $(ACCA)_{2\text{-}3}$ (66), which is present on both sides of the center of symmetry of the 3' (left end) dimer bridge, but neither the GAA nor the TC side is cut (204), suggesting that a cellular protein is needed to activate NS1 nicking. Christensen et al. (54,55 and 56) have recently purified the cellular factor and called it parvovirus initiation factor (PIF). In the presence of purified PIF, NS1 cleaves the GA/TC side of the dimer bridge. Although the position of the nick has been mapped (77), it is not yet clear whether a specific sequence at the site of the nick is recognized as in the case of AAV.

PIF is a heterodimer composed of related 96- and 79-kd polypeptides (56); it binds coordinately to two ACGT motifs (see Fig. 2) that overlap the ATF site just upstream of the NS1 binding site (55). The PIF subunits (p96 and p79) have recently been cloned (56), and PIF has been identified as a member of a family of transcription factors that contain the KDWK amino acid motif. Its role in transcription of MVM is not clear, but it appears to be another example of how a virus can recruit a cellular transcription factor to promote the synthesis of DNA. Together, PIF and NS1 protect virtually all of the minimal origin sequence (50 bp) required for NS1 nicking of the dimer bridge, and they meet at the position of the TC dinucleotide (55). The actual sequence of the TC dinucleotide is not essential but the spacing is, because insertion of any third nucleotide (as on the GAA side of the bridge) abolishes nicking and subsequent DNA synthesis (77). In the case of MVM, the NS1 binding site (ACCA) occurs many times in the MVM genome. Thus, some mechanism must confine NS1 nicking to the right hairpin and the left-end dimer bridge, and prevent nicking within the 3' hairpin or at other locations within the MVM genome. PIF (and the spacing between the PIF site and the NS1 site) apparently serves this function by stimulating nicking on only one side, the TC side, of the 3' bridge. Presumably, the same mechanism inhibits nicking of the same 3' origin sequence when it is in the hairpin configuration.

Cotmore et al. (67,79) have identified the sequences and cellular protein required for nicking at the right (5') end of MVM (see Fig. 2). In this case, NS1 must nick and resolve a covalently closed hairpinned species to continue DNA synthesis. The cellular cofactor required to activate NS1 in an *in vitro* reaction is either HMG1 or HMG2 (HMG1/2) (79). In the absence of either protein, NS1 does not nick the hairpin. HMG1/2 preferentially binds bent DNA, cruciform DNA, or four-way junctions (i.e., Holliday intermediates). Three sequence elements are essential for nicking the 5' hairpin (see Fig. 2): (a) an NS1 binding site at the very end of the hairpinned 5' end, just beyond the AGA bubble, (b) an NS1 binding site near the nick site, approximately 110 bp upstream of the first NS1 site, and (c) a specific sequence at the nick site just inboard of the second NS1 site (67). In the presence of HMG protein, the NS1 sites appear to form a higher-order nucleoprotein complex, in which the intervening sequence is distorted in a way that suggests the formation of a double helical loop. Thus, NS1 carries out a similar reaction at the left-left dimer junction and the hairpinned right end, two distinct sequences, by interacting with two different cellular proteins.

Although the two NS1 sites and the nicking site appear to be the essential sequences required for endonuclease activity in the right hairpin, Costello et al. (64) showed that *in vivo* the presence of the unpaired AGA triplet within the left hairpin was important for efficient DNA replication. Absence of the bubble significantly reduced replication of the mutant MVM genome, but replacement of the bubble with a different sequence restored DNA replication, suggesting that the sequence of the bubble was not important but the actual presence of unpaired bases was. Additionally, in both 3' dimer bridge nicking and 5' hairpin nicking, ATP is required for the reaction to proceed. This may simply reflect the fact that NS1 binding requires ATP, unlike AAV Rep. Alternatively, it may mean that the DNA helicase activity of MVM must modify the DNA substrate prior to nicking. The nature of the modification, however, is not clear. In the 5' hairpin, a potential cruciform structure could form that would include the NS1 site at the tip of the hairpin. Mutation analysis of this cruciform structure, as well as the companion NS1 site that is present just inboard of the right end hairpin (not shown in Fig. 2), indicated that neither was essential for nicking the 5' hairpin (67).

In addition to the sequences within the 5' palindrome, two other sequences at the right end of the genome but inboard of the palindrome have been shown to have an effect on DNA replication. One of these is a 65-bp repeat between nucleotides (nts) 4,760 and 4,850 of MVM, each of which contains multiple copies of a CC(C/A) repeat (243). Deletion of this sequence reduces DNA replication in some cell lines by as much as 100-fold, and a similar sequence has been mapped in H1. The second sequence is located between nts 4,489 and 4,695 (37). This region has been referred to as an internal replication sequence (IRS). Mutation of this region has identified two sequence blocks that reduce DNA replication about fivefold, and several cellular proteins that bind specifically to this region of the genome have been detected. It is not clear what role these internal sequences play in DNA replication, but some groups have suggested that they may act as internal origins that initiate DNA replication via a cellular primase (37, and refs. therein). Initiation of DNA replication close to the 5' end has been postulated to be a possible alternative mechanism for unwinding and refolding the right-end palindromic sequence, a step that is normally energetically unfavorable (37).

Christensen et al. (54) have identified some of the other cellular factors that are required *in vitro* for replication when a 3' dimer bridge origin is used. In addition to PIF, DNA synthesis required PCNA, RPA (the cellular single-strand DBP), and a partially purified fraction that contains DNA polymerases. PCNA is an accessory protein for DNA polymerases d and e, suggesting that one of these two polymerases is involved in MVM DNA replication. *In vitro* studies using purified DNA polymerases or polymerase inhibitors have not definitively identified the cellular DNA polymerase used *in vivo*, but they do show that the viral single-stranded DNA can be converted into a double-stranded RF *in vitro* using a variety of DNA

polymerases (73).

Any model of DNA replication must be able to account for the particular features found in mature virion DNA, as well as for intracellular structures (73). In particular, the model in Figure 7 is somewhat more complex than that illustrated earlier for AAV, because the rodent parvoviruses, which have been studied in most detail, have terminal sequence arrangements in which the 5' terminal sequence of the virion is inverted during replication but the 3' terminal sequence is not. Additionally, the rodent viruses tend to package only one of the two strands. There are some exceptions to these features among the other autonomous parvoviruses, and presumably this means that alternative mechanisms for parvovirus DNA replication will be uncovered. BPV DNA, for example, shows evidence that both terminal sequences are inverted during replication, although the majority of the virion strands have the 3'-terminal sequence in the same orientation (255). An additional complexity of replication is the generation of variant genomes similar to those seen with AAV (73). The first represents internal deletions of varying extents, and the second is a duplex hairpin molecule representing segments from either the left or the right end of the molecule. Virions containing the incomplete genomes can function as DI particles.

Protein Synthesis

Synthesis of the NS proteins appears to occur first, because the NS protein transcripts appear earlier in the course of infection than the transcripts for the structural proteins, and because one or both of the NS proteins regulate gene expression (91,175,231). Both NS1 and NS2 are phosphorylated subsequent to translation (73). NS1 binds poorly to its recognition site, but binding can be detected in the presence of ATP (53). Binding, ATPase, and DNA helicase activity are modulated by phosphorylation of NS1 (89,203,205). Phosphorylation is on serine and threonine residues by the lambda isoform of protein kinase C and possibly other PKC family members. Dephosphorylated protein has lower nickase and helicase activity but enhanced binding activity, and it retains its ability to regulate transcription. Thus, the phosphorylation state of the NS1 protein seems to determine whether it will be used for transcriptional control or DNA replication. The ATPase binding site and several helicase motifs have been mapped with specific mutations (142), as has a metal coordination site (204). NS1 self-associates prior to nuclear localization (206), and at least one interaction domain has been mapped between amino acids 261 and 280 (220). MVM NS2 is required for efficient virus growth in a host-range-dependent manner. Although not required in hamster, transformed rat, monkey, or human cell lines, it is essential for growth in mouse cell lines and in the whole animal (36,44,198,199). An earlier report suggested that NS2 is required for efficient translation of MVM mRNA (199), but subsequent studies suggested that it was more likely to be involved in particle assembly (68). NS2 also has been shown to interact with 14-3-3 proteins and CRM1 (26,33,207).

The capsid proteins are thought to be acetylated at the N termini (143). VP2 has also been reported to be phosphorylated (250). Synthesis of coat proteins is marked by two unusual phenomena. The first is that the major coat protein, VP2, is proteolytically cleaved to produce VP3 during entry into the cell, and it can be cleaved *in vitro*, but only in the context of full virions (271). The ratio of VP2 to VP3 depends on the time course of the infection. The second is that, as in the case of the AAV capsid proteins VP2 and VP3, the initiator AUG for MVM VP1 is several hundred bases (the whole of the NS2 second exon) downstream from the cap site. The AUG codon for VP2 is another several hundred nucleotides farther downstream. The reason for this is not clear. When expressed in insect cells, the coat proteins can self-assemble to form particles (34,60,146). In the case of B19, the unique region of VP1 (the N terminus) appears to project from the surface of the particle (238).

Oncolysis

Autonomous parvoviruses are normally species restricted. However, transformation of nonpermissive cells, including human cells, with a variety of viral and cellular oncogenes or chemical carcinogens can render the cells permissive for productive infection by rodent parvoviruses such as MVM (reviewed in refs. 234 and 235). In cell culture experiments, infection of transformed cells with MVM or H1 leads to lytic death of the culture; in animal experiments, infection of tumor cells inhibits tumor formation or progression and leads to improved animal survival. A variety of experiments have led to the conclusion that the cytotoxic effect of MVM (or H1) on tumor cells is the result of expression of NS1. Notably, Mousset et al. (195) isolated a Fisher rat fibroblast cell line (FR3T3) that expressed NS1 under the control of an inducible mouse mammary tumor virus (MMTV) promoter. When NS1 was induced in this cell line, no cell death was seen. However, when the same cell was transformed with c-Ha-*ras* or the polyoma oncogenes, the cells died within a few days after induction of NS1. Many but not all oncogenes produced similar NS-induced cytotoxicity. For example, in one study (242), FR3T3 cells transformed with SV40, v-*myc*, and v-*src* were susceptible to MVM killing, but cells transformed by BPV1 were not. If the BPV-transformed cells were supertransformed with Ha-*ras*, they became susceptible to the MVM cytotoxic effect. Although NS1 expression appears to be sufficient for tumor cell killing, NS2 expression had a synergistic effect in some cell types (30).

NS1 expression in nontransformed and transformed cells leads to cell accumulation in the G2 phase of the cell cycle, suggesting that at least part of the cytotoxic phenotype has to do with alteration of the cell cycle (208), but the essential trigger for tumor cell killing is not yet clear. In this respect, Rayet et al. (224) have recently shown that H1 infection of human U937 tumor cells downregulates c-*myc* and activates the CPP322 ICE-like protease (believed to be involved in apoptosis). This is similar to what happens when these cells undergo killing due to tumor necrosis factor (TNF)-a, and it suggests that the TNF pathway may be one of the signaling pathways involved in tumor cell cytotoxicity. It is worth noting that the oncosuppressive effect of parvovirus infection is potentially of clinical value. For example, Faisst et al. (100) have demonstrated that mice with severe combined immunodeficiency disease (SCID), injected with human tumor cells and then infected with H1, showed a dose-dependent regression of the tumors.

SUMMARY AND CONCLUSION

In terms of physical structure, genetic map, autoregulation, and close dependence on the state of the intracellular milieu, the autonomous parvoviruses are closely related to the dependoviruses. There are significant stretches of homology at the amino acid sequence level in both structural and NS proteins of viruses of both genera, and the details of the

biochemical reactions involved in DNA synthesis illustrate how the same RCR reaction can be modified to produce quite different overall outcomes. This produces a continuous spectrum of properties among the viruses of both genera—for example, LuIII and BPV encapsidate both strands, the 3' terminal palindrome of the BPV shows evidence of inversion during replication, and B19 contains TRs. However, there remain several major differences at the biologic level. It seems certain that latent infection plays a major role in the replication cycle of AAV, whereas this is not the case with the autonomous parvoviruses. They do frequently cause cryptic infections, and perhaps these are the biologic equivalent of the AAV latent infection. Another major difference, of course, is that all of the autonomous viruses are serious pathogens in their normal hosts, whereas AAV has yet to be associated with disease. The key difference between the genera seems to be that autonomous viruses simply wait for the cell to enter S phase, relying entirely on their ability to infect dividing cells or tumor cells. In contrast, AAV relies on a helper virus, which can induce S phase in a variety of terminally differentiated, nondividing cells, to expand its host range; but this comes at a price—the helper virus is not always available.

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CHAPTER 70 Parvoviruses

Marshall E. Bloom and Neal S. Young

As a subject of study, parvoviruses appeal to diverse groups. Parvoviruses interest veterinarians because they are responsible for many serious diseases in animals. Some parvovirus infections, like feline panleukopenia and Aleutian mink disease, are models of human illness. Microbiologists have been concerned with parvoviruses as contaminants of cell culture; later, molecular biologists studied them intensively because their simple genomes make them attractive for the dissection of gene expression and regulation. B19, the first pathogenic human parvovirus, has entered the medical curriculum as an agent of human disease, and the adeno-associated viruses (AAVs) have become attractive vehicles for therapeutic gene delivery.

This chapter primarily describes pathogenesis and medical aspects of parvovirus B19. AAV is briefly described because of its potential in gene therapy. The viruses described in this chapter are members of the subfamily *Parvovirinae* of the family *Parvoviridae* (25,26). B19 and the related simian parvoviruses are placed in the genus *Erythrovirus,* whereas all other presently characterized autonomous parvoviruses are in the *Parvovirus* genus (type species, minute virus of mice [MVM]). The defective AAVs are classified in the *Dependovirus* genus. The chapter begins with an abbreviated survey of the autonomous animal parvoviruses because these agents and the diseases that they cause are common and provide insight into B19 infections. Chapter 69 should be consulted for the molecular biology of the *Parvoviridae.* A number of excellent review articles and monographs have dealt with parvoviruses, and these sources are cited liberally throughout the chapter.

HISTORY OF PARVOVIRUSES

In the first part of the 20th century, there were numerous epidemics of enteritis, panleukopenia, and congenital cerebellar ataxia in feline populations (195,196). In 1947, a similar syndrome of enteritis suddenly appeared in ranch mink, and by 1952, it was concluded that the same hardy viral agent caused disease in both species (234). In 1959, a small hemagglutinating stable virus was inadvertently recovered from rat tumor lysates and named *rat virus* (RV) (109). At about the same time, another virus with similar characteristics, denoted *H-1,* was shown to cause a complex syndrome of craniofacial anomalies in newborn hamsters (218). By the mid-1960s, investigators had determined that feline panleukopenia virus (FPV), mink enteritis virus, H-1, and RV were all members of the same family of small, nonenveloped viruses with single-stranded DNA genomes. Defective parvoviruses were first recognized as common contaminants of adenovirus stocks in the mid-1960s and named AAV (15,25,99) because replication required co-infection with an adenovirus (27,28 and 29). The generic designation, parvovirus (*parvus* means small) was proposed in 1965 (25,127) and formally accepted in 1970 (14).

Subsequently, parvoviruses have been associated with a broad array of diseases in animals (25,75,196). In addition, persistent and inapparent infections of animals and cell cultures were also described, indicating that parvovirus infection was not inextricably linked to overt disease (196). In the late 1970s, a new syndrome of viral enteritis and myocarditis in puppies and dogs swept rapidly across the world. The virus was named *canine parvovirus* (CPV) and was closely related to FPV and mink enteritis virus (221,222). The rapid spread of CPV was enhanced by the remarkable physical stability of parvoviruses (25,177). In 1980, another parvovirus was shown to be the cause of a chronic immune complex disease in mink known as *Aleutian disease* (37,38). Finally, in the early 1980s, a human parvovirus, B19, was found to cause several disease states, including fifth disease, polyarthropathy, transient aplastic anemia, and erythroblastosis fetalis.

In 1984, a parvovirus, RA-1, was isolated from mice neonatally infected with samples from rheumatoid arthritis patients (198,208). The mice developed a syndrome of spinal and skeletal deformities. No additional work has been reported on RA-1, and the genomic sequence, true provenance, and nature of this agent remain unclear. In addition, a newly described human DNA virus with a single-stranded genome (TTV) appears to *not* be a parvovirus (159,197). This makes B19 the only parvovirus known to cause clinical disease in humans.

GENERAL FEATURES OF AUTONOMOUS PARVOVIRUS INFECTIONS

Several principles provide a basis for understanding the pathogenesis of parvoviral infections and diseases

(25,75,195,196). First, the autonomous parvoviruses have a near-absolute requirement for rapidly dividing cell populations in S phase of the cell cycle (25,75,161). This principle does not generally apply to AAV infections because the co-infecting helper viruses circumvent the dependence for replication upon the cell cycle (27,28). Second, identifying targets for virus infection can often explain the precise pathogenesis (170). For example, targeting of the developing cerebellum by FPV leads to congenital ataxia (195). Third, short segments of viral genes, usually in the capsid coding sequences, govern the host range or pathogenicity of parvoviruses (17,36,58,75,220,221). In addition, parvovirus infections are often persistent and in some cases asymptomatic (100,195). In this section, several parvovirus systems are used as abbreviated examples of these principles. This is not a comprehensive recitation, and many viruses are not mentioned but are dealt with elsewhere (25,75,100,162,170,195,196,222).

The preference for rapidly dividing cell populations as targets for viral replication suggests that fetal and newborn animals would be highly susceptible after transplacental infection. Several parvoviruses are responsible for fetal wastage, including porcine parvovirus (PPV) (101,132,162,196) (Fig. 1**A**), bovine parvovirus (196,209), H-1 parvovirus (196), goose parvovirus (45,196), parvovirus B19 (57) (see Fig. 1**B**), and ADV (41).

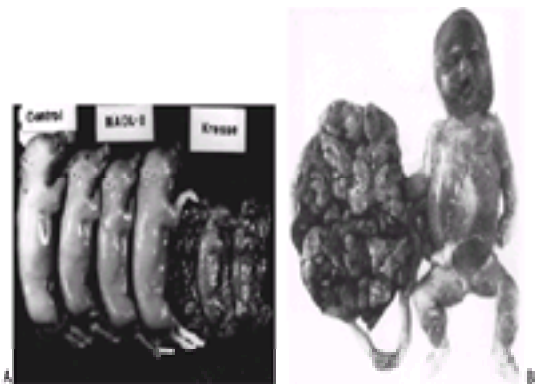

FIG. 1. A: Fetal loss in pigs. Animals were inoculated *in utero* with two strains of porcine parvovirus, including the virulent Kresse strain; shown here are dead and mummified 23-day fetuses. (Photograph courtesy of Tom Molitor.) **B:** Human hydropic fetus and placenta after intrauterine parvovirus B19 infection. (From ref. 57, with permission.).

Some infections in late gestation or the immediate newborn period are not fatal but lead to severe sequelae. Rodent parvoviruses and FPV induce a congenital cerebellar ataxia by lytically targeting cells in the differentiating cerebellum (195,218). The destruction of the developing cerebellum may be mediated by virus-induced apoptosis (157). Another example is the development of craniofacial and dental deformities in hamsters infected either *in utero* or in the early postnatal period by rodent parvoviruses (195,196,218). This syndrome results because the cell populations susceptible to virus replication are found in developing osteogenic tissue (185). A fatal perinatal myocarditis results from CPV infection *in utero* or in very young puppies (170). In contrast, a strain of MVM infects renal vascular endothelial cells and lymphocytes in baby mice (100). Finally, newborn mink infected with ADV develop a severe, often fatal, respiratory distress syndrome subsequent to permissive infection of the type 2 alveolar pneumocytes, which proliferate in the newborn period (6,37). It is apparent that the disease patterns are diverse; however, because the organs and cell types mentioned divide rapidly only in developing animals, the same viruses do not usually target the same tissues in adult animals.

Parvoviral diseases in older animals fall into two categories: those due to direct effects of acute virus infection on replicating target tissues, and those due to the subsequent immune response. In the acute category (reviewed in 25,37,75,170,195, and 196) are (a) enteritis induced in a number of species, but probably best known in CPV-infected dogs; (b) panleukopenia in cats (FPV), swine (PPV), and bovines; (c) hepatitis in hamsters and geese, and (d) erythrocyte aplasia in humans (B19). The pathogenesis of these diseases directly reflects the target organ in which virus replicates and is often different from that induced by the same virus during early development.

Although a detailed description of the immunologic features of parvovirus infections is beyond the scope of this chapter, several points are relevant to discussion of B19. Vaccination is very effective against the CPV-FPV family (170); however, other parvoviruses, including ADV, can persist in the face of robust immune responses (37,100,196). Several disorders result from the host response to infection (37,63,195,196,216). The most dramatic of these is probably the chronic immune complex disorder caused by persistent infection by ADV, which is worsened by vaccination (1,37). Another example is the ability of certain PPV isolates to induce immunologically mediated vesicular skin lesions in late-gestation swine fetuses (24,61,117). In addition, the immune response is responsible for fifth disease and the polyarthropathy caused by B19 infection (235).

Studies from a number of autonomous parvovirus systems, including MVM, CPV-FPV, PPV, and ADV, indicate significant differences in host range and pathogenicity among closely related strains or isolates (17,36,58,75,100,158,162,195,216,220,221 and 222). Because the structures for several parvoviruses have been solved (3,5,58,130,223), it has been possible to map the sequences controlling these aspects of viral biology to specific regions on the viral capsids (Fig. 2). The viral sequences governing pathogenicity map primarily to the capsid proteins, and structural studies model the determinants onto the surface of the capsid near the threefold axis of symmetry (3,5,36,58,130). The same general region of the capsid is also involved in mediating the pronounced differences in host range among members of the CPV-FPV family and MVM (3,58,222). Although the capsid genes appear to be the major viral determinants of host range and pathogenicity, nonstructural gene products also play a role in some systems (54,147,226).

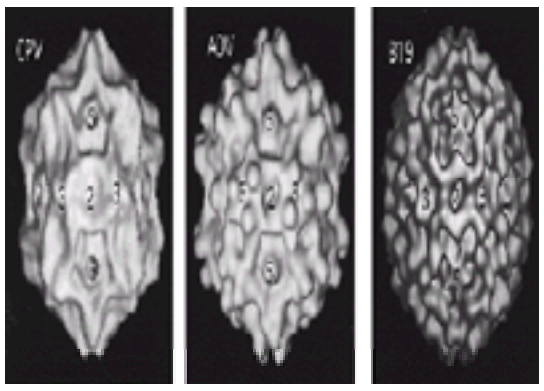

FIG. 2. Comparison of three autonomous parvoviruses: reconstructed images of Aleutian mink disease parvovirus (*ADV,* 22 Å), canine parvovirus (*CPV,* 22 Å), and parvovirus B19 (*B19,* 26 Å). These three viruses produce very different diseases in their hosts. Although the threefold (*3*), fivefold (*5*) and twofold (*2*) axes of symmetry are readily apparent, differences in the surface topography are evident. Surface regions related to host range, pathogenicity, antigenicity, and receptor binding are located near the threefold axes. (Adapted from refs. 5,130, and 223, with permission.).

Viral persistence is another facet of many autonomous parvoviruses infections both in cell cultures and in animal hosts. The mechanisms involved remain undefined (25,100,188,196) but may involve restricted infection (37). The defective AAVs persist by integrating into the cellular genome (28), but there is no evidence that autonomous parvoviruses use this strategy for persistence.

The animal models described in this section should be kept in mind when reading about B19. Many features of B19 infections have definite parallels in the animal models.

PARVOVIRUS B19

History

In the mid-1970s, Yvonne Cossart, a virologist working in London, was investigating assays for hepatitis B virus (73). When material from false-positive reactions was examined in the electron microscope, parvovirus-like particles were seen (Fig. 3). The name B19 derives from the blood bank code of a viremic donor.

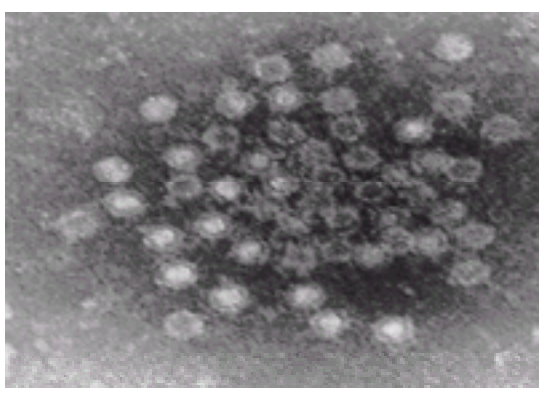

FIG. 3. Electron micrograph of Aleutian mink disease parvovirus (*ADV*) capsids immune-complexed with antiviral antibody. The antibody molecules can be observed bridging and aggregating the 25-nm virions. Images like this provided Yvonne Cossart the initial clues to the presence of B19 parvovirus particles in sera.

The infection was initially thought to be asymptomatic (73), and 30% of normal adults (70,194) were found to have antibody, suggesting common exposure in childhood. However, a 1981 survey of sera archived at University Hospital in London identified antigenemia or seroconversion in children with sickle cell anemia suffering from transient aplastic crisis (TAC) (171,194), suggesting an etiologic role for B19 in an acute disease of presumed infectious origin. Two years later, parvovirus B19 infection was implicated in an outbreak of fifth disease in London (11), linking the virus to a common childhood exanthem. In 1984, parvovirus B19 was found in a hydropic infant (13), and the known propensity of parvoviruses to cause fetal wastage led to the association of B19 infection with hydrops fetalis (57,95). The spectrum of B19-associated diseases was expanded when persistent infection with parvovirus B19 was diagnosed in an anemic, immunosuppressed patient (122). These findings clearly implicated B19 as the cause of a number of important human medical problems.

The amount of virus present in acute-phase sera allowed characterization of the virus as a proper member of the *Parvoviridae* family (64,212). In the mid-1980s, the genome was cloned and sequenced (73,192), and virus was cultivated *in vitro* in human bone marrow cells (166). Human erythroid progenitors remain the most convenient productive culture system, and the molecular biology and infectious cycle have been described in these cells. Expression of parvovirus B19 capsid proteins using recombinant technology has been useful for development of clinical assays for specific antibody (103) and as a human vaccine reagent (18,134).

In the early 1990s, an outbreak of severe anemia in cynomolgus monkeys was found to be due to infection with a simian

parvovirus (154). This was the first description of a B19-like virus in nonhumans and led to the present animal model.

Infectious Agent

As noted earlier and in Chapter 69, the autonomously replicating parvovirus B19 is classified in the *Erythrovirus* genus, of which it is the type species (26). The genus name connotes the tightly restricted host range for cells of the erythroid series. Parvovirus B19 shares genetic features with both autonomous and defective parvoviruses. The closest relatives to parvovirus B19 are parvoviruses of cynomolgus monkeys (44,154,156), rhesus monkeys (52), and pig-tailed macaques (52). All of these are about 60% to 80% homologous to B19, and the simian parvovirus has a cellular host range and biology similar to B19 (44,156). A new parvovirus was recently isolated from the sera of healthy Manchurian chipmunks (240). The chipmunk agent is about 50% homologous to parvovirus B19. This may be the first nonprimate erythrovirus to be described, but firm ranking must await confirmation of its genomic organization, biology, and host range.

Despite the characterization of many isolates of B19, little variation in B19 has been identified. The two prototype B19 isolates, Au and Wi, differ by only 41 bases (51). The sequence divergence is generally less than 1%, with even less variation in regions corresponding to epitopes (80). In a Japanese study, viruses were isolated at two different times separated by 5 years (224). The sequence of the predominant genome differed slightly at the two times, but there was no evidence of geographically restricted distribution of viral genotypes (224). Although various isolates can be divided into groups based on restriction enzyme digestion, there is no obvious correlation with disease patterns. However, observations from other parvovirus systems indicate that host range and pathogenicity can be governed by minor sequence variation (17,36,220); hence, this issue presently remains open.

Cell Culture Systems

Parvovirus B19 has a pronounced tropism for human erythroid cells *in vitro.* Cultures of human bone marrow (167,206), peripheral blood (186), fetal liver (238), and a few leukemic cell lines (144,193) have been used to reproduce the effects of the virus on natural target cells and to determine functional correlates of virus structure (Fig. 4). Permissive viral replication occurs at high levels only in erythroid progenitor cells (166), and replicative form DNA can be detected in the bone marrow of infected patients (118,121,122). Erythroid colony formation by the late erythroid progenitor cell (erythroid colony forming unit, and the more primitive erythroid burst-forming unit) is strongly inhibited by infection, and susceptibility of marrow cells increases with erythroid differentiation (214). B19 can also replicate in cynomolgus monkey bone marrow cells (86). In all culture systems, productive virus propagation is dependent on the presence of erythropoietin (193), suggesting that cell susceptibility is related to the sustained effects of erythropoietin on erythroid differentiation. The immunophenotype of infected cells is typical of the normoblast or pronormoblast (140,141). Myelopoiesis, formation by the granulocyte-macrophage colony forming unit, is unaffected even by high concentrations of virus *in vitro* (142,206).

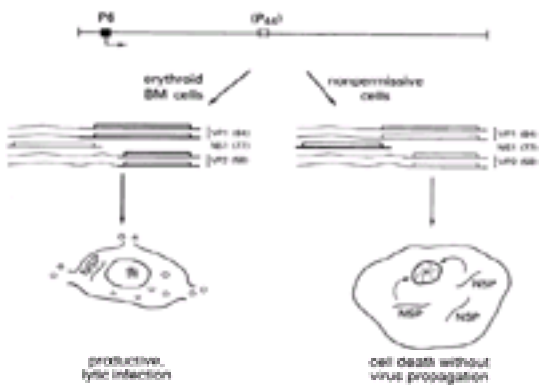

FIG. 4. Schema showing two routes to cell death after parvovirus B19 infection. Permissive cells allow full-length transcription of both the nonstructural and the capsid gene RNAs. Capsid RNAs are the predominant RNA species in erythroid cells. Nonpermissive cells have a functional RNA-processing block in the middle of the genome that results in relative overexpression of nonstructural protein RNA and cell death without viral propagation.

Interestingly, a “block” to replication in nonpermissive cells is a consequence of aberrant transcription (116,126) (see Fig. 4), leading to the messenger RNA (mRNA) for NS1 being the most abundant species (126). This is in contrast to permissive infections, in which the capsid protein mRNA predominates (165). Limited expression of NS1 in the absence of detectable DNA replication can suppress megakaryocytopoiesis and inhibit hematopoietic colony formation by both simian and human parvoviruses (85,86,205). Predominant expression of NS1 could explain virus-induced inhibition of megakaryocytic progenitor proliferation *in vitro* and reduction of platelets and white blood cell numbers in patients, even in the absence of productive viral infection.

The cytotoxicity induced by B19 is the result of expression of NS1 (165) and may reflect NS1-induced apoptosis (125,139) (see Fig. 4). Cotransfection of the *NS1* gene and a selectable gene into certain nonpermissive cells abrogates colony formation by these cells (164), whereas expression of the capsid protein genes alone has no effect on cell proliferation (105). It remains to be determined whether B19 cytotoxicity results solely from *NS1* expression.

Pathogenesis and Pathology

Human Infection

Experimental parvovirus B19 infection is an acute, biphasic illness and is similar to natural infection (10,174) (Fig. 5).

After intranasal inoculation of volunteers, virus can first be detected in serum at days 5 and 6, and levels peak at days 8 and 9. Viremia is accompanied by nonspecific, influenza-like symptoms, including fever, malaise, and myalgia. Red-cell aplasia coincides with viremia, leading to a drop in hemoglobin, reticulocytopenia, and modest lymphopenia and neutropenia. Patients with transient aplastic crisis develop symptoms early in the course of infection, when 10^{8} to 10^{14} genome copies per milliliter of virus DNA may circulate (12,65). Appearance of specific immunoglobulin M (IgM) and IgG antibodies 10 to 14 days after inoculation is accompanied by classic fifth disease symptoms; in volunteers, both a generalized erythematous eruption and joint inflammation occur. The clinical manifestations of fifth disease are coincident with the development circulating immune complex formation (10,87), and patients therefore present to medical attention after the peak of viremia. Administration of commercial immunoglobulin preparations to patients with persistent B19 infection produces classic fifth disease symptoms (118,122) strongly suggesting that immune complex formation and deposition play a prominent role in fifth disease.

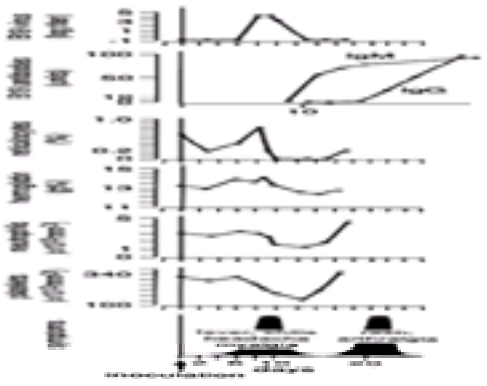

FIG. 5. Course of human parvovirus infection in normal volunteers. (Adapted from ref. 10, with permission.).

In infected volunteers, B19 is found in the nasopharynx, and the major route of transmission is probably through the upper airway (10). There is little evidence of virus excretion in feces or urine (10), but fomites may also be common source of infection, owing to the hardiness of parvoviruses (177). Infection can also be acquired from contaminated blood products (51,143).

Because parvovirus B19 has an extreme tropism for erythroid progenitor cells *in vitro,* by extrapolation, the major site of viral replication in patients has been assumed to be the adult marrow and the fetal liver, the site of erythropoiesis during development. As expected with the intense viremia, B19 DNA may be detected in any tissue perfused by blood, but replication in adults has been documented only in marrow cells (118,121,122).

Consistent pathologic changes have been confined to hematopoietic tissues: giant pronormoblasts in bone marrow aspirates (84,120,122) and fetal liver tissue sections (7,57), accompanied by depletion of later erythroid precursor cells. Erythroid precursor cells infected with parvovirus B19 are readily identified as giant pronormoblasts (84,120,122) by characteristic light microscopic (167) (Fig. 6) and electron microscopic (243) (Fig. 7) morphologic changes. The presence of these abnormal cells in the bone marrow of patients with transient aplastic crisis has been recognized since 1948 (163), and the morphology can be reproduced in culture (167). Less frequently, dysplastic changes in myeloid and megakaryocytic cells are observed.

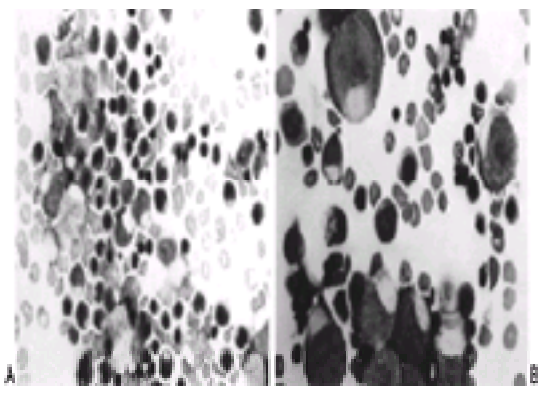

FIG. 6. Cytopathic giant pronormoblasts in normal bone marrow cultured for 48 hours after inoculation with parvovirus B19.

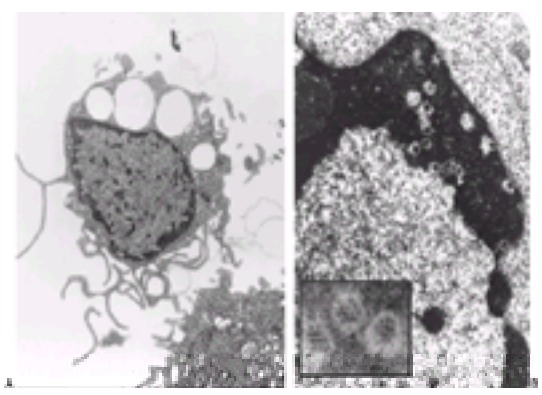

FIG. 7. Ultrastructure of parvovirus-infected human erythroid progenitor cells. **A:** Cell showing pseudopod formation, vacuolization, and margination of nuclear chromatin. **B:** On higher power, crystalline arrays of viral particles (*inset*) are present in lacunae with the marginated chromatin.

That B19 is likely transmitted through the nasopharynx (10,174), together with the fact the virus can be transmitted vertically from mother to fetus, suggests that nonerythroid cells may also be targets for B19 replication. For example, fetal cardiac myocytes can be infected (151,173), and B19 DNA and antigen have been demonstrated in skin biopsies (153,187). Some of these cell types may bear the parvovirus B19 receptor (42). Other nonerythroid cells might also be infected by means of uncharacterized receptors. In addition, virus contained in immune complexes might be able to infect Fc receptor–bearing immune cells (macrophages) through an antibody-dependent enhancement of infection (79,106). This mechanism would allow B19 to infect cells involved in regulating immune reactions (37,106). B19 gene products can induce the cytokine interleukin-6 in some cells (136,213), and cytokines might play a role in the immunopathogenesis of some B19 syndromes (fifth disease, polyarthropathy) (37).

Animal Models

A simian parvovirus (erythrovirus) was identified in a cynomolgus monkey colony (154). Severe chronic anemia and persistent simian parvovirus infection were occurring in monkeys immunosuppressed by an active concurrent infection with the type D simian retrovirus (52,120,152). This clinical picture was similar to persistent B19 infection in humans. About 50% of animals in the colony had serologic evidence of prior infection (52); similar viruses have also been identified in rhesus monkeys and pig-tailed macaques (52). Studies of natural and experimental infections of healthy cynomolgus monkeys indicate that simian parvovirus causes an acute disease remarkably similar to that seen in humans (47,155). The clinical symptoms range from inapparent to very mild; however, the obvious arthropathy seen in humans has not been noted (155,156). Monkeys would seem an ideal model to study B19 infections, however, lack of susceptible cell lines and expenses associated with primate work limit their usefulness. The recent detection of an erythrovirus-like parvovirus in Manchurian chipmunks (156) may provide an alternative model.

Cellular Receptor

A cellular receptor for parvovirus B19 has been identified as globoside or erythrocyte P antigen (42,46,72). Globoside is glycosphingolipid tetrahexoseceramide; high-affinity binding of virus (Ka = 10^{10}/M) is specific for the carbohydrate moiety and not the lipid portion. The importance of this receptor is confirmed because individuals who genetically lack P antigen (p phenotype) are not susceptible to parvovirus B19 infection (46). In tissue culture, either excess soluble globoside or anti-P monoclonal antibody can protect erythroid progenitors from infection. The binding of globoside to empty capsids of B19 has been resolved to 26 Å by cryoelectron microscopy (60). The receptor molecules dock into depressions on the threefold axes of the capsids, adjacent to sequences identified as neutralizing epitopes (see Fig. 2).

Globoside is expressed on mature erythrocytes and erythroid precursors. Other cells contain this protein, but it is uncertain whether the globoside is displayed on the cell surface or can function as a B19 receptor (72). The existence of coreceptors or multiple receptors on different cell types, as suggested for AAV (135,175,210,211), might partly explain a presumed respiratory route for infection and the induction of B19-associated disease in nonhematopoietic organ systems.

Immune Response and Key Antigens

Both virus-specific IgM and IgG antibodies to the B19 capsid are made after experimental (10) and natural (182) parvovirus B19 infection (see Fig. 5). IgM antibody appears at about 10 to 12 days (10) and can be found in serum for several months (9). IgG appears about 2 weeks after inoculation (10) and probably persists for life (10). IgA antibodies can also be detected and presumably play a role in protection against infection by the natural nasopharyngeal route (81). Transient aplastic crisis resulting from B19 infection is a singular event in the life of a sickle cell patient, further implying durable, lifelong immunity (190). The important role of antibody can also be deduced by the efficacy of commercial immunoglobulin to cure or control chronic infection in immunodeficient patients (84,118,120,152).

In immunocompetent people, the early antibody response is to the major capsid protein VP2, but as the response matures, reactivity to the minor capsid protein VP1 dominates (119,128). By analogy with the three-dimensional structure of canine parvovirus, some of the B19-neutralizing epitopes defined by murine monoclonal antibodies reside on the surface of the virion (see Fig. 2) (4,58,223,241), but the relationship between neutralizing activity and antibody titer is not simple (40). Some linear neutralizing epitopes are present on the VP1-unique region and are on the outside of the capsid (179). Human monoclonal antibodies against both the VP1-unique region and VP2 have potent neutralizing activity (89), confirming that humans recognize neutralizing epitopes on both VP1 and VP2.

Patients with persistent B19 infection uniformly lack strong reactions to capsid antigens on immunoblot analysis of antiviral antibodies and typically contain only anti-VP2 antibody (119). Congenitally immunodeficient patients may show normal reactivity on enzyme-linked immunosorbent assay (ELISA) testing for IgG and IgM parvovirus-specific antibodies (119). These results suggest that the antibody against linear epitopes detected by immunoblotting is functionally important for effective viral clearance (40), but it must be recognized that these results come from a limited number of studies.

The role of antibody against the major nonstructural protein NS1 has recently been examined (39,227,229,230), but the presence of antibody to this viral protein has not been convincingly linked to a particular syndrome. A human monoclonal antibody against NS1 has been derived but had very weak neutralizing activity (89).

Antibody may play a dual role in B19 infections. On the one hand, antibody can clear virus from the system in both acute and persistent infections. On the other hand, antiviral antibody may be important in producing the immune complex–mediated manifestations of B19 infections.

Because most patients with persistent B19 infection have T-cell as well as B-cell immune deficits, it is probable that a cellular component to the normal response must exist. Few studies have examined cellular immunity to B19 infection (119,228).

Clinical Syndromes

Fifth Disease and Polyarthropathy

Most B19 infections are unrecognized. In investigations of B19 outbreaks, 25% of infected patients report no symptoms, and up to 50% recall no rash (62,235), the most obvious clinical sign.

Acute infection with parvovirus B19 causes the common childhood exanthem, fifth disease (erythema infectiosum) (11,53,62). Children with fifth disease usually are not very ill. The rash is a characteristic “slapped-cheeks” facial erythema and a lacy, reticular evanescent maculopapular eruption over the trunk and extremities (Fig. 8 and Fig. 9). Fifth disease occurs after the viremic phase of infection and results from the host immune response to infection. Because the virus is readily transmitted, an epidemic may be recognized in the community. In a single patient, however, fifth disease is often confused with other childhood exanthems.

FIG. 8. “Slapped-cheek” rash in a child with typical erythema infectiosum or fifth disease. (Photograph courtesy of Mary Anderson.).

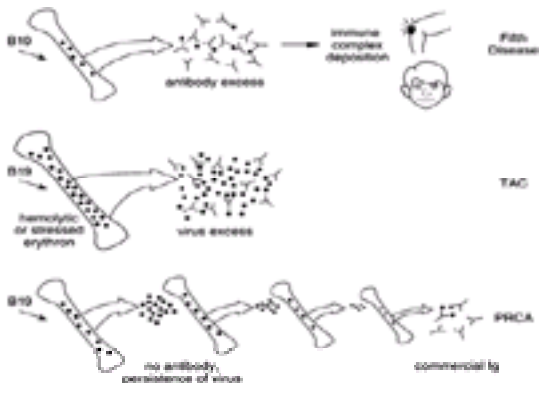

FIG. 9. Pathogenesis of diseases resulting from parvovirus B19 infection in children and adults. (TAC, transient aplastic crisis; PRCA, pure red-cell aplasia.).

Adults with acute B19 infection, especially women, more commonly suffer a symmetric polyarthropathy than a rash (235) (see Fig. 9). The patient's symptoms, including the distribution of joints affected, the presence of frank inflammation, and even a positive rheumatoid factor test, can mimic acute rheumatoid arthritis (148,233). This syndrome also follows the viremic phase of the infection and is most likely a consequence of the host immune response. Parvovirus-induced arthropathy can persist for weeks, months, or years. In one arthritis clinic, 12% of new patients had evidence of recent parvovirus infection (233).

Any association of B19 with chronic joint diseases, particularly rheumatoid arthritis, remains controversial. Despite numerous studies, a convincing link has yet to be demonstrated (55,68,133,148,150,181,203,213). In addition, B19 DNA is also found in normal synovia (55,202).

Transient Aplastic Crisis and Hematologic Disorders

In patients with underlying hemolytic disorders, acute B19 infection causes transient aplastic crisis (TAC) (see Fig. 9), an abrupt cessation of red blood cell production, characterized by reticulocytopenia, absent erythroid precursors in the marrow, massive viremia (12,65), and a precipitous worsening of anemia (reviewed in 49 and 242). TAC was the first

clinical illness associated with B19 infection (171). Temporary depression of erythropoiesis is likely a constant feature of acute B19 infection in all patients (10,49), although it is generally of no significance in the absence of underlying hemolysis or erythroid compartment stress. TAC results only when a shortened red-cell life span is coupled with failed erythropoiesis. Examples of predisposing conditions are sickle cell anemia, hereditary spherocytosis, enzymopathies, thalassemias, and acquired hemolytic anemias (reviewed in 53 and 242). Conditions of erythroid stress, such as hemorrhage and iron deficiency or bone marrow transplantation, have also been associated with TAC.

Ultimately, the condition is self-limiting. Nevertheless, the patient may be acutely ill with the symptoms of severe anemia (49,189). Community outbreaks can occur (191), and B19 infection should be a leading diagnosis in any patient with acute cessation of erythropoiesis. Resolution of the crisis, first manifested by reticulocytosis, begins about 7 to 10 days after onset, concomitant with the appearance of antiviral antibody and viral clearance.

B19 infection has also been less convincingly implicated in other hematologic disturbances (51,53). Fifteen cases of virus-associated hemophagocytic syndrome in adults and children have been firmly linked to B19 infection (50).

Fetal Infection and Hydrops Fetalis

The demonstration in 1984 (13) that B19 could be a cause of nonimmune hydrops fetalis led to significant public concern. *In utero* infection may be persistent and characterized by severe anemia, high-output cardiac failure, and often death (8,236) (Fig. 10; see Fig. 1**B**). Erythroblasts in the fetal liver exhibit clear signs of B19 infection, including cytopathology, viral DNA, and antigen (7,57,74). In addition, fetal myocarditis occurs (57,137,151,173) and may contribute to the pathogenesis. The risk for a fatal outcome is greatest during the first two trimesters.

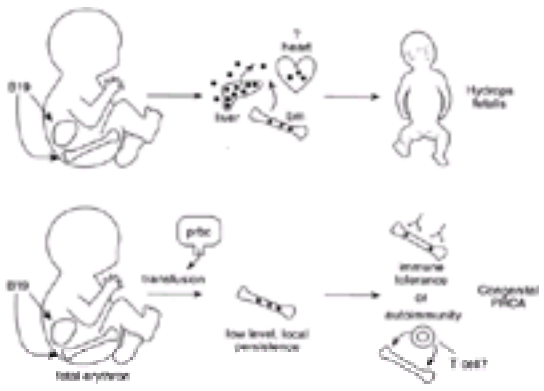

FIG. 10. Pathogenesis of diseases resulting from fetal infection with parvovirus B19. The fetal erythron consists of the liver and bone marrow. Packed red blood cells (*prbc*) are used for intrauterine transfusion. The severe anemia of congenital pure red cell aplasia (*PRCA*) can be a consequence of nonfatal fetal infection with B19.

Fetal infection need not be fatal but may persist after birth as pure red-cell aplasia (PRCA) (23,43,51,83,237). The data suggest a severe chronic anemia accompanied by persistent viral DNA in the bone marrow but not in the blood (43,51) (Table 1; see Fig. 10). Intravenous immunoglobulin did not ameliorate the anemia (43). Analysis of the effectiveness of intrauterine immunoglobulin treatment is complicated because fetal B19 infections may often have no adverse outcomes (8,83).

Syndrome	Clinical features	Host characteristic
Fifth disease	Cutaneous eruption, arthralgia/arthritis	Children
		Adults
Transient aplastic crisis	Severe acute anemia	Hemolysis
Pure red-cell aplasia	Chronic anemia	Immunodeficiency
Hydrops fetalis	Fetal anemia, heart failure	Mid-trimester fetus
Congenital red-cell aplasia	Aregenerative chronic anemia	Treated hydrops fetalis

TABLE 1. *Parvovirus B19 diseases*

Exposure of the fetal immune system to virus might result in tolerance to viral antigens and the absence of an antibody response despite persistent virus production. However, there is no direct evidence demonstrating this phenomenon.

Pregnant women are commonly exposed to B19 through other children in a variety of settings (225). About 30% of maternal infections are vertically transmitted to the fetus, and fetal death is thought to occur in 2% to 10% of maternal infections (93,176,225). The demonstration of IgM antibody to B19 in the cord blood of healthy infants suggests that fetal infection may sometimes have no adverse consequences (8,83). Systematic studies have failed to provide evidence of congenital abnormalities after fetal B19 infection (51,108).

Persistent Infection

Many patients with persistent parvovirus B19 infection suffer from the hematologic disease PRCA (see Fig. 9). Persistent infection has been associated with a wide variety of congenital and acquired immune disorders, including congenital immunodeficiency (120,152), acquired immunodeficiency syndrome (2,84), leukemia in remission (51,118), and organ transplantation (51). Clinically, the anemia is severe, and the patients become dependent on transfusions. The marrow contains the characteristic giant pronormoblasts, and there may be associated neutropenia (122). Persistently infected patients fail to mount a neutralizing antibody response to the virus and do not exhibit the immune complex–mediated symptoms of fifth disease. The diagnosis is established by detection of parvovirus B19 genome in serum, blood, or bone marrow cells by dot-blot hybridization or PCR (2,51,65,66,77). Administration of immunoglobulin can be beneficial and ameliorative, if not frankly curative, in cases of PRCA; however, evidence of its utility in clearing persistent infection in other settings is less well established (84,118,120,152).

Other Disease Associations

Infection with parvovirus B19 has also been implicated in a variety of other diseases, although the causal links are weak in many instances. A limited number of cases of myocarditis have been reported concomitant with B19 infection, an association worthy of additional study because P antigen is present on myocardial cells (72,137,151,173). B19 has also been suggested as a cause of hepatitis or fulminant liver failure associated with aplastic anemia (48,123,149,200). This is a provocative finding, too, because P antigen and globosides are present in fetal liver cells (42,72).

B19 infection has also been associated with vasculitic syndromes (53,82), including polyarteritis nodosa, Henoch-Schönlein purpura, vasculitis, and Kawasaki disease. The presence of P antigen on endothelial cells (42) and the ability of B19 to cause immune complex deposition (153) may explain involvement of the virus in this type of syndrome, but additional studies are needed to determine the significance of parvovirus in these disorders. The causal role of B19 is also uncertain in case reports linking infection to meningitis and neurologic diseases (16,51,53,152,160,232). The relevance of these associations is complicated by the fact that the serum and tissues of some immunocompetent patients can contain detectable B19 DNA for extended periods of time.

Epidemiology

Prevalence and Incidence

Human infection with parvovirus B19 is common. IgG antibody against B19 persists for life (9,10) and is the most convenient marker for past exposure. By the age of 15 years, about 50% of children have detectable IgG antibody to the virus (51). Infection continues to occur throughout life, and in excess of 90% of elderly people have antibody (67). The annual seroconversion rate for women of childbearing age is estimated at 1.5% (110). Similar patterns of prevalence are generally noted in different countries (51).

The high antibody prevalence is not matched by a correspondingly high frequency of viremia. Only about 1 in 20,000 to 40,000 units of blood contains high titers of B19 in epidemic seasons (69). One study using polymerase chain reaction (PCR) as a detection test found B19 DNA in 1 of 3,000 units of pooled samples from blood donors (131). Another study using PCR found B19 DNA in 11 of 9,568 (0.1%) blood donor units, of which 3 contained no measurable B19 IgG (102).

Origin and Spread of Epidemics

An infectious basis for TAC and fifth disease was suspected long before the discovery of parvovirus B19 based on epidemiology. Both fifth disease and TAC are seasonal, with peak occurrence in late winter, spring, and summer (51). Epidemics of both syndromes occur concurrently (62,182), and both appear to cycle in 3- to 4-year periods (190).

Attack rates are high during outbreaks. Between 10% and 60% of susceptible schoolchildren develop fifth disease in school outbreaks (62,110,209); and for adult school and daycare personnel, parvovirus infections occurred in 20% to 30% of susceptible individuals (51,90). Sibling-to-sibling transfer is probably a major path of transmission (110). Nosocomial transmission probably also occurs and is different to distinguish from community acquired infections (22). Patients with acute or persistent infection should be considered infectious, and appropriate precautions should be employed to limit transmission.

Although the prevalence of viremia is low, infection can be transmitted by blood and blood products (143). Because of the resistance of parvoviruses to heat and solvents (177), a fraction of virus can withstand the inactivation treatments usually employed for blood products (51,143). In addition, viral particles escape filtration because of their small size, and as a consequence, the virus in a single positive unit of blood can be readily spread throughout the pooled preparations.

Diagnosis

Diagnostic laboratory assays are useful to distinguish the rash of fifth disease from other viral exanthems, to determine the presence of protective antibodies or recent exposure in pregnancy, to distinguish parvovirus infection from other causes of erythropoietic failure, and to establish the diagnosis of persistent infection in an anemic patient.

IgM directed against parvovirus B19 can be detected 10 to 12 days after infection (10,182) and is a convenient marker for recent or ongoing infection. The most sensitive methods use the capture technique in radioimmunoassay or ELISA formats (51,70). Testing for IgG is useful for seroprevalence studies but is not helpful in diagnosing acute infection because it simply indicates past exposure to virus. Determination of IgG avidity may also prove useful in gauging recent

exposure (91,201).

No method is suitable for routine virus isolation; hence, detection usually relies on probing for the presence of the viral genome. During acute uncomplicated infections in immunocompetent patients, B19 DNA is only detectable for 2 to 4 days by dot-blot hybridization (see Fig. 5) (2,12,65,76). The sensitivity of B19 DNA detection has been greatly increased by the use of PCR (56,66,81,111). However, confusing results can be a problem in diagnosing acute disease because of the ease of contamination, long-term persistence of low levels of virus in uncomplicated infections, and false-positive results. Because of these issues, DNA hybridization is more certain than PCR, and the results are easier to interpret (2). DNA can also be detected by *in situ* hybridization of tissues (141), but this is not a useful routine screening test.

In the event of B19 infection or seroconversion during pregnancy, hydrops can be detected by ultrasound and fetal infection determined by PCR (219). Treatment of hydropic infants with intrauterine transfusions has been reported, although this procedure is fraught with risks (83,172,219).

Prevention and Control

Treatment

Antiviral therapy in the form of commercial immunoglobulin preparations is effective in PRCA associated with persistent infection, although not all persistent infections are terminated with this treatment. Immunoglobulins are administered by daily intravenous infusion at a dose of 0.4 g/kg for 5 to 10 days (84,119,120). Relapses may occur in patients with human immunodeficiency virus as a result of the high virus burden, but such patients can respond to a second course (84). Serial measurement of serum virus levels may be helpful in predicting relapse. The recent development of human monoclonal antibodies that neutralize B19 infection *in vitro* (89) may presage therapy without the numerous risks associated with human blood products.

Vaccines

Prospects for a parvovirus B19 vaccine are good. A vaccine derived from self-assembled capsids expressed in recombinant baculoviruses induces a robust neutralizing antibody response in experimental animals, particularly when used with adjuvant (18,40,104). Phase 1 trials of one B19 vaccine commenced in 1995; however, the target population for such a vaccine remains to be determined (71).

Perspectives

B19 is the only parvovirus with demonstrated pathogenicity for humans. The virus is remarkable for its extremely narrow tropism, and productive infection has only been demonstrated in human erythroid progenitor cells. Viral tropism for productive infection appears to be regulated by expression of its cellular receptor, erythrocyte P antigen or globoside, and an intracellular block in transcription in nonpermissive cells. The virus is cytotoxic to host cells through action of the nonstructural protein. Because the nonstructural protein is expressed in nonpermissive cells, abortive infection in these cells might nevertheless result in cell death.

The pattern of disease that follows B19 infection reflects the balance among virus, target cell, and the immune response (see Fig. 5, Fig. 9, and Fig. 10). Bone marrow depression occurs early, during viremia. Normally, virus replication is terminated by a neutralizing antibody response. The immune response produces the clinical manifestations of fifth disease, in children a rash illness and in adults a rheumatic syndrome; these symptoms are coincident with antibody formation and are probably immune complex mediated. In both persistent infection in children and adults and *in utero* infection, failure to mount a neutralizing antibody response allows B19 to persist and cause a chronic anemia. However, it must be recognized that the role of cellular immune responses is poorly understood in B19 infection. Cellular responses to B19 may play a role in viral clearance and may explain why some patients treated with immunoglobulin fail to be cured. Congenital infection is characterized by low-level infection of marrow, and the chronic anemia may be the result of indirect, perhaps immune-mediated, host responses.

Persistent infection is treatable with immunoglobulin preparations that contain neutralizing antibody. Recombinant molecular technology will allow development of a vaccine (see Table 1). The safety and effectiveness of a B19 vaccine will have to be evaluated in coming years before widespread application is contemplated.

An unresolved issue is the implication of B19 in a number of conditions that do not directly involve the erythroid system. A firm causal role for involvement in rheumatoid arthritis, hepatitis, and other disorders has not yet been demonstrated, but further study is warranted. A possible pathogenetic mechanism of these diseases might be mediated by expression of nonstructural proteins in nonpermissive cells, leading either to direct cytopathology or induction of inappropriate cytokine responses. Alternatively, minor sequence differences between different viral isolates might predispose to induction of specific syndromes. As noted under “General Features of Autonomous Parvovirus Infections,” this is the case for many animal parvovirus systems.

The development of better *in vitro* and *in vivo* models would facilitate studies of erythrovirus infections. In addition, a permanent cell line permissive for high-level productive replication would make it practical to prepare viruses chimeric between isolates and look for virally encoded pathogenicity determinants. A readily available, small animal model would enable investigators to compare chimeric viruses and also to study pathogenesis under tightly controlled conditions.

ADENO-ASSOCIATED VIRUSES

The adeno-associated viruses (AAVs) are helper-dependent parvoviruses and are assigned to the *Dependovirus* genus of the subfamily *Parvovirinae* of the family *Parvoviridae* (26). As implied by the genus name, these ubiquitous viruses are

defective and originally were thought to replicate only in the presence of a helper adenovirus. It is now recognized that various herpes viruses (27,88,183), human papillomavirus (231), and vaccinia virus (183) can also supply helper functions for AAV. AAV can establish long-term latent infections in the absence of helper virus and be rescued by addition of helper virus. In addition, under certain genotoxic conditions, AAV can be induced to replicate to a limited extent without helper virus.

A brief discussion of AAV infections in a general virology textbook is warranted because the AAV are attractive and promising vector systems for gene therapy (28,94,112). To understand some of the potential problems in the development of these therapies, an understanding of AAV infections is helpful.

History

The AAV were initially observed in the mid-1960s as 20-nm particles, contaminating preparations of adenoviruses (15,99), and it was rapidly determined that the particles were defective viruses and not adenovirus breakdown products. Studies soon revealed that antibodies to several distinct serotypes of AAV (98) were common in human populations and that the viruses could be isolated from a variety of clinical specimens (20,33). It is now appreciated that AAV are extremely widespread in the animal kingdom and may occur in every species that has an adenovirus (reviewed in 29 and 177).

Because of their small genome size and relatively simple genomic organization, AAVs were the subject of numerous studies on DNA replication, DNA structure, gene expression, and gene regulation (27,28,207). Earlier work had suggested that AAV could initiate a latent infection in cell lines and be rescued hundreds of passages later by adding helper virus (113). When these observations were restudied with modern molecular biology, it was discovered that AAV persisted by integrating into chromosomal DNA in a nonrandom fashion (113,114 and 115). These findings, coupled with the apparent lack of pathogenicity, directly led to investigations of AAV as possible gene therapy vectors (28,112,146). Successful application of AAV vectors in gene therapy studies (78,94,107,199,215) has led to a renaissance in this interesting virus system.

Infectious Agent

Cell Culture Systems

AAV can be grown productively in a variety of continuous and primary cell lines, including HeLa cells, KB cells, African green monkey kidney cells and human embryo fibroblasts, provided a helper adenovirus or herpes virus is co-infected (21,28,33,135,145). Limited replication of AAV in the absence of helper virus can occur in some cells pretreated with a variety of genotoxic stresses (heat shock, ultraviolet irradiation, gamma-irradiation, carcinogens) (28,183,239). AAV can be rescued from latently infected cells by adenovirus superinfection (28,88,113,115).

Biologic Characteristics

There are six serotypes of primate AAV (denoted AAV1 to AAV6) currently described (20,88,99), with AAV2 being the type species (26). These can be discriminated by a variety of capsid-based antibody assays, including complement fixation, immunofluorescence, and neutralization (88,99). All AAVs exhibit the stability characteristic of parvoviruses (177).

All serotypes except AAV5 were initially found as contaminants of adenovirus stocks. AAV5 was isolated from a condylomatous genital lesion (20), and the natural helper for AAV5 may be a herpesvirus (88). AAV4 appears to be a monkey parvovirus (34,59,178). DNA sequence information is available for AAV2, AAV3, AAV4, AAV5, and AAV6 (19,59,145,180,207). A detailed description of the relatedness at both the DNA and protein level is beyond the scope of this chapter, but suffice it to say that AAV5 is the most distantly related to other members of the AAV (19). The divergent capsid residues have been modeled on the virion surface near the region involved with receptor binding, tropism, and neutralizing epitopes for other parvoviruses (5,19,58,180) (see Fig. 2).

Pathogenesis

Human Infections

Of necessity, AAV infections must initially occur in the presence of an adenovirus or herpesvirus infection. Based on viral isolation studies, the most likely routes of infection would appear to be respiratory, fecal-oral, and direct conjunctival inoculation (31,33,184). Although not conclusively demonstrated, sexual transmission has also been suggested (88,96,231). A discussion of latent AAV infections is not included in this chapter.

Animal Models

AAV infections are easy to study in a variety of convenient animal systems. In early studies, rhesus monkeys were used to examine possible teratogenicity of AAV1 (30), and mice were used to study virus distribution in various tissues after transplacental infection (124). The recent interest in AAV as a gene therapy vehicle has led to a large number of animal studies, including mice (199), rats (78,107,168), rabbits (94), and dogs (97,199).

Receptors

The AAVs have extremely broad cellular tropisms and can infect across species barriers (21,146,175,211). However, the efficiency of infection can vary among cell lines, and not all cells that bind AAV can be productively infected (59,135,146,175,211). Infection with these small, nonenveloped icosahedral viruses may involve participation of more

than one cell surface molecule. Molecules suggested as receptors or coreceptors are heparan sulfate proteoglycan (135,211), human fibroblast growth factor receptor 1 (175), and a_vb_5 integrin (210).

These results, along with others implying that different serotypes of AAV use different receptors (59), have direct implications for gene therapy studies. To direct gene expression to specific target cell populations, it will be crucial to ensure that the transducing virus can infect the desired cell type. Efficient transduction may require matching surface macromolecules on desired target cells with type-specific transducing virions (175,210). It may be necessary to develop hybrid capsid structures that bind to ligands on the target cell membranes.

Epidemiology

Virus Isolation and Molecular Epidemiology Studies

Infectious isolates of AAV2, AAV3, and AAV5 have been obtained from humans in the presence of numerous adenovirus serotypes (20,31,33). As might be expected, given the defective nature of AAV, all subjects were experiencing concomitant adenovirus infections. AAV3 was isolated during an outbreak of adenovirus type 3 conjunctivitis among children and adults (184). Virus was found in conjunctival, throat, and fecal specimens. As noted previously, a single isolation of AAV5 has been made from a male with a flat condylomatous genital lesion (20).

Evidence of AAV infection has also been sought using molecular epidemiology tools. AAV DNA, primarily AAV2, has been detected in peripheral blood cells, cervical biopsy samples, and tissues from spontaneous abortions (92,96,138,217,231). The significance of these identifications is unclear.

Seroepidemiology

Serum antibodies to AAV1, AAV2, AAV3, and AAV5 are extremely common (32,34,35,88,129,169,178,204). Average titers peak by the age of 10 years, at which time more than 60% of the population have neutralizing antibody to AAV1, AAV2, or AAV3, and more than 50% retain antibody into adulthood. The age pattern of seroconversion to AAV parallels acquisition of antibodies against adenoviruses. The seroepidemiology of AAV5 may be significantly different (88). The peak average titers occurred between 15 and 20 years of age, and about 50% of adults in the former West Germany tested positive (88).

In contrast, antibody to AAV4 is detected much less frequently (34), with peak incidence of about 10% between 2 and 5 years of age. The exception seems to be military recruits who have received respiratory vaccines prepared in monkey cells (178).

Despite the high seroprevalence to AAV, no study has conclusively linked AAV infection to any human disease. In addition, no evidence associating AAV with congenital infections or malformations has been found (30). Several surveys have found a lower incidence of anti-AAV antibodies in cancer patients (88,96,129).

Studies in cell cultures and in animal systems indicate that the presence of neutralizing antibody to a particular serotype of AAV reduces the efficiency of gene transduction (94,180). Efforts to deliver genes using AAV2 may have to circumvent the high prevalence of antibody to that serotype. It is the high prevalence of antibody to AAV that has stimulated investigators to develop gene therapy vectors from a variety of serotypes.

Perspectives

The AAVs were studied initially as useful tools for unraveling viral and cellular molecular biology. Once the ability of AAV to integrate into the cellular genome was appreciated, interest expanded to new groups of investigators.

The AAV have high potential as agents of gene delivery. Their imminent application as vectors in animals and humans will likely sustain and enhance interest in AAV. Their utility may ultimately be restricted because only relatively short sequences can be encapsidated in the small virions.

Additional problems will likely arise from the high prevalence of neutralizing antibody. This issue may be circumvented by developing vectors from a variety of AAV serotypes or by preparing vectors with hybrid capsids. Elucidation of the capsid structure will aid in addressing these problems.

Targeting of genes to specific cell types will require comprehensive analysis of virus–receptor interactions. It may be necessary to engineer the capsid surface to promote binding to receptors molecules on specific cell types.

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CHAPTER 71 The Family *Herpesviridae*: A Brief Introduction

Bernard Roizman and Philip E. Pellett

Who made the world I cannot tell;
'Tis made, and here I am in hell.

My hand, though now my knuckles bleed,
I never soiled with such a deed.

A. E. Housman, No. XIX in *More Poems*

DEFINITION

Inclusion in the Family *Herpesviridae*

Membership in the family *Herpesviridae* is based on the architecture of the virion (Fig. 1). A typical herpesvirion consists of a core containing a linear double-stranded DNA, an icosadeltahedral capsid approximately 100 to 110 nm in diameter containing 162 capsomeres with a hole running down the long axis, an amorphous-appearing, sometimes asymmetric material that surrounds the capsid and is designated the tegument, and an envelope containing viral glycoprotein spikes on its surface. A hotly debated issue is whether inclusion in the family should also be based on DNA sequence conservation. Thus viruses have been isolated from poikilothermic animals that retain conserved herpesvirus structural features, but their DNAs appear to have little if any similarity to the herpesviruses of homoiothermic animals. All of these viruses are presently included in the family.

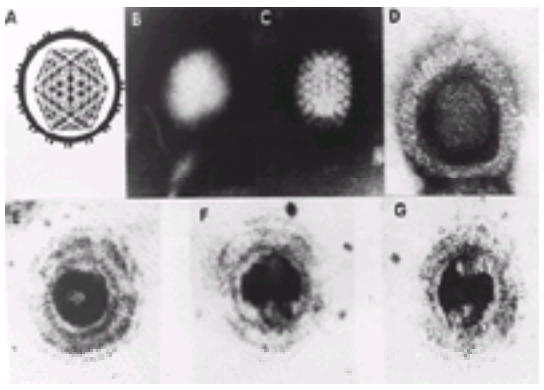

FIG. 1. The morphology of herpesviruses as visualized by transmission electron microscopy. **A:** Schematic representation of the herpesvirion seen through a cross section of the envelope with spikes projecting from its surface. The sides of the icosadeltahedron forming the capsid show a twofold symmetry. The irregular inner perimeter of the envelope is meant to represent the occasional asymmetric arrangement of the tegument. **B:** An intact, negatively stained HSV-1 virion. The intact envelope is not permeable to negative stain. The diameter of the virion is approximately 120 nm. **C:** An HSV-1 capsid exposed to negative stain and showing twofold symmetry matching the diagrammatic representation of the capsid in A. **D:** HSV-1 capsid containing DNA permeated with uranyl acetate. The electron micrograph shows the presence of threadlike structures 4 to 5 nm wide on the surface of the core. **E, F, G:** Electron micrographs of thin sections of HSV-1 virions showing the core cut at different angles. The preparation was stained with uranyl acetate and counterstained with lead citrate. The DNA core preferentially takes up the stain and appears as a toroid with an outer diameter of 70 nm and an inner diameter of 18 nm. The toroid appears to be suspended by a fibrous cylindrical structure. The micrographs show the toroid seen looking down the hole **(E)**, in cross section **(F)**, and from the side **(G)**. (The electron micrographs shown in panels **D** to **G** are from ref. 22, with permission.)

Distribution in Nature

Herpesviruses are highly disseminated in nature and most animal species have yielded at least one herpesvirus and frequently several diverse herpesviruses upon examination. Inasmuch as few herpesviruses naturally infect more than one species, the number of herpesviruses in nature is likely to exceed the approximately 130 herpesviruses identified to date. Thus far, nine herpesviruses have been isolated from humans [herpes simplex virus 1 (HSV-1), herpes simplex virus 2 (HSV-2), human cytomegalovirus (HCMV), varicella-zoster virus (VZV) and Epstein-Barr virus (EBV), and *Human herpesviruses 6A*, *6B*, *7*, and *8* (HHV-6A, HHV-6B, HHV-7, and HHV-8)]. The known herpesviruses are listed in Table 1.

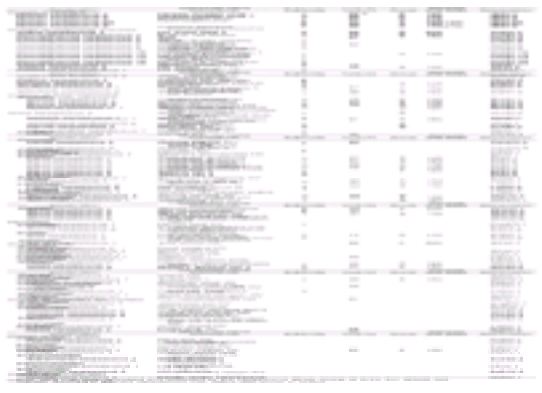

TABLE 1. *Viruses of the family* herpesviridae[a]

Biologic Properties

The known herpesviruses appear to share four significant biologic properties:

1. All herpesviruses specify a large array of enzymes involved in nucleic acid metabolism (e.g., thymidine kinase, thymidylate synthetase, dUTPase, ribonucleotide reductase), DNA synthesis (e.g., DNA polymerase, helicase, primase), and processing of proteins (e.g., protein kinases), although the exact array of enzymes may vary from one herpesvirus to another.
2. The synthesis of viral DNAs and capsid assembly occurs in the nucleus. Capsids are enveloped as they transit through the nuclear membrane. A contentious issue is the fate of the herpesvirion as it transits the cytoplasm, whether it undergoes internal maturation/single envelopment or whether it undergoes successive de-envelopment and envelopment (reviewed in ref. 18).
3. Production of infectious progeny virus is invariably accompanied by the destruction of the infected cell.
4. The herpesviruses examined to date are able to remain latent in their natural hosts. In cells harboring latent virus, viral genomes take the form of closed circular molecules, and only a small subset of viral genes are expressed. Latent genomes retain the capacity to replicate and cause disease upon reactivation. The precise molecular mechanisms that lead to reactivation from the latent state are not fully understood and may differ from one virus to another. Latency differs from chronic infection in that infectious progeny are not present. Conversely, the capacity to reactivate differentiates latency from abortive infection.

Herpesviruses also differ with respect to their biologic properties. Some have a wide host-cell range, multiply efficiently, and rapidly destroy the cells they infect (e.g., HSV-1, HSV-2). Others have a narrow host cell range (EBV, HHV-6) or have a long replicative cycle (HCMV). Although all herpesviruses remain latent in a specific set of cells, the exact cell in which they remain latent varies from one virus to another. For example, whereas latent HSV has been detected only in neurons of dorsal root ganglia, latent EBV has been found primarily in B lymphocytes. Herpesviruses differ substantially with respect to the clinical manifestations of diseases they cause.

NOMENCLATURE AND CLASSIFICATION

Nomenclature

A formal binomial nomenclature is not currently applied. The International Committee on the Taxonomy of Viruses (ICTV)-endorsed nomenclature consists of designation of herpesviruses by serial Arabic number and the family (most cases) or subfamily (for primates and some animals) of the natural host of the virus (e.g., *Human herpesvirus 7*, *Cercopithecine herpesvirus 1*; see Table 1). The species number is not intended to imply anything about the relationship between a virus and other herpesviruses that infect the same host species (e.g., HHV-7 and HHV-8 are members of different subfamilies) or between similarly numbered viruses that infect different host species (e.g., EHV-2 and BHV-2 are members of different subfamilies). A recent nomenclature change that applies to all viruses is italicization, plus capitalization of the first letter of the first word of the names of ICTV-recognized virus species (61); this system will be used in this chapter.

Classification

The members of the family *Herpesviridae* were initially classified by the Herpesvirus Study Group of the ICTV into three subfamilies (i.e., the *Alphaherpesvirinae*, the *Betaherpesvirinae*, and the *Gammaherpesvirinae*) on the basis of biologic properties and before DNA sequences of the individual members of the family were known (50,51). The same study group classified a small number of herpesviruses into genera based on DNA sequence homology, similarities in genome

sequence arrangement, and relatedness of important viral proteins demonstrable by immunologic methods. Whereas a few genes [e.g., homologs of glycoprotein B of HSV-1 (46,64), or of glycoprotein H (23)] are conserved among members of different subfamilies, nucleic acid and protein sequence similarities are particularly useful for the classification of viruses that are closely related (genera).

Alphaherpesvirinae

The members of the subfamily *Alphaherpesvirinae* were classified on the basis of a variable host range, relatively short reproductive cycle, rapid spread in culture, efficient destruction of infected cells, and capacity to establish latent infections primarily but not exclusively in sensory ganglia. This subfamily contains the genera *Simplexvirus* (HSV-1), *Varicellovirus* (VZV), *Marek's disease-like virus*, and *Infectious laryngotracheitis-like virus.*

Betaherpesvirinae

A nonexclusive characteristic of the members of the subfamily *Betaherpesvirinae* is a restricted host range. The reproductive cycle is long and the infection progresses slowly in culture. Infected cells frequently become enlarged (cytomegalia) and carrier cultures are readily established. The virus can be maintained in latent form in secretory glands, lymphoreticular cells, kidneys, and other tissues. This subfamily contains the genera *Cytomegalovirus* (HCMV), *Muromegalovirus* (murine cytomegalovirus), and *Roseolovirus* (HHV-7).

Gammaherpesvirinae

The experimental host range of the members of the subfamily *Gammaherpesvirinae* is limited to the family or order to which the natural host belongs. *In vitro*, all members replicate in lymphoblastoid cells, and some also cause lytic infections in some types of epithelioid and fibroblastic cells. Viruses in this group are usually specific for either T or B lymphocytes. Latent virus is frequently demonstrated in lymphoid tissue. This subfamily contains two genera: *Lymphocryptovirus* (EBV), and *Rhadinovirus* (AtHV-2 and SaHV-2).

Since this classification system was adopted, nucleotide sequence analyses (detailed later) support and have expanded the platform on which the system rests. The issues that the new data pose are dealt with at the end of this chapter.

ARCHITECTURE

Structural Components

The Core

The core of the mature virion contains the viral DNA in the form of a torus (21,22,44). In some herpesvirions, the torus appears to be suspended by a proteinaceous spindle consisting of fibrils embedded in the underside of the capsid and passing through the hole of the torus. The precise arrangement of the DNA in the torus not known.

The Capsid

The structural features of the capsid (i.e., its 100-nm diameter and 162 capsomeres) are characteristic of all herpesviruses. The pentameric capsomeres at the vertices have not been well characterized. The hexameric capsomeres are 9.5 × 12.5 nm in longitudinal section; a channel 4 nm in diameter runs from the surface along their long axis (65).

The Tegument

The tegument, a term introduced by Roizman and Furlong (54) to describe the structures between the capsid and the envelope, has no distinctive features in thin sections, but it may appear to be fibrous on negative staining (42,43,65). Structural polarity across the tegument has been visualized by immunoelectron microscopy (59), indicating that it is an ordered structure. The tegument is sometimes distributed asymmetrically, and its thickness may vary depending on the location of the virion within the infected cell; when the amount is variable, there is more of it in virions accumulating in cytoplasmic vacuoles than in those accumulating in the perinuclear space (21). The available evidence suggests the amount of tegument is more likely to be determined by the virus than by the host (36).

The Envelope

Electron microscopic studies on thin sections have shown that the outer covering of the virus, the envelope, has a typical trilaminar appearance (19). It appears to be derived from patches of altered cellular membranes (1,21,43). The presence of lipids was demonstrated by analyses of virions (3) and by the sensitivity of the virions to lipid solvents and detergents (57). The herpesvirus envelope contains numerous protrusions of glycoprotein that are more numerous and shorter than those appearing on the surface of many other enveloped viruses (65). The number and relative amounts of viral glycoproteins vary; HSV specifies at least 11 glycoproteins (see Chapter 72); the copy number of individual glycoproteins can exceed 1,000 per virion.

The Virion

Herpesvirions have been reported to vary in size from 120 to nearly 300 nm (reviewed in ref. 54). The variation is in part due to variability in the thickness of tegument. Another major source of variability is the state of the envelope. Intact envelopes are impermeable and generally retain the quasi-spherical shape of the virion during preparation for electron microscopy (see Fig. 1). Damaged envelopes are permeable to negative stains; permeated virions have a

sunny-side-up-egg appearance with a diameter much larger than that of intact virions.

The precise number of polypeptide species contained in the virions is not known and may vary from one virus to another. Estimates generally range from 35 to 45 polypeptides.

Herpesvirus DNAs

Size, Conformation, and Base Composition

Herpesvirus DNAs extracted from virions and characterized to date are linear and double stranded, but they circularize immediately upon release from capsids into the nuclei of infected cells.

Variable features of herpesvirus DNAs include their length and base composition. The length of herpesvirus DNAs varies from approximately 120 to 250 kbp (see Table 1). The variability in genome lengths of different herpesviruses is distinct from the polymorphism in the size of DNAs of individual viruses. Variation in the size of the genome of any one herpesvirus appears to be minimal, but not insignificant. Thus, herpesvirus genomes contain terminal and internal reiterated sequences that can vary in copy number, leading to variations in genome length of more than 10 kbp. Spontaneous deletions also occur; they have been noted in HSV and EBV strains passaged outside the human host (e.g., EBV strain P3HR1, HSV-1 strain HFEM). Recently, a highly passaged strain of HCMV was found to lack a segment encoding at least 19 genes present in wild-type isolates (10).

The base composition of herpesvirus DNAs varies from 31% to 75% total G+C content (see Table 1). Furthermore, herpesvirus DNAs vary with respect to the extent of homogeneity of G+C content across the length of the genome. The extent of inhomogeneity in the base composition varies from minimal (e.g., HSV) to very extensive. For example, the DNAs of SaHV-2 and AtHV-2 (6) consist of reiterated sequences of high G+C content at the genomic termini and unique segments of low G+C content.

Sequence Arrangements in Herpesvirus DNAs

An interesting feature of herpesvirus DNAs is their sequence arrangement. The sequence arrangement shown in Figure 2 emphasizes the presence and location of reiterations of terminal sequences greater than 100 bp. According to this scheme, the herpesviruses can be divided into six groups designated by the letters A to F. In the genomes of viruses comprising group A and exemplified by channel catfish herpesvirus, a large sequence from one terminus is directly repeated at the other terminus. In the group B genomes exemplified by herpesvirus saimiri, the terminal sequence is directly repeated numerous times at both termini, and furthermore, the number of reiterations at the termini may vary. In the group C genomes exemplified by EBV, the number of direct terminal reiterations is smaller, but there may be other, directly repeated and unrelated sequences greater than 100 bp that subdivide the unique (or quasi-unique) sequences of the genome into several well-delineated stretches. In group D genomes exemplified by VZV, the sequence from one terminus is repeated in an inverted orientation internally. In these genomes, the domain consisting of the stretch of unique sequences flanked by inverted repeats (small, or S, component) can invert relative to the remaining sequences (large, or L, component), so that the DNA extracted from virions or infected cells consists of two equimolar populations differing solely in the orientation of the S component relative to the L component. In group E viral genomes exemplified by those of HSV and HCMV, sequences from both termini are repeated in an inverted orientation and juxtaposed internally, dividing the genomes into two components each of which consists of unique sequences flanked by inverted repeats. In this instance, both components can invert relative to each other, and DNA extracted from virions or infected cells consists of four equimolar populations differing in the relative orientation of the two components. The genomes comprising group F are exemplified by tupaia herpesvirus; the terminal sequences are not identical and are not repeated either directly or in an inverted orientation. Although the potential of inverted repeat sequences to cause inversions and gene expansion is well appreciated, their role and the advantage they confer to viral replication are not understood.

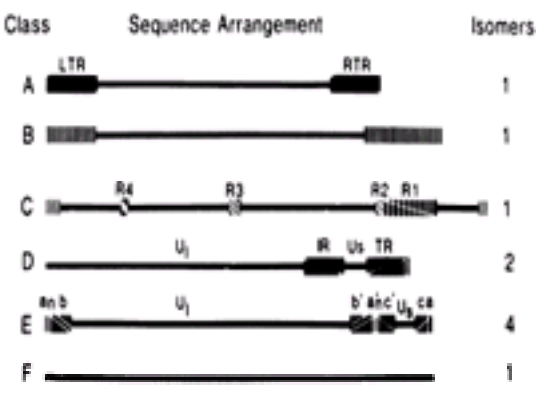

FIG. 2. A schematic diagram of the sequence arrangements in the six classes of genomes of the viruses comprising the family *Herpesviridae*. The genomes A, B, C, D, E, and F are exemplified by the channel catfish herpesvirus, herpesvirus saimiri, Epstein-Barr virus, varicella-zoster virus, herpes simplex viruses, and tupaia herpesvirus, respectively. In the schematic diagram, the *horizontal lines* represent unique or quasi-unique regions. The reiterated domains are shown as *rectangles* and are designated as left and right terminal repeats (LTR and RTR) for group A, repeats R1 to R4 for internal repeats of group C, and internal and terminal (IR and TR) repeats of group D. The termini of group E (e.g., HSV) consist of two elements. One terminus contains *n* copies of sequence *a* next to a larger sequence designated as *b*. The other terminus has one directly repeated *a* sequence next to a sequence designated *c*. The terminal *ab* and *ca* sequences are inserted in an inverted orientation (denoted by *primes*) separating the unique sequences into a long (U_L) and short (U_S) domains. Terminal reiterations in the genomes of group F have not been described. In group B, the terminal sequences are reiterated numerous times at both termini. The number of reiterations at each terminus may vary. The components of the genomes in classes D and E invert. In class D, the short component inverts relative to the long. Although (rarely) the long component may also invert, most of the DNA forms two populations differing in the orientation of the short

component. In the class E genomes, both the short and long components can invert and viral DNA consists of four equimolar isomers.

All herpesvirus genomes examined to date contain at their termini signals for packaging the DNA into capsids (16).

Genetic Architecture

Herpesvirus Genes

Most herpesvirus genes contain a promoter/regulatory sequence spanning 50 to 200 bp upstream of a TATA box, a transcription initiation site 20 to 25 bp downstream of the TATA box, a 5' nontranslated leader sequence of 30 to 300 bp, a single major open reading frame (ORF) with a translation initiation codon that meets the host requirements for efficient initiation, 10 to 30 bp of 3' nontranslated sequence, and a canonical polyadenylation signal with standard flanking sequences. Exceptions exist and are important. Genes without a TATA box [e.g., HSV-1 $g_1$34.5 gene (12)] and initiation from a second in-frame methionine (34) have been reported. In the case of HSV late genes, the promoter-regulatory sequences may be located 3' to the TATA box (35).

Gene overlaps are common. Thus, except for head-to-head gene arrangements, the promoter-regulatory sequences of 3' genes are located within the coding domain of the 5' gene. In several instances, a second transcriptional unit is embedded in the coding sequence to yield a truncated polypeptide initiated at an internal methionine and colinear with the carboxyl-terminal domain of the larger protein [e.g., HSV U_L26 and U_L26.5 (33)]. The resulting proteins thus share a domain of identical amino acid sequence yet can differ markedly in their function. Finally, ORFs can be expressed that are situated entirely antisense to each other [e.g., HSV-1 $g_1$34.5 and ORFs P and O (32)]. A common feature of herpesvirus genomes is clusters of 3' co-terminal transcripts, each designed to express a different ORF. Within these clusters, coding domains seldom overlap by more than a few codons of the same or opposite strand. Although most genes are transcribed by RNA polymerase II, at least a small set of transcripts (EBERs) made in cells infected with EBV are synthesized by RNA polymerase III (27). Most herpesvirus genes are not spliced; that is, their primary transcription product is the mature mRNA sequence, minus polyadenylation. Nonetheless, every herpesvirus encodes at least a handful of spliced genes. One strategy is to use splicing to enable differential regulation of a gene at different parts of the virus life cycle. In some cases, this means that the same ORF is accessed from different promoters. Herpesviruses also encode noncoding RNAs (e.g., the Ori_S RNA and latency-associated transcripts of HSV and the EBERs of EBV).

Herpesviruses encode between 70 (the smallest genome) and 200 (the largest genome) genes. These estimates are rooted in studies of viral mutants, transcriptional mapping, analysis of the translation products of particular transcripts, and computational analyses of protein coding potential. Current estimates probably understate the number of gene products. Noncoding RNA have not been included in this count, and proteins can be encoded by very small ORFs that have not yet been identified as functional. Alternative splicing, translational frameshifting, and antisense ORFs, and tissue-specific transcription are only now beginning to be fully appreciated. Elucidation of the true genetic complexity encoded in herpesvirus genomes remains one of the major objectives of herpesvirus research.

Gene Functions

Two characteristics clearly define the functions of herpesvirus gene products. First, they frequently have multiple disparate functions that may or may not be related. For example, HSV infected cell protein no. 0 (ICP-0) acts as a promiscuous transactivator, but at a molecular level it localizes sequentially first in the nucleus and later in the cytoplasm, and it interacts with several host proteins located in both compartments (20,31). Even some of the smaller proteins (e.g., HSV-1 U_S11 and $g_1$34.5) have been ascribed more than one function. The decision as to which function is performed by a particular protein may be determined by the nature of posttranslational modifications, to which many herpesvirus proteins are subjected. Full assessment of the multifunctional nature of viral proteins and their dependence on interactions with other viral proteins requires that they be studied in the context of infection with wild-type or mutant viruses.

The second characteristic is related to the role of viral-encoded proteins in viral replication. For HSV, more than 50% of the ORFs are dispensable for growth in cells in culture. For the most part, however, these genes are not dispensable for viral replication or for reactivation from the latent state in experimental animal systems. On the other hand, in assays involving establishment of latency, no virus gene has been found to be indispensable. The herpesvirus genes (other than those involved in entry, DNA replication, packaging of viral genomes, and envelopment) function in creating a suitable environment for viral replication, blocking host defenses to infection, and, where proven, establishment of the latent state. Assessment of the full set of functions of viral proteins in human hosts is hampered by the fact that no experimental system can fully mimic the biology of the natural host.

Genes involved in viral replication encode proteins required for entry into the cell, regulation of viral gene expression, nucleotide metabolism, synthesis of viral DNA and structural proteins, and virion assembly. Regulatory genes are specifically adapted to the requirements of the cell type inhabited by the virus *in vivo.* Some subsets of herpesviruses (e.g., the alphaherpesviruses) share clear homologs of regulatory genes such as a4, a0, and a27. Other groups of herpesviruses have important regulatory genes that have little obvious sequence similarity, although they may occupy similar genomic locations and even share splicing patterns (e.g., the immediate-early genes of the betaherpesviruses).

Every herpesvirus studied to date establishes latent infection in a specific population of cells. The mechanisms differ from one virus to the next. There appears to be no general strategy or shared genetic component for establishment, maintenance, or termination of the latent state, even though latency is the cornerstone of herpesvirus biology. Whereas

members of some genera of herpesviruses encode several proteins that are expressed during maintenance of the latent state (e.g., EBV), others (e.g., HSV) have no absolutely required expression of the viral genome during this phase of latency.

The cellular and organismal host ranges of herpesviruses differ enormously, from very wide (e.g., HSV) to very narrow (e.g., EBV, HHV-6B). The host range is determined only in part by the availability of receptors. Little is known about the cell-specific factors that enable herpesviruses to replicate in specific hosts or cell types.

Herpesviruses are able to alter the cellular environment to suit their needs. These alterations include shutting off or stimulating host cell macromolecular synthesis, inducing or inhibiting host cell DNA replication, or immortalizing the host cell. Parenthetically, although many herpesviruses encode genes that can transform cells in experimental systems outside the context of viral infection, in only a few cases, such as the immortalization of B cells by EBV in the process of establishing its latent repository, is transformation or immortalization an integral part of the *in vivo* life cycle.

The environment in which herpesviruses replicate is decidedly unfriendly to the virus. An armamentarium of viral functions is encoded by herpesviruses to fend off both cellular and organismal reactions to the presence and expression of viral genes (reviewed in ref. 63). At the cellular level, the virus blocks the induction of programmed cell death and the activation of the interferon pathway (e.g., by activation of protein kinase R, which leads to shutoff of protein synthesis). At the organismal level, the response is highly varied and depends in part on the cells in which the virus replicates. Here the responses range from blocking the presentation of antigenic peptides on the surfaces of antigenic cells, to molecular mimicry, in which herpesvirus-encoded proteins perform some, but not all, functions of immunomodulators. A common consequence is a delay in the elimination of the infected cell—long enough to enable the virus to replicate, colonize the host, and become available for transmission to another nonimmune individual. The range of genes evolved to this end varies from one genus to another, with limited conservation among members of subfamilies. The diversity of approaches is a remarkable testimony both to the depth and complexity of host defenses and to the adaptive prowess of the virus. That the virus wins in the infected cell but normally allows the host to survive attests to an evolutionary necessity, since viruses that kill their host ultimately disappear.

Regulation of Gene Expression

Herpesvirus genes belong to any one of several regulatory classes; the more they are studied, the more complex appears to be the system that regulates their expression. Standing apart are genes expressed during latency, and even here, as in the case of EBV, there are multiple stages of latency that differ with respect to the genes that are expressed. In cells productively infected with HSV, viral genes form at least four groups that differ with respect to the order of gene expression and the mechanisms that drive it. These are the a (immediate-early) genes, which require no prior viral protein synthesis for their expression; the b (early) genes, whose expression is totally independent of viral DNA synthesis; g_1 (partial-late) genes, whose expression is augmented by the onset of viral DNA synthesis; and g_2 (true-late) genes, whose expression is totally dependent on viral DNA synthesis. The immediate-early, early, and late nomenclature is rooted in early phage studies; it has the virtue of instant recognition but does not fully describe the events taking place in the infected cell. Both systems are used widely and interchangeably. Herpesvirus gene expression during productive infection represents a classic regulatory cascade, the basic features of which are conserved across the family.

Gene Relatedness and Arrangement

Complete genomic sequences have been determined for approximately 20 herpesviruses; smaller segments have been sequenced for many others. From this information, several fundamental principles of herpesvirus genetic architecture have been deduced.

Herpesvirus gene sequences are related to each other in ways that reflect the longstanding, biology-based classification of herpesvirues into subfamilies and their respective genera, as described later. These relationships are substantial and easy to detect with any of the widely available tools for sequence analysis. Thus the nucleotide sequence of a 200-bp polymerase-chain-reaction product amplified from the viral DNA polymerase gene by the use of degenerate consensus primers is sufficient to establish the identity of a herpesvirus and place it into the appropriate subfamily. Higher resolution and more robust classification can be obtained by expanding the size of the comparison sequence and by analyzing more than one gene. Comparisons of the available herpesvirus glycoprotein B (gB) sequences are shown in Figure 3 and Figure 4. The gB tree shown in Figure 3 provides a good representation of the current state of the taxonomy of the alpha-, beta-, and gammaherpesviruses down to the genus level.

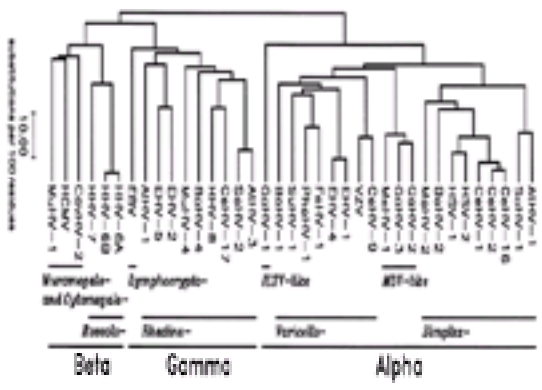

FIG. 3. Relationships among herpesvirus gB sequences. This phylogram was constructed from aligned sequences by the UPGMA method. Virus abbreviations are as listed in Table 1. (Figure courtesy of Yuan-Xiang Meng, Centers for Disease Control and Prevention.)

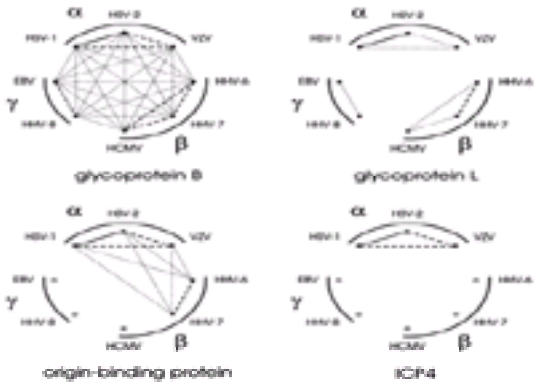

FIG. 4. Clustering of conserved genes among the human herpesviruses. Viruses encoding the indicated protein are shown by *filled dots*; *empty dots* indicate viruses that do not encode the protein. Degrees of similarity are indicated by the *lines* connecting the viruses; *solid*, *dashed*, and *dotted lines* represent >70%, 40% to 70%, and <40% identity, respectively. Glycoprotein B is highly conserved and is represented in all of the viruses; glycoprotein L is conserved within subfamilies; the origin-binding protein is conserved among the alphaherpesviruses and a subset of betaherpesviruses; ICP4 is found only in the alphaherpesviruses. The HHV-6A and HHV-6B counterparts of these proteins are highly related to each other and have similar distances to the other viruses, and they are represented here as a single entity.

Approximately 26 genes, referred to as the herpesvirus core genes, are conserved across the alpha-, beta-, and gammaherpesviruses (Table 2). Each of these genes belongs to one of seven core gene blocks, which encode 2 to 12 genes. Within each gene block, gene order and polarity are conserved. Across the family, the core gene blocks are found in various permutations of order and orientation. In general, block arrangement, and thus gene order, is conserved at the subfamily level. The arrangements of the conserved gene blocks in the genera containing human herpesviruses are summarized in Figure 5.

Gene regulation
HSV-1 ICP27 homolog
Nucleotide metabolism
Ribonucleotide reductase, large subunit
Uracil DNA glycosylase
dUTPase
DNA replication
Alkaline exonuclease
Helicase/primase (3 subunits)
DNA polymerase
ssDNA binding
Virion
Maturation
Transport?
Terminase/packaging
DNA packaging
Scaffold protease
Capsid
Major capsid protein
Minor capsid protein
Capsid (2)
Tegument
Large tegument protein
Envelope
Glycoprotein B
Glycoprotein H
Glycoprotein M
Four genes of unknown function

TABLE 2. *Genes conserved among the alpha-, beta-, and gammaherpesviruses*

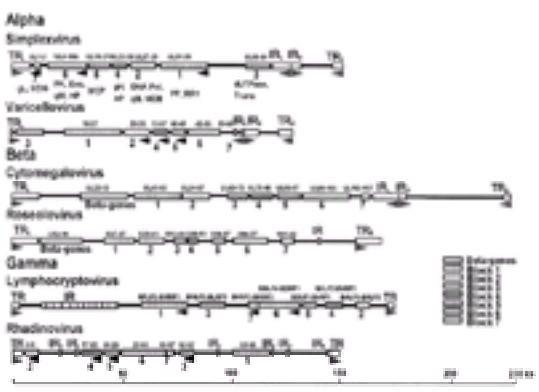

FIG. 5. Conserved gene blocks of the *Alpha*-, *Beta*-, and *Gammaherpesvirinae* (adapted from refs. 7,11,24,39, and 45, with permission). Each genus is represented by a member in its prototype genomic arrangement. Gene nomenclatures are from the following references: *Simplexvirus* (38), *Varicellovirus* (14), *Cytomegalovirus* (11), *Roseolovirus* (17,24,29), *Lymphocryptovirus* (4), and *Rhadinovirus* (55). The seven conserved herpesvirus sequence blocks (block 1 through block 7), plus an additional sequence block (beta-genes) found only in betaherpesviruses, are diagramed. ORFs bounding the conserved sequence blocks are indicated. Gene blocks inverted relative to the betaherpesviruses are indicated by the *black arrowheads.* Structures of genomic termini are indicated. TRL, IRL, IRS, and TRS denote inverted repeats flanking the L or S component, respectively. *Hatched arrowheads* indicate directionality of terminal repeats. Gene product abbreviations: gL, glycoprotein L; UDG, uracil-DNA glycosylase; PK, phosphotransferase; Exo, alkaline exonuclease; gM, glycoprotein M; HP, helicase/primase complex; MCP, major capsid protein; gH, glycoprotein H; AP, protease; DNA Pol, DNA polymerase; gB, glycoprotein B; PP, polymerase-associated processivity factor; RR1, ribonucleotide reductase large subunit; and Trans, herpesvirus transactivator. (Figure provided courtesy of Geraldina Dominguez, Centers for Disease Control and Prevention.)

Some genes are conserved at the subfamily level. Thus alphaherpesviruses encode related latency-associated genes, glycoprotein D, a tegument-associated protein that induces transcription from a-genes, and a transcriptional regulatory protein related to HSV a4 (see Fig. 4). Betaherpesviruses encode a block of 14 genes that have no counterparts elsewhere in the family, sets of diversified genes that are related to HCMV U_S22, and highly divergent immediate-early genes that nonetheless have shared evolutionary history. Gammaherpesviruses encode more homologs of cell genes than do other herpesviruses, plus proteins needed to maintain latent genomes in dividing cells.

An interesting variation on the linkage of gene sets to subfamilies is offered by the origin-binding protein (OBP) and its binding site. OBP binds to its cognate sites in origins of lytic replication and serves to nucleate the DNA replication machinery and play a role in initiating DNA replication. Only alphaherpesviruses and members of the *Roseolovirus* genus of the betaherpesviruses encode OBP homologs and OBP sites (28) (see Fig. 4). Other betaherpesviruses and the gammaherpesviruses do not encode OBP homologs or sites; they initiate DNA replication by another mechanism.

Gene content (the presence or absence of homologous genes) has been used to deduce phylogenetic relationships among the herpesviruses that are consistent with phylogenies based on gene sequences (40). In addition, comparisons of the presence or absence of members of gene families from virus to virus enable the construction of reciprocal trees that reveal the groups of genes encoded in each of the possible combinations of subfamilies (Fig. 6). This amplifies some of the relationships shown in Figure 4 and illustrates both the depth of shared evolutionary history and the breadth of divergence among these viruses.

FIG. 6. Reciprocal tree showing clustering of groups of related genes (each group being a cluster of orthologous groups, or COG) based on the phylogenetic profile of each group, as identified from a collection of 13 complete herpesvirus genome sequences (40). The tree was constructed by identifying 104 COGs among the genes encoded by the 13 viruses and then tabulating for each virus the presence or absence of a member for each of the COGs. Clusters among the phylogenetic profiles were identified by the UPGMA method and the results plotted as shown. COGs common among, or specific to, various combinations of the herpesvirus subfamilies are evident. As also shown in Figure 4, the COG for some highly conserved genes (e.g., glycoprotein B) included members from all of the viruses except for channel catfish virus (IHV), and some functionally related proteins are split among more than one COG because their sequences are very different [e.g., glycoprotein L (see Fig. 4)], and other genes are represented in only a small subset of the viruses [e.g., ICP4 (see Fig. 4)]. (From ref. 40, with permission.)

Herpesviruses generally encode at least one gene of obvious host origin; HHV-8 has acquired 12 (55). Examples include thymidylate synthase and bcl-2. These genes appear to have been acquired by retrotransposition events from cDNAs, given that they are most often encoded on the viral genome as continuous ORFs that do not require splicing. Some of these genes appear to have been acquired independently by different herpesvirus lineages (again, bcl-2 and thymidylate synthetase). Host-acquired genes tend to be located toward the viral genomic termini and between core gene blocks. In some cases, the host-acquired genes retain a function similar to its cellular counterpart; expression from the viral genome allows the desired product to be produced when needed by the virus. In other cases, the host-acquired gene has been modified to alter its function—for example, the HHV-8 homolog of the cellular D-type cyclin does not respond to signals that inhibit the cellular version, rendering the viral version constitutively active and capable of transforming cells (2).

HERPESVIRUS EVOLUTION

The origins of herpesviruses are the subject of interesting but largely unverifiable speculations. The presence of cellular homologs in many viruses indicates that herpesviruses acquired many of their genes from cellular genomes. The similarity between mechanisms and pathways of replication of herpesviruses and those of DNA phages cannot be explained totally on the basis of convergent evolution, and it places the ancestry of herpesviruses at the early stages of the evolution of parasitism. The similarities in the structure of herpesviruses suggest that they have arisen from a common ancestor. The ancestral virus evolved into at least two highly diverged lineages, one represented by the alpha-, beta-, and gammaherpesvirus subfamilies that infect birds and mammals, the other represented by the herpesviruses of poikilothermic animals. Although there is very little obvious genetic similarity between the latter herpesviruses and the rest of the family, their capsid structures are well conserved (5). Because the capsid sizes of all herpesviruses are nearly identical, it could be predicted that the capsid evolved to contain the largest herpesvirus genomes, which are conserved today only among some members of the betaherpesviruses.

The ancestral herpesvirus of birds and mammals must have encoded at least some of the core genes that are shared by the contemporary progeny. The three major lineages of these viruses emerged prior to the mammalian radiation (37).

According to one analysis, the alphaherpesviruses emerged prior to the beta- or gammaherpesviruses (37); a different method suggested that HHV-6 is closest to being a progenitor herpesvirus and that the alphaherpesviruses emerged relatively recently (30). The evolutionary distances between closely related herpesviruses that infect different species [e.g., the rhadinoviruses HHV-8 (in humans) and HVS (in squirrel monkeys)] suggest that viral speciation occurred at approximately the same time as did host speciation (37, 41). Thus herpesviruses have an intimate shared evolutionary history with their hosts.

In the past decades, a large number of rational but unprovable mechanisms have been used to explain the divergence of herpesviruses. These include (a) acquisition of and subsequent evolution of chunks of cellular DNA, (b) transposition of viral or cellular DNA or RNA, (c) gene duplication and evolutionary divergence, (d) shifts in base and dinucleotide composition in response to viral DNA polymerase mutation rates or concentrations of intracellular nucleotide precursors and pools, and (e) standard mutagenic processes (8, 13, 15, 25, 37, 50, 58).

An important point is that herpesviruses are well adapted to their hosts. In the natural immunocompetent host, fatal infections are rare; from the point of view of viral survival, fatal infections are counterproductive as they do not lead to transmission of virus. This is not always the case when herpesviruses infect a heterologous host, such as the frequently fatal outcomes of infection of cattle with pseudorabies virus, of humans with simian B virus, or infection of African or Asian elephants with herpesviruses from the other species (49). Thus, in addition to adaptation to specific cellular niches, many of the auxiliary genes must also function in striking the optimal balance between virus growth in the host and a combination of avoidance and stimulation of host responses, so that the virus can persist in populations of organisms over an evolutionary time scale.

TRENDS AND ISSUES IN HERPESVIRUS NOMENCLATURE AND CLASSIFICATION

Naming of Genes and Their Products

The ICTV has established a framework for naming herpesviruses but not viral genes or their products. This has led to several problems: The nomenclature applied to viral genes and their products is a hodgepodge, difficult to follow, and potentially misleading. Historical precedents, self-interests, and common usage maintain nomenclatures whose time has long past. The complexity of the problems facing us may be summarized with a few examples:

The HSV-1 virion transactivator was first identified as a component of the virion and designated by its migration in denaturing gels relative to that of other virion proteins [VP16 (56)]. Subsequently, it was designated by its migration in denaturing gels relative to other infected cell proteins [ICP25 (26)]. Still later, it was designated by its apparent molecular weight in denaturing polyacrylamide gels [vmw65 (9)], by its best-known function [a-trans-inducing factor or a-TIF (47)], or by the position of the gene encoding it [U_L48 (38)]. Another example is the major regulatory protein of HSV-1, which was designated initially as ICP4 and subsequently as vmw175 and a4.

HSV is the most extensively studied herpesvirus, but it is not the only one beset by problems in the nomenclature of its genes and proteins. For example, with the exceptions of the proteins expressed during latent infection, EBV genes and their products are named after the DNA fragment in which the gene maps. This nomenclature precludes all but the most dedicated from visualizing the position of the gene.

The least precise current designations are based on apparent molecular weight. For example, ICP4 (vmw175), is expressed in several forms that differ in electrophoretic mobility in denaturing polyacrylamide gels. In addition, the apparent molecular weights relative to standards may vary depending on the composition of the gel. Furthermore, although the HSV-2 homolog has a higher apparent molecular weight, it is sometimes designated by the apparent molecular weight of its HSV-1 counterpart.

The naming of homologous genes based on their HSV-1 counterparts enables a relational database but leads to a loss of information and may be misleading. For example, glycoprotein B is encoded as U_L28 in HSV, U39 in HHV-6B, U_L55 in HCMV, orf8 in HHV-8, and so on. A designation such as gB(HHV-6B) addresses sequence homology but not map position. A real hazard rests in the assumption that the functions of gB are fully conserved from virus to virus. Many herpesvirus proteins perform multiple functions; proteins sharing a conserved block of amino acids may diverge in the functions encoded in other domains. To join in nomenclature by finite commonalities or splinter by fine distinctions is a dilemma that must be solved on middle ground; future nomenclatures may have to allow for the multifunctionality of viral proteins. Ideally, protein names would convey key information useful to the field, such as major function, kinetic class, and genome map location.

Speciation

The definition of viral species accepted by the ICTV is “A virus species is a polythetic class of viruses that constitutes a replicating lineage and occupies a particular ecologic niche” (60). Consistent with this, herpesvirus are defined as distinct species if “(a) their genomes differ in a readily assayable and distinctive manner across the entire genome (e.g., restriction endonuclease cleavage site patterns obtained with many enzymes) and not merely at a specific site (e.g., small number of genes or small number of restriction endonuclease sites) and (b) if the virus can be shown to have distinct epidemiologic and biologic characteristics” (53). Several circumstances have arisen over the past few years that at first glance seemed to challenge these definitions, but, as illustrated below, the definitions allow resolution of the issues.

The EBV variants EBV-1 and EBV-2 differ markedly in several nuclear antigen (EBNA) gene sequences (Chapter 74). The differences lead to differences in some biologic properties, including transforming potential. However, (a) the variants do not occupy distinct ecologic niches, (b) the differences map to a small number of genes, and (c) intermediates carrying one variant allele at one locus and the other variant allele at another locus have been detected. Thus the EBV variants must be recognized as allelic variants of the same species. At least two alleles of the HHV-8 K15 gene, which

maps at the right end of the unique segment of the genome, have been identified (48). As for the EBV variants, recombinants have been detected between viruses encoding the different K15 alleles, thus this represents another example of intraspecies allelic variation. It is possible that the EBV and HHV-8 allelic variants represent early steps in speciation. The process of adaptation to a specialized niche might ultimately result in lineages characterized by additional mutations elsewhere in their genomes, a unique epidemiology, and ultimately reduced biologic fitness of recombinants between different lineages.

HHV-6A and HHV-6B are independent lineages that may represent relatively recent products of this process. Although they are closely related, (a) these viruses differ in a readily differentiable manner across their entire genomes, (b) they differ significantly in their epidemiology and association with disease, and (c) if recombinants occur, they do not appear to persist in nature. Thus by all criteria, they represent distinct species.

Species Nomenclature

The current host name–serial number nomenclature system was devised in an attempt to rectify problems associated with earlier systems in which viruses were named based on their disease associations, discoverer, geographic source, or whatever inspired the discoverer. Some viruses ended up with multiple names and some names were applied to multiple viruses. Of the various vernacular names in use, the most objectionable were disease associations, because viruses either rarely cause a specific disease, or they cause multiple and quite different clinical entities, or the disease for which the virus is named is caused by multiple agents. The system adapted in 1973 (51) was designed to create an orderly system in which each virus would be named unambiguously and independently of classification or properties (which at that time were largely unknown). Like all nomenclatures and classifications, it was meant to be provisional, meaning that it could in time be supplanted by a relational scheme. It was designed to bring herpesviruses into a cohesive order, but unlike a binomial nomenclature, it was not meant to supplant vernacular names grandfathered by time and usage.

The challenges to the current nomenclature system are twofold. First, there is no formal connection between nomenclature and classification. The naming of HHV-6A and HHV-6B reflects, among other things, an attempt to introduce a degree of classification into a system designed to preclude such attempts. An immediate effect is to compromise the status of these viruses as independent species.

The second challenge is based on the absence of any connection in the formal system between nomenclature and disease association, and the understandable desire to make such associations. An example is the designation of HHV-8 as Kaposi's sarcoma–associated herpesvirus on the basis of its association with Kaposi's sarcoma; others include VZV and the malignant catarrhal fever viruses. Although in these cases the disease association was already or ultimately proven, that questions remain after more than 30 years over the etiologic role of EBV in Burkitt's lymphoma (an association made at the time of EBV discovery), as well as the possibility of making an incorrect or incomplete linkage between name and disease, should inspire caution as designations are chosen for the yet-to-be discovered herpesviruses.

Fortunately, as noted, names and classifications are forever provisional.

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CHAPTER 72 Herpes Simplex Viruses and Their Replication

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> Dans l'ignorance ou nous vivons du processus exact de l'herpes, nous voyons chaque generation medicale creer une theorie adaptee aux idees, aux decovertes du moment.
>
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Herpes simplex viruses (HSVs) were the first of the human herpesviruses to be discovered and are among the most intensively investigated of all viruses. Their attractions are their biologic properties, particularly their abilities to cause a variety of infections, to remain latent in their host for life, and to be reactivated to cause lesions at or near the site of initial infection. They serve as models and tools for the study of translocation of proteins, synaptic connections in the nervous system, membrane structure, gene regulation, gene therapy, and a myriad of other biologic problems, both general to viruses and specific to HSV. For years, their size and complexity served as formidable obstacles to intensive research. More than 40 years passed from the time of their isolation (341) until Schneweiss (542) demonstrated that there were in fact two serotypes of HSV, HSV-1 and HSV-2, whose formal designations under International Committee on Taxonomy of Viruses (ICTV) rules are now human herpesviruses 1 and 2 (508). Not until 1961 were practical plaque assays published (513), and only much later were the genome sizes and the extent of homology between these two viruses reported. This chapter recounts well established facts, but its main emphasis is on burning issues, the problems whose time has come.

To the preface to the third edition of this book, which we affirm but do not repeat here, we add an additional comment. The field has grown enormously since the dawn of studies on the molecular biology of HSV more than 40 years ago. Studies on HSV are entering, at last, a most exciting period for two reasons. First, the words *structure* and *function* are beginning to have an operational meaning. Second, HSV gene products have become powerful probes for the study of cellular metabolic pathways. Host factors crucial to virus multiplication and, potentially, to latency are being identified. The armamentarium for a major assault on the mysteries underlying the biology of these viruses is in place and reflects the contributions of many laboratories over many, many years.

At the same time, the field and the literature have grown enormously. The task before us was far greater for not having followed Ludwig Wittgenstein's dictum that “Whereof one cannot speak, thereof one must be silent,” but chapters of citations bereft of commentary are dull. The decision to avoid assembling an annotated bibliography was in large part dictated by the space available to us.

VIRION STRUCTURE

The HSV virion (Fig. 1) consists of 4 elements: (a) an electron-opaque core, (b) an icosadeltahedral capsid surrounding the core, (c) an amorphous tegument surrounding the capsid, and (d) an outer envelope exhibiting spikes on its surface (509). The core contains the double-stranded DNA (dsDNA) genome wrapped as a toroid (171) or spool (702) and

possibly in a liquid crystalline state (42). The tegument is largely unstructured, except for some icosahedral structure around the pentons (702). The capsid is composed of 162 capsomers (676) arranged in a T=16 icosahedral symmetry. Finally, the envelope consists of a lipid bilayer with about 12 different viral glycoproteins embedded in it.

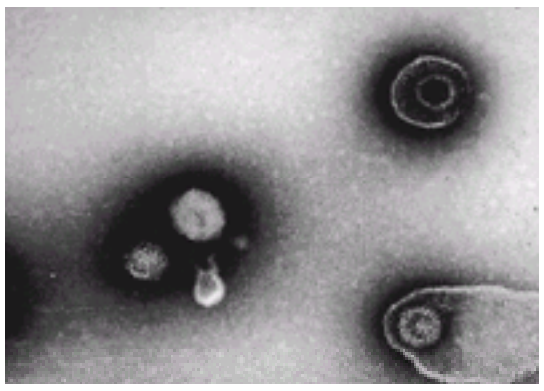

FIG. 1. Electron micrograph of negatively stained HSV virion particles. The *lower left* shows a capsid released from the envelope, revealing the icosahedral structure of the capsid shell. At the *lower right* is shown a virion with a distended envelope. The *upper right* shows a more intact particle, in which stain has penetrated to the core of the particle revealing the multiple layers of the virion, including the envelope as the outer layer, then the dark tegument layer, the capsid shell, and the dark core.

The dry masses of herpes simplex virions, full nucleocapsids, empty nucleocapsids, and cores were calculated from permeability of virions to electrons to be $13.33 \pm 2.56 \times 10^{-16}$ g, $7.55 \pm 1.11 \times 10^{-16}$ g, $5.22 \pm 1.10 \times 10^{-16}$ g, and $2.07 \pm 0.95 \times 10^{-16}$ g, respectively. The average mass ratios of the virion, full capsid, and core to DNA are 8.1, 4.6, and 1.25, respectively. The experimentally derived ratio of virus mass to DNA is 10.73 ± 0.96, from which it has been calculated that the virion contains 19.4×10^{-16} g of protein (216). A similar value was derived from counts of virions in purified virus preparations (R. W. Honess and B. Roizman, unpublished data), although the error in these determinations was higher. This ratio was used in the calculation of the polypeptide content of HSV virions by Heine and colleagues (216).

Virion Polypeptides

Early studies on purified HSV-1 virions suggested that they contain more than 30 distinct proteins, which were designated as virion polypeptides (VP) and given serial numbers (216,589). All of the virion proteins were made after infection, and no host proteins could be detected in purified virion preparations. Of the about 30 known and another 10 suspected virion proteins (Table 1), at least 11 are on the surface of the virion (accessible to antibody), and at least 10 are glycosylated. The glycoproteins are gB (VP7 and VP8.5), gC (VP8), gD (VP17 and VP18) and gE (VP12.3 and VP12.6), gG, gH, gI, gK, gL, and gM. The presence of gJ (U_S5) and gN ($U_L49.5$) in virions has not been demonstrated. Virion envelopes also contain at least two (U_L20 and U_S9) and possibly more (U_L24, U_L43, and U_L34) nonglycosylated intrinsic membrane proteins. Stannard and associates (599) reported that spikes projecting from envelopes are, as was expected, the viral glycoproteins, and that the latter were nonrandomly distributed.

TABLE 1. *HSV genes and their functions*

Gibson and Roizman (188,189) described three kinds of capsids: those that lack DNA and were never enveloped (type A), those that contain DNA and were never enveloped (type B), and those that contain DNA and were obtained by deenveloping intact virions (type C). In the current nomenclature, the term *A capsid* refers to capsids without an internal toroidal structure; those with internal scaffolding proteins but without DNA are designated as *B capsids*; and those with DNA have been designated as the C capsids. The A capsids are a defective byproduct (558) rather than precursors of the B capsids. A short-lived assembly intermediate termed the *procapsid* has recently been described (636).

The empty (A) capsids consist of four proteins: VP5 (U_L19), VP19C (U_L38), VP23 (U_L38), and a smaller Mr 12,000 protein described subsequently (95) and often referred to as VP26 (U_L35) (110,439). VP5 was estimated to be present in ratios of 850 to 1,000 per virion, that is, about six per hexameric capsomere (216,509,647), but Schrag and co-investigators (543) suggested that VP5 is a component of both pentameric and hexameric capsomeres. VP19C and VP5 appear to be linked by disulfide bonds (706) and are present in about similar ratios per virion (216). Braun and associates (45) showed

that VP19C, identified as the infected cell protein (ICP) 32, bound to DNA and may be involved in anchoring the viral DNA in the capsid. The HSV-2 counterpart has also been mapped (694). VP26 is located at the hexon tips. B-type capsids differ from the A type in that they contain three proteins: VP21, VP22a, and VP24. VP22a corresponds to ICP35e-f, the product of the open reading frame (ORF) U_L26.5 cleaved at the carboxyl terminus by the protease resident in VP24 (333,334). VP21 corresponds to ICP35b (amino acids 248 to 610) of the protease Pra, the nascent U_L26 gene product. VP24 corresponds to the N-terminal domain (codons 1 through 247) of Pra designated as Prn, the smallest form of the protease encoded by the ORF U_L26. Pra is cleaved by the autologous protease between ala247 and ser248 and between ala610 and ser611 (126,331). The ICP35 family of proteins described by Braun and colleagues (45) plays a vital role in capsid assembly and encapsidation of viral DNA (178,462,493,558). Gibson and Roizman (189) suggested that VP21 is an internal capsid protein. Newcomb and Brown (404,405) demonstrated that VP19C and VP23 are on the surface of the capsid and form triplex structures that link the hexon capsomers and that VP22a is in the interior of the capsid and forms a ringlike structure that is quantitatively removed by 2.0 molar (M) guanidine hydrochloride.

Type C capsids were reported to contain a smaller protein—VP22—but not VP22a, and it has been suggested that the proteins are related (189). Sherman and Bachenheimer (558) and Rixon and colleagues (493) suggested that the VP22 found in the C-type capsids may not be related to VP22a. Depending on the procedure for stripping the envelope, the C-type capsids may contain variable amounts of tegument proteins. C capsids retain VP24 (the protease).

Cryoelectron microscopy has provided moderately high-resolution models of the HSV nucleocapsid and virion (42,543,702). These studies have shown that the HSV nucleocapsid is composed of three layers: the outer layer arranged in a T=16 symmetry, an intermediate layer organized in a T=4 lattice, and an inner layer consisting of genomic DNA (543). The same study concluded that the outer and intermediate layers are organized so that channels along their icosahedral twofold axes coincide, forming a direct pathway and potential channel between the DNA layer and the exterior of the virion (543). Another study of intact virions has shown that the channel is plugged, possibly with tegument proteins (702). These authors suggested that the VP1-3 tegument proteins regulate the transport of DNA through the channel.

In the interval between 1965 and 1974, a large number of articles dealt with the structure and morphogenesis of herpesvirus capsids; a list of citations and review of that literature was published by Roizman and Furlong (509). On the basis of the electron microscopic appearance of the capsid and the core, as many as eight distinct capsid forms were identified (509). Although the various forms probably reflect different stages of capsid assembly, the reagents necessary to relate the various forms to specific proteins are only now becoming available. The use of specific antibody reagents, viral assembly mutants, cryoelectron microscopy, and methods for *in vitro* as well as *in vivo* assembly hold out the prospect of relating specific proteins, protein assemblies, and capsid forms in a comprehensive view of capsid morphogenesis.

The space between the undersurface of the envelope and the surface of the capsid was designated as the tegument (509); thus, it contains the rest of the virion proteins (see Table 1). The most notable of the proteins associated with the space between the underside of the envelope and the capsid are the a-trans-inducing factor (aTIF; ICP25; VP16), VP11-12 (U_L46), VP13-14 (U_L47), the virion host shutoff (vhs) protein (U_L41); the product of the U_S11 gene; and a very large protein (VP1-2) associated with a complex that binds to the terminal *a* sequence of the viral genome (22,79,483,521,522). Extensive discussion of the various types of capsids and virions can be found elsewhere (227,509,604).

Several publications have reported on the production of “light particles” that are devoid of DNA. These particles consist of enveloped tegument-like structures (371,492,616). They appear to contain, in addition, other nonstructural proteins previously associated with virions (e.g., ICP4) (690). Little is known of their synthesis beyond the facts that they do not appear to be uniform in size and that the capsid is not an essential trigger for envelopment or egress.

Viral DNA

Like other herpesvirus DNAs, the bulk of packaged HSV DNA is linear and double stranded (24,272,444). In the virion, HSV DNA is packaged in the form of a toroid (171) or a spool (702). The ends of the genome are probably held together or are in close proximity inasmuch as a small fraction of the packaged DNA appears to be circular and the bulk of the linear DNA circularizes rapidly in the absence of protein synthesis after it enters the nuclei of infected cells (445). DNA extracted from virions contains ribonucleotides, nicks, and gaps (26,169,677).

Based on physical characterization studies, the HSV genome was originally estimated to be about 150 kilobase pairs (kbp), with a G+C content of 68% for HSV-1 and 69% HSV-2 (24,272). Complete sequencing of the HSV-1 strain 17 genome described the genome as 152,260 base pairs (bp) (accession number X14112) (363); minor updates altered this sequence to 152,261 bp (132). Complete sequencing of the HSV-2 strain HG52 genome defined it as 154,746 bp (accession number 286099) (132). However, these numbers include only single copies of the *a* sequence at the ends of the L component and do not take into account the variation in the size of the *a* sequences (200 to 500 bp each) or the variable number of direct repeats present throughout the genome but especially in the inverted repeats flanking the L and S components.

The HSV genome can be viewed as consisting of two covalently linked components, designated as L (long) and S (short) (Fig. 2). Each component consists of unique sequences bracketed by inverted repeats (650). The repeats of the L component are designated *ab* and *b'a',* whereas those of the S component are *a'c'* and *ca* (see Fig. 2). The number of *a* sequence repeats at the L-S junction and at the L terminus is variable; thus, the HSV genome can then be represented as follows:

$$\mathbf{a_L a_n b\text{-}U_L\text{-}b'a'_m c'\text{-}U_S\text{-}ca_S}$$

where a_L and a_S are terminal sequences with unique properties (described later), and a a_n and a_m are terminal *a* sequences directly repeated 0 or more times (n) or present in one to many copies (m) (335,503,505,650,655). The structure of the *a* sequence is highly conserved, but it consists of a variable number of repeat elements. In the HSV-1 (F) strain, the *a* sequence consists of a 20-bp direct repeat (DR1), a 65-bp unique sequence (U_b), a 12-bp direct repeat (DR2) present in 19 to 23 copies per *a* sequence, a 37-bp direct repeat (DR4) present in 2 to 3 copies, a 58-bp unique sequence (U_c), and a final copy of DR1 (388,389). The size of the *a* sequence varies from strain to strain, reflecting in part the number of copies of DR2 and DR4. The structure of the HSV-1 (F) *a* sequence can be represented as follows:

$$\mathbf{DR1—U_b—DR2_n—DR4_m—U_c—DR1}$$

with the adjacent *a* sequences sharing the intervening DR1. Linear virion DNA contains asymmetric terminal *a* sequence ends. The terminal *a* sequence of the L component (a_L) contains a truncated DR1 with 18 bp and one 3' nucleotide extension, whereas the terminal *a* sequence of the S component (a_S) ends with a DR1 containing only 1 bp and one 3'-overhanging nucleotide (390). The two truncated DR1 sequences form one complete DR1 upon circularization.

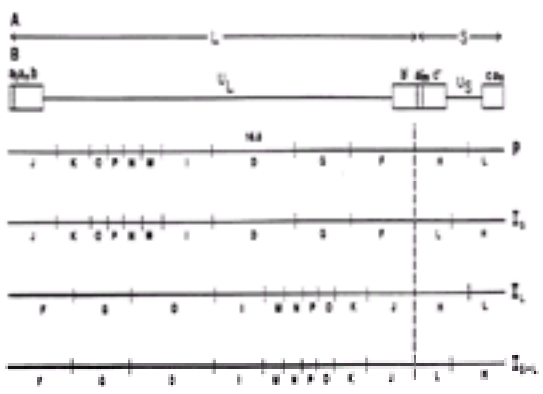

FIG. 2. Schematic representation of the arrangement of DNA sequences in the HSV genome. **A:** The domains of the L and S components are denoted by the *arrows.* The second line shows the unique sequences (*thin lines*) flanked by the inverted repeats (*boxes*). The letters above the second line designate the terminal *a* sequence of the L component (a_L), a variable (*n*) number of additional *a* sequences, the *b* sequence, the unique sequence of the L component (U_L), the repetitions of the *b* sequence and of a variable (*m*) number of sequences (a_m), the inverted *c* sequence, the unique sequence of the S component (U_S), and finally the terminal *a* sequence (a_S) of the S component. **B:** The HindIII restriction endonuclease map of HSV-1 (F) strain for the P, I_S, I_L, and I_{SL} isomers of the DNA. Note that, because HindIII does not cleave within the inverted repeat sequences, there are four terminal fragments and four fragments spanning the internal inverted repeats in concentrations of 0.5 and 0.25 M, respectively, relative to the concentration of the viral DNA.

The L and S components of HSV are found inverted relative to one another, to yield four linear isomers. Populations of unit-length DNA from wild-type virus-infected cells consist of equimolar concentrations of the four predicted isomers (see Fig. 2) (120,211). The isomers have been designated as P (prototype), I_L (inversion of the L component), I_S (inversion of the S component), and I_{SL} (inversion of both S and L components) (211,395,396). The first evidence for the existence of repetition of terminal sequences in inverted orientation was based on electron microscopic studies of denatured HSV-1 DNA allowed to self-anneal (554). Electron microscopic analyses of denatured molecules allowed to self-anneal and of partial denaturation profiles of HSV DNA revealed that the terminal repeats are also repeated internally and that the repeats flanking the L component differ from those of the S component in size and sequence arrangement (650). The demonstration that restriction endonucleases that cleave outside the inverted repeats yield four terminal fragments, each present in half of the molecules (also called half molar or 0.5M fragments), and 4 L-S component junction fragments that are each present in one fourth of the molecules (also called *quarter molar* or 0.25M fragments) (211) (see Fig. 2), as well as analyses of the partial denaturation profiles of Wadsworth and coauthors (650), supported the conclusion that the L and S components can invert relative to each other.

The internal inverted repeat sequences are not essential for growth of the virus in cell culture; thus, mutants from which portions of unique sequences and most of the internal inverted repeats have been deleted have been obtained in all four arrangements of HSV DNA (253,446). The genomes of these mutants do not invert and are frozen in one arrangement of the L and S components, but all retain their viability in cell culture.

Other Constituents

Polyamines

The search for polyamines in the virion evolved from the observations that HSV capsid assembly requires addition of arginine to the medium (352,620) and that the capsid does not contain highly basic proteins, which would neutralize the negative charges on viral DNA to allow proper folding within the capsid. Highly purified virions contain the polyamines spermidine and spermine in a nearly constant ratio of 1.6 ± 0.2:1, or about 70,000 molecules of spermidine and 40,000 molecules of spermine per virion (187,190). The polyamines appear to be tightly bound and cannot be exchanged with exogenously added, labeled polyamines. Disruption of the envelope with nonionic detergents and urea removed the spermidine but not the spermine. The spermine contained in the virion is sufficient to neutralize about 40% of the DNA phosphate (190). Parenthetically, proteins have been noted in association with the toroidal structure (171) in the capsid,

and a capsid protein has been reported to bind DNA (45).

The compartmentalization of spermine and spermidine may reflect the distribution of polyamines in the infected cell. It is of interest to note that after infection, the conversion of ornithine to putrescine appears to be blocked, but the synthesis of spermine and spermidine does not appear to be affected (190).

Lipids

It has been assumed that HSV acquires the envelope lipids from its host. The hypothesis that the lipid composition of the viral envelope is determined by the host was supported by the observation that the buoyant density of the virus was host cell dependent on serial passage of HSV-1 alternately in HEp-2 and chick embryo cells (588). More recent studies (642) suggest that the virion lipids are similar to those of cytoplasmic membranes and different from those of nuclear membranes of uninfected cells. This conclusion assumes that the lipid content of nuclear membranes remains unchanged during infection notwithstanding the change in the overall surface area of nuclear membranes and lack of *de novo* synthesis of cellular proteins in infected cells.

Herpes Simplex Virus Polymorphism

Intertypic Variation

Although the genetic maps of HSV-1 and HSV-2 are largely colinear, they differ in the locations of restriction endonuclease cleavage sites in their genomic DNAs and in the apparent sizes of their proteins. Thus, analyses of HSV-1 × HSV-2 recombinants took advantage of the intertypic differences in the sizes of the proteins and the locations of restriction endonuclease cleavage sites to determine the initial locations of viral genes on the linear map of HSV genomes (354,395,396,463). Comparison of the complete DNA sequences of an HSV-1 genome and an HSV-2 genome confirmed the colinearity of the genetic maps (132).

Intratypic Variation

The first evidence of intratypic polymorphism emerged from studies of virion structural proteins and indicated that nonglycosylated proteins vary sufficiently in electrophoretic mobility to be used as strain markers (436). Although specimens from epidemiologically related individuals appeared to yield similar electrophoretic profiles, the usefulness of virion proteins as markers for molecular epidemiologic studies was limited by the effort required to purify virions for such analyses.

At the DNA level, differences between HSV-1 strains appear to result from (a) base substitutions, which may add or eliminate a restriction endonuclease cleavage site, and on occasion change an amino acid; and (b) variability in the number of repeated sequences present in a number of regions of the genome (e.g., g $_1$*34.5,* U_{s11}) (82,494). The restriction endonuclease cleavage patterns of a given strain are relatively stable, whereas the number of repeats is not (53,210,507,632). Thus, no changes in restriction endonuclease patterns were noted in isolates from the same individual over an interval of 13 years or in genomes of an HSV-1 strain passaged serially numerous times in cell culture. However, restriction endonuclease site polymorphism was readily noted in isolates from epidemiologically unrelated individuals (202,520). On the basis of these properties, restriction endonuclease site polymorphism was used in several epidemiologic studies of HSV transmission in the human population (53,507,520), and blind restriction endonuclease analysis of virus isolates has been used to trace the spread of infection from patients to hospital personnel (51), from patient to patient (328), and from hospital personnel to patient (3,52). Sakaoka and associates (529) reported on clustering of divergent sites along geographically and racially distinct areas. Although these authors concluded that the evolution of HSV-1 may be host dependent, it seems more likely that random mutations were conserved and dispersed in different populations.

GENOME STRUCTURE AND ORGANIZATION

Viral Genes: Pattern of Organization and Expression

Distribution and Organization of Transcriptional Units

The HSV DNA encodes by current count about 90 unique transcriptional units (see Table 1). The HSV-1 sequence and gene map can be found at website http://www.stdgene.lanl.gov/. At least 84 of these transcriptional units encode proteins. As described in more detail later, these genes are classified into at least three general kinetic classes: a, or immediate early; b, or early; and g, or late. The key elements of genome organization are as follows:

1. With three known exceptions, each viral transcript encodes a single protein. In the first exception, a single transcript serves as the template for both ORF P and ORF 0 proteins. Protein synthesis is initiated from a single methionine but diverges between the 1st and 35th codons to yield different polypeptides (480). Second, the U_L*26* gene encodes a polypeptide that cleaves itself to form two proteins. The amino-terminal product functions as a protease. The carboxyl-terminal product, ICP35a,b, is a component of the capsid scaffolding. The third exception is the messenger RNA (mRNA) that encodes the U_L3 protein. This RNA, 2.9 kb in size, contains the ORFs of U_L1, U_L2, and U_L3, but U_L1 and U_L2 proteins are encoded by distinct mRNAs (569). The mechanism of expression of U_L3 protein is unknown.
2. Many clusters of transcriptional units are 3' co-terminal. In addition, these clusters may be arranged either head-to-head, head-to-tail, or tail-to-tail. The precise transcription initiation sites are not known for all transcriptional units. In many instances, but especially late in infection, transcription termination sites are ignored, with the consequence that run-on transcripts of giant size are present along with the properly terminated transcripts. Some of the 3' co-terminal mRNAs initiate within the body of an expressed ORF and encode only the carboxyl-terminal

portion of the protein encoded by the ORF. For example, the mRNA encoding $U_S1.5$ initiates within the ORF expressing ICP22, and the amino acid sequence of $U_S1.5$ is identical to that of the carboxyl-terminal domain of ICP22. The same situation exists for U_L26 and $U_L26.5$.

3. Some of the expressed ORFs are antisense to each other. These include *ICP34.5* and *ORF P*, *U_L43* and *$U_L43.5$*, and *gB* and *$U_L27.5$*. The existence of additional antisense ORFs cannot be excluded.
4. Few of the transcripts accumulating in infected cells arise as a consequence of splicing of RNA. In only two cases, those encoding ICP0 and U_L15, are the introns within coding domains. The intron of U_L15 is transcribed antisense to U_L15 and encodes two ORFs, U_L16 and U_L17 (363). The intron of ICP0 mRNA is stable and transported into the cytoplasm but appears to play no role in productive infection because substitution of the wild-type *ICP0* gene with an intronless *ICP0* gene has no effect on its known functions (147). In all other cases (genes encoding ICP22, ICP47, and gC), the introns are in the 5'-noncoding domains. In one instance, that of the gene encoding ICP34.5, an intron is present in the coding domain of HSV-2 but not that of HSV-1 (362,482).
5. Several transcripts appear not to encode expressed ORFs. Those best known are the latency-associated transcripts (LATs) described in the section dealing with latency and the *Ori_S* mRNA that is expressed late in infection and is 3' co-terminal with the mRNA encoding ICP4. The functions of these RNAs in productive infection are not known.

Functional Organization of Herpes Simplex Virus Genomes

a genes map near the termini of the L and S components (9,258,259,347,396,400,463,661,662). The a*0* and a*4* genes map within the inverted repeats of the L and S components, respectively, and are therefore each present in two copies per wild-type genome. However, a single copy of each is sufficient inasmuch as mutants lacking the internal inverted repeats are viable but are attenuated for growth in animals (446).

With few exceptions, b and g genes are scattered in the unique sequences of both the L and S components. The exceptions are the g_1*34.5* and *ORF P* genes located in the reiterated sequences flanking the L component between the terminal *a* sequence and the a*0* gene (2,84,307). At present, only two functional gene clusters are strikingly apparent, but their significance is uncertain: The b genes specifying the DNA polymerase and the ICP8 single-stranded DNA (ssDNA)-binding protein flank the L component origin of DNA synthesis (*Ori*L) (98,226,478), and the g genes specifying membrane glycoproteins D, E, G, I, and J map next to each other within the unique sequences of the S component (2,165,311,490,510,526,584,662).

Application of Genetic Techniques to the Identification of Gene Product Function: Genes Essential and Nonessential for Growth in Cell Cultures

Key to the identification of viral functions and mapping of viral genes encoding these functions are temperature sensitive (ts) and null mutants. Earlier studies identified about 30 complementation groups (668)—an extraordinary accomplishment in itself given the difficulties inherent in the selection and testing of the numerous mutants produced by many laboratories. The ts mutants have been enormously helpful in mapping genes. Nevertheless, this approach to identification and mapping of viral functions suffers from several problems: (a) the phenotypes of viruses containing mutations in nonessential genes cannot be readily differentiated from that of the wild type parent; (b) conditional lethal (e.g., ts) mutants produced by general mutagenesis of the viral genome may contain a large number of silent nonlethal mutations in both essential and nonessential genes; (c) the phenotypes of mutations introduced into domains shared by more than one gene cannot be readily attributed to the malfunction of a specific gene product; and (d) although the usefulness of ts mutants is in part dependent on their efficiency of plating at permissive and nonpermissive temperatures, tight mutants with high permissive-to-nonpermissive ratios may well contain more than one point mutation. Although the presence of multiple mutations in a single gene should not affect the mapping or identification of the gene function, it does present a problem in mapping the functional domains of the gene.

An alternative to the random or fragment-specific substitution of bases in DNA is localized mutagenesis of the viral genome. Much of the HSV genetic work thus far has used a co-transfection procedure involving the introduction of infectious viral DNA with a viral DNA fragment that contains a marker or mutation to be introduced into the viral genome by a double crossover event (279,526,611). This has been used for marker rescue mapping of viral ts mutants, introduction of markers into a viral genome (marker transfer) (279,526), and introduction of mutations into a viral genome (86,98).

A protocol for site-specific insertion and deletion of viral genes was reported by Post and Roizman (454). It was based on selection of recombinants generated by double recombination through homologous flanking sequences between an intact viral DNA molecule and a DNA fragment containing an insertion or deletion and a selectable marker. The selectable marker used in these studies was the viral thymidine kinase (*tk*) gene because (a) it can be deleted from the HSV genome without affecting the growth of virus in cell culture, (b) a plasmid-borne *tk* gene can be altered so that it cannot recombine by double crossover to repair the deletion in the genomic *tk* gene; (c) viruses carrying a functional *tk* gene can be selected against by plating viral progeny in the presence of nucleoside analogs phosphorylated by the viral thymidine kinase (TK) (e.g., Ara T), and (d) viruses expressing the TK gene can be selected for by plating the virus on TK-negative cells in medium containing methotrexate or aminopterin, which blocks the conversion of thymidine monophosphate (TMP) from deoxyuridine monophosphate (dUMP) by thymidylate synthetase and precludes the *de novo* pathway of TMP synthesis. This procedure permits the selection of viable mutants with deletions or insertions in genes that appear to be nonessential for growth in cells in culture.

A second popular approach for mutagenesis of a specific gene is to replace the gene with *Escherichia coli lacZ* coding sequences so that the b-galactosidase enzyme is expressed by the recombinant virus. The recombinant virus can be identified by the formation of blue plaques in the presence of chromogenic substrates such as X-GAL (194). If the recombinant virus can be isolated and propagated on normal cells, such as Vero cells, the mutated gene is considered to be nonessential. If the viral gene is essential for growth in that cell line, stable complementing cell lines have often been

used to propagate these null mutant viruses (122). In this protocol, the gene to be mutated is transfected into and stably maintained in a cell line that serves as the complementing cell line. The complementing cell line is then transfected with intact viral DNA and the mutated DNA fragment. The progeny of transfection are screened for mutants that form blue plaques or multiply only in the complementing cells (122,326,424).

In recent years, several other techniques have come into vogue that promise to simplify greatly the construction of mutants. The first involves the use of overlapping cosmids that contain viral DNA sequences that encompass the entire genome. After transfection into cells, viral gene expression from the overlapping cosmids enables recombination at the overlapping ends to reconstitute the intact genome (104). The second technique is based on the use of bacterial artificial chromosomes to propagate the viral genome in bacterial cells. Mutations can be introduced into the HSV sequences, and introduction of the recombinant bacterial artificial chromosome into mammalian cells yields infectious virus (235). Both techniques enable construction of multiple mutants. The genomes cloned as bacterial accessory chromosomes are amenable to random insertional mutagenesis by transposons. Finally, an adjunct to all methods designed to inactivate viral genes is the use of baculoviruses carrying the target gene under a viral promoter. Such genes are expressed only in infected cells and enable selection of mutants lacking essential genes (691).

The genes known to be dispensable for viral replication in cells in culture are listed in Table 1. Of importance, most of the dispensable genes are required for replication in experimental animal systems, and in no instance has a virus lacking a dispensable gene been isolated from human lesions (although viruses that fail to react with a specific monoclonal antibody are readily isolated).

Genes dispensable for viral replication in cells in culture fall into several groups whose products are involved in entry of HSV into cells, regulation of gene expression, posttranslational modification of proteins, exocytosis, inhibition of host response to infection, and spread of virus from cell to cell.

In a special category are deletion mutants, whose ability to multiply in cells is species dependent. One example of such mutants is the a22^{-} virus, which grows well in Vero and HEp-2 cell lines but not in human fibroblast strains or in rodent cell lines (547). In the nonpermissive cells, the virus fails to express g2 genes efficiently. Another example of a cell-specific gene is g $_1$*34.5*, which enables HSV to multiply in human cells but is dispensable in Vero, baby hamster kidney cells, and so forth. In the absence of the gene, there is total shutoff of protein synthesis before significant amounts of virus are synthesized (78,80,81).

It could be predicted that viral genes that specify products whose functions are identical and interchangeable with those of cellular genes would be dispensable, at least in cells that express these functions. In this category are the *tk* gene and the genes specifying ribonucleotide reductase. The observation that some virion proteins are dispensable for infection and replication of virus at least in cell culture presented was puzzling for many years. Although we cannot exclude the possibility that cells express proteins with similar functions that complement the deletion mutants, a more likely scenario is that cells in culture express many more genes than cells *in situ* in animal organs. The exceptions to date are polarized epithelial cells and neuroblastoma cells, in which the gene products are sorted differently or which differ from other cells in culture with respect to the nature of the genes that are expressed. HSV may carry a set of genes that enables the virus to enter (e.g., the dispensable glycoproteins), multiply (e.g., g $_1$*34.5*) or egress (e.g., U_L*20*) from a wide variety of human cells. Because these genes are not required for replication in all cells, there exists the formal possibility that functional analogs of the viral gene are encoded and may be expressed by cells. The obvious examples are the *tk,* ribonucleotide reductase, and so forth. The less obvious homologs are the cellular protein kinases that substitute for some of the viral enzymes and the homolog of U_L20, which enables virus to egress from a variety of cell lines other than Vero cells. The complexity involved in defining the function of the numerous genes whose products are dispensable for viral replication in at least some cells in culture is offset by the fact that these genes are excellent probes of cellular functions that, at least in some instances, complement the missing viral function.

VIRAL REPLICATION

Overview of Replication

It is convenient to begin this section on viral replication with a bird's eye view of the major events of HSV replication (Fig. 3).

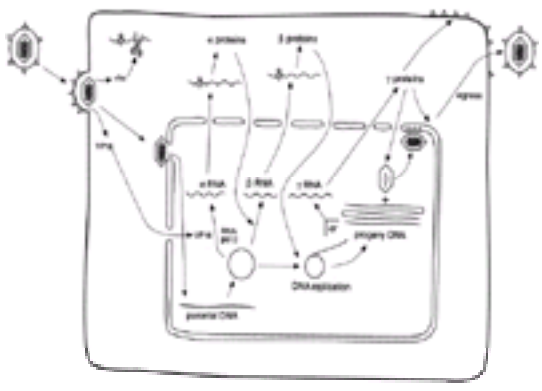

FIG. 3. Diagram of the replication cycle of HSV. At the *upper left,* the virion binds to the cell plasma membrane, and the virion envelope fuses with the plasma membrane, releasing the capsid and tegument proteins into the cytoplasm. The vhs protein acts to cause degradation of mRNAs. The capsid is transported to the nuclear pore, where the viral DNA is released into the nucleus. The viral DNA circularizes and is transcribed by host RNA polymerase II to give first the IE or a mRNAs. IE gene transcription is stimulated by the VP16 tegument protein. Five of the six IE proteins act to regulate viral gene expression in the nucleus. They transactivate E or b gene transcription. The E proteins are involved in replicating the viral DNA molecule. Viral DNA synthesis stimulates L or g gene expression. The L proteins are involved in

assembling the capsid in the nucleus and modifying the membranes for virion formation. The filled capsid buds through the inner membrane to form an enveloped virion, and the virion exits from the cell by mechanisms described in the section on Virion Assembly and Egress.

To initiate infection, the virus must attach to cell surface receptors. Fusion of the envelope with the plasma membrane rapidly follows the initial attachment. The deenveloped tegument–capsid structure is then transported to the nuclear pores, where DNA is released into the nucleus.

Transcription of the viral genome, replication of viral DNA, and assembly of new capsids take place in the nucleus. Viral DNA is transcribed throughout productive infection by host RNA polymerase II, but with the participation of viral factors at all stages of infection. The synthesis of viral gene products is tightly regulated: Viral gene expression is coordinately regulated and sequentially ordered in a cascade fashion. The gene products studied to date form at least five groups as a result of both transcriptional and posttranscriptional regulation.

Several of the gene products are enzymes and DNA-binding proteins involved in viral DNA replication. The bulk of viral DNA is synthesized by a rolling-circle mechanism, yielding concatemers, which are cleaved into monomers during the process of nucleocapsid assembly.

Assembly occurs in several stages. After packaging of DNA into preassembled capsids, the virus matures and acquires infectivity by budding through the inner lamella of the nuclear membrane. The transit of virions from the space between the inner and outer nuclear membranes to the subcellular space is less well defined. It has been suggested that the virion envelope is processed by transit through Golgi stacks or by being deenveloped and then reenveloped at the trans-Golgi network. In fully permissive tissue culture cells, the entire process takes about 18 to 20 hours.

Virus Attachment and Entry

Entry of HSV into cells is effected in three stages (Fig. 4). The first involves the attachment of the virion to the surface of the cell. The second step involves the interaction of gD with one of several cellular receptors. In the last step, the viral envelope and the plasma membrane fuse to release the capsid–tegument structure into the cytoplasm. Most of the information on this process is based on studies of nonpolarized cells in which the membrane proteins interacting with envelope proteins are most likely randomly distributed. Attachment and entry of viruses into polarized cells—most epithelial cells in the human body—may differ in detail.

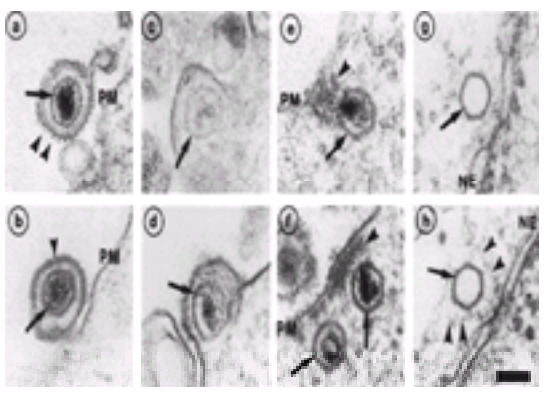

FIG. 4. Entry and uncoating of HSV-1. Shown are ultrathin Epon sections of Vero cells infected with HSV-1 at an MOI of 500 in the presence of cycloheximide (*CH*). After 2 hours of virus binding at 4°C, the cells were either fixed immediately (***a*** and ***b***), or warmed up for 30 (***c*** to ***f***) or 60 (***g*** and ***h***) minutes. Binding (***a*** and ***b***). The main morphologic features of the intact virus bound to the plasma membrane (*PM*) are the viral envelope (***b,*** *arrowhead*) with the viral spikes (***a,*** *arrowhead*), the viral capsid (*arrow*), and the electron-dense viral DNA genome within the capsid. Fusion (***c*** and ***d***). Upon warming up, the viral envelope fuses with the plasma membrane, and the capsid (*arrow*) and the tegument proteins are released into the cytosol. Release of the capsid (***e*** and ***f***). Preparations, which display a prominent contrast of the tegument, show that the tegument (*arrowheads*) stays behind at the PM. Binding to the nuclear pore (***g*** and ***h***). At later time points, the capsids (*arrows*) have arrived at the nuclear envelope (*NE*), where they are exclusively located in close apposition to the nuclear pore complexes. Occasionally, fibers emanating from the pores (*arrowheads*) are visible, to which the capsids appear to bind. Almost all of the capsids at the nuclear pores appear empty and have lost the electron-dense DNA core. Bar, 100 nm. (From ref. 578, with permission).

The initial attachment to nonpolarized cells involves the interaction of the viral envelope gC, and, to a lesser extent gB, with the glycosaminoglycan moieties of cell surface heparan sulfate (560,686). Although gC confers the greatest efficiency for attachment in these systems, it is not essential for either entry or viral replication (216).

Attachment to heparan sulfate enhances infection but is not an absolute requirement for HSV infection of cells because cells devoid of heparan sulfate but expressing other glycosaminoglycans (e.g., chondroitin sulfate) can be infected. Cells devoid of both of these types of glycosaminoglycans can also be infected, although at a reduced efficiency (19). The second, sequential step, designated here as binding to a coreceptor, involves the interaction of gD with one of several cellular molecules that belong to three structurally unrelated molecular families. As is often the case, they have been given different designations based on their cellular function or their function in viral infection.

The first of these coreceptors is a member of the tumor necrosis factor (TNF) receptor family originally called *herpes virus entry mediator* (HVEM) (392) but renamed HveA after other receptors were discovered (659). HveA is present primarily in lymphoid cells, and thus it is unlikely to function as the major mediator of HSV entry into human cells. It serves as a receptor for entry of some selected HSV-1 strains, HSV-2, but not for the related alphaherpesvirus, pseudorabies (PRV) virus (392,670).

The second family of coreceptors belongs to the immunoglobulin superfamily and is related to the poliovirus receptors (183). The first identified members of this family were named HveB (659) and HveC (183) and herpesvirus immunoglobulin-like receptor (HIgR) (92). This family includes several isoforms present in both human and nonhuman cells that are encoded by mRNAs generated by alternative mRNA splicing. More recently, these cellular proteins have been shown to act as intercellular adhesion molecules and to be localized at adhesion junctions where their C-terminal domains bind to L-afadin, a PDZ-binding protein that anchors these molecules to cytoskeleton and adherens junctions. On the basis of their cellular function, they have been named *nectins* (617); therefore, the designations that appear now in the literature are nectin-1a (HveC, Prr1), nectin-1b (HIgR), nectin-2a (HveB, Prr2), and nectin-2d (91,340,586). Nectin-1a and nectin-1b, two splice variants with a common ectodomain, are broadly expressed in cells of epithelial, fibroblastic, neural, and hematopoietic origin; in keratinocytes; and in human tissues that are targets of HSV infection, including skin, brain, and spinal ganglia (92,183). They mediate entry of all HSV-1 strains tested, HSV-2, PRV, and bovine herpes virus 1 (183). They also mediate cell-to-cell spread of HSV (91). Nectin-2a (HveB) and nectin-2d, also splice variant isoforms, mediate the entry of HSV-2, PRV, and certain viable mutants of HSV-1, but not of wild-type HSV-1 (340,659). The distribution of this general class (immunoglobulin superfamily) of receptors in human tissues reflects the susceptibility of cells to infection and is likely to account for both entry and spread of virus from cell to cell.

Homologs of human nectins have been found in a wide variety of species. The murine homolog of human nectin-1 also mediates entry of HSV-1 and HSV-2 into murine cells (382). This raises the possibility that the ability to use the animal homologs of the nectin-type proteins can account for the broad host range of HSV in cell cultures and animals of different species. Interestingly, this receptor activity was reported to function independently of gD binding (382).

The third family has to date only one member: 3-*O*-sulfated heparan sulfate (563). The 3-*O*-sulfated heparan sulfates are broadly distributed on human cells and tissues and mediate efficient HSV-1 but not HSV-2 entry (563). Whether they can mediate entry into human cells remains to be determined. It is interesting that either protein molecules or specific sites on heparan sulfate can serve as receptors for HSV entry.

The last step in entry is the fusion of the viral envelope with the plasma membrane of the host cell. There is overwhelming acceptance of the hypothesis that productive infection results from the entry of virus mediated by fusion of the envelope and plasma membranes (394). Key evidence is the demonstration that gI and gE—the viral Fc receptors—can be detected on the surface of cells immediately after penetration of the virus (430). Conversely, a body of evidence suggests that entry by endocytosis does not result in productive infection. Thus, cells expressing gD internalize virus by endocytosis, but viral replication does not ensue (58,703).

Current evidence indicates that virus–cell fusion requires the participation of gD (326), gB (536), and the gH-gL heterodimer (164). The exact role of these proteins in the fusion process is unknown, but one could speculate that the binding of gD to the cellular receptors leads to a rearrangement of viral proteins on the virion surface, a conformational change in their structure, or both, so that gB, gH/gL, or both can promote fusion. An additional observation underlying the hypotheses is that cells are infected with wild-type virus aggregate (*syn*$^+$ phenotype), whereas certain viral mutants cause the cells to fuse (called either *syn*$^-$ or *syn* phenotype). *Syn*$^-$ mutations map in the genes encoding gB, gH, and gK and in the U_L24 membrane protein (526). The data suggest that these proteins may form a complex and that mutations in any one of the syn proteins cause a rearrangement or a conformational change in the complex, resulting in cell fusion (526). For detailed reviews on this topic, see references 57 and 585.

Studies on viral entry into polarized cells have progressed to a much lesser extent. Current data are based on studies of viral entry into a few polarized cell lines in culture and on corneal cells *in vivo.* These data indicate that attachment to the apical surface is gC dependent, whereas entry through the basolateral surface is gC independent (548), and that gG, a viral glycoprotein with no other known function, plays a key role in the postattachment entry on the apical surface but is not essential for basolateral entry (633).

The transition from attached to penetrated virus, as measured by the loss of susceptibility to neutralization, inactivation by acidic pH, or proteases, is very rapid and occurs within minutes (121,236).

Transport to the Cell Nucleus

After fusion of the envelope to the plasma membrane, some tegument proteins remain in the cytoplasm (e.g., vhs, U_S11) whereas others are either transported to the nucleus (VP16) or may remain associated with the capsid (e.g., VP1-2), as discussed later in the text. The capsid with associated tegument structures is then transported through the microtubular network to the nuclear pore. The extent of our knowledge of virus transport is limited but can be described as follows:

HSV capsids have been observed bound to microtubules in neurons (343,344,433). In cultured cells, antibodies specific for capsid proteins co-localize with microtubules after viral entry (578). Microtubule depolymerizing agents such as colchicine and nocodazole block the transport of nucleocapsids to the cell nucleus, and antibody labeling of dynein, the microtubule-dependent motor, showed that it was attached to HSV capsids (578). These authors proposed that incoming capsids bind to microtubules and use dynein to propel them to the cell nucleus. The precise mechanism of loading capsid–tegument structures onto the cytoplasmic dynein motors is unknown, but two observations are relevant. First, shortly after infection, the microtubules at the junction with the plasma membrane become disrupted (657), suggesting the possibility that the loading of the capsid–tegument structures may affect the interaction of microtubules or associated proteins with cognate structures in the plasma membrane. More extensive rearrangements of the microtubular network

take place late in infection. Second, recent studies have shown that the U_L34 protein binds the amino-terminal domain of the intermediate chain 1a of cytoplasmic neuronal dynein and that, in the presence of nocodazole, which causes the depolymerization of dynein, U_L34 protein is not transported to the nucleus (692). Finally, because the normal dynein motor terminus is at the microtubule-organizing center, it is not clear how the nucleocapsids ultimately come in apposition with the nuclear pore complex.

After intracytoplasmic transport, the nucleocapsids accumulate at the nuclear envelope and associate with the nuclear pore complexes (21,578) (see Fig. 4). At the pore, the nucleocapsid releases its DNA into the nucleus, leaving an empty capsid at the cytoplasmic side of the complex. Little is known about the mechanisms of HSV DNA release and transport through the nuclear pore into the nucleus. The HSV-1 ts mutant *ts*B7 is completely blocked for viral gene expression at the nonpermissive temperature (NPT) (278) because it is defective for the release of DNA from capsids at the NPT (21). The ts mutation in *ts*B7 maps in the *VP1/2* gene (21), suggesting that this large tegument protein plays a role in DNA release at the nuclear pores. These results also suggest that the VP1/2 tegument protein is transported to the nuclear pore with the capsid; nevertheless, it is still conceivable that the defective protein is unable to be released in the cytoplasm and thus is kept artifactually with the nucleocapsid during transport through the cytoplasm and blocks uncoating at the nuclear pore. Recent *in vitro* results have shown that binding of the capsid to the nuclear pores is blocked by wheat germ agglutinin, antinuclear pore complex antibodies, or antibodies against importin b (422). Thus, the available data suggest that the interaction of capsid–tegument structures with the nuclear pore proteins causes a structural change in the former, with the consequence that viral DNA is extruded from the capsids into the nucleus; however, the molecular details remain to be defined. The presence of intact, empty capsids for at least 2 hours after exposure of cells to the virus suggests that the extrusion of the DNA does not coincide with complete dissociation of the capsid.

Some of the functions of viral tegument proteins and of genes expressed after infection suggest that the environment inside the host cell is decidedly hostile to the incoming virus. The tegument proteins are introduced into the cell concurrently with the capsid, and some play a key role in creating an environment that allows efficient viral replication. Although the functions of many tegument proteins remain elusive, it seems clear that their distribution varies early in infection. For example, two of these proteins, VP16 and U_L41, are described in detail in later sections, but they promote a gene transcription in the nucleus and shutoff of host protein synthesis in the cytoplasm, respectively. These two virion proteins play a clear role in preparing the host cell for efficient viral replication.

Table 1 lists a large number of viral proteins reported to be components of the viral tegument. With the two exceptions listed previously, the functions of the tegument proteins are not readily discernible, largely because many are either dispensable for viral replication or their function as tegument proteins is different from that expressed in the course of productive infection after *de novo* synthesis. For example, U_S11, genetically engineered to be expressed early in infection, blocks the shutoff of protein synthesis induced by activated protein kinase R (PKR) (66). Virions carry about 2,000 copies of U_S11 protein; after infection, these are associated with polyribosomes and 60S ribosomal subunits and do not block the shutoff of protein synthesis caused by activated PKR in cells infected with $g_134.5$-negative mutants. Similarly, ICP22 and other a proteins are posttranslationally modified by both the U_L13 and U_S3 viral protein kinases. However, there is no evidence that ICP22 is modified by the viral kinases introduced into the infected cell as tegument proteins (421,471,472). Thus, the functions of many of the tegument proteins remain to be identified. Nevertheless, the process of entry and release of tegument proteins can be harmful to the cell because cells arrested at the stage of release of tegument proteins can undergo apoptosis (176,700).

The Fate of Viral DNA upon Entry into the Nucleus

The bulk of the incoming viral DNA circularizes rapidly after infection and in the absence of viral protein synthesis (179,445), suggesting that under these conditions, circularization of the genome is promoted by cellular proteins or viral proteins brought into the infected cell in the virion. It has been suggested that the viral DNA ends are held together in the virion and are rapidly ligated by cellular enzymes (445). Because viral DNA is nicked and contains ribonucleotides (see Genome Structure and Organization), repair, circularization, and conversion to the appropriate conformation must then take place. These events may explain the involvement of the RCC1 (regulator of chromosome condensation) gene product in early events in viral infection reported by Umene and Nishimoto (638), but this remains to be verified. It has also been suggested that circularization may result from recombination between direct repeats in the terminal *a* sequences (37), consistent with the hypothesis that fluctuation in the number of *a* sequences could be the consequence of recombination between free ends (390). Such recombination events undoubtedly take place because the number of such repeats varies within the DNA populations arising from a single plaque. It is likely, however, that such recombination events also occur during viral DNA synthesis and packaging.

Leinbach and Summers (317) reported that HSV DNA remains in a nonnucleosomal form during the course of productive infection of cells.

Overview of Productive Infection Gene Expression

During productive infection, more than 80 HSV proteins are expressed in a highly regulated cascade fashion (Fig. 5) in a number of coordinately expressed groups of gene products (230), and several viral proteins play a role in regulation of viral gene expression. Transcription of viral DNA takes place in the nucleus, and as would be expected, all viral proteins are synthesized in the cytoplasm. The host RNA polymerase II is responsible for transcription of all viral genes on the viral DNA during infection (8,102), although viral gene products may modify its activity and structure. Very soon after infection, about 2 to 4 hours postinfection (hpi) at a multiplicity of infection (MOI) of 10 to 20 or in the absence of other *de novo* synthesized viral proteins, the viral a or immediate-early (IE) genes are expressed. These consist of six proteins designated as ICP0, ICP4, ICP22, ICP27, ICP47, and $U_S1.5$. Although transcription of a genes requires no prior viral protein synthesis, an HSV protein brought in with the virion tegument, VP16 (also called aTIF and Vmw65), stimulates the transcription of the a genes, as described later. Five of the six a gene products, including ICP4, ICP0, ICP27, ICP22, and

U_S1.5, stimulate E gene expression in at least some types of cells. As described later, HSV infection inhibits host transcription, RNA splicing and transport, and protein synthesis to facilitate the transition from cellular to viral gene expression.

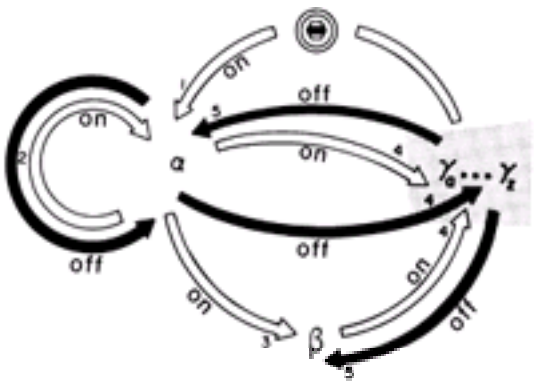

FIG. 5. Schematic representation of the regulation of HSV gene expression. *Open* and *filled arrows* represent events in the reproductive cycle that turn gene expression "on" and "off," respectively. 1. Turning on of a gene transcription by a-TIF, a g protein packaged in the virion. 2. Autoregulation of a gene expression. 3. Turning on of b gene transcription. 4. Turning on of g gene transcription by a and b gene products through transactivation of g genes, release of g genes from repression, and replication of viral DNA. Note that g genes differ with respect to the stringency of the requirement for DNA synthesis. The heterogeneity is shown as a continuum in which inhibitors of viral DNA synthesis are shown to have minimal effect on g_a gene expression but totally preclude the expression of g_z genes. 5. Turning off of a and b gene expression by the products of g genes late in infection.

Expression of the next set of HSV genes, the b or early genes, requires at least the presence of functional ICP4 but not the onset of viral DNA synthesis. Between about 4 to 8 hpi, the b proteins are expressed at peak rates. Among these are proteins involved in replication of the viral DNA and nucleotide metabolism. These viral proteins promote viral DNA replication, and expression of g, or late, genes is then stimulated. Two general groups of b proteins have been identified, although rigid criteria for classification into the two groups have not been defined. The b1 genes are expressed within a short time after or almost concurrently with the onset of synthesis of a proteins and are exemplified by *U_L29* encoding ICP8, the single-stranded protein, and *U_L39* encoding ICP6, the large subunit of ribonucleotide reductase. The b_2 genes are expressed with more delay after a protein expression and are exemplified by *U_L23* encoding thymidine kinase. Some of the b genes require ICP27 for their expression, and this may correlate with the later kinetics characteristic of b_2 genes.

The g, or late, genes are expressed at peak times after viral DNA synthesis has commenced, and their expression is enhanced by viral DNA synthesis. They have been lumped for convenience into two groups, one called g_1, early-late, or leaky-late, and one called g_2, late, or true-late, although in reality, they form a continuum differing in their timing and dependence on viral DNA synthesis for expression (98,101,259,565,651). The typical g_1 gene (e.g., the genes encoding ICP5, gB, gD, and ICP34.5) is expressed relatively early in infection and is stimulated a few fold by viral DNA synthesis. The relatively abundant major capsid protein ICP5 is made both early and late in infection. In contrast, typical g_2 genes (e.g., *U_L44*, the gene encoding gC, *U_L41, U_L36, U_L38*, and *U_S11*) are expressed late in infection and are not expressed in the presence of effective concentrations of inhibitors of viral DNA synthesis.

Based on our limited knowledge of the structure of the promoter and regulatory regions of HSV genes at this time, there appear to be differences in the general organization of the promoter and regulatory sequences of typical genes in each class (Fig. 6). In general, the a gene promoters contain numerous binding sites for cellular transcription factors (see Fig. 6, *ICP4* gene) upstream of a TATA box. The b gene promoters thus far studied contain binding sites for two or three cellular transcription factors upstream of the transcriptional start site (see Fig. 6, *TK* gene). The g_1 gene promoters contain only one or two upstream binding sites (see Fig. 6, *ICP5* gene), whereas the g_2 promoters consist of a TATA box, an initiator element, and a downstream activator element, each of which may vary considerably from cellular homologs (see Fig. 6, *gC* or *U_L44* gene). The level of transcription of viral genes increases in general for the later kinetic classes of genes (699), likely because large amounts of late proteins are needed for virion assembly. This increased level of transcription is apparently due to increased template number and virus-induced modification of the transcription apparatus that is operating on the viral DNA molecule.

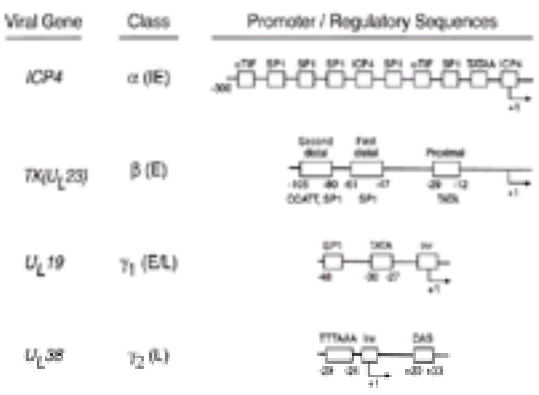

FIG. 6. Diagram of the structure of prototypic HSV gene promoters. The promoter and regulatory sequences of prototypic

genes of a, b, g_1, and g_2 kinetic classes are diagrammed.

We would like to emphasize that although there are prototype genes that allow the definition of four post-a classes of genes, there is likely to be a continuum of genes from b_2 to g_1 genes, in terms of their kinetics and requirements for viral gene expression. First, b and g gene expression in the context of the viral genome requires the participation of five of the six a proteins: ICP0 (528), ICP4 (279,456), ICP27 (527), ICP22 (454,547), and US1.5 (421). Only a few viral genes have been completely characterized with regard to their requirement for these a proteins; thus, we do not know how the requirement for these genes correlates with the kinetic classes already defined. Second, the differences between b and g_1 genes are difficult to quantify and are not perfectly delineated. In principle, the expression of b genes is not augmented by the onset of DNA synthesis, whereas the expression of g_1 genes is reduced in the presence of inhibitors of DNA synthesis. However, expression of some of the b genes is inhibited slightly by inhibitors of viral DNA synthesis (639), whereas some g_1 genes are expressed early and are affected only minimally by inhibitors of DNA synthesis (e.g., *gB*) (478). Nevertheless, it is useful to consider b and g gene expression separately while discussing events in the course of productive infection.

The *ORF O* and *ORF P* genes present a special case. These genes are repressed by ICP4 and therefore are not expressed during productive infection or in the presence of functional ICP4. These genes have been designated as pre-a genes in recognition of the special requirements for their expression (307).

Expression of a Genes

Upon entry into the cell nucleus, the HSV genome, presumably in a circular form, localizes to intranuclear sites near the cellular ND10 structures where transcription of a genes is thought to take place (358). The a gene promoters contain numerous binding sites for cellular transcriptional factors. The major characteristic that differentiates a genes from other viral genes is the presence of the consensus sequence 5'-GyATGnTAATGArATTCyTTGnGGG-3' in one to several copies within several hundred base pairs upstream of the cap site (173,346,347 and 348). This sequence binds Oct-1 but acts as a response element to a complex of the viral protein VP16, host cell factor (HCF) or C1, cellular Oct-1, and other transcriptional factors to promote the expression of the a genes (185,292,293,295,418,459,634).

VP16

VP16 was first described as a virion protein number 16 (VP16) by Spear and Roizman (589) and as ICP25 during the enumeration of proteins accumulating in cells after infection (230). The first evidence that HSV encodes a function that transactivates a genes after infection was reported by Post and co-investigators (453). Subsequently, this virion factor, named a gene transactivating factor (a-TIF) was shown to be a component of the tegument capable of inducing genes carrying the cognate promoter response element, even in the absence of the release of viral DNA into the nucleus (22). VP16 was identified as the virion protein responsible for the transactivation of a genes by Campbell and colleagues (61), and the gene was sequenced by Pellett and associates (432). Upon release from the tegument, VP16 binds to a cellular protein called host cell factor (HCF) or C1 (265,297,603,688). It has been reported that HCF carries VP16 into the nucleus (302), where the VP16–HCF complex binds to Oct-1 bound to viral DNA, forming the activator complex.

The functional organization of VP16 involves several domains of the protein: Residues 378 to 389 are required for interaction with the POU domain of Oct-1 (197,198,297,603), residues 173 to 241 are required for binding of the complex to DNA (603), and the acidic carboxyl-terminal residues 411 to 490 are required for transactivation (634). The C-terminal region likely activates transcription by interaction with host transcription factors, possibly the TFII-B (327) and TFII-D general transcription factors (612). Little is known about the portions of VP16 needed for assembly into virions other than the observation that the amino-terminal 389 residues are sufficient for interaction with the vhs protein, another tegument component (570).

As illustrated in the case of the gene encoding ICP4 (see Fig. 6), a promoters also contain binding sites for other cellular transcriptional activators (e.g., Sp1) in addition to Oct-1. The precise mix of cellular and viral response elements varies between different a gene promoter and regulatory sequences, and their relative contribution to the abundance and duration of transcription has been investigated in only a few instances. In rapidly dividing cells, VP16 increases the accumulation of transcripts by about fourfold and is not a critical factor in viral gene expression (592). Presumably, Oct-1, Sp1, and other cellular transactivating factors account for the basal level of a gene expression. The requirement for VP16 may be different in resting cells or differentiated cells in which these transcriptional factors are unavailable or tightly regulated.

Shutoff of a Gene Expression

The mechanism of the shutoff of a gene transcription is not entirely clear, but three mechanisms are thought to play a role:

1. ICP4 represses its own gene by binding to its cognate DNA binding site located across the transcription initiation site (see Fig. 6). The original consensus sequence of the binding site reported by Faber and Wilcox (154) was ATCGTCNNNNYCGRC, but binding sites vary considerably with respect to degeneracy and affinity for ICP4. The strongest binding sites are those located across the transcription initiation sites of the *ICP4* and the *ORF P* and *ORF O* genes (154,296,399). The effectiveness of the binding site in repressing transcription in the context of the viral genome varies depending on the distance from the transcriptional initiation site. The further downstream it is, the less the repression. Also, displacement by half of a helical turn is more effective in minimizing repression than that by a full helical turn (320). In addition, whereas mutagenesis of the ICP4-binding site across the transcription

initiation site resulted in a 10-fold increase in accumulation of transcripts, the added mutagenesis of the upstream and more distant binding site had barely a twofold effect on transcript accumulation (320). It is less clear whether ICP4 represses the expression of the other a genes. ICP4 binds upstream of the *ICP0* gene transcriptional initiation site (294), and ICP4 represses the *ICP0* promoter in transfected cells (181). Nevertheless, the specific effect of ICP4 on *ICP0* gene expression in infected cells has not been defined by construction of a mutant virus with the binding site in the *ICP0* gene mutated. Consensus ICP4-binding sites are not evident in or near the promoters of the other a genes.

2. During productive infection, b gene products down-regulate a gene expression (230). One b protein identified as a candidate is ICP8, the ssDNA-binding protein encoded by the b_1 gene U_L29. ICP8 decreases the expression of all genes from the parental viral DNA molecules (191, 192), in particular transcription of the gene encoding ICP4 (193). The mechanism of this effect of ICP8 is not known, but it may involve the sequestration of viral DNA molecules in replication complexes, changes in the conformation of viral DNA, or displacement of DNA to sites of DNA synthesis away from those required for transcription.
3. In cells infected with *vhs* mutants, the transition from a to b and subsequently to g protein synthesis lags behind that observed in wild-type virus-infected cells. One function of *vhs* is to synchronize sequential viral gene expression. Underlying this process is the expectation that the a transcripts would overwhelm and outcompete the b mRNAs with respect to translation initiation.

Activation of b Gene Expression

The b_1 and b_2 groups of polypeptides reach peak rates of synthesis about 5 to 7 hours after infection (230). The b_1 proteins, exemplified by polypeptides ICP6 (the large component of the viral ribonucleotide reductase (239) and ICP8 (the major DNA binding protein (98), appear very early after infection and in the past have been mistaken for a proteins (90). They are differentiated from the latter by their requirement for functional ICP4 protein for their synthesis (230, 231). The b_2 polypeptides include the viral TK and DNA polymerase. The appearance of b gene products signals the onset of viral DNA synthesis, and most viral genes involved in viral nucleic acid metabolism appear to be in the b group. The b genes are heterogeneous with respect to time of peak synthesis, although those defined as b_2 are generally made later in infection.

Unlike a genes, b or g genes do not share common promoter elements that instantly predict the timing, duration, or abundance of gene expression. Rather, the overall impression is that they appear to be made up of a diversity of elements, and both the organization and the context in which these elements are placed determine the expression of the gene.

ICP4

ICP4 is required for all post-a gene expression (90, 130, 279, 456, 660), and the effect of ICP4 is exerted at the transcriptional level, as demonstrated by nuclear runoff assays (193). Despite the extensive studies of ICP4 through transfection studies, viral genetic studies, and biochemical studies, the mechanism by which ICP4 transactivates post-a genes remains elusive; however, three lines of investigation are noteworthy.

The first centers on the DNA-binding activity of ICP4. As noted earlier in the text, ICP4 binds to both consensus and nonconsensus sites as measured by gel shift assays (294, 384, 385). Binding of ICP4 to specific DNA sites seems unlikely to explain its transactivation activity because the strongest affinity of ICP4 is for sites known to repress rather than activate transcription (e.g., at the transcription initiation sites of the *ICP4* or *ORF P* genes), and destruction of the sole sequence-specific binding site of ICP4 upstream of the transcription initiation site of the *ICP4* gene attenuated repression in the context of the viral genome (320). With regard to the role of DNA-binding in the transactivation function of ICP4, early mutational analysis studies showed that the sequence-specific binding activity of ICP4 correlated extensively with its ability to activate b gene expression in infected cells (556). In addition, insertion of ICP4 binding sites was reported to render reporter genes responsive to ICP4, albeit in transfected cells (622). However, at least one viral mutant has been obtained in which the DNA-binding activity of ICP4 was greatly reduced but the ability to activate b genes in infected cells was nearly unchanged (555). Furthermore, most post-a genes do not have recognizable consensus binding sites for ICP4, and mutation of consensus sites has not affected the level of expression of the surrounding genes in the viral genome (572). Thus, the bulk of the data from studies of infected cells have failed to correlate ICP4 DNA binding with transactivation of b genes.

As noted earlier, gel shift studies have shown that ICP4 binds to several nonconsensus b and g gene sequences (241, 384, 385, 427, 428). It was postulated that, by binding to DNA sequences near promoters, ICP4 might stabilize the binding of cellular transcription factors to the b gene promoters (427, 428).

The second line of studies has centered on the interaction of ICP4 with transcriptional factors. ICP4 has been shown to form complexes *in vitro* with TATA-binding protein, TFII-B, and TAF250 (63, 573), and most current models for ICP4 transactivation involve interactions with cellular transcription factors to stimulate transcription. Again, given the ability of ICP4 to both activate and repress gene expression, it is not clear whether the binding of cellular transcriptional factors represents the transactivation or repressive function of ICP4.

The mechanism of activation of b genes has also been approached in studies attempting to map the cis-acting sequences needed for activation by virus infection or ICP4. The most extensively studied b gene promoter is that of the U_L23 or *tk* gene. Extensive mutagenic analysis of the promoter showed that the sequences needed for basal level transcription (369) or activated transcription (94) were the same (see Fig. 6): (1) a proximal signal from nt –12 to –29 containing a TATA box and (2) two distal signals from nt –47 to –61 and –80 to –105 containing an SP1 transcription factor–binding site, an SP1 site, and a CCAAT transcription factor (CTF) site, respectively. These studies also supported the hypothesis that ICP4 transactivates b gene promoters through interactions with cellular basal transcription factors.

Considerable work has been devoted to defining the functional organization of the ICP4 molecule. Residues 143 to 210 and 800 to 1298 are required for transactivation (431,556); residues 263 to 487 are required for DNA-binding (556,683); residues 723 to 732 are required for nuclear localization (123,398); residues 309 to 489, including the DNA-binding domain, are required for dimerization (557,682); and residues 171 to 251, including the serine-rich domain from residues 175 to 198, are required for phosphorylation (123,687).

ICP4 is modified extensively by phosphorylation, poly(ADP-ribosyl)ation *in vitro* (461) and *in vivo* (27), and nucleotidylylation (30). The latter modification, carried out by casein kinase II (386), is signaled by the sequence RPRA/S-R shared with the nucleotidylylated proteins ICP0, ICP22, ICP27, and the products of the U_L21, U_L31, U_L47, and U_L49 genes (28). The available evidence suggests that nucleotidylylation of some a proteins requires the presence of proteins made later in infection (27).

Posttranslational processing of ICP4 is associated with translocation into the nucleus. ICP4 forms numerous spots in two-dimensional gels (1) and at least three bands named ICP4a, b, and c in one-dimensional SDS-PAGE gels (437). The slower one-dimensional electrophoretic forms are due to posttranslational modifications. They appear at about the time when ICP4 associates with the nucleus (158), and they do not form with ts mutant ICP4 molecules that are unable to localize into the nucleus (279,456). ICP4 is probably phosphorylated at multiple sites, as suggested by the complex pattern of phosphopeptides generated from it (687). The serine-rich region from residues 142 to 210 contains many potential sites for phosphorylation, and this region has been shown to be phosphorylated. The serine-rich region or its phosphorylation is essential for efficient phosphorylation of the rest of the molecule (687).

Residues 515 to 520 of ICP4, gly-tyr-gly-ala-ala-gly, match an adenosine triphosphate (ATP)-binding motif, gly-X-gly-X-X-gly, characteristic of the catalytic domain of certain protein kinases (687). ICP4 has a tightly associated protein kinase activity (687), but it is not known if this activity is intrinsic to ICP4.

There is considerable indirect evidence that the various isoforms of ICP4 may have different properties and functions. The isoforms of ICP4 differ in their affinities for different DNA-binding sites (385). A later study reported that dephosphorylated forms of ICP4 bound to a promoters, whereas phosphorylated forms bound to b and g promoters (429). The transition from one isoform to another may well be dependent on the function of other a proteins inasmuch as ICP27 causes a shift of ICP4 to a faster migrating form (485,613) and stimulates the transactivation of g genes by ICP4. Some of the ICP4 isoforms cycle phosphate on and off (675), an activity that may be related to the protein kinase activity tightly associated with the protein (687).

In summary, ICP4 performs the functions of a transcriptional activator but, except for its repressive functions, does not behave like a classic transcriptional regulator. The many isoforms accumulating in the infected cells suggest that its different functions may be dependent on the posttranscriptional modification and the context of the cognate DNA-binding site. A perhaps simplistic view summarizing the current models of ICP4 action is that it represses transcription by binding to consensus sequences near sites of transcriptional initiation but activates transcription by binding to transcription factors and perhaps nonspecifically to DNA.

ICP27

ICP27 is an essential (360,527), multifunctional a protein that is required for expression of several classes of post-a genes (360,489,527,639), viral inhibition of RNA splicing (206,208), and inhibition of transcription of many host genes (593). ICP27 localizes into the nucleus independently of other viral proteins and, at early times, has been reported to be distributed diffusely in the nucleus or with small nuclear ribonucleoprotein particles (snRNPs) (441,534) and in replication compartments at later times of infection (114).

Functional domains mapped on the 512-residue ICP27 molecule include an amino-terminal acidic region essential for viral replication from residues 12 to 63 (487), an N-terminal nuclear localization signal from residues 110 to 137 (375), an N-terminal leucine-rich nuclear export signal from residues 7 to 15 (532), a methylated internal RGG box required for RNA-binding (378), a C-terminal transactivator region from residues 262 to 512 and a transrepressor region from 434 to 512 (533), and a zinc finger motif from 483 to 508 (646).

Early studies on viral gene expression concluded that ICP27 is required only for viral DNA replication and late gene expression (485,527), but recent results have shown that ICP27 is also needed for efficient expression of certain b genes (365,530,639), in particular the less abundant viral DNA replication proteins (639). Thus, ICP27 is required indirectly for viral DNA replication to promote the expression of viral gene products that are essential for viral DNA synthesis. These studies have shown that ICP27 increases the mRNA levels for these b genes, but it has not been determined whether this effect is transcriptional or posttranscriptional. Further discussion of the mechanisms by which ICP27 stimulates g gene expression and inhibits RNA splicing and host transcription is presented in the sections that follow.

ICP0

ICP0 is not essential for viral replication but promotes viral infection and viral gene expression, especially at low multiplicities of infection (MOI) (528,610). At low MOIs, cells infected with ICP0 deletion mutants yield about 10- to 100-fold less virus than cells infected with wild-type virus. ICP0 null mutant viruses are able to enter cells and express a proteins but are defective for progression through the productive cycle. Therefore, the particle-to-plaque-forming unit (PFU) ratios of ICP0 null mutant virus stocks are significantly higher than those of wild-type virus stocks (610). The phenotype of ICP0 null mutants is MOI dependent because at MOIs of 5 and higher, ICP0 null mutants give viral yields similar to those of wild-type virus.

The early literature on ICP0 clearly established that it is a nonspecific (sometimes called promiscuous) transactivator because in transfected cells, it induces the expression of HSV a, b, and g promoters (148,182,419,476) as well as

heterologous promoters (401). ICP0 null mutants are defective for expression of a, b, and g mRNAs (74), a defect manifest at the level of RNA synthesis (260). Because ICP0 does not bind DNA directly (153), it appears to be affecting transcription indirectly. Functional analysis of mutant proteins has defined an N-terminal C3HC4 zinc-binding domain or RING finger motif (146). ICP0 is phosphorylated by both viral (U_L13) and cellular kinases in infected cells (420) and is nucleotidylylated (29).

The mechanisms by which ICP0 stimulates viral gene transcription in infected cells have not been defined, but a number of interactions of ICP0 with cellular proteins and organelles have been observed, and these may ultimately lead to an understanding of the mechanism of action of ICP0.

1. Early in infection, ICP0 localizes in the nucleus. Beginning 3 hours after infection, ICP0 is translocated to the cytoplasm (primary human embryonic lung fibroblasts). The translocation is nearly complete by 7 to 9 hours, depending on multiplicity of infection. Translocation is blocked by inhibitors of DNA synthesis, suggesting that the translocation required g_2 proteins (644).
2. During the nuclear phase, ICP0 localizes at or near nuclear structures known as *nuclear domain 10* (ND10) structures and causes the loss of the ND10 component PML and the disorganization of the ND10 structures (151,356,357). The function of these cellular structures is not known, but several viral DNA genomes are believed to target to these structures early in infection (243,244). A key component of ND10 is PML, named after promyelocytic leukemia, in which an aberrant form consisting of PML fused to rectinoic acid receptor-a accumulates. Certain forms of PML are lost after HSV infection. A simple model for this viral effect on the host cell is that the ND10 structures are part of a host cell repression system that the virus evades by causing disruption of the structure.
3. ICP0 interacts with components of the protein degradation machinery (150,152). ICP0 binds to a ubiquitin-specific protease named HAUSP and increases its localization to ND10 sites (152). ICP0 and HSV infection causes a loss of PML isoforms that are known to be conjugated to SUMO-1 or PIC1 (150). Thus, it has been hypothesized that ICP0 binds to HAUSP and stimulates the cleavage of SUMO-1 from PML and possibly other proteins, allowing their ubiquitination and subsequent degradation (150). ICP0 is also required for the loss of the regulatory subunit of the cellular DNA-dependent protein kinase (313), which may be a result of this same activity.
4. In dividing cells, ICP0 associates with kinetochores and causes the degradation of centomeric protein C (CENP-C) (149). This protein plays a key role in the assembly of the kinetochore, and non–virus-induced degradation of this protein results in delayed transit ion of metaphase to anaphase. The zinc finger is required to bind to the kinetochore, but the actual structure to which it binds is unknown; thus, the mutation that abolishes the degradation overlaps the site required for binding of the ubiquitin-specific protease and may be a manifestation of the interaction of ICP0 with the latter protein.
5. ICP0 also binds several other proteins. These include cyclin D3, BMAL-1, elongation factor-1d (eF-1d), and a protein currently known as p60. In cells infected with wild-type virus, the levels of cyclin D3 and D1 decrease with time after infection (136,269,581). However, the rate of decay in cyclin D3 levels in cells infected with wild-type virus is slower than in cells infected with ICP0-negative mutant or a mutant whose ICP0 (D199A) does not bind cyclin D3. In addition, in cells infected with wild-type viruses, cdk4, the partner of D cyclins, is activated because p21, the cdk4 inhibitor, is degraded; whereas in cells infected with the D199A mutant, cdk4 is not activated, and p21 levels remain unchanged. The D199A mutant showed reduced capacity to replicate in stationary contact-inhibited cells and reduced neurotoxicity in mice infected by a peripheral route (643). The D199A co-localizes with PML and causes the disaggregation of ND10, but unlike the wild-type virus, it is not transported out of the nucleus into the cytoplasm. Neither wild-type nor the D199A mutant activates cdk2. Analyses of the interaction of cyclin D3 with ICP0 led to two significant observations. First, cyclin D3 co-localizes with wild-type ICP0 but not with the D199A mutant during the nuclear phase of ICP0. Second, overexpression of cyclin D3 accelerates the transport of ICP0 to the cytoplasm (644). The picture that emerges is that by stabilizing cyclin D3, ICP0 also stabilizes the cyclin-dependent kinase cdk4 and cyclin D1, another partner of cdk4. The function of cdk4–cyclin D complexes is not known. By analogy with the apparent function of activated cdc2, the activated function of cdk4–cyclin D complexes may lead to more rapid activation of viral DNA synthesis, synthesis of late proteins, and translocation of ICP0 into the cytoplasm. The apparent preoccupation of HSV with D cyclins is underscored by the observation that members of other herpesvirus subfamilies either induce a D-type cyclin (568) or encode a cyclin D homolog (70,261,324,411).
6. ICP0 has been shown to interact with eF-1d (267). The interaction of ICP0 with eF-1d, an elongation factor responsible for adenosine diphosphate (ADP)-ATP exchange, appears to be significant inasmuch as a truncated ICP0 polypeptide containing the binding site interfered with *in vitro* synthesis of a reporter protein. The significance that HSV places on eF-1d is underscored by the observations that U_L13 phosphorylates eF-1d and that this protein is also phosphorylated in cells infected with representative betaherpesviruses and gammaherpesviruses. The protein p60 binds ICP0 and ICP22 and is discussed in conjunction with the latter protein. ICP0 appears to have other functions, which are dealt with in the section on cell death.
7. ICP0 has also been postulated to have an effect similar to that of a histone deacetylase inhibitor (224), potentially resulting in increased acetylation of histones and increased gene expression from DNA condensed in nucleosomes. It seems more likely that alterations of histone acetylation would affect transfected genes or viral reactivation as compared with HSV productive infection because the former forms of DNA are wrapped in nucleosomes whereas HSV DNA during productive infection is not.

In summary, ICP0 is emerging as a multifunctional protein directed primarily toward modification of cellular functions to promote viral gene expression and viral productive infection. Precisely how the sum total of all of these modifications account for the function of ICP0 remains to be ascertained.

ICP22 and $U_S1.5$

As noted previously, the $U_S1.5$ transcription unit resides within the coding sequence of the gene encoding ICP22, and the amino acid sequence of the corresponding protein is identical to the carboxyl-terminal two thirds of the amino acid

sequence of ICP22 (64). Like ICP22, U_S1.5 is expressed as an a protein.

Viral mutants that fail to express the carboxyl-terminal half of ICP22 (454) have been studied extensively and exhibit several phenotypes, such as (a) restricted growth in primary human fibroblasts, rabbit, mouse, and rat cells (549); (b) attenuated virulence by intracerebral inoculation in mouse; (c) reduced expression of some (e.g., U_S11, U_L38, U_L41) but not all g_2 (e.g., gC) proteins; (d) abolished posttranslational modification of the C-terminal domain of the large subunit of RNA polymerase II, which takes place in some but not all cell lines (338); (e) reduced stability of ICP0 mRNA (64); and (f) increased stability of several cell cycle proteins (e.g. cyclins A and B) (4). The same phenotype is exhibited by a mutant defective for the U_L13 protein kinase (471,472). ICP22 also interacts with p60, a protein that also binds ICP0, and with p78, a cell cycle protein of unknown function (50).

ICP22 and U_S1.5 are phosphorylated by the U_L13 and U_S3 viral protein kinases, and ICP22 is nucleotidylylated by casein kinase II. Sequential deletions have established a relatively detailed functional map of ICP22 (421) and made apparent three significant features of its structure and organization. First, a mutant lacking the sequences unique to ICP22 (i.e., not shared with U_S1.5) behaved like the wild-type parent with respect to expression of g_2 genes but was avirulent on intracerebral inoculation of mice. Second, two sets of sequences are required for the phosphorylation of ICP22 by U_L13 and U_S3. One is within the carboxyl-terminal domain shared with U_S1.5; whereas the other highly homologous sequence is within the amino-terminal portion of ICP22 that is not shared with U_S1.5. Alteration of either sequence abolishes the phosphorylation of ICP22. Mutagenesis of the amino-terminal domain enables wild-type expression of g_2 proteins, but alteration of the carboxyl-terminal sequence caused a reduction in the expression of the selected g_2 proteins, similar to that seen in cells infected with deletion mutants in ICP22 (452). These studies indicate that U_L13 and ICP22 and U_S1.5 proteins act independently and either jointly or sequentially to regulate several aspects of HSV gene expression.

Synthesis and Processing of Viral Proteins

Viral proteins appear to be made on both free and bound polyribosomes. Many HSV proteins are posttranslationally modified, and these modifications include cleavage, phosphorylation, sulfation, *N*-linked and *O*-linked glycosylation, myristoylation, ADP-ribosylation, and nucleotidylylation (514). With the exception of some glycoproteins, the extent to which processing is a requirement of virus growth rather than the consequence of an encounter between cellular or viral enzymes and molecules resembling natural substrates remains uncertain. Much of this processing is by cellular enzymes and pathways (reviewed in 514), but some enzymes are virus encoded. The HSV-encoded protease is discussed later in the section on capsid assembly.

Herpes Simplex Virus Protein Kinases

HSV specifies at least three protein kinases, the large subunit of the ribonucleotide reductase (RR1), U_L13, and U_S3.

RR1

A protein kinase activity was first reported to be associated with the large subunit of the ribonucleotide reductase (ICP6) of HSV-2 but not of HSV-1 (87). Later studies showed autophosphorylation activity with the HSV-1 RR1 protein as well (99). Whereas the ribonucleotide reductase activity is associated with the conserved carboxyl-terminal domain, the protein kinase activity maps near the amino terminus (7,99). The large subunit of ribonucleotide reductase is therefore a multifunctional protein. The substrate of this protein kinase activity is not known.

U_S3

McGeoch and Davison (364) predicted that U_S3 encodes a protein kinase on the basis of its sequence, a prediction borne out by biochemical (166) and genetic studies (470). A major substrate of this enzyme is an intrinsic membrane protein exposed on the surface of infected cells and encoded by *U_L34*. The U_L34 protein contains the amino acid motif recognized by the U_S3 protein kinase; thus, substitution of the serine or threonine residues within this motif with alanine resulted in a loss of phosphorylation, but the virus grew poorly and revertants were readily detected. It is noteworthy that four prominent phosphoproteins unrelated to the U_L34 protein appear in lysates of baby hamster kidney cells infected with the U_L3-negative mutant or with mutants in the amino acid motif recognized in U_L34 by the U_S3 kinase. The anti-U_L34 serum copreciptates the four phosphoproteins along with the U_L34 protein. It would appear that the four phosphoproteins compensate in some fashion for the absence of phosphorylation by U_S3 kinase. U_S3 also phosphorylates the U_L3 protein and ICP22 (353,421,452). The major targets of U_S3, however, may well be host proteins, as discussed in the section on apoptosis.

U_L13

Smith and Smith (577) and Chee and coauthors (73) reported that the sequence of the *U_L13* ORF contains the signature amino acid motif common to other protein kinases and is shared among alphaherpesviruses, betaherpesviruses, and gammaherpesviruses. Cunningham and colleagues (105) reported on the properties of a new kinase very similar to the protein kinase activity demonstrated in tegument–capsid structures described by Lemaster and Roizman (319) and ascribed the new kinase to the product of the *U_L13* gene. Studies of U_L13- and U_L13-U_S3-negative mutants led to the conclusion that the U_L13 protein kinase affects the phosphorylation and processing of the gene products of ICP22 and U_S1.5, ICP0, and U_L47 and affects the accumulation of the a0, U_L26 and U_L26.5 (protease and its substrate), U_L38 encoding the VP19C capsid protein, U_L41, and U_S11 gene products (471,472).

Although the U_L13 protein kinase is associated with structural proteins, the enzyme brought into the infected cell by the

virion does not phosphorylate the newly synthesized ICP22. The phenotype of the U_L13-negative virus is similar to that described for the a22-negative mutant by Sears and coauthors (6,471,547). As noted earlier in the text, U_L13 protein kinase phosphorylates EF-1d. This function appears to be highly conserved among herpesvirus subfamilies.

A large number of other proteins (e.g., ICP4, gE; see Table 1 for a more detailed list) are phosphorylated in the course of the reproductive cycle. The kinases responsible for the phosphorylation and the role of phosphorylation in the functions of these gene products have not been elucidated.

Adenosine Diphosphate Ribosylation

Preston and Notarianni (461) reported that ICP4 and VP23 are poly(ADP-ribosyl)ated in isolated nuclei, a significant finding that nevertheless left unanswered the question of whether this reaction actually takes place in the infected cell. Blaho and coworkers (27) reported that antibody specific for poly(ADP-ribose) reacts with ICP4 extracted from cells late in infection, in effect answering the question in the affirmative. However, the poly(ADP-ribose) added to ICP4 was digested by poly(ADP-ribose) glycohydrolase, but only after denaturation of the protein. In contrast, poly(ADP-ribose) added to ICP4 in isolated nuclei was readily removed from the native protein by the glycohydrolase. The results indicate that ICP4 is poly(ADP-ribosyl)ated and suggest that, in the isolated nuclei, the poly(ADP-ribose) is added either by elongation of existing chains or to novel sites.

Nucleotidylylation of Viral Proteins

An initial report by Blaho and Roizman (30) showed that ICP4 is both guanylated and adenylated. The label transferred by a ^{32}P-ATP or a ^{32}P-guanosine triphosphate (GTP) is associated with the slowly migrating forms of the protein. Conclusive evidence for the nucleotidylylation emerged from transfer of a ^{3}H-labeled purine ring from ATP to ICP4. Subsequent studies revealed that ICP0, ICP22, ICP27, and the proteins encoded by *U_L21*, *U_L31*, *U_L47*, and *U_L49* are also nucleotidylylated and that all these proteins share the sequence R/PRAPS/R (27,28). Nucleotidylylation is carried out by casein kinase II. U_L21 is dispensable for growth in cultured cells (15), U_L31 cofractionates with the nuclear matrix (71) and appears to be an important component of the nuclear membrane of the infected cell (Baines, personal communication), and U_L47 may interact with VP16 (368). U_L49 is a virion protein (VP22/3?) that is labeled in cells with [^{32}P]orthophosphate and also is (ADP-ribosyl)ated.

Modification of Membrane-associated Proteins

Of the 11 predicted glycoproteins, at least 10 have been studied in sufficient detail to demonstrate the presence of oligosaccharide chains. The 11th, gJ, remains elusive, although recent studies indicating that gJ plays a role in blocking apoptosis are likely to lead to more extensive studies of this protein (254,700). In addition, it has been reported recently that the products of the U_L47 gene, VP13-14, contain *O*-linked polysaccharide chains and are phosphorylated (383).

At least one protein, the product of the *U_L11* gene, has been shown to be myristoylated (349). Deletion mutants in the *U_L11* gene show an impairment in egress from infected cells (17).

Viral DNA Replication

Once the b proteins are expressed, several of these proteins localize into the nucleus and assemble onto the parental viral DNA molecules in punctate structures called *prereplicative sites* located near ND10 structures (243,639) (Fig. 7A), where viral DNA synthesis initiates on the circular molecule, probably to give theta replicative forms. As viral DNA synthesis progresses, the progeny DNA molecules and replication complexes accumulate in the nucleus in globular structures called *replication compartments* (475) (see Fig. 7B). Viral DNA synthesis proceeds within these areas of the nucleus. At some point, presumably relatively early after its initiation, viral DNA synthesis switches from a theta replication mechanism to a rolling-circle mechanism, the latter producing concatemeric molecules. Thus, most of the viral progeny DNA molecules that accumulate in the infected cell nucleus are “head-to-tail” concatemers containing the four sequence isomers described previously (245).

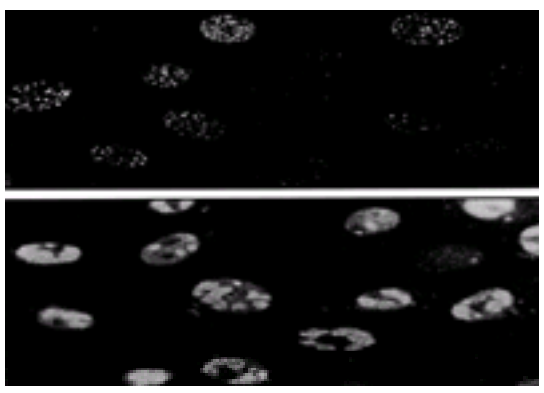

FIG. 7. Prereplicative sites and replication compartments. Immunofluorescence micrographs showing the localization of the ICP8 to prereplicative sites (**A**) when viral DNA synthesis is inhibited and to replication compartments, and (**B**) when viral DNA replication is proceeding. (Micrographs courtesy of E. McNamee.)

Seven viral proteins are required for viral DNA replication. Phenotypic analysis of viral ts mutants first identified two

virus-encoded gene products required for viral DNA synthesis, the viral DNA polymerase (469) and the ICP8 ssDNA-binding protein (98), and transfection studies showed that seven viral genes are sufficient to replicate a viral origin sequence co-transfected into cells (69,681). The gene products of the other five genes were identified through biochemical purification and genetic studies. The seven gene products are the viral DNA polymerase (U_L30) and its accessory protein or processivity factor (U_L42), an origin-binding protein (U_L9), the ICP8 ssDNA-binding protein (SSB; U_L29), and the helicase–primase or primeosome complex of three proteins, U_L5, U_L8, and U_L52. Host cell factors are presumably also involved in viral DNA synthesis, but these have not been identified, in large part because HSV DNA replication involving origin-dependent initiation and synthesis has not been achieved *in vitro.* Nevertheless, host enzymes, including the DNA polymerase a-primase, DNA ligase, and topoisomerase II, are probably also required.

The origins of viral DNA synthesis were identified through mapping of sequences found in defective viral genomes (168,336) and sequences needed for plasmid DNA amplification in transfection studies (390,648,669). The origins include *oriS,* a sequence located in the *c* sequences bounding the S component and therefore present in two copies in the viral genome, and *oriL,* a sequence located between the divergent transcription units of the genes for two viral DNA replication proteins, ICP8 and DNA polymerase.

Both *oriS* and *oriL* are palindromic structures, *oriL* being a 144-bp palindrome (281,337,477,669) and *oriS* being a shorter palindrome of 45bp (116,390,607,608) that center around AT-rich regions of 18 and 20 bp, respectively. Inverted repeats that contain binding sites for the U_L9 protein, called Box I, are located on either side of the AT-rich region of *oriL.* The *oriS* contains a Box I sequence to the 5' side of the AT-rich region and a similar sequence with a 10-fold lower binding affinity for U_L9, called Box II, located 3' to the AT-rich region. In *oriL,* the Box I sequences are flanked by another homologous sequence, called Box III, which has greatly reduced affinity for U_L9. The *oriS* contains one copy of Box III flanking the 5' Box I. The *oriL* is notoriously unstable when cloned into plasmids; hence, little genetic analysis has been performed on this sequence. Box I is required for *oriS* function, and mutations in Box II greatly reduce DNA replication. Although Box III shows weak binding to U_L9 *in vitro,* mutations in Box III reduce replication by about fivefold in transfection assays.

The reasons for three potential origins of replication in the viral genome are not apparent at this time. Neither origin is specifically required for viral replication. A mutant virus with a deletion in *oriL* is viable and showed normal burst size and latent infection (450). In addition, mutant viruses with both *oriS* sequences deleted showed at most a fourfold reduction in viral yields and only slightly delayed viral DNA synthesis (240). It has been suggested that one of these origins may represent vestigial origins from the L and S components of the viral genome (514) and that one of these origins, *oriL,* may function in reactivation from latent infection (207).

Viral DNA Replication Proteins

The properties and functions of the seven essential HSV DNA replication proteins are summarized here but reviewed in detail elsewhere (37).

Origin-binding Protein, U_L9, OBP

The U_L9 origin-binding protein forms a homodimer and binds specifically to the sequence CGTTCGCACTT (139,140,283,284). U_L9 also has ATP-binding and DNA helicase motifs that are essential for viral replication. Binding of U_L9 to origin sequences induces a bend in the DNA and formation of a single-stranded stem-loop structure. The addition of the HSV ssDNA-binding protein, ICP8 (see later), allows U_L9 to unwind Box I of *oriS* if an 18-nucleotide single strand tail is present 3' to Box I. Thus, ICP8 may provide a ssDNA region in the AT-rich region, from which U_L9 can separate the strands. As an origin-binding protein, U_L9 would play a role in origin-dependent synthesis originating from the circular molecules, but once synthesis has converted to a rolling-circle mode, U_L9 would presumably not be needed as an origin-binding protein.

Single-Stranded DNA-Binding Protein, ICP8, U_L29.

ICP8 is an abundant protein in HSV-infected cells and was first identified as the major DNA-binding protein in HSV-infected cells (23). ICP8 binds preferentially to ssDNA (310,525), and this ssDNA-binding (SSB) function is essential for viral DNA replication. ICP8 also exhibits a helix-destabilizing activity but can also catalyze the renaturation of complementary single strands (134). Probably as a consequence of the latter two activities, ICP8 can promote strand transfer (43), and this may contribute to the high frequency of homologous recombination observed in infected cells. In addition to its SSB function, ICP8 interacts with several other viral DNA replication proteins, as evidenced by its physical interaction and stimulation of U_L9 helicase activity (36,38,162), stimulation of the $U_L5/8/52$ helicase activity (155,201,619), functional interaction with DNA polymerase (77,218), and coimmunoprecipitation with U_L9, U_L8, and pol proteins (639). Consistent with these numerous interactions, ICP8 is required for localization of other viral proteins and cellular proteins to prereplicative sites in infected cell nuclei (54,112,329,342). As a result of its size and numerous protein interactions, ICP8 is likely to play a scaffold role in assembly of HSV DNA replication complexes (112). ICP8 also exerts a repressive effect on IE and E gene expression under certain conditions (191,192 and 193), possibly as a consequence of promoting the formation of DNA replication complexes on viral DNA.

DNA Helicase–Primase Complex

This complex, which contains the protein products of the *U_L5, U_L8*, and *U_L52* genes, was first identified as a helicase activity from infected cells (103). The complex unwinds short oligonucleotides annealed to single-stranded M13 DNA in the 5' to 3' direction. U_L5 has ATP-binding and DNA helicase motifs, and these are essential for DNA replication (704). U_L52 has a proposed divalent metal-binding motif that is conserved in DNA polymerases and primases, and this motif is

required for DNA replication. A complex of U_L5 and U_L52 has DNA-dependent ATPase, helicase, and primase activities; hence, this constitutes the core enzyme. U_L8 promotes the nuclear localization of this complex and, in concert with ICP8, stimulates optimal activities of the core enzyme. Thus, the holoenzyme can unwind a 2.3-kbp nicked plasmid in the presence of ICP8. The primase activity produces oligoribonucleotides 6 to 13 bases in length, and the preferred template sequence is 3'AGCCCTCCCA, with synthesis initiating at the first C.

DNA Polymerase

The HSV DNA polymerase has been studied extensively, partly as a result of its potential as a target for antiviral drugs. A new DNA polymerase activity was detected in HSV-infected cells in the 1960s (270), but formal genetic proof that it was virus encoded was not provided until the late 1970s when temperature-sensitive and drug-resistant mutations affecting the properties of the polymerase were isolated and mapped to the viral genome (469). The HSV DNA polymerase holoenzyme is a heterodimer of the 136-kd U_L30 protein complexed with the 65-kd U_L42 protein. The U_L30 protein contains the polymerase activity, and it contains three sequence motifs that are homologous and align with sequence motifs I, II, and III of other DNA polymerases (186). The U_L30 protein also has an intrinsic 3'-5'-exonuclease activity, which can serve as a proofreading activity. U_L42 increases the processivity of the U_L30 DNA polymerase. The interaction between U_L30 and U_L42 is essential for viral DNA replication *in vivo,* and these interaction sites are being investigated as possible targets for antiviral compounds.

The HSV DNA polymerase has a broader substrate specificity than cellular polymerases, and this has allowed the development of compounds that specifically inhibit the viral DNA polymerase. These compounds are discussed in Chapter 15 and Chapter 73.

Based on the available information, a model for HSV DNA replication (Fig. 8) has been formulated (reviewed in 37). The first steps in HSV DNA replication involve the binding of the U_L9 protein to the origin sequences and the looping and distortion of the origin sequences by U_L9. ICP8 then binds to U_L9 or ssDNA regions, and the U_L9 helicase activity unwinds the DNA. The helicase–primase complex is then recruited to the origin by interactions with U_L9 or ICP8. Leading-strand synthesis involves the unwinding of the DNA and synthesis of a primer by the HSV helicase–primase complex, from which leading-strand synthesis can be accomplished by the HSV pol-U_L42 holoenzyme. Alternatively, primers may be synthesized by the cellular polymerase a-primase. Lagging-strand synthesis is then accomplished by primer synthesis and pol-U_L42 extension of the DNA strand. Because the progeny DNA is largely head-to-tail concatemers (245), it is believed that although synthesis initiates from the theta form, it is rapidly converted to a rolling-circle replicative mechanism, by which concatemeric molecules can be synthesized.

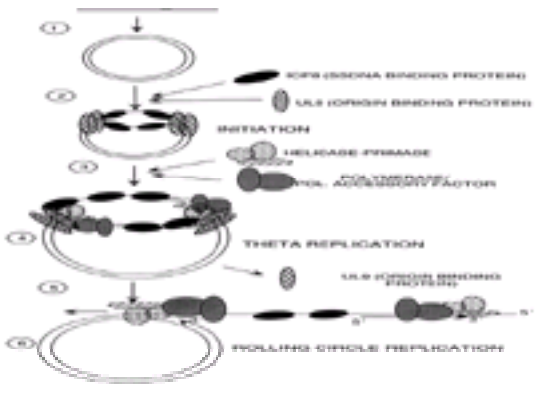

FIG. 8. Diagram of a model of HSV DNA replication, with the following steps: 1. Input DNA is circularized upon entry into the nucleus. 2. U_L9 (the origin-binding protein) initially binds to specific elements in the origin (either ori_L or ori_S) and begins to unwind the DNA. U_L9 then recruits ICP8 (the single-stranded DNA-binding protein) to the unwound single-stranded DNA. 3. U_L9 and ICP8 recruit the five remaining viral DNA replication proteins to the replication forks. 4. The helicase–primase proteins and the viral polymerase complex assemble at each replication fork for initial rounds of theta-form replication. 5. Replication switches from theta to rolling-circle mode by an unknown mechanism. U_L9 is not necessary for rolling-circle replication because it is not origin dependent. 6. Rolling-circle DNA replication produces long head-to-tail concatamers of viral DNA, which are cleaved into monomeric molecules during packaging. (Diagram courtesy of E. McNamee.)

Nonessential Viral Gene Products Involved in Viral DNA Synthesis and Postsynthesis Modification

Several other HSV gene products, including the thymidine kinase, ribonucleotide reductase, deoxyuridine triphosphatase, and uracil DNA glycosylase, are not essential for viral replication in cultured cells but are likely to be essential for nucleotide metabolism and viral DNA synthesis and repair in resting cells, such as neurons. The corresponding host cell enzymes are not expressed in resting cells, and it is likely that the virus has evolved to encode these enzymes to optimize its own DNA synthesis in these cells.

Thymidine Kinase

This enzyme was first identified as a deoxythymidine kinase (273), giving rise to its name. In fact, it phosphorylates pyrimidines and even purine nucleosides. In addition, it has a thymidylate activity. Its broad substrate specificity allows it to phosphorylate nucleoside analog molecules, which then can serve as antiviral compounds (172). The HSV TK consists of a homodimeric complex of the U_L23 protein product, the structure of which has been solved (47). HSV presumably

encodes a TK activity to provide nucleoside triphosphate precursors for DNA synthesis in resting cells, such as neurons, where the cellular enzyme is not expressed (see later). The viral enzyme leads to an increase in deoxythymidine triphosphate (dTTP) pools relative to that seen in uninfected cells or early in infection. This complicates the use of radioactive thymidine for labeling of viral DNA synthesis in infected cells because the specific activity of the labeled DNA will be lower at later times of infection.

Deoxyuridine Triphosphatase

Deoxyuridine triphosphatase (dUTPase) hydrolyzes dUTP to dUMP and pyrophosphate, preventing the incorporation of uracil into DNA and generating dUMP, the precursor of dTTP. The HSV enzyme is a monomer of the U_L50 protein product (464) and appears to be most essential for viral replication in the nervous system (473).

Ribonucleotide Reductase

Ribonucleotide reductase catalyzes the reduction of ribonucleoside diphosphates to the corresponding deoxyribonucleoside diphosphate. The HSV enzyme consists of a complex of the U_L39 and U_L40 proteins as an a_2b_2 tetramer (14). The HSV ribonucleotide reductase is not subject to the same allosteric controls as the cellular enzyme; thus, the HSV enzyme is not inhibited by the increased dTTP pools in HSV-infected cells. The HSV ribonucleotide reductase is required for viral replication in nondividing cells (194). The large subunit has an intrinsic serine-threonine kinase activity that is separable from the ribonucleotide reductase activity. The role of the protein kinase activity is not known, although there has been one report that the HSV-2 protein kinase activity is required for IE gene expression and virus growth (574).

Uracil N-Glycosylase

This enzyme removes uracil bases from DNA, bases that arise in DNA by the deamination of cytosine to form uracil, by the cleavage of the *N*-glycosidic bond linking uracil to the deoxyribose sugar. The site is then repaired so that a mutagenic event converting a G-C base pair to an A-T base pair does not occur. The HSV enzyme is encoded by the U_L2 gene (62). This gene product is not essential for replication in growing cells in culture, but it plays a role in viral pathogenicity and reactivation from latent infection (474).

Alkaline Nuclease

This enzyme is a phosphoprotein encoded by the U_{L12} gene (20,458), has endonuclease and exonuclease activities, and is active at pH 9.0 to 10.0 (270,271,625). As described later, this nuclease plays a role in DNA maturation and encapsidation. It is believed to interact with the ICP8 (625), possibly to allow the nuclease to act directly on viral replication intermediates or products. The HSV nuclease may be required to resolve concatenated viral DNA for packaging (553).

Viral DNA Recombination

Homologous recombination is very efficient in HSV-infected cells, and multiple crossover events between co-infecting viral genomes are apparent in progeny viruses, even between HSV-1 and HSV-2 genomes (396). Viral DNA replication is required for this high level of homologous recombination, and the time courses of DNA replication and recombination are parallel in cells transfected with viral *oriS* plasmids (133). The mechanism by which replication of the DNA promotes recombination is not known, but the single-stranded regions or the concatemeric molecules might be targets for cellular or viral recombination machinery. In addition, ICP8 is known to promote strand transfer; hence, it might play a role in recombination in infected cells.

Inversion of the genomic L and S segments also involves recombination between the terminal repeats and the internal inverted repeats. As described previously, HSV virion DNA contains four populations of molecules bearing the four orientations of the L and S components of the genome. This novel feature of the genome of HSV and certain other, but not all, herpesviruses (see Chapter 71) has long intrigued the field, and interest has been further piqued by the observation that viruses without internal repeats are viable, indicating that inversion is not essential for viral replication (253,446). These mutant viruses are less virulent, but the role of the internal repeats as protein-coding sequences versus their structural role as repeated sequences has not been separated. The L–S junction sequences and, in particular, the *a* sequences promote high-efficiency inversion in that insertion of a copy of these sequences at other sites in the genome leads to inversion of these sequences (83,387,388 and 389). Duplication of certain other viral sequences at a second site in the viral genome can lead to additional inversion events (448,663), but these events appear to be less efficient than *a* sequence–mediated inversion. The 95-bp Uc-DR1 sequence is specifically required for inversion at the *a* sequence (133), but it remains to be determined if this event involves site-specific recombination or a hot spot for recombination. This may be resolved by the further purification of the activity needed for inversion (49).

Activation of Late Viral Transcription

Once viral DNA replication has initiated, expression of g genes is increased (232), largely as a result of increases in their transcription (193,666,699). For some genes called g^1, early-late, or leaky-late genes, this represents an increase in expression that was already occurring under early conditions. These include the genes encoding ICP5 (U_L19), gB (U_L27), and gD (U_S6). Other genes, called g^2, late, or true-late genes, are transcribed at significant levels only after viral DNA synthesis. These include U_L38, *gC* (U_L44), U_S11, U_S9, and $U_L49.5$. Whereas g^2 genes are unambiguously defined as requiring the onset of viral DNA synthesis for their expression, the g^1 group lumps together all genes whose expression is enhanced by the onset of viral DNA synthesis but in reality contains a continuum of genes that either resemble g genes but are expressed late in infection or are partially expressed in the absence of DNA synthesis but kinetically are not

different from the g_2 genes.

Late transcription takes place in replication compartments within the infected cell nucleus, as evidenced by (a) the localization of ICP4 (280,481), pol II (323,488), and ICP22 (323) to replication compartments at late times; and (b) RNA-pulse labeling of replication compartments at late times (443). Thus, the transition from b to g gene transcription involves a change in the nuclear location of transcription from sites near ND10 domains to replication compartments. This is likely to play a significant role in this change in viral gene transcription.

Although the complete mechanism for stimulation of late viral gene expression has not been defined, it is known that transcription of late genes increases upon viral DNA synthesis (193), that the alteration in the viral DNA template is cis-acting (359), and that the viral proteins ICP4, ICP27, and ICP8 are required. The cis-acting effect on the template could be due to changes in the viral DNA molecules themselves by exposure of single-stranded regions or by conversion from a circular form to a linear form. Alternatively, the cis-acting effect could be due to proteins tightly bound to the viral parental DNA that do not exchange to other DNA molecules in the infected cell.

As a general rule, while sequences upstream of transcription initiation are sufficient to endow a reporter gene with the kinetics of b gene expression both in transfected cells and in the context of the viral genome (94,666), this is not generally true of g genes (359,565). In the context of the viral genome, expression of reporter genes as g_2 genes also requires regulatory elements present in the 5'-transcribed noncoding domains (229). Even then, such chimeras are expressed as b genes in cells transfected with the chimera and then superinfected with the virus (565).

The HSV g_1 promoters have not been studied as extensively as some of the other viral promoters, but the *ICP5* (*U_L19*) gene promoter elements have been defined (see Fig. 6). The essential elements of the minimal *ICP5* promoter are an SP1-binding site at –48, a TATA box at –30, and and an essential cis-acting element between –2 and +10 whose sequence resembles the human immunodeficiency virus (HIV) initiator element (238). A cellular factor that binds at the cap site has been identified (237). The g_1 gene promoters are heterogeneous in that the minimal *VP16* gene promoter from base pair –90 to +6 contains an E Box (CACGTG) at –85, a CAAT box at –77, and an SP1 site at –48 as well as a different initiator element (325).

Analyses of the g_2 viral gene promoters have shown that the upstream sequences consist of a TATA box with few other upstream transcription factor–binding sites (163,228,229,257) and with additional sequences needed for activation within the 5'-untranslated region (200,229,359,601,667). For example, the g_2 *U_L38* gene promoter (see Fig. 6) contains three elements: (a) an unusual TATA element with the sequence TTTAAA at –31, (b) a consensus initiator element at the transcriptional start site, and (c) a downstream activation sequence (DAS) from base pair +20 to +33 that is required for normal levels of gene expression (200). The DAS appears to increase transcriptional initiation (199), and several other HSV g genes, including *U_S11, gC (U_L44), gB (U_L27), L/ST,* and *U_L49.5*, have similar downstream control elements in their promoter (reviewed in 199). In addition, the *gC* gene DAS can partially substitute for the *U_L38* DAS (199), suggesting that common mechanisms may act on the different DAS sequences. One cellular protein purified on the basis of binding to the DAS has been identified as the DNA-binding subunit of the DNA-dependent protein kinase (440). Given that HSV infection leads to the degradation of the catalytic subunit of DNA-PK and loss of kinase activity (313), this effect may free up the DNA-binding subunit for interaction with viral DNA.

In addition to ICP4, g gene expression requires ICP27 (489,527). As described previously, ICP27 stimulates expression of viral DNA replication proteins and thereby viral DNA synthesis (639), but it is also specifically required for g gene expression (489). The mechanism by which ICP27 stimulates g gene expression has not been completely defined because there is evidence that its effects may be both transcriptional and posttranscriptional. Posttranscriptional effects of ICP27 were initially inferred from its stimulation of reporter gene expression in transfected cells in which the stimulation appeared to be dependent on sequences in the 3'-untranslated region of the reporter gene (72,535). Similarly, a late polyadenylation factor (LPF) was reported to be dependent on ICP27 (370). In infected cells, g viral transcription was not inhibited when cells infected with the ICP27 ts mutant *ts*LG4 were shifted to the nonpermissive temperature (575). Based on this result, it was concluded that ICP27 stimulated late gene expression by posttranscriptional mechanisms. Nevertheless, the *ts*LG4 ICP27 may not be thermolabile for transcriptional functions upon shift to the NPT.

In addition, ICP27 was shown to bind to RNA (46,242,377), and one report indicated sequence specificity in this binding activity (46), whereas others failed to observe this specificity (242,377). A portion of the nuclear ICP27 can shuttle from the nucleus to the cytoplasm in infected cells (376,442,579). This activity requires an RNA-binding sequence, an export sequence, and an export control sequence on ICP27 (580). A correlation has been reported between the ability of ICP27 to shuttle from the nucleus to the cytoplasm and its ability to stimulate gene expression, suggesting that ICP27 stimulates gene expression by shuttling late mRNAs from the nucleus to the cytoplasm (579).

Not all studies, however, support the hypothesis that the effect of ICP27 on viral gene expression is solely a consequence of posttranscriptional effects. ICP27 has been shown to bind to ICP4 (426), to alter the phosphorylation of ICP4 (485,613,687), and to localize to replication compartments (115). These properties are all consistent with effects on transcription of late HSV genes.

The ability of ICP27 to promote g viral gene expression is likely a distinct function derived from its ability to stimulate viral DNA synthesis because at least one mutant virus, *n*504, separates these two functions. This mutant is defective for g gene expression, in particular g_2 proteins, while maintaining normal levels of viral DNA synthesis (486). This unique mutant virus has been used further to demonstrate that ICP27 stimulates transcription of g genes independently of its role in stimulating b gene expression and viral DNA replication (252). This study found that ICP27 promotes transcription of at least two g_2 genes, *U_L44* and *U_L47*, in infected cells, as assayed by *in vivo* pulse labeling of RNA, without any apparent effects on transport or stability of these transcripts.

ICP27 has also been reported to stimulate the use of weak polyadenylation sites (365) and downstream polyadenylation

sites (203). Thus, ICP27 appears to have roles in transcription, processing, and transport of late mRNAs in infected cells, a multifunctional role similar to the HIV Rev protein. Interestingly, ICP27 has been reported to interact with the hnRNP K protein, which also plays a role in both transcriptional and RNA processing and transport events, and with casein kinase 2 (649). ICP27 may stimulate late transcription or RNA processing by binding to nascent RNA transcripts and promoting transcriptional elongation like HIV Tat protein or by altering the phosphorylation of viral and cellular proteins involved in transcription and RNA processing and transport.

ICP8 has also been found to stimulate g gene expression from progeny DNA templates (76,177). This ability to stimulate late gene expression correlates with the ability of the virus to form large replication compartments in infected cells (374). Thus, transcription of g_2 genes such as *gC* may require the movement of viral progeny DNA to new sites in the nucleus in a process promoted by ICP8.

Finally, the accumulation of a subset of g_2 proteins exemplified by U_S11, U_L41, and U_L38 is significantly reduced in the absence of the carboxyl-terminal portion of ICP22 or $U_S1.5$, or U_L13 proteins (421,452,471). The same two proteins, $U_S1.5$ and U_L13, are required for degradation of cyclins A and B and for the activation of cdc2. The connection may be a requirement for cdc2 for the expression of U_S11, and so forth, inasmuch as in cells transfected with dominant negative cdc2 and infected with wild-type virus, a, b and g_1 proteins are expressed, but U_S11 is not (6).

The ultimate goal of viral late gene expression is to express large amounts of viral structural proteins for assembly of progeny viral particles.

Viral Capsid Assembly

After synthesis of the g capsid proteins, they are localized into the infected cell nucleus, where capsid assembly occurs. Empty shells containing an internal scaffolding are assembled first, and the scaffolding is lost upon DNA encapsidation or insertion of viral DNA into the capsid. Our knowledge about the mechanisms of assembly of the HSV capsid has come from several lines of experimentation: (a) study of infected cell complexes and structures, (b) assembly of capsid structures from extracts of insect cells infected with baculoviruses expressing HSV scaffolding proteins, (c) study of protein localization using immunofluorescence, and (d) genetic analysis of the functions of capsid and scaffolding proteins. Each of these approaches has contributed important information to our understanding of this process (reviewed in 227 and 604).

Immunofluorescence studies have shown that the initial stages of assembly of at least some capsid proteins occur in the cytoplasm (412,491). VP5, the major capsid protein, VP26, the outer tip of hexons, and VP23, a triplex protein are incapable of nuclear localization on their own, but VP5 can be carried into the nucleus by VP19C, a capsid triplex protein, or pre-VP22a, a scaffolding protein. VP23 localizes into the nucleus with VP19C, consistent with their interaction in triplexes, and VP26 localizes into the nucleus only when it is expressed with both VP5 and VP19C or pre-VP22.

Electron microscopic studies have shown that the final assembly of capsids occurs in the nucleus. Three types of capsids, called *A-, B-,* and *C-capsids,* have been identified from infected cell nuclear extracts by sucrose density gradient ultracentrifugation (189). All three types of capsids are about 120 nm in diameter, with an outer shell consisting of hexons and pentons made up of VP5, the major capsid protein. The capsomers are linked by triplex structures composed of the two minor capsid proteins VP19C (U_L38) and VP23 (U_L18), each complex consisting of one molecule of VP19C and two molecules of VP23 (409). C-capsids contain viral DNA and can mature into infectious virions by budding through the nuclear membrane (434). A- and B-capsids lack DNA, but B-capsid cavities are filled with VP22a and VP21, the cleaved forms of the scaffolding protein, and a viral protease, VP24 (189,404). The internal proteins, including the scaffolding proteins and protease, are removed upon encapsidation of DNA (111,189). A-capsids are not filled with DNA or scaffolding protein and are believed to be abortive forms that result from failed attempts to package DNA.

In the nucleus, the VP5–pre-VP22a complexes come together as a result of self-interactions of pre-VP22a molecules. The triplex proteins are then added to form a partial capsid. As hexons and pentons are added, the partial capsid assembles into a round procapsid (406). The procapsid structures undergo a structural transformation and become angular and polyhedral (88,630,636). It is controversial whether the round or the polyhedral B capsids are the structure into which viral DNA is encapsidated (312,435,636). Much of our knowledge of the capsid assembly process has come from analysis of structures formed in insect cells infected with baculoviruses expressing HSV capsid or scaffolding proteins (621,631) and in extracts from these infected cells (408). In these studies, a series of baculovirus recombinants were constructed that each express one capsid or scaffolding protein, and B-capsids with normal structure were formed when insect cells were co-infected with viruses encoding VP5, VP19C, VP23, and U_L26 or $U_L26.5$ gene products. When the U_L26 and $U_L26.5$ gene products were left out, no intact capsids were formed, indicating that these gene products are needed to form a scaffold structure around which the shell proteins could form a closed capsid. Similar phenotypes were observed with HSV strains in which the U_L26 and $U_L26.5$ genes were mutated (124). In the baculovirus studies, when the U_L26 gene was omitted, large cored B-capsids were observed because in the absence of the maturational protease, the $U_L26.5$ protein is not cleaved. Thus, cleavage of the $U_L26.5$ scaffolding protein leads to condensation of the core. When the $U_L26.5$ gene was omitted, intact capsids were observed, but no core structure was apparent. Viruses mutated for $U_L26.5$ can still produce infectious virus, but progeny virus yields are reduced by 10^2- to 10^3-fold relative to wild-type virus (178). These data indicate that the U_L26 gene products can serve as scaffolding proteins, but they form a different core and one that is not as effective as cores containing $U_L26.5$ gene products.

In vitro assembly of B-capsids can occur in extracts when the insect cell extracts from cells individually infected with baculoviruses expressing VP5, VP19, VP23, U_L26, and $U_L26.5$ gene products are mixed and incubated (406). *In vitro* assembly of procapsids requires only the purified viral components VP5, VP19C, VP23, and a scaffolding protein; hence, assembly of procapsids does not require any cellular proteins (407). It has been hypothesized that procapsids may be

involved in the early stages of viral DNA packaging, much like the prohead structures of bacteriophages (227).

Although the other capsid proteins are encoded in separate transcriptional units (see Table 1), the scaffolding proteins and maturational protease are encoded by the overlapping *U_L26* and *U_L26.5* genes, which encode a complex set of gene products involved in formation of a core for capsid assembly and for capsid maturation (331,333,334,462). The *U_L26* gene encodes a 635–amino acid residue precursor protein with an intrinsic protease activity that cleaves either autoproteolytically or in trans after residues 247, the R site, and 610, the M site, in the precursor molecule. The amino-terminal 247-residue fragment is VP24, which retains protease activity. The fragment from residues 248 to 610 is VP21, which can serve as a scaffolding for capsid assembly. The *U_L26.5* gene mRNA initiates within the *U_L26* gene and encodes a protein that is read in the same reading frame as U_L26. The U_L26.5 protein, pre-VP22a, is equivalent to the C-terminal 329 residues of U_L26 and is also subject to cleavage by the U_L26 protease at the M site near its C-terminus to give a 304-residue protein, which is known as VP22a. The pre-VP22a protein functions as the major scaffolding protein for capsid assembly. The carboxyl-terminal 14 residues of pre-VP22a (and VP21) are recognized by VP5 during capsid assembly (233).

The protease activity encoded by the *U_L26* gene is a serine protease (332) whose activity is required for virus assembly (178). Cleavage at the R site is required for viral infectivity (496), and cleavage at the M site is believed to be involved in release of the scaffolding protein from the capsid interior. Mutagenesis studies have identified two histidines and two glutamic acid residues essential for proteolytic activity, but a conserved cysteine was dispensable in the 247–amino acid polypeptide (331,332). Proteases have been targets for drug development in many biologic systems, and considerable effort has been devoted to identifying specific inhibitors of the HSV protease as possible antiviral compounds. The crystal structures of the HSV-1 and HSV-2 proteases have been solved (234).

Assembly of capsids may take place within specialized infected cell nuclear structures called *assemblons* (403,658), or they may be assembled within replication compartments at sites near the sites of viral DNA replication (85,88,114,309,373). However, there is more general agreement that the next step in viral replication, encapsidation, occurs in replication compartments (15,309,658).

Encapsidation of Viral DNA

Encapsidation of HSV DNA involves a process in which cleavage of HSV progeny DNA concatemers into unit-length monomers and packaging of the monomers is linked, as originally shown for pseudorabies virus (304,305). This process is not well defined, but the concatemeric progeny molecules are likely fed into the capsid concomitant with displacement of the scaffolding molecules, VP21 and VP22a, from the capsid. The viral DNA concatemer is believed to be cleaved upon encapsidation of a length of DNA that fills the capsid or when a “headful” (a term that originated with bacteriophage head assembly) of DNA has been inserted. Varmuza and Smiley (645) first mapped signals for cleavage and packaging within the U_b and U_c domains of the *a* sequences, and Deiss and colleagues (118) mapped these precisely and designated the two DNA packaging elements as *pac1* and *pac2,* respectively.

Concatemers are cleaved into unit-length molecules during packaging (118,262,335,445,559,582,608,609,645). Cleavage occurs site specifically within the DR1 sequences of the *a* sequences (388) and involves two site-specific breaks at defined distances from the *pac1* and *pac2* packaging signals (118,571,645). The maturational process duplicates the sequences between these two cleavage sites, so that cleavage at sites bearing only one *a* sequence leads to two molecules with terminal *a* sequences (119,571,645). Several models have been proposed to explain the cleavage and metabolism of viral DNA during encapsidation (118,645). In general, these models propose that a packaging complex binds to the DNA and scans for a U_c sequence (Fig. 9). Cleavage occurs at a DR1 element proximal to the U_c sequence, and then the packaging complex scans the DNA as it is packaged until a directly repeated junction is encountered. Alternatively, two cleavages at L- and S-termini produce a monomer molecule. The amplification of the *a* sequence may occur by staggered nick-repair or gene conversion. The terminal *a* sequence generated by the cleavage may be recombinogenic in either a single-stranded or double-stranded form and promote inversions or *a* sequence amplification. In any event, the process must yield the virion form of the genome with an L-terminus containing one or more *a* sequences and a 3' single-base overhang, a unit-length genome with an L and S component, and an S-terminus containing a single *a* sequence and a 3' single-base overhang.

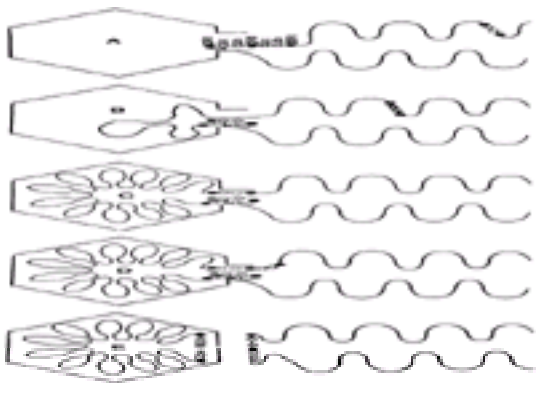

FIG. 9. Packaging of HSV-1 DNA. This general model predicts that (a) proteins attach to components of the *a* sequence, probably U_c; (b) empty capsids scan concatemeric DNA until contact is made in a specific orientation with the first protein-U_c sequence (capsid A); (c) the DNA is then taken into the capsid B until a “headful” or contact is made with a sequence whose nucleotide arrangement is in the same orientation (i.e., one genome equivalent in length away) is encountered (capsid C); (d) the packaging signal requires nicking of both strands from signals on opposite sites of a DR1 sequence. In the absence of two adjacent *a* sequences (capsid D), the juxtaposition of the *a* sequences would result in duplication of the *a* sequence (capsid E). (Model based on refs. 118 and 645.)

Encapsidation of viral DNA requires several viral gene products, including the *U_L6, U_L15, U_L25, U_L28, U_L32, U_L33, U_L36,* and *U_L37* gene products, but the mechanisms of this process remain to be defined. Several biochemical activities that may be involved in this process have been identified, including virus-encoded proteins that bind to the *pac2* site (79) and a virus-encoded endonuclease that introduces double-stranded cuts in the U_c domain of the *a* sequence (135,679).

Virion Assembly and Egress

After encapsidation of full-length viral genomic DNA molecules, the nucleocapsids are capable of budding through the inner nuclear membrane (648). Interactions between capsid and tegument proteins and between tegument proteins and viral glycoproteins in the inner nuclear membrane promote this budding process. It is clear that nucleocapsids acquire a tegument layer upon budding through the inner nuclear membrane, although the tegument of virions in the internuclear membrane space appears less dense than that of extracellular virions (184,596). This apparent change in the tegument during egress of the virions may be due to maturation or exchange of tegument layers, as discussed later. Some tegument proteins play essential roles in the assembly process. VP16 has been reported to be required for envelopment at the inner nuclear membrane (665) or for another step downstream of nuclear envelopment (397). In addition, the *U_L11* gene product increases the efficiency of envelopment (17,349).

Knowledge of the localization patterns of the tegument proteins could be informative as to the site of tegument assembly, but thus far, the results on tegument protein localization are complex. VP16 has been reported to localize to the cytoplasm and the nucleus (141) and at early times to the nucleus in diffuse patterns or replication compartments and later times also to a perinuclear distribution (303). Thus, VP16 could be available for nuclear or cytoplasmic assembly of tegument structures. The potential role for VP16 in replication compartments remains to be explored. The localization patterns of the VP22 tegument protein also show complex results, but this protein has displayed some novel properties. In some studies VP22, was shown to localize primarily to the cytoplasm in a perinuclear pattern in infected cells (141), whereas others have observed significant accumulations of VP22 in nuclei (451). The latter study found that nuclear VP22 is phosphorylated. Thus, VP22 could also assemble into tegument structures in either the nucleus or cytoplasm. Interestingly, VP22 has the property of intercellular transport in transfected and infected cells (142). VP22 is found in the cytoplasm of the cell where it is synthesized, but it is localized to the nucleus of the surrounding cells where it traffics. Intercellular trafficking occurs by a nonclassic, Golgi-independent mechanism apparently involving microfilaments. The function of this unique property in viral infection is not known, but applications of this property to delivery of proteins and other molecules inside of cells are being actively sought (127).

The route for egress of the virion particle from the space between the inner and outer nuclear membranes to the exterior of the infected cell is controversial. Two general pathways have been hypothesized for virion egress (Fig. 10), originally based on electron microscopic evidence. In pathway A, often called the *reenvelopment pathway,* the enveloped particle fuses with the outer nuclear membrane, resulting in deenvelopment of the nucleocapsid and its entry into the cytoplasm. The nucleocapsid then buds into the trans-Golgi network, and the enveloped particle is then released through secretory vesicles. The early electron microscopic evidence for this pathway came from observations of naked nucleocapsids in the cytoplasm of cells infected with HSV or a frog herpesvirus (566,596). In pathway B, called the *lumenal pathway* by Enquist and associates (144), the enveloped particle moves through the cytoplasm in the lumen of the endoplasmic reticulum (ER) or in vesicles either to the trans-Golgi network or Golgi vesicles where final maturation of the virion glycoproteins occurs. The early electron microscopic evidence for this pathway was that cytoplasmic viral particles largely showed enveloped particles in vesicles (109) or in tubular membrane structures (545). Further evidence interpreted in favor of this pathway came from studies involving monensin treatment of HSV-infected cells (256). Monensin, an ionophore that inhibits the secretory pathway, blocked viral egress and caused accumulation of virions in cytoplasmic vesicles. Thus, it appeared that egress involved the secretory pathway. Studies on the lipid content of virions have shown that virions are similar to cytoplasmic membranes rather than nuclear membranes, supporting the reenvelopment model (642); however, as noted earlier, the comparison is between virion envelopes and nuclear membranes of uninfected cells. The assumption underlying this comparison may not be tenable in light of the many modifications known to take place in the nuclear membrane.

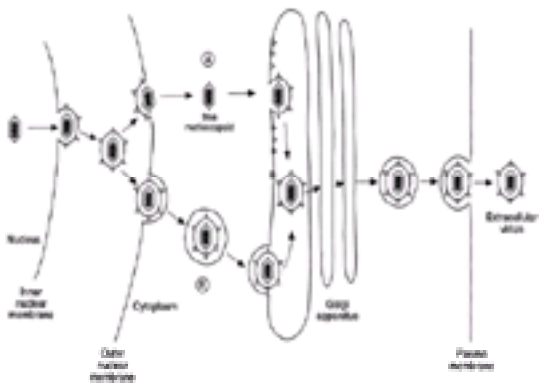

FIG. 10. Diagram of pathways of egress. HSV capsids bud through the inner nuclear membrane, forming an enveloped virion particle. Egress of the virions from the host cell may occur by either of two general pathways. **A:** The envelope fuses with the outer nuclear membrane, de-enveloping the capsid and releasing it into the cytoplasm. The capsid then buds into the Golgi apparatus, forming an enveloped virion, which is transported to the surface by vesicular transport. **B:** The virion particle buds through the outer nuclear membrane and is transported by vesicular movement through the Golgi apparatus to the exterior of the cell.

The major new type of evidence favoring the reenvelopment model comes from genetic studies of virus release in cells infected with viruses expressing viral glycoproteins with novel targeting signals. A virus encoding a mutant glycoprotein H with an ER retention signal released normal numbers of extracellular particles that contained no gH and that were not infectious (48). Similarly, viruses encoding gD molecules with ER retention or ER-retrieval signals produced 10- to 100-fold less extracellular virus, whereas viruses encoding gD molecules with Golgi-retention or Golgi-retrieval signals formed normal amounts of extracellular infectious virus (671). Preliminary results with a viral mutant encoding an ER-retained gD indicate that viral particles within the nuclear membranes contain gD, whereas extracellular virions do not (A. Minson, personal communication). These studies argue that virus acquires its final membrane in a compartment that contains Golgi-targeted gD but from which ER-retrieved gD is excluded. However, the published data have not shown that gD or gH reach the nuclear membranes.

Whatever scheme is eventually shown to be correct, it must take into account two additional observations. First, in many cell types, the Golgi apparatus is fragmented and dispersed, a phenomenon that may be related to rearrangement of the cytoskeleton (59,657). Curiously, the Golgi apparatus is not fragmented in cells infected with *syn*$^-$ mutants. The second observation is that the U_L20 gene product plays an important role in viral egress from the infected cell. In cells in which the Golgi apparatus is fragmented, virions accumulate in the space between the inner and outer nuclear membrane, whereas the glycoproteins are transported to the trans-Golgi network (18).

In addition to spreading from one cell to the next through the extracellular space, HSV can spread directly from cell to cell, likely through cell junctions. The gE and gI glycoproteins are required for this mode of spread (128), possibly because the gE–gI complex can target to cell junctions and mediate movement of virions through these junctions (129).

THE FATE OF THE INFECTED CELL

Viruses require living cells for their replication. The longer the infected cells remain alive, the more progeny viruses are made, and ultimately the more the virus spreads. From an anthropomorphic viewpoint, viruses, unlike most bacteria, benefit from maintaining the cell alive. Cells productively infected with herpesviruses do not survive. Cell death is the result of not only irreparable injury caused by viral replication but also cellular responses to infection. This section deals with changes induced by the virus. Almost from the beginning of the reproductive cycle, the infected cells undergo major structural and biochemical alterations that ultimately result in their destruction.

Structural Alterations

Changes in Host Chromatin

As described in detail elsewhere (509), one of the earliest manifestations of productive infection is in the nucleolus. In infected cells, the nucleolus becomes enlarged, is displaced toward the nuclear membrane, and ultimately disaggregates or fragments. Concurrently, host chromosomes become marginated, and later in infection, the nucleus becomes distorted and multilobed. The numerous protrusions and distortions have in the past been mistaken for amitotic division (263,546). Margination of the chromosomes may or may not be linked with the chromosome breakage reported by numerous investigators (reviewed in 509).

Virus-induced Alteration of Cellular Membranes

Duplication and Folding of Intracellular Membranes

Changes in the appearance of cellular membranes and, in particular, of nuclear membranes are characteristic of cells late in infection. Deposition of material (tegument proteins?) on the inner surface facing the nucleoplasm or cytoplasm, but not in the space between inner and outer lamella or in the cisternae of the ER, results in the formation of thickened patches along the membranes. Ultimately, the patches in the nuclear membrane coalesce and fold upon themselves to give the impression of reduplicated membranes (100,145,314,361,393,413,455,544,562,680).

Fragmentation and Dispersal of Golgi Stacks. HSV also causes a cell-type–dependent fragmentation and dispersal of Golgi vesicles. In cells in which this occurs, the Golgi vesicles fragment into myriad small vesicles that become dispersed throughout the cytoplasm (59). Disruption of microtubules is essential but not sufficient to induce the fragmentation of the Golgi apparatus. (13). In cells in which the Golgi apparatus is fragmented and dispersed, transport of virions from the nuclear membrane to the extracellular space requires the U_L20 gene product (18). In cells in which the Golgi stacks remain intact (e.g., in 143 TK-negative cells and in cells undergoing polykaryocytosis), the U_L20 protein plays a less prominent role in virion transport. In these cells, the Golgi stacks aggregate in what appears to be a central location. The distribution of viral glycoprotein D matches the distribution of the Golgi stacks, suggesting that the aggregated Golgi stacks funnel viral glycoproteins and viral particles to a limited region of the plasma membrane of the polykaryocytes rather than directing exocytic flow in a more dispersed fashion as seen in *syn*$^+$ (wild-type) virus-infected cells exhibiting fragmented and dispersed Golgi (657).

Insertion of Viral Proteins into Cellular Membranes. The first inkling that herpesviruses modify cellular membranes was based on the observation that mutant strains can differ from wild-type strains with respect to their effects on cells. For example, although wild-type viruses usually cause cells to round up and clump together, some mutant viruses cause cells to fuse into polykaryocytes (137,504). These observations led to the prediction that herpesviruses alter the structure and antigenicity of cellular membranes—a prediction fulfilled by the demonstration of altered structure and antigenic specificity (495,512,513,519) and the presence of viral glycoproteins in the cytoplasmic and plasma membranes of infected cells (217,517,518,587).

Polykaryocytosis. Both HSV-1 and HSV-2 cause infected cells to round up and adhere to each other. Some viral mutants

cause cells to fuse into polykaryocytes, and this fusion may be cell-type specific or cell-type independent (137, 225,526). Polykaryocytosis has been studied for several reasons: (a) as a probe of the structure and function of cellular membranes reflected in the “social behavior of cells,” (b) as a tool for analyses of the functions of viral membrane proteins, and (c) as a model of the initial interaction between HSV and susceptible cells that results in the fusion of the viral envelope and the plasma membrane (60,504,583,584). Cell fusion induced by HSV also requires conditions that favor processing of high mannose glycans to complex glycans, but in this regard, it is not clear whether complex glycans must be present only on viral glycoproteins present on the surface of the infected recruiter cells, or on both the recruiter cells and on the uninfected cells being recruited into polykaryocytes (60,583,584).

Polykaryocytosis can be viewed as an aberrant manifestation of the interaction of altered membrane domains of infected cells and unaltered membranes of juxtaposed cells (504). Genetic analyses have shown that syncytial (*syn*) mutations that confer the capacity to fuse cells map in at least five and possibly more loci within the viral genome (16,41,117,330,350,447,449,523,526,689). These loci map within the domains of the *gB, gK, gL, U_L24*, and *U_L20*. One interpretation of these observations is that the membrane proteins form complexes whose structure and conformation become altered by mutations in any of the component polypeptides, and that the changes in the conformation are similar to those that occur in the envelopes of virions interacting with the plasma membrane (526).

Rearrangement of the Microtubular Network

Changes in the microtubular network are apparent very early in infection. Thus, the microtubules at the junction of the network with the plasma membrane appear to be disrupted in cells exposed to either *syn^+* (wild-type) or *syn^-* virus either in the presence or absence of cycloheximide. The overall arrangement of microtubules, however, is similar to that of uninfected cells. Late in infection, microtubules rearrange to form parallel bundles surrounding the nucleus. In polykaryocytes induced by the virus, microtubules form parallel bundles extending along the axis of recruitment of new cells (657).

Formation of Intranuclear Inclusion Bodies

Electron microscopy of herpesvirus-infected cells showed electron-translucent intranuclear inclusions surrounded by marginated and compacted cell chromatin (108,545) (see Fig. 9 in Chapter 7). Light microscopic observation of nuclear inclusions shows an hourglass appearance of the inclusions at early times and an eosinophilic staining at later times (402,576). Immunofluorescence experiments using antibodies specific for HSV DNA replication proteins have shown that viral DNA replication proteins accumulate in intranuclear foci by 3 hours after infection and that these foci enlarge into globular nuclear structures called *replication compartments* (112,475). The replication compartments are likely to be equivalent to (a) the translucent nuclear inclusions seen by EM and (b) the early nuclear inclusions seen by light microscopy.

These replication compartments are located in the interior of the nucleus as shown by EM (108,545) and by confocal microscopy (113). Viral DNA synthesis, late gene transcription, capsid assembly, and DNA encapsidation may all occur within these nuclear factories. Thus, the accumulation of replication proteins, progeny viral DNA, and nucleocapsid components within these replication compartments may cause the nuclear cytopathic effect described as *nuclear inclusion bodies.*

Host Macromolecular Metabolism

Background

It has been known for many years that HSV shuts off host RNA, DNA, and protein synthesis rapidly after infection (511). Thus, host DNA synthesis is shut off (511), host protein synthesis declines rapidly (506,614,615), and glycosylation of host proteins ceases (572). Host macromolecular metabolism is altered in infected cells in at least four different ways: (a) mRNA present in infected cells at the time of infection is degraded, and this degradation continues for at least several hours; (b) transcription appears to be turned off; (c) cellular proteins are selectively degraded or stabilized; and (d) cellular proteins are redirected to perform novel tasks.

Shutoff of the Synthesis of Cellular Products

HSV host shutoff occurs in two stages: degradation of host mRNA and inhibition of further synthesis and processing of host mRNAs.

Virion Host Shutoff and Degradation of RNA

The existence and properties of a virion lost shutoff (*vhs*) function were documented in several stages. The first stage, documented initially by Fenwick and Walker (160) and by Nishioka and Silverstein (414,415 and 416), involves structural proteins of the virus and does not require *de novo* protein synthesis. Thus, HSV shutoff of host protein synthesis occurs in physically or chemically enucleated cells (158). Furthermore, the shutoff was effected by density gradient purified virus but not by purified virus inactivated by heating or neutralization with antibody. The shutoff is faster and more effective in HSV-2– than in HSV-1–infected cells, and this observation permitted the initial mapping of the genetic locus that confers upon HSV-1 × HSV-2 recombinants the accelerated shutoff characteristic of HSV-2 (157).

The second stage began with the isolation of *vhs^-* mutants that failed to shut off host polypeptide synthesis in HSV-infected cells (483). Viral mutants of HSV-1 that were defective for shutoff of host cell protein synthesis were identified. The *vhs* function was initially mapped to 0.52 to 0.59 map units (157). Isolation of a mutant defective in this function (483) allowed further mapping of the gene responsible. Mapping studies by Oroskar and Read (425) and Kwong and colleagues (301) have identified sequences mapping from 0.604 to 0.606 map units on the viral genome (*U_L41* ORF)

as being responsible for the *vhs*⁻ phenotype of the mutants. *U_L41* RNA is expressed as a g_1 gene (170). The products of *U_L41* are an abundant phosphoprotein of Mr 58,000 and a less abundant, more extensively phosphorylated protein of apparent Mr 59,500. Only the faster, migrating protein is found in virions (484). Conclusive evidence that vhs acts in the absence of other viral proteins emerged from studies of Zelus and associates (698), but the question of whether it acts directly as a ribonuclease or indirectly to activate a cellular nuclease remains unresolved. Virions have a ribonuclease activity that is inactivated by anti-vhs antiserum (698). The observation that vhs has weak sequence similarity to the fen-1 family of nucleases suggested that it has intrinsic nuclease activity. Consistent with this view, Elgadi and associates (138) reported that the vhs protein induces endoribonucleolytic cleavage of mRNAs *in vitro,* whereas Karr and Read (264) noted that vhs causes the degradation of the 5' end of mRNAs before that of the 3' end. Recently, it has been reported that vhs interacts with the translation factor eIF-4H and that this interaction is essential for the RNase activity and perhaps for targeting of vhs to polyribosomes for selective degradation of mRNAs (S. Read, personal communication; J. Smiley, personal communication). The complex appears to cause the decapping of cellular mRNAs from the 5' terminus.

In cells infected with a *vhs*⁻ mutant, host protein synthesis is not shut off, at least early in infection, and the duration of a and b protein synthesis is prolonged compared to wild type. Both of these effects have been shown to be due to a stabilization of host and viral mRNAs in cells infected by *vhs*⁻ mutants; thus, vhs protein accelerates the degradation of both cellular and viral mRNAs (300,425) and serves to inhibit translation of cellular mRNA. Combined with the viral effects of inhibition of host transcription and RNA splicing, the cellular mRNA pools are not replenished, and as viral mRNA accumulates, it is preferentially translated. It has been suggested that vhs serves to facilitate the transitions from a to b to g protein synthesis by shortening the life of viral mRNA (300,425). As infection progresses, the degradation of RNA by vhs appears to be reduced or blocked altogether. The most direct observation in support of this conclusion is that the half-lives of viral mRNAs increase with time after infection (159). A compelling rationale for the turnoff of vhs function is that the vhs protein accumulates in appreciable amounts late in infection. If the nascent protein were functional, it would be expected that it would totally degrade viral mRNA concurrent with its accumulation in the infected cell. VP16 and vhs interact (570), and this interaction blocks the degradation of RNA by vhs (308). Thus, assembly of vhs into tegument complexes not only allows the virion to bring vhs into the cell and inhibit host translation but also serves to modulate the activity of vhs at later times of infection when mostly viral mRNA is present in the cell. The conditions required for the dissociation of vhs and VP16 proteins at the outset of infection remain to be defined.

Shutoff of Transcription

To facilitate the transition from cellular to viral gene transcription, HSV infection causes a rapid decrease in host RNA synthesis (209,654). Transcription by all three cellular RNA polymerases declines to less than 50% of uninfected cell levels by 4 hours postinfection (460). Repression of host cell poll II transcription requires multiple HSV genes, the specific gene dependence varying with the cellular gene (593). HSV infection causes changes in the phosphorylation of RNA pol II (593) in some cell lines, and this effect on the host cell requires ICP22 and U_L13 protein kinase. However, the relationship of this change in pol II to inhibition of host gene transcription and promotion of viral gene transcription remains to be defined.

Inhibition of Splicing of Messenger RNA

Early in infection, some of the intranuclear ICP27 localizes to spliceosomes, causing a redistribution of host snRNPs (441,534). Two cellular snRNP proteins, U1 and a 70-kd protein, coprecipitate with ICP27, and ICP27 causes an increase in their phosphorylation (533). Phosphorylation of U1 protein impairs its ability to stimulate RNA splicing. Thus, this effect of ICP27 may contribute to its ability to inhibit RNA splicing. This effect on splicing has little or no effect on viral RNA synthesis because three of the genes encoding spliced RNAs are a genes (a *0*, a*22*, and a*47*). Late in infection, ICP27 is localized to replication compartments and shuttles to the cytoplasm, and at this stage, it may no longer repress RNA splicing, allowing U_L15 RNA to be spliced.

Selective Degradation of Cellular Proteins

Destruction or posttranslational modification of cellular protein is implied by the changes in microtubular network, nucleoli, mitochondria, Golgi apparatus, ND10 structures, and so forth. Recent studies have shown that HSV causes both accelerated destruction and stabilization of cellular proteins.

Accelerated destruction of specific proteins in infected cells was described earlier. These included PML, the kinetochore protein CENP-C (149), and cyclins A and B (4). The destruction of CENP-C is mediated by ICP0, whereas the destruction of cyclins A and B requires two functions, that of ICP22 and of U_L13 protein kinase (5). Additional evidence for selective destruction of cellular proteins is likely to emerge given the increasing prominence of viral–cellular protein interactions in current research. What differentiates normal turnover of cellular proteins from selective degradation is evidence that specific viral proteins mediate this degradation.

Selective Stabilization and Activation of Cellular Proteins

As noted earlier, ICP0 stabilizes cyclin D1 and D3 and activates cdc2 cell cycle kinase. The stabilization of cyclins D1 and D3 is mediated by ICP0 (269,644). Thus, wild-type ICP0 binds cyclin D3 and stabilizes it. Mutants lacking ICP0 or carrying an alanine substitution in place of aspartic acid 199 fail to bind cyclin D3 and are less pathogenic when administered to mice at a peripheral site (643). The D199A mutation is pleiotropic in the sense that both cyclin D3 and cyclin D1 are destabilized and degraded within a few hours after infection (643). Moreover, the D199A mutant is not transported from the nucleus into the cytoplasm (644). The stabilization of cyclin D3 and D1 is surprising in light of the observation that cdk2 is inactive (136). The pRB protein is hypophosphorylated, E2F-pRB and E2F-p107 complexes accumulate, E2F-dependent gene expression decreases, and E2F proteins are posttranslationally modified and inactive (5,6,136,223,423,581), all suggesting that S-phase genes are unlikely to be activated.

The hypothesis that HSV uses cyclins D1 and D3 for purposes other than to induce S phase is supported indirectly by another observation. Thus, 5 to 9 hours after infection, cyclins A and B are destroyed, but their catalytic partner, cdc2, is activated (5), prompting the question of how catalytic subunit regulation occurs in the absence of the normal cyclin subunit. In addition, cdc2 is required for the expression of a subset of g_2 genes exemplified by *U_S11* (6). More recent studies indicate that cdc2 can phosphorylate ICP0 and ICP22 *in vitro* and in the context of infected cells (S. J. Advani and B. Roizman, manuscript in preparation). Activation of cdc2 is mediated by ICP22 and the U_L13 protein kinase.

The conscription of cdc2 to phosphorylate viral proteins is not an isolated phenomenon. As noted elsewhere in this chapter, the $g_1$34.5 protein binds and redirects protein phosphatase 1 to dephosphorylate eIF-2a (215). As analyses of viral–cellular protein interactions progress, many more examples of diversion of cellular proteins to meet viral needs will emerge. Equally significant, posttranslational modifications of cellular proteins are also likely to be commonplace as more and more cellular proteins are studied in depth in the context of infected cells. The distinction between specific and nonspecific modification will depend on the viral genes that are involved in the process and evidence that they play a role in viral gene expression. A case in point is the phosphorylation of translation factor EF-1d in infected cells by the U_L13 protein kinase. The specificity of this reaction was reinforced by the observation that EF-1d is hyperphosphorylated in cells infected with representative alphaherpesviruses, betaherpesviruses, and gammaherpesviruses and that in cytomegalovirus (CMV)-infected cells, the phosphorylation of EF-1d is mediated by the U_L97 protein, the CMV homolog of U_L13 protein kinase (268). An additional example of the activation of cellular kinases is the activation of p38 and JNK stress-activated kinases, which augments viral yield (372,697).

Effects of Herpes Simplex Virus Infection on the Cell Cycle Machinery

HSV infection causes several changes in the cell cycle machinery. First, it causes a block in the cell cycle in either the G_2 (96) or the G_1 (112) phase of the cell cycle. The G_1 phase block is distinct from the viral inhibition of cell DNA synthesis because early to mid G_1 phase events are blocked, including pRB protein phosphorylation and induction of cyclins and cyclin-dependent kinases (136,581). The net effect of HSV infection on cells is to lower the level of G_1 phase–specific functions, including cyclin D3 protein levels (581) and cdk2 kinase activity (136). This is interesting in light of several reports indicating that HSV infection requires cyclin-dependent kinases (539,540 and 541). Clearly, we have much more to learn about the interactions of HSV with the cell cycle machinery.

VIRULENCE

Background

During infection of humans, viral disease includes primary and recurrent epithelial lesions as well as disseminated disease and encephalitis. In studies on the molecular basis of disease induced by HSV, the end point of the research objective—disease—is often taken to be synonymous with the destruction of central nervous system (CNS) tissue. In healthy, immunocompetent humans, encephalitis occurs rarely but with catastrophic results (672). In experimental animals, it is frequently a major component of the disease. Neurogrowth, the ability to grow in nervous system tissue, as measured solely by intracerebral inoculation of virus, is the most commonly measured aspect of virulence. Direct injection of virus into the CNS measures the capacity of the virus to destroy an amount of CNS tissue that will result in death before the immune system blocks further virus spread. Because in most instances destruction of the CNS and death are related to virus multiplication, in quantitative terms, the growth of the virus in the CNS is measured in terms of the amount of virus required to reach a specific level of mortality (50% of inoculated animals).

A second attribute of virulence is invasiveness—the capacity to reach a target organ from the portal of entry. To disseminate to the target organ, it is necessary for the virus to multiply at peripheral sites. In experimental systems, virulence is composed of (a) peripheral multiplication, (b) invasion of the CNS, and (c) growth in the CNS. Peripheral growth and invasiveness into the CNS can be quantified by measuring the amount of virus recovered at the peripheral site and in the CNS as a function of the quantity of virus inoculated at a peripheral site (e.g., footpad, eye, ear).

The sum total of events defined under the heading “virulence” reflects two distinct sets of viral functions. The first comprises viral genes responsible for access to and injury of the cells whose destruction is responsible for the disease. The second, less well appreciated, are viral genes and gene functions that turn off host responses to infection.

Viral Functions that Contribute to Viral Invasiveness and Replication

Several types of studies have been done to define genes required for neurovirulence or neuroinvasiveness. They may be summarized as follows:

1. An important question is whether the virus that causes adult encephalitis is different from that which establishes recurrent labial infections. Viral isolates taken either from CNS tissues of encephalitis patients, from facial or genital isolates of the same patients, or from normal individuals have been compared in mice inoculated intracranially or through peripheral routes with respect to neurogrowth. Although the encephalitis isolates appear to have slightly lower average PFU/LD_{50} values as compared with nonencephalitis strains, no correlation was apparent between encephalitis in humans and neurogrowth or neuroinvasiveness in experimental animals (R. J. Whitley, personal communication). However, as a variant of this approach, infection of neurons in culture has indicated that virus isolates from encephalitis cases are better able to infect and be transported to the cell body than isolates from peripheral lesions (25).
2. Numerous studies have been undertaken to assess the relative abilities of various viral mutants to grow in the CNS after direct intracerebral inoculation. What has emerged from the accumulated data is that so-called neurovirulence factors are not the exception, but the rule. Almost all virus mutants tested to date, including a number of HSV-1 ×

HSV-2 recombinants, many ts viruses, a variety of spontaneous mutants, and myriad recombinant viruses with deletions in one or more genes, exhibit LD_{50} values following intracerebral inoculation ranging from 100- to 100,000-fold higher than those of the parental strains. Experiments using nongenetically engineered viruses mapped loci implicated in “neurovirulence” to a number of regions, including the *tk* gene and sequences around the right terminus of the L component (68,161,249,250 and 251,282,465,524,598,626,627). Recombinant viruses with elevated intracerebral LD_{50} values include those with deletions in the genes encoding ICP22, U_S2, U_S3 protein kinase, U_L13, U_L16, U_L24, gG, gJ, gE, gI, ribonucleotide reductase, thymidine kinase, $g_134.5$, U_L55, and U_L56 (56,379,380,547,664,673; E. Kern, B. Meignier, J. Baines, R. J. Whitley, A. Sears, and B. Roizman, unpublished results). Most of the attenuated deletion mutants listed previously also exhibit impaired growth in peripheral tissues (e.g., cornea) as assayed either by the occurrence of epithelial lesions or by quantitation of infectious virus in the tissues, indicating that the genes deleted are required generally for growth in differentiated or polarized cells, not specifically for growth in neurons or in CNS tissues (44,246,247; A. Sears, B. Meignier, and B. Roizman, unpublished results). In fact, the only genes that have to date been shown *not* to be required for neurovirulence in the murine model are *gC, U_S9*, *U_S10*, *U_S11*, and a*47* (417; R. J. Roller, B. Roizman, unpublished data). Because the function of a*47* appears to be human cell specific (695), it is conceivable that if the *U_S9* to *U_S11* genes share in this property, their function will be inapparent in the mouse.

The conclusion that emerges is that all HSV genes are important in enabling optimal viral replication and viral spread in the CNS. The special importance of some genes is nevertheless obvious. Mutations in *gD* that restrict or expand the host range are likely to have a significant impact on viral pathogenicity. Another, as yet not fully defined function that plays a significant role in viral pathogenesis is that which enables capsid–tegument structures to be transported retrograde in axons leading to sensory neurons or the CNS.

Viral Functions that Block Host Responses to Infection

Of the 84 ORFs identified to date, more than half are dispensable for replication in cells in culture. Some (e.g., thymidine kinase) are not essential in dividing cells but must be present in nondividing cells to enable viral replication. A large number of the “dispensable” genes as well as some “essential” genes, however, appear to target host responses to infection. These genes may be divided into two groups: (a) those that block *de novo* synthesis of cellular gene proteins that may adversely affect viral replication, and (b) those that block the function of preexisting proteins residing in cells after infection. Examples of the former are vhs, the product of U_L41 gene, and ICP27. The former causes degradation of mRNA present in infected cells, whereas the latter mediates an inhibition of transcription and splicing during the initial stages of infection. Included in this list are the other viral gene functions that block transcription of cellular genes discussed earlier in the text. This section concerns viral gene functions that block the activation of proteins preexisting in the cell at the time of infection.

ICP34.5, U_S11 protein, and the RNA-dependent Protein Kinase (PKR)

One of the principal but easily defeated host defenses is PKR. This enzyme resides in small amounts in uninfected cells but its expression is induced by interferon. PKR is activated by some forms of dsRNA. Activated PKR causes the phosphorylation of the a subunit of eIF-2. Phosphorylated eIF-2a causes total shutoff of protein synthesis. In cells infected with a $g_134.5$-negative mutant, protein synthesis is shut off after the onset of viral DNA synthesis concomitant with the phosphorylation of eIF-2a and the appearance of complementary viral RNA capable of self-annealing to form dsRNA (80,248,288). All viruses that cause the synthesis of RNA with extensive secondary structure must block PKR from shutting off protein synthesis. The manner in which HSV does this is quite different from that of other viruses: ICP34.5 binds protein phosphatase 1a and redirects it to dephosphorylate eIF-2a (215). The virus totally ignores the activated PKR. The domain of ICP34.5 responsible for this function resides in the carboxyl terminus of ICP34.5 and exhibits two attributes. First, it contains the motif of the protein phosphatase accessory protein necessary to direct the phosphatase to specific targets. Mutagenesis of two key amino acids within this motif precludes ICP34.5 from causing the dephosphorylation of eIF-2a (214). Second, the carboxyl-terminal domain of ICP34.5 is highly homologous to the corresponding domain of a conserved mammalian protein known as growth arrest and DNA damage 34 (GADD34) protein. The GADD34 domain substitutes for the corresponding ICP34.5 domain in blocking the effects of PKR (213).

Mohr and Gluzman (391) serially passaged a $g_134.5$-negative mutant and obtained a phenotypic revertant. The revertant showed a spontaneous deletion of the coding sequence of a *47* and the adjacent promoter of *U_L11* gene such that the a promoter of a*47* became juxtaposed to the *U_L11* gene. The net consequence was that *U_L11* was expressed earlier in infection (212). Subsequent studies showed that PKR interacted physically with *U_L11 in vitro* and that the capacity of U_S11 protein to block activation of PKR was dependent on the time and order of additional U_S11 protein. Exposure of PKR to U_S11 protein before its activation was effective in precluding the phosphorylation of eIF-2d, whereas activated PKR responded poorly to added U_S11 protein (67).

As noted in Chapter 71, the *$g_134.5$* gene is conserved only in HSV-1, HSV-2, and simian B virus, the implication being that *$g_134.5$* gene is a late acquisition by these herpesviruses. Therefore, the HSV progenitor may have dealt with PKR differently, perhaps by U_S11 protein expressed earlier in infection.

Apoptosis

Several HSV-1 deletion mutants have now been shown to induce classic apoptosis, characterized by chromatin condensation, release of cytochrome C from mitochondria, fragmentation of cellular DNA, vacuolization, and blebbing of the cytoplasm. The mutants shown to cause programmed cell death in at least one cell line include a *4^-*, a*27^-*, *ts*B7 carrying a ts lesion in the U_L36 protein and maintained at the nonpermissive temperature, and *$(gD, gJ)^-$* mutant viruses. Conversely, wild-type HSV-1 blocks apoptosis induced by osmotic shock, thermal shock, Fas ligand, and TNF-a

(11,12,176,255,287,321,564). The salient features of the available data may be summarized as follows:

1. Apoptosis is cell-type dependent. For example, the *d*120 mutant lacking the a*4* gene induces apoptosis in all continuous cell lines (175,176) tested but not in human embryonic lung cells (175,701). *ts*B7 at the nonpermissive temperature induces programmed cell death in Vero cells but not in the human SK-N-SH cells derived from a malignant glioma (176).

   The pathway leading to programmed cell death is also cell-type dependent. For example, the *d*120 mutant induced caspase 3–independent apoptosis in SK-N-SH cells but caspase 3–dependent apoptosis in HEp-2 cells. Recently, a novel caspase-independent pathway mediated by a mitochondrial protein designated *apoptosis-inducing factor* (AIF) was described. In induced cells, AIF is translocated from mitochondria into the nucleus. In induced human embryonic cells, AIF is translocated into the nucleus, but apoptosis does not ensue.

   These observations suggest that different cells react differently to the same stimulus, that mutants differ in the stimuli they induce, and that wild-type virus blocks all viral activators of programmed cell death to achieve its optimal replication.
2. Little is known of the mechanism by which HSV mutants induce apoptosis. It has been reported that HSV degrades Bcl2 (355), and indeed overexpression of Bcl2 in HEp-2 cells blocks apoptosis induced by the *d*120 mutant (174). The delay in the cytopathic effects induced by wild-type HSV-1 suggests that the cytopathic effects observed in cells undergoing productive infection may at least in part be due to apoptosis. Consistent with this view is the observation that the cytopathic effect of *d*120 virus was more extensive than that caused by wild-type virus, whereas mutants lacking both a*0* and a*4* genes were far less cytopathic (531,684).
3. There is also a paucity of data on the mechanism by which HSV blocks apoptosis. One observation of particular interest was that in SK-N-SH cells treated with sorbitol, (osmotic shock) HSV blocked fragmentation of DNA but not activation of caspase 3 or release of cytochrome C (175,176). Viral genes shown to date to block apoptosis are *gD, gJ,* and the protein kinase U_S3 (254,322,700). ICP4 and ICP27 deleted from respective viruses most likely block apoptosis by enabling the synthesis of proteins made late in infection. The *gD* and *gJ* genes were able to block apoptosis only in cells infected with a mutant lacking both genes but not in cells exposed to TNF-a or Fas ligand (703); therefore, they may be necessary and sufficient in some circumstances but not in all systems studied to date.

In essence, cell death appears to be a complex phenomenon resulting in part from injury to the cell and in part from the cell response to infection. A major cause of cell death even in wild-type virus-infected cells may result from exhaustion of mitochondria and ultimately necrosis, if the levels of ATP have been totally exhausted, or apoptosis, if the cell can still react to the injury caused by viral gene products.

Herpes Simplex Virus and the Immune System

York and colleagues (695) were the first to report that HSV blocks the presentation of antigenic peptides by class I major histocompatibility complex (MHC) proteins. As the story unfolded, it became clear that ICP47, a small protein encoded by the a*47* (U_S12) gene binds to the transporter associated with antigen processing (TAP) and blocks the transport of peptides into the ER. ICP47 appears to have a high affinity for human and porcine TAP but not for murine homologs of the transport proteins. It may explain why the anti-HSV response in mice is so much more effective than in humans.

There is no current evidence that HSV blocks presentation by class II MHC proteins.

LATENCY

The ability of HSV to establish a lifelong latent infection in the human host is the most intellectually challenging aspect of HSV biology. The available data support the following sequence of events: The virus enters nerve endings and is transported retrograde to the nucleus of sensory nerves innervating mucosal epithelium (Fig. 11). In latently infected neurons, viral genomes acquire the characteristics of endless or circular DNA (167,381,497,498), and no replicating virus can be detected in the sensory ganglia innervating the site of inoculation. In a fraction of neurons harboring latent HSV, the virus is periodically reactivated. Infectious virus is carried anterogradely to peripheral tissues by axonal transport (97), usually to cells at or near the site of initial infection (65,106,195,502). Depending on several factors, including the host immune status, the reactivation may be asymptomatic or lead to a recurrent lesion, which may vary considerably in severity from punctate lesions that are invisible to the naked eye to severe, debilitating lesions in immunosuppressed individuals.

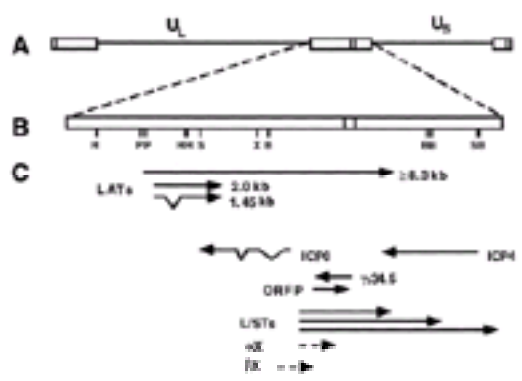

FIG. 11. Stages of HSV infection of the host. 1. HSV is introduced onto a mucosal surface or a break in the skin; it replicates productively in epithelial cells at the site of inoculation and spreads through the tissue. Virus enters sensory neuron axons and is transported to the cell body in a ganglion. 2. Virus either replicates productively or establishes a latent infection in the neuronal cell nucleus. Viral DNA is circular and in a nucleosomal form. Upon neuronal damage or activation, the virus reactivates and undergoes at least a limited productive cycle. Capsids are transported by

anterograde transport to the axonal termini and virions are released. 3. Reactivated virus causes a recurrent infection of the mucosal tissue, causing the shedding of virus.

Viral DNA fragments have been detected in CNS and other tissues; thus, to differentiate between presence of defective genomes and latent infections, the operational definition of latent infections has historically embodied the requirement that wild-type virus must be able to reactivate from latent state under appropriate experimental conditions. Operationally, this meant that virus could be detected after incubation of intact ganglionic tissue with suitable susceptible cells, but not by inoculation of the susceptible cells with homogenized ganglia (597). This definition obviously also included the ability to reactivate and replicate to detectable levels in the ganglia. Because virus has been reactivated from sensory and autonomic ganglia only, this definition restricts viral latency to only these tissues. More recently, the definition of latency was extended to include viruses that can be detected in sensory ganglia several weeks after infection by *in situ* hybridization (Fig. 12) with probes for latency-associated transcripts (described later) or by assays of viral DNA in ganglia (93,266,316,318,345,623). At the present time, the only technique for proving that a virus is incapable of establishing latent infections is the inability to detect viral DNA by PCR assays of DNA extracted from whole ganglia (378a, 478a). A possible new standard is the identification of neurons containing viral DNA by *in situ* PCR (143,600), although the results to date with this technique have been variable (378,478a). This redefinition of latent virus enables an assessment of the roles of specific genes in the establishment and maintenance of latency, separate from their roles in reactivation. The downside of the revised definition is that it equates the presence of viral DNA with a *bona fide* latent state defined biologically as at least capable of reactivation. Because viral DNA has been detected in the CNS and even at peripheral sites, the redefinition arguably extends the sites of viral latency beyond currently proven sites.

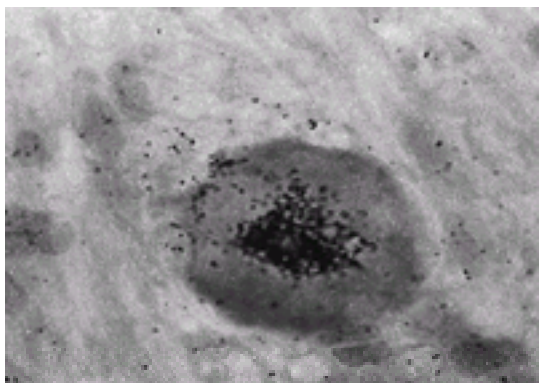

FIG. 12. Detection of HSV stable latency-associated transcript (LAT) RNA in latently infected murine trigeminal ganglion neurons by *in situ* hybridization. Mice were infected with HSV-1 after corneal scarification. At 30 days postinfection, the mice were sacrificed, and trigeminal ganglia were frozen and sectioned. The sections were hybridized with a ^{3}H-labeled DNA probe detecting the LAT RNA as described (93). (Micrograph courtesy of Magdalena Kosz-Vnenchak.)

The clinical aspects of latent infection and reactivation are discussed in Chapter 73. This section concerns the molecular biology of latency. The key issues are how the virus establishes latency, how it is maintained, and what makes it reactivate spontaneously.

Herpes Simplex Virus Latency in Experimental Systems

The Experimental Models

The most useful animal model systems are mice, guinea pigs, and rabbits. In all three species, inoculation of wild-type virus results in viral replication at the peripheral site, retrograde transport of virus to the nucleus of dorsal root neuron, followed ultimately by establishment of latency. In all three systems, a fraction of sensory neurons replicate the virus after entry into the neuron (274,275,276 and 277,366,367,466,467,637,656,678). It is not known whether acute viral replication also occurs in humans or whether this replication reflects the animal system, route of inoculation, induction of host transactivating factors, or the large amount of virus used in the inoculum to attain a high percentage of latently infected ganglia.

Explantation of dorsal root ganglia causes the latent virus to replicate and spread from cell to cell. There are several key differences in the animal models. In rabbits, both HSV-1 and HSV-2 reactivate spontaneously. The rabbit eye model is particularly useful for studies of spontaneous reactivation because the virus is readily detected in lacrimal secretions. The guinea pig is a useful model of HSV-2 genital infections. The virus reactivates spontaneously, causing lesions containing small amounts of virus, although the acute and recurrent phases are somewhat artificially defined. The virtues of the mouse model are twofold. First, highly inbred strains differ with respect to their susceptibility to infection, and knock-out mice have been particularly useful in studies of immune responses to infection (339). Second, spontaneous reactivations of virus resulting in the appearance of infectious virus either in ganglia or at peripheral sites are very rare (34,35,221,222,605). Here, the key issue is spontaneous reactivations resulting in abortive replication. As discussed later in the text, if HSV reactivates to cause abortive infections in dorsal root ganglia ever so rarely, it would have a significant impact on our understanding of the mechanism of reactivation. As in other systems, latent virus in mice can be induced to reactivate, leading to appearance of virus in sensory ganglia or at peripheral sites by peripheral tissue trauma, ultraviolet light exposure, hyperthermia, or administration of drugs that stimulate prostaglandin synthesis.

Establishment of Latency: Initial Stages

Studies on neurons cultured *in vitro* indicate that viral capsids are transported by retrograde axonal transport involving microtubules; thus, drugs that disrupt neuronal microtubule structures, or that are known to inhibit retrograde transport of certain compounds, also inhibit the ability of the virus to move from the peripheral endings of neurons to the nuclei (291). Electron microscopic studies indicate that in neurons infected in cell culture, the viral particle being transported is the unenveloped nucleocapsid or nucleocapsid–tegument structure (344,433).

Several important facets of latent infections relate to the role of virus multiplication, both at the portal of entry and in the neurons harboring the virus.

1. As noted previously, HSV must have access to the nerve endings to establish latency; therefore, it could be expected that the greater the number of peripheral cells that become infected and support virus multiplication, the larger the number of neurons that will harbor latent virus. The relevant phenomenon in humans is that the frequency of reactivations resulting in recrudescences of lesions is related to the severity of lesions caused by the first infection. In the model discussed subsequently, the frequency of recurrences would be determined in part by the number of neurons harboring virus.
2. Because wild-type viruses multiply in sensory neurons during the first 7 to 10 days after peripheral inoculation, most of the information available to date on the establishment of the latent state is based on studies done 2 weeks or more after infection and therefore adequately reflects maintenance rather than establishment. Studies with mutants lacking essential genes have been very revealing in several respects.

First, viruses lacking the essential a *4* and a*27* genes, totally unable to replicate in cultured cells in the absence of complementing viral genes, were shown to be able to establish and maintain latent infections but were unable to reactivate (266,552). The fact that no deletion mutant totally failed to establish latency raises the question of whether any viral gene is essential for this process and suggests that no viral function is absolutely required for the establishment of latency. Nevertheless, viral gene products may affect the levels of DNA persisting in latent viral ganglia. For example, recent studies have shown that an HSV-2 U_L5-negative, U_L29-negative mutant virus is defective for stable maintenance of viral DNA in trigeminal ganglia (107).

Second, either viral latency is determined by cellular functions or virion proteins and no viral gene function expressed *de novo* is required to establish latency, or viral DNA entering neurons is retained in an episomal form irrespective of its ability to express any function, and therefore at least some of the deletion mutants may not be truly latent but merely retained in dorsal root neurons. Differentiation between these two alternatives requires an analysis of dorsal root ganglia during the first few hours after infection. Alas, the small number of neurons harboring these mutants makes effective analyses of the early events in the establishment of latency very difficult. In essence, no gene has been identified as essential for establishment of latency based on analyses of latent virus present during the maintenance phase, although certain viral functions, such as the LATs and the missing functions in the *dl*5-29 mutant, appear to increase the efficiency of latent infection.

Function(s) of Latency-associated Transcripts

The major viral gene products expressed during latent infection are the LATs (606). This transcriptional unit is expressed from the *b* repeat sequences of the L component (Fig. 13), and the more than 8-kb transcript is antisense to ICP0 mRNA and possibly ICP4 mRNA. The viral promoter driving expression of this transcriptional unit functions efficiently in sensory neurons, and expression of LAT ensues rapidly after viral infection of sensory neurons (285,595). Latent infection is favored in certain subsets of murine trigeminal ganglion sensory neurons, as evidenced by expression of LATs, whereas productive infection is favored in other types of the sensory neurons, as evidenced by expressing of lytic antigens (351). LAT-negative mutant viruses exhibit increased acute expression of viral a, b, and g genes in sensory neurons as compared with rescued or wild-type viruses (180), suggesting that LATs in some way down-regulate lytic gene expression in neurons. The threefold increase in neurons expressing lytic genes with LAT-negative viruses correlates with the similar decrease in latently infected neurons with LAT-negative viruses (628). Thus, LAT may play a role in promoting latent infection by down-regulation of lytic gene expression. The mechanism of this effect could include antisense down-regulation of ICP0 or ICP4 mRNA, structural effects of the transcript on the viral genome, or expression of a protein product.

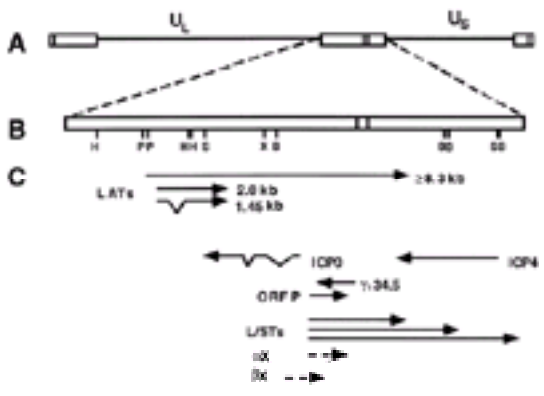

FIG. 13. Map of the latency-associated transcript (LAT) transcriptional unit. Map of the HSV-1 genome showing the LAT transcriptional unit. **A:** HSV-1 genome in the prototype orientation; U_L and U_S denote the unique sequences of the long (L) and short (S) components of the genome, respectively, and the *open boxes* denote repeat sequences. **B:** Expanded view of the L-S junction region with restriction endonuclease cleavage sites: B, BamHI; H, HpaI; P, PstI; S, SalI; X, XhoI. **C:** Locations and orientations of transcripts of the L-S junction region are denoted by *solid arrows* and include the LATs, ICP0, ICP4, $g_1$34.5 (78,82), ORF P (306), L/STs (693), and partially mapped transcripts (*dashed arrows*) aX and bX

(39,40).

A promising observation that did not live up to its expectation is that ORF P and ORF O were encoded in the domain transcribed by the 8.3-kb LAT. The transcript was detected late in infection and in nuclear runoff experiments, but not in cells treated with cycloheximide from the time of infection (40,693). The transcript or the protein it encodes has been detected early in infection in cells infected with a *4*-negative virus (693) or at 39°C, the temperature at which ICP4 of a wild-type virus is nonfunctional (306). Although attempts to detect the transcript in latently infected neurons have been unsuccessful (693), the results remain inconclusive because both the transcript and the protein are made in very small amounts. In addition, Yeh and Schaffer (693) failed to detect the 8.3-kbp LAT, which contains the sequences contained in the *ORF P* transcript. The transcription initiation site contains a high-affinity binding site for ICP4, and the genes are expressed only in the absence of functional ICP0 (480). Both ORF O and ORF P proteins are expressed from a single mRNA; thus, they share the amino-terminal 1 to 35 codons but diverge thereafter owing to RNA editing or undetected splicing. Overexpressed ORF P protein co-localized with spliceosomes and reduced the accumulation of ICP22 and ICP0, whereas the ORF O protein bound ICP4 and interfered with its binding to cognate sites on HSV DNA. Notwithstanding these attributes, mutagenesis of the initiator methionine, which abrogated the synthesis of ORF P and ORF O, had no effect on the ability of the virus to replicate in experimental animal systems and only a slight effect on the amount of virus recovered from latently infected ganglia (479). *ORF P* and *O* gene products may contribute to the establishment but are not key determinants of latency.

In the absence of detailed analyses of the events occurring during the first few hours after entry of viral DNA into dorsal root ganglia, one key hypothesis regarding the establishment of latency remains unchallenged. In essence, the hypothesis predicts that latency is established by default in the absence of initial sustained expression of a genes. The lack of a gene expression is likely explained by several mechanisms acting in combination: (a) lack of nuclear forms of host factors necessary for a gene transcription (298,640), (b) hormonally regulated repression of viral gene expression (674), and (c) LAT-mediated repression of a gene expression (180). The hypothesis that lack of a gene expression is central to latency is further supported by two sets of observations.

The first set stems from attempts to establish latency in cultured cells *in vitro.* Initial attempts were based on suppression of viral multiplication by high temperatures or antiviral drugs. Although these early attempts used nonphysiologic means and lacked the power of modern analytic tools, they did show that the HSV resident in these cells could be reactivated by superinfection with unrelated human CMV. More recent studies in so-called quiescent systems have yielded similar information, but in this instance, the power of the analytic tools applied to the system indicate that HSV does remain quiescent (nonreplicating) if a gene expression immediately after entry of the virus into the cell is repressed. The mechanism of repression after the initial stages of infection is unknown.

The second set of supporting data comes from two series of experiments. In chronologic order, the first was based on the hypothesis that the tegument protein aTIF or VP16 does not reach the neuronal nucleus. This possibility was tested using a recombinant virus that contained an insertion of a second copy of the a *TIF* gene under the control of the mouse metallothionein (MT) promoter (548). Despite expression of the chimeric a*TIF* gene in latently infected sensory neurons, latent infection continued. Furthermore, expression of the same chimeric gene in transgenic mice also failed to prevent establishment or maintenance of latency. *In situ* hybridization with probes specific for Oct-1 demonstrated detectable levels of Oct-1 mRNA in the sensory neurons of the MT–aTIF transgenics (V. Hukkannen and B. Roizman, unpublished data), indicating that the presence of both aTIF and Oct-1 was insufficient to prevent latent infection. A formal explanation as to why MT–aTIF failed to induce viral multiplication emerged from studies of Kristie and co-investigators (298), who showed that HCF or C1, the cellular cofactor required for enhancement of a gene expression, along with Oct-1 and aTIF, resides in the cytoplasm and is translocated into the nucleus only under conditions of reactivation, such as explantation of ganglia in culture.

Maintenance Phase of Latency: The Latency-associated Transcripts

Extensive studies on ganglia harboring latent HSV have been rewarded by an extreme paucity of evidence for viral gene expression. The only transcripts regularly detected in a large fraction of latently infected neurons have been designated as the LATs (606). They constitute a family of transcripts mapping to the inverted repeats flanking the U_L sequence (see Fig. 13). The full-length 8.3-kb transcript accumulates at low levels in latently infected neurons, whereas the 2.0- and 1.5-kb introns processed from a full-length transcript are abundant and accumulate in the nucleus (125,290,499,594,606,652,653,705). These introns are highly stable as a result of unusual lariat structures (156,500,685,696). The literature on LATs is enormous but fortuitously does lend itself to a brief summation. Viruses deleted in various domains of LATs have been reported to establish latency at normal levels (250,315,602) or at threefold reduced levels (628). Several studies have shown reduced explant reactivation of virus from ganglia latently infected with LAT-negative mutant viruses (31,220,250,315,551,602), although in many instances, LAT-negative mutant viruses show reduced replicative ability (31; Garber et al., manuscript in preparation; Lagunoff and Roizman, unpublished studies). The region of the LATs associated with decreased reactivation has been mapped to a 348-bp sequence in the 5' end of LAT (33,219).

Notwithstanding several reports (131,624), the stable 2.0- and 1.5-kb LATs have not been shown unambiguously to express ORFs. The functions attributed to LATs have been numerous and varied. Thus LATs, have been reported to (a) block productive infection gene expression by blocking the transcription of the gene encoding ICP0 located antisense or by other mechanisms (75,180), (b) enable reactivation of virus from latent state, and (c) maintain virus in latent state. Each of these putative mechanisms has been disputed (32,75,89,180,196,221,315,316,628,635). The most recent function attributed to LATs is that they protect neurons from apoptosis. Thus, a virus deleted for LAT was reported to induce apoptosis in rabbit trigeminal ganglia at slightly higher levels than the wild-type virus (438). The substance of the report is that LATs do spare the neurons from destruction but not the mechanism by which this takes place. This report

requires confirmation. If LATs do protect neurons from apoptosis, the question arises as to how RNA does this, and whether reactivation is the cause or consequence of failure of LAT to protect neurons from programmed cell death. Irrespective of the final determination of the functions of LATs, current data suggest that LATs exert a quantitative effect on latency rather than an absolute role.

Copy Number of Viral DNA in Latently Infected Neurons

In a different category from LATs is the observation that in trigeminal ganglia harboring latent virus, there are between 0.1 and 1 viral genome equivalents per cell genome (55,468,497,498). This datum posed an intriguing question. Heretofore, the number of neurons harboring virus was thought to be about 3% of total neurons. Rodahl and Stevens (501) indicated that under some circumstances, the number can be higher; however, neurons account for only about 10% of the total cells in a sensory ganglion. Unless viral genomes are contained in every single cell in a ganglion, including glial cells, it is obvious that, as was first calculated a number of years ago (516), each latently infected neuron must contain more than one viral genome.

Two series of experiments indicate that, as was predicted earlier (516), replication of viral DNA by viral enzymes is not necessary for the establishment of latent infection and is not responsible for the attainment of the high copy number. In these experiments (567,590,591), mice were infected by the footpad route, and assays of LAT, lytic antigens, and DNA copy number were done. Although there was a correlation between levels of lytic replication and the level of viral DNA per LAT-positive neuron, even those ganglia that showed little or no sign of viral antigen expression still had DNA copy numbers of greater than 20 viral genomes per LAT-positive neuron. To account for the high number of viral genomes per cell harboring latent virus, it is then necessary to postulate that (a) more than one viral genome can enter a single neuron during the establishment of latency, or (b) viral genomes are amplified by the cellular machinery during latency (516). In support of the second of these hypotheses, at least one host-dependent origin of DNA replication embedded within the viral genome has been identified (550). A second, symmetrically arranged copy was also found (A. E. Sears, personal communication). As discussed later, one hypothesis governing these studies is that one of the cellular polymerases amplifies resident viral genomes at a low rate. The dogma, however, has been that there is no DNA replication machinery in neurons.

On the other hand, evidence in favor of the high copy number being due to viral DNA replication functions includes (a) the constant level of viral DNA during latent infection by viruses incapable of viral DNA replication in neurons as determined by quantitative PCR (289,641) and (b) the general correlation between ganglionic replication and latent DNA load (629).

Reactivation of Virus from Latent State

The issues central to understanding viral reactivation from latent state are fivefold: (a) Do the events surrounding viral recrudescences in humans provide a clue to the events leading to reactivation? (b) Is there a specific viral gene whose function is unambiguously required for viral reactivation? (c) What is the order of expression of viral genes in the course of reactivation? (d) What is the significance of the observation that not all neurons harboring virus reactivate all at once? and (e) What is the fate of the neuron in which virus reactivated? It is convenient to consider these questions in the order stated.

a. In humans, latent virus is reactivated after local stimuli, such as injury to tissues innervated by neurons harboring latent virus, or by systemic stimuli, such as physical or emotional stress, hyperthermia, exposure to ultraviolet light, menstruation, hormonal imbalance, and so forth, which may reactivate virus simultaneously in neurons of diverse ganglia (e.g., trigeminal and sacral). In experimental systems, multiplication of latent virus has been induced by physical trauma to tissues innervated by the neurons harboring virus (10,204), by iontophoresis of epinephrine (299) or other chemicals or drugs (204,205,561), by transient hyperthermia (537,538), or by corneal scarification (640). The most plausible common denominator is that injury or stimulation of cells innervated by dorsal root neurons harboring latent virus is a common trigger of recrudescence of lesions caused by reactivated virus. The nature of the transduced signal is unknown.
b. It is a common practice to test deletion mutants for ability to establish latent infections and to reactivate. Not surprisingly, a large number of deletion mutants either failed to reactivate or reactivated poorly. Included in this list are virtually all of the genes essential for viral replication in cell culture but also mutants lacking thymidine kinase, ICP0, ribonucleotide reductase, $g_1$34.5, and so forth. A central problem with these claims is that neuronal cells harboring virus are relatively nonpermissive and devoid of enzymes necessary for viral nucleic acid metabolism. In the context of experimental tools based on explantation of sensory ganglia, it is virtually impossible to differentiate between genes required for optimal replication in tissues *in vivo* and those dedicated to reactivation of virus from latent state.
c. For years, the prevailing hypothesis concerning the regulation and order of gene expression during acute infection and reactivation was that it matched the program of viral gene expression taking place in infected cells in culture, that is, a genes are expressed first, followed by b and g genes. Recent evidence supporting the idea that a genes are expressed first is the rapid translocation (within minutes) of the HCF or C1 transcription factor from the cytoplasm to nucleus upon explantation of trigeminal ganglia (298). Nevertheless, theoretical arguments and experimental observations have suggested that viral gene expression may be regulated differently in neurons. Theoretical considerations have argued that HSV depends to a large extent on cellular factors for its gene expression, that the order of viral gene expression depends on available factors, and that in neuronal cells, the available factors differ from those of run-of-the-mill cultured cells (457). The experimental observations are of two kinds. First, Kosz-Vnenchak and associates (286) reported that high-level expression of a and b genes depended on viral DNA synthesis during acute ganglionic infection and in explanted ganglia. It was postulated that, in neurons, low levels of a and b gene expression preceded viral DNA synthesis and g gene expression, which then further activated a and b gene expression. The latter hypothesis was supported using reverse transcription PCR (RT-PCR) and PCR methods to detect viral RNA and DNA levels, respectively (289). Nevertheless, it has been argued that viral gene expression may not be detectable in the initial infected neuron and that viral spread amplifies the signal so that high-level a and b gene expression can be detected. By this hypothesis, viral DNA synthesis is

needed for progeny virus formation and spread and not direct induction of a and b gene expression. The currently available experimental data cannot rule out this alternative explanation for the *in vivo* data. However, one study of infections of sensory neurons in culture has supported the idea that viral gene expression is regulated differently in neurons. Nichol and colleagues (410) observed that, in HSV-infected sensory neurons in culture, viral DNA synthesis stimulates a and b gene expression. These results support the hypothesis of Kosz-Vnenchak and associates (286) that viral DNA synthesis stimulates a and b gene expression in neurons, but further studies are needed to rule out the possibility that viral spread accounts for the results *in vivo* and in explanted tissue. Second, Tal-Singer and colleagues (618) reported that they could detect b gene expression before a gene expression during explant reactivation. In this study, they used RT-PCR to detect viral transcripts, but they did not show that the assays for each of the transcripts was equally sensitive; therefore, it is conceivable that the differences in kinetics of appearance reflected sensitivity of the detection assay rather than the true order of appearance. The order of expression of HSV genes may be different during reactivation, but additional studies are needed to adequately document this.

d. Unlike the reactivation of varicella-zoster virus, not all neurons harboring latent HSV reactivate at once. The current consensus is that the recurrent lesions caused by reactivated virus are the reservoir for transmission of virus from person to person. Because transmission depends on physical contact between infected and uninfected individuals, frequent protracted recurrences would enhance transmission far more than a single synchronous transmission. One hypothetical model detailed elsewhere (45,515) that could explain asynchronous reactivation takes into account the DNA copy number per latently infected neurons. Briefly, the model is that each stimulus leading to reactivation causes an increase in the copy number of viral DNA. The stimulus may be local or systemic. At a certain point when the viral DNA copy number becomes very high, the virus reactivates either by diluting a repressor (copy number effect) or by competing more effectively for a scarce host factor necessary for basal level of viral gene expression (multiplicity effect). This model predicts that the decrease in recurrence rate reflects exhaustion of neurons that are stimulated to increase viral DNA copy number and, therefore, the larger the initial burden of latent virus, the more frequent or longer lasting interval of recurrences. This DNA level threshold could be achieved by host replicative mechanisms (516) or by viral replicative mechanisms (286).
e. The fate of the neurons after viral reactivation is a hotly debated topic. The proponents of the notion that neurons survive to reactivate again and again base their arguments on two observations. The first is that local anesthesia is not a sequelae in patients suffering from frequent recurrences at the same site. A formal rebuttal of this argument is that nerve endings from adjacent unaffected neurons grow into the healed area and reestablish a network. The second and more weighty observation is that women with recurrent genital lesions shed virus in the interim between clinically manifest lesions. The shed virus appears to originate from punctate, microscopic lesions that arise frequently and for long intervals. If each microscopic lesion arose from a reactivated neuron, there would not be enough neurons to support viral reactivation over many years if the neurons perished as a consequence of this process. The questions that have not been resolved yet is whether each lesion is the consequence of reactivation and whether chronic infections of mucosal tissues characterized by the observed microscopic lesions can be ruled out.

The studies on the molecular biology of viral replication and especially the effects of viral gene products on cellular structures and gene products detailed in this chapter do not support the hypothesis that neurons expressing viral gene products necessary for productive infection can survive and continue to function. A strange and unsupported model that satisfies the requirement that the neuron survive reactivation is that the neuron transport anterograde to peripheral sites not virions but viral DNA replicated by cellular enzymes.

CONCLUSIONS

There is an apocryphal story of three blind people asked to deduce the nature of an elephant. Each touched a different part and the identity became elusive. This probably was the case with herpesvirus research 50 years ago. In the interim, thousands of investigators explored every facet of HSV biology. We know what it consists of and the end result of infection: thousands of replicas and a very dead cell. The mass of data has produced a giant mosaic with huge gaps, but the designer's intent and general strategy of HSV design emerges. What is apparent is the following:

1. The overall strategy is total control of its own replication and of the cellular environment in which replication takes place.
2. A large fraction of viral coding sequence is devoted to changing the environment of the cell. The objectives of viral functions are the shutoff of *de novo* cellular gene expression, scavenging existing cellular proteins to redirect them to either block the host or to assist in viral replication, and complete dedication to the task of producing lots of viral progeny.
3. Many functions overlap. For example, several viral genes independently target *de novo* synthesis of cellular gene products. Many viral objectives are reached by accretion rather than by a single function of a viral gene.
4. Many, if not most, HSV proteins are multifunctional. In some instances, the functions can be mapped to blocks of amino acids. There is sufficient evidence to postulate that the function performed by individual proteins is determined by posttranslational modifications. Indeed, viral proteins are extensively posttranslationally modified.
5. Other members of the herpesvirus family with a much more restricted host range encode homologs of cellular genes. HSV encodes instead sequences that interact with and stabilize or redirect cellular proteins. The net effect is that whereas a homolog basically performs a single function, viral proteins containing amino acids blocks that redirect cellular proteins can perform multiple functions. This design presents a curious problem: It is much easier to identify a homolog than to detect the intent or function of an amino acid block that interacts with a similar cellular protein.
6. Latent infection of sensory neurons by HSV is likely to be a complex process in which numerous factors, including missing host cell factors, cellular repressor molecules, and viral functions, may each play a role in this alternative infection pathway.

Notwithstanding the many problems, the virus is no match to the effort, modern technology, and ingenuity of the blind investigators.

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CHAPTER 73 Herpes Simplex Viruses

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Knowledge of human herpes simplex virus (HSV) infections has increased dramatically since the initial historical descriptions of the disease in a variety of patient populations and the histopathology of induced lesions. Advances in our understanding of the natural history and pathogenesis of HSV infections have been paralleled by several major achievements. First, sensitive and specific serologic tests allow the distinction between HSV type 1 (HSV-1) and type 2 (HSV-2) infections. Two of these tests are now licensed. Second, antiviral drugs have proven to be selective and specific inhibitors of viral replication and are clinically efficacious. Such drugs as acyclovir, valaciclovir, and famciclovir have provided probes for assessing disease pathogenesis in both the normal and immunocompromised host. The unequivocal establishment of the value of therapy has had a major impact on altering the spectrum of human disease and has implications for long-range control of HSV infections. Third, polymerase chain reaction (PCR) detection of HSV DNA has contributed immensely to our knowledge of disease pathogenesis, as well as to the diagnosis of disease, particularly HSV infections of the brain. Furthermore, the ever-evolving knowledge of the molecular biology of HSV has been unusually striking over the past several years. This is best exemplified by the elucidation of the events of viral replication (especially induction of replication), including viral gene products and biologic functions, the translocation of proteins, gene regulation, membrane structure, and virus entry molecules. Our current level of biomedical knowledge sets a stage for (a) the application of molecular biology to the evaluation of human disease, (b) the development of genetically engineered vaccines, (c) the development of antiviral therapeutics predicated on newly identified site-specific molecular targets, and (d) the use of genetically engineered HSV for gene and tumor therapies (140,446). As with the study of other viruses, there is the potential for applying genetically engineered probes, which would make an understanding of the molecular pathogenesis of human disease even more attainable. This chapter will summarize the current status of our knowledge of HSVs as human pathogens.

HISTORY

HSV infections of humans have been documented since ancient Greek times (332,556). Records of human HSV infections began with descriptions of cutaneous spreading lesions thought to be of herpetic etiology, as noted in the writings of Hippocrates and reviewed by Nahmias and Dowdle (332). Scholars of Greek civilization define the word *herpes* to mean to creep or crawl, in reference to the spreading nature of the visualized skin lesions (43,556). The Roman scholar Herodotus associated mouth ulcers and lip vesicles with fever and defined this association as “herpes febrilis” (311). Most likely, many of these original observations were predicated on Galen's deduction that the appearance of such lesions was an attempt by the body to rid itself of evil humors, and this possibly led to the name of “herpes excretins” (556). These original descriptions of skin lesions probably show little resemblance to later reports (in the 19th and 20th

centuries) of these infections (312).

As noted by Wildy (556), Shakespeare was probably cognizant of recurrent HSV labial lesions. In Romeo and Juliet, Queen Mab, the midwife of the fairies, stated, “O'er ladies lips, who straight on kisses dream, which oft the angry Mab with blisters plagues, because their breaths with sweetmeats tainted are.”

In the 18th century, Astruc, physician to the king of France, drew the appropriate correlation between herpetic lesions and genital infection (203). By the early 19th century, the vesicular nature of lesions associated with herpetic infections was well characterized. However, it was not until 1893 that Vidal specifically recognized human transmission of HSV infections from one individual to another (556).

Several observations made at the beginning of the 20th century brought an end to the early era of imprecise descriptions of HSV infections. First, histopathologic studies described multinucleated giant cells associated with herpesvirus infections (517). Second, the unequivocally infectious nature of HSV was recognized by Lowenstein in 1919 (289). He demonstrated experimentally that virus retrieved from the lesions of humans with HSV keratitis or from the vesicles of HSV labialis produced lesions on the cornea of the rabbit. These corneal lesions were similar to those encountered in humans with eye infection. Furthermore, vesicle fluid from patients with herpes zoster failed to produce similar dendritic lesions in the rabbit eye model. These observations were actually attributed to earlier investigations by Grüter, who performed nearly identical experiments around 1910 but did not report them until much later (169).

Investigations reported between 1920 and the early 1960s focused on the biologic manifestations of HSV and the natural history of human disease. During the first four decades, the host range of HSV was expanded to include a variety of laboratory animals, chick embryos, and, ultimately, cell culture systems. Expanded animal studies demonstrated that transmission of human virus not only could occur with the rabbit, as noted, but also could lead to infections of either the skin or the central nervous system (CNS) (reviewed in refs. 127,346,543,545, and 550).

Host immune responses to HSV were first described in the early 1930s. The first studies were performed by Andrews and Carmichael and defined the presence of neutralizing antibodies to HSV in the serum of previously infected adults (14). Subsequently, some of these patients developed recurrent labial lesions, albeit less severe than those associated with the initial episode. This observation led to the recognition of a unique biologic property of HSV, namely, the ability of these viruses to recur in the presence of humoral immunity, a characteristic known as reactivation of latent infection. Only individuals with neutralizing antibodies developed these recurrent vesicular lesions, a paradoxical finding given the classic lessons of infectious diseases provided by measles and rubella, in which antibodies were normally associated with protection from subsequent episodes of disease. By the late 1930s, infants with severe stomatitis were reported to shed a virus thought to be HSV (126) and developed neutralizing antibodies during the convalescent period (71). Later in life, some of these children had recurrent lip lesions.

The medical literature of the 1940s and 1950s was replete with articles describing specific disease entities such as primary and recurrent infections of mucous membranes (e.g., gingivostomatitis, herpes labialis, and genital HSV infections, and such skin manifestations as herpetic whitlow or eczema herpeticum) (453), keratoconjunctivitis (159), neonatal HSV infection, visceral HSV infections of the immunocompromised host, and HSV encephalitis (474). Recently, the clinical spectrum of HSV infection was expanded to include Kaposi's varicella-like eruption, infections of the immunocompromised host, and an association with erythema multiforme.

Laboratory advances have provided a foundation for better understanding the epidemiology and natural history of infection. One significant advance was the detection of antigenic differences between HSV types. Although suggested by Lipschitz (282) on clinical grounds over 70 years ago and by others from laboratory observations (379,447), in 1968 antigenic and biologic differences were demonstrated between HSV-1 and HSV-2 by Nahmias and Dowdle (332). These investigators demonstrated that HSV-1 was more frequently associated with nongenital infection (infection above the belt), whereas HSV-2 was associated with genital disease (infection below the belt). Today, the principle of type specificity and location is not as predictable as it was 30 years ago. Nevertheless, this observation was pivotal for many of the early clinical, serologic, immunologic, and epidemiologic studies.

Other critical advances that have contributed to our understanding of the natural history of HSV infections include the following: First, successful antiviral therapy was established unequivocally for HSV encephalitis (552) and, subsequently, for genital HSV infections (63,108,109,112) and HSV infections in the immunocompromised host (313,433; reviewed in refs. 535 and 544). Second, differences between strains of HSV were demonstrated by restriction endonuclease technology—an important epidemiologic tool (66). Third, type-specific antigens have been applied to seroepidemiologic surveys (411). Fourth, an understanding of the genotypic properties HSV has been applied to phenotypic studies. Such efforts have advanced our understanding of latency, trophism, and virulence at a molecular level. Fifth, the engineering of HSV and the expression of specific genes are providing the technology for experimental vaccines (410), as well as for the use of such viruses for gene therapy. Finally, extensive effort has been devoted to the study of latency with incremental advances (defined in ref. 492, and reviewed in ref. 413).

INFECTIOUS AGENT

HSVs are members of a family of viruses whose genomes consist of a single large double-stranded DNA molecule, as reviewed in Chapter 72 (556). The herpes simplex virion consists of four components: (a) an electron-dense core containing viral DNA; (b) an icosadeltahedral capsid; (c) an amorphous, at times eccentric, layer of proteins, designated tegument, which surrounds the capsid; and (d) an envelope. The capsid consists of 162 capsomeres and is surrounded by the tightly adhering tegument. The envelope surrounds the capsid–tegument structure and consists of at least 10 glycosylated and several nonglycosylated viral proteins, lipids, and poly-amines. Viral DNA contains at least 152 kbp. The variability in size is due chiefly to the variation in the number of reiterations of specific terminal and internal sequences. The DNA of HSV-1 and HSV-2 DNAs consists of two covalently linked components, designated as L (long) and S (short). Each component is composed of unique sequences (U_L or U_S, respectively) flanked by relatively large

inverted repeats. The inverted repeat sequences flanking U_L are *ab* and *b'a'*, whereas those flanking U_S are *a'c'* and *ca*. The two components can invert relative to one another to yield four populations of DNA molecules differing solely in the relative orientation of these DNA sequences (414).

Current studies indicate that HSV-1 and, by extension, HSV-2 encode at least 84 different polypeptides (Table 1) (527). Of this number, 14 map in the U_S, four in each of the sequences flanking U_L, one in each of the sequences flanking U_S, and the remainder in U_L. The genes mapping in the repeats are present in two copies per genome.

Ab Genome domains	U_L Open reading frames	B'a'c' U_S ca *N*
L component		
Ab, b'a'	α0, γ34.5, ORF-P, O	8
U_L	U_L1–56, 8.5, 9.5, 10.5, 12.5, 20.5, 26.5, 27.5 43.5, 49.5	65
S component		
A'c', ca	α4	2
U_S	U_S1–12, 1.5, 8.5	14
	Total	89
	Total single copy	84

S, short; L, long; ORF, open reading frame.

TABLE 1. *Polypeptides encoded by herpes simplex viruses 1 and 2*

To initiate infection, HSV must attach to cell surface receptors, fuse its envelope to the plasma membrane, and allow the de-enveloped capsid to be transported to the nuclear pores. The DNA is released into the nucleus at the pore. The key events in viral replication that occur in the nucleus include transcription, DNA synthesis, capsid assembly, DNA packaging, and envelopment. Viral surface glycoproteins mediate attachment and penetration of the virus into cells. They also elicit host immune responses to the virus. Currently, at least 10 viral glycoproteins (designated gB, gC, gD, gE, gG, gH, gI, gK, gL, and gM) are known, and an eleventh (gJ) has been reported. Two, gC and gB, are required for attachment to the cell surface, whereas gD is required for entry of virus into cells. Two more, gE and gI, form a rather potent Fc receptor; gE is also required for basolateral transmission of virus in polarized cells and for efficient expression of late genes. It is of note that the deletion of gH allows only a single cycle of viral replication. This principle is being applied to the development of infectious vaccines.

The synthesis of viral gene products, both RNA and proteins, takes place in three sequential periods. The first set consists of six proteins known as a or immediate-early proteins. Of these, five regulate the reproductive cycle of the virus and one blocks the presentation of antigenic peptides on the infected cell surfaces. The a proteins are essential for the synthesis of the second set of viral gene products, known as b or early proteins. Most of the b proteins are responsible for viral nucleic acid metabolism and are the main target of antiviral chemotherapy (e.g., viral thymidine kinase, viral DNA polymerase). These enzymes are essential for activation of acyclovir and penciclovir and subsequent inhibition of viral replication (437). HSV viruses resistant to these medications have been described (115, and reviewed in ref. 545).

The third class of proteins (g or late) are for the most part the structural components of the virion. They assemble to form the capsid and the tegument, and they incorporate into nuclear membranes for eventual envelopment of virions. In the process of viral assembly, a procapsid is formed from scaffolding and capsid proteins. The scaffolding proteins consist of a protease and its substrate, with the protease cleaving the substrate during capsid assembly. The newly synthesized DNA is cleaved into unit-length molecules and packaged into the virions. Generally, envelopment favors capsids containing DNA. Capsids containing DNA attach to the nuclear surface of the inner nuclear membrane and are rapidly enveloped and released into the space between the inner and outer nuclear membranes. Here, the virions become encased in transport vesicles and are transported through vesicles formed by fragmented and dispersed Golgi stacks to the extracellular space. This process takes approximately 18 hours.

Several aspects of the viral replication cycle and of the viral proteins are relevant to the pathogenesis of human diseases, and to the present and future development of antiviral chemotherapy:

1. At least five viral proteins play a key role in ensuring a robust expression of viral genes and the mobilization of cellular proteins necessary for efficient synthesis of viral DNA and proteins. The first of these, aTIF (for trans-inducing factor) or VP16, is a protein, packaged in the tegument, that augments the basal level of expression of a genes. Like many other viral proteins, aTIF is both a transactivator and an essential structural protein of the virion.

The second, an a protein known as the infected cell protein (ICP4), binds directly to both high- and low-affinity sites on viral DNA. ICP4 acts both as a transactivator at low-affinity sites and as a potent repressor at high-affinity sites located at the transcription initiation sites of its own gene and that of a few others.

Another a protein, ICP0, acts a promiscuous transactivator of genes introduced by infection or transfection. It does not bind DNA. Recent studies indicate that it binds an array of cellular proteins. The functions of the interactive proteins suggest that ICP0 has at least two roles: (a) to stabilize important cell cycle regulatory proteins (e.g., cyclin D3), forcing the cell to the brink of the S phase (but not allowing cell division), and (b) to maintain a vigorous protein synthesis for its own use.

ICP27, another a protein, also has several functions. The most remarkable is control of posttranslational processing of RNA. Unlike cellular genes, the vast majority of viral genes lack introns. IPC27 blocks the splicing of mRNA. Although it

reduces the amounts of spliced viral mRNAs, the net effect is on host mRNAs and results in diminished cellular protein synthesis. In addition, ICP27 acts as a transporter for late RNA from the nucleus to the cytoplasm and, as a consequence, regulates the expression of late proteins. ICP22 is another multifunctional a protein. This protein has revealed few of its functions, but it appears to regulate the longevity of ICP0 mRNA. It also is involved in the use of specific splice acceptor sites of ICP0 mRNA.

2. The tegument proteins function to enhance the capacity of HSV to replicate efficiently. In addition to the aTIF described, two viral proteins play an important role in viral infection. The first, *vhs*, is the product of the gene U_L41. It induces an RNase activity, which degrades all mRNA. Because viral transcription occurs at a very high rate, viral protein synthesis is less affected than cellular protein synthesis. The tegument also includes a protein kinase encoded by U_L13. Although dispensable in cell culture, this kinase is essential for viral replication in experimental animal systems, and its substrate is both viral and cellular proteins.

3. Viral proteins interact with cellular proteins, either to stabilize them and redirect their function, or to block their function. For example, as noted, ICP0 binds to and prolongs the half-life of cyclin D3. The two other types of interactions of viral and cellular proteins are involved in blocking host cell response to infection. Small peptides derived from degradation of cellular DNA are normally transported through the endoplasmic reticulum to the cell surface. In the first of the two interactions that interfere with function, HSV blocks the presentation of antigenic peptides by encoding an a protein, ICP47, that binds to TAP1/TAP2, the proteins that bind to and translocate peptides into the endoplasmic reticulum.

A second antiviral host defense mechanisms is based on the activation of dsRNA-dependent protein kinase (PKR). Activated PKR phosphorylates the a subunit of a translation initiation factor (eIF-2a) and shuts off protein synthesis. Nearly all viruses make dsRNA, either as part of their replicative cycle or because both strands of the nucleic acid that are transcribed activate PKR, and all of them have evolved functions to block the shutoff of protein synthesis. HSV has evolved a totally different technique: The protein encoded by the viral $g_1 34.5$ gene binds protein phosphatase 1 and redirects its activity from common substrate to dephosphorylate eIF-2a. The rate of dephosphorylation of eIF-2a in cells infected with wild-type virus increases 3,000-fold compared to that of uninfected cells. Expression of several viral functions, including the stabilization and prolonged function of cyclin D3, is likely to induce apoptosis. HSV has evolved at least one gene, the protein kinase expressed by the U_S3 gene, to block apoptosis induced by the virus as well as that induced by other agents.

4. Viral replication is highly deleterious to the integrity of the infected cell. Cellular chromosomes are marginated to the nuclear membrane and degraded, the nucleolus falls apart, the Golgi is fragmented and dispersed, and the microtubules are rearranged. All of these events are designed to create the environment for viral DNA synthesis, to enhance the glycosylation and exocytosis of virions, and to prevent a host response to infection.

5. Of the 84 genes known to be encoded by HSV-1 and HSV-2, at least 45 are dispensable for growth in cells. These 45 include genes whose products enable the virus to attach and enter both apical and basolateral aspects of polarized cells, to fine-tune expression of individual genes, to provide efficient cell-to-cell spread, to sort proteins and viral exocytosis, to increase the precursor pool and repair viral DNA, and to block cellular and immune responses to viral infection. It should be stressed that these genes are not truly dispensable: Viruses lacking them have not been isolated from people, and viruses from which these genes have been deleted by genetic engineering frequently exhibit reduced capacity to multiply and spread in experimental animals. On the other hand, the observation that so many HSV genes are dispensable and can be replaced by non-HSV genes sustains the expectation that a live attenuated vaccine capable of acting as a vector for foreign gene expression is feasible.

PATHOLOGY AND PATHOGENESIS

Pathology

The pathologic changes induced by the replication of HSV are similar for both primary and recurrent infection but vary in the quantitative extent of cytopathology. The histopathologic characteristics of a skin lesion induced by HSV are shown in Figure 1. These changes represent a combination of virally mediated cellular death and associated inflammatory response. Changes induced by viral infection include ballooning of infected cells and the appearance of condensed chromatin within the nuclei of cells, followed by subsequent degeneration of the cellular nuclei, generally within parabasal and intermediate cells of the epithelium. Cells lose intact plasma membranes and form multinucleated giant cells. With cell lysis, clear (referred to as vesicular) fluid containing large quantities of virus appears between the epidermis and dermal layer. The vesicular fluid contains cell debris, inflammatory cells, and, often, multinucleated giant cells. In dermal substructures, there is an intense inflammatory response, usually in the corium of the skin, more so with primary infection than with recurrent infection. With healing, the vesicular fluid becomes pustular with the recruitment of inflammatory cells, and then it scabs. Scarring is uncommon but has been noted in some patients with frequently recurrent lesions. When mucous membranes are involved, vesicles are less likely to be prominent. Instead, shallow ulcers are more common because the vesicles rapidly rupture as a result of the very thin cornified epithelium. Nevertheless, the histopathologic findings of these shallow ulcers are similar to those of the skin.

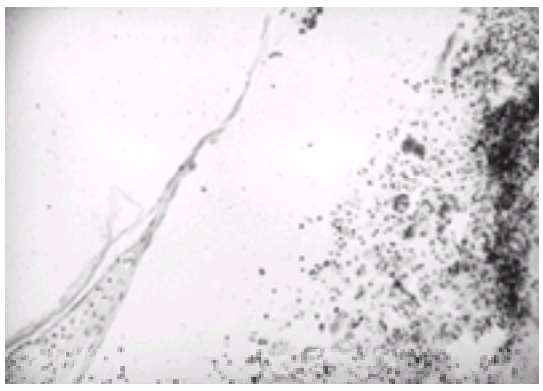


FIG. 1. Histopathology of an HSV infection.

Vascular changes in the area of infection include perivascular cuffing and areas of hemorrhagic necrosis (Fig. 2). These histopathologic findings become particularly prominent when organs of the body other than skin are involved, as is encountered with HSV encephalitis or disseminated neonatal HSV infection. In such cases, widespread areas of hemorrhagic necrosis, mirroring the area of infection, become most prominent. When the brain is involved, oligodendrocytic involvement and gliosis are common, as is astrocytosis, but these changes develop late in the disease course. Local lymphatics can show evidence of infection with intrusion of inflammatory cells, which allows the draining of infected secretions from the area of viral replication. The intensity of the inflammatory response is significantly less with recurrent disease. As host defenses develop, an influx of mononuclear cells can be detected in infected tissue.

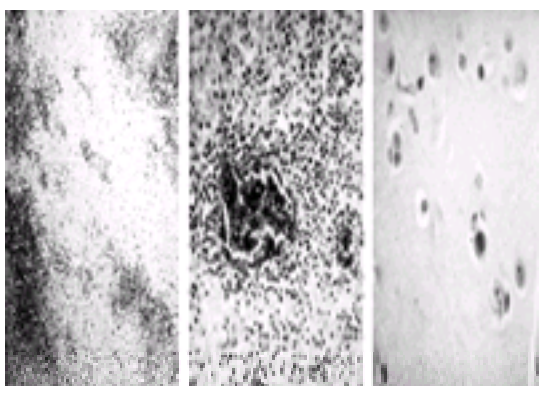


FIG. 2. Perivascular cuffing with areas of hemorrhagic necrosis of the brain.

Pathogenesis of Human Infection

The pathogenesis of HSV infections is understood through knowledge of the events of replication, including virulence, and establishment of latency in both animal models and humans. The pathogenesis of human disease depends on intimate, personal contact of a susceptible individual (namely, one who is seronegative) with someone excreting HSV. The trigeminal ganglion becomes colonized and harbors latent virus. Acquisition of HSV-2 infection is usually the consequence of transmission by genital contact. Virus replicates in the genital, perigenital, or anal skin sites with seeding of the sacral ganglia. An overview of these sites of infection appears in Figure 3.

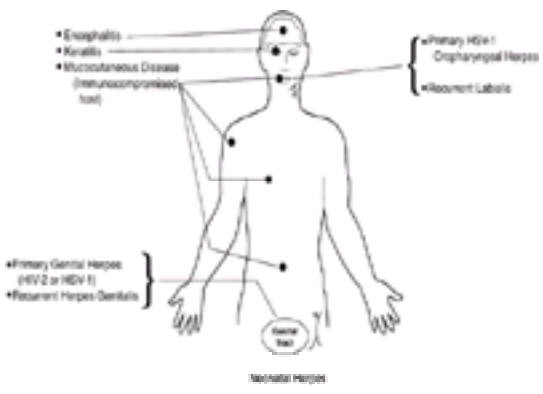


FIG. 3. Sites of HSV infection and disease.

Accumulated clinical experience suggests that after primary infection, replication of virus at the portal of entry, usually oral or genital mucosal tissue, results in infection of sensory nerve endings; the virus is transported to the dorsal root ganglia, as noted previously (32,34,493,495). Figure 4 illustrates the events of primary infection. The more severe the primary infection, as reflected by the size, number, and extent of lesions, the more likely it is that recurrences will ensue. However, experimental evidence to substantiate this hypothesis is lacking. In large part, this notion is attributed to the large quantity of virus present when primary disease is extensive and clinically overt as compared with those infections

that are asymptomatic and usually undetected. The resulting cutaneous lesions contain infectious virus.

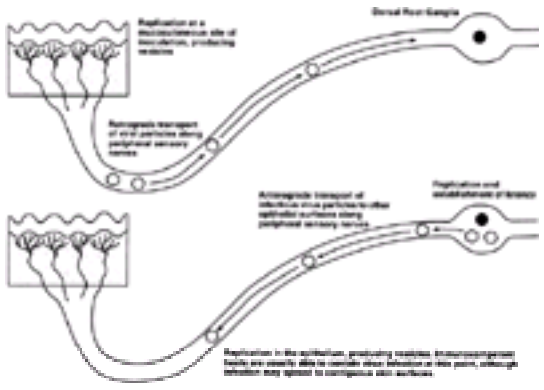


FIG. 4. Schematic diagram of an HSV infection.

Thus, virus must come in contact with mucosal surfaces or abraded skin for infection to be initiated. With viral replication at the site of infection, either an intact virion or, more simply, the nucleocapsid is transported by neurons to the dorsal root ganglia, where latency is established. These events were demonstrated in a variety of animal models (reviewed in ref. 194). Transport of the virion is by retrograde axonal flow (103). The fundamental principle of disease pathogenesis, then, is the propensity of virus to replicate at mucosal surfaces, to be transported to dorsal root ganglia, and to become latent. Although replication can sometimes lead to disease and can infrequently result in life-threatening CNS infection, the host–virus interaction leading to latency predominates. After latency is established, a proper stimulus will cause reactivation to occur; virus becomes evident at mucocutaneous sites (Fig. 5), appearing as skin vesicles or mucosal ulcers. Primary infection can spread beyond the dorsal root ganglia, thereby becoming systemic; however, this event is unusual. Such circumstances include disseminated neonatal HSV infection with multiorgan involvement, multiorgan disease of pregnancy, and, rarely, dissemination in immunosuppressed patients. It is likely that widespread organ involvement is the consequence of viremia in a host incapable of limiting replication to mucosal surfaces.

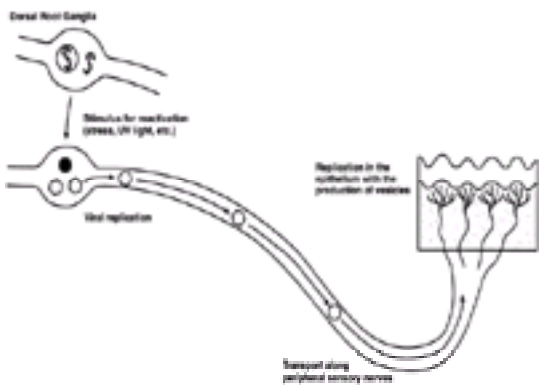


FIG. 5. Schematic diagram of latency and reactivation

Animal models provide insight into the transport of virus to the ganglia by retrograde axonal flow (103,195,261). Both histopathologic (using immunofluorescence staining of targeted cells) and electron-microscopic studies have unequivocally established axonal transport as a mechanism for translocation of the virus from a peripheral site to a central one. Virus is transported by the sensory nerves to central axons, where translocation of the capsid containing viral DNA occurs at a rate of approximately 10 mm/hr. This rate is similar to that of retrograde axoplasmic flow (104,261). If axonal flow is interrupted by severing the nerve or by chemical manipulation, translocation of virus from a peripheral mucocutaneous site to the associated ganglia will not occur.

After transport, virus replicates for several days in the associated sensory ganglia that innervate the site(s) of inoculation (103,193,194). Once replication in the sensory ganglia is complete, antiviral drugs have no effect on the elimination of virus from its latent state. Thus, one significant conclusion is that latent virus does not multiply; therefore, it is not susceptible to drugs that affect viral DNA synthesis. Numerous studies have attempted to eliminate latent HSV by treatment with nucleoside analogs, but all were unequivocal failures. It must be reemphasized that the mechanisms by which HSV establishes a latent state and maintains it are not yet well understood.

Operative definitions of the nature of the infection are of pathogenic relevance. Human definitions will be applied to both systems. For individuals susceptible to HSV infections (namely, those without preexisting antibodies), the first exposure to either HSV-1 or HSV-2 results in primary infection. The epidemiology and clinical characteristics of primary infection are distinctly different from those associated with recurrent infection, as will be noted later. After the establishment of latency, a recurrence of HSV is known as recurrent infection. This form of infection results in vesicular lesions of the skin such as HSV labialis or recurrent HSV genitalis. An individual with preexisting antibodies to one type of HSV (i.e., HSV-1 or HSV-2) can experience a first infection with the opposite virus type (i.e., HSV-2 or HSV-1, respectively) at a different site. Under such circumstances, the infection is known as an initial infection rather than as a primary infection.

An example of an initial infection occurs in those individuals who have preexisting HSV-1 antibodies (acquired after HSV gingivostomatitis) and then acquire a genital HSV-2 infection. Reinfection with a different strain of HSV can occur,

although it is extremely uncommon in the normal host. When this does occur, it is called exogenous reinfection.

A fundamental question that arises from the appearance of recurrent lesions, either of the lip or of the genital tract, is, Can exogenous reinfection with the same or opposite strain of virus account for “apparent” recurrent HSV infections? Clinical evidence, as early as 1968, had suggested that this was a possibility (332). However, in 1979, further speculation regarding exogenous reinfection was clarified by an important observation in an animal model. Namely, it was possible to reinfect both mice and *Cebus* monkeys with HSV-2 by the genital route after they had recovered from prior genital infection at the same site and with the same virus type (284). Unequivocal demonstration of exogenous reinfection became possible only after the development of restriction endonuclease technology, as described later.

UNIQUE BIOLOGIC PROPERTIES OF HSV

HSV has two unique biologic properties that influence human disease: neurovirulence and latency. The propensity of HSV to invade and replicate in nervous system tissue can result in profound disease with severe neurologic devastation. This is especially the case for HSV encephalitis. Alternatively, virus can infect sensory ganglia without resulting in significant disease. When this occurs, latency is established, thus providing a reservoir for virus and allowing its transmission to susceptible persons if reactivated.

Neurovirulence

Neurovirulence encompasses both neuroinvasiveness from peripheral sites and the ability of HSV to replicate in neuronal cells. While this property may be the function of numerous genes, the deletion of one gene in particular, $g_1$34.5, removes the ability of HSV to both invade and replicate in the CNS (93). The molecular basis for the failure of $g_1$34.5-deleted mutants to multiply in CNS is unclear and probably different from the function of the protein in directing the dephosphorylation of eIF-2a. Regardless, the inability of viruses with this gene deletion to replicate in the CNS and their significantly decreased ability to establish latency provide a foundation for the use of this virus as a vector for gene therapy and the development of a genetically engineered vaccine.

Mutations affecting neuroinvasiveness have also been mapped in genes encoding glycoproteins. These findings are not unexpected. Access to neuronal cells from usual portals of entry into the body requires postsynaptic transmission of virus and, therefore, a particularly vigorous capacity to multiply and to sort the virions to appropriate membranes. In addition, because neuronal cells do not make cellular DNA, they lack the precursors for viral DNA synthesis that are also encoded by those viral genes that are dispensable for replication in cell culture.

Latency

The ability of HSV to establish latency has been recognized biologically since the beginning of the 20th century. Following entry and infection of nerve endings, both HSV-1 and HSV-2 are transported by retrograde movement to the nuclei of sensory ganglia (494). The available evidence indicates that the virus multiplies in a small number of sensory neurons. In the majority of the infected neurons, the viral genome remains in an episomal state for the entire life of the individual. Reactivations occur following a variety of local or systemic stimuli such as physical or emotional stress, fever, exposure to ultraviolet (UV) light, tissue damage, and immune suppression.

Little is known regarding the mechanisms by which the virus establishes and maintains a latent state or is reactivated. In fact, disagreements exist on the fate of neurons in which latent virus becomes reactivated (reviewed in ref. 412). The relevant issues may be summarized as follows:

1. Sensory neurons harboring virus contain nuclear transcripts arising from the sequences *ab* and *b'a'* flanking U_L. To date, two sets of transcripts, designated latency-associated transcripts (LAT) have been detected. The most abundant transcripts arise from 2.0 domain mapping 3' to the gene encoding ICP0. This domain encodes two RNAs, a 2.0-kb and a 1.5-kb RNA. Both RNAs are nuclear and both have characteristics of stable introns. They do not appear to encode proteins. The other RNA detected to date is an 8.5-kb species of much lower abundance. This species appears to include the 2.0-kb sequence contained in the stable intron. The issue then is if the 2.0-kb RNA and the 1.5-kb RNA are introns, where is the product of the splicing event? Furthermore, deletion of the domain of the 2.0-kb RNA or of its promoter decreases the number of neurons harboring latent virus and the frequency of reactivation, but it does not abolish either process.

Recent studies on the 8.5-kb DNA domain transcribed during latency led to the discovery of two open reading frames (ORFs) totally repressed by ICP4 during productive infection. These ORFs, designated ORF P and ORF O, are antisense to the $g_1$34.5 gene. ORF P protein has been shown to bind p32, a protein involved in splicing. In cells in which ORF P is derepressed, ICP0 and ICP22, two proteins made from spliced mRNAs, are grossly underproduced early in infection. Late in infection, these two proteins accumulate concomitant with extensive posttranslational processing of ORF P protein. The ORF O protein binds to ICP4 and blocks it from binding its cognate site on DNA *in vitro*.

The mechanism whereby HSV establishes latency remains obscure, but probably not for long. It is likely that establishment of latency is based on several events occurring simultaneously. These could well be expression of viral genes that block a proteins from being made, absence of factors that enable high-level expression of a genes, and cell factors that do not favor or actually participate in the repression of viral gene expression.

2. As noted before, replication of HSV-1 and HSV-2 results in the destruction of the infected cell. It has been suggested that reactivation of latent virus in sensory neurons does not destroy the neuron harboring the virus. This suggestion is based on the observation that patients do not suffer from local anesthesia at the site of frequent, multiple recurrences. Alternatively, nerve endings from adjacent tissues innervated by other neurons could extend into the site of the healed lesion. Finally, it has also been suggested that small amounts of virus could remain latent in peripheral tissues. The

answer to this puzzle has not been forthcoming.

Further study of the biology of latency is indicated. Patients treated for trigeminal neuralgia by sectioning a branch of the trigeminal nerve can develop herpetic lesions along the innervated areas of the sectioned branch (117). This association was also suggested by Goodpasture (166). Carlton and Kilbourne (78), Pazin et al. (375,376), and others (141) have demonstrated that microvascular surgery of the trigeminal nerve tract, performed to alleviate the pain associated with tic douloureux, resulted in recurrent herpetic lesions in over 90% of seropositive individuals.

Latent virus has been retrieved from the trigeminal, sacral, and vagal ganglia of humans (29,30,32,34). In humans, the frequency of isolation of HSV from ganglia was described by Baringer (29,30,32) and others (34,528). In one study, virus isolation was attempted from the trigeminal ganglia of 90 cadavers and was documented in 44 individual situations. In 26 of these cases, HSV was isolated bilaterally from both ganglia. The retrieval of virus from the trigeminal ganglia helps explain the observation of vesicles that recur at the same site in humans, usually the vermilion border of the lip. Similarly, when 68 sacral ganglia were co-cultivated, only nine were positive and all for HSV-2. Baringer extended these observations to successfully retrieve virus from the thoracic ganglia of one patient whose trigeminal ganglia yielded HSV-1 bilaterally (29,30,32). A model of reactivation appears in Figure 5.

Reactivation of latent virus appears to depend on an intact anterior nerve route and peripheral nerve pathways. Induction of viral replication by axonal injury was reported by Walz et al. (526). They reactivated latent ganglionic infection by sectioning a peripheral nerve, resulting in the appearance of virus within the ganglia 3 to 5 days after the surgical manipulation. Not surprisingly, if an attempt is made to excise the lesions induced by HSV, vesicles will still recur adjacent to the site of excision (234,526). Recurrences occur in the presence of both cell-mediated and humoral immunity. Recurrent herpes labialis is three times more frequent in febrile patients than in nonfebrile controls.

At present, no data support the contention that recurrent infections are the result of chronic viral production at the site of recurrence. Skin biopsies performed at the site of recurrent lesions, but between episodes of disease, for purposes of virus isolation have not resulted in the retrieval of virus by standard laboratory procedures, as noted previously (412). However, during the prodrome of recurrent HSV labialis, virus can be retrieved by needle biopsy or cell scrapings from apparently normal tissue (483).

Models of Disease Pathogenesis

Animal models have been used to study the pathogenesis of HSV infections, especially in the evaluation of latency, vaccines, and antiviral agents. Although these models have used routes of infection encountered in human disease (i.e., mucosal and skin inoculation), only a select few approximate the pathogenesis of human disease. Human models of disease have been reported as well.

Animal model studies become the only system in which the pathogenesis of infection outside the human can be delineated; however, herein lies a series of fundamental problems. These studies were reviewed by Kirchner and Kern, among others (229,230,241). The animal species (indicative of susceptibility), virus type, route of infection, state of immune competence, and specific viral strain will all influence disease pathogenesis (314,316). Disease progression alone can be selectively modulated by the specific strain of animal selected. The route and site of inoculation become especially important in distinguishing one disease state from another. Models of primary HSV infection vary from system to system (e.g., susceptibility of the mucosa for replication of HSV). The strengths and weaknesses of the most common models are summarized next.

Both encephalitic (virulence) and latency models have been defined for inoculation of HSV-1 by the eye route, which is the best route to study latency. However, the end points of virulence and latency have distinctive differences for predicting human disease. After ocular inoculation by corneal scarification, replication of virus in the eye peaks within 48 hours and declines over the next 6 days. Virus appears in the trigeminal ganglia approximately 1 day after inoculation, with peak replication occurring 4 to 6 days after inoculation. If a sufficient quantity of virus and/or a virulent strain of virus is inoculated onto the eye, brain infection follows replication of virus within the trigeminal ganglia. The extent of disease (localized to the eye and trigeminal ganglia, not invasive disease) depends on a variety of factors, as noted earlier. Invasive brain infection is a rare event, if it has ever occurred, after human eye infection. Nevertheless, the trigeminal ganglion is routinely involved with human eye infection (239,247).

For HSV-1 infections of the skin, mice (particularly the hairless mouse) (516) and rabbits or guinea pigs with abraded or punctured skin were used to study the events of replication and disease pathogenesis and, more frequently, to evaluate antiviral therapies. Although in each system the nature, duration, and histopathology parallel human infection, there are notable differences. The hairless mouse, guinea pig, and rabbit are all artificial models of human infections, particularly for recurrent infections of humans. Regardless, as a screen for antiviral drug evaluations, they are of value.

HSV-2 mucosal infections have been studied most extensively in the guinea pig model. Both HSV-1 and HSV-2 have been used in this model. Intravaginal inoculation of HSV into female Hartley guinea pigs appears to be the most predictive of all the animal models for human disease, although not totally so. Reasons for these inconsistencies include endogenous host defenses, the artificial nature of infection, and viral strain differences. Moreover, these animals tend to experience continual recurrences of lesions, but retrieval of HSV from these lesions is most variable. This propensity for an enhanced frequency of recurrent lesions that are usually culture negative for virus has raised questions regarding the validity of the model for studies of the pathogenesis and molecular basis of latency of HSV-2. Nevertheless, it is the best available model.

Models of life-threatening disease have been developed for both HSV-1 and HSV-2. Intranasal inoculation of HSV-1 or HSV-2 into young (3-week-old) BALB/c mice leads to CNS and visceral (usually lung) disease, which is considered similar to neonatal HSV infection. However, inoculation of older mice with similar quantities of either of these two viruses may not result in any evidence of disease, or, if it does lead to disease, it might be encephalitis but certainly not

overwhelming multiorgan disease. Intracerebral inoculation of virus provides an unnatural route of infection even for the study of antiviral therapeutics; however, it is used to screen new drugs.

Accurate and predictive models of human HSV encephalitis have been described using rabbit (440,441) and mouse models. The resultant disease leads to focal localization of infection in the rabbit brain, as compared with diffuse pancortical infection of the CNS. Virus is inoculated directly into the olfactory bulb or upon abraded nasal epithelium of the rabbit over the nerves from the olfactory bulb. Virus can be traced along the olfactory tract to the anterior-frontal region of the rabbit brain. This region correlates with the temporal lobe of humans. Immunosuppression after subclinical infection can result in focal reactivation (498).

Over the past several years, human models of both labial and genital herpes have been reported. Spruance et al. have induced reactivation of HSV labialis by exposure of the vermilion border of the lip of seropositive individuals to UV light (482). The resulting lesions and course of infection are similar to those that occur in nature. Lesions develop in approximately 70% of UV-exposed volunteers after approximately 3 days. This model has been exploited for the evaluation of antiviral therapies (415).

A UV model was developed by Rooney et al. for individuals with HSV-2 infection of the buttocks and thigh. The incidence of reactivation is lower, approximately 35%, and the incubation period longer, about 5 days (416).

EPIDEMIOLOGY

Orolabial HSV Infections

HSV disease ranges from the usual case of mild illness, undiscernible in the majority of patients, to a few cases of sporadic, severe, and life-threatening disease in infants, children, and adults. Although HSV-1 and HSV-2 are usually transmitted by different routes and involve different areas of the body, there is a great deal of overlap between the epidemiology and clinical manifestations of infections. HSV infections are distributed worldwide and have been reported in both developed and underdeveloped countries, including remote Brazilian tribes (46). Animal vectors for human HSV infections have not been described; therefore, humans remain the sole reservoir for transmission of these viruses to other humans. Virus is transmitted from infected to susceptible individuals during close personal contact. There is no seasonal variation in the incidence of infection. Because infection is rarely fatal and because these viruses become latent, over half of the world's population probably has recurrent HSV infections and, therefore, the capability of transmitting HSV during episodes of productive infection.

Primary HSV-1 infections usually occur in the young child, less than 5 years of age, and are most often asymptomatic. The mouth and lips are the most common sites of HSV-1 infections; however, any organ can become infected with this virus. With clinical illness, gingivostomatitis usually is the manifestation. Primary infection in young adults has been associated with only pharyngitis and a mononucleosis-like syndrome (163,303).

After the implication of HSV as the etiologic agent responsible for gingivostomatitis (126) and its confirmation by Burnett and Williams (71), virologic screening became a useful tool for epidemiologic surveys. Dodd et al. (126) determined that primary infection led to the shedding of virus in mouth and stool and from the former site for as long as 23 days (average, 7 to 10 days). This observation has been recently confirmed in a controlled trial of acyclovir for gingivostomatitis (9). Neutralizing antibodies begin to appear between days 4 and 7 after the onset of disease and peak in approximately 3 weeks. In these studies, virus could be isolated from the saliva of asymptomatic children (68). Viral shedding was documented in approximately 20% of the children in the age range of 7 months to 2 years. Despite no overall race or sex differences in the study, the young African-American children had higher rates of both neutralizing antibodies and virus isolation than did their white counterparts. Virus shedding in children less than 6 months of age was uncommon, as has been noted in more recent studies (216). In older children, 3 to 14 years of age, presumed asymptomatic shedding was documented in 18%. Virus retrieval decreased with advancing age; in individuals over 15 years of age, the frequency of excretion was 2.7%. These frequencies of shedding are similar to contemporary cross-sectional surveys, ranging from 2% to 5% (83,129,246,257,280,365,491,567).

The influence of age is underscored by the results of two other studies (121,133). A thorough clinicoserologic study of primary herpetic oral infection was performed in Yugoslavia. Of the 18,730 children attending outpatient clinics, evidence of oral herpetic infection was found in approximately 13% over a 10-year period. Children 1 to 2 years of age were most commonly afflicted, accounting for over half of all cases. Neither difference in sex nor seasonal variation was detected.

Most children experience asymptomatic infection. Cesario et al. (83) reported a study of 70 seronegative children who had evidence of primary infection. Only 6 of the 70 children, or less than 10%, had clinical symptoms associated with illness. In an Australian study, Anderson and Hamilton (12) found that 67.4% of seronegative children (29 of 43) developed HSV antibodies over a period of 1 year; but less than one third of these children had symptomatic infections.

Geographic location, socioeconomic status, and age are the primary factors that influence the acquisition of HSV infection, regardless of the mode of assessment (126,397,450,489,505). In developing countries, seroconversion occurs early in life, but with some minor differences between selected populations. When classic antibody assays have been used to assess status of infection among Brazilian Indians and children of New Orleans, HSV antibodies were found in over 95% of children by 15 years of age (26,47). In Uruguay and lower socioeconomic populations of the central United States, the appearance of antibodies occurred at similar but lower frequencies (47). These lower rates of acquisition were particularly evident in the poor African-American communities of Atlanta and Houston (334,335,402). By 5 years of age, approximately one third of patients seroconverted; this frequency increased to 70% to 80% by early adolescence. Predictably, presumed middle-class individuals of industrialized societies acquire antibodies at a later age. Seroconversion occurred over the first 5 years of life in 20% of children, followed by no significant increase until the second and third decades of life, at which time the prevalence of antibodies increased to 40% and 60%, respectively (434,531), as noted in other studies (163,300,472). Seroconversion of susceptible university students occurs at an annual

frequency of approximately 5% to 10% (161,163).

Using type-specific serologic assays, the seroprevalence of HSV-1 and HSV-2 infections was redefined using sera obtained from two randomized National Health and Nutrition Examination Surveys (NHANES) (148,331,340). The seroprevalence of HSV-1 is more common than its counterpart HSV-2. For the United States, a summary of these HSV-1 seroprevalence appears in Figure 6. These data indicate that by 5 years of age, over 35% of African-American children versus 18% of white children are infected with HSV-1. There were no significant differences in seroprevalence between the two NHANES studies performed on sera collected in 1978 and then again in 1988 to 1994. Subsequently, through adolescence, African-Americans had approximately a twofold higher prevalence of antibodies to HSV-1 than did whites, and females had a slightly higher prevalence of HSV-1 antibodies than did males. Furthermore, the female prevalence was slightly higher for HSV-1 antibodies than the male. By 40 years of age, both African-Americans and whites had similarly high prevalences of antibodies, which may be the consequence of a cohort effect; that is, these individuals had a higher rate of acquisition of HSV-1 earlier in life.

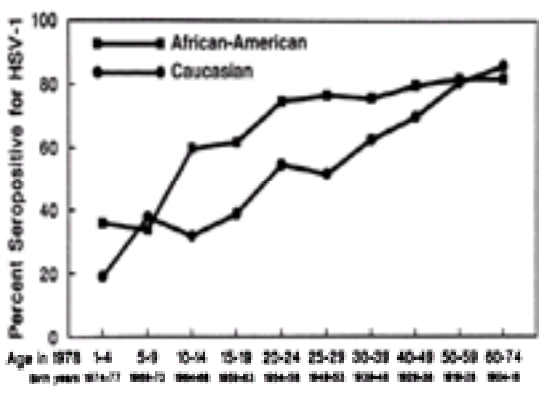


FIG. 6. Seroprevalence of HSV-1 by age.

When similar analyses were performed on populations from a variety of countries around the world, notable differences were detected. For example, only 5 of 18 countries surveyed had an HSV-1 antibody prevalence of less than 70%. These included white individuals in the United States (U.S. NHANES participants) as well as selected populations residing in Pittsburgh, Pennsylvania; Birmingham, Alabama; Atlanta, Georgia; Japan; Lyon, France; and Sweden. An antibody prevalence in excess of 95% was detected in Spain, Italy, Rwanda, Zaire, Senegal, China, Taiwan, Haiti, Jamaica, Uganda, and Costa Rica. Seroprevalence was determined for adults between the ages of 20 and 40 years. Thus, a high prevalence of antibodies to HSV-1 exists worldwide and, therefore, a high rate of infection, but there is significant country-to-country variation (545).

In summary, these studies demonstrate a significantly lower prevalence of antibodies during childhood, adolescence, and even later in life among relatively middle and upper socioeconomic classes. Primary infection occurs much earlier in life, generally very early, in children of underdeveloped countries as well as in those of lower socioeconomic classes, whereas in developed countries and more affluent classes, primary infection may be delayed until adolescence or perhaps even adulthood. The frequency of direct person-to-person contact, indicative of crowding encountered with lower socioeconomic status, appears to be the major mediator of infection.

Recurrent HSV-1 Infection

The largest reservoir of HSV-1 infections in the community is recurrent herpes labialis. Studies performed to assess the frequency and severity of recurrent infection in the immunocompetent host are limited. Baseline frequencies for recurrent herpes labialis have been defined from a study performed in Philadelphia. A positive history of recurrent herpes labialis was noted in 38% of 1,800 graduate students (460,461). New lesions occurred at a frequency of one per month in 5% of the students and at intervals of 2 to 11 months in 34% of the students. Recurrences of one per year or less often were found in 61%. The predictable frequency of recurrence is relatively constant, approximately 33% in several studies (156,458,459). Exceptions to this rate of recurrence were reported by Young et al. (567) and others (129), who reported lower frequencies of recurrences of approximately 16%, but under vastly different (namely, lower) socioeconomic conditions. Ironically, one other study found that recurrences occur more frequently among the more socially privileged (459), suggesting more attention to relatively trivial medical illnesses.

Recurrent infection may occur in the absence of clinical symptoms. Asymptomatic excretion of HSV in normal children after recurrence is about 1% (183,186) and varies from approximately 1% to 5% in the normal adult (163,180,181,280,456). Nearly 1% of pregnant women and nursery personnel excrete HSV at any time (181), providing a source of virus for transmission to the newborn. Asymptomatic excretion of virus is not limited to the healthy adult; excretion of HSV in renal transplant recipients, without signs or symptoms of disease, occurs in nearly one third of seropositive patients (372,373). Studies of closed populations have documented a high frequency of recurrent HSV excretion in the absence of symptoms (83). A summary of seroprevalence of HSV-1 infections by geographic locale and over time is illustrated in Figure 7.

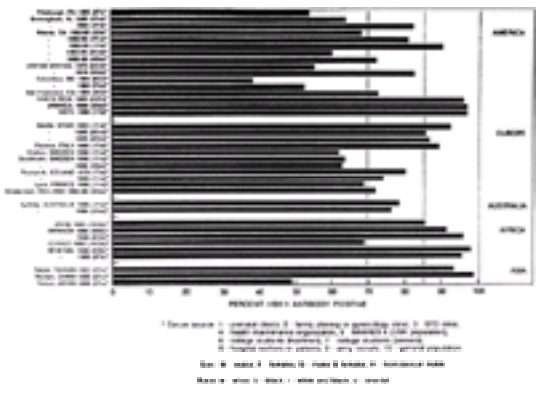


FIG. 7. Seroprevalence of HSV-1 by specific populations.

Unusual Epidemiologic Infections

Clustered outbreaks of human HSV infections have been reported (216,450); however, there is no indication from either clinical or molecular epidemiologic studies that these viruses cause epidemic diseases. Most of the studies reported involve families in which several individuals within the family had HSV infection at approximately the same time. No index case could be identified, although recurrent labial lesions of one family member were frequently incriminated.

Outbreaks within hospitals have been identified; however, here also no clear epidemic nature of the disease could be determined. The frequent occurrence of HSV infections among renal transplant recipients within the 4-week period immediately after surgery was reported (373). Pugh et al. (386) described an outbreak of eczema herpeticum in a large group of hospitalized patients within an 8-day period; in this case, hospital conditions (particularly the lack of attention to nursing details, such as hand-washing) were incriminated as being responsible for virus transmission.

Outbreaks of herpetic stomatitis have been reported within orphanages (172,216), where the attack rate for clinically apparent primary infections was approximately 75% of the susceptible patients. Similarly, an outbreak of a larger number of cases than expected of HSV encephalitis was recognized in Boston; however, no common virus was identified (174). Interestingly, increased transmission of HSV in day care centers has not yet been reported to be a problem (444).

Genital HSV Infections

Because infections with HSV-2 are usually acquired through sexual contact, antibodies to this virus are rarely found before the age of onset of sexual activity. Although most genital HSV infections are caused by HSV-2, a recognizable and ever-increasing but variable proportion is attributable to HSV-1 (107,221,473,559). The distinction in virus type is not insignificant, because genital HSV-1 infections are usually both less severe clinically and less prone to recur (107,404). Sexual transmission is the primary route of the spread of HSV-2 (120,213,214 and 215,368).

Clinics treating sexually transmitted diseases (STDs) provide one setting for acquiring prevalence data on genital HSV infections, particularly those in the United States, England, and Sweden (36,142,215,343). The number of new cases of genital HSV infections has been conservatively estimated to be approximately 500,000 individuals per year (343). Because genital HSV infections are not reportable diseases in the United States, these rates are estimates (205). The number of new cases of recognized STDs was used to determine estimates of the number of cases of genital herpes. If the incidence of new cases is calculated from a ratio of 5 to 10 cases of gonorrhea to every 1 case of genital HSV infection, approximately 300,000 new cases would occur yearly in the United States (210,211,343). A report by Corey et al. (110) suggests a much higher rate of infection, namely, a ratio of 1 case of genital herpes infection to 2.2 cases of gonorrhea. Such estimates would increase the frequency of primary disease. Current estimates of individuals with genital herpetic infection in the United States alone range from as low as 10 million to as high as 30 to 40 million (110,205). Predicated on newer serologic methods for detection of prior HSV-2 infection, as will be described later, a more accurate estimate range is 40 to 60 million infected individuals in the United States (293).

Cytopathologic screening by Papanicolaou staining of cervical scrapings or virus isolation from cervicovaginal swabs has determined the prevalence of genital HSV infection to range from 0.09% to 0.24% in normal women (127,129,213,244,346,519,558). In contrast, rates of infection in individuals routinely attending clinics for STD clinics vary between 0.002% and 3.3% and even 7.0%, depending on the population studied (130,346). These studies were performed over a decade ago, when the prevalence of the disease was much lower.

Women were reported to have higher rates of infection than were men, particularly prostitutes and others with multiple sex partners. Several studies estimate that the risk of susceptible females for contracting HSV from infected males is 80% after a single contact (333,381,398,399). However, the best data that define the rate of transmission emanate from couple studies of promising vaccines. In putative monogamous relationships, the risk of transmission from a seropositive male to a seronegative female is approximately 20% annually (59). If the female is seropositive for HSV-1, the risk is somewhat diminished to 15%. On the other hand, the risk of transmission from an HSV-2 seropositive female to a seronegative male is about 50% annually. The risk decreases to 5% if the male is HSV-1 seropositive (309). These data suggest that preexisting HSV-1 antibodies influence the frequency of HSV-2 acquisition. An interesting observation was made that relates the frequency of HSV shedding to the age of the prostitutes. The most active prostitutes (age range, 20 to 29 years) were most likely to excrete HSV (12%), whereas those in the older age groups had a decreased frequency (6%) of viral excretion. As would be expected, one study found that the incidence of genital HSV infections in both indigent women and those of middle and upper socioeconomic classes appeared significantly lower (0.3% and 0.2%, respectively) (354) than that found among women attending STD clinics.

As with HSV-1 infections of the mouth, HSV-2 can be excreted in the absence of symptoms at the time of primary, initial, or recurrent infection (138,392), provided a reservoir exists for transmission of infection. Among women evaluated prospectively after their first episode of genital herpes, asymptomatic shedding was detected in approximately 12%, 18%, and 23% of those with primary HSV-1, primary HSV-2, and nonprimary HSV-2 infection, respectively (248). Among women with established genital HSV-2 infection, asymptomatic shedding was detected on 1% of all days cultures were obtained (57). Shedding of virus occurs more frequently in the first year following genital infection than in subsequent years (524). Thus, asymptomatic shedding provides a significant source for virus transmission. The use of PCR to detect virus shedding markedly increases these rates, as is discussed later.

The appearance of type-specific HSV-2 antibodies correlates positively with the onset of sexual activity (3,335,402). Crowded living directly parallels increased antibody prevalence, as well (35,346). Historically, if HSV-2 antibodies are sought in healthy women, there is a wide discrepancy in prevalence, ranging from 10% in individuals in the United States to 77% in Ugandans (394). However, overall in the United States, the seroprevalence of HSV-2 is about 22%, a 30% increase over the past decade (148). As many as 50% to 60% of women at lower socioeconomic levels in the United States and elsewhere develop antibodies to HSV-2 by adulthood (148,341). Antibodies to HSV-2 are virtually nonexistent in nuns (335,397,558). This compares with similar data from Birmingham, Alabama, of 11.4% in 1985 and 28% in 1990. Sera collected from pregnant women in the mid 1980s from Padova, Italy; Orebro, Sweden; and Stockholm, Sweden, showed seroprevalence rates varying between 8% and 28%. Relevant to the importance of these serologic observations is the potential for these women to reactivate HSV-2 at the time of delivery and transmit infection to their infants. In contrast, 10% to 20% of women in higher socioeconomic groups are seropositive (18,300). Seroprevalence continues to increase over time, indicating widespread acquisition of HSV-2 infection.

Using type-specific antigens for HSV-2, antibodies were demonstrated in 35% of middle-class women receiving care through the Atlanta Health Maintenance Organization (274,336). Seroprevalence of HSV-2 increases from 6.9% at 15 to 29 years of age, to 23.4% by 60 years of age. When the populations are analyzed according to race, these same percentages were 4.6% and 19.7% for whites and 21.8% and 64.7% for African-Americans, respectively. Factors found to influence acquisition of HSV-2 include gender (greater for women than men), race (more frequent among African-Americans than whites), marital status (more for divorced individuals than single or married individuals), and place of residence (more in the city than in the suburbs). Importantly, the number of sexual partners greatly influences acquisition of infection (398,399 and 400). A recent NHANES III study has clarified the seroprevalence of HSV-2 in the population of the United States (Table 2 and Table 3). For all classes, the seroprevalence of HSV-2 was 21.9%. This represents an increase of 30% over a 10-year period (Table 3); it is graphically illustrated in Figure 8.

Variable	Overall[a]		Whites		Blacks		Mexican-Americans	
	Sample size	Percent prevalence (95% CI)	Sample size	Percent prevalence (95% CI)	Sample size	Percent prevalence (95% CI)	Sample size	Percent prevalence (95% CI)
Sex								
Both sexes	13,284	21.9 (20.3–23.6)	4737	17.6 (15.7–19.6)	3884	45.9 (42.9–47.0)	3991	22.3 (21.2–23.5)
Male	6,407	17.9 (15.6–20.2)	2363	14.3 (12.3–16.1)	1798	34.7 (32.3–37.0)	1992	19.2 (17.9–20.6)
Female	6,987	25.6 (24.0–27.3)	2544	20.2 (18.3–22.2)	2386	55.1 (52.7–57.5)	1999	25.7 (24.3–27.2)
Age (yr)								
12–19	2,398	5.6 (4.3–7.2)	905	4.5 (2.9–6.8)	868	8.7 (6.4–11.9)	870	5.4 (4.0–7.3)
20–29	2,792	17.2 (15.6–18.7)	675	14.7 (12.3–18.1)	801	33.6 (30.3–37.2)	1072	14.9 (12.9–17.3)
30–39	2,567	27.9 (24.8–31.2)	762	21.9 (18.7–25.6)	694	54.4 (50.3–58.6)	703	28.7 (26.3–31.4)
40–49	2,061	28.6 (25.5–30.0)	724	19.9 (15.4–24.1)	534	58.9 (53.7–62.4)	612	33.9 (28.4–38.3)
50–59	994	25.1 (21.5–29.4)	458	19.4 (15.6–24.1)	271	62.7 (54.4–72.3)	185	42.5 (31.5–47.1)
60–69	1,369	24.3 (22.2–26.1)	480	19.2 (17.7–24.3)	258	76.8 (72.3–81.5)	316	38.7 (32.5–46.8)
≥70	1,367	27.7 (24.6–31.1)	995	23.5 (20.5–27.0)	360	74.3 (67.6–81.6)	146	44.8 (38.8–51.2)

[a]Totals differ from the sums for whites, blacks, and Mexican-Americans because other races and ethnic groups are included in the overall totals.
NHANES III, National Health and Nutrition Examination Surveys III; CI, confidence interval.

TABLE 2. *HSV-2 seroprevalence in NHANES III (1988 to 1994) according to sex, age, and race or ethnic group*

Category of subjects	NHANES II		NHANES III	
	Sample size	Age-adjusted percent seroprevalence (95% CI)	Sample size	Age-adjusted percent seroprevalence (95% CI)
All races and ethnic groups[b]				
Both sexes	9,897	16.8 (14.7–17.4)	13,284	20.8 (19.2–22.6)
Men	1,681	13.4 (11.7–15.4)	6,407	17.3 (15.5–19.5)
Women	1,916	18.4 (16.5–20.5)	6,987	24.2 (22.7–25.7)
Whites				
Both sexes	2,153	12.7 (11.2–14.3)	4,737	16.5 (14.7–18.5)
Men	1,021	10.7 (8.7–13.0)	2,363	14.1 (11.6–17.2)
Women	1,132	14.6 (12.4–17.0)	2,344	18.7 (17.0–20.6)
Blacks				
Both sexes	1,130	43.6 (40.0–47.6)	3,884	47.6 (45.4–49.9)
Men	406	34.1 (30.0–38.7)	1,798	37.3 (34.8–40.0)
Women	635	51.4 (47.6–55.6)	2,086	55.7 (53.3–58.2)

[a]Seroprevalence has been age-adjusted to the 1980 census. The age range is 12 years.
[b]Totals differ from the totals of whites plus blacks because other races and ethnic groups are included in the category of all races and ethnic groups.
CI, confidence interval.

TABLE 3. *Changes in age-adjusted HSV-2 seroprevalence between NHANES II (1976 to 1980) and NHANES III (1988 to 1994)*[a]

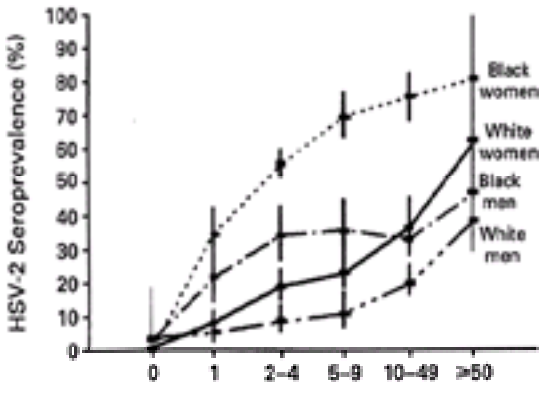


FIG. 8. Seroprevalence of HSV-2 by specific populations.

The highest prevalence of antibodies to HSV-2 in the United States was identified in female prostitutes (75%), as would be expected, a rate almost identical to that of prostitutes in Tokyo (340). Seropositivity among female prostitutes in Dakar, Senegal, was even higher, 95.7% in 1985. Homosexual men have seroprevalence rates to HSV-2 varying from a high of 83.1% in San Francisco (1985 to 1986) to lows of 21.6% in Seville, Spain (1985 to 1986); 24.2% in Tokyo (1988); and 50.0% in Amsterdam (1986). A comparison of HSV-1, HSV-2, or dual-antibody assessments by population is shown in Figure 9. Numerous seroprevalence studies have recently been reported (56,259,340,361,429).

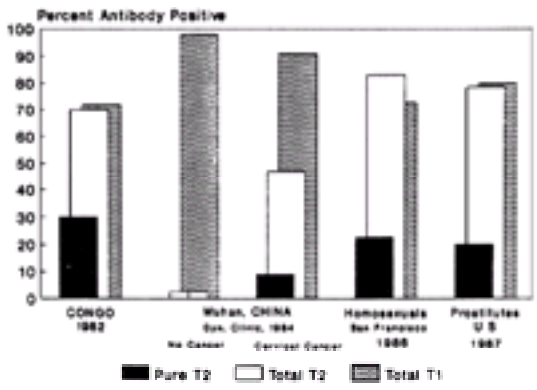


FIG. 9. Seroprevalence of HSV-1 and HSV-2 by selected country.

Some countries have surprisingly low HSV-2 seroprevalence rates. For example, in Italy, the seroprevalence was high in STD clinics (26%), but it was significantly lower in obstetric populations of Modena (5.3%) (118). These figures are in sharp contrast to data from the United States.

As noted, the number of different sexual partners has been found to correlate directly with acquisition of HSV-2, as shown in Figure 10. For heterosexual women living in the United States having one partner, the probability of acquisition of HSV-2 is less than 10%. The probability increases to 40%, 62%, and greater than 80% as the number of lifetime sexual partners increased to 2 to 10, to 11 to 50, or to greater than 50, respectively. For heterosexual men, similar data are 0% for one lifetime sexual partner and 20%, 35%, and 70% for each of the subsequent three risk groups. In contrast, for homosexual men, seroprevalence increases from greater than 60% to 90% for those with 11 to 50 and more than 50 partners. Thus, multiple sexual partners (“polypartnerism”), irrespective of sexual preference, correlates directly with acquisition of HSV-2 infection (340).

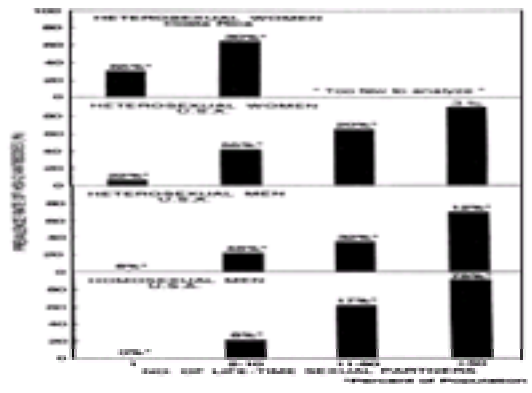


FIG. 10. Seroprevalence of HSV-2 as a function of the number of sexual partners.

The incidence of HSV-2 infection has been assessed prospectively. Among low-risk individuals, namely college students, the rate of acquisition was approximately 2% per year over 4 years, compared with 4% per year for homosexual men in San Francisco (340). The incidence of HSV-2 infection during pregnancy is about 2.5% per gestation but was reported by other investigators to be as low as 0.58% (53). Acquisition of HSV-2 infection between monogamous sexual partners with

discordant infection status is 10% to 15% yearly (309).

HSV-2 infection, by the nature of its being an ulcerative disease, is associated with the acquisition of both human immunodeficiency virus type 1 (HIV-1) and human T-cell lymphotropic virus type 1 (HTLV-1). Several case-control studies performed in Baltimore, San Francisco, Seattle, and Central and South Africa indicated increased risk ratios by one-and-one-half- to over twofold (54,88,97,200,201,226,340,484). One study, however, refutes this conclusion (238). The association between acquisition of HIV-1 and HSV-2 has been documented in heterosexual populations, as well (201).

Recurrent HSV-2 Infection

As with orolabial lesions, recurrent genital infection is the largest reservoir of HSV-2. Factors responsible for reactivation of genital HSV infections cannot be unequivocally gleaned from retrospective studies. Recurrent HSV-2 infection, like HSV-1, can be either symptomatic or asymptomatic. A recurrence is usually associated with a shorter duration of viral shedding and fewer lesions (107). The frequency of recurrences varies somewhat between men and women, with calculations of 2.7 and 1.9 per 100 patient-days, respectively (107). The actual number of days of viral excretion has been clarified in studies assessing detection of HSV DNA by PCR. Wald and colleagues (525) documented viral excretion in a select group of motivated women to be about 6% annually. Utilizing PCR, DNA could be detected in nearly 18% of days—a threefold increase over viral culture. Notably, there was an association between detection of virus by PCR (but not by culture) and person-to-person transmission.

Overall, several studies have reported a frequency of recurrence as high as 60% (3,85). The type of genital infection (HSV-1 or HSV-2) is predictive of the frequency of recurrence (91,404). HSV-1 infection recurs less frequently than does HSV-2 infection for reasons that are entirely unclear (37,267). The changing prevalence of HSV-2 infections is illustrated by surveys of representative populations (Table 4).

Site of study	Year of study	Prevalence of HSV-2 antibodies (%)
Different individuals		
Reykjavik, Iceland (pregnant women)	1970	4.1
	1985	23.4
Lyon, France (pregnant women)	1977–1978	10.7
	1985	17.3
Orebro, Sweden (pregnant women)	1982	11.8
	1985	16.1
Zaire		
Kinshasa	1959	21
	1985	60
Rural areas	1959	6
	1985	33
Same individuals		
Alabama (pregnant women)	1985	6-mo acquisition rate = 2%
South Carolina (university students)	1983	0.4 (acquisition rate = 2%/year)
	1987	7.0
San Francisco (homosexual men)	1978	44 (acquisition rate = 4%/year)
	1985	72

TABLE 4. *Prevalence of HSV-2 antibodies in different populations*

Molecular Epidemiology

Hayward et al. (184) found that fragments resulting from the cleavage of HSV DNA by restriction endonuclease enzymes define fingerprints for the virus. Analyses of numerous HSV-1 and HSV-2 isolates from a variety of clinical situations and widely divergent geographic areas demonstrated that epidemiologically unrelated strains yielded distinct HSV DNA fragment patterns. In contrast, fragments of HSV DNA derived from the same individual obtained years apart, such as monogamous sexual partners, or after short and long passages *in vitro*, had identical fragments after restriction endonuclease cleavage.

This technique has been applied to specimens collected from a variety of patients with a genital HSV infection in order to assess potential strain differences or similarities after digestion with various endonucleases. Analyses of patterns of HSV DNA fragments showed that isolates from the same patient or their respective sexual partners could be either the same or different (66,67). Stated differently, a given patient might well have nonidentical isolates obtained from lesions at adjacent sites. This finding indicates that an individual can be infected with multiple, different strains. No doubt, similar findings will become apparent for orolabial HSV-1 infections when properly studied. The use of endonuclease restriction enzymes has unequivocally demonstrated that a target organ, in this case the genitalia, is not immune to reinfection. The frequency of this occurrence in large-scale studies is low (269,443). From a public health viewpoint, this is an extremely important issue. If exogenous reinfection occurred frequently (regardless of the actual incidence), both the potential of vaccination and the methods for vaccination would have to be seriously reassessed to provide immunity to target organs. Protection of mucosal sites from the initiation of replication after primary infection becomes exceedingly important. Clearly, an exogenous source of virus does not explain the majority of instances of apparent reactivation.

Maternal Genital HSV Infections

Genital HSV infection in the pregnant woman is as common as in age-matched, nonpregnant women. However, it must be considered separately from nonpregnant populations because of the risk to the fetus and newborn, as was recently reviewed (540).

An uncommon problem encountered with HSV infections during pregnancy is that of widely disseminated maternal disease. As first reported by Flewett et al. (149) in 1969 and by others subsequently (10,167,190,377,566), HSV disease can involve multiple visceral sites, in addition to cutaneous dissemination. In a limited number of cases, dissemination after primary oropharyngeal or genital infection has led to life-threatening disease, such as necrotizing hepatitis with or

without thrombocytopenia, leukopenia, disseminated intravascular coagulopathy, and encephalitis. The mortality rate among these pregnant women is reported to be greater than 50%. Fetal deaths also have occurred in more than 50% of cases, although mortality did not necessarily correlate with the death of the mother. Surviving fetuses were delivered by cesarean section either during the acute illness or at term, and none had evidence of neonatal HSV infection. Cumulative experience suggests that factors associated with pregnancy may place both mother and fetus at increased risk for severe infection, possibly because of altered cell-mediated immunity (378,509). Fortunately, with the advent of safe antiviral therapy, specifically the availability of acyclovir, if HSV disease appears progressive in the pregnant woman, treatment is initiated.

From several prospective trials, the major risk to the fetus is maternal primary or initial genital HSV infection (58,262). Thus, identification of the woman at risk for primary infection (seronegative for HSV-2) is of paramount importance. Studies of the discordant serologic status of sexual partners and rates of acquisition of genital HSV-2 infection begin to define this problem, as recently summarized (383). With primary or initial genital infection occurring at rates from 0.5% to 10% per year, precise risks are not clear (62,383); however, incidence according to maternal infection is becoming clearer. If a pregnant woman experiences initial or primary genital infection in the last trimester of gestation, the likelihood of transmission varies between 30% and 50%. On the other hand, recurrent maternal infection is associated with a transmission of 3% or less (59). These different rates of transmission have direct implications on strategies to prevent neonatal disease or at least ameliorate it if the child is inadvertently delivered through an infected birth canal.

Maternal primary infection before 20 weeks gestation in some women has been associated with spontaneous abortion (336). Although the original estimate of spontaneous abortion after a symptomatic primary infection during gestation was thought to be as high as 25%, it is likely that this calculation is high because of the very small number of women followed. Precise data indicating the true risk of spontaneous abortion after primary infection during gestation are not available at this time. Furthermore, the contribution of primary maternal genital infection to spontaneous abortion must be weighed above that of a routine rate of fetal loss of approximately 20%. Infection that develops later in gestation has not been associated with the termination of pregnancy (168,178,338). Notably, morbidity of the fetus born to a woman with primary infection was documented with manifestations of either neonatal HSV disease or severe intrauterine growth retardation as compared with controls (60).

Recurrent infection is the most common form of infection during gestation. The frequency of HSV recurrences during gestation should be of concern for women with a known history of infection. Transmission of infection to the fetus is most frequently related to the actual shedding of virus at the time of delivery. Because HSV infection of the fetus is usually the consequence of contact with infected maternal genital secretions at the time of delivery, the determination of viral excretion at this time is important. The actual incidence of viral excretion at delivery was suggested to be 0.01% to 0.39% for all women, irrespective of past history (338,392,504,521), and perhaps as high as 0.6% (16,58).

Several prospective studies have evaluated the frequency and nature of viral shedding in pregnant women with a known history of genital herpes. In a predominantly white, middle-class population, documented recurrent infection occurred in 84% of pregnant women with a history of recurrent disease (521). Moreover, asymptomatic viral shedding occurred in at least 12% of the recurrent episodes. Viral shedding from the cervix occurred in 0.56% of symptomatic infections and in 0.66% of asymptomatic infections. These data are similar to those obtained in other populations (16,51,392). The incidence of cervical shedding in pregnant women with asymptomatic HSV infection was reported to average approximately 3% (182). However, the observed rate of shedding among pregnant women with asymptomatic infection has varied more than that among nonpregnant women (from 0.2% to 7.4%), depending on the study population and trial design (51,168,170,179,392). The frequency of recurrences has not been shown to be different from one pregnancy to the next for a given woman (179). Overall, these data indicate that the frequency of cervical shedding is low, rendering the risk of transmission of virus to the infant similarly low when the infection is recurrent in nature (338). The frequency of shedding does not appear to vary by trimester during gestation (179,521). No increased incidence of premature onset of labor is apparent in these prospective studies. Regardless, given the high seroprevalence of infection, a significant degree of protection for the fetus must exist, or the incidence of neonatal disease would be significantly higher.

Importantly, most infants who develop neonatal disease are born to women who are completely asymptomatic for genital HSV infections at the time of delivery and have neither a past history of genital herpes nor a sexual partner reporting a genital vesicular rash (338,543,551,555). These women account for 60% to 80% of all women whose infected children become clinically ill. An analysis of demographic characteristics of those women delivering children who developed neonatal HSV infection has been summarized (543). Only 27% of these women had either a history of or evidence of recurrent lesions indicative of HSV infection during the current pregnancy. Furthermore, only half of this latter group of women reported genital HSV infection in their sexual partners.

HOST IMMUNOLOGIC RESPONSE

The natural history of HSV infections is influenced by both specific and nonspecific host defense mechanisms, as recently reviewed (287). Animal models have provided the majority of data that address the relationship between host defense and disease pathogenesis. Host genetic background, macrophages, natural killer cells, specific T-cell subpopulations, specific antibodies, and lymphokine responses have been implicated as important host defenses against HSV infections. Notably, the relative value of each varies according to the route of viral inoculation, among other factors. Specifically, intraperitoneal and intracerebral inoculation most likely require host defense mechanisms that are different from those needed for the control of ocular infections.

Studies performed by Lopez (285,286) have confirmed the relative importance of host genetics on disease pathogenesis when virus is inoculated by the peritoneal route to inbred strains of mice. Differences in outcome are notable according to the virus type studied (80). For example, when HSV-1 was inoculated into inbred strains of mice, resistance was inherited as a dominant trait, involving two loci, and not restricted to the histocompatibility complex. If HSV-2 was used, however, resistance patterns were less apparent and implicated macrophage replication of virus as the major determinant of

outcome (319,320,321,322 and 323).

Experiments on macrophage function in response to HSV infection in animal models have been conducted for some time. Macrophage activation and inactivation experiments can either reduce or increase mortality, respectively (241,299,326,448,488,571). Such findings have been particularly well characterized in a newborn mouse model. However, the relevance of these observations to the pathogenesis of human infection remains to be established, particularly for neonatal HSV infection. Macrophage responses are dependent on lymphokine modulation, at least after intraperitoneal inoculation.

Both animal model and human studies have provided a basis for understanding humoral and specific cell-mediated immune responses. With the appearance of nonspecific inflammatory changes, paralleling a peak in viral replication, specific host responses can be quantitated but vary from one animal system to the next (247,260,358). In the mouse, delayed-type hypersensitivity responses can be identified within 4 to 6 days after disease onset, followed by a cytotoxic T-lymphocyte (CTL) response and by the appearance of both immunoglobulin (Ig)M- and IgG-specific antibodies (223,245,247,352,560,569). Host responses in humans tend to be more delayed, developing approximately 7 to 10 days later than in the mouse. Immunodepletion studies identified the importance of CTLs in resolving cutaneous disease (240,350). Adoptive transfer of CD8+ or HSV-immune CD4+ T cells also reduced viral replication or provided protection upon challenge (52,278,350,454). Furthermore, the characterization of the CD8+ immune response in genital lesions has been correlated with disease resolution.

T-cell lymphocyte subsets were examined for host susceptibility to infection, including those responsible either for H2-restricted cytotoxicity or for *in vitro* or adoptive transfer of delayed-type hypersensitivity (256,352,353,387). These latter cells have a requirement for both the IA and the H2 K/D regions (351). Similar specific T cells are under investigation in humans. Notably, studies using a specific infected cell polypeptide product (ICP4) have identified its requirement for mediation of T cells (297). Humoral immunity to HSV infection was evaluated exhaustively in disease pathogenesis. An increasing body of data suggests that prior HSV-1 infection, with its subsequent host response, has a somewhat protective effect on the acquisition of HSV-2 infection (309). Polyclonal antibodies were used to alter disease lethality, particularly in the newborn mouse (58,125,212,223,255,263,302,360,403,463). In addition, these antibodies were used to limit progression of both neurologic and ocular disease (119,223,302,359,360). With the development of monoclonal antibodies to specific infected cell polypeptides, especially the envelope glycoproteins, protection from lethality (27,125,311,403) can be achieved. As an aside, human trials of a human monoclonal antibody in combination with antiviral drugs will be explored for neonatal herpes. Success in the animal model was accomplished with both neutralizing and nonneutralizing antibodies. Antibody-dependent cell-mediated cellular cytotoxic host responses also have correlated with improved clinical outcome, as will be noted later for neonatal HSV infections. Taken together, these observations are extremely relevant for vaccine development.

The dissection of all arms of host immune responses during human disease has resulted in intense investigative efforts to attempt to develop effective vaccines. Clearly, an understanding of host immune responses must weigh the clinical observation of recurrent lesions in the presence of both humoral and cell-mediated immunity against the shortened duration of recurrent lesions. There is no resolution of this enigma at this time.

As with studies of strains of disease susceptibility in inbred strains of mice, a plethora of reports have incriminated or refuted human leukocyte antigen (HLA) associations with HSV infections (48,276). For recurrent fever blisters, these studies have included HLA-A1, HLA-A2, HLA-A9, HLA-BW16, and HLA-CW2 (48,84,98,352,570). Recurrent ocular HSV infections were associated with HLA-A1, HLA-A2, HLA-A9, and HLA-DR3 (98,314,315). These confusing and conflicting associations can be faulted by population selection bias, as reviewed by Rawls (393).

Humoral antibody responses of humans appear to parallel those documented after systemic infection of mice and rabbits. As classically described, IgM antibodies appear transiently and are followed by IgG and IgA antibodies, which tend to persist over time (42,139,157,220,264). Neutralizing and antibody-dependent cellular cytotoxic antibodies generally appear 2 to 6 weeks after infection and persist for the lifetime of the host (249,250,324,391,399,401). Analyses of antibody responses by immunoblot and immunoprecipitation have elucidated host responses to infected cell polypeptides and correlated these responses with the development of neutralizing antibodies (17,38,40,41,134,315 and 136,536,572). Soon after the onset of infection, antibodies to structural proteins are followed by those directed against gD, gB, ICP4, gE, gG-1 or gG-2, and gC (218). Both IgM and IgG antibodies can be demonstrated, depending on the time of assessment after disease onset.

When attempts are made to define the basis for frequent recurrences, research efforts have focused on cell-mediated immune responses. Lymphocyte blastogenesis studies demonstrate reactivity within 4 to 6 weeks after the onset of infection and sometimes as early as 2 weeks (113,371,391,423,424,425 and 426,457,487,499). With recurrences, boosts in blastogenic responses can be promptly defined; however, these responses, as after primary infection, decrease with time. Nonspecific blastogenic responses do not correlate with a history of recurrences. As noted, HSV-1 and HSV-2 antigens are cross-reactive in these assays. Specific CTL responses are under investigation. It has been suggested but not confirmed that cytotoxic cells are depressed in patients with frequent recurrences (510). Nevertheless, a similar case can be made for depression of natural killer cells. As noted, recent studies have demonstrated a role of envelope glycoproteins in the establishment of cell-mediated immunity (512). As with humoral responses, the appearance of lesions correlates with a boost in cytotoxic responses.

Lymphokine production was incriminated in the pathogenesis of frequently recurrent genital and labial HSV infection. Notably, several investigators have recognized a decrease in both gamma-interferon production and natural killer cells during the disease prodrome (116,366,455). No reproducible data from selected populations confirm these observations.

Host response of the newborn to HSV must be defined separately from that of older individuals. Impairment of host defense mechanisms is a cause of the increased severity of some infectious agents in the fetus and the newborn. Factors that must be considered in defining the host response of the newborn include the mode of transmission of the

agent (viremia versus mucocutaneous infection without blood-borne spread), and the time of acquisition of infection. Two broad issues are of relevance: (a) protection of the fetus by transplacental antibodies and (b) definition of host response of the newborn.

Host response to HSV infection may well influence (a) the acquisition of disease, (b) the severity of infection, and (c) host resistance to the development, maintenance, and subsequent reactivation of HSV. Clearly, humoral immunity does not prevent either recurrences or exogenous reinfection. Thus, it is not surprising that transplacentally acquired antibodies from the mother are not totally protective against newborn infection but with conflicting results (219,256,499,500,555,562). Furthermore, the quantity of neutralizing antibodies is higher in those newborns who do not develop infection when exposed to HSV at delivery (384,562). The key issue, then, is to what extent these antibodies are protective and whether specific antibody classes or subclasses provide greater protection than others. Transplacentally acquired neutralizing antibodies seem to either prevent or ameliorate infection in exposed newborns, as do antibody-dependent cell-mediated cytotoxicity antibodies (384,562). Nevertheless, the presence of antibodies at the time of clinical presentation with disease does not necessarily influence the subsequent outcome (219,543,551).

Humoral responses of IgM and IgG antibodies have been well characterized. Infected newborns produce IgM antibodies (as detected by immunofluorescence) specific for HSV within the first 3 weeks of infection. These antibodies increase rapidly during the first 2 to 3 months, and they may be detectable for as long as 1 year after infection. The most reactive immunodeterminants are the surface viral glycoproteins, specifically gB and gD. Humoral antibody responses were elaborated recently using contemporary immunoblot technology, and the patterns of response are similar to those encountered in adults with primary infection (219,499). These immunoblot studies indicate that the severity of infection correlates directly with the number of antibody bands to defined polypeptides. Children with more limited infection—namely, infection of the skin, eye, and/or mouth—have fewer antibody bands than do children with disseminated disease. Total neutralizing antibodies in babies with disseminated infection are low (499).

Cellular immunity is considered important in neonatal host response to HSV infection. Newborns with HSV infections have a delayed T-lymphocyte proliferative response compared with that of older individuals (499). Most infants had no detectable T-lymphocyte responses to HSV 2 to 4 weeks after the onset of clinical symptoms (390,499). Other investigators have observed delayed blastogenic responses to HSV (371). The correlation between these delayed responses may be of significance in evaluating outcome to neonatal HSV infection. Specifically, if the response to T-lymphocyte antigens in children who have disease localized to the skin, eye, or mouth at the onset of disease is significantly delayed, disease progression may occur at a much higher frequency than it does in babies with a more appropriate response (90,499).

Infected newborns have decreased alpha-interferon production in response to HSV antigen when compared with adults with primary HSV infection (499). The importance of the generation of alpha-interferon on the maturation of host responses, particularly the elicitation of natural killer cell responses, remains to be defined (64,252). Lymphocytes from infected babies have decreased responses to gamma-interferon generation during the first month of life (69,252,499). These data taken together would indicate that the newborn has a delayed immune response by assessment of both cell-mediated immunity and lymphokine generation.

Antibodies plus complement and antibodies mixed with killer lymphocytes, monocytes, macrophages, or polymorphonuclear leukocytes will lyse HSV-infected cells *in vitro* (418). Antibody-dependent cell-mediated cytotoxicity has been demonstrated to be an important component of the development of host immunity to infection (251). However, the total population of neonatal killer lymphocytes seems lower than that found in older individuals. The type and quantity of antibodies present, as well as the immaturity of monocytes and macrophages of human neonates, are greater than those of adults (15,198,253,254,288,317,514,570). These findings are supported by data from animal models.

CLINICAL MANIFESTATIONS OF DISEASE

Primary/Recurrent Oropharyngeal Disease

The clinical symptomatology of primary HSV-1 infections varies greatly. Manifestations of infection range from totally asymptomatic to any combinations of fever, sore throat, ulcerative and vesicular lesions, gingivostomatitis, edema, localized lymphadenopathy, anorexia, and malaise. Asymptomatic infection is the rule rather than the exception. The incubation period ranges from 2 to 12 days, with a mean of approximately 4 days.

Symptomatic disease in children is characterized by involvement of the buccal and gingival mucosa (Fig. 11). The duration of clinical illness may be from 2 to 3 weeks, with fever of 101°F to 104°F. Children with symptomatic primary infection are often unable to swallow liquids because of the significant edema of the mucosal membranes and the associated pain. Lesions within the mouth evolve from vesicles, oftentimes not witnessed with intraoral disease, to shallow ulcerations on an erythematous base. They then slowly heal. In contrast to cutaneous lesions, intraoral lesions are not generally associated with the development of scabs. Submandibular lymphaden-opathy is commonly associated with primary HSV gingivostomatitis but rarely with recurrent infections. Other findings include sore throat and mouth, malaise, tender cervical lymphadenopathy, and an inability to eat. A clinical distinction should be drawn between intraoral gingival lesions and lip lesions, which are indicative of presumed primary and recurrent infections, respectively.

FIG. 11. Herpes simplex gingivostomatitis.

With the onset of primary HSV infections later in life, a common finding is HSV pharyngitis in association with a mononucleosis syndrome. Under such circumstances, ulcerative tonsilar lesions on an erythematous base with associated submandibular lymphadenopathy are common (163). The differential diagnosis of both primary HSV gingivostomatitis and pharyngitis focuses on other mucosal lesions of the oropharynx. These would include herpangina (caused by coxsackieviruses), candidal infections of the mouth, Epstein-Barr virus–induced mononucleosis, lesions induced by chemotherapy or radiation therapy, and Stevens-Johnson syndrome.

The onset of recurrent orolabial lesions is heralded by a prodrome of pain, burning, tingling, or itching, which generally lasts for less than 6 hours followed within 24 to 48 hours by vesicle formation (480,481,483,567). The natural history of recurrent herpes labialis is illustrated in Figure 12 (479). Vesicles appear most commonly at the vermilion border of the lip and persist in most patients only 48 hours at the longest (Fig. 13). Vesicles generally number three to five. The total area of involvement usually is localized, being less than 100 mm^2, and lesions progress to the pustular or ulcerative and crusting stage within 72 to 96 hours. Pain is most severe at the outset and resolves quickly over 96 to 120 hours. Similarly, the loss of virus from lesions decreases with progressive healing over 2 to 3 days (26,483). Healing is rapid, generally being complete in 8 to 10 days. The frequency of recurrences varies among individuals (483). The factors responsible for recurrences in humans are both highly variable and poorly defined but include fever, stress, and exposure to UV light (452,459).

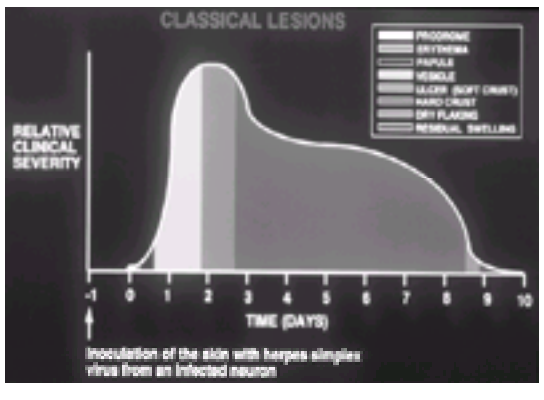


FIG. 12. The natural history of herpes simplex labialis.

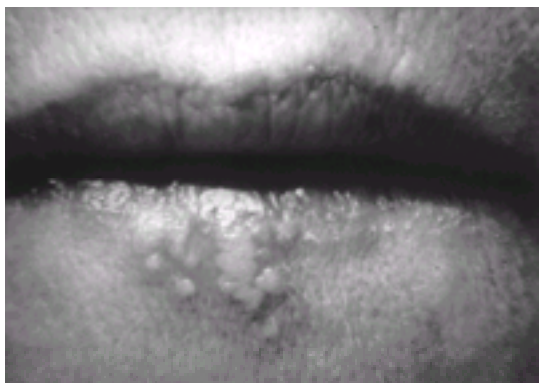


FIG. 13. Recurrent herpes simplex labialis.

Primary/Initial/Recurrent Genital Disease

The most severe clinical expression of genital herpes is encountered with primary infection. After acquisition of HSV infection at an abraded site, macules and papules, followed by vesicles, pustules, and ulcers, appear. The duration of lesions averages 3 weeks. There are both similarities and differences in the clinical symptomatology between men and women with infection (105,107).

Primary infection is associated with (a) larger quantities of virus replicating in the genital tract (more than 10^6 viral

particles per 0.2 mL of inoculum) and (b) a period of viral excretion that may persist for an average of 3 weeks. Systemic complications in men are relatively uncommon; however, aseptic meningitis can develop. Paresthesias and dysesthesias that involve the lower extremities and perineum can result from genital herpetic infection. Primary infections can be associated with fever, dysuria, localized inguinal adenopathy, and malaise in both men and women. The severity of primary infection and its association with complications are statistically higher in women than in men, for unknown reasons (63,107). Systemic complaints are common in both sexes, approaching 70% of all cases. Extragenital lesions are common.

In women with primary infection, lesions appear on the vulva and are usually bilateral (Fig. 14), with the cervix being invariably involved (Fig. 15). The actual frequency of primary cervical infection in the absence of vulvar infection is unknown. Lesions usually are excruciatingly painful, associated with inguinal adenopathy and dysuria, and may involve the vulva, perineum, buttocks, cervix, and/or vagina. A urinary retention syndrome occurs in 10% to 15% of female patients, and as many as 25% of women will develop aseptic meningitis.

FIG. 14. Genital HSV infection (female).

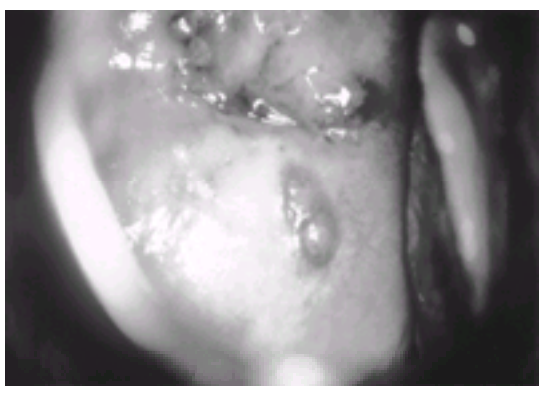


FIG. 15. Cervical HSV infection.

In men, primary genital HSV infections are most often associated with vesicular lesions superimposed on an erythematous base, usually appearing on the glans penis or the penile shaft (Fig. 16). The total number of lesions can vary significantly from six to 10 to many more. Extragenital lesions of the thigh, buttocks, and perineum can occur.

FIG. 16. Genital HSV infection (male).

Complications after primary genital herpetic infection have included sacral radioculomyelitis, which can lead to urinary retention, neuralgias, and meningoencephalitis (74,191,197,468,507). Primary perianal and anal HSV-2 infections, as well as associated proctitis, are common in male homosexuals (165,207). Like HSV-1 infections, many primary HSV-2 illnesses are subclinical, involving the mouth (172) or the uterine cervix (213,214,564).

Nonprimary but initial genital infection (i.e., occurring in an individual with preexisting antibody) is less severe symptomatically and heals more quickly than primary infection. The duration of disease is closer to 2 weeks. The number

of lesions, severity of pain, and likelihood of complications are significantly decreased. Preexisting antibodies to HSV-1 have an ameliorative effect on disease severity of HSV-2 (8,110,224,267,399,400,443,525) but do not appear to prevent infection (111,525).

Recurrent genital herpes is the mildest form of disease. With recurrent genital herpetic infection, a limited number of vesicles, usually three to five, appear on the shaft of the penis in men or as simply a vulvar irritation in women (6). The duration of disease, approximately 7 to 10 days, parallels that encountered with recurrent HSV labialis. Neurologic or systemic complications are uncommon with recurrent disease; however, paresthesias and dysathesias occur.

The virologic manifestations are also different for recurrent infection compared with primary infection. Virus is shed for an average of only 2 to 5 days and at lower concentrations (approximately 10^2 to 10^3 per 0.2 mL of inoculum in tissue culture systems) in women with recurrent genital infection. Recurrent genital herpetic infection in both men and women is characterized by a prodrome (which is a useful marker for therapeutic trials) (290) and localized irritation.

The major problem with recurrent genital HSV infection is the frequency of the recurrences, which varies from one individual to the next. Anecdotally, the severity of primary infection correlates directly with the frequency of recurrences; that is, the more severe the primary infection, the more likely and frequent the recurrences. Notably, all patients suffer from recurrences. One third of patients are estimated to have recurrences in excess of eight or nine times per year, one third will have two to three per year, and the remaining third will have four to seven (107). Clearly, with recurrences, either symptomatic or asymptomatic, transmission of infection to sexual partners can occur with intimate contact. Recent studies suggest that HSV DNA can be detected by PCR (99,496) three to five times more frequently than the isolation of virus. The implications of these data forces a reanalysis of our understanding of subclinical recurrences, especially the education of our patients, including behavioral interventions and use of condoms to prevent transmission of infection.

Neonatal HSV Infections

Incidence of Newborn Infection

Although centers caring for infants with neonatal HSV infections have observed fluctuations in disease incidence, the estimated rate of occurrence is approximately 1 in 3,000 to 1 in 5,000 deliveries per year (338,339). An increase in the number of cases of neonatal HSV infection has been noted in some areas, with rates approaching 1 in 1,500 deliveries (502), but this is unusual. Neonatal HSV infection occurs far less frequently than do genital HSV infections in the adult child-bearing population. Several countries do not appear to recognize a significant number of cases of neonatal HSV infection, probably reflecting their low overall prevalence of HSV-2 infection. As a contrast, serologic studies in central Africa indicate that women have a high frequency of antibodies to HSV-2, but the first case of neonatal herpes was reported only relatively recently (5,505). On the other hand, in the United Kingdom, the seroprevalence of HSV-2 is relatively low and, in parallel, so is the incidence of neonatal HSV infection. Why the United Kingdom has a distinctly lower seroprevalence of HSV-2 is not well understood.

Factors That Influence Transmission of Infection to the Fetus

At least four factors influence transmission of infection from mother to fetus. First, the type of maternal genital infection at the time of delivery is crucial. The duration and quantity of viral excretion and the time to total healing vary with primary, initial, and recurrent maternal genital infection: Primary infection is most and recurrent infection is least severe (105,107). Data generated from investigators in Seattle and supported by two other investigative teams suggest a risk of transmission of 30% with maternal primary or initial infection, compared with 3% or less with recurrent infection (58). In part, this reflects prolonged viral excretion during the third trimester of gestation, as well as an inadequate transplacental antibody response. Second, paralleling the type of maternal infection, the mother's antibody status to HSV at delivery influences the severity of infection as well as the likelihood of transmission. Transplacental maternal neutralizing and antibody-dependent cell-mediated cytotoxic antibodies appear to have at least an ameliorative effect on acquisition of infection for babies inadvertently exposed to virus at delivery (21,338,384,561,562). Third, the duration of ruptured membranes is an important indicator of risk for acquisition of neonatal infection. Observations by Nahmias et al. (338) indicate that prolonged rupture of membranes (longer than 6 hours) increases the risk of acquisition of virus, probably the consequence of ascending infection from the cervix. Fourth, certain forms of medical intervention in the labor and delivery suites may increase the risk of neonatal HSV infection. Specifically, fetal scalp monitors can be a site of inoculation of virus (225,369). Such devices should be relatively contraindicated in women with a history of recurrent genital HSV infection.

Times of Transmission of Infection

Infection of the newborn can be acquired at one of three times: *in utero*, intrapartum, or postnatally. Certainly, the mother is the most common source of infection for the first two of these routes of transmission of infection to the newborn. Although intrapartum transmission accounts for 75% to 80% of all cases, the other two routes must be considered in a child with suspect disease, for both public health and prognostic purposes. The first group of babies become infected *in utero* (28,150,176,204,477). Using stringent diagnostic criteria (namely, identification of infected babies within the first 48 hours of life who have virologic confirmation of infection), over 30 babies have been identified in the world's literature with symptomatic congenital disease. Other reports have included, as evidence for *in utero* acquisition of disease, the presence of HSV lesions at birth, detection of elevated IgM in cord blood, and neonatal viremia (1).

In utero infection can occur as a consequence of either transplacental or ascending infection. Placental necrosis and inclusions in the trophoblast can occur (158,160,557). Ascending infection, with histopathologic evidence of chorioamnionitis, was identified as an alternative route for *in utero* infection.

The second and most common route of infection is that of intrapartum contact of the fetus with infected maternal genital secretions. Most likely, 75% to 80% of infected babies acquire HSV infection by this route. Factors favoring intrapartum

transmission of infection were noted ealier.

The third route of transmission is postnatal acquisition. Even though HSV-1 is associated with genital lesions, postnatal transmission of HSV is increasingly suggested because 15% to 20% of neonatal HSV infections are caused by this virus type (338). In fact, more recent data from the National Institutes of Allergy and Infectious Diseases (NIAID) Collaborative Antiviral Study Group (CASG) indicate that the frequency of babies with neonatal HSV-1 infections has increased to nearly 30% (543). This observation should be weighed against the recognition that HSV-1 infections are becoming increasingly common in the United States and now account for about 25% to 30% of new cases of genital herpes. Regardless, relatives and hospital personnel with orolabial herpes are a reservoir of virus for infection of the newborn. The documentation of postnatal transmission of HSV by molecular epidemiologic tools has focused attention on such sources of virus for neonatal infection (128,133,152,175,233,279,281,503). The documentation of identical isolates from babies born to different mothers in a nursery by restriction endonuclease technology is troublesome (175) but, fortunately, rare. Postnatal transmission from mother to child was documented as a consequence of nursing on an infected breast (133,233,431,503). Father-to-baby transmission was documented from lesions of herpes labialis (128,563).

Clinical Presentation

Clinical presentation of babies with neonatal HSV infection is a direct reflection of the site and extent of viral replication. Although reported cases of asymptomatic infection in the newborn exist, neonatal HSV infection is almost invariably symptomatic and frequently lethal. Classification of newborns with HSV infection is mandatory for prognostic and therapeutic decisions. Babies with congenital infection should be identified within 48 hours after birth. Babies who are infected intrapartum or postnatally with HSV infection can be divided into three categories: those with (a) disease localized to the skin, eye, and mouth; (b) encephalitis with or without skin involvement; and (c) disseminated infection that involves multiple organs, including the CNS, lung, liver, adrenals, skin, eye, and/or mouth (334,553).

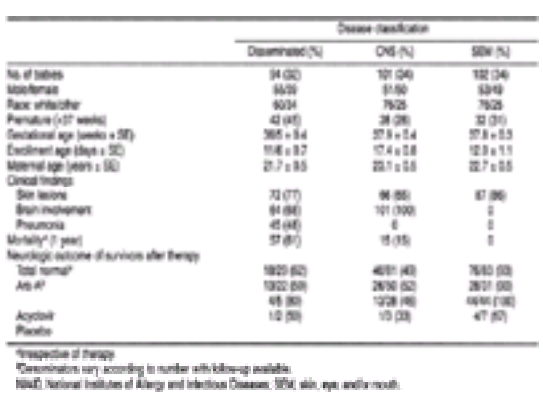
Intrauterine Infection

Intrauterine infection results in a group of severely afflicted babies; disease is apparent at birth and is characterized by the triad of skin vesicles or skin scarring, eye disease, and the far more severe manifestations of microcephaly or hydranencephaly. Often, chorioretinitis alone or in combination with other eye findings, such as keratoconjunctivitis, is a component of the clinical presentation. The frequency of occurrence of these manifestations has been estimated to be about 1 in 200,000 deliveries (28).

A small group of children will have skin or eye lesions that are present at the time of delivery. These children are frequently born to women who have had prolonged rupture of membranes. The babies have no other findings of invasive multiorgan involvement. The mothers of these babies often had prolonged rupture of membranes for as long as 2 weeks before the onset of labor and delivery. The prognosis for successful antiviral therapy in this group of babies is better than in the children who are born with hydranencephaly.

Disseminated Infection

Table 5 summarizes the disease classification of 291 babies with neonatal HSV from the NIAID CASG studies (see also Fig. 17). Babies with the worst prognosis for both mortality and morbidity have disseminated infection. Children with disseminated infection are usually brought to tertiary care centers for therapy at 9 to 11 days of age; however, signs of infection usually begin an average of 4 to 5 days earlier. Historically, this group of babies has accounted for approximately one half to two thirds of all children with neonatal HSV infection. However, with the early introduction of antiviral therapy, this figure was reduced to about 23% (see Fig. 16).



	Disease classification		
	Disseminated (%)	CNS (%)	SEM (%)
No. of babies	94 (32)	101 (34)	102 (34)
Male/female	66/28	81/20	63/39
Race: white/other	80/24	76/25	76/26
Premature (<37 weeks)	42 (45)	38 (38)	32 (31)
Gestational age (weeks ± SE)	38.5 ± 0.4	37.9 ± 0.4	37.8 ± 0.3
Enrollment age (days ± SE)	11.6 ± 0.7	17.4 ± 0.8	12.3 ± 1.1
Maternal age (years ± SE)	21.7 ± 0.5	23.1 ± 0.6	22.7 ± 0.5
Clinical findings			
Skin lesions	72 (77)	66 (65)	67 (66)
Brain involvement	64 (68)	101 (100)	0
Pneumonia	45 (48)	6	0
Mortality* (1 year)	57 (61)	15 (15)	0
Neurologic outcome of survivors after therapy			
Total normal*	18/29 (62)	46/81 (40)	76/83 (53)
Anb A*	13/22 (59)	26/56 (52)	28/31 (90)
	4/6 (80)	12/28 (46)	48/48 (100)
Acyclovir	1/2 (50)	1/3 (33)	4/7 (57)
Placebo			

*Irrespective of therapy.
†Denominators vary according to number with follow-up available.
NIAID, National Institutes of Allergy and Infectious Diseases; SEM, skin, eye, and/or mouth.

TABLE 5. *Demographic and clinical characteristics of infants enrolled in NIAID collaborative antiviral study*

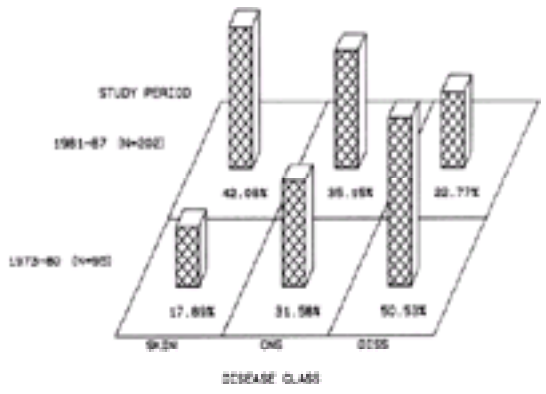


FIG. 17. Change in disease classification of neonatal HSV infection. Patients were enrolled in the NIAID Collaborative Antiviral Study Group and grouped according to disease class.

The principal organs involved after disseminated infection are the liver and adrenals. Infection can involve multiple other organs, including the larynx, trachea, lungs, esophagus, stomach, lower gastrointestinal tract, spleen, kidneys, pancreas, and heart. Constitutional signs and symptoms include irritability, seizures, respiratory distress, jaundice, bleeding diatheses, shock, and, frequently, the characteristic vesicular exanthem, which is often considered pathognomonic for HSV infection. Encephalitis appears to be a common component of this form of infection, occurring in about 60% to 75% of children with disseminated infection. The vesicular rash, as described later, is particularly important in the diagnosis of HSV infection. However, over 20% of children having disseminated infection will not develop skin vesicles during the course of their illness (19,543). Mortality in the absence of therapy exceeds 80%; all but a few survivors are impaired. The most common cause of death in babies with disseminated disease is either HSV pneumonitis or disseminated intravascular coagulopathy.

Encephalitis

Babies with infection of the CNS alone or in combination with disseminated disease present with the findings indicative of encephalitis in the newborn. Overall, nearly 90% of babies with disseminated disease have evidence of acute brain infection. Brain infection can occur in one of two fashions: either as a component of multiorgan disseminated infection or only as encephalitis with or without skin, eye, and mouth involvement. Nearly one third of all babies with neonatal HSV infection have only the encephalitic component of disease. The pathogenesis of these two forms of brain infection is very likely different. Babies with disseminated infection probably seed the brain by a blood-borne route, resulting in multiple areas of cortical hemorrhagic necrosis. In contrast, babies who present with only encephalitis probably develop brain disease as a consequence of retrograde axonal transport of virus to the CNS. Two pieces of data support this contention. First, babies with disseminated disease have documented viremia and are hospitalized earlier in life than are those with only encephalitis: at 9 to 10 days versus 16 to 17 days, respectively. Second, babies with encephalitis are more likely to receive transplacental neutralizing antibodies from their mothers, which may prevent viremia, allowing only intraneuronal transmission of virus to the brain.

Clinical manifestations of either encephalitis (alone or in association with disseminated disease) include seizures (both focal and generalized), lethargy, irritability, tremors, poor feeding, temperature instability, bulging fontanelle, and pyramidal tract signs. Whereas babies with disseminated infection often have skin vesicles in association with brain infection, the same is not true for the baby with encephalitis alone. This latter group of children may have only skin vesicles in approximately 60% of cases at any time in the disease course (19,501,537,543,563). Cultures of cerebrospinal fluid yield virus in 25% to 40% of all cases. Anticipated findings on cerebrospinal fluid examination include pleocytosis and proteinosis (as high as 500 to 1,000 mg/dL). Although a few babies with CNS infection demonstrated by brain biopsy were reported to have no abnormalities of their cerebrospinal fluid, this occurs only rarely. Death occurs in 50% of babies with localized CNS disease who are not treated, and it is usually related to brain system involvement. With rare exceptions, survivors are left with severe neurologic impairment (537,546).

The long-term prognosis, after either disseminated infection or encephalitis, is particularly poor. As many as 50% of surviving children have some degree of psychomotor retardation, often in association with microcephaly, hydranencephaly, porencephalic cysts, spasticity, blindness, chorioretinitis, or learning disabilities. It is unclear at this time whether visceral or CNS damage can be progressive after initial clearance of the viral infection, a possibility suggested by long-term assessment of children with skin, eye, or mouth disease (338,543,550) and, more recently, in a group of babies with more severe disease (171).

Recently, subclinical infection of the CNS has been detected in babies with disease apparently localized to the skin, eye, and mouth. These children had normal cerebrospinal fluid assessments but PCR-detectable HSV DNA at the onset of disease (235). Subsequently, a subset of these children developed neurologic impairment. These findings stress the often subtle presentation of CNS disease.

A comment regarding the diagnosis of encephalitis is indicated. In as many as 40% of children with disease localized to the CNS (e.g., the 2- to 3-week-old baby with cells and protein in the cerebrospinal fluid), other diagnostic clues, such as skin vesicles (the classic sign of HSV disease) may not be present. For the neonate with cerebrospinal fluid findings indicative of infection, HSV must be considered along with bacterial pathogens (e.g., group B *Streptococcus*, *Escherichia coli*). A reasonable diagnostic approach, if all antigen and Gram stain studies are negative and so are the PCR evaluations, would be serial cerebrospinal fluid examinations to document progression in protein and mononuclear cell counts.

Skin, Eye, and Mouth Infection

Infection localized to the skin, eye, and/or mouth is associated with lower mortality, but significant morbidity can occur. When infection is localized to the skin, the presence of discrete vesicles remains the hallmark of disease. Clusters of vesicles often appear initially on the part of the body that was in direct contact with the virus during birth. With time, the rash can progress to involve other areas of the body as well, particularly if viremia occurs. Vesicles occur in 90% of children with skin, eye, or mouth infection. Children with disease localized to the skin, eye, or mouth generally present at about 10 to 11 days of life. Babies with skin lesions invariably will experience recurrences over the first 6 months (and longer) of life, regardless of whether therapy was administered. Although death is not associated with disease localized to the skin, eye, and/or mouth, approximately 30% of these children eventually develop evidence of neurologic impairment (537,546,550). The skin vesicles (Fig. 18) usually erupt from an erythematous base and are usually 1 to 2 mm in diameter. Other manifestations of skin lesions have included a zosteriform eruption (329).

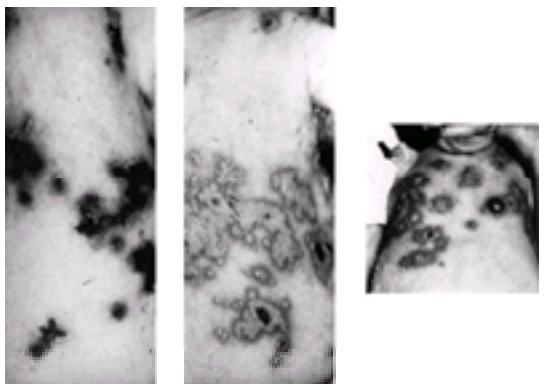


FIG. 18. Vesicular rash of neonatal HSV infection.

Infections involving the eye may manifest as keratoconjunctivitis or, later, as chorioretinitis. The eye may be the only site of HSV involvement in the newborn (546). These children present with keratoconjunctivitis or, surprisingly, evidence of microphthalmia and retinal dysplasia.

Long-term neurologic impairment has been encountered in children whose disease appeared to be localized to the skin, eye, and/or mouth. The significant findings include spastic quadriplegia, microcephaly, and blindness. Important questions regarding the pathogenesis of delayed-onset neurologic debility are raised by such clinical observations. Despite normal clinical examinations in these children, neurologic impairment has become apparent between 6 months and 1 year of life. As noted earlier, these children had detectable HSV DNA in the cerebrospinal fluid at presentation, implying subclinical infection of the CNS. The clinical presentation occurs in a manner similar to that associated with congenitally acquired toxoplasmosis or syphilis.

Prognostic Factors

Recently, the NIAID CASG completed an assessment of factors that predict long-term prognosis (Table 6) (541). The most significant predictors of both mortality and morbidity were disease classification: Babies with the most severe disease had the worst outcome. A low level of consciousness also predicted a more severe outcome. By disease classification, the poorest prognostic factors for babies with disseminated disease were, in addition to the preceding, the development of HSV pneumonia or disseminated intravascular coagulopathy. With encephalitis, prematurity and seizures predicted a poor outcome. Finally, for babies with skin, eye, or mouth disease, frequently recurrent HSV-2 cutaneous lesions defined a group at risk for a poor neurologic outcome.

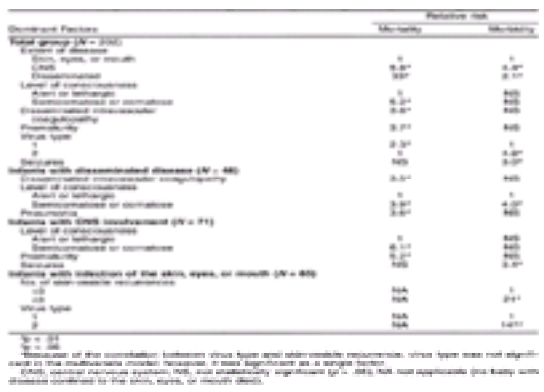


TABLE 6. *Prognostic factors identified by multivariate analyses for neonates with HSV infection*

Herpes Simplex Keratoconjunctivitis

Viral infections of the eye are usually caused by HSV-1 beyond the newborn age (45,364,449). Approximately 300,000 cases of HSV infections of the eye are diagnosed yearly in the United States. These infections are second only to trauma as the cause of corneal blindness in this country. Primary herpetic keratoconjunctivitis is associated with either unilateral

or bilateral conjunctivitis, which can be follicular in nature, followed soon thereafter by preauricular adenopathy. HSV infection of the eye is also associated with photophobia, tearing, eyelid edema, and chemosis, accompanied by the pathognomonic findings of branching dendritic lesions (Fig. 19). Less commonly, with advanced disease the infection is associated with a geographic ulcer of the cornea (Fig. 20). Healing of the cornea can take as long as 1 month, even with appropriate antiviral therapy.

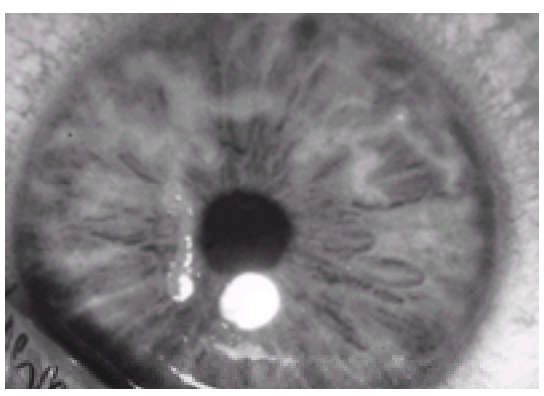


FIG. 19. Dendritic lesions of herpes simplex keratoconjunctivitis.

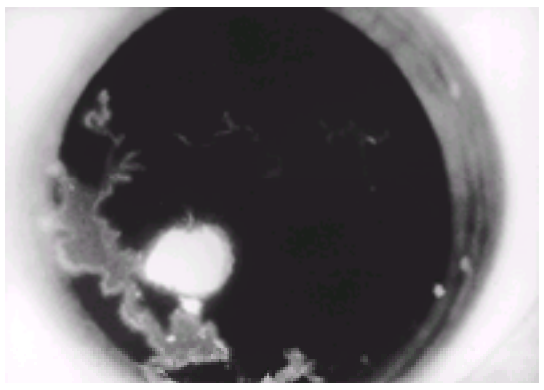


FIG. 20. Geographic corneal ulcer caused by HSV infection.

Recurrent HSV infections of the eye are common. Recurrences parallel that described for herpes labialis. Most frequently, recurrences are unilateral in involvement, but a small percentage of cases involve both eyes. Characteristically, either dendritic ulceration or stromal involvement occurs. Visual acuity is decreased in the presence of the ulcers; with progressive stromal involvement, opacification of the cornea may occur. Repeated individual attacks may last for weeks or even months after appropriate antiviral therapy. The route and pathogenesis of infection remain unknown. Progressive disease can result in vision loss and even rupture of the globe.

Skin Infections

Skin infections caused by HSV generally manifest as eczema herpeticum in patients with underlying atopic dermatitis (386,506,533). The lesions can be either localized, resembling herpes zoster with a dermatomal distribution, or disseminated. The latter occurs commonly in Kaposi's varicella-like eruption (422). HSV infections of the digits, known as herpetic whitlow, have been reported and are particularly common among medical and dental personnel (417). Herpetic infections of the hand have been described since 1909 and are particularly problematic for hospital personnel. The estimated incidence is 2.4 cases per 100,000 population per year, and the cause may be HSV-1 or HSV-2. An increasing incidence of HSV-2 herpetic whitlow has been recognized among health care professionals (162).

The prevalence of HSV skin infections in Skaraborg, Sweden, was assessed among approximately 7,500 individuals over 7 years of age and was found to be about 1% (186). In another Swedish study performed in dermatology clinics, 2% of men and 1.5% of women attending clinics in Gothenburg over a 6-year period had evidence of herpetic skin infections (137). In addition to individuals with atopic disease, patients with skin abrasions or burns appear particularly susceptible to HSV-1 or HSV-2 infections, and some may develop disseminated infection (151). Disseminated HSV infections were also reported among wrestlers, and this is referred to as herpes gladitorium (534). Other skin disorders associated with extensive cutaneous lesions include Darier's disease and Sezary's syndrome (185,199). As would be predicted, localized recurrences followed by a second episode of dissemination were observed (363). HSV infections of either type were found to trigger erythema multiforme (532). The association of HSV DNA in skin lesions of erythema multiforme is as high as 80%.

Infections of the Immunocompromised Host

Patients compromised by either immune therapy, underlying disease, or malnutrition are at increased risk for severe HSV infections. Renal, hepatic, bone marrow, and cardiac recipients are all at particular risk for increased severity of transplant HSV infection (283,327,372,554). An example of cutaneous dissemination after shaving, in a renal transplant recipient, is shown in Figure 21. Virus was most likely autoinoculated to multiple skin sites by the razor blade. In organ transplant recipients, the presence or quantity of HSV antibodies before treatment predicts the individual at greatest risk for recurrence (373). These patients may develop progressive disease involving the respiratory tract, esophagus, or even

the gastrointestinal tract (258,325). The severe nature of progressive disease in these patients appears to be directly related to the amount of immunosuppressive therapy (389). Esophagitis is a common occurrence in the immunocompromised host and can be caused by HSV, cytomegalovirus, or *Candida albicans.* Notably, acyclovir-resistant HSV disease will occur in the immunocompromised host and can be progressive. Reactivation of latent HSV infections in these patients can occur at multiple sites, and healing in these patients with severe progressive disease occurs over an average of 6 weeks (549).

FIG. 21. Cutaneous dissemination of HSV infection in an immunosuppressed host.

Since the first reports of acquired immunodeficiency syndrome, the severity of HSV clinical disease in these severely immunocompromised hosts was noted (294). Herpetic disease, even more exaggerated than in the cancer patient, has been reported and is an essential target for antiviral therapy. Although conflicting reports have suggested no enhanced severity of HSV infection in these patients (428), disease appears to occur more frequently and can lead to resistance with requisite therapy.

Asymptomatic excretion of HSV can occur even in the immunocompromised host, as is best exemplified in HIV-infected individuals (435,436,525). Parenthetically, ac-quisition of HSV infection from a transplanted organ (kidney) has been reported (131).

Infections of the Central Nervous System

Herpes simplex encephalitis is one of the most devastating of all HSV infections (Fig. 22), being the most common cause of sporadic, fatal encephalitis in this country (362). The Centers for Disease Control estimate the incidence of herpes simplex encephalitis to be approximately 40 to 50 cases per year; however, disease occurrence is undoubtedly higher (553). From data collected prospectively, the incidence of severe hemorrhagic focal encephalitis is approximately 1 in 200,000 individuals per year, for a national annualized rate of approximately 1,250 cases in the United States.

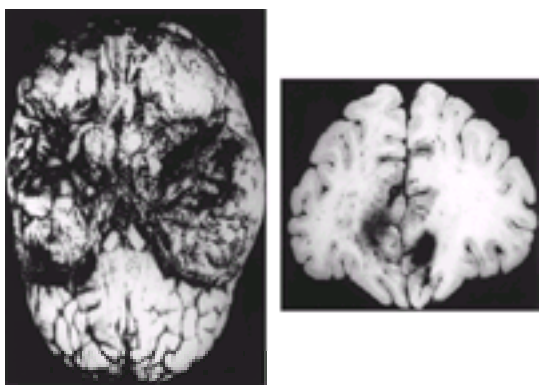


FIG. 22. Coronal section of brain from a patient with herpes simplex encephalitis.

The manifestations of HSV encephalitis in the older child and adult are indicative of the areas of pathology in the brain. These include primarily a focal encephalitis associated with fever, altered consciousness, bizarre behavior, disordered mentation, and localized neurologic findings. These clinical signs and symptoms generally are associated with evidence of localized temporal lobe disease, as demonstrated by neurodiagnostic procedures (552,553). There are no characteristic series of findings pathognomonic for herpes simplex encephalitis; however, a progressively deteriorating level of consciousness, fever, an abnormal cerebrospinal fluid formula, and focal neurologic findings in the absence of other causes should make this disease highly suspect. Other diagnostic evaluations should be initiated immediately because other treatable diseases mimic HSV encephalitis (542). Mortality in untreated patients is in excess of 70%, and only 2.5% of all patients return to normal neurologic function.

Pathogenesis

The pathogenesis of HSV encephalitis, particularly as it relates to the source of virus responsible for brain disease, has been considered for some time. HSV isolates obtained from both the brain and the lip or mouth of patients with virologically confirmed HSV encephalitis were examined by restriction endonuclease analyses for identity (548). These studies demonstrated that only approximately 65% of patients excreting virus from two sites had identical isolates. When

the viruses were proven identical, invariably the patient was experiencing a primary infection (as defined serologically). Thus, virus excreted from the mouth of patients with HSV encephalitis may be identical to that of the brain or may be entirely different. The NIAID CASG observed that approximately half of the patients with HSV encephalitis (confirmed by brain biopsy and virus isolation) appear to have primary infections and that the remaining patients appear to have recurrent infections (523,546,548).

Diagnosis

Standard neurodiagnostic procedures are used in the evaluation of patients with suspect HSV encephalitis and include cerebrospinal fluid examination, electroencephalogram, and one or more scanning procedures such as technetium, computed tomography, or magnetic resonance imaging. Characteristic abnormalities of the cerebrospinal fluid include elevated levels of cells (usually mononuclear) and protein. Red blood cells are found in most (but not all) cerebrospinal fluids obtained from patients with HSV encephalitis. Upon serial cerebrospinal fluid examination, protein and cell counts increase dramatically. The electroencephalogram generally localizes spike and slow-wave activity to the temporal lobe. A burst suppression pattern is considered characteristic of HSV encephalitis. Imaging will allow for localization of disease to the temporal lobe. Early after onset, only evidence of edema is detectable, if at all. This finding is followed by evidence of hemorrhage and a midline shift in the cortical structures.

One of many areas for further investigation is the elucidation of the spectrum of HSV infections of the brain. Historically, diagnosis of CNS infections was accomplished only by brain biopsy (542); however, PCR assessment of the cerebrospinal fluid has replaced this diagnostic approach. Serologic procedures performed on serum or cerebrospinal fluid are not helpful early in the disease course when therapeutic decisions are mandatory (344). Therefore, noninvasive diagnostic procedures have been developed. Such procedures used in the past have included detection of HSV-specified thymidine kinase, DNA polymerase, and DNase (89) or detection of HSV-excreted glycoproteins (87) in the cerebrospinal fluid (270). The detection of viral DNA by PCR is now the diagnostic method of choice, but it must be performed by a reliable laboratory. Its utility will be described later in the section, Diagnosis. The spectrum of HSV infection of the CNS will be defined with the availability of such assays as PCR.

Other Neurologic Syndromes

In addition to encephalitis, HSV can involve nearly all anatomic areas of the nervous system. Crain and Nahmias (114) have reviewed the association of HSV with meningitis, myelitis, and radiculitis, among others. Aseptic meningitis is a common occurrence in individuals with primary genital HSV infections. The relationship between HSV infections of the brain and chronic degenerative disease, psychiatric disorders, or Bell's palsy requires further definition (31,95,102,299,328).

Other Forms of Infection

Other forms of infection should be noted briefly. HSV was isolated from the respiratory tract of adults with adult respiratory distress syndrome and acute onset bronchospasm (515). Both were associated with increased mortality and morbidity.

Association with Malignancy

Over a century ago, it was recognized that cervical carcinoma rarely developed in nuns and that it occurred at a much higher rate in married versus single women (407). Nahmias et al. associated HSV-2 with cervical squamous cell carcinoma, as have other investigators (2,4,23,72,93,155,222,301,335,341,345,395,396,409,420,421). This subject was reviewed extensively (308,342,408,427). These observations were predicated on (a) the site of cancer and the known propensity of HSV-2 to infect this site and (b) a plethora of antibody determinations that support the association (3,7,81,82,91,94,147,206,208,232,298,370,470,471). These serologic studies often failed to consider other STDs (154,231). Thus, HSV-2 infection is only a marker of behavior that predisposes to human papillomavirus infection, the more obvious cause of cervical carcinoma.

DIAGNOSIS

The appropriate use of laboratory tools is essential if a diagnosis of HSV infection is to be achieved (reviewed in ref. 106). Virus isolation remains the definitive diagnostic method. If skin lesions are present, a scraping of skin vesicles should be made and transferred in appropriate virus transport media to a diagnostic virology laboratory. Clinical specimens should be shipped on ice for inoculation into cell culture systems (e.g., foreskin fibroblasts, Vero cells) that are appropriate for the demonstration of the cytopathic effects characteristic of HSV replication (446). Cytopathic effect tends to develop within 24 to 48 hours after inoculation of specimens containing infectious virus. The shipping and processing of specimens should be expedited.

In addition to skin vesicles, other sites from which virus may be isolated include the cerebrospinal fluid, stool, urine, throat, nasopharynx, and conjunctivae. In infants with evidence of hepatitis or other gastrointestinal abnormalities, it also may be useful to obtain duodenal aspirates for HSV isolation. The virologic results of cultures from these anatomic sites should be used in conjunction with clinical findings to define the extent of disease in the newborn and immunocompromised host.

Typing of an HSV isolate can be accomplished by one of several techniques, which may not be routinely available. Because outcome with treatment does not appear to be related to the virus type, identification is only of epidemiologic and pathogenetic importance and, therefore, not usually necessary.

Every effort should be made to confirm infection by viral isolation. Today such efforts are both reasonable and the expected standard of care. In the absence of diagnostic virology facilities, cytologic examination of cells from the

maternal cervix or from the infant's skin, mouth, conjunctivae, or corneal lesions may be useful in making a presumptive diagnosis of HSV infection. These methods have a sensitivity of only approximately 60% to 70% and therefore should not be the sole diagnostic determinant for infection in the newborn (50,341). Similar methods in adults with genital herpes have a slightly higher sensitivity and specificity (268). Cellular material obtained by scraping the periphery of the base of the lesions should be smeared on a glass slide and promptly fixed in cold ethanol. The slide can be stained according to the method of Papanicolaou, Giemsa, or Wright before examination by a trained cytologist. Deployment of either a Giemsa or a Tzanck smear most likely will not demonstrate the presence of intranuclear inclusions. The presence of intranuclear inclusions and multinucleated giant cells are indicative, but not diagnostic, of HSV infection. Electron-microscopic assays are continuing to be developed (275). PCR, as described later, is used to detect HSV DNA in lesions or genital and swabs.

Serologic diagnosis of HSV infection is not of great clinical value. Therapeutic decisions cannot await the results of serologic studies. Until recently, the inability of the commercially available serologic assays to distinguish between antibodies to HSV-1 and HSV-2, as well as to denote the presence of transplacentally acquired maternal IgG, as opposed to endogenously produced antibodies, makes the assessment of the neonate's antibody status difficult during acute infection. Serial antibody assessment may be useful if a mother without a prior history of HSV infection has a primary infection late in gestation and transfers little or no antibody to the fetus. The most commonly used tests for measurement of HSV antibodies are complement fixation, passive hemagglutination, neutralization, immunofluorescence, and the enzyme-linked immunosorbent (ELISA) assays. The more recent development of type-specific antibody assays will very likely replace many of the older systems. Recently, two tests have been licensed by the Food and Drug Administration that utilize detection of antibiotics to gG-2 as the basis for type specificity (22). Importantly, however, diagnostic tests for such life-threatening disease as HSV encephalitis will require improvement.

The experience with PCR indicates that it is a useful tool for diagnosis of HSV encephalitis (11,24,25,122,236,237,385,419,513). Using primers from an HSV DNA sequence that was common to both HSV types 1 and 2 (either the glycoprotein B or the DNA polymerase gene), successful identification of HSV DNA in the cerebrospinal fluid has been reported by several investigators. The largest clinical study to date has used cerebrospinal fluid specimens obtained from patients undergoing brain biopsies for HSV encephalitis. This study conclusively supports PCR as the diagnostic method of choice for CNS infection (271).

Importantly, PCR evaluation of cerebrospinal fluid can be used to follow therapeutic outcome in patients with HSV encephalitis. A recent publication (237) identified persistence of HSV DNA in the cerebrospinal fluid of newborns with suspect HSV encephalitis as a predictor of neurologic outcome. These data taken together suggest the utility of PCR for diagnosing HSV encephalitis. The evaluation of cerebrospinal fluid specimens obtained from patients with biopsy-proven HSV encephalitis and those with other proven diseases indicates a sensitivity of more than 95% at the time of clinical presentation, and a specificity that approaches 100% (271). Presumptive false-positive PCR evaluations were encountered when the site of disease was not properly determined via biopsy. Similarly, false-negative assessments can be found when there is contamination of hemoglobin in the cerebrospinal fluid or the presence of inhibitors.

PCR also has been used to detect HSV DNA in lesions. It has a sensitivity and a specificity that warrant careful evaluation under these conditions (100,101,177). The results of all control subjects were negative in this assay system. Additionally, PCR analyses of cerebrospinal fluid specimens will redefine the spectrum of HSV infections of the CNS (271,438,439).

PREVENTION: CONTROL OF HSV INFECTIONS

Two avenues exist for the control of HSV infections. One approach is that of antiviral chemotherapy. It is discussed in detail in Chapter 72. The alternative approach is one of prevention. For the purposes of this discussion, prevention will be considered from two perspectives. The first is an evaluation of the practical issues that surround the prevention of neonatal HSV infections. The second perspective is a discussion of the current developments in vaccines to prevent primary HSV infection or to influence the course of frequently recurrent disease.

Genital HSV Infections

Because of the rising incidence of genital herpes and neonatal herpes, and the increasing association of herpes with acquisition of HIV, every effort should be made to prevent HSV-2 infections. Until a vaccine is proven efficacious, educational efforts must be developed for adolescents and those at greatest risk. The use of condoms should be promoted.

Neonatal HSV Infection

With the increased awareness of neonatal HSV infection as a consequence of genital herpes, along with its recognition as a cause of severe and often fatal disease, methods of prevention have attracted particular attention. Surgical abdominal delivery is associated with decreased transmission of infection when membranes are ruptured less than 4 hours, but cesarean section has not been proven efficacious when membranes are ruptured for longer periods of time. Nevertheless, when membranes are ruptured up to 24 hours, there is still a time frame within which cesarean section may be of value. Although the recommendation seems logical, no data exist from adequately performed clinical trials to fully support it.

For women with a past history of genital HSV infection, a careful vaginal examination at presentation to the delivery suite is of paramount importance. Although visualization of the cervix is often difficult, speculum examination for documentation of recurrent lesions is extremely important and should be attempted in all women.

Clearly, identifying the women who excrete HSV at delivery and then optimizing either prophylactic protocols with safe and acceptable antiviral agents or delivery by cesarean section remains the optimal way to manage genital infection at

the time of delivery. Unfortunately, at the present time only isolation of virus in tissue culture will document the excretion of virus. Alternative and nontissue culture–based diagnostic approaches are being developed to expedite identification of women at risk for delivering an infected baby (96,406), including PCR detection of HSV DNA. Those at greatest risk should be identified. The detection of type-specific antibodies to gG-2 will be of value in identifying those women at greatest risk (337). Importantly, assessing discordant serologic status between sexual partners may assist in prevention of maternal primary infection.

Hospital Staff

At many institutions, a policy that requires transfer or provision of medical leave for nursery personnel who have a labial HSV infection is impractical and causes an excessive burden to be placed on those attempting to provide adequate care. Temporary removal of personnel who have cold sores is advocated for some clinical services. As noted previously, individuals with herpetic whitlow carry a high risk of viral shedding. These individuals should be removed from the care of newborns at risk for acquiring neonatal HSV infection because even gloves may not prevent transmission of infection. Education regarding the risk of transmission of virus and the importance of hand washing when lesions are present should be repeatedly emphasized to health care workers. In addition, hospital personnel should wear masks when active lesions are present.

Prevention of Other HSV Infections

At the present time, the only mechanism for prevention of mucocutaneous HSV infection is to avoid contact with infected secretions. There are no proven effective postexposure antiviral therapies or vaccines.

VACCINE DEVELOPMENT

Vaccination remains the ideal method for prevention of viral infection; however, prevention of HSV infections introduces unique problems because of recurrences in the presence of humoral immunity. Nevertheless, protection from life-threatening infection can be achieved in animal models with avirulent, inactivated, or subunit glycoprotein vaccines, each of which has unique differences. Excellent reviews summarize HSV vaccine development (539).

Human HSV Vaccine Development

Only in the past 10 years has there been a resurgence in the vaccine effort directed against HSV infections. These recent efforts have focused on the application of molecular techniques to antigen preparation and delivery. Three of the more promising HSV vaccines continue to be assessed. The first is based on the production of sufficient quantities of recombinant HSV-2 gB or gD to be used as subunit vaccines. The second is a genetically engineered, live, attenuated HSV-2 deletion mutant with carefully selected gene deletions to remove putative neurovirulence sequences. The third deletes an essential glycoprotein, gH, which allows only a single cycle of infectious virus replication.

Although the development of these vaccines is exciting, it has taken much effort. Earlier investigations provided important lessons from which future human trials will benefit; they will be reviewed before considering the newer, genetically engineered products. These approaches include the use of (a) wild-type virus, (b) inactivated or killed virus, (c) subunit vaccines, and (d) live vaccines. Each will be discussed.

Wild-Type Virus Vaccines

Numerous clinical investigators have attempted to alter the pattern of recurrences by inoculation of (a) autologous virus, (b) virus from another infected individual, or (c), in one set of experiments, virus recovered from an experimentally infected rabbit (356,357,374). In each circumstance, the inoculation of virus led to evidence of infection at the site of injection in as many as 40% to 80% of patients (146,291). Efficacy was reported with limited numbers of patients but without the use of matched controls (164,291,367). In some of these cases, inoculation led to recurrences of latent infection (49,273,356,374). In large part, live viruses were abandoned as immunogens on the grounds that many patients failed to develop lesions at the site of inoculation; therefore, it was concluded that the virus did not produce an “adequate take” (44,55,153). The analogy that was used was the cutaneous “take” required after smallpox immunization. At the present time, inoculation of either autologous or heterologous virus is considered unacceptable.

Inactivated (or Killed) Virus Vaccines

Killed viruses have been used as vaccines in a variety of animal model systems, often with good results, as reviewed elsewhere (123,173,304). Unfortunately, when these vaccines were administered to patients with preexisting HSV infection in order to alleviate recurrences, most studies failed to include an appropriate control group. Under such circumstances, significant bias can be introduced because patients may experience 30% to 70% decrease in the frequency of recurrent lesions, as well as improvement in severity, simply from having received placebo (39,209,227,228,490,518).

The initial inactivated vaccines were made from phenol-treated tissues obtained from infected animals (44,55,153). Because of the potential for demyelination after the administration of animal proteins, these vaccines attracted little biomedical attention. Subsequently, UV light inactivation of purified virus derived from tissue culture experiments provided greater impetus for the vaccine field. During the past 20 years, numerous reports have appeared in the literature regarding either the success or failure of such approaches. As reviewed by Meignier (304), viral antigens obtained from amniotic or allantoic fluid, chorioallantoic membranes, chick cell cultures, sheep kidney cells, and rabbit kidney cells that were inactivated either by formalin, UV light, or heat were used in a series of vaccine studies in thousands of patients (33,61,65,86,123,132,143,187,188,202,209,242,292,330,347,348 and 349,382,432,442,445,462,475,520,529). With one exception (330), each of these studies reported significant

improvement in as many as 60% to 80% of patients (132,347,348 and 349). Notably, however, no controls were used.

As these studies progressed, several important observations were made. First, despite repeated inoculations, antibody titers (as measured by neutralization or complement fixation) remained unchanged in the majority of patients (65,188,405) or demonstrated only slight increases (475,520). Second, although few side effects were reported, some authors noted concern that, in patients with keratitis, autoimmune phenomena might make the herpetic disease worse (189,277,382,445,476).

Placebo-controlled studies that have used inactivated vaccines are few (13,228,476,530). The results from these studies differ widely, even when the same vaccine was used. A conclusion from these investigations was that there may be some initial benefit for patients with recurrent infection; however, long-term benefit could not be established.

The only prospective study of prevention of HSV infections using this vaccine approach was performed by Anderson et al. in children living in an orphanage (13). In this study, 10 children received vaccine and 10 the placebo; nevertheless, HSV gingivostomatitis developed in an equal number of patients in both groups on long-term follow-up.

In summary, the perception of the biomedical research community was that these vaccines did not meet accepted standards for licensure. Very likely, the potential risks of inactivated vaccines, especially as a cofactor in the development of cervical carcinoma—a prevalent notion at the time of these studies—may have contributed to the abandonment of this approach. Finally, new techniques in recombinant DNA technology have made these vaccines obsolete.

Subunit Vaccines

Subunit vaccines evolved from attempts to remove viral DNA and eliminate the potential for cellular transformation, to enhance antigenic concentration and induce a stronger immunity, and, finally, to exclude any possibility of contamination with residual live virus (145). Available subunit vaccines were prepared using a variety of methods for antigen extraction from infected cell lysates by detergent and subsequent purification.

The immunogenicity of envelope glycoproteins, free of viral DNA, was demonstrated in animals (76,77,310,465). The results of studies in humans are disappointing. As recently reported, envelope glycoproteins do not convey protection to uninfected sexual partners of individuals with genital HSV infection (20,111,568).

These subunit vaccines, as well as others, have been studied in a variety of animal models, including mice (124,243,245,266,272,305,469), guinea pigs (265,485,486,511,522,565), and rabbits (75). Neutralizing antibodies can be detected in these systems in varying amounts, as can ELISA antibodies in some systems (192). In these systems, the quantity of neutralizing antibody appears to correlate with the degree of protection upon challenge. Challenge in the experimental systems was accomplished in mice (68,192,243,245,266,305,464,469), rabbits (75,79,388,451), and guinea pigs (451,485,486,511). Each of these systems used a variety of different routes of challenge as well as dosages. Challenge sites included skin, lip abrasion, intravaginal tissue, intradermal ear pinna, intradermal tissue, foot pad, intraperitoneal tissue, ocular tissue, and subcutaneous sites. Thus, interpretation of these results is extremely difficult, especially in light of the lack of clinically efficacious data in humans. Although there are many conflicting studies, generally the subunit vaccines elicited some protection, as evidenced by the amelioration of morbidity and reduction in mortality in the immunized animals. The predictability of these models for human studies is most disappointing. Furthermore, several injections are required to induce protection, and they must include adjuvant. The necessity for an appropriate adjuvant has recently been emphasized (430,508). Protection in the rodent is significantly easier than in higher primate species, as previously noted. This may be because HSV is not indigenous to rodent species; therefore, the protection studies may be totally irrelevant when evaluating human responses.

Vaccination of primates, specifically rhesus monkeys (75), chimpanzees (75,76), and *Cebus* monkeys (196), can induce neutralizing antibodies, which can lead to an anemnestic response after subsequent injection months later. The degree of protection in these animals remains unclear.

Subunit vaccines have failed in human experiments despite initial promising results. Both HSV-1 and HSV-2 antigens have been prepared in human diploid cells and chicken embryo fibroblasts cells for vaccine purposes. At least two studies have led to evidence of protection as defined by immune responses (466,467). Nevertheless, other experiments have suggested that these vaccines offer very little benefit (20,76,568).

Extensive vaccine experience was reported for individuals receiving the Merck Sharpe & Dohme (196) glycoprotein envelope subunit vaccine (20,568). In this study, conducted in sexual partners of patients known to have genital herpes, the number of individuals developing herpetic infection was nearly the same for both placebo and vaccine recipients, indicating that the vaccine was not effective.

Studies have been completed with one of two gB and/or gD recombinant vaccines in humans. Initially, the gD-2 construct was administered to 24 human volunteers at the National Institutes of Health for reactogenicity and immunogenicity. These studies demonstrated an increase in the geometric mean antibody titer to gD-2; however, lymphocyte blastogenic responses were not consistent between populations. Either 30 µg or 100 µg of purified gD-2 was used as the immunogen with alum (497). Extensive rodent experiments using the guinea pig genital herpes model have explored the utility of using viral glycoproteins gB and gD as vaccine components. If the dose is optimal, a mixture of gB and gD can protect against both primary and spontaneous recurrent disease after intravaginal viral inoculation (485). However, to demonstrate this effect it was necessary to use complete Freund's adjuvant, a component not acceptable for human administration. More recently, investigators demonstrated that gD combined with a lipophilic muramyl tripeptide affords a high level of protection from HSV disease (70,430); however, this adjuvant was abandoned because of reactogenicity. In a series of experiments, investigators were able to demonstrate that the amount of antigen in the vaccine affects the host response. An important observation from these studies is that the quantity of neutralizing antibodies elicited by

immunization and the total HSV antibody titer (as measured by ELISA) were higher after vaccination than after natural infection and, furthermore, that these antibody titers correlated with protection from disease (217,485).

These initial studies were expanded by further experiments using muramyl tripeptides with gB and gD, to determine protective effects against recurrent infection. These studies show the development of immune responses similar to those of natural infection (497). The use of these subunit vaccines for therapy of recurrent genital herpes appeared to decrease the frequency of recurrence (538). Unfortunately, the failure of the gB and gD vaccine in a well-performed clinical trial is most disappointing (111) and has resulted in one vaccine company abandoning the approach.

Live Virus Vaccines

For decades, live virus vaccines have been considered most likely to provide a high level of protection, as was the case with numerous viral pathogens such as measles, mumps, and rubella. The rationale for the selection of live virus vaccines is that because they replicate in the recipient, the resulting immunity should be longer lasting. Moreover, these vaccines usually require smaller quantities of antigen and, therefore, should be more economical. Several approaches to live virus vaccines have been attempted, including HSV mutants, heterologous herpesviruses, antigens expressed in non-HSV viral vectors, and genetically engineered viruses.

Genetically Engineered HSV

Molecular biology makes it possible to modify, almost at will, the genome of large DNA viruses and to construct genetically engineered attenuated live viruses (410). The technology developed by Post and Roizman (318,380) has been used to construct recombinant HSV as a prototype of herpes vaccines (306). These vaccines were engineered with the objectives that they should be attenuated for both primary inoculation and reactivation of latent virus; they should protect against HSV-1 or HSV-2 infections; they should provide serologic markers of immunization distinct from wild-type infections; and they should serve as vectors to express immunogens of other human pathogens.

The construction of these viruses was based on the use of a type 1 HSV, HSV-1(F), as a backbone to avoid the presence of the sequences of HSV-2 reported to be associated with transformation in cell cultures. The genome was deleted in the domain of the viral thymidine kinase gene and in the junction region of the U_L and U_S segments in order to excise some of the genetic loci responsible for neurovirulence as well as to create convenient sites and space within the genome for insertion of other genes. Finally, a type 2 HSV DNA fragment encoding the HSV-2 glycoproteins D, G, and I was inserted in place of the internal inverted repeat. The purpose of the type 2 genes was to broaden the spectrum of the immune response and to create a chimeric pattern of antibody specificities. The resulting recombinant, designated as R7017, was further altered by insertion of the thymidine kinase gene next to the HSV-2 DNA fragment (R7020). Because this virus expresses thymidine kinase, it is susceptible to antiviral chemotherapy with acyclovir. When analyzed by restriction enzyme digestion, the DNA of the recombinants has a unique pattern that facilitates their unambiguous identification.

When evaluated in rodent models, the two constructs appeared considerably attenuated in their pathogenicity and ability to establish latency and were capable of inducing protective immunity. The recombinants did not regain virulence, nor did they change DNA restriction enzyme cleavage patterns when subjected to serial passages in the mouse brain (336). It is remarkable that the thymidine kinase–negative virus R7017 behaved similarly to the thymidine kinase–positive virus R7020.

These results were corroborated by studies in owl monkeys (*Aotus trivirgatus*) (380). Although administration of 100 plaque-forming units of wild-type virus by peripheral routes was fatal to the monkeys, recombinants given by various routes in amounts at least 10^5-fold greater were innocuous or produced mild infections, even in the presence of immunosuppression by total lymphoid irradiation (307).

When R7020 was evaluated in humans in a reactogenicity study, human immune responses were elicited at low levels (73). Unfortunately, production difficulties precluded the development of higher dosages of vaccine.

The application of these principles to HSV-2 constructs has been undertaken and assessed in animal models (478). The definition of the $g_1$34.5 gene of HSV-1 as a mediator of CNS replication provides a potential vaccine construct (92,547). Furthermore, deletions of $g_1$34.5 significantly decrease the ability of this construct to establish latency (547). Because a homologous $g_1$34.5 gene exists in HSV-2, the opportunity to create an HSV-2 vaccine exists. Other creative approaches to the manipulation of HSV are underway for vaccine development (144,307,355,478).

More recently, a vaccine deleted in gH has been studied in phase I studies in the United Kingdom. This vaccine, known as the HSV-DISC vaccine, undergoes a single cycle of replication. Because gH is deleted, no further replication is possible. The results of this phase I study have not been reported at this time.

CONCLUSION

Tremendous progress has been made in our understanding of the molecular biology, natural history, and therapy of HSV infections; however, it is apparent that a great deal remains to be learned. The molecular pathogenesis of these infections, along with the prevention of resultant disease by means of molecularly derived vaccines, remains a principal goal for future research efforts. The prevention of HSV disease remains a high priority, especially in light of the failure of one of two subunit vaccines. Furthermore, as we learn about the role of HSV infection in programmed cell death, new avenues of advance will occur. One such avenue is the use of HSV and its genes for the treatment of brain tumors (295,296).

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CHAPTER 74 Epstein-Barr Virus and Its Replication

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CLASSIFICATION

Epstein-Barr virus (EBV) was discovered in 1964 (192). Beginning in the 1940s, Denis Burkitt, a British missionary surgeon, observed and treated children with previously undescribed extranodal lymphomas. He noted that the lymphomas were frequent in regions of equatorial Africa with holoendemic malaria and rarely occurred elsewhere. Burkitt wrote and spoke widely about the unusual epidemiologic and clinical features of this lymphoma, raising the specter of an infectious etiology (81). After hearing Burkitt speak about this new disease at Bristol University, Tony Epstein arranged collaborations to obtain tumor biopsies and succeeded in culturing the lymphoma cells. Epstein, Achong, and Barr identified a herpesvirus in electron micrographs of the tumor cells growing in culture. They showed that the virus differed from the known human herpesviruses in being unable to replicate in other cultured cells and in being nonreactive with antibodies to other known human herpesviruses. EBV became the first candidate human tumor virus.

EBV is now the prototype of the gamma subfamily of potentially oncogenic herpesviruses. The gamma herpesvirus subfamily includes both the gamma 1, or *Lymphocryptovirus* (LCV), and gamma 2, or *Rhadinovirus* (RDV), genera. EBV is the only human LCV, and the recently discovered Kaposi's sarcoma–associated herpesvirus (KSHV) is the only human RDV (93). Herpesvirus saimiri (HVS), an RDV that was discovered in New World primates and found to cause lymphomas in experimental infection, was the previous prototype RDV (10). Many Old World primate species have their own endemic LCV, and recent evidence suggests that some New World primate species also have endemic LCVs (61, 207, 244, 246, 313, 457, 474, 676). In contrast, RDVs have been identified not only in many primate species but also in many subprimate mammalian species (9, 11, 188, 858). At this stage of accumulation of LCV and RDV DNA sequences, RDV DNAs are more diverse than LCV DNAs. The endemicity of RDVs in a broader range of mammalian species and their greater genome divergence are evidence that RDVs evolved earlier than the LCVs. Given the many similarities of the LCV and RDV genomes and the restriction of LCVs to primates, LCVs are likely to have evolved from an early primate RDV. This would explain the presence of LCVs only in primates.

The LCV genomes are very similar to each other in structure and gene organization. In general, their DNAs are composed of colinearly homologous sequences. The EBV genome organization is shown in Figure 1 and compared to that of KSHV. The schematic diagram is based on the published analyses of KSHV DNA sequences. The LCVs share structural features such as similar 0.5-kbp terminal (TR), 3-kbp internal (IR1), and short internal (IR2, IR4) tandem direct repeats (142, 143, 313, 314, 316, 474, 714). The LCV open reading frames (ORFs) also encode colinearly homologous, antigenically related, structural and nonstructural proteins (384, 648, 690).

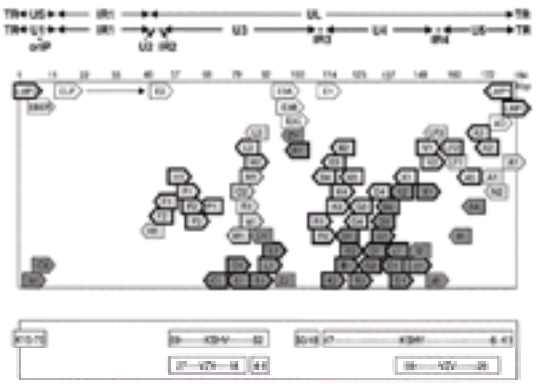

FIG. 1. Schematic depiction of the linear EBV genome, and comparison with the genomes of KSHV (712) and VZV (154, 155). At the *top* of the figure, the terminal repeat (TR), internal repeat (IR1-4), and the largely unique sequence domains (U1-U5) of the EBV genome are depicted in proportion to their overall size. The position of the cis-acting element for episome maintenance and replication in latent infection, oriP, is indicated. The origins for EBV DNA replication in lytic infection are in U3 and U5 just to the right of IR2 and IR4. The P3HR-1 EBV strain is deleted for DNA between 45 and 52 kbp in the indicated map. EBV open reading frames (*ORFs*) are indicated based on the size of the encoding *Bam*HI fragment (A, B, C, etc., in decreasing size), using the convention of Barrell et al. (36, 209). The origins and direction of the reading frames are indicated in the *large panel* according to scale. However, the size of the text box

and end of the ORFs are not to scale. ORFs expressed in EBV latent infection are in *clear text boxes*, immediate-early in *dark gray*, early in *light gray*, and late in *medium gray.* ORFs that are conserved in KSHV are indicated by *thick outline* and by blocks shown in the *lower panel.* The numbers in the lower panel refer to homologous ORFs of KSHV (712) r VZV (154), which are displayed by their corresponding EBV map location.

LCV genomes include genes that are shared by most herpesviruses, genes that are shared among gamma herpesviruses but not with other herpesviruses, and a number of genes that are characteristic only of LCVs (see Fig. 1). The genes that are shared with most herpesviruses include genes that encode for proteins that are involved in nucleotide metabolism, genes that encode for proteins that replicate viral DNA, genes that encode for proteins that comprise the structural components of the virion, and genes that encode for proteins that package viral DNA, modify the infected cell, and enable the virus envelope to fuse with the cell membrane. Despite base compositions ranging from 43% guanine plus cytosine for varicella-zoster virus (VZV) to 71% guanine plus cytosine for herpes simplex virus (HSV), herpesvirus DNAs have large regions with colinear, distant homology at the predicted protein level (see Fig. 1) (36,154,155,442). For example, one large conserved domain, homologous to VZV ORFs 28 to 59 (see Fig. 1), encodes for the major DNA binding protein, DNA polymerase, glycoprotein (gp) B (gB), thymidine kinase (TK), and gH. Similarities notwithstanding, base-pair conservation even in the homologous domains is inadequate to permit EBV DNA to hybridize to the homologous HSV, VZV, cytomegalovirus (CMV), or even KSHV DNAs. Furthermore, antigenic cross-reactivity between EBV and other human herpesviruses is rare, even among proteins encoded by the most conserved genes. The genes that are shared among the gamma herpesviruses and have more limited or less obvious representation in the genomes of other herpesviruses include genes that encode several immediate-early or early regulators of viral gene expression, an antiapoptotic Bcl-2 homolog, and two integral membrane proteins that in EBV are designated LMP1 and LMP2. LCVs and RDVs also have analogous, but nonhomologous, cis-acting DNA sequences and transacting nuclear proteins that are necessary and sufficient for persistence of the genomes as episomes in dividing cells (41,910). The genes that are well conserved among only LCVs include a set of genes that encode nuclear and integral membrane proteins that are important for latent B-lymphocyte infection and cell growth transformation, genes that encode two small nonpolyadenylated RNAs, genes that encode an interleukin (IL)-10 homolog, and a gene that encodes a glycoprotein that binds to a B-lymphocyte surface protein, CD21.

Overall, the LCV and RDV genomes are much more closely related to each other than to the genomes of the alpha or beta herpesviruses (36,154,155,712). Of the 75 KSHV predicted ORFs, 54 are colinearly homologous to ORFs of EBV (see Fig. 1). Only one of these 54 is out of place relative to its position in EBV. The mean amino acid identity to EBV among the 54 KSHV ORFs is 35%, with only two less than 21%.

Nevertheless, LCV and RDV are clearly distinct genera. The sequence homology between EBV and KSHV is insufficient to detect significant cross-reactive antibody or T-cell responses. Primates including humans can be persistently systemically infected by viruses of both genera. Whereas LCVs are able to efficiently immortalize B lymphocytes of their natural host, RDVs lack similar activity. At the genome level, RDVs have very highly reiterated terminal direct repeats and lack long internal direct repeats. Most RDVs have a common set of cellular genes that include dihydrofolate reductase, interferon regulator factors, G-protein coupled receptors, chemokine analogs, and a cyclin homolog, none of which have been found in LCVs.

The gamma herpesvirus subfamily classification was initially established not on the basis of similarity in genome organization but on the basis of similarity in biologic properties. EBV and KSHV have particularly limited host ranges for *in vitro* infection and are associated with human malignancies. Much of the interest in these viruses is because of their association with cancer. These and other gamma herpesviruses also establish latent infection in lymphocytes, but they are not unique in this regard; beta herpesviruses such as HHV-6, HHV-7, and CMV latently infect lymphocytes. Although the oncogenic properties of LCVs and RDVs were historically important in segregating them into the gamma herpes subfamily, the nucleotide and protein sequence relationships provide a more enduring basis for phylogenetic classification.

Taxonomists have renamed EBV as human herpesvirus 4 (HHV4). Although most authors still use the name EBV, some indexing services have adopted HHV4, so both entries should be used when doing bibliographic searches.

VIRUS STRUCTURE

Like other herpesviruses, EBV has a toroid-shaped protein core that is wrapped with DNA, a nucleocapsid with 162 capsomeres, a protein tegument between the nucleocapsid and the envelope, and an outer envelope with external glycoprotein spikes (172,173,192,659). The major EBV capsid proteins are 160, 47, and 28 kd, similar in size to the major HSV-1 capsid proteins (172,173). EBV has a range of envelope glycoproteins, including several that are homologs of relatively well conserved herpesvirus glycoproteins. These include homologs of HSV gH, gL, gB, gM, and gN, which are also known as EBV gp85 or BXLF2, gp25 or BKRF2, gp110 or BALF4, gp84/113 or BBRF3, and gp15 or BLRF1, respectively. However, the most abundant EBV envelope and tegument proteins are 350/220 and 152 kd, respectively, and these are not homologous to major envelope and tegument proteins of HSV-1 (172,173,597,668,669,820,822).

GENOME STRUCTURE

The EBV genome is a linear, double-stranded, 184-kbp DNA composed of 60 mole percent guanine or cytosine (see Fig. 1) (36,142,253,254 and 255,379,405,662,663,673). The characteristic features of EBV and most other LCV genomes include the following (see Fig. 1): (a) a single overall format and gene arrangement (142,147,253,254 and 255,671); (b) tandem, reiterated, 0.5-kbp direct repeats of the same sequence at both termini (TR) (255,304,430); (c) six to 12 tandem reiterations of 3-kbp, internal direct repeats (IR1) (103,104,253,254,305), and (d) short and long, largely unique sequence domains (U_S and U_L) that have almost all of the genome coding capacity. Although largely unique DNA, U_L and

U_S include perfect and imperfect tandem DNA repeats, most of which are within ORFs. A duplicated region, D_L, near the left end of U_L, consists of multiple, highly conserved, G-C-rich, tandem 125-bp repeats and 2 kbp of adjacent unique DNA, all of which have extensive homology to the D_R region, near the right end of U_L. D_R consists of multiple, highly conserved, G-C-rich, tandem, 102-bp repeats and 1 kbp of nearby unique DNA (149,311,313,314,316,351,378,463). The D_L and D_R repeats are within ORFs. Because the D_L repeat is 125 bp and not divisible by three, sequential iterations are translated in different reading frames, resulting in an unusual protein. D_L and D_R also include the origins for initiation of viral DNA replication in lytic infection.

The reiteration frequency of the EBV tandem perfect repeats becomes variable during viral DNA replication, with the average number of repeats being identical to the parent genome, and most of the progeny having a number of repeats that is identical or similar to that of the parental genome. When EBV infects a cell, the genome becomes an episome with a characteristic number of TRs, dependent on the number of TRs in the parental genome, with variation introduced during viral DNA replication and the unique cleavage and joining events of the single infecting viral genome. If the infection is nonpermissive for viral replication and permissive for latent infection and continued cell growth, each EBV episome in progeny infected cells will usually have the same number of TRs as the parent cell. Homogeneity or heterogeneity in the number of TRs is therefore useful in determining whether a group of latently infected cells arose from a single common progenitor infected cell or from multiple progenitors (76,674). The number of 3-kbp IR1 repeats also varies among EBVs and among progeny of EBV replication, but this is more difficult to assess as a marker of heterogeneity because of the 18- to 30-kbp size of the total IR1 element. Other, smaller, imperfect or less highly reiterated repeats within ORFs are more stable during replication but differ sufficiently among different virus isolates that the size of the encoded proteins can be used to uniquely identify each isolate.

Because of the limited host range for EBV replication *in vitro* and the difficulty in obtaining large amounts of viral DNA for molecular biologic investigation, EBV was the first herpesvirus whose complete genome fragments were cloned into *Escherichia coli* (33,77,142,216,656,673). EBV was also the first herpesvirus to be completely sequenced (36,301,632). Since the EBV genome was sequenced from a *Bam*HI fragment library, genes, promoters, ORFs, and polyadenylation sites are frequently referenced to the corresponding *Bam*HI fragment (36). Thus, the EBV DNA polymerase gene is frequently referred to as BALF3 for *Bam*HI A fragment, third leftward ORF. Exons of spliced mRNAs are also frequently designated by the *Bam*HI DNA fragment that encodes the exon.

Two EBV types circulate in most populations. The two types are now usually designated 1 and 2, although A and B were widely used in the past. Type 1 is far more common in most populations. EBV type 2 is nearly as common as type 1 in equatorial Africa and New Guinea, and it is also not infrequent among people infected with human immunodeficiency virus (HIV) (4,30,144,147,206,288,311,419,426,549,710,725,740,741,761,901,903,904,920). The only significant differences between the genomes of the two types are in the genes that encode for the EBV nuclear proteins [or antigens (EBNA)] LP, 2, 3A, 3B, and 3C. The type 1 and 2 alleles for EBNA-2, -3A, -3B, and -3C differ in predicted primary amino acid sequence by 47%, 16%, 20%, and 28%, respectively (4,144,723). EBNA-LP differs less overall (144,381). Other than these differences, the genomes of various isolates of type 1 or type 2 EBV strains from widely different geographic areas have single base changes and repeat reiteration frequency differences from prototype genomes, with an overall difference in nucleotide sequence between type 1 and type 2 isolates outside of the EBNA-LP, -2, -3A, -B, or -3C ORFs of less than 4%. Differences among individual type 1 or type 2 isolates are more in the range of 1% (266,267 and 268,269,288,419,549). Similarities in the numbers of DNA repeats at various sites in the EBV genome among EBV isolates from a given geographic area have been used to group isolates into strains and to epidemiologically track EBV infection. Similar strain variations have been noted among both type 1 and type 2 isolates, consistent with the notion that these polymorphisms evolve regularly in nature. Persistent infection with more than one EBV type or strain is not unusual, particularly for more promiscuous and immunocompromised people. Intertypic recombinants have been identified in the oropharynx and lymphocytes of immunocompromised people and less commonly from otherwise healthy people (5,266,269,288,419,549,761,860,861,862 and 863,901,903).

GENERAL ASPECTS OF EBV INFECTION OF CELLS *IN VITRO*

The *in vitro* host range for efficient EBV infection is restricted to primary human B lymphocytes (323,660). EBV readily infects B lymphocytes derived from peripheral blood, tonsils, or fetal cord blood (29,319,403,697,883,897,921). B lymphocytes at earlier stages of development from adult or fetal bone marrow or fetal liver, as well as leukemic and non-EBV-infected Burkitt's lymphoma (BL) cell lines, can also be infected, although the efficiency of infection is lower than with peripheral blood (20,35,85,87,175,195,294,435,528,529,815). Fully differentiated plasma cells cannot be infected. The outcome of infection of primary human B lymphocytes with EBV is nonpermissiveness for virus replication and conversion of the infected cells to continuous proliferation into long-term lymphoblastoid cell lines (LCLs) (323,660). The seminal observation that latent EBV infection efficiently causes perpetual B-lymphocyte proliferation was critically important in establishing that EBV has oncogenic potential (323,563,607,608,660).

EBV can also establish latent infection in other cell types, including T or natural killer (NK) cells, although the efficiency is low, even compared to non-EBV-infected BL cells, which are usually 10-fold less susceptible to infection than primary B lymphocytes (397,398,745). Primary epithelial cells (763,918) are also relatively resistant to infection, whereas some epithelial cell lines, including HEK293 (213), gastric carcinoma cell lines (916), and biliary, laryngeal, hepatocellular, colon, and bladder carcinoma cell lines (365), can be infected with efficiencies that approach those of BL cells.

Wild-type virus can be obtained from human saliva or from infected human B lymphocytes growing in culture. Most adults have been infected by EBV and intermittently shed virus in saliva (245,902). They also have EBV in a latent state in approximately 1 in 10^5 to 10^6 of their peripheral blood B lymphocytes (608). When 10^6 to 10^7 peripheral blood B lymphocytes from an EBV-seropositive person are put in culture in the absence of functional T lymphocytes, EBV-infected B lymphocytes are likely to grow out, without adding exogenous virus (608,685,821). These cell lines are then a source of infectious EBV. Interestingly, the recovery of EBV-infected cells from such cultures in the absence of exogenously added virus can be blocked by inhibitors of virus replication or the addition of neutralizing antibody (687,819). Explantation to culture can apparently cause a rare EBV infected cell to become permissive for virus

replication. The newly replicated virus can then infect other B cells in the culture, and thereby initiate a latent, immortalizing infection, whereas the *in vivo* latently infected cell cannot spontaneously convert to a transformation type of latent infection. Virtually all cell lines that arise from cultures of normal blood cells are EBV immortalized B LCLs.

EBV infection of primary B cells, *in vitro*, results in about 10% of the cells becoming latently infected and proliferating as immortalized or “transformed” latently infected LCLs (319,783). Virus replication in LCLs is usually minimal or undetectable. The presence of the “latent” virus in each of the infected cells can be readily detected using antibodies to any of the eight different virus proteins that are characteristically expressed in LCLs. The eight viral encoded proteins include six nuclear proteins (the EBNAs) and two integral membrane proteins (the LMPs) (Fig. 2). In this context, the virus genome is hardly inactive and the infection is latent only in the absence of lytic virus infection.

	I	II	III
EBNA-1	Qp	Qp	Wp, Cp
EBNA-2, LP, 3A-C	-	-	+
LMP1, LMP2	-	+	+
EBERs	+	+	+
BARTs	+	+	+
Example	EBV-BL	HD, NPC	LCL, EBV-BL

FIG. 2. Types of EBV latent infection observed in Burkitt's lymphoma (*BL*) cells growing in culture (types I or III), or in nasopharyngeal carcinoma or Hodgkin's disease cells *in vivo* (type II). Type III latency is also characteristic of human B lymphocytes that have been infected with EBV and are proliferating as a consequence of latent EBV infection.

The standard conditions for clonal infection of primary B lymphocytes with EBV are to add less than 50 transforming units of EBV to 10^7 T-cell-depleted lymphocytes, and to then plate the infected cells in 96 wells of a microtiter tray in 0.1 to 0.15 mL of a complete growth medium consisting of the defined constituents of RPMI 1640 (Sigma Chemical Company, St. Louis, MO) and 10% to 20% fetal calf serum. Each infectious virus will give rise to a macroscopically visible cell line by 5 weeks of incubation. The virus titer is usually defined by this most sensitive assay of EBV infection. If the purpose is to study the biochemical events of primary infection, lymphocytes are infected with three transforming units of virus per cell and kept in bulk culture. Higher multiplicities are rarely used because of the practical difficulty of obtaining high-titer EBV preparations. For the cloning of virus or latently infected cells, putatively infected cells are fed every 2 weeks, and the assay is scored after 6 to 8 weeks, by which time an infected cell will usually have multiplied into more than 10^5 progeny cells. The cloning efficiency can be modestly improved by culturing the infected lymphocytes on irradiated human diploid fibroblasts, which enriches the culture conditions beyond those provided initially by the 10^5 uninfected lymphocytes, residual macrophages, and complete growth medium.

In latently infected B lymphocytes, EBV expresses not only the six EBNAs and two LMPs but also two small nonpolyadenylated RNAs (or EBERs) and highly spliced *Bam* A rightward transcripts, or BARTs (see Fig. 2). These viral gene products maintain the latent infection and cause the previously resting B lymphocytes to enter cycle and continuously proliferate. The effect on cell growth is immediate, and most infected B cells enter the first round of cell DNA synthesis 48 to 72 hours after EBV infection (12,594,595 and 596). The target cell that is most amenable to conversion to an LCL has a resting B-lymphocyte phenotype (22).

Aside from the presence of the EBV genome and its gene products, the EBV-infected proliferating B lymphocytes are similar to lymphocytes proliferating in response to antigen, mitogen, or IL4 and anti-CD40 in their expression of a similar repertoire of “activation”-associated proteins, their secretion of immunoglobulin (Ig), their high level of expression of adhesion molecules, and their adherence to each other (42,67,85,86 and 87,363,635,636,785,826,828,867,868 and 869). EBV-infected B lymphocytes may continue to secrete immunoglobulin for months or even years in culture. Some cells can continue to differentiate and undergo class switching in culture when exposed to IL4 (243,373).

Most non-EBV-infected BL cell lines can be infected with EBV *in vitro* (85,87). The growth of non-EBV-infected BL cells *in vitro* is substantially the result of a characteristic reciprocal chromosome translocation involving the c- *myc* and Ig heavy or light chain loci. This results in constitutive c- *myc* expression (141; for review see ref. 531). Other changes are presumed to be necessary for the development of BL *in vivo*, including changes in chromosome 1 (52). Still other genetic changes occur in BL cells during growth *in vitro*. BL cells frequently grow as single cells and can regrow from true single-cell end-point dilution. They are similar to germinal-center B lymphocytes in their expression of surface markers including IgM, and they differ from LCLs in their high-level expression of Bcl-6 and CD10 and low-level expression of activation markers and adhesion molecules. When EBV infects BL cells *in vitro*, the virus usually initially expresses the same program of latency as in LCLs. This type of latency, characteristic of LCLs and marked by the expression of the six EBNAs, two LMPs, two EBERs, and BARTs, is termed latency III (21,271,708) (Fig. 2 and Fig. 3). Latency III EBV gene expression causes the cells to grow in tight clumps, express activation markers, down-regulate Bcl-6 and CD10, and otherwise assume the phenotype of LCLs (1,57,133,134,321,453,527,867,868 and 869,871,873). Over the ensuing months of culture, however, some of the cells may lose the EBV genome and revert to a germinal center BL phenotype. Some BL cell lines, infected with EBV *in vitro* and maintained in culture for several months, remain EBV infected and continue in a latency III phenotype. Others convert to a latency 1 phenotype and express only EBNA-1, EBERs, and BARTs (see Fig. 3). Some BL cells retain the EBV genome as an episome, whereas in other cells, the EBV genome can be integrated into the cell genome (318,361,467,543). Some BL cells with integrated EBV DNA maintain a latency III

phenotype.

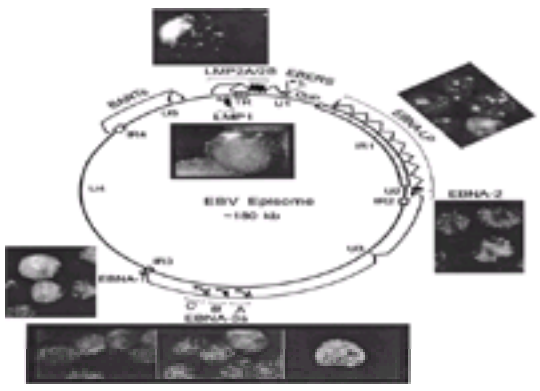

FIG. 3. EBV episome, transcripts, mRNAs, and proteins in type III latent B-lymphocyte infection. Largely unique (U1-U5) and repetitive internal (IR1-4) or terminal (TR) repeat DNA segments are indicated. The origin for latent infection EBV episome replication, oriP, is also indicated. Exons encoding EBV nuclear proteins (EBNA-1, -2, -3A, -3B, -3C, and -LP) and EBV integral membrane proteins (LMP1, -2A, or -2B) are indicated by *vertical lines* radiating from the circular episome map. EBNA transcription is shown as it is first initiated from the IR1 or Wp promoter. In many infected lymphocytes, after expression of EBNA-LP and EBNA-2, the Cp promoter in U1 dominates EBNA transcription. Alternative polyadenylation and splicing lead the various EBNA mRNAs. EBNA-LP is encoded by repeating exons from IR1 and two short unique exons from U2. The EBERs are two small, nonpolyadenylated RNAs transcribed in latent or productive infection. LMP1 is transcribed in the direction opposite to the EBNAs, LMP2A and -B, EBERs, and BARTs. The typical intracellular localizations of the proteins that are known to be expressed in type III latency are shown on the EBV DNA map on the *left.* The proteins are identified by immune fluorescence microscopy using specific antisera. The smaller episome drawing on the *right* shows the terminal exons of the differentially spliced Bam A fragment rightward transcripts that are likely to encode the proteins provisionally designated RPMS1 and BARF0 in all three types of latent infection (234,422,716,766). LMP2A also has an upstream EBNA-2 response element, whereas LMP1 and LMP2B share the same EBNA-2 response element.

As described in Chapter 75, most EBV-infected BL tumors have a latency I phenotype. When these cells are explanted to culture, they initially maintain latency I and express only EBNA-1, EBERs, and BARTs. Paradoxically, on continued culture, many of the *in vivo*–infected BL cells switch to a latency III phenotype and grow in clumps similar to LCLs. Most *in vivo*–infected BL cells never lose their EBV episomes when the cells are grown *in vitro*, whereas BL cells infected *in vitro* may lose their EBV genomes entirely or may have only integrated EBV DNA (361,527,871). The persistence of EBV episomes in most *in vivo*–infected BL cell lines that have been maintained for years in culture is compatible with the possibility that the episome is important for cell proliferation or survival. Cells of other B- or even T-lymphocyte malignancies that can be grown as continuous cells lines *in vitro*, and that express low levels of EBV receptors, usually express only EBNA-1, EBERs, and BARTs when infected with EBV *in vitro*.

With a frequency characteristic of each EBV-infected B-lymphocyte line, some progeny latently infected proliferating cells growing *in vitro* become spontaneously permissive for virus replication. Primary human B-lymphocyte-derived cell lines (LCLs) vary from zero to a few percent of cells permissive for EBV replication. *In vitro*–infected BL cell lines tend to be even less permissive for virus replication, whereas *in vivo*–infected BL cell lines are frequently substantially more permissive for virus replication than LCLs. The lack of permissivity of *in vitro*–infected BL cells for EBV replication is in part related to the poor persistence of EBV episomes in such cells, as lytic replication probably requires episomal viral DNA.

In all instances, permissively infected cells exhibit the characteristic cytopathic changes associated with herpesvirus replication, including formation of an intranuclear inclusion in the center of the nucleus at the site of lytic infection DNA replication, margination of nuclear chromatin, assembly of capsids within the nucleus near the nuclear membrane, budding of virus through the nuclear membrane, and formation of cytoplasmic vesicles containing enveloped virus (189,190,191,192 and 193,261,659). As with other herpesviruses, cell macromolecular synthesis is inhibited early in EBV replication (247).

The frequency with which latently infected lymphocytes become permissive for virus replication can sometimes be influenced by specific culture conditions. Transient exposure to drugs that inhibit host macromolecular synthesis, cell starvation, arginine-free media, inhibition of DNA methylation with azacytidine, exposure to butyric acid (which inhibits histone deacetylase), treatment with phorbol ester to stimulate protein kinase C (PKC), exposure to ionophores (which increases intracellular free calcium), or cross-linking of surface Ig can each increase the permissivity of some EBV-infected cells for lytic infection (45,152,350,464,717,802,804,805,896,898,899).

Analyses of cultures of a latently infected BL tumor–derived cell line that is particularly permissive for virus replication led to the identification of defective EBV genomes in these cultures (332,559,678). The defective genomes express an EBV immediate-early protein, BZLF1 or Z (381,383). The expression of Z following heterologous gene transfer is now frequently used to induce lytic EBV replication in latently infected cells (129,130,264,564). Not all cells in which Z is expressed go on to lytic infection (129).

The most sensitive method for recovering EBV from a latently infected cell line that is largely nonpermissive for virus replication is to lethally irradiate 10^4 induced cells and co-cultivate them with 10^5 primary B lymphocytes in 150 µL of complete medium in a microwell (566,567). This allows maximal contact between the rare cell that becomes permissive for lytic EBV infection and uninfected B lymphocytes. Under such conditions, the irradiated cells also provide feeder

effects and enhance the growth of the newly infected primary B lymphocytes. In the absence of autologous or heterologous feeder cells, EBV-transformed B lymphocytes do not usually regrow from suspensions of single cells in 100 µL of rich growth medium.

The induction of virus replication in latently infected B lymphocytes remains the principal way to study EBV replication. Fortunately, latently infected BL and marmoset cell lines have been identified in which a substantial fraction of the cells can be rendered permissive for lytic virus infection (137,138,333,566,567,802,804,805). These cell lines are essential for studying lytic infection *in vitro*, and they are the best source of EBV for studying virus structure or cell infection. After induction of lytic EBV infection in B lymphocytes in culture, 2 to 3 days are required for maximal late gene expression. Maximal titers of extracellular virus are reached at 4 to 5 days.

Since epithelial cells *in vivo* are fully permissive for lytic EBV infection (606,760), considerable effort has been made to adopt EBV to growth in epithelial organ cultures or transformed epithelial cell lines (763). Primary cultures or even some continuous cultures can be infected, particularly when incubated with cells that are producing virus, but the infection is inefficient and little virus results (759). The efficiency of infection has been slightly improved by expression of the B lymphocyte EBV receptor in an epithelial cell line (481). More cells are infected but the infection is largely abortive. Epithelial cell cultures, especially of HEK293 cells, have recently been useful for EBV genetic studies because of the relative ease of transfection of these adherent cell cultures with plasmid DNAs that include the entire EBV genome. The small amount of virus recovered from such cells can then be expanded in B lymphocytes. Surprisingly, the EBV receptor on some HEK293 cells appears to be the same as the normal B-cell receptor (213).

MOLECULAR BIOLOGY OF VIRAL INFECTION OF CELLS*IN VITRO*

Adsorption

The *in vitro* host range restriction of LCVs to B lymphocytes is partly the result of a restriction in high-level expression of a cell surface protein, CD21, which is an efficient receptor for EBV and for the C3d component of complement (214,456,878,879). Efficient B-lymphocyte infection correlates with the stages of B-lymphocyte development in which CD21 is expressed (364,390). Monoclonal antibodies to CD21 block virus infection (214). Furthermore, purified CD21 binds to EBV and can block infection (604,605). Moreover, CD21 expression on heterologous cells confers the ability to adsorb EBV (6,481). CD21 is an immunoglobulin superfamily glycoprotein, and it consists of 15 or 16 imperfect repeats of a 60-plus amino acid domain, a transmembrane domain, and a short carboxyl-terminal cytoplasmic domain (588,878). EBV binding activity maps to the two amino-terminal domains (537,582,587,604).

The most abundant EBV outer envelope glycoprotein, gp350/220, is the CD21 ligand (602,603,810,811,880). Gp350 and gp220 are translated from abundant late-replication-cycle nonspliced and spliced mRNAs that are transcribed from the same gene (50). CD21 is the only B-lymphocyte surface protein that binds to gp350/220 (810). The gp350/220–CD21 binding affinity is 1.2×10^{-8} M (811). Soluble gp350/220 can saturate B-lymphocyte receptors and block virus infection, confirming a key role for gp 350/220 in virus adsorption (604,811). A gp350/220 peptide sequence EDPGFFNVEI is similar to the peptide sequence EDPGKQLYNVEA through which the C3d component of complement binds to CD21. Peptides containing the LYNVEA C3d sequence block C3d binding to CD21 (456), and peptides containing the EDPGFFNVEA sequence block EBV infection (602), whereas mutant gp350/220 molecules that lack VE in the EDPGFFNVE peptide do not bind to CD21 (811). The EBV receptor on epithelial cells that lack CD21 has not been identified, in part because of the less efficient adsorption of EBV to primary or continuous epithelial cell lines than to B lymphocytes. Recently, the derivation of a virus that has an insertion into gp350/220 codon 79, which should functionally inactivate gp350/220, has resulted in a virus that can still infect B lymphocytes and epithelial cells, albeit with reduced efficiency. This indicates that gp350/220-mediated adsorption to CD21 is not absolutely required for lymphocyte or epithelial infection (375).

Penetration and Uncoating

Wild-type EBV infection of primary B lymphocytes is initiated by gp350/220 adsorption to CD21 on the B-lymphocyte plasma membrane, aggregation of CD21 in the plasma membrane, and internalization of EBV into cytoplasmic vesicles (91,601). CD21 is part of a complex that includes CD19 and associated intracellular tyrosine kinases, similar to the surface Ig (sIg)–associated signal transducing cell surface proteins. EBV aggregation of CD21 probably results in CD21-mediated tyrosine kinase signal transduction, and this may have an activating effect on the B lymphocyte (536,541). Infected cells begin to synthesize RNA, enlarge, express activation and adhesion molecules, clump, and secrete Ig soon after exposure to virus (12,24,363,810). CD21 ligation also increases NF-kB activation and may up-regulate the EBNA Wp promoter at the start of EBV infection (782). The precise role of these effects in initial infection is somewhat uncertain, because inhibitors of cell protein synthesis, of tyrosine kinases, or of PI3 kinase do not block initial EBNA RNA transcription (756).

The EBV gH homolog, gp85, is the second most abundant envelope glycoprotein (177,178,310,779). EBV gp85 is part of two virion glycoprotein complexes: a heterodimeric complex with the EBV gL homolog, gp25, a product of BKRF2, and a heterotrimeric of gp85, gp25, and gp42, the product of BZLF2. EBV gL is all that is necessary for gp85 processing (666,905). Studies with a monoclonal antibody to gp85 that does not affect virus adsorption to B lymphocytes but that blocks EBV envelope fusion with B lymphocyte membranes, provided an initial indication that gp85 is particularly important in envelope fusion with cellular membranes (289,480,568). This role of gp85 is similar to that of HSV-1 gH, which mediates envelope fusion with the cell membrane and thereby enables nucleocapsid exocytosis into the cytoplasm (260).

The heterotrimeric complexes of EBV gH/gL and gp42 (905) have a unique role in mediating a coreceptor interaction in B lymphocyte infection by gp42 engagement of human leukocyte antigen (HLA) class II, D_R, D_P, and a subset of D_Q alleles (286,287,479,581,776,875,876). Epithelial cells do not express HLA class II, and antibody to gp42 blocks B-lymphocyte infection but not epithelial infection (480). The lack of effect of gp42-specific antibody on epithelial infection suggested

the possibility that gp42 has an insignificant or different role in epithelial infection. Recent studies with an EBV recombinant that does not express gp42 confirm a key role for gp42 in efficient B-lymphocyte infection and a noncritical role in epithelial cell infection (581,875,876).

EBV gH and the gH/gL heterodimer have still another role in epithelial infection. An EBV recombinant that does not express gp85 adsorbs normally to B lymphocytes and is not only deficient in fusion with B-lymphocyte membranes but is unable to adsorb to epithelial cells (581,623). These latter studies confirm the importance of gp85 in fusion with B-lymphocyte membranes and indicate a unique role for gp85/gp25 dimeric complexes in interaction with a putative epithelial cell receptor or coreceptor.

Early Intracellular Events in Infection

Little is known about EBV capsid dissolution, genome transport to the cell nucleus, or DNA circularization. By analogy with other DNA viruses that replicate in the nucleus, the cytoskeleton is likely to mediate EBV capsid transport to the nucleus or to nuclear pores (140). A protein similar in size to the HSV virion tegumentary protein VMW 65, or VP16, has been detected in purified EBV preparations and remains associated with cells after infection (12). HSV VP16 is a key trans-inducer of immediate-early lytic virus gene expression (see Chapter 73). The EBV BPLF1 ORF is colinearly 30% homologous to the HSV VP16 ORF. EBV BPLF1 could therefore be a transactivator of lytic EBV replication in epithelial cells. The role of BPLF1 in lymphocyte infection is even less predictable. The initial latent infection promoter, Wp (12,721,773,855), has high constitutive activity in B lymphocytes (852).

The first evidence that herpesvirus genomes circularize in infected cells arose from the observation that almost all EBV-infected cells contain covalently closed circular EBV episomes (3,496). In primary B lymphocytes, EBV genome circularization precedes or coincides with earliest virus gene expression and may require activation from G0 to G1 (12,363). Whether the putative viral protein that recognizes EBV TR for cleavage and packaging (see later section, Late Gene Expression, Cleavage, Packaging, Envelopment, and Egress) facilitates the joining of the linear virion EBV TRs to produce genome circularization is not known. A cellular protein complex that includes Sp1 has also been identified that can recognize the ends of the DNA and could be involved in genome circularization (790,791). By 16 hours after infection, each infected cell contains one EBV episome. In some experiments, the addition of inhibitors of DNA, RNA, or protein synthesis to cells at the time of infection blocked genome circularization, indicating that active cell macromolecular synthesis is required for circularization. Inhibition of infected cell protein synthesis does not, however, prevent viral gene transcription (756).

Latent Infection in Primary B Lymphocytes and Growth Transformation

Viral Gene Expression in Latent Infection

EBV infection of primary human B lymphocytes *in vitro* results in type III latency (see Fig. 2) and transformation of the infected cell into a proliferating lymphoblast capable of long-term growth as an LCL. In defining the genes expressed in latent infection, it is important to recognize that most latently infected cell cultures have a small number of cells that are spontaneously permissive for virus replication. Since virus gene expression in lytic infection is at a much higher level than in latent infection, lytic-cycle RNAs or proteins are frequently detectable in predominantly latently infected B-cell lines. Proof that an EBV gene is expressed in latent infection requires the demonstration that the specific virus gene product is in most latently infected cells in a culture. This has usually been accomplished by defining an mRNA sequence whose abundance is not affected by the number of permissively infected cells (212,317,427,428,852,853), deriving monospecific antibody to the putative protein product using heterologous antigen expression systems (324,325,326,327,328 and 329,511,650,651 and 652,870) or peptide synthesis (169,170 and 171), and using the antibody to identify the protein in latently infected cells by immune microscopy (324,325,327,329,511,650,652,870).

The time course of events in *in vitro* primary B-lymphocyte infection varies somewhat, depending on the virus inoculum and the physiologic state of the lymphocytes. The EBV genome circularizes in the infected cell nucleus within 12 to 16 hours of infection (see Fig. 3). At about the same time, the Wp promoter in the 3-kbp, Bam or IR1, long internal direct repeat element initiates rightward transcription (12,16,362,594,595,702). The B-cell specificity of the W promoter derives at least in part from the presence of upstream BSAP/Pax5 sites (830). As is true of the other herpesviruses, EBV does not encode an RNA polymerase, and host cell RNA polymerase II transcribes viral mRNAs. By nuclear run-on or transient transfection analysis, the initial EBNA promoter, Wp, and the more upstream promoter, Cp, which is activated by EBNA-2, are the strongest EBV pol II promoters in latent EBV infection (719,720,852). The promoter in the leftmost repeat appears to dominate, as most of the EBNA cDNAs have multiple exons from downstream copies of the W repeat (62,63,64,65 and 66,719). The simplest model for the domination of the leftmost W repeat element is a positive transcriptional element in the upstream U1 region (see Fig. 2).

Wp promotes the transcription of the first EBV mRNAs, and these are differentially spliced to encode EBNA-LP and EBNA-2, two proteins that coordinately up-regulate transcription from viral and cellular promoters, including Wp and an activatible upstream surrogate, Cp (66,198,886,887 and 888). The initial mRNAs have a 40-base first exon, W0, which ends with an AT (12,721). The W0 exon is spliced to a 66-base W1 exon or to a 5' 5-base truncated W01 exon. The W01 exon begins with a G, creating a translational start site, whereas the W1 exon has no translational start site. The W1 exons are followed by a 132-base W2 exon, by repeating 66-base and 132-base W1W2 exons derived from successive reiterations of the long internal repeats, by two short exons, Y1 and Y2, and then by one long exon (YH) from the unique DNA (Bam Y and H) downstream of Bam W long internal repeats (see Fig. 3) (12,719,870). The resulting mRNAs translate either EBNA-LP from the W01 translational start site or EBNA-2 from a translational start site near the 5' end of the YH exons of those alternatively spliced RNAs that lack the W01 translational initiation site. EBNA-LP derives its name from the fact that it is a nuclear protein encoded by the potential 5' ORF in the leader of the EBNA-2 mRNA.

The efficient translation of EBNA-2 from only those mRNAs that lack the EBNA-LP translational start site and therefore have a long untranslated 5' leader is surprising, because 40s ribosomal subunits usually associate with mRNAs at the 5'

cap and then move along the untranslated leader without a stabilizing 60s subunit, until either disengaging or encountering a translational initiation codon (444). Translation of the 5' EBNA-LP ORF would be expected to stabilize mRNA interaction with the ribosome and yield more efficient translation of the 3' EBNA-2 ORF. Although expected, an independent ribosome entry sequence upstream of the EBNA-2 ORF has not been defined, and this enigma has not been resolved.

EBNA-LP and EBNA-2 reach the level that is maintained in transformed B lymphocytes (LCLs) by 24 to 32 hours after infection (12). EBNA-2 has a well-characterized role in up-regulating virus and cell gene expression, including expression of the virus-encoded EBNAs, LMP1 and LMP2, and the cell-encoded CD23, CD21, c-*fgr*, and c-*myc* proteins (Fig. 4) (1,12,127,199,200,437,844,865,867,868,870,871,925,930). Up-regulation of CD23, CD21, and c-*fgr* is observed following transient or stable expression of EBNA-2 in non-EBV-infected BL cells. C-*myc* expression is also up-regulated in primary B-lymphocyte infection at the time that EBNA-LP and EBNA-2 are expressed, and c-*myc* expression is continuously dependent on EBNA-2 expression (12,394). EBNA-2 also up-regulates various LMP1 (1,199,388,764,848,873), LMP2 (460,461,930,931), and Cp (322,386,498,499,793,895) promoter constructs following transient transfection with an EBNA-2 expression vector into non-EBV-infected B-lymphoma cells.

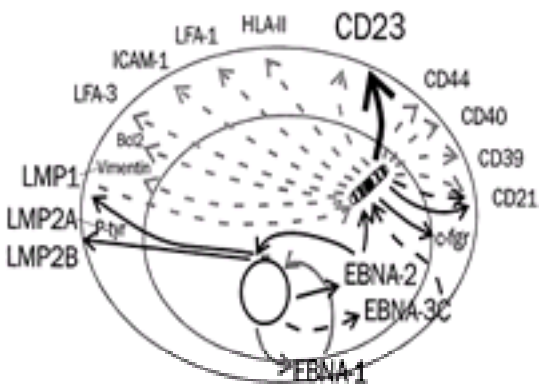

FIG. 4. Expression of EBNAs or LMPs in BL lymphoblasts provides indications of the biochemical mechanisms by which these proteins effect changes in lymphocyte growth or survival. EBNA-1 binds to oriP and to chromosomes and enables the EBV episome to partition to progeny cells. EBNA-2 specifically up-regulates cellular CD23, CD21, c-*myc*, and c-*fgr* expression and viral EBNA, LMP1, and LMP2 expression. EBNA-3C can also up-regulate CD23 expression, although the effects are much less than those of EBNA-2. LMP1 patches in the cell plasma membrane where it is associated with and induces higher level expression of vimentin. LMP1 has dramatic effects on BL cell growth; it functionally activates homotypic adhesion; it up-regulates ICAM 1, LFA 1, and LFA 3; it up-regulates bcl2; it raises the lymphocyte intracellular free calcium; it up-regulates activation markers including transferrin receptor, HLA-II, CD21, CD23, CD39, CD40, and CD44; and it down-regulates CD10 and bcl-6. LMP1 also alters lymphoblast TGF-beta responsiveness. Each of these effects is cell-line dependent, and most effects are not evident in all BL cell lines. LMP2A constitutively activates B-cell receptor tyrosine kinase signalling pathways at a low level and desensitizes B lymphoblasts to further B-cell receptor–type tyrosine kinase signal transduction.

EBNA-2 has three critical components to its transcriptional up-regulating effects (see Fig. 3) (125,296,842,894). First, the specificity of EBNA-2 effects on certain virus and cell promoters and not others is caused by an EBNA-2 domain that interacts with cell-sequence-specific DNA binding proteins (125,276,322,388,895). The repertoire of cellular transcription factors includes RBP-Jk, a transcription factor expressed in all cells (276,322,895), and PU.1, a hematopoietic transcription factor that is particularly important in B lymphocytes (388). Second, once it is near promoter sites, EBNA-2 has an acidic activating domain that recruits basal and activation-associated transcription factors, including transcription factor IIB (TFIIB), TAF40, TBP, transcription factor IIH (TFIIH), and CBP/p300 (123,124,839,840 and 841,874). Much of the EBNA-2 acidic domain is complexed with a cell nuclear protein, p100, that has coactivating effects with EBNA-2 (840). P100 is a scaffolding protein for the acidic domain, for TFIIE, for c-*myb*, and for PIM-1. Third, efficient assembly of multiple transcription factors at promoter sites appears to require multiple EBNA-2 acidic domains. EBNA-2 has two separate domains in its amino terminus that can mediate homotypic association, thereby enabling EBNA-2 to assemble multiple transcription factors near a promoter that has RBP-Jk or PU.1 sites (894). The ability of EBNA-2 to self-associate may account for the large size of EBNA-2 complexes in cell extracts.

Because EBNA-LP is transcribed along with EBNA-2 at the initiation of EBV infection in primary B lymphocytes and because it is a nuclear protein, it is a prime candidate for cooperation with EBNA-2 in the regulation of virus and cell gene expression (see Fig. 4). Indeed, EBNA-LP substantially enhances EBNA-2 up-regulation of virus and cell gene transcription (295,609). The effect is evident in transient expression assays with artificial LMP1 promoter constructs and with endogenous LMP1 expression in EBV-infected BL cells that are otherwise in latency I and fail to express LMP1 until EBNA-2 and EBNA-LP are coexpressed in these cells following gene transfer. EBNA-LP has no promoter-specific effects alone and stringently requires EBNA-2 for activation of specific promoters. Surprisingly, the EBNA-LP effect requires only the EBNA-2 acidic domain near a promoter site and expression of the repeating domains of EBNA-LP (295). Thus, EBNA-LP is likely to biochemically interact with the EBNA-2 acidic domain or with another factor that binds to the acidic domain.

Probably as a consequence of EBNA-2 effects on a response element in the unique DNA in the EBV Bam C fragment that is upstream of the long internal repeat, EBNA promoter usage in many EBV-infected primary B lymphocytes moves from Wp to the nearby Cp latency promoter (295,386,789,882,885,914). In those cells in which the Cp promoter is turned on and supplants Wp, two short exons downstream of the Cp promoter, C1 and C2, replace the short W0 exon in the EBNA mRNAs (66). As with the W0 exon, the C2 exon ends in an AT that is spliced to either the 66-base or the 61-base internal-repeat-derived exon, W1 or W01, resulting in an mRNA leader incapable or capable, respectively, of translating EBNA-LP. All resulting C- or W-initiated mRNAs have the same repeating 66- and 132-base exons in their leader

(62,63,64,65 and 66,387,653,699,721,773). A potential splice donor near the beginning of the EBNA-2-encoding YH exon but preceding the EBNA-2 initiation codon is now activated in some transcripts, and some RNA molecules are spliced to a far downstream acceptor in Bam U (62,65,66,653,721,773). The U exon is then spliced to any of four alternative acceptor sites of exons that begin the ORFs that encode for the amino terminus of EBNA-3A, EBNA-3B, or EBNA-3C, or for EBNA-1 (see Fig. 3). As a consequence of alternate 5' splicing of the Cp- or Wp-initiated RNAs, some mRNAs will still encode EBNA-LP. As a consequence of partial use of the alternative donor site between the beginning of the YH exon and the EBNA-2 initiation codon, some RNAs that do not encode for EBNA-LP or for EBNA-2 are spliced to the U exon and now encode for EBNA-3A, -3B, -3C, or -1 instead of EBNA-2. Alternative acceptor site usage after the U donor site determines the frequency with which transcripts encode EBNA-3A, -3B, -3C, or -1 (see Fig. 2).

The simplest model to explain the turn-on of EBNA-3A, -3B, -3C, and -1 expression is that EBNA-LP and EBNA-2 up-regulation of the EBNA-2 response element upstream of the Cp promoter leads to higher level transcription of the EBNA mRNAs by Cp or by Wp in those cells that do not switch to Cp. In a partial test of this hypothesis, cell lines were derived that were infected with an EBV recombinant that is deleted for the EBNA-2 response element upstream of the Cp promoter. As expected from the model, cells transformed with the response-element-deleted virus were biased toward initial Wp promoter usage relative to cell lines transformed with wild-type virus (915). Not surprisingly, given the need for EBNA-3A, -3C, and -1 for the establishment and maintenance of transformation (469,837), stable EBNA transcripts were not different in cell lines transformed by mutant and wild-type virus. Surprisingly, the mutant virus was not markedly impaired in transformation efficiency (914). A corollary of the model is that Cp dominates over Wp after EBNA-LP and -2 expression, because the EBNA-2 response element is upstream of the Cp promoter and the increased activity of the Cp promoter overrides and interferes with the 3-kb-downstream Wp promoter. In support of this aspect of the model, mutations that down-modulated Cp activity or reversed Cp direction resulted in up-regulation of Wp activity (664).

Whether the net effect of EBNA-LP and -2 is to up-regulate Cp or Wp, increased transcription by either or both promoters probably accounts for transcripts that extend past the polyadenylation site downstream of the EBNA-2 ORF and through the polyadenylation sites downstream of the EBNA-3A, -3B, -3C, or -1 ORFs. Presumably, the efficiency of polyadenylation at each site determines the relative amount of each of these downstream EBNA mRNAs. The subsequent processing of the transcripts is likely to be determined by the characteristics of their polyadenylation sites. The EBNA mRNAs are all at low abundance, with the EBNA-3 or EBNA-1 mRNAs estimated to be several copies per cell.

EBNA-1 expression probably further activates EBNA transcription by enabling EBNA-1 to bind to the EBNA-1-dependent enhancer component of oriP that is 3.5 kb upstream of Cp (241,665,683). The up-regulation of the Cp and Wp promoters first by EBNA-2 and EBNA-LP and then by EBNA-1 would be anticipated to result in unbridled up-regulation of EBNA mRNA transcription were it not for the repressive effect of EBNA-3A, -3B, and -3C expression on transcription mediated by EBNA-2 and EBNA-LP activation of the Cp enhancer (389,534,692,693,864,926). EBNA-3A, -3B, and -3C bind more strongly to RBP-Jk than EBNA-2 and associate with much of the RBP-Jk in the cell. Because the EBNA-3s have no demonstrable positive effects on the Cp enhancer, their net effect is to limit EBNA-2-mediated effects on this element. EBNA-3C has more complicated effects on the LMP1 promoter and can, under certain circumstances, up-regulate LMP1 expression (927).

Concomitant with up-regulation of the Cp and Wp promoters, EBNA-2 and EBNA-LP also up-regulate cell promoters, including c-*myc*, CD23, and the virus LMP1, -2A, and -2B promoters (12). LMP2 transcription is in the same direction as EBNA transcription, and the two LMP2 promoters bracket the LMP1 promoter, with the LMP2A promoter being 3.3 kbp 5' of the LMP2B promoter (see Fig. 3) (462,465,724). LMP2A and -2B transcription are regulated through EBNA-2 response elements upstream of their promoter (925,930). LMP1 transcription is in the opposite direction, and the LMP1 transcript is entirely within the first intron of, and antisense to, the LMP2A transcript (212). The LMP1 promoter upstream regulatory sequence is also the upstream regulatory sequence of the LMP2B promoter. The LMP1 and -2B promoters regulate transcription in opposite directions and are separated by only 266 bases.

LMP2A and -2B differ only in their first exons. The LMP2A first exon is 419 bases and the LMP2B first exon is 167 bases. The LMP2A exon encodes an amino-terminal 120-amino-acid cytoplasmic signaling domain of LMP2A, whereas the LMP2B first exon is noncoding. The remaining LMP2 exons are common to both LMP2A and -2B and are transcribed from U1. By 32 hours after infection of primary B lymphocytes, all EBNA and LMP mRNAs are expressed (12). Cell DNA synthesis follows the onset of LMP1 expression. By 48 hours after primary human B-lymphocyte infection *in vitro*, all of the EBNAs and LMP1 are near the levels that are maintained consistently through latent infection (12,16,362,594,595,700,702).

EBER expression lags by approximately 24 hours and does not reach substantial levels until 70 hours after infection (12). The EBER-1 and EBER-2 RNAs are mostly transcribed by cell RNA polymerase III, although polymerase II may also be involved (32,340,342,376,475,701). The EBERs have typical intragenic control regions common to pol III transcripts and upstream Sp1, ATF, and TATA box elements characteristic of pol II transcriptional sites (342). At steady-state levels in latently infected B lymphocytes, the EBERs are the most heavily transcribed and most abundant EBV RNAs. The EBERs are estimated to accumulate in cell nuclei at about 50,000 copies per cell (32,340,427,428,660,720,842).

The delineation of the highly spliced EBNA and LMP1 transcripts through Northern blot, S1 nuclease mapping, cDNA cloning and sequencing, and nuclear run-on studies explains the observations that latently infected cell nuclei have transcripts from most of the EBV genome, yet cytoplasmic polyadenylated polyribosomal mRNA is much less complex (146,303,306,427,428,628). Nuclear run-on studies indicate that almost the entire rightward or clockwise strand of the genome 3' to the Wp promoter is transcribed, although the rate of transcription drops off somewhat downstream of EBNA-2 and again downstream of EBNA 1 (see Fig. 3) (719). Transcription, per se, does not regulate EBV gene expression. As indicated previously, the differential use of latent infection polyadenylation sites probably determines upstream splicing and thereby regulates synthesis of the six EBNA mRNAs from the same promoter.

Each EBNA primary transcript has multiple copies of IR1 or Bam W–encoded RNA, and each copy has a 500-base segment that can form an extensively base-paired structure (104). This unusual sequence could have a cis-acting role in processing of EBNA transcripts, but such a role has not as yet been identified. Analysis of a defective EBV DNA indicates

that the corresponding DNA sequence can be a site for inversion within the EBV genome (381). This suggests that extensive base-pairing may occur within this nucleotide sequence under physiologic conditions.

Latent Infection Proteins

EBNA-LP

Because the number of copies of the IR1 repeat varies among EBV isolates, and because IR1-derived exons W1 and W2 encode repeating 22- and 44-amino-acid segments of EBNA-LP (Fig. 5), the size of EBNA-LP is frequently different in cells infected by different EBV isolates (169,215,872). The 44-amino-acid repeat domain encoded by W2 exons has two runs of basic amino acids, RRHR or RRVRRR, which are responsible for EBNA-LP nuclear localization (648). Aside from the N-terminal repeat domain, EBNA-LP has a unique 45-amino-acid carboxyl-terminal sequence. EBNA-LP is phosphorylated at multiple sites, probably at least once within each 44-amino-acid repeat, because the number of isoelectric forms varies with the W1W2 exon repeat number (651). The EBNA-LP 44-amino-acid repeat domain has a near consensus casein kinase II serine phosphorylation site. *In vitro*, p34cdc2 can phosphorylate this serine as well as a C-terminal unique serine. Casein kinase II can also phosphorylate only the unique C-terminal serine (433). EBNA-LP is highly phosphorylated in G2/M, consistent with a role for p34cdc2 in phosphorylation of EBNA-LP *in vivo* (433). At the present time, the effects of phosphorylations on EBNA-LP functionality are uncertain.

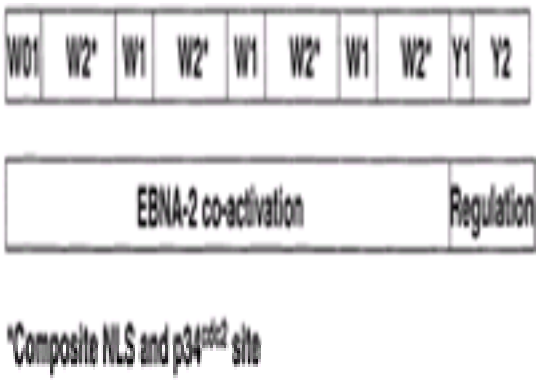

FIG. 5. Schematic diagram of the repeat and unique exon and domain structure of EBNA-LP. The EBNA-LP repeat domain can coactivate transcription mediated by the EBNA-2 acidic domain. The unique carboxyl terminus of EBNA-LP modulates EBNA-LP effects.

EBNA-LP is more associated with the nuclear matrix fraction than the other EBNAs (651). In immune microscopy of LCLs or of non-EBV-infected BL lymphoblasts in which EBNA-LP is expressed alone by gene transfer, EBNA-LP localizes to nuclear dots that correspond to sites of ND10 proteins (800,870). Some EBNA-LP is also diffusely spread through the nucleus. RNA *in situ* cytohybridizations with EBV IR1 DNA reveal similar intranuclear structures (466). These observations suggest the possibility that EBNA-LP could associate with EBNA transcripts and play a role in RNA processing.

EBV recombinant molecular genetic analyses of EBNA-LP have focused on the carboxyl-terminal two exons. Mutations in the repeating exons of EBNA-LP are more difficult to construct. Specific deletion of DNA encoding the carboxyl-terminal two exons, or placement of a nonsense codon at the beginning of the last two exons, has resulted in EBV recombinants that are deficient in primary lymphocyte transformation into LCLs (291,525). In two series of experiments that resulted in many wild-type EBV recombinant LCLs, only two LCLs were obtained that were infected with recombinants that are deleted for the carboxyl-terminal two exons, and these LCLs were deficient for growth. Transfection of one of these LCLs with an expression vector for wild-type EBNA-LP enabled the transfected LCLs expressing wild-type EBNA-LP to grow more rapidly than the LCLs that did not express wild-type EBNA-LP (13). Despite growth of infected primary B lymphocytes on fibroblast feeder layers, the frequency of derivation of nonsense recombinant transformed LCLs was less than 5% of wild-type, and infected cell growth was slower than with wild-type recombinants. One mutant recombinant infected LCL was obtained that had wild-type growth characteristics, and that LCL proved to be infected with a revertant (525). Passage of the deletion or nonsense EBNA-LP recombinants into other primary B lymphocytes further confirmed the reduced transformation phenotype (525). Infection of multiple primary B lymphocytes with higher-titer mutant recombinant virus enabled better outgrowth of LCLs infected with the mutant EBNA-LP EBV recombinants, presumably because of an enabling effect of cross-feeding among the multiple mutant EBV-infected cells (525). The mutant virus–infected LCLs were more highly differentiated toward immunoglobulin secretion, compatible with the possibility that wild-type EBNA-LP inhibits differentiation. In other experiments, transient expression of EBNA-LP and EBNA-2 in primary B lymphocytes co-stimulated with gp 350 resulted in induction of cyclin D2 (755). Thus, overall, these experiments favor the model that EBNA-LP has a key role in up-regulating cell gene expression critical for LCL outgrowth.

The effects of EBNA-LP on cell gene expression are likely to be similar to EBNA-LP effects in strongly coactivating viral promoters with EBNA-2. EBNA-LP coactivates EBNA-2-mediated transcription from the Cp and LMP1 promoters in transient transfection assays with reporter constructs, and it also coactivates EBNA-2 up-regulation of LMP1 expression in latency 1 EBV–infected BL cells (295,609). The EBNA-LP 22- and 44-amino-acid repeat domains have most of the activity of wild-type EBNA-LP, exerting very similar effects on EBNA-2 up-regulation of the Cp and LMP1 promoters or of a simple multimerized RBP-Jk site position near a basal promoter (295). Interactions with DNA sequence-specific transcription factors other than RBP-Jk are not critical for EBNA-LP coactivation with EBNA-2. EBNA-LP coactivation with EBNA-2 does not even require that EBNA-2 interact through RBP-Jk, because EBNA-LP robustly coactivates transcription mediated by the EBNA-2 acidic domain alone fused to the Gal-4 DNA binding domain when cells are

co-transfected with a minimal promoter/reporter plasmid that has multiple upstream Gal-4 binding sites (295).

The unique 3' exons of EBNA-LP encode 11- and 34-amino-acid carboxyl-terminal domains. Whereas the repeating exons are efficient coactivators, addition of 11 or even 35 of the carboxyl-terminal residues results in nearly complete loss of coactivating effects. Addition of the entire 45 carboxyl-terminal amino acids restores wild-type coactivation. These data indicate that the first 35 carboxyl-terminal amino acids have a strong negative regulatory effect on coactivation, and that the last 10 amino acids effectively silence the negative regulatory effect (295). The negative effect of the stop codon mutation that prevented expression of the last 45 amino acids on primary B-lymphocyte transformation (525) is evidence of the importance of this complex regulatory domain for efficient lymphocyte growth transformation. Differential phosphorylation of EBNA-LP by p34cdc2 or casein kinase II may be important in the natural regulatory effects of the carboxyl-terminal domain.

An effect of EBNA-LP on Rb and p53 has been suggested based on *in vitro* biochemical interactions and co-localization of EBNA-LP with Rb as detected with one monoclonal antibody but not with others (385,797). However, EBNA-LP is not associated with Rb family proteins or p53 in cell lysates, and expression of EBNA-LP in transgenic mice had no discernible effect on development or tumor incidence. The EBNA-LP transgenic mice died of heart failure, which was likely to be due to a toxic effect of EBNA-LP expression or to an insertion site effect (355). Immunoprecipitations of EBNA-LP from B lymphoblasts have resulted in the identification of the DNA-PK catalytic subunit, an AKAP 95 homolog HA95, HSP70, and HSP27 as EBNA-LP-associated proteins (434,526,799).

EBNA-2

Early studies identified a transformation-incompetent but replication-competent laboratory strain of EBV, P3HR-1 (565,598). The search for the biochemical basis of the nontransforming phenotype led to the discovery and characterization of EBNA-2 (Fig. 6). P3HR-1 is deleted for the DNA that encodes RNA and protein in latently infected cells, and the principal ORF in the DNA deleted from P3HR-1 encodes EBNA-2 (144,311,327,427,675).

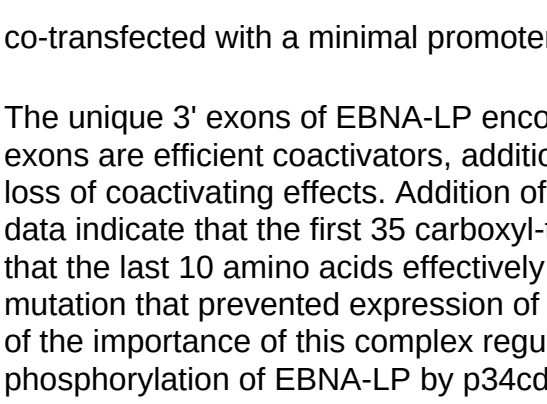

FIG. 6. Schematic depiction of the biochemical mechanisms by which EBNA-2 and EBNA-LP up-regulate transcription by the LMP1 promoter in B lymphocytes. EBNA-2 is an acidic transcriptional transactivator that biochemically interacts with the cellular sequence-specific DNA binding proteins RBP-Jk and PU.1 to bring it to specific promoters, including the LMP1 promoter. The EBNA-2 amino terminus has two domains that can mediate homotypic association with other EBNA-2 molecules. The EBNA-2 acidic domain interacts with TFIIB, TAF40, TFIIH, TBP, p100, P300/CBP, and EBNA-LP in stimulating transcription. The EBNA-2 acidic domain is extensively associated with p100, which is a scaffolding protein for c-*myb* and PIM-1.

Superinfection of latently infected cells with P3HR-1 induced replication of the endogenous viral genome, resulting in the production of virus that could transform primary B lymphocytes into latently infected LCLs. Restriction endonuclease analyses of the viral DNA in the resultant LCLs for polymorphisms that are characteristic of the P3HR-1 genome indicated that many of the genomes were partly from the P3HR-1 EBV. All of the viral DNAs were wild type at the site of the P3HR-1 deletion, supporting the hypothesis that the EBNA-2-encoding DNA is critical for LCL outgrowth (765).

More precise recombinant EBV molecular genetic experiments done by transfection of cells that are infected with the P3HR-1 EBV with molecularly cloned wild-type EBV DNA fragments that span the deletion resulted in homologous recombination and restoration of the deleted DNA segment in 1 in 10^5 progeny virus (126,290). The recombinant virus could be easily recovered because of its unique ability to transform primary B lymphocytes into LCLs. Transfection with cloned EBV DNA fragments deleted for part of the EBNA-2 ORF, or containing a stop codon one third of the way into the ORF, repeatedly failed to restore transforming ability, whereas wild-type DNA or DNA with a small marker deletion restored transforming ability. Recombinant EBVs with a nontransforming EBNA-2 mutation could also be recovered by infection of EBV-negative BL cells (527). Thus, several different genetic analyses proved that EBNA-2 is essential for primary B-lymphocyte growth transformation.

EBNA-2 also differs more extensively between EBV types 1 and 2 than any other latent infection–associated protein, and that difference results in a differential ability to cause LCL outgrowth (7,126,144,722,723,835). Infection of primary human B lymphocytes with EBV type 2 rarely gives rise to rapidly growing LCLs (689). Restoration of the P3HR-1 deletion with prototype type 1 or type 2 EBNA-2 genes indicated that EBNA-2 is the principal determinant that enables type 1 EBV to more efficiently transform primary B lymphocytes than type 2 EBV strains (126). Primary B lymphocytes infected with the type 2 P3HR-1 EBV that had its deletion cured with type 1 EBV DNA proliferated into LCLs as rapidly as lymphocytes transformed by type 1 EBV strains. In contrast, recombination of cloned type 2 EBNA-2 DNA into the site of the P3HR-1 deletion resulted in the deficient transformation phenotype characteristic of type 2 EBV strains (126).

Comparison of the nucleotide and predicted amino acid sequence of the EBV type 1 and 2 EBNA-2s, or the corresponding sequences of other LCVs, reveals conserved or divergent domains that in general correspond with sequences that are more or less critical in genetic and biochemical analyses (see Fig. 6) (123,124 and 125,144,276,296,322,498,500,647,840,842,894,895): (a) The amino-terminal 57 amino acids contain a motif like the immunoreceptor tyrosine-based activation motif (ITAM), have an overall negative charge, and are highly conserved between types 1 and 2. (b) The polyproline repeat of type 1 EBNA-2 is usually longer than that of type 2. (c) The 46 amino acids carboxyl-terminal to the polyproline repeat are well conserved between types 1 and 2, with 30 identical amino acids. (d) Only 52 of the next 149 amino acids are identical between types 1 and 2. (e) Carboxyl-terminal to this highly divergent region is a 46-amino-acid domain with 26 type-common amino acids. (f) A 26-amino-acid imperfect Arg-Gly repeat, of which 13 amino acids are Arg or Lys, is highly conserved. (g) In the 121-amino-acid carboxyl terminus, 84 amino acids are identical in types 1 and 2, and 16 are acidic. And (h) near the carboxyl terminus is a Lys-Arg-Pro-Arg sequence that can provide nuclear localization. In EBNA-2, the Arg-Gly repeat region near the carboxyl terminus can provide nuclear localization (125).

EBNA-2 is associated with nucleoplasmic, chromatin, and nuclear matrix fractions and localizes to large nuclear granules (327,651). It is phosphorylated on serine and threonine residues and may undergo significant posttranslational modification other than phosphorylation, as the size of the nascent protein is 10 kd smaller than stable intranuclear EBNA-2 (265,870). The basis for this modification is not known. EBNA-2 extracted from lymphoblasts or even from baculovirus-infected sf-9 cells is heterogeneous in velocity sedimentation and gel elusion chromatography. EBNA-2 can form homotypic complexes the size of tetramers or larger, and some of the EBNA-2 complexes in lymphoblasts are larger than 1 MDa (265,850,891,892). The size of EBNA-2 complexes is at least in part due to the ability of EBNA-2 to homotypically self-associate through two separate domains: the amino-terminal 57 amino acids before the polyproline domain and the 120 amino acids after the polyproline domain.

EBNA-2 is a specific transactivator of cell and viral gene expression, a property first described in delineation of the mechanism for up-regulated expression of CD23 (865), a B-lymphocyte-specific surface protein expressed in high abundance on EBV-transformed or antigen-activated primary B lymphocytes (362,429). EBNA-2 transactivates CD23 expression through an EBNA-2 responsive element upstream of the CD23 promoter (867,868). Subsequently, EBNA-2 has been shown to also up-regulate the cellular CD21 (25,127,868), c-*fgr* (437), and c-*myc* mRNAs (394), as well as the viral LMP1 (1,199,200,248,873), and LMP2A and -B (930) and Bam C promoter mRNAs (386,793). Curiously, EBNA-2 also transactivates the HIV LTR, and the transactivation is dependent on the NF-kB and PU.1 sites in the LTR, despite the absence of NF-kB sites in other EBNA-responsive cis-acting elements (730).

EBNA-2 response elements upstream of the CD23, LMP1, LMP2A, and Bam C promoters have been defined by deletional analyses and by positioning the response elements near a heterologous basal promoter (199,386,461,498,546,848,870,873,930). The LMP1 promoter, EBNA-2 response element, is largely restricted in EBNA-2 responsiveness to human B lymphocytes (200) and was not observed in epithelial cells, probably because of the role of the B-lymphocyte-specific factor, PU.1 in EBNA-2 activation of the LMP1 promoter (388,764).

Analysis of EBNA-2 using recombinant EBV molecular genetics with marker rescue of primary B-lymphocyte transforming activity in the EBNA-2-deleted, nontransforming, P3HR-1 EBV background has identified three essential EBNA-2 domains, and these domains are also critical for LMP1p or Cp promoter up-regulation. The essential domains correspond to a requirement for either of the amino-terminal domains that mediate homotypic association, the domain between 280 and 337 that mediates interaction with cellular DNA binding proteins, or the domain between amino acids 420 and 464 that is an acidic activation domain (123,124,125 and 126,296,840,842,894,895).

The EBNA-2 acidic domain is similar but not identical to the prototype VP16 acidic domain (123,124 and 125). Despite little colinear homology, both acidic domains include similarly placed critical hydrophobic residues, a phenylalanine in VP16 and a tryptophan in EBNA-2. A fusion of 28 amino acids of the EBNA-2 acidic domain to the Gal-4 DNA-binding domain is nearly as strong in activation of basal reporter constructs with multiple upstream Gal-4 binding sites as the core VP16 14 amino acids, whereas the corresponding EBNA-2 14 amino acids have only 25% of the VP16 activity. Despite their greater activity in transient assays, the VP16 14 amino acids can substitute for the corresponding EBNA-2 residues when expressed from an EBV recombinant (123). The EBNA-2 acidic domain is more active in BL cells than in Chinese hamster ovary (CHO) cells, and the VP16 acidic domain is more active in CHO cells than in BL cells, evidence of EBNA-2 selectivity for B lymphocytes (124). The EBNA-2 acidic domain shares with VP16 an affinity for TFIIB, TAF40, TFIIH, RPA70, and p300/CBP, but it has much less affinity for TBP (839,840 and 841,874). In contrast, the EBNA-2 acidic domain has greater affinity for p100 than does VP16, and the EBNA-2 acidic domain is highly complexed with p100 in LCLs (840). P100 is a scaffolding protein for c-*myb* and PIM-1 (150,476) and also can interact with TFIIE (840). Given the extent of association between the EBNA-2 acidic domain and p100, and the potential for p100 to recruit TFIIE, c-*myb*, and PIM-1, p100 is likely to have an important role in regulating EBNA-2-mediated transcription. Although p300 and CBP are less stably associated with EBNA-2, p300 and CBP are also likely to be critical for EBNA-2-mediated transactivation because of their intrinsic histone acetylase activities, because of their ability to recruit PCAF histone acetylase, and because of their interaction with other transcription factors. Much of the substantial histone acetylase activity that can bind to the EBNA-2 acidic domain is associated with p300/CBP (874). CBP has been specifically implicated in EBNA-2 activation of the c-*myc* promoter, whereas p300 appear to be repressive (377).

The second domain of EBNA-2 that is critical for transformation mediates an interaction and high-level association with a cellular sequence-specific DNA binding protein, RBP-Jk, also known as CBF1 (276,322,389,895). The EBNA-2 amino acid sequence that binds to RBP-Jk includes amino acids 290 to 337. Although PWWPP is the core sequence that interacts with RBP-Jk, and mutations of the WW to FF or SS are sufficient to ablate binding, deletion of amino acids 292 to 310 also significantly disrupts the ability of EBNA-2 to associate with RBP-Jk (276,296,497,895). The WWP sequence in EBNA-2 closely mimics the WFP sequence in the Notch-receptor intracellular domain that normally mediates tight association of the Notch intracellular domain with RBP-Jk for up-regulating HES1 in tissue development (808). The WW to SS mutation ablates EBNA-2 ability to up-regulate the Cp promoter, reduces the ability to activate the LMP-1 promoter by 50%, and ablates the ability of the specifically mutated EBV recombinant to transform primary B lymphocytes into

LCLs (895). These point mutation data are strong evidence for the key role of RBP-Jk association in promoter choice, promoter regulation, and LCL outgrowth; the data also support the hypothesis that RBP-Jk-mediated promoter regulation is linked to the transformation of resting B lymphocytes into LCLs.

RBP-Jk has intrinsic repressive activity that is at least in part mediated by interaction with SKIP, a component of the SMRT, Sin3A, and HDAC 2 corepressor complex (928,929). Notch and EBNA-2 may partly activate transcription from RBP-Jk-responsive promoters by interacting with SKIP and interfering with SMRT interaction with SKIP. The interaction with SKIP may also stabilize Notch and EBNA-2 interaction with RBP-Jk.

RBP-Jk recognizes the nucleotide sequence GTGGGAA (276,322,499). This sequence is part of the EBNA-2 responsive elements that have been implicated in Cp, LMP1, LMP2A, CD23, IL6, and c-*fgr* promoter regulation in LCLs (174,284,386,848,870,931). Although existing data favor the possibility that the PRF site in the c-*myc* promoter is critical in EBNA-2 promoter regulation (377), the first intron of c-*myc* has a GTGGGAA sequence that could also, or alternatively, mediate EBNA-2 up-regulation. The latter possibility might also explain the role of constitutively activated Notch receptors in human T-cell leukemia and in inducing mouse T-cell leukemia (185,640).

EBNA-2 enjoys a high-level continuous association with RBP-Jk in LCLs (389). Further, a multimerized Cp sequence that contains the RBP-Jk binding site or a multimerized minimal synthetic RBP-Jk binding site can convey EBNA-2 responsiveness to minimal promoters (295,499). However, analyses of EBNA-2-mediated activation of those EBNA-2-responsive enhancer elements that have been most extensively studied from the LMP1p and Cp promoters show that responsiveness requires at least one other cellular sequence-specific DNA binding protein, PU.1 for LMP1 (388) and AUF1 for Cp (238). Mutation of the sole RBP-Jk site in the LMP1 EBNA-2-responsive enhancer element, or point mutation of the EBNA-2 WW to SS so that EBNA-2 cannot interact with RBP-Jk, only reduces EBNA-2 responsiveness 50%. In contrast, mutation of the PU.1 binding site ablates EBNA-2 responsiveness (388). The EBNA-2 domain that interacts with PU.1 appears to include the site that interacts with RBP-Jk, and more-carboxyl EBNA-2 amino acids, because bacterial fusion proteins that include these EBNA-2 amino acid sequences can efficiently remove PU.1 from nuclear extracts (388). However, unlike RBP-Jk, which readily coimmunoprecipitates with EBNA-2 from LCL cell lysates, PU.1 does not coimmunoprecipitate with EBNA-2 (388). The targeting of PU.1 by EBNA-2 in transcriptional activation is an important aspect of EBV adaptation to lymphoid cells, because PU.1/Spi1 is a key Ets family regulator of the IgH enhancer (600). The role of AUF1 binding to CBF2 in complementing EBNA-2 interaction with CBF1 also seems to involve a AUF1 functional interaction with EBNA-2 that may be independent of EBNA-2 interaction with CBF1. AUF1 is a component of the cellular DNA binding protein complex that interacts with CBF2, and a highly multimerized CBF2 can be activated by EBNA-2 expression (238). Further, EBNA-2 activation through CBF2 is indirectly enhanced by protein kinase A (PKA) (238), as has been shown in regulation through an AUF1 site in the EBNA-2-responsive CD21 promoter (834). Although direct interaction or association of EBNA-2 with AUF1 has not been demonstrated, EBNA-2 activation of a minimal promoter through a mutimerized CBF2 element is coactivated by AUF1 overexpression and PKA activation (238).

The third EBNA-2 domain that is important for primary B-lymphocyte growth transformation appears to be either of the domains that are amino- or carboxyl-terminal to the polyproline repeat. Either domain, amino-terminal amino acids 1 to 57 or carboxyl-terminal amino acids 97 to 210, or almost all the prolines, can be deleted with only a moderate loss of primary B-lymphocyte growth transformation and Cp promoter activation, but deletion of *all* the prolines is incompatible with the mutant EBV initiating primary B-lymphocyte growth transformation (894). Biochemical studies indicate that either domain can mediate homotypic association. Homotypic association is likely to be critical to the ability of EBNA-2 to form large homotypic and larger heterotypic complexes (265,850,891,892) that can facilitate simultaneous recruitment of multiple transcription factors and enhance transcription. This probably explains the unusual effect of the deletion of both domains.

Although not essential per se for transformation or transcriptional activation, the domain that is carboxyl-terminal to the polyprolines has also been implicated in an interactions with human SNF5/Ini1 and the DEAD box protein DP103. Ini1 and DP103 interact with EBNA-2 amino acids 112 to 344 in yeast two-hybrid screens (281,891). DP103 is a DEAD box protein with imputed RNA helicase activity that appears to have transcription-repressive effects (629). *In vitro*, translated EBNA-2 amino acids 1 to 210 bind to Gst-Ini1, and a small fraction of phosphorylated EBNA-2 coimmunoprecipitates with Ini1 from 1.5- to 2-MDa fractions of cell lysates. Consistent with EBNA-2 recruitment of Ini1 to specific sites, immunoprecipitation of chromatin fragments with Ini1 antibody enriches for promoter DNA sequences downstream of LMP2A-derived enhancer construct or CD23 first-exon DNA in EBNA-2-positive cells (892). Thus, EBNA-2 interaction with Ini1 could be physiologically significant.

Deletion of the EBNA-2 DNA segment encoding the Arg-Gly repeat domain RGQSRGRGRGRGRGRGKG, amino acids 337 to 354, results in EBV recombinants that transform primary B lymphocytes with decreased activity. The transformed cells grow slowly and require maintenance at a high density. The Arg-Gly repeat has an intrinsic ability to bind to histone H1, to many other proteins (including EBNA-1), and to nucleic acids, especially poly(G). The strong interaction of the Arg-Gly domain with histone H1 is consistent with the observed effect of deletion of the Arg-Gly domain on release of a significant fraction of EBNA-2 that is in the 0.4 M salt–extractable chromatin fraction to the isotonic salt–extractable nucleoplasmic fraction in LCLs transformed by the mutant recombinant EBV. EBNA-2 deleted for the Arg-Gly domain is better than wild-type EBNA-2 at transactivating the LMP1 promoter and is as active as EBNA-2 in transactivating the *Bam*HI-C promoter. These data are most compatible with a model in which the Arg-Gly domain of region 3 is a modulator of EBNA-2 interactions with chromatin and activities in up-regulating gene expression.

EBNA-3A, -3B, -3C

The EBNA-3 genes are tandemly placed in the genome (see Fig. 3) and are transcribed from the far-upstream Cp or Wp EBNA promoters. The EBNA-3A, -3B, and -3C ORFs share colinear distant homology and are each encoded in the 3' terminal short and long exons of their mRNAs (36,62,64,65,325,329,387,395,418,651,653,721). Latently infected cells have only a few copies of each EBNA-3 mRNA. EBNA-3A, -3B, and -3C have repeating polypeptide domains near their carboxyl termini, representing unusual examples of domain amplifications within ORFs of genes that are themselves amplified from a common progenitor. Type 1 EBNA-3A, -3B, and -3C are 944, 938, and 992 amino acids, respectively.

The type 2 EBNA-3A, -3B, and -3C proteins are 925, 946, and 1,069 amino acids and are 84%, 80%, and 72% identical to their type 1 counterparts (725).

The EBNA-3 proteins (see Fig. 5) are remarkably hydrophilic, with up to 20% charged amino acids. Each has several localized concentrations of arginines or lysines, which could be responsible for nuclear localization; heptad repeats of leucine, isoleucine, or valine that could facilitate hydrophobic homo- or heterodimerization; and LXLXXL motifs that could enable interaction with nuclear hormone receptors. Despite the very low abundance of the three mRNAs in latently infected cells, EBNA-3A, -3B, and -3C are stable proteins. The EBNA-3s accumulate in intranuclear clumps, sparing the nucleolus (325,651,653). With differential salt extraction, EBNA-3A, -3B, and -3C partition to the nuclear matrix, chromatin, and nucleoplasmic fractions (651).

Although significantly divergent in nucleotide and amino acid sequence, type 1 and type 2 EBNA-3A, -3B, and -3C have similar effects in B-lymphocyte growth transformation (835). EBV recombinants made in a type 2 EBV P3HR-1 background with type 1 EBNA-LP and EBNA-2 have the robust primary B-lymphocyte transformation phenotype characteristic of type 1 EBV strains. Replacement of the P3HR-1 type 2 EBNA-3s with type 1 EBNA-3s does not alter infected B-cell outgrowth into LCLs, or the ability of the recombinant to replicate in response to induction of permissive infection. Thus, EBNA-3 type specificity does not affect latent or lytic virus infection of B lymphocytes *in vitro*.

EBNA-3B is not critical for latent infection of primary B lymphocytes, for LCL outgrowth, for cell survival, or for lytic virus replication (836). EBV recombinants with a nonsense codon after codon 109 of the 938 type 1 EBNA-3B codons are indistinguishable from wild-type recombinants in latent infection of primary B lymphocytes *in vitro*, in LCL outgrowth, and in lytic infection. The finding that EBNA-3B is not essential for any aspect of lymphocyte infection *in vitro* is surprising because EBNA-3B epitopes are frequently recognized by human immune cytotoxic T lymphocytes (see Chapter 75). Immune recognition would be expected to provide strong *in vivo* selection against a nonessential gene. Consistent with the lack of effect of the putative null EBNA-3B mutation on lymphocyte infection and LCL outgrowth *in vitro*, and the expectation that EBV strains with altered or absent EBNA-3B genes would be expected to emerge under immune selection, an EBV mutant with a truncated EBNA-3B ORF was recently discovered in a transplant patient with EBV-associated lymphoproliferative disease who was undergoing therapy with anti-EBNA-3B cytotoxic T lymphocytes. Nevertheless, the consistent presence of EBNA-3B in EBV isolates from normal people is evidence that EBNA-3B is important in natural infection, one aspect of which may be to contribute to immune recognition.

In contrast to the lack of phenotypic association with truncation of the EBNA-3B ORF, a nonsense codon after codon 302 of EBNA-3A or codon 365 of EBNA3C results in EBV recombinants that are unable to transform primary B lymphocytes (835). These experiments indicate that EBNA-3A and -3C are critical for B-lymphocyte growth transformation or survival, and that they differ significantly from EBNA-3B in their roles in EBV infection. Subsequent experiments with EBV recombinants that have a missense mutation beginning at EBNA-3A codon 304 confirm a critical role for EBNA-3A in LCL outgrowth and survival (412). However, two of more than 40 LCLs that were obtained in this latter study eventually grew in culture without wild-type EBNA-3A expression, indicating that EBNA-3A is not absolutely required for continued LCL proliferation or survival. Recombination of rhesus LCV EBNA-3A, -3B, and -3C, or -3B and -3C, into EBV results in recombinants that cannot transform human B lymphocytes into LCLs, consistent with the critical role of EBNA-3A and -3C in LCL outgrowth (384).

Surprisingly, EBNA-3A, -3B, and -3C are similar to EBNA-2 in being able to bind to and stably associate with RBP-Jk (389,446,534,692,693,864,926). In associating with RBP-Jk, the EBNA-3s effectively compete for RBP-Jk with EBNA-2 and Notch and limit EBNA-2 strong up-regulation through RBP-Jk (389,446,468,534,692,693,864,926). An RBP-Jk domain as small as amino acids 125 to 181 can bind to EBNA-3C, and amino acids 172 to 223 of EBNA-3C, which are relatively well conserved in all three EBNA-3 family members, can interact with RBP-Jk (926). EBNA-3A amino acids 224 to 556 also bind well to RBP-Jk, and amino acids 1 to 138 can bind at a low level (122,693).

Because of their role in LCL outgrowth, more attention has been given to the biochemical properties of EBNA-3A and -3C than to EBNA-3B. When expressed alone in B-lymphoma cells, EBNA-3s effect modest changes in cell gene expression. EBNA-3C up-regulates CD21 expression, but not that of CD23, CD10, CD30, CD39, CD40, CD44, or cellular adhesion molecules in BJAB cells (868), whereas EBNA-3B up-regulates vimentin, CD40, and Bcl-2 expression and down-regulates CD77 in DG75 cells (751,752).

EBNA-3C can increase the levels of LMP1 expression, which fall off normally in RAJI cells that arrest in G1 when they grow to saturation (17). EBNA-3C is unique among the EBNA-3s in its ability to coactivate the LMP1 promoter with EBNA-2 (534,927). This effect can occur independently of the RBP-Jk binding site in the LMP1 promoter and of the RBP-Jk site in EBNA-3C. The effect depends on the LMP1 promoter PU.1 site and appears to be mediated by an interaction of the bZIP domain of EBNA-3C with the Ets domain of PU.1 or Spi-B. When fused to the Gal-4 DNA binding domain, EBNA-3C glutamine- and proline-rich amino acids 724 to 826 can activate transcription from a Gal-4-responsive promoter (534). However, EBNA-3C also has repressive effects. Full-length EBNA-3C fused to the Gal-4 DNA binding domain represses transcription from a variety of promoters in a variety of cell types, and the effect is independent of the presence of RBP-Jk sites on the promoter plasmid (40,71). The repressive effects map to amino acids 280 to 525; the larger proline- and glutamine-rich regions corresponding to amino acids 580 to 992 also have repressive activity (40). EBNA-3C also represses transcription from the Cp promoter. In this instance, the effect depends on interaction with RBP-Jk and on the EBNA-3C repression domain. The repressive effect of EBNA-3C is in part attributable to an interaction and association with HDAC1, but that interaction maps to a region of EBNA-3C that includes the RBP-Jk binding domain (679). EBNA-3C has been reported to cooperate with oncogenic Ras in transforming rat embryo fibroblasts, and a low level of *in vitro* interaction with RB has been detected (633). In NIH3T3 fibroblasts or human U2OS cells compelled to arrest by serum withdrawal, EBNA-3C expression can inhibit the accumulation of p27(K1P1) and can cause bi- and multinucleated cells by abrogating the mitotic spindle checkpoint (634). EBNA-3C amino acids 365 to 509 avidly bind prothymosin-alpha, a 112-amino-acid nuclear protein that can interact with histone (128). Although only about 2% of EBNA-3C coimmunoprecipitated with ProT-a and much less than 1% of ProT-a coimmunoprecipitated with EBNA-3C, EBNA-3C appeared to co-localize with ProT-a in nuclei (128). These data indicate that EBNA-3C could be

involved in transcriptional effects through interaction with ProT-a.

EBNA-3A also represses the activity of the Cp promoter in B cells and in epithelial cells, and this activity largely depends on the domain through which EBNA-3A interacts with RBP-Jk (122). Using Gal-4 EBNA-3A fusion proteins and Gal-4-responsive promoters, the EBNA-3A-repressive domain has been mapped to 143 amino acids that do not interact with RBP-Jk and can mediate down-regulation of transcription (71,122). EBNA-3A also has a potential weak activation domain that is evident when EBNA-3A lacking the amino acid 100-to-364 repressive domain are fused to the Gal-4 DNA binding domain and expressed in B lymphoblasts along with a Gal-4-responsive promoter (122). The activation effects appear to be B-cell specific and are not evident in epithelial cells. EBNA-3A used as bait in a yeast two-hybrid search for interacting proteins curiously retrieved the carboxyl part of the epsilon subunit of the chaperonin T-complex protein 1, and the p38 subunit of the aryl hydrocarbon receptor complex (401,402). EBNA-3A binds to p38 and causes p38 to partially localize to the nucleus (401).

EBNA-1

EBNA-1 was initially identified as an EBV nuclear neoantigen that is present in all EBV-infected cells, regardless of the state of EBV infection (682). This was the first evidence that EBV encoded a nuclear protein in latently infected cells, perhaps similar to T antigen in simian virus 40 (SV40)-transformed cells. Soon thereafter, EBNA-1 was shown to associate diffusely with mitotic chromosomes (625). EBNA-1 is unique among the EBNAs in this regard (274,652). The significance of EBNA-1 association with chromosomes emerged later from the discovery that EBV has a cis-acting element, termed oriP, that enables the persistence of episomes in EBV-infected cells or in any human cells in which EBNA-1 is expressed (906,910). EBV and other oriP-containing episomes associate randomly with human chromosomes in cells that express EBNA-1 (159,299,753). The unique random association of EBNA-1 with chromosomes, the association of EBV or other oriP plasmid DNA with chromosomes in EBNA-1 expressing cells, and the need for EBNA-1 in oriP persistence, position EBNA-1 as the key mediator of EBV DNA binding to chromosomes and episome persistence. Indeed, EBNA-1 is essential for EBV episome maintenance (469).

OriP has at least two components: a family of 20 copies of a 30-bp repeat, FR, that can be an EBNA-1-dependent enhancer, and a dyad symmetry, DS, of four copies of the 30-bp repeat, two in tandem and two in a larger dyad symmetry, that are required for episome maintenance (683,684). A key component of the mechanism by which this system works is that EBNA-1 binds specifically to the 30-bp repeat (most strongly to the 20×30-bp repeat element) and then to the dyad symmetry element, and to another site within the EBNA transcript (681). An EBNA-1 expression vector and oriP can be combined with a cassette for positive selection to make a plasmid into which any gene can be cloned to enable its maintenance as an episome in human cells (784). The EBNA-1 and oriP system has been widely exploited to achieve heterologous gene expression. In one use, oriP enabled plasmids containing 150 to 200 kbp fragments of random human DNA to persist as multicopy episomes in EBNA-1-expressing cells. Two such plasmids could persist in the same cell (792). Although essential for oriP enablement of plasmid persistence, excess EBNA-1 expression does not increase oriP-replicated plasmid copy number. Cellular controls limit DNA replication to one initiation event per DNA molecule per S phase (2,909), and equal numbers of plasmids are distributed to cell progeny (787).

EBNA-1 is encoded by the 2-kb 3'-terminal exon of spliced mRNAs. In type 3 latency, the 3.5-kb EBNA-1 mRNA is initiated at the Cp or Wp promoter and is highly spliced, and the primary transcript is about 100 kb (see Fig. 3) (62,63,64,65 and 66,317,326,327,719,774,788). Transcription from the Wp promoter is initially regulated by cellular factors. Wp is up-regulated and Cp turned on by EBNA-2 and -LP. EBNA-1 then further up-regulates Wp and Cp. Eventually, high-level expression of EBNA-3s down-modulates the strong up-regulating effects of EBNA-2 and EBNA-LP and prevents runaway EBNA transcription (295,468,665,692,693,788,793,886). In latency type 1 or 2, the EBNA-1-specific 2.4-kb mRNA is primarily initiated at n62423, with secondary sites at 62392 and 62340 in the prototype B95 sequence (36,611,612,613 and 614,733). This latency type 1 and 2 Qp promoter lacks a recognizable TATAA element. Transcription appears to be positively regulated by an unknown factor, LBP-1 (611), by IRF1 and 2 (613,731), and by E2F (612,794), which bind to three distinct sites bracketing the n62423 initiation site. The two EBNA-1 binding sites just 3' to the transcriptional start site are the principal elements that self-regulate EBNA-1 transcription in latency type 1 and 2 (720,732,794). Similar regulatory sites are conserved in the baboon LCV (711).

EBNA-1 from the prototypical B95 EBV strain consists of 641 amino acids. The protein has a high proline content, is charged, and migrates on denaturing polyacrylamide gels with an apparent size of 76 kd. From amino to carboxyl terminus, EBNA-1 has four components: (a) an amino-terminal 89 amino acids, which include amino acids 32 to 83 and are Arg rich, (b) amino acids 90 to 327, which are an irregular copolymer of Gly and Ala, (c) amino acids 328 to 386, which are Arg rich and include a nuclear localization sequence at amino acids 379 to 386, and (d) carboxyl-terminal amino acids 387 to 641, which include amino acids 459 to 607 that bind DNA and dimerize. The two Arg-rich regions of EBNA-1 amino acids 1 to 379 can each bind to chromosomes and together reconstitute the chromosome association characteristic of EBNA-1 (360,532). Whereas EBNA-1 amino acids 379 to 641 appear to permit initial accumulation of oriP-containing plasmids in cells, the chromosome-associating domains are necessary for long-term plasmid maintenance, presumably because of their role in interacting with chromosomal proteins (349) and partitioning oriP episomes to progeny nuclei. Cellular chromosomal proteins, HMG-I amino acids 1 to 90, or histone H1 can functionally substitute for EBNA-1 amino acids 1 to 378 in enabling long-term episome persistence (360).

Identification of the biochemical interactions of the EBNA-1 carboxyl-terminal domain with oriP is facilitated by the high expression level of the EBNA-1 carboxyl terminus in bacteria- or baculovirus-infected sf9 cells (230,574). EBNA-1 amino acids 450 to 641 or 607 have similar DNA binding and dimerization activity to full-length EBNA-1 (26,27,391,573,681). The core amino acids for DNA interaction are amino acids 459 to 500, particularly 462 to 477, whereas the core residues for dimerization are amino acids 501 to 532 and 554 to 598. A dimer of an EBNA-1 oligopeptide corresponding to amino acids 458 to 478 can bind DNA, albeit nonspecifically (99,100). Surprisingly, amino acids 450 to 641 fused to the pyruvate kinase ORF and the EBNA-1 nuclear localization signal (NLS) are sufficient for wild-type EBNA-1 transcriptional activation through FR upstream of a minimal TK promoter in Vero cells, and the carboxyl-terminal acidic domain is critical for activation (26).

Each 30-bp EBNA-1 dimer-binding site is a partial dyad similar to TAGGATAGCATATGCTACCCAGATCCAG (27,391,681). Relative to the center of each half of the dyad, nucleotides 3 to 8 are most critical for EBNA-1 binding (27). EBNA-1 has a high affinity for its cognate sequence, and interactions with the cognate sequence can be demonstrated even after protein denaturation and renaturation on cellulose nitrate. EBNA-1 not only dimerizes on 30-bp elements but also forms higher-order oligomers. Oligomerization of EBNA-1 on templates that have EBNA-1 binding sites separated by intervening sequence induces looping-out of the intervening DNA (230,259,548,781). On oriP, EBNA-1 first saturates FR and then binds to DS, looping out the intervening DNA (230). EBNA-1 amino acids 322 to 377 are important for efficient looping of EBNA-1 bound to FR and DS, or for linking multiple oriP DNA molecules to each other in a larger complex (228,518,519 and 520). Only DNA-bound EBNA-1 can participate in the large, linked EBNA-1 DNA complexes.

The binding of EBNA-1 to oriP results in two thymine residues, 64 bp apart in the region of dyad symmetry, becoming reactive to potassium permanganate, indicative of a helical distortion. EBNA-1 binding to DS lengthens interstrand H-bonds for three base-pairs centered over the permanganate-sensitive thymine base and three potential intrastrand H-bonds are formed between adjacent bases. Dimethyl sulfate protection studies indicate that EBNA-1 binds on the opposite face of the helix from the reactive thymines (229,307). Analyses on oriP in EBV-infected cells indicate that similar permanganate- and dimethyl sulfate–reactive sites exist *in vivo* (344).

Crystal structures of the EBNA-1 carboxyl-terminal 255-amino-acid dimer have been resolved at 2.5 Å, and of the dimer of cognate DNA at 2.4 and 2.2 Å (58,59 and 60). The EBNA-1 DNA binding domain has two structural motifs: a core domain that mediates protein dimerization and is similar to the DNA binding domain of the papillomavirus E2 protein, and a flanking domain that mediates sequence-specific contacts. Genetic and biochemical studies of the EBNA-1 core domain residues, which are structural homologs of the E2 residues that mediate sequence-specific DNA binding, implicate the corresponding EBNA-1 residues in DNA binding. The EBNA-1 core domain, when expressed in the absence of the flanking domain, has sequence-specific DNA binding activity, and the flanking domain residues contribute to the DNA binding. Thus, both the core and the flanking domains of EBNA-1 play direct roles in DNA recognition (132).

Only seven to eight copies of the 30-bp repeats are required for full activity of FR in transcription or episomal DNA replication (893). EBNA-1 binding to FR may effect up-regulation of the Wp and Cp promoters and of the far-upstream LMP1 and LMP2B promoters (241,665). The requirement for FR to enhance transcription and for episomal DNA replication can be partially replaced by a tandem duplication of DS (893). Tandem duplication of DS decreases its ability to serve as an origin (655). FR also arrests and functionally terminates the replication of episomes containing oriP in cells expressing EBNA-1 (167,240).

FR increases the retention and enhances transcription of oriP plasmids in cells, whereas DS is the site of initiation of plasmid DNA replication (300). DS supports EBNA-1-dependent replication in the absence of the FR (908). DNA synthesis begins in DS and proceeds bi-directionally from DS (240). Plasmids containing two copies of DS are not amplified relative to wild-type oriP (431). Two EBNA-1 binding sites in DS appear to be adequate for initial DNA replication (300,908). The ease of unwinding the 65-bp dyad symmetry of two EBNA-1 binding sites in DS, or relative nucleosome sparing in DS (i.e., relative to other sites in oriP) may contribute to the preferential use of DS as an origin (744,882). DS is not stringently required for episome maintenance, and other sequences can substitute for DS in initiation of DNA synthesis (109), including another site in oriP, designated Rep* (432). DS is also a preferential but not exclusive site for EBV genome replication, and deletion of DS does not prevent EBV episome persistence (501,617).

At least one cellular cofactor that is not conserved in rodent cells is required for DS origin function and not for FR enhancer activity, as EBNA 1 enhances transcription in mouse or human cells, whereas oriP episomes do not replicate in rodent cells (893). Replication in rodent cells can be restored by replacing DS with a rodent DNA origin; such vectors are still dependent on the enhancer function of EBNA-1 and the family of repeats (447). Similarly, human cell DNA that contains a putative origin for DNA replication can functionally substitute for DS in oriP to allow persistence as an episome in human cells (448,542). A human cell cDNA has been identified that encodes a protein that interacts specifically with EBNA-1 cognate DNA, although the relevance of this protein to oriP function in DNA synthesis has not been established (924).

The ability of Rep*, human cell DNA origins, or rodent cell DNA origins to substitute for DS in the context of oriP in an EBNA-1-expressing cell, calls into question the fundamental role of EBNA-1 in DNA replication versus episome persistence (8). Indeed, oriP increases the conversion of transfected plasmid DNA to Dpn1 resistance about 10-fold in the absence of EBNA-1 at 48 hours after transfection and about 20-fold in the presence of EBNA-1. This DNA had gone through two cycles of DNA replication as evidenced by Dpn1 resistance and by bromodeoxyuridine incorporation. The major EBNA-1 effect occurred at 96 hours after transfection, at which point Dpn-resistant DNA disappeared from cells that lack EBNA-1. Further, DS was not specifically required for persistence; FR with a triplicate of Rep* DNA replicates and persists as well as wild-type oriP in cells that express EBNA-1.

EBNA-1 amino acids 90 to 327 are an irregular glycine–alanine repeat domain. The DNA encoding this domain is similar to repeat sequences in cell DNA, and similar polypeptides may be encoded from the cell genome (312). EBV isolates frequently differ in the length of this repeat. This difference has been useful in identifying the EBNA-1 ORF and typing EBV isolates (204,267,268 and 269,326). Deletion of the repeat does not affect EBNA-1 stability or function (907). EBNA-1 expressed in murine cells does not engender a specific cytotoxic T-cell response in mice (847). The failure to recognize EBNA-1 as foreign is due to the EBNA-1 Gly-Ala repeat, which inhibits EBNA-1 processing through proteosomes (477). Because proteosome-mediated degradation is necessary for antigen processing into major histocompatibility complex (MHC) class I molecules for presentation at the cell surface, epitopes in EBNA-1 are poor targets for attack by CD8 cytotoxic T cells. Fusion of the Gly-Ala domain to the EBNA-3B ORF down-regulates CD8 recognition of EBNA-3B-expressing cells. Other proteins are also protected from proteosome degradation by fusion to the glycine–alanine repeat (746). The length of the repeat and particularly the alanines are critical for this effect. Primate LCVs have shorter Gly-Ala repeats that do not have a similar effect on proteosome processing, leaving open the

possibility that the Gly-Ala repeat domain may have yet another role in EBV infection.

EBNA-1 is very stable and is phosphorylated on serine residues in at least two separable domains in the carboxyl half of the molecule (308,652,658). The role of these phosphorylations in EBNA-1 function has not as yet been established through genetic analyses. Further, EBV DNA in human cells that express EBNA-1 associates with the nuclear matrix. The fragment of EBV DNA that associates with the matrix includes oriP, potentially linking oriP to a site for DNA replication or transcription. EBNA-1 can also interact with diverse nucleic acid substrates *in vitro*, including EBER-1 and HIV-TAR (768).

EBNA-1 is the only EBNA that continues to be transcribed when cells are activated to lytic infection (317,706,873,877). Cp and Wp promoter activities cease in lytic infection, and the downstream Fp promoter is activated (614). The lytic mRNA initiates in an Fp promoter just upstream of the Qp promoter and otherwise has the same sequence as the latent infection RNA.

LMP1

Despite being a weak promoter for the LMP1 gene relative to the Cp and Wp EBNA promoters, as revealed by nuclear run-on assays, LMP1 mRNA is almost 10 times more abundant than the EBNA mRNAs in latently infected B lymphocytes (212,720). In epithelial cells, LMP1 transcription is mediated by an upstream promoter that initiates transcription from multiple TATA-less sites in the nearest copy of TR, resulting in a 3.5-kb transcript with a long, largely untranslated 5' exon (251,715). The LMP1 transcripts have two short introns (44,212). The EBNA proteins are remarkably stable, whereas a significant fraction of LMP1 has a short half-life. The primary amino acid sequence of LMP1 is that of an integral membrane protein and includes at least three domains: (a) a 20-amino-acid, arginine- and proline-rich, hydrophilic amino terminus lacking the characteristics of a signal peptide; (b) six markedly hydrophobic 20-amino-acid, alpha-helical, transmembrane segments, separated by five reverse turns, each eight to ten amino acids in length; and (c) a 200-amino-acid carboxyl terminus, rich in acidic residues (Fig. 8). LMP1, translated *in vitro*, posttranslationally inserts into cell membranes consistent with the expected membrane insertion properties of the three pairs of highly hydrophobic transmembrane segments joined by short reverse turns (490,492,724). Although LMP1 has little primary sequence homology to other proteins, aspects of its three-domain organization are similar to some other integral membrane proteins such as erythrocyte membrane band three or the *mas* oncogene.

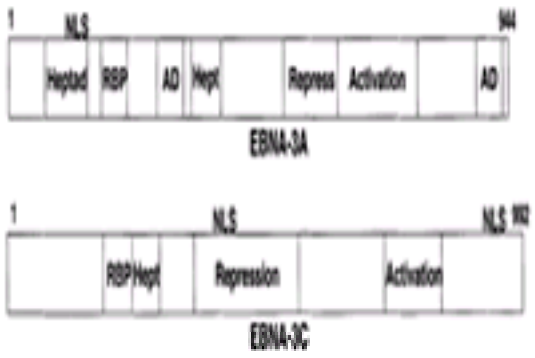

FIG. 7. Schematic diagram of the EBNA-3A and EBNA-3C open reading frames. Indicated are domains that associate with RBP-Jkappa as well as domains that have activating or repressive effects when fused to the Gal-4 DNA binding domains and assayed for transcriptional up- or down-regulating effects in transient transfection assays with a promoter that has multiple upstream Gal-4 binding sites.

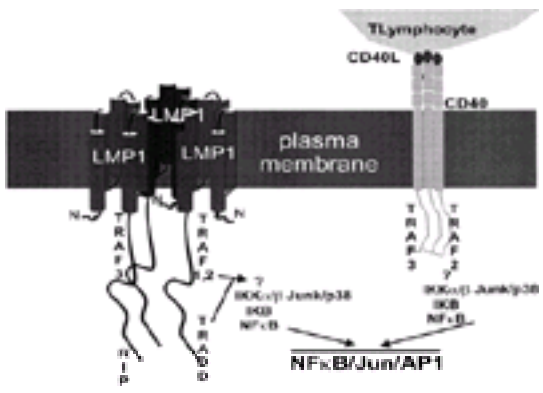

FIG. 8. Schematic diagram of biochemical mechanisms by which LMP1 effects cell growth and survival pathways by constitutively mimicking an activated tumor necrosis factor (TNF) receptor such as CD40. The six hydrophobic transmembrane domains of LMP1 enable LMP1 to aggregate in the plasma membrane. The amino-terminal cytoplasmic domain is fully dispensable for primary B-lymphocyte growth transformation, and its primary function is in tethering the amino terminus of the first transmembrane domain to the cytoplasmic side of the plasma membrane. The transmembrane domains and the carboxyl-terminal cytoplasmic domain are essential for primary B-lymphocyte growth transformation. The carboxyl-terminal cytoplasmic domain is composed of 200 amino acids and it has two essential components: One is the first 44 amino acids, which associate with TNF receptor-associated factors. The second is the carboxyl-terminal 30 amino acids, which associate with the TNFR-associated death domain proteins, TRADD and RIP, without propagating a death signal. Both domains activate the IkB kinases causing phosphorylation of IkB, IkB degradation, and NF-kB activation. Both domains also activate Junk and p38 kinases. The rest of the carboxyl-terminal cytoplasmic domain can be deleted without loss of B-lymphocyte transforming activity.

Immunofluorescence microscopy (324,492), membrane fractionation (523), and live-cell protease cleavage studies (492) indicate that about half of the cellular LMP1 is in the plasma membrane. LMP1 is less abundant in other cytoplasmic membranes. Live-cell protease cleavage studies also indicate that both the N- and C-termini are on the cytoplasmic side of the plasma membrane and only three short reverse-turn domains are on the extracellular side of the plasma membrane (492).

LMP1 is phosphorylated on serine and threonine residues, at a ratio of 6:1, and is not phosphorylated on tyrosines (38,490,524,589,591). Half or more of LMP1 is associated with the cytoplasmic cytoskeleton as defined by resistance to extraction with buffers supplemented with nonionic detergents (490,492,524,589,591). The half-life of LMP1 has been measured by determining the time to reach stable levels of ^{35}S-methionine label in LMP1 and by determining the rate of decay of LMP1 in the presence of protein synthesis inhibitors. The data do not precisely agree, possibly because of an effect of the protein synthesis inhibitors in increasing total LMP1 turnover, or differences in the cell lines studied (38,490,524,589,591). Clearly, nascent, nonionic, detergent-soluble LMP1 has a half-life of less than 2 hours and is converted to a more stable cytoskeleton-associated form. Cytoskeletal LMP1 is phosphorylated, and soluble LMP1 is not (490,524,589,591). LMP1 is ultimately cleaved near the beginning of the carboxyl-terminal cytoplasmic domain, resulting in a transiently stable soluble C-terminal domain of about 25 kd (589,591). The principal serine and threonine phosphorylation sites are near each other in the 25-kd cleavage product (591).

LMP1 constitutively forms discrete patches in the plasma membrane, which are often further organized into a single caplike structure, which localizes to the uropod (324,492). LMP1 aggregates in patches in LCLs or other cell plasma membranes in the absence of exogenous growth factors; the aggregation appears to be an intrinsic property of the multiple hydrophobic transmembrane domains (490,492). Vimentin co-localizes with the LMP1 cap in the latently infected B-lymphocyte plasma membranes (490). Vimentin is not ordinarily found in a plasma membrane patch and is drawn into this patch by LMP1. Vimentin relocalizes into perinuclear rings and coils when cells are treated with colcemid, and LMP1 then relocalizes to the vimentin rings (490). Thus, once LMP1 is associated with vimentin, vimentin can further direct LMP1's localization. LMP1 aggregates in patches and caps in the plasma membrane of cells that lack vimentin or other EBV proteins and does not require vimentin for its signal transducing effects (489,491,866). Thus LMP1 interacts with itself or with other cell membrane proteins to form large, noncovalently linked membrane complexes. LMP1 is partially associated with cholesterol-rich membrane microdomains (31).

In single gene transfer experiments under heterologous promoters, LMP1 has transforming effects in continuous rodent fibroblast cell lines (39,865,866,590). In Rat-1 or NIH3T3 cells, LMP1 alters cell morphology and enables cells to grow in medium supplemented with a low concentration of serum. In Rat-1 cells, LMP1 also causes loss of contact inhibition, so that cells heap up in monolayer culture (865). Rat-1 or BALB/c 3T3 cells lose anchorage dependence and grow in soft agar after LMP1 expression (39,866). Rat-1 cells expressing LMP1 are tumorigenic in nude mice, whereas control Rat-1 cells are nontumorigenic (865). The growth of BALB/c 3T3 cells in soft agar correlates quantitatively with the extent of LMP1 expression up to the levels ordinarily expressed in LCLs (866). Expression at higher levels results in toxicity. The hypothesis has been put forward that the toxic effects of LMP1 expressed at high levels may be a useful surrogate marker for LMP1 biologic activity in rodent fibroblasts; LMP1 mutants that have transforming activity when expressed at levels similar to LCLs, are usually toxic when expressed at higher levels (538). Comparison of the transforming properties of LMP1 with those of D1LMP1 indicates that D1LMP1 does not have these effects (37,866). D1LMP1 lacks the amino-terminal cytoplasmic domain and the first four transmembrane domains, inserts into cell membranes, and does not patch; these data indicate that the amino terminus and the first four transmembrane domains are essential for LMP1 transforming effects in BALB/c 3T3 cells. Addition of the 25-amino-acid N-terminal cytoplasmic domain to the amino terminus of D1LMP did not restore the ability to cause anchorage-independent growth of BALB/c 3T3 cells (37,538), indicating that abnormal transforming phenotype of D1LMP is not caused by an absence of the amino-terminal cytoplasmic domain. Carboxyl-terminal cytoplasmic domain truncations have been variable in rodent fibroblast effects but consistently unable to cause loss of contact inhibition in Rat-1 cells (590). These data suggest that the carboxyl-terminal cytoplasmic domain is important for loss of contact inhibition in Rat-1 cells.

LMP1 also dramatically alters the growth of EBV-negative BL lymphoblasts or even primary B lymphocytes when expressed stably or transiently at the appropriate level in such cells following gene transfer (57,321,489,645,646,649,709,867,868). LMP1 induces many of the changes usually associated with EBV infection of primary B lymphocytes or with antigen activation of primary B lymphocytes, including cell clumping; increased villous projections; increased vimentin expression; increased cell surface expression of CD23, CD39, CD40, CD44, and class II MHC; increased IL10 expression; decreased expression of CD10; and increased expression of the cell adhesion molecules LFA-1, ICAM-1, and LFA-3 (867). Not only does LMP1 increase plasma membrane expression of adhesion molecules, but it also functionally activates adhesion and induces higher levels of LFA-1 mRNA (866,867). D1LMP1 has none of these effects, confirming the importance of the amino terminus and multiple transmembranes for domains for phenotypic effects (491,867). LMP1 has also been shown to protect B lymphocytes from apoptosis (270,321,709). These effects are mediated in part through LMP1 induction of bcl-2 (321,539,707). LMP1 effects in BL cells or rodent fibroblasts vary with the particular cell background and particularly in B lymphoblasts with the basal level of activation molecule expression. LMP1 induction of B-lymphocyte adhesion molecules may be important, as homotypic B-lymphocyte adhesion or adhesion to other cells can facilitate cell growth through autocrine or paracrine growth factors and ligands expressed on other cell surfaces. Also, adhesion is important for the immune elimination of EBV-transformed B lymphocytes *in vivo*, and therefore for continued virus infection. Despite the robust and pleotropic effects of LMP1 expression in human B-lymphoma cells, the effects of deletion mutants that partially affect the membrane-patching properties of LMP1 on each of the phenotypes is intermediate without a clear loss of some effects and the retention of others (491).

LMP1 expression also alters cell growth and gene expression in multipotent hematopoietic stem cells and epithelial cells (156,201,202,346). The effects of LMP1 on epithelial cell growth are evident with transgenic expression of LMP1 in the skin of mice: LMP1 induces epidermal hyperplasia and alters keratin gene expression (885). In monolayer cultures, LMP1 alters keratinocyte morphology and cytokeratin expression (201). In stratified air–liquid interface raft cultures of

immortalized epithelial cell lines, LMP1 inhibits cell differentiation (156). In C33 cervical carcinoma cells, LMP1 expression induces matrix metalloproteinase 9 expression; matrix metalloproteinases are believed to be important in tissue invasion (807,917).

The ability of EBV recombinants specifically mutated in LMP1 to transform primary B lymphocytes into LCLs is a definitive assay for EBV transforming effects, because primary human B lymphocytes grow out as LCLs only in response to EBV infection. This assay has proven to be quite useful for defining the critical components of LMP1. As expected from the transforming effects of LMP1 expression in rodent fibroblasts and B-lymphoma cells, LMP1 is essential for primary B-lymphocyte growth transformation (409). EBV recombinants with mutations in LMP1 that result in amino-terminal truncation of the cytoplasmic and first transmembrane domain have a nontransforming phenotype (409). This form of LMP1 is expressed as a stable protein, localizes to the plasma membrane, but does not aggregate in the plasma membrane, indicating the importance of plasma membrane aggregation in LMP1 effects on primary B-lymphocyte transformation into LCLs (409,491). Similarly, expression of the last three transmembrane domains and the carboxyl-terminus resulted in a nontransforming phenotype; this form of LMP1 localizes to all cytoplasmic membrane without evidence of accumulation in the plasma membrane or aggregation (409,491). The effects of these mutations are not caused by specific interactions mediated by the amino-terminal cytoplasmic domain, because deletion of any part of the amino-terminal cytoplasmic domain results in at least 10% residual transforming efficiency, and the LCLs have a normal growth phenotype (368). The most severe effects of mutation in the amino-terminal cytoplasmic domain are associated with deletion of all of the arginines, which appears to interfere with membrane positioning effects of the transmembrane domains. In contrast, expression of the amino-terminal 187 amino acids of LMP1, which includes the amino-terminal cytoplasmic domain and all of the transmembrane domains but not the carboxyl-terminal cytoplasmic 200 amino acids, ablates primary B-lymphocytes transformation (411).

Specifically mutated EBV recombinants that allow expression of the amino-terminal 231 amino acids, which includes only the first 45 of the 200 amino acids of the carboxy-terminal cytoplasmic domain, results in LCL outgrowth that depends on fibroblast feeder layers (409). The LMP1 1 to 231 recombinant EBV is nearly wild type in the initiation of foci of LCL outgrowth, but most of these foci cannot be expanded to LCLs; their growth is unusually dependent on a high cell density (410). In contrast, an EBV recombinant deleted for DNA encoding only the first 15 amino acids of the cytoplasmic carboxyl terminus is unable to transform primary B lymphocytes (367). These data indicate that the first 15 amino acids of the carboxyl-terminal cytoplasmic domain include part of a site whose first 45 amino acids are essential for LCL outgrowth and even sufficient, in the context of the transmembrane domains, for initial B-cell transformation. Further, these data indicate that efficient LCL outgrowth requires the more carboxyl-terminal 155 amino acids. The first mutations of two of the three LMP1 carboxyl-terminal amino acids that were tested in an EBV recombinant defined a second site of up to 35 amino acids that is important for efficient transformation of primary B lymphocytes (370). Despite the fact that multiple sites between the first 45 and last 35 amino acids of the LMP1 carboxyl terminus have been implicated in various biologic or biochemical processes, specific deletion of all of the LMP1 codons for amino acids 232 to 351 results in an EBV recombinant that is indistinguishable from wild-type LMP1 in primary B-lymphocyte growth transformation, LCL outgrowth, LCL growth characteristics, or lytic EBV replication (371). Thus, the nontransforming phenotype resulting from the deletion of the first 15 codons of the cytoplasmic carboxyl terminus, the robust initial transformation phenotype of the LMP1 amino acids 1 to 231, the deficient LCL outgrowth phenotype of the recombinant with two of the last three carboxyl-terminal amino acids mutated, and the wild-type phenotype of the recombinant deleted for codons 232 to 351 indicate that LMP1 carboxyl-terminal residues 187 to 231 and 352 to 386 are the only residues that are critical for LMP1 cytoplasmic signalling effects in the conversion of primary B lymphocytes to LCLs. Therefore, these two domains have been given the acronym TES1 and 2, for transformation effector sites 1 and 2.

Investigations of the biochemical bases of LMP1 effects in up-regulating expression of B-lymphocyte activation markers, adhesion molecules, HIV, or A20 (an antiapoptosis factor) led to the identification of NF-kB activation as one basis for LMP1 signalling effects (292,453). The domains of LMP1 required for NF-kB activation are the multiple transmembrane domains and the carboxyl-terminal 55 amino acids or the carboxyl-terminal 36 amino acids and first 45 amino acids of the cytoplasmic carboxyl terminus; the amino-terminal cytoplasmic amino acids are not specifically required (356,578). The amino-terminal 45 and carboxyl-terminal 36 amino acids of the cytoplasmic carboxyl terminus are therefore designated C-terminal NF-kB activation regions 1 and 2, or CTAR1 and -2 (356). The need for the LMP1 transmembrane domains could be replaced in various ways, including expression of CD2-CTAR1 and -2, or CD2-CTAR2 chimeric fusions in cells, and adding anti-CD2 antibody to induce aggregation, confirming the important constitutive homoaggregating role of the LMP1 transmembrane domains (226,252).

The biochemical mechanisms that underly the transformation effector sites and NF-kB activating domains were initially united by the yeast two-hybrid-based discovery that LMP1 TES1/CTAR1 interacts with, and in the context of the LMP1 transmembrane domains constitutively associates with, human tumor necrosis factor (TNF)-associated cell cytoplasmic factors hTRAF3 and hTRAF1 (593); the mouse TRAF1 and -2 were described several months before by their interaction with the type 2 TNF-receptor cytoplasmic domains (703). LMP1 TES1 is structurally and functionally homologous to the TNF receptor (TNFR), CD40 (593), which, when activated, induces effects in B lymphocytes that are similar to LMP1 (42,101,585). The structural basis for LMP1 and CD40 engagement with TRAFs and many of the downstream effects are equivalent (74,164,166,180,181,182,183 and 184,225,227,302,408,424,572,667,726,798,854,911). LMP1 and CD40 share a PXQXT motif (164,227) that interacts with a shallow canyon on the surface of TRAF trimers (911,912). The biochemical effect is to stabilize TRAF1 and -2 and TRAF3 and -5 homo- and heterodimers on LMP1 TES1/CTAR1 and in the cytoplasm (164,166). TRAF trimers that incorporate TRAF2 and TRAF5 are particularly implicated in forward signalling from TNFRs. One aspect of LMP1 signalling through TRAFs is that the LMP1 PXQXT domain has a higher affinity for TRAF1 and -3 than for TRAF2 and -5 (164,166). TRAF1 is expressed at low levels in B lymphocytes and is then induced by LMP1 expression, whereas in epithelial cells or other cell types expression of TRAF1 may be minimal or not inducible, and this may affect the level of LMP1 signalling through TES1/CTAR1 in these cells.

TES2/CTAR2 also engages TNFR signalling pathways and ultimately signals through TRAFs (369,372). A yeast two-hybrid screen with TES2 bait retrieved the TNFR-I associated death domain protein, TRADD (369). The LMP1 carboxyl-terminal 11 amino acids interact with and constitutively associate with TRADD and RIP (369,372). LMP1 engages a small component of the death domain and does not propagate death domain complex recruitment or induce

cell death. Much of the signal that LMP1 transmits through TRADD and RIP appears to then be mediated by TRAFs, which are known to mediate nondeath signalling from TRADD and RIP. Dominant negative mutants of TRAFs block NF-kB activation from both TES1/CTAR1 and TES2/CTAR2 (408). Further, dominant negative mutants of the NF-kB-inducing kinase, NIK, or of the IKK inducing kinases, IKK a or b block TES1/CTAR1 and TES2/CTAR2-mediated NF-kB activation (Fig. 8) (798). However, NIK is probably only one component of the kinases that mediate signalling downstream of the TRAFs. NIK is probably not even the principal kinase that connects TRAFs to the IKKs. Furthermore, signalling downstream of the TRAFs activates not only IKKs and NF-kB, but also JUNK and *jun*/*fos*, p38 and AP-1, and CDC42 (180,182,184,302,423,667). Thus, while LMP1 signalling through TRAFs mediates most or all of LMP1's effects, aspects of these pathways are as yet incompletely delineated. One incompletely understood aspect of LMP1 signalling is the preferential role of TES1/CTAR1 over TES2/CTAR2 in the activation of EGF receptor expression in epithelial cells, or of TRAF1 expression in B lymphocytes (166,569,570,571 and 572). NF-kB activation is an important component of many of these transcriptional activations and is critical to LCL survival (82). Despite continued high level expression of Bcl-2 and Bcl-XI, inhibition of NF-kB activation results in apoptosis of LCLs.

LMP1 and EBNA-1 are the only latent infection–associated genes that are also transcribed in lytic EBV infection. In some latently infected BL cells such as Raji, LMP1 expression can be increased by treatment with activators of PKC, which increase early lytic cycle promoter activity (68,464,704). The promoter for full-length LMP1 expression in lytic infection has not been characterized. In late lytic infection, a promoter in the third LMP1 exon transcribes the part of the LMP1 ORF that encodes the last two transmembrane domains and the entire LMP1 cytoplasmic domain (353). This smaller integral membrane protein, referred to as D1LMP1, localizes to cytoplasmic membranes and does not have the transforming or cell activating properties of LMP1 and does not associate with vimentin or other cytoskeletal elements (490,491,523). The detection of LMP1 but not D1LMP1 in preparations of purified virus and newly infected cells indicates that LMP1 may be incorporated into virions (194,523). This raises the possibility that virion-associated LMP1 could have an effect on the growth of newly infected cells.

The expression of LMP1 in three lineages of transgenic mice under control of the immunoglobulin heavy-chain promoter and enhancer results in low-level expression in normal lymphoid tissue and an increase in B-cell hyperplasia and lymphomas (449). The incidence of lymphoma increased significantly with age with lymphomas developing in 42% of transgenic mice over 18 months of age. The lymphomas are monoclonal or oligoclonal by IgH rearrangement, and LMP1 is expressed at LCL levels in the lymphomas. These data indicate that LMP1 alone can be oncogenic in B lymphocytes *in vivo*. B cells of transgenic mice expressing LMP1 under the control of immunoglobulin promoter/enhancer–displayed enhanced expression of activation antigens, spontaneously proliferated and produced antibody. LMP1 expression in transgenic mice in CD40-deficient or normal backgrounds mimics CD40 signals to induce extrafollicular B-cell differentiation and blocks germinal center formation (854).

LMP2A and -2B

The cloning and sequencing of cDNAs (462,465,724) to 2.3- and 2.0-kb cytoplasmic polyadenylated RNAs (856) from latently infected cells provided the first evidence for two integral membrane proteins that were designated LMP2A and -2B, or TP1 and -2 (Fig. 9). (231,511). The first exons of LMP2A and -2B are the only unique exons; all other exons are shared by LMP2A and -2B. The first LMP2A exon is predicted to encode for a 119-amino-acid hydrophilic amino-terminal cytoplasmic domain. The first LMP2B exon is short and lacks a methionine codon. Translation of LMP2B initiates at a methionine codon at the beginning of the common second exon, before the first transmembrane sequence (724). The remaining LMP2A and -2B exons are predicted to encode 12 hydrophobic integral membrane sequences separated by short reverse turns and a 27-amino-acid hydrophilic carboxyl-terminal domain. Monospecific antisera generated against LMP2 fusion proteins localize LMP2A to a patch in the plasma membrane of latently infected B lymphocytes, where it co-localizes with LMP1 (510,511). LMP2A localizes to the same site in the plasma membrane of B-lymphoma cells whether or not LMP1 is expressed in the same cell.

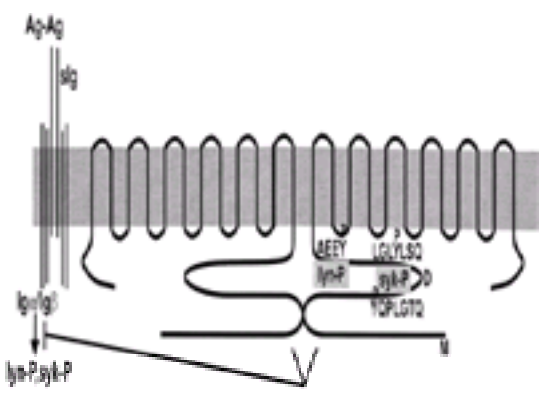

FIG. 9. Schematic diagram of the biochemical mechanisms by which LMP2 constitutively effects tyrosine kinase signalling pathways, inducing low-level activation and high-level global desensitization.

LMP2A is a substrate for and is associated with B-lymphocyte src family tyrosine kinases. Antiphosphotyrosine antibodies localize sites of constitutive tyrosine phosphorylation in the plasma membrane of cells that express LMP2A (510). The sites of tyrosine phosphorylation coincide with LMP2A, indicating that LMP2A is one of the few localized stable tyrosine phosphorylated proteins in BL cells or LCLs (510). LMP2A is stably associated with src family tyrosine kinases and with syk, unlike the sIg B-cell receptor complex, which markedly increases its association with these kinases when cross-linked by antigen (78,80,88,510). The first 167 amino acids of LMP2A, which include the amino-terminal cytoplasmic domain and the first two transmembrane domains, are able to associate with and be phosphorylated by these kinases (80,510). Among src family tyrosine kinases, LMP2A exhibits specificity for fyn and lyn in B lymphocytes

(80).

Despite the interaction of LMP2A with B-lymphocyte tyrosine kinases, LMP2A and -2B do not effect EBV transformation of primary B lymphocytes into LCLs or LCL survival. LMP2A and -2B have been extensively mutated in EBV recombinants (425,512,513 and 514,698,771). Except for one anomalous result (73), the specifically mutated recombinants transform primary B lymphocytes with wild-type efficiency. The resultant LCLs grow the same as wild-type EBV-transformed LCLs under standard culture conditions, in reduced serum, in soft agarose, and in mice with severe combined immunodeficiency disease (SCID) (425,512,513 and 514,698,771). Virus replication is also normal following treatment of latently infected lymphocytes with chemical inducers of lytic infection (425,512,513 and 514). The specific mutations include insertion of an amber nonsense linker after codon 19 of the LMP2A ORF, which results in ablation of expression of LMP2A and continued expression of LMP2B (512); insertion of an amber nonsense linker after DNA encoding the first five transmembrane domains, which results in ablation of expression in the last seven transmembrane domains and the carboxy-terminus of LMP2A and -2B (513); and deletion of DNA encoding the first five transmembrane domains of LMP2A and -2B, which results in truncation of LMP2A after the amino-terminal cytoplasmic domain and the addition of missense codons (514).

LMP2A expression in BL lymphoblasts constitutively blocks the signal transduction events that would follow the cross-linking of sIgM, CD19, or class II MHC on cells that do not express LMP2A (552,555). This blockade is similar to the desensitization that occurs after sIg signal transduction (78,88). LMP2A effects depend on the LMP2 transmembrane domains that mediate aggregation in the cell plasma membrane and on the LMP2A amino terminal cytoplasmic domain. The simplest model that would account for this activity is that LMP2 transmembrane domain–mediated aggregation of LMP2A results in phosphorylation of LMP2A, constitutive binding of src and syk, a constitutive low level of forward signaling, and turn-on of a down-regulatory kinase or phosphatase that globally blocks other src family–associated receptor tyrosine kinase–mediated signal transduction. Indeed, the basal tyrosine phosphorylation of many cellular proteins, including lyn, syk, and the p85 subunit of PI3 kinase, are higher in LCLs transformed by EBV recombinants that express wild-type LMP2A than in LCLs transformed by EBV recombinants that do not express LMP2A. Further, cross-linking of sIg on wild-type EBV–transformed LCLs results in abrogation of tyrosine kinase signalling at the first steps of src family and syk phosphorylation, whereas cross-linking of sIg on LMP2 null mutant recombinant–transformed LCLs results in normal sIg protein tyrosine kinase signal transduction, including transient phosphorylation of lyn, fyn, syk, p85, Vav, PLCgamma, SHC, and Grb2 (552). The amino terminus of LMP2A has a YXXL$(N)_7$YXXL sequence that in the B-cell receptor–associated molecules Ig alpha and Ig beta binds syk, and a YEEI sequence that binds lyn or other src family tyrosine kinases (88). When these LMP2A domains are fused to the CD8 surface and transmembrane domains and CD8 is cross-linked on cells that express the chimeric protein, the LMP2A domains transmit an increase in intracellular free calcium and are then refractory to further stimulation, as is characteristic of sIg cross-linking (49). The key roles for the LMP2 putative src family PTK binding site at Y112 and the putative syk binding site at the ITAM motif have been directly demonstrated by expression of the appropriate mutant proteins in BL cells and by examining sIg signal transduction in LCLs transformed by specifically mutated EBV recombinants (235,236 and 237). Both Y112 and the ITAM are stringently required for B-cell receptor desensitization.

The effect of LMP2A expression on LCLs is to block lytic infection that would occur in the absence of LMP2A following cross-linking of sIg on the surface of LCLs. Cross-linking of sIg on primary B lymphocytes transformed by LMP2 null mutant recombinants results in normal sIg-mediated signal transduction and activation of EBV replication (553,554). The LMP2A blockade to lytic activation can be bypassed by activating PKC and raising intracellular free calcium with phorbol ester (TPA) and calcium ionophore treatment. Treatment of wild-type or mutant transformed cells with TPA and calcium ionophore results in similar inductions of lytic infection (554). Because LMP2A is expressed in most latently infected B lymphocytes *in vivo*, one role may be to keep EBV infection latent in these cells when they encounter T lymphocytes or other potentially activating signals; a corollary of this hypothesis is that activation of lytic EBV infection in a latently infected lymphocyte could still be triggered by a cytokine released from an epithelial cell that might trigger a G protein–coupled receptor or JAK kinase signal transduction pathway (758).

A second role for LMP2 may be to provide survival and antidifferentiation signals to B cells that are not in type 3 latency. LMP2A expression in transgenic mice under control of IgH chain promoter and enhancer results sIg-negative B cells escaping from the bone marrow and colonizing peripheral lymphoid organs, bypassing normal B-lymphocyte developmental checkpoints (84). This indicates that LMP2A has sufficient constitutive forward signaling activity to affect the biology of normal B cells. The LMP2A effect is also evident in RAG 1(–/–) mice and is dependent on the LMP2A ITAM (83,547). Thus, LMP2A expression could have a role in latency type 2–infected lymphocytes in enhancing cell survival, in lymphoid organ persistence, or in blocking differentiation. As described in Chapter 75, LMP2A is also expressed in type 2 latency in Hodgkin's disease and nasopharyngeal carcinoma. Constitutive forward signalling effects of LMP2 could also be important in the pathogenesis of these malignancies. The effects of LMP2 are, however, subtle. No effect on epithelial differentiation or survival is evident with transgenic expression of LMP2A in normal mouse epithelium under control of the keratin 14 promoter (509). Effects on cell survival are evident with LMP2A expression in the human keratinocyte cell line, HaCaT. LMP2A-expressing cells are hyperproliferative in raft cultures, differentiation is inhibited, and cloning efficiency in soft agar is substantially increased (737). Cells that grew in soft agar formed aggressive, poorly differentiated tumors in nude mice. PI3 kinase and AKT were activated in the LMP2A-expressing cells and tumors. Thus, in the appropriate cell type, LMP2A can have survival-enhancing effects.

EBERs

The EBV-encoded, small, nonpolyadenylated RNAs are by far the most abundant EBV RNAs in latently infected cells. Estimates of abundance place the EBERs at 10^4–10^5 copies per cell (32,340,343). EBER-1 is transcribed by RNA polymerases II and III, whereas EBER-2 is solely transcribed by RNA polymerase III (340,341 and 342). The EBERs localize to the cell nucleus, where they are complexed with the cellular proteins La and EBER-associated protein (EAP) (475,832,833). The EBER RNAs have stable secondary structures that persist in RNA La protein complexes (256). La protein is associated with the 3' terminus of the EBER RNAs (256), whereas EAP is associated with the stem-loop 3 structure (832,833). Most of the EBER-1 is precipitable with antibody to EAP. EAP is L22 ribosomal and about half of the EAP in EBER-expressing cells is in EBER-1 ribonucleoprotein (RNP) particles in the nucleoplasm (831). The EBER

RNPs are predominantly in the nucleoplasm. L22 is associated with chromosomal translocation in leukemia, raising the possibility L22 association with EBERs could be of functional significance. EBER-1 and -2 have extensive primary sequence similarity to adenovirus VA1 and VA2 and cell U6 small RNAs, both of which also form similar secondary structures and complex with La protein (256).

EBER-1 or -2, adenovirus VA, and U6 cell RNAs may have similar primary sequences, secondary structures, and association with La protein in order to accomplish similar functions. Based on the known functions of VA and U6 RNAs, two alternative roles have been proposed for the EBERs. In adenovirus infection, VA1 RNA acts in the cytoplasm to directly inhibit activation of the interferon-induced protein kinase PKR, which would, in the absence of VAI RNA, phosphorylate protein synthesis initiation factor eIF-2 alpha and block translation (736). EBER-1 and -2, provided by transfection into cells or substituted in to the adenovirus genome, can partially complement the replication of adenovirus with null mutations in VA1 and -2 (53,54). EBER-1 can also inhibit PKR *in vitro*, at levels that are similar to VA1 RNA (116,117,118 and 119,747). Moreover, EBV-mediated growth transformation is sensitive to high levels of interferon, and the EBV Bam W latency 3 EBNA transcript can activate PKR, so a role for the EBERs in inhibiting PKR could be important (179). However, evidence that PKR is inhibited in latency 1, 2, or 3 infected cells has not been obtained.

Small nuclear RNAs have long been suspected to be involved in RNA splicing, partly because of base complementarity to splice sites. U6 RNA has been shown to be base-paired to U4 RNA in particles required for *in vitro* RNA splicing. Six of seven identical nucleotides to those through which U6 binds to U4 are in a single-strand loop in EBER-2 RNA (256).

EBV recombinants specifically deleted for the EBER genes have been compared with wild-type recombinants in primary B-lymphocyte infection (797). EBER-deleted virus was as able to initiate primary B-lymphocyte infection and growth transformation as wild-type virus. No difference could be found in the growth of LCLs infected with EBER-deleted or control virus or in the permissivity of these cells for lytic virus infection following induction.

To further evaluate the hypothesis that the EBERs could have an antiinterferon effect, the effect of the EBERs on the replication of an interferon-sensitive virus in EBV-transformed human B lymphocytes was determined (796). Replication of vesicular stomatitis virus (VSV) in human lymphocytes is sensitive to interferon. However, interferon had a similar inhibitory effect on VSV replication in LCLs infected with EBER-deleted or wild-type virus (796). Further, lytic wild-type EBV replication could not be inhibited by high levels of interferon, and replication in cells infected with EBER-deleted virus was not more sensitive to interferon. Moreover, EBER-deleted virus was not more sensitive to the antitransforming effects of interferon (796). Once established as LCLs, neither EBER-deleted nor wild-type EBV–infected LCLs were sensitive to potential antiproliferative effects of interferon. Thus, the role of the EBERs in EBV infection is not obvious from studies in B lymphocytes *in vitro* and may emerge from testing of the mutants in other cell types or in an *in vivo* model.

Recent data indicate that EBER expression may increase the tumorigenicity of BL cells or non-EBV-infected nasopharyngeal carcinoma cells, in which EBERs have been specifically expressed (440,441,818). Surprisingly, EBER expression in Akata BL cells resulted in increased Bcl-2 levels (440).

Other Viral Gene Products Expressed in Latent EBV Infection: BARF0, RPMS1, A73, and BHRF1

Complementary-strand Bam A rightward transcripts, also known as CSTs, BARTs, or BARF0 RNAs, are differentially spliced RNAs that were identified in the cloning of cDNAs from RNA of latently infected nasopharyngeal carcinoma cells grown in nude mice (98,251,334,399,716,766). CSTs have also been identified in latently infected BL cells (75,766), latently infected lymphocytes from human peripheral blood or marmoset tumors (97,922), and early primary infection of B cells *in vitro* (75) and LCLs (75,766). These RNAs initiate at n 150645 in the B95 sequence (36,716,766). Transfection of EBV DNA sequences corresponding to n 150199–151283 results in expression of the first exon in HeLa cells (766). Three ORFs in the differentially spliced CSTs may encode protein in latently infected cells, BARF0, RPMS1, and A73. BARF0 is potentially encoded in the most 3' ORF of the CSTs. Humans have antibody to the 173-amino-acid BARF0 polypeptide, consistent with its expression at some point in EBV infection (251). Two differentially spliced forms of BARF0 are described, BARF0 and the further spliced ORF, RK-BARF0 (234,421). Both are encoded by CST exon 7 (766). RK-BARF0 has a signal peptide sequence and interacts with the Notch4 ligand binding domain in yeast two-hybrid, coimmunoprecipitation, and confocal microscopy assays (452). RK-BARF0 expression can induce translocation of part of Notch4 to the nucleus and can turn on LMP1 expression in latency 1 infected BL cells.

RPMS1 is encoded in an ORF formed by the fourth exon and components of the fifth exon of some CSTs, includes a WWP motif similar to EBNA-2, interacts with RBP-Jk amino acids 310 to 500 in yeast two-hybrid assays, and blocks EBNA-2 transactivation of a promoter with a multimerized upstream RBP-Jk site (766). Expression of RPMS1 alone does not up- or down-regulate promoters. Thus, RK-BARF0 can potentially up-regulate promoters with upstream RBP-Jk sites, and RPMS1 would modulate that activity.

The third potential CST ORF, A73, is formed by components of exons 6 and 7 (766). A73 is expressed as a cytoplasmic protein that can interact with the cell RACK1 protein in yeast two-hybrid assays, and it can associate with a small fraction of RACK1 in 293 cells. A73 could potentially affect RACK1 modulation of PKC or src tyrosine kinase signaling.

Although the CSTs are likely to be important in latent EBV infections, infections of primary B lymphocytes with EBV recombinants that are deleted for the DNA that encodes the CSTs efficiently results in LCLs that can be readily expanded *in vitro* (412,691,694). Thus, at this first level of analysis, CSTs are not critical for EBV transformation of primary B lymphocytes *in vitro* or for LCL outgrowth or survival.

Some EBNA-type Bam W transcripts from various latently infected cells include BHRF1 at their 3' end (34,721). BHRF1 is expressed at high levels early in lytic infection, it is a bcl-2 homolog, and it has antiapoptotic effects (320,339,641). These antiapoptotic effects are likely to be quite important for efficient lytic infection in most cell types. Expression at even a low level in latent infection could be of significance. However, searches for the protein in latently infected cells using

polyclonal or monoclonal antibodies that readily detect the protein in lytically infected cells fail to detect BHRF1 in LCLs or type 3 latently infected BL cells (641). BHRF1 protein has been detected transiently after refeeding growth-arrested Raji cells but not in similarly treated LCLs (G. Pearson, personal communication).

Consistent with the failure to detect BHRF1 protein expression in latently infected LCLs and the abundant expression of antiapoptotic Bcl-2 family proteins in these cells (82,321,775), EBV recombinants with a nonsense mutation or deletion of the BHRF1 ORF are fully competent for the transformation of primary B lymphocytes into actively growing LCLs (471,530). Mutant recombinant virus replicated in LCLs could be recovered from these cells, and it transformed primary B lymphocytes with efficiencies similar to wild-type virus. BHRF1 is therefore not critical for outgrowth or survival of latency 3 infected LCLs or for EBV replication in such cells.

Role of Latency 3 Infection Proteins in LCL Outgrowth and Survival

EBV-transformed primary B lymphocytes are nearly indistinguishable from B lymphocytes proliferating in response to polyvalent antigen and T-cell help or IL4, IL10, and CD40 in their growth and display of molecular markers of adhesion, activation, and differentiation (23,24,42,85,263,337,363,585,635,661,688,824,825,829,913). The growth and survival of LCLs is highly dependent on autocrine and paracrine signalling mediated by IL5, IL6, IL10, TNF-beta, and transforming growth factor (TGF) (19,48,79,165,168,183,186,197,263,330,345,436,445,544,584,599,638,639,705,728,729,778,812,829,845,846,859). The major characteristics that distinguish LCLs from B lymphocytes proliferating in response to polyvalent antigen and T-cell help are the expression of EBV latency 3–associated genes, immortality, and autonomy. For heuristic purposes, two hypotheses are considered here and in Chapter 75. First, the unique set of genes that LCVs use to cause LCL outgrowth evolved to enable these viruses to rapidly expand the number of latently infected B lymphocytes in the course of primary infection and to periodically reexpand that pool to further the persistence of virus infection *in vivo*. Second, the principal constraint in evolution of these genes is that they use normal B-cell growth pathways and leave the cell susceptible to checkpoint controls, external regulatory signals, and immune surveillance, so as to minimally damage the host. LCVs encode EBNAs to constitutively activate and regulate transcription from Notch- and PU.1-responsive promoters in cell DNA, including the c-*myc*, c-*fgr*, CD21, and CD23 promoters. EBV regulation of c-*myc* is significantly more akin to c-*myc* regulation by B-cell growth stimulatory processes. Constitutive c-*myc* expression in an LCL radically alters the cell phenotype (135,508,657). Constitutive c-*myc* expression in LCLs negatively regulates endogenous c-*myc*, alters adhesion and activation molecule expression so that the cells have the characteristics of a BL cell, increases susceptibility to NK cell cytotoxicity, and increases tumorigenesis in nude mice.

EBV has evolved EBNA-2 responsive cis-acting transcriptional regulatory sequences that bind RBP-Jk and PU.1 to autoregulate EBNA and LMP transcription from the viral genome. LMP1 constitutively activates the TNFR/CD40 downstream signalling pathways to co-stimulate cell growth and survival through NF-kB, junk, and p38 map kinase activation. These two pathways are depicted in Figure 10. *In vivo*, EBV regulation of LCL growth can be overridden by the microenvironment of infected cells: simply resuspension of the dispersed cells in dilute culture medium stops growth for weeks until the medium is adequately conditioned. Some of the mechanism by which the normal cell and the EBV-transformed cell regulate progress through the cell cycle, maintain telomeres through multiple rounds of expansion, and escape programmed cell death are unclear and present substantial future challenges in this area of EBV investigation. A substantial body of genetic and biochemical data described in this chapter and in the references indicate that the EBV latency-associated genes do not inactivate normal cell checkpoint controls such as Rb or p53 (18,89,366). EBNAs and LMPs do have “transforming effects.” EBNA-2, expressed under control of the SV40 promoter and enhancer in transgenic mice, caused islands of kidney tubular hyperplasia at 20 weeks in all animals (843). At 50 weeks, multiple foci of microscopic renal tubular adenocarcinomas were evident; eventually, 90% of the transgenic mice has renal tubular adenocarcinomas. *In vitro*, EBNA-2 lowers the serum dependence of Rat-1 cells (148). EBNA-2 also exerts strong activating effects on genes that are regulated by Notch: Constitutive Notch activation in T cells induces leukemia (185,640). Moreover, EBNA-2 is involved in up-regulation of c-*myc* in LCLs (394). However, EBNA-2 has not been reported to have SV40 virus–like T antigen, or adenovirus-like E1A effects in collaborating with activated Ras in primary rodent cell immortalization. As described earlier, LMP1 has multiple activated Ras–like effects on immortalized rodent fibroblast cell lines, and transgenic LMP1 expression in mice results in increased incidence of lymphoma. LMP1 is not reported to substitute for activated Ras in complementing T or E1A in primary rodent fibroblast transformation. EBNA-1 has had a c-*myc*-like effect in inducing lymphomas in transgenic mice (884). However, the one lineage with this robust effect on lymphoma incidence did not express EBNA-1 in the transgenic normal lymphocytes. Only one study ascribed an E1A-like effect to one of the EBNAs in primary rat fibroblast immortalization experiments (633). The potential basis for the latter effect has not as yet emerged from intensive investigation of EBNA-3C.

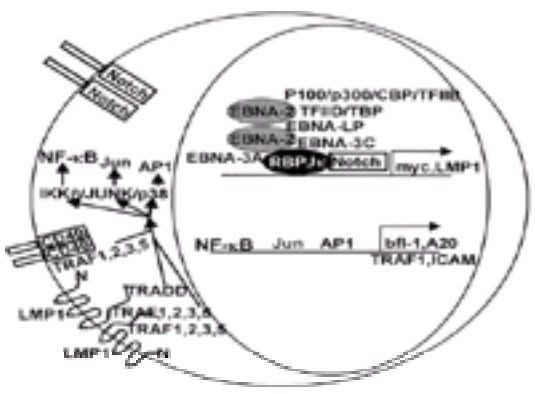

FIG. 10. Schematic diagram of the biochemical mechanisms by which EBNA-2, -LP, -3A, and -3C and LMP1 collaborate to induce primary B-lymphocyte growth transformation and survival. EBNA-2, -LP, -3A, and -3C use the nuclear anchor for Notch signaling and the B-cell Ets family protein PU.1 to constitutively up-regulate cellular and viral promoters including the c-*myc* promoter, the LMP1 promoter, and the EBNA promoter. LMP1 constitutively activates TNFR-associated factor signalling, inducing NF-kB, Jun/Fos, and AP-1.

Persistence of EBV DNA in Latently Infected Cells

The circularization of linear input EBV DNA in the nuclei of acutely infected lymphocytes requires 12 to 24 hours (12,362). Cells do not enter S phase until all of the EBNAs and LMPs are expressed. Stable latently infected proliferating lymphocytes characteristically contain multiple copies of EBV episomes (3,400,496,616,786,809). Most LCLs have about 10 episomes per cell. The range of episome copy number in BL cells is greater. Raji cells have 50 episomes per cell and the number has been stable over 30 years in culture (2,26). EBV episomes are replicated early in S phase by cell DNA polymerase (2,283,293). The initial amplification of circular EBV DNA is at the start of latent infection and requires early S phase DNA synthesis, as prior to S phase each infected cell has only one episome (12,786).

Although EBV DNA usually persists in cells as an episome, the EBV genome can also integrate into chromosomal DNA, and both integrated and episomal DNA coexist in some cells (159,282,318,361,393,467,543,889). In one EBV-infected human Burkitt tumor cell line, EBV DNA has persisted by integrating on chromosome one at IP35 (318,467). The entire EBV genome is integrated into A-T-rich cell DNA through the EBV TR (543). More than 15 kb of cell DNA were deleted by the integration of the 170-kb complete EBV genome. The entire block of cell DNA with the integrated EBV genome has then been duplicated as an inverted DNA domain (467). Another example is a latently infected fetal human LCL that contains an EBV genome integrated at chromosome 4q25 (318,543). In this instance, integration appears to be through a site within U1 (315,318), and there are several EBV genomes integrated in tandem (362). In the first instance, integration through TR favors the hypothesis that integration occurred prior to circularization; however, in the second instance, integration through U1 and the finding of tandem repeats of the EBV genome are more consistent with the hypothesis that integration occurred after initial circularization. Integration occurs frequently when BL cells are infected with EBV *in vitro* and extensively passaged (361). In contrast, infection of BL cells with EBV and selection for cells infected with virus almost always results in cell lines that contain episomal EBV DNA (527,528 and 529,869). The genome persists as an episome in such cell lines for at least 1 year. Clearly, integration is not a regular feature of EBV infection or EBV induced cell growth transformation. However, because LMP2 is the only EBV gene that spans the TR, and LMP2 is not required for cell transformation, EBV may be able to transform cells with the genome persisting solely as integrated DNA.

Episomal DNA is likely to be necessary for lytic cycle EBV DNA replication, because lytic herpesvirus DNA replication characteristically proceeds from a circular DNA intermediate (for review, see Chapter 72). Circular EBV DNA copy number increases in lytic infection (747a). Lytic EBV replication has not been observed in cells that contain only integrated EBV DNA, and virus has not been recovered from such cells in response to known inducers of virus replication.

In the establishment of latent infection, the EBV genome undergoes progressive methylation (203,347,374,429,458,459,478,540,577,615,650,695,718,801,813,814) and associates with chromosomal proteins so as to give a nucleosomal pattern after micrococcal nuclease digestion (176,654,744,747b). Regulatory domains involved in maintaining latent infection such as oriP tend to be undermethylated in latently infected cells relative to DNA that is expressed only in lytic infection (196,203,205,347,374,546,575,576,615,695). Extensive methylation of lytic genes and their regulatory elements probably helps to maintain latency by inhibiting lytic gene expression. Treatment of latently infected cells with drugs that reduce DNA methylation increases the frequency of spontaneous activation to lytic infection (51,540,696).

Induction of Lytic Infection

Lytic EBV infection is usually studied by inducing latently infected BL cells to become permissive for lytic virus replication (51,350,516,680,717,933). Various induction strategies give characteristic, although not precisely reproducible, levels of permissivity for each latently infected B cell line. Autocrine TGF-beta may participate in lytic reactivation, as antibody to TGF-beta reduces the responsiveness of cells to lytic induction (168). Phorbol esters are among the most reproducible and most broadly applicable inducers. The effect is probably mediated by PKC activation of *jun-fos* interaction with AP-1 sites upstream of the immediate-early virus genes (28,139,210,211,218,219,221,222,223 and 224,464,473,486,817). The rapidity of activation of immediate-early EBV genes by phorbol ester is most consistent with the activation of a preexisting transcriptional coactivator of lytic cycle gene expression, or with inactivation of an active repressor of lytic infection. PKC does activate BZLF1 itself, and the effect of PKC could be to increase background activity of the BZLF1 promoter through autostimulation (47). Among latently infected LCLs, marmoset LCLs tend to be more inducible than adult human lymphoblasts, and neonatal human lymphoblasts are least inducible for virus replication (558,561,562,566,567, and for review see ref. 556). A few cell lines can be consistently induced to permit lytic virus replication in 10% of the cells (333,567,802,803). One cell line, Akata, can be induced to 20% to 50% lytic infection by cross-linking sIg; it is currently the best source for large quantities of EBV (803). SIg cross-linking activates phospholipase-C-gamma. Phospholipase-C-gamma releases IP3 and diacylglycerol, which mobilize calcium from intracellular stores and activate PKC. Treatment of cells with calcium ionophore and phorbol ester induces a rise in intracellular free calcium and activates PKC but is only a partial surrogate for sIg cross-linking, compatible with the possibility that other pathways activated by sIg cross-linking may have a role in the induction of lytic infection in Akata cells (95,136,137,139,257,505,507,748,772). Several elements upstream of the BZLF1 promoter seem to mediate the sIg response: Sp1, Sp3, myocyte enhancer factor 2D, ATF-1, and ATF-2 (505). The ATF-1 and ATF-2 binding sites correspond to a site that is regulated in part by CAM kinase IV (95). The initial activation of Z may be dependent on two 7-bp dyad symmetry elements separated by 27 bp. These sequences are similar to a reversible YY1-dependent silencing element in adeno-associated virus and may contribute to the lack of Z expression during latency (583). Other negative regulatory elements are more upstream from the transcriptional initiation site (739). Changes in the BZLF1 promoter histone acetylation appear to be critical for BZLF1 activation (380).

The responsiveness of Akata cells to sIg cross-linking is due in part to stable latency 1 infection. The absence of LMP2A enables sIg cross-linking induced signal transduction and induction of lytic EBV infection. In latency 3–infected lymphocytes where LMP2A is expressed, calcium ionophore and TPA can be used to induce virus replication (554). An

alternative strategy is to use cells infected with EBV recombinants with specific null mutations in the LMP2A gene (551,554).

A second approach to studying virus replication is to induce lytic replication by superinfection of Raji cells with the P3HR-1 EBV (55,597,670). Raji is an EBV-infected Burkitt tumor–derived cell line that has an unusually high EBV episome copy number and is responsive to this type of induction. The endogenous Raji genome has at least two deletions and is defective for EBV DNA replication and late gene expression (301,656). Thus, EBV late genes are expressed in Raji cells only after superinfection. The P3HR-1 virus is efficient in inducing lytic infection in Raji because of defective virus genomes in most noncloned P3HR-1 cell cultures (129,311,559,564,701). An abundant class of defective virions from noncloned P3HR-1 cells has rearranged EBV DNA molecules in which the Z or R and Z immediate-early transactivator(s) are downstream of the latent infection cycle IR1 EBNA gene promoter and are, therefore, expressed in latently infected cell lines following superinfection (110,311,381,382 and 383,564,677,678). The induction of productive infection following high multiplicity superinfection is more rapid and synchronous than following chemical induction. Maximal late virus protein expression and enveloped virus release requires 48 to 96 hours.

Following induction, cells that have become permissive for virus replication undergo cytopathic changes characteristic of herpesviruses, including margination of nuclear chromatin (406), inhibition of host macromolecular synthesis, replication of viral DNA at the center of the nucleus, assembly of nucleocapsids at the nuclear periphery, nucleation of nucleocapsids, and envelopment of virus by budding through the inner nuclear membrane (158,247,261,262,631,727). Virus gene expression follows a temporal and sequential order (for review, see refs. 209 and 803). Some virus mRNAs are expressed early after induction despite the inhibition of new protein synthesis. These RNAs are classified as immediate-early (630). Most early lytic virus genes that are expressed before the onset of virus DNA replication are dependent on the immediate-early BZLF1 or BRLF1 protein transcription factors for their expression. Genes are formally classified as early if their expression is not affected by inhibition of viral DNA synthesis. Late proteins are expressed temporally later but are formally categorized as late based on the marked reduction in their mRNA abundance in the presence of viral DNA synthesis inhibitors.

Immediate-Early Genes and Their Targets

In typical herpesvirus infection, immediate-early genes are defined by their transcription following infection in the presence of inhibitors of protein synthesis (for review, see Chapter 72). Characteristically, a virion tegument protein contributes to the activation of immediate-early gene expression. *In vivo*, EBV replicates in epithelial cells of the oropharynx before infecting B lymphocytes. Infection of epithelial cells lines *in vitro* is not efficient, and studies of the biochemical events of synchronous lytic infection in permissive epithelial cell cultures has not been feasible. Only B lymphocytes can be efficiently infected by EBV, and the outcome of infection is nonpermissivity for virus replication and the expression of the EBNA-2 and EBNA-LP mRNAs. EBNA-2 and -LP are likely to be immediate-early gene products in primary B lymphocytes, given the constitutive activity of the Wp EBNA promoter and the indications that EBNA-LP and -2 are the first EBV mRNAs expressed in these cells, but independence of transcription from new protein synthesis has not been demonstrated. Similarly, the constitutive activity of the Bam Q EBNA-1 promoter in BL cells (718) and the early expression of EBNA-1 mRNA in infected BL cells may be in the mode of an immediate-early gene.

Because synchronous lytic EBV infection *in vitro* can be studied only by induction of permissivity in cells that are already latently infected, the definition of immediate-early lytic infection–associated gene expression must be modified to acknowledge the potential direct or indirect effects of EBV latent infection genes on transcription from the activated EBV genome or the potential effect of the induction strategy on the transcription of specific EBV genes. Moreover, chemical induction may fail to activate an immediate-early gene whose transcription in lymphocytes or primary epithelial cells depends on a virion trans-acting factor. Thus, our current definition of BZLF1 and BRLF1 as the principal immediate-early viral genes may be incomplete.

P3HR-1 superinfection of Raji cells and inhibition of protein synthesis with anisomycin results in accumulation of 1-kb BZLF1, 2.8-kb BRLF1, and 4.1-kb BI'LF4 mRNAs (55). Induction of lytic infection in Akata cells by sIg cross-linking and incubation of the cells in the presence of moderately high concentrations of cycloheximide (50 µg/mL) results in relatively abundant expression of the 1-kb BZLF1 and 2.8-kb BRLF1 mRNAs, confirming that these are immediate-early gene products (803). Cycloheximide resistant 1.6- and 4.1-kb mRNAs from Bam M and A, respectively, are also expressed in induced Akata cells, but these mRNAs are much less abundant in cycloheximide-treated cells than in nontreated cells. In the absence of cycloheximide, the Bam M and A mRNAs are most abundant from 4 to 12 hours after induction, whereas the BZLF1 and BRLF1 mRNAs are expressed earlier. Thus, the M and A mRNAs appear to be early but not immediate-early genes. The Bam A mRNA is encoded by BALF2, which is the EBV homolog of HSV-1 ICP8. ICP8 is also expressed very early in HSV infection and is less sensitive to viral protein synthesis inhibition than other early virus genes. Another immediate-early gene is encoded from the BI'LF4 ORF (533).

The first evidence that BZLF1- and BRLF1-encoded proteins are key transactivators of lytic EBV gene expression (Fig. 11) came from the discovery that highly defective P3HR-1 virus preparations that induce lytic infection in EBV-infected BL cells express BZLF1, or BZLF1 and BRLF1, under control of the Wp promoter (381,382 and 383,557,564). BZLF1 expression from heterologous vectors broadly activates lytic infection (272,381,382 and 383). BZLF1 and BRLF1 coordinately up-regulate expression from early EBV promoters (96,105,106). BI'LF4 also has transactivating effects, although these effects are restricted to specific types (533). Consistent with these *in vitro* observations, BZLF1 is expressed in advancing margins of lytic EBV infection in oropharyngeal epithelia (919).

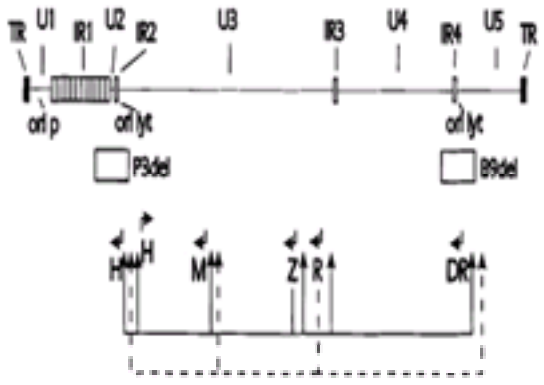

FIG. 11. Immediate-early lytic EBV infection. In latently infected B lymphocytes, the promoters for BZLF1 and BRLF1 can be activated by physiologic changes, by pharmacologic treatment, or by heterologous Z overexpression. In newly infected epithelial cells, the BZLF1 and BRLF1 promoters are presumed to be activated by virion-associated transactivators. BZLF1 and BRLF1 are transcribed, translated, and localized to the nucleus, where they coordinately further up-regulate their promoters and turn early genes, including BHLF1, BHRF1, D_R, BSMLF1, and BALF2 (not shown).

Two key early promoter regulatory elements within D_L and D_R (duplication, left and right) are coordinately up-regulated by the BZLF1- and BRLF1-encoded proteins. D_L and D_R encode abundant early mRNAs (149,232,351,357,620,621) and include the origins for lytic viral DNA replication within their promoter upstream regulatory domains (291). BZLF1 and BRLF1 synergistically up-regulate the bidirectional BHLF1 and BHRF1 D_L promoters in transient transfection assays (96,105,106,131). Promoter activation has a strong positive effect on DNA synthesis from the neighboring D_L origin. The cis-acting domains of D_R and D_L have been partially dissected and have many similarities including multiple BZLF1 binding sites (96,105,106,297,486,735). The promoter proximal domain (–79 to –221 bp relative to BHLF1) confers strong promoter activity in HeLa or Vero cells only when BZLF1 is provided in trans (736). ZBP-89 and Sp1 bind synergistically to a site near the promoter, stimulate transcription, and interact with the viral DNA polymerase and processivity factor (46). PKC phosphorylation of BZLF1 S186 increases BZLF1 transactivation and DNA replication effects (47). A DNA segment 639 bp upstream of the BHLF1 cap site is a position- and orientation-independent enhancer composed of one component with constitutive activity in fibroblasts or epithelial lines but not in B lymphocytes, and a second component, TTGTCCCGTGGACAATGTCC, which is responsive to the BRLF1 transactivator. Although BZLF1 interaction with this site has negligible effects on transcription from the BHRF1 promoter, BRLF1 activates transcription and BZLF1 strongly up-regulates transcription with BRLF1 (131). Regulation of the D_R element appears to be different, in that BZLF1 does not strongly up-regulate the BZRF1 effects on D_R (279).

The BSMLF1 and BMRF1 promoters are also coordinately regulated by BZLF1 and BRLF1 (336,672). Direct BRLF1 binding to the BMRF1 promoter upstream DNA is essential for BMRF1 coordinate up-regulation by the BZLF1 and BRLF1 transactivators (672). BRLF1 transactivation of the BSMLF1 early promoter is also mediated by activation of an upstream sequence CCGTGGAGAATGTC similar to the activation sequence in BHLF1 (277,413). *In vitro* translated R binds directly to this R response elements by interacting with two sequences catGTCCCtctatcatGGCGCagac within the element that are close to an optimal R binding site (277,278,280). The BZLF1 protein interacts with a TPA response element upstream of the BSMLF1 promoter (506).

The BZLF1 protein interacts with NF-kB, p53, and virion proteins (285,338,925). NF-kB interaction inhibits the transactivating effects of BZLF1. Similarly, the BZLF1 coiled-coil dimerization domain interacts with the carboxyl-terminal portion of p53, the two proteins associate *in vivo*, and both proteins are transcriptionally inactivated by the association. BZLF1-mediated inhibition of p53 could be important in preventing p53-induced apoptosis in response to lytic EBV DNA replication. Later in lytic infection, BZLF1 becomes associated with virion structural proteins; BZLF1 has not been detected in virus (404). The interaction of BZLF1 with NF-kB, p53, and virion structural proteins could down-modulate BZLF1 activity late in infection so as to minimize the toxic effects of high-level BZLF1 expression.

A minor immediate-early RNA encodes an unusual spliced product of the BRLF1 and BZLF1 bi-cistronic transcript that could also down-modulate BZLF1 activity (239,742). This RNA has the beginning of the BRLF1 ORF spliced in-frame to the DNA binding and dimerization domains of BZLF1. Because the BRLF1 activation domain is at the C-terminus and the BZLF1 activation domain is at the N-terminus, the spliced product lacks an activation domain and when dimerized with Z, it represses Z activity (239).

BZLF1 can down-regulate the EBNA Cp promoter, perhaps facilitating the transition from latent to lytic infection (415,754). The action of BZLF1 on Cp appears to be indirect, involving the up-regulation of *fos* by BZLF1 and the down-modulation of the positive effect of a glucocorticoid response element, which maps about 1 kbp upstream of Cp (450,754,757). Glucocorticoid withdrawal from culture media increases permissivity for replication, indicative of a mutual antagonism between Cp function and lytic reactivation. The Cp promoter may antagonize lytic infection, in part because Cp transcription is antisense to BZLF1 and BRLF2 transcription.

BZLF1 probably autoregulates its own promoter and the BRLF1 promoter (218). The Z promoter has several AP1-like upstream elements that mediate activation in the presence of low levels of Z and repression in the presence of high levels of Z. Differential Z binding to the various upstream sites could be mediated by changes in Z abundance during lytic infection.

BZLF1 mRNA is composed from three exons, each encoding a separate functional domain (215,485). The first exon encodes amino acids 1 to 167, which constitute a transactivating domain when fused to a Gal-4 DNA binding domain

(107,218,483,560). The activating domain can recruit TAFs and stabilize TFIID association with TATA elements through protein–protein interactions (483,484,488). Replacement of the BZLF1-activating domain with the VP16 acidic domain results in enhanced transactivation of early promoters (48). The second exon encodes amino acids 168 to 202, a sequence that includes a strongly basic domain with homology to a conserved region of the c-*fos* and c-*jun* family of transcriptional modulators (211). This domain confers the ability to interact with AP1-related sites in DNA (94,220,482,483,485,486) and also targets Z to the nucleus (550). A BZLF1 intron also has homology to the 3' untranslated c-*fos* sequences, suggesting that BZLF1 relatively recently evolved from c-*fos* (211). The third exon encodes for amino acids 203 to 245, a sequence that includes a near-perfect leucine and isoleucine heptad repeat capable of coiled-coil dimer formation (94,211,219,220,443,483). Like c-*fos*, BZLF1 is a dimerizing DNA binding protein that binds directly to AP1-related sequences (211,220,222,223 and 224,483,485,486). Dimerization may facilitate high affinity interaction with templates having two or more Z binding sites (92).

BZLF1 is multiply phosphorylated by casein kinase II, PKA, and PKC on serines 173, 186, and 336 (47,136,439). The phosphorylation regulates DNA binding activity and transcriptional activating effects. Phosphorylation of S186 in response to PKC activation stimulates transcription but decreases affinity for cognate DNA (47).

The R transactivator is also a DNA sequence-specific acidic transactivator and has distant homology to c-*myb* (280,521,522). C-*myb* can interact synergistically with Z in transactivating the BMRF1 promoter in lymphocytes (414). Two domains of R when fused to the Gal-4 DNA binding domain have transactivating activity for Gal-4 response elements upstream of basal promoters (297,298). Amino acids 416 to 519 are weakly activating and are required only for activation in B lymphocytes, whereas the carboxyl-terminal amino acids 520 to 605 are a potent acidic transactivating domain similar to VP16.

Early Lytic Infection

EBV early replicative cycle genes are operationally differentiated from late genes by their persistent transcription in the presence of inhibitors of viral DNA synthesis. By this criterion, at least 30 EBV mRNAs are early mRNAs, and almost 30 EBV mRNAs are late mRNAs (see Fig. 1) (36,43,55,56,209,210,249,250,301,352,353 and 354,358). Early and late mRNAs are intermingled through most of the EBV genome. Frequently, different promoters initiate nested transcripts, which begin with different ORFs and terminate at the same polyadenylation site, so that the longer mRNAs include all of the shorter mRNAs; the primary reading frames of the shorter mRNAs are 3' to the primary reading frame in the longer mRNAs and are likely to remain untranslated or inefficiently translated in this context. Some early and late genes are spliced, and others are not. Minor spliced mRNA species have frequently been discerned when genes have been studied in detail.

Because of the difficulty of doing genetic studies of EBV, proteins encoded by EBV early or late genes have usually been identified and assigned functions from predictions based on the ORF sequence, from the temporal class and abundance of the RNA or the size of its *in vitro* translation products, or from functional assays of *in vitro* translated or *in vivo* expressed proteins. The function of many EBV genes was first suggested from comparison of predicted primary amino acid sequences with those of other herpesvirus proteins of known function. Subsequent studies have generally confirmed these tentative functional assignments, and they frequently revealed unique as well as common attributes of the EBV proteins.

Aside from the two immediate-early proteins BZLF1 and BRLF1, the EBV early proteins BSMLF1 and BMRF1 may also transactivate expression of other early EBV genes. Both are moderately abundant early nuclear proteins (113,114). BMRF1 activity has been demonstrated only by co-transfection with SV40 promoter driven by chloramphenicol acetyl transferase reporter gene DNA into BHK cells (624). BSMLF1 is a promiscuous transactivator of gene expression that acts synergistically with BZLF1 or BRLF1 in inducing higher-level expression in transient expression assays (413,415,416,487,624,890). The level of CAT reporter gene mRNA does not increase in cells co-transfected with BSMLF1-expressing plasmids (417). BSMLF1 does not effect increased transcription in nuclear on assays (72,713). Instead, BSMLF1 increases cytoplasmic transport of unspliced mRNAs, and this accounts for the promiscuity of its effects in transactivation (72,713). Both cytoplasmic transport and promiscuous transactivation depend on BSMLF1 association with CRM1.

Two other EBV early proteins are quite abundant. One encodes the 135-kd BALF2 protein (357). This protein has primary amino acid sequence homology to the major HSV DNA binding protein ICP8, and it is important in DNA replication (217). A second RNA that is less abundant encodes BHRF1 (34,641). BHRF1 encodes an 18-kd protein that consists of a predicted hydrophobic signal peptide, a 135-amino-acid hydrophilic domain that contains two potential N-linked glycosylation sites, a hydrophobic potential transmembrane domain, and a short carboxyl-terminal cytoplasmic domain. BHRF1 has extensive colinear homology with *bcl-2*, a cell gene activated by the chromosome 14/18 translocation in human follicular B-cell lymphomas (120). Immunofluorescent microscopy with specific sera reveals nuclear membrane and cytoplasmic localization compatible with endoplasmic reticulum or mitochondrial residence (641). *Bcl-2* is important in lymphocyte survival because of its role in stabilizing mitochondrial membrane potential and preventing apoptotic death, and BHRF1 appears to have similar effects in preventing apoptotic cell death in lytic EBV infection (320). The effects of BHRF1 are evident with lytic replication of EBV in type 1 latency–infected BL cells, but they are not evident when lytic commences in type III latency–infected LCLs because of their high-level expression of other antiapoptotic proteins (82). EBV recombinants with a stop codon early in BHRF1, or with deletions of the BHRF1 ORF, are fully able to initiate or maintain cell growth transformation, enter lytic cycle, and produce virus even in media with low serum or low cell concentration (471,530). BHRF1 is expected to have a similar role in epithelial infection. EBV BALF1 also has significant homology to *bcl-2* and is likely to have similar antiapoptotic effects (535).

Among the EBV early genes identified by their homology to early genes of other herpesviruses are several that are linked to DNA replication. These include DNA polymerase (BALF5), major DNA binding protein (BALF2), ribonucleotide reductase (BORF2 and BARF1), thymidine kinase (BXLF1), and alkaline exonuclease (BGLF5). These genes are

distributed through the long EBV U_L domain (see Fig. 1).

The EBV DNA polymerase has been extensively purified and is 117 kd (396). Partially purified DNA polymerase is associated with several other EBV nuclear proteins, including the 50-kd protein encoded by BMRF1 (420,481). The catalytic subunit expressed in insect cells from a baculovirus vector has 3'-to-5' proofreading exonuclease activity in addition to DNA polymerase activity (851). Reconstitution of baculovirus-expressed polymerase with a 2:1 molar ratio of baculovirus-expressed BMRF1 enhances the double-strand 3'-to-5' exonuclease activity fourfold (849) and the DNA polymerase activity tenfold (848). BMRF1 also markedly enhances polymerase processivity and results in full-length products (848). Unlike cellular polymerases, the EBV polymerase is active in 100mM ammonium sulfate or in KCl and is sensitive to inhibition by ethyl maleimide, phosphonoacetic acid, phosphonoformic acid, arabinofuranosyl-thymine, and acycloguanosine triphosphate (14,102,108,121,143,144,145,146,147,148 and 149,151,152 and 153,275,344,345 and 346,348,625,626 and 627,786). The EBV DNA polymerase also differs from HSV DNA polymerase in its salt sensitivity and its relative resistance to amphidicolin. The EBV DNA polymerase only weakly distinguishes between 2-amino-purine nucleoside trip-hosphate and dATP, so that 2-amino-purine is preferentially incorporated into viral as opposed to cell DNA (273).

The EBV, VZV, and HSV ribonucleotide reductases are extensively colinearly homologous. The large HSV subunit is 140 kd, whereas the EBV subunit is 85 kd, and VZV is predicted to be 87 kd (250). The EBV, VZV, and HSV large and small subunits have domains that are common to ribonucleotide reductases from a wide variety of prokaryotic and eukaryotic species (90,606). The HSV-1 large subunit has an amino-terminal domain that is absent from other ribonucleotide reductases. Surprisingly, the EBV ribonucleotide reductase large subunit is confined to multiple discrete regions in the cytoplasm of productively infected cells (258). The large subunit is a delayed early protein that accumulates in cells approximately 4 hours after the MRF1 nuclear early protein (258,641). Acetone fixation of cells destroys the immunologic reactivity of the large subunit, suggesting that it may be a major component of the restricted early antigen complex originally identified by EBV immune human sera from African patients with BL (see Chapter 75).

The existence of an EBV-specific deoxypyrimidine kinase was inferred from the co-induction of EBV lytic replication and a new kinase activity in P3HR-1 cells (157,777). An EBV-induced dTK and dCK activity elutes as a single peak on DEAE-cellulose, away from the cellular dTK and dCK activities. Gene transfer of EBV DNA, or of the EBV DNA fragment with homology to HSV TK, into TK-mammalian cells or into TK-*E. coli* restores TK activity mapping the kinase activity to this DNA fragment (502,503 and 504). The EBV TK is similar to HSV TK in accepting a broad range of nucleosides or nucleoside analogs as substrates and has some antigenic cross-reactivity with the HSV TK (503).

The D_L and D_R EBV DNA segments (see Fig. 1) that have strong early promoter activity are also origins of lytic infection viral DNA replication (290,730,734). Using transient transfection to activate lytic EBV replication co-transfection of either of these EBV DNA segments on plasmids results in replication of the plasmid DNAs in the lytically infected cells. An essential 1.4-kb core element replicates with considerably reduced efficiency (290). The core 1.4-kb origin has numerous inverted repeat elements that could serve as the definitive origin. Even within the 1.4-kb segment, at least three domains contribute to the replicative activity. One domain can be functionally replaced in part by the CMV immediate-early promoter and enhancer, suggesting that this domain is important for lytic origin function because of transcriptional enhancement (163). Interestingly, when cells containing lytic origin plasmids are induced to lytic EBV infection, plasmid copy number increases slightly and linear concatemers are produced. With induction of lytic EBV replication, episome copy number increases, and labeled thymidine, initially incorporated into circular EBV DNA, chases into very large, presumably linear concatemeric EBV DNA (115,757,762). Other LCVs have D_L and D_R elements; these are anticipated to have similar roles in DNA replication (313,314,316,474). The herpesvirus Papio element has transcriptional activation and DNA replication sites that are similar to those in EBV (714).

Considerable progress has been made in constituting an *in vitro* minimal replication system similar to the Challberg HSV system (217). The components include BALF5, which encodes the core DNA polymerase; BALF2, the single-strand DNA binding protein; BMRF1, the processivity factor; BSLF1 and BBLF4, the primase and helicase complex; BBLF2/3, a spliced primase helicase complex component; and BKRF3, the uracil DNA glycosylase. A surprising aspect of the EBV DNA replication genes is the extent to which they are dependent on expression of the BZLF1, BRLF1, and BSMLF1 transactivators. EBV DNA replication also requires topoisomerase 1 and 2 activity (407). The redistribution of BZLF1 and BMRF1 gene products to the same nuclear site may identify the site at which viral DNA is replicated (806).

Late Gene Expression, Cleavage, Packaging, Envelopment, and Egress

The EBV TR has obvious similarities to the HSV “a” sequence (543,580,767,769,770). Both EBV TR and the HSV “a” sequence are directly repeated at both ends of the DNA. Both sequences are high in G+C composition, with runs of three to five Cs. In both HSV and EBV, there is a short extra oligonucleotide segment (D_R1) where the repeat joins the unique DNA. The EBV D_R1 oligonucleotide GCATGGGGGG brackets the multiple TR copies (543,580). In HSV-1, D_R1 is the site of cleavage of head full-length DNA for packaging into virions (767,769,770). There seems to be little sequence specificity for D_R1, because the nonhomologous oligonucleotides of HSV-1, HSV-2, and CMV are interchangeable (770). HSV or CMV cleavage and packaging appear to occur 33 bases 3' to a conserved sequence of $C(G)_{6–8}TGT(G/T)(T)_3NG(C/G)(G)_6(G/C)(T/C)$. A similar sequence $C(G)_5TGT(T)_2CCT(G)_5CC$ occurs 25 bases before the EBV D_R1 and is the key cleavage packaging recognition sequence for EBV. The EBV rightmost TR acquires a short unique sequence from the leftmost TR during cleavage and packaging, and that sequence becomes the left end of the DNA (932). Plasmids containing the EBV latent infection episome origin, the EBV lytic origin, and the EBV terminal repeat sequence are stably maintained as an episome in latently infected cells, are replicated into linear concatemers by EBV DNA polymerase when lytic infection is induced, and are cleaved and packaged into infectious virions (291,871). Cell lines have recently been derived that are infected with a TR-negative EBV that cannot itself be packaged, but they are suitable hosts to support the replication and packaging of plasmids that include oriLyt and EBV DNA termini.

The viral glycoprotein genes that have been identified so far are all late genes

(50,208,262,310,359,451,454,455,480,517,610,622,643,644,905). These have been more intensively studied than other late genes because of their potential importance in antibody-mediated immunity to virus infection (70,289,331,472,479,480,568,581,631,776,875,876). Current knowledge of nonglycoprotein late genes is quite limited. Among the nonglycoprotein late genes, the major nucleocapsid protein is almost certainly encoded by BcLF1 based on homology to the major HSV capsid-protein gene (545). Further, BcLF1 encodes a 4.5-kb late mRNA that, translated *in vitro*, yields a 150-kd protein, identical in size to the major EBV nucleocapsid protein (357). Similarly, BNRF1 probably encodes a major 140-kd virion nonglycoprotein. The NRF1 4.1-kb late mRNA, translated *in vitro*, produces a 140-kd protein, identical in size to the virion component (357). Because the BNRF1 predicted sequence has no major hydrophobic domains or glycosylation sites, it is more likely to encode a tegument protein than an integral membrane protein. Another late RNA includes an ORF BXRF1 homologous to the VZV basic virion core protein gene (154,232).

The known (gp25) EBV glycoprotein genes are BLLF1 (gp350/220), BALF4 (gp110), BXLF2 (gp85), BKRF2, BZLF2 (gp42), BILF2 (gp55/80), BDLF3 (gp150), BLRF1 (gp15), BBRF3 (gp84/113), and BILF1 (gp64) (50,245,262,273,310,359,517,621). Other glycoproteins may be encoded by BFRF1 and BMRF2. BILF2 expressed from a recombinant vaccinia virus is 55 to 80 kd. A 78-kd glycoprotein has been identified in EBV lytically infected cells and in virus using antiserum from recombinant vaccinia virus–infected rabbits or EBV-immunized mice. The BILF2 glycoprotein corresponds to an abundant glycoprotein in purified virus preparations. Antibody to BILF2, however, fails to neutralize virus infectivity (517).

BDLF3 cDNA cloning and sequencing confirm that BDLF3 mRNA is not spliced (245). The predicted amino acid sequence includes a hydrophobic amino terminus, consistent with a signal peptide, a hydrophilic external domain with nine potential N-linked glycosylation sites, and a single hydrophobic transmembrane domain. Translation of the 0.9-kb mRNA *in vitro* yields a 32-kd protein that is processed by dog pancreas microsomes to 60 kd, and the size of the fully processed glycoprotein in EBV infected cell is 150 kd. Disruption of gp150 expression for the context of a specifically mutated EBV indicates that gp150 is not critically important for lymphocyte infection.

Because HSV-1 gB is a major virion and infected cell surface glycoprotein, the homologous EBV BALF4 ORF is of interest (187,262,642). Both BALF4 and HSV-1 gB are predicted from their primary amino acid sequence to have amino-terminal cleavable signal peptides, external domains of 650 amino acids with four potential N-linked glycosylation sites, three potential membrane-spanning domains, and a 150-amino-acid cytoplasmic domain (642). BALF4 encodes two late mRNAs: an abundant 3-kb RNA that is not spliced and a scarce 1.8-kb RNA (262). The BALF4-encoded 3-kb mRNA translates a 93-kd protein that is glycosylated to 110 kd (261,262). Gp110 is one of the most abundant late EBV proteins (187,261,262). The glycosylation is probably both N- and O-linked, because N-glycanase, which cleaves N-linked oligosaccharides, only reduces gp110 to 105 kd (261,262). Endoglycosidase H has a similar effect on almost all of the gp110. Because endoglycosidase H cleaves only N-linked oligosaccharides that have not been modified in the Golgi, this suggests that most of the gp110 is not Golgi processed. A small fraction of gp110 is processed to a form compatible with a size of 120 kd. This 120-kd glycoprotein is not endo-H sensitive and is partially incorporated into virus (Alfieri and Kieff, manuscript in preparation). The intracellular distribution of gp110 closely parallels that of an endoplasmic reticulum resident protein (261,262). Immune light or electron microscopy localizes gp110 to the inner and outer nuclear membranes and to cytoplasmic membranes frequently surrounding enveloped virus, but not to the Golgi or plasma membrane. Further, despite the high abundance of gp110 in the inner nuclear membrane through which nucleocapsids bud to acquire their initial envelope, only small amounts of gp110 are in enveloped extracellular virus, as seen by immune electron microscopy (261,262). Protein and sugar stains of polyacrylamide gel–separated proteins from purified virus confirms that gp110 is not a major virion glycoprotein. Thus, EBV gp110 is a very abundant glycoprotein in the nuclear and endoplasmic reticular membranes and may have a critical role in initial envelopment, but only a small amount of gp110 is in the definitive virion envelope.

In contrast to gp110, gp85 and gp350/220 are processed much more efficiently through the Golgi and are found on the virus and in the plasma membrane of lytically infected cells (50,172,173,261,262,309,310,335,517,597,618,619,621,622,668,669 and 670,780,822,823,827).

Gp85 is encoded by XLF2, which has significant colinear homology to HSV-1 gH (310,622), whereas gp350/220 is encoded by LLF, which has only a small region of distant homology to HSV-1 gC (50). Gp85 is a relatively minor virus component, important in fusion of the virus and cell membranes (568), whereas gp350/220 is the dominant external virus glycoprotein that mediates virus binding to the B-lymphocyte receptor, CD21 (see preceding). Interestingly, gp85 expressed in NIH3T3 cells localizes to the internal cytoplasmic and nuclear membranes, whereas gp350/220 localizes to the Golgi and plasma membrane (262,881). In fact, gp85 requires another virus protein to bring it to the plasma membrane of lytically infected lymphocytes, where it is characteristically found, whereas gp350/220 possesses the necessary signals for efficient Golgi transport, processing, and plasma membrane insertion. Gp85 complexes with the BKRF2-encoded protein, which enables its transport to the plasma membrane (905). Two homologous glycoproteins, gH and gL, heterologomerize and have similar functions in HSV replication: EBV gp85 (gH) has a dual role both in fusion and in coreceptor recognition. A third glycoprotein, gp42 or BZLF2, is a component of gH–gL complexes. BZLF2 binds to several HLA class II complexes and is critical for B cell infection. Overexpression of gp42 results in saturation of gH/gL complexes with gp42, a virus that can infect only B lymphocytes and not epithelial cells. Thus, BZLF2 could have a critical role in directing the virus toward B lymphocytes versus epithelial cells. In B lymphocytes, gp42 may have still another role in down-modulatory class II expression.

Although gp350/220 is the most abundant viral protein in the lytically infected cell plasma membrane and in the virus envelope, only small amounts of gp350/220 accumulate in the lytically infected cell nuclear membrane (261). EBV gp350/220 is extensively N- and O-glycosylated (261,743,822,823,827,881). Gp350 nascent protein has an apparent size of 135 kd, and 37 potential N-linked glycosylation sites, whereas mature glycoprotein has an apparent size of 350 kd. Approximately half of the glycosylation is due to N linkage and half to O linkage. Virtually all of the N-linked oligosaccharides are complex, with tri- and tetra-antennary chains, whereas most O-linked chains are probably di- or tri-N-acetyl lactosamine, consistent with extensive Golgi processing (743).

Early electron microscopic observations of lymphocytes lytically infected with EBV revealed the presence of

nucleocapsids in the cytoplasm. Although this could occur as a consequence of nuclear disruption late in infection or as a consequence of reinfection of the infected cell by released virus, an alternative hypothesis is that virus acquires an initial envelope as it buds through the nuclear membrane, that de-envelopment occurs within cytoplasmic vesicles resulting in release of nucleocapsids into the cytoplasm, and that re-envelopment occurs at the plasma membrane, where the virus acquires a definitive envelope rich in gp350/220 and gH/gL. Consistent with this hypothesis, the plasma membrane and virus are both rich in gp350/220, whereas the nuclear membrane is relatively deficient (261). Even if this hypothesis is correct, late in infection, as the cell deteriorates, partially enveloped or initially enveloped virus may be released in substantial quantity. The relative importance of these various pathways in natural infection is difficult to evaluate. A second possible role for high-level plasma membrane gp350/220 expression is in saturation of lymphocyte CD21 and HLA class II, so that virus can be completely released from and not reabsorbed to the lytically infected cells. A price may be paid for such efficiency, however, because antibody to gp350/220 and gH/gL may fix complement and cause lysis of infected cells *in vivo*.

Gp350/220 is not only the most abundant viral protein in the lytically infected cell plasma membrane, it is also one of the most abundant late viral proteins and the most abundant protein on the outer surface of the virus. Surprisingly, disruption of the gp350/220 ORF is not lethal to the virus, and both lymphocytes and epithelial cells can be infected with reduced efficiency. Most EBV neutralizing antibody response is directed to gp350/220 (827). Gp350/220 is therefore an important component of any prospective EBV vaccine that would aim to engender neutralizing antibody (592,618). Significant parts or all of the protein have been expressed in *E. coli*, in *Saccharomyces cerevisiae*, and in mammalian cells, and a recombinant VZV that expresses Gp350/220 under control of the gpI promoter has been constructed (50,604,738,811,881,923). Gp350 is expressed in large amounts in recombinant VZV-infected cells, and it is incorporated into the infected cell plasma membrane and into the VZV outer envelope (515). Gp350/220 has also been inserted into vaccinia capable of under control of the p8.5 promoter (592). Injection with purified EBV gp350/220, or infection with vaccinia expressing gp350/220, protects cottontop tamarins against a lethal, lymphomagenic EBV challenge (592).

BCRF1 is a close colinear homolog to the human IL10 gene, with nearly 90% colinear identity in amino acid sequence (345,857). BCRF1 is expressed only late in EBV replication despite its location in the middle of the EBNA regulatory domain between oriP and the Cp promoter. IL10 has B-cell growth factor activity (586), and some data have suggested that BCRF1 could be important in latent EBV infection (579). However, nonsense or deletion mutations involving BCRF1 have no effect on the ability of the recombinants to initiate growth transformation, to maintain wild-type latent infection and transformed cell growth, or to lytically infect induced B lymphocytes *in vitro* (795). Thus, the role of BCRF1 is in lytic infection *in vivo*. The principal role of IL10 in mice and humans appears to be as a negative regulator of macrophage and NK cell functions that otherwise positively regulate TH1 cytotoxic T lymphocytes (586). BCRF1 has most of the activities of human IL10 *in vitro*.

Thus, BCRF1 is likely to act to blunt the initial NK and T-cell cytotoxic response to EBV infection in epithelial cells and B lymphocytes. One important aspect of that response is the release of gamma interferon. The initiation of cell growth transformation is quite sensitive to interferon (242,795). A mixture of lytically infected EBV recombinant transformed LCLs with normal peripheral blood mononuclear cells results in low interferon release and efficient transformation of primary B lymphocytes, whereas a mixture of lytically induced BCRF1 null mutant recombinant infected LCLs with peripheral blood mononuclear cells results in high-level gamma interferon release and inefficient transformation of the primary B lymphocytes (795). A second aspect of the NK and CD8 cytotoxic responses is the direct cytotoxic component. Blunting of that component is likely to be important in both primary EBV infection and in reactivation of lytic infection in B lymphocytes. To create an analogous murine model for this possible effect, infection of C57BL/6 mice with vaccinia virus expressing beta galactosidase was compared with infection of C57BL/6 mice with vaccinia virus expressing murine IL10. The NK- and vaccinia-specific CD8 cytotoxic responses were lower in the mice infected with vaccinia virus expressing IL10, although there was no difference in virulence or in antibody response (438). Thus, virally expressed IL10 appears to have an effect on the initial interferon and NK and CD8 cytotoxic responses and may have a local effect on these responses to reactivated virus infection.

Defective Virus

The complete EBV genome is usually maintained as an episome in latently infected lymphocytes, including BL lymphoblasts when there is restricted EBV gene expression. However, in several instances, incomplete genomes have been detected in latently infected BL cells growing in culture or in subclones of such cells. The three best-studied examples are the EBV genomes in the Raji, P3HR-1, and Daudi cell lines (69,110,111 and 112,162,301,311,378,381,382 and 383,392,426,675). The Raji cell has 50 EBV episomes, each of which is deleted for EBNA-3C and for BALF2, the 135-kd, major DNA binding protein. As a consequence of the deletion of the BALF2 gene, the EBV genome in Raji cells is not replicated by the Raji DNA polymerase when lytic infection is induced. Thus, no late genes are expressed and the infection is abortive. Expression of BALF2 in Raji cells allows lytic infection to proceed, and virus is produced.

P3HR-1 and Daudi have fewer EBV episomes and are deleted for a single EBV DNA fragment that includes some of the IR1 copies, all of U2 and IR2, and several hundred base pairs of U3. The P3HR-1 EBV deletion appears to have arisen on *in vitro* passage, because the parent cell lines from which P3HR-1 was cloned are not deleted (4,426,677). The EBV genome in P3HR-1 can be induced to full lytic infection and virus is produced. Thus, EBV genes deleted from P3HR-1, including EBNA-LP, EBNA-2, and HLF1, are not necessary for EBV replication in B-lymphoma cells *in vitro*. The resulting P3HR-1 or Daudi virus cannot transform normal B lymphocytes. These cell lines carrying minimally defective EBV genomes are useful for EBV recombinant genetic studies (see later). Interestingly, similar defectives arise during EBV infection in epithelial cells *in vivo* (637,762).

Aside from the minimally defective EBV DNA, some of the EBV DNA in P3HR-1 cells is more highly defective. There are several formats for the more highly defective DNAs and several biologic properties have been delineated (110,112,311,381,382 and 383,816). Analysis of P3HR-1 subclones reveals that most cells contain only the minimally defective EBV P3HR-1 genome (332). A rare cell contains both the minimally and the more defective P3HR-1 EBV genomes. The more defective genome is spread by cell-to-cell transmission following spontaneous reactivation of lytic EBV replication (559). Reactivation occurs in cells containing the defective genomes because the defective genomes

contain the ZLF1 immediate-early transactivator under control of the IR1 latent infection cycle promoter (381,382 and 383). One population of defective genomes appears to be centered about the IR1 palindrome (272,381,382 and 383). In the highly defective DNA, the left arm of the palindrome is a complete inverted copy of the right arm. The IR1 sequence to the right of the palindrome now also extends from the palindrome in the opposite direction into a new linkage with the right end of Bam Z. A second new linkage joins the left of Bam Z to Bam S and Bam M. A third new linkage joins Bam M to Bam B1, W1I1 and *Bam*HI A. *Bam*HI A is presumably linked to TR. The lytic ori in *Bam*HI B1 and the TR presumably provide the necessary cis-acting lytic EBV DNA replication origin and packaging signals, respectively, to ensure the perpetuation of the defective virus in the P3HR-1 cell cultures. The specific and selective inclusion of BZLF1, BSMLF1, and BI'LF1 probably arises because of the adjuvant effect of these transactivators on lytic EBV replication.

GENETICS

Attempts at constructing EBV recombinants have been hindered by the limitation in the EBV host range to human B lymphocytes and the nonpermissivity of these cells for EBV replication. The development of strategies for switching latently infected cells into lytic infection has partially alleviated this major experimental barrier. The first attempt at developing an EBV recombinant genetics system was to infect replication-defective Raji cells with P3HR-1 EBV stocks, and to plate the resultant virus onto primary B lymphocytes (111,233). Raji EBV cannot replicate its own genome in lytic infection because of deletion of the major single-strand DNA binding protein BALF2, but it has EBNA-LP and EBNA-2. Raji is also deleted for EBNA-3C, which in later experiments turned out to be a critical gene for B-lymphocyte growth transformation. The P3HR-1 EBV genome is lytic replication competent, but it is transformation incompetent because of the deletion of a DNA segment that encodes EBNA-LP and EBNA-2. Infection of Raji cells with P3HR-1 EBV induced partial permissivity for lytic infection, and replication of both genomes ensued. Because Raji cells contain 50 episome copies of EBV DNA, most of the transforming virus that came out of the infection should have been simply Raji EBV genomes. However, the few transforming viruses that were obtained in transformed primary B-lymphocyte-derived cell lines were in fact recombinants between Raji and P3HR-1 that had restored the deleted EBNA-LP and EBNA-2 encoding fragment, providing initial genetic evidence that EBNA-2 or EBNA-LP is essential for transformation (765). In retrospect, these results also provided an indication that the Raji EBV genome was itself transformation incompetent because of the EBNA-3C deletion. Recent attempts to exploit the EBNA-3C deletion in Raji to assay the ability of wild-type or specifically mutated LBNA-3C genes to marker-rescue a transforming EBV amma from Raji have met with very limited success because of the limited virus from Raji cells even when BALF2 is provided by a heterologous expression vector.

More recently, the entire EBV genome has become amenable to molecular genetic manipulation. In these experiments, specifically mutated recombinant EBV genomes have been obtained by transfecting latently infected lymphocytes with specifically mutated recombinant EBV DNA fragments that had been cloned and amplified in *E. coli.* When virus replication is then induced, the mutant EBV DNA fragment undergoes homologous recombination with replicating viral DNA (125,126,290,368,409,411,470,471,512,513,514,525,527,529,530,694,795,797,835,836,837 and 838,871). Parental and recombinant virus can be used to infect primary B lymphocytes or non-EBV-infected BL cells. Because primary B lymphocytes depend on EBV infection for their ability to grow *in vitro*, a mutation in an essential transforming gene will only be recoverable in primary B lymphocytes if the cells are co-infected with a defective EBV that is wild type for the mutated gene. The usual source for defective EBV is P3HR-1 cells, because 10 $^{7–8}$ P3HR-1 EBVs can be obtained from 10^7 P3HR-1 cells, and the virus is wild type except for the EBNA-LP and EBNA-2 deletion. BL cells are not dependent on EBV for their growth in culture and therefore are potentially useful for recovery of recombinants with mutations in essential transforming genes (527,528,529 and 530,871). BL cells or primary B lymphocytes can be made dependent on recombinant virus for their growth by including a positive selection marker such as SV40 promoter-driven hygromycin phosphotransferase in the transfected recombinant EBV DNA. Only cells infected with a recombinant virus will then grow out under selective conditions (871). Alternatively, cells infected with the replication-competent but transformation-defective P3HR-1 EBV can be used as the source of a parent virus strain. When these cells are transfected with a wild-type EBV DNA fragment spanning the EBNA-LP and EBNA-2 deletion, a small number of transformation-competent wild-type EBNA-LP and EBNA-2 P3HR-1 EBV recombinants result, and these can be specifically isolated in primary B-lymphocyte-derived cell lines, because only the recombinants will be able to cause LCL outgrowth (125,126,290).

Because transfection with a wild-type EBV cosmid DNA fragment that includes EBNA-LP and EBNA-2 into P3HR-1 EBV-infected cells and induction of lytic infection results in homologous recombination between the transfected DNA and the replicating P3HR-1 EBV with restoration of transforming ability to recombinant P3HR-1 genomes, the effect on transformation of specific EBNA-LP or EBNA-2 mutations or of EBNA-LP or EBNA-2 type-specific differences could be determined (125,126,290,525). Recombination with a wild-type EBV cosmid DNA fragment that spans the deletion uniformly resulted in transforming recombinants. The number of transforming recombinants was sufficiently consistent in a clonal transformation assay that differences in transformation efficiency could be detected. Some deletion mutations within the EBNA-2 ORF or a stop-codon mutation resulted in no transforming recombinants, demonstrating that EBNA-2 is essential for lymphocyte growth transformation. EBNA-2 was also shown to account for the differences between high-transforming type 1 EBV strains and low-transforming type 2 EBV strains (126). Analysis of the phenotype of 11 linker insertion and 15 deletion mutations within the EBNA-2 ORF revealed four separable domains that are essential for transformation of primary B lymphocytes (125). Subsequent experiments with EBV recombinants containing EBNA-LP deletion or stop-codon insertion mutants revealed a markedly reduced B-lymphocyte transformation efficiency.

The use of large cosmid DNA fragment for EBNA-2/EBNA-LP marker rescue of transformation in P3HR-1 cells enables specific mutations to be made in genes mapping near EBNA-LP and EBNA-2 and within the flanking EBV DNA that is common to both the P3HR-1 and transfected EBV DNA fragment. Deletion of the EBER-encoding DNA, or mutation of the BHLF1 or BHRF1 ORFs in the cosmid fragment, resulted in many of the transforming recombinants being also mutant in the adjacent gene (530,797). The resultant mutant EBV recombinants transform cells with wild-type efficiency and replicate following induction.

This led to the observation that almost as high a frequency of incorporation into recombinant EBV genomes occurs with a second, nonlinked, cosmid EBV DNA fragment transfected along with the EBNA-LP and EBNA-2 cosmid DNA into P3HR-1 cells (835,836 and 837). Using this strategy of second-site homologous recombination, mutations could be made

on any EBV DNA fragment, and, after transfection into P3HR-1 cells, harvest of the resultant virus, and clonal infection of primary B lymphocytes, a substantial fraction of the resultant LCLs are infected with mutant recombinants. The frequency of incorporation of nonlinked second EBV DNA fragments is directly related to transfected fragment size. Fragments of 4 to 5 kb are incorporated with about 20% to 30% of the efficiency of 40-kb fragments, the latter being incorporated into about 10% of the EBV DNAs that recombine with the EBNA-LP/2-positive selection marker.

An alternative strategy for constructing EBV recombinants, which is particularly useful for making deletions in the EBV genome, is to use the P3HR-1 cells as a lytic infection transcomplementing and packaging cell line for cosmid cloned EBV DNA fragments (694,837). Approximately 50% of all transforming recombinants from cells transfected with five overlapping EBV DNA fragments representative of the entire genome consist of the transfected cosmid DNAs without any evidence for recombination with the P3HR-1 EBV genome. For studying the transforming EBV genes, only two and a half cosmid-cloned EBV DNA fragments are necessary, because all of the genome between EBNA-1 and LMP1 is unnecessary and most of the noncoding exons and introns of the EBNA mRNAs can be deleted.

Although positive selection for transformation from the transformation-negative P3HR-1 parent strain is useful for making specifically mutated recombinants in most genes, there are limitations. First, nontransforming mutations in EBNA-LP or EBNA-2 cannot be recovered, and the effect of the mutation can be ascertained only by the failure to generate a transforming recombinant from P3HR-1. Second, nontransforming mutations in other essential or critical transforming genes such as EBNA-3A or -3C or LMP1 can be recovered only when the wild-type EBNA-3A, -3C, or LMP1 is provided to the infected primary B lymphocyte. In many instances, this can be conveniently provided by nonrecombinant P3HR-1 virus, as 10^{7-8} nonrecombinant P3HR-1 EBVs are in each preparation of 50 to 200 recombinant EBVs. Because the virus preparation is used to infect 10^{7} primary B lymphocytes, 50% of the primary B lymphocytes are initially infected with nonrecombinant P3HR-1. Any cell infected with a recombinant therefore has a 50% chance of being initially co-infected with a nonrecombinant. Both genomes can stably persist in cells and function, although the nonrecombinant P3HR-1 genome is usually lost over a few months in continuous culture of the resultant LCL if it is not required to maintain cell growth transformation. The characterization of the mutant recombinant genome in co-infected cells is more complicated than in cells infected with a single EBV genome. Furthermore, in some instances, the mutant recombinant can be lost, because the mutant recombinant with its mutated EBNA-3A, EBNA-3C, or LMP1 gene and its wild-type EBNA-2 and EBNA-LP genes undergoes secondary recombination with the co-infecting P3HR-1 EBV, which is wild type for EBNA-3A, EBNA-3C, or LMP1 but deleted for EBNA-2 and EBNA-LP. The secondary recombinant genome could be fully wild type. Third, the efficiency of second-site recombination is about 10%. Many recombinants are obtained that are not mutant at the second site, and these must be identified by polymerase chain reaction for the mutated DNA.

To circumvent some of these problems, non-EBV-infected BL cells or primary B lymphocytes can be used to isolate and recover mutant EBV recombinants carrying a selectable marker (470,471,527,528 and 529,871). BL cells can be infected with EBV *in vitro*. The efficiency of infection varies among BL cell lines and is frequently 10% of the efficiency of primary B-lymphocyte infection. Mutant recombinant virus can be recovered from such cells. Because the BL cells are not dependent on EBV for their ability to grow *in vitro*, mutant recombinants deleted for an essential transforming gene can be transferred to non-EBV-infected BL cells and marker-rescue experiments similar to those done in P3HR-1 cells can be carried out. A limitation of this strategy has been alleviated with the derivation of EBV-negative Akata BL cells, which retain EBV episomes and can be induced to become permissive for EBV replication (749).

Recently, several alternative strategies have been developed for making EBV recombinants. One strategy uses targeted homologous recombination to insert mutations, cassettes for expression of positive selection markers, or bacterial replicons into wild-type EBV episomes in cells that can be readily induced to lytic infection, such as Akata or B95 cells (750). The resultant virus can then be harvested and passaged into EBV-negative Akata cells, induced to replicate in these cells, and used for subsequent genetic studies (365,900). EBV recombinants with positive selection markers inserted using this strategy have been used to study the *in vitro* host range for EBV infection, and to show that EBV gp85 is essential for B lymphocyte and epithelial cell infection *in vitro*, that gp42 is critical for B-lymphocyte infection *in vitro*, and that gp150 deletion enhances the efficiency of epithelial cell infection (70,365,581,623,875,900).

A second strategy is a further modification, importing the entire EBV genome into *E. coli* using F plasmids and recombination-deficient cells. Targeted insertional mutations can then be made in cells that have recombination functions. F plasmids containing the entire EBV genome and a positive selection marker for mammalian cells have been recovered from *E. coli* and transferred to human epithelial 293 cells. These plasmids can be induced to replicate in the infected 293 cells and small amounts of virus can be obtained to study the effects of specific mutations on virus infection, replication, or cell growth transformation (160,161). This system should improve the efficiency of EBV genetic studies, assuming that the EBV genome proves to be stable in the recombination-deficient *E. coli.* Initial experiments that demonstrate the utility of the system for investigating the dependence of EBV on gp350 for B-lymphocyte or epithelial cell infection indicate that the system is useful (375).

INHIBITORS OF VIRUS REPLICATION

Lytic EBV infection in B lymphocytes *in vitro* and in EBV infection *in vivo* (see Chapter 75) are quite sensitive to acycloguanosine, which is a substrate for the EBV deoxynucleoside kinase (14,15,494,502) and an inhibitor of EBV DNA polymerase. Other inhibitors of herpesvirus DNA polymerases such as phosphonoacetic acid and phosphonoformic acid are also useful for *in vitro* inhibition of EBV DNA polymerase in B lymphocytes, so as to distinguish early from late gene expression (153,493,495,686). Because of the limited clinical benefit in inhibiting EBV replication with acycloguanosine in patients with acute primary EBV infection (see Chapter 75), there has been little effort to develop other specific inhibitors of lytic EBV infection.

BRIEF SUMMARY

In the first decade of EBV research, a great deal was learned about the biology of EBV infection *in vitro* and *in vivo*. The virus was found to cause infectious mononucleosis and to be latent in most adult humans. The tight linkage to BL and

NPC, the rare occurrence of lymphoproliferative disease in young children and renal transplant recipients, and the ability of the virus to efficiently immortalize lymphocytes *in vitro* and to induce lymphomas in marmosets provided a biologic framework indicating that EBV can be oncogenic under unusual circumstances. The discovery of cell lines that could be induced to replicate EBV led in the second decade to increasingly sophisticated biochemical analyses. The structure of the genome was determined, the genome cloned, the transcriptional program in latent and lytic infection worked out, and the entire genome sequenced. EBNA became a protein on an acrylamide gel, and then more than one protein. EBV mRNAs in latently infected and growth-transformed B lymphocytes were cloned, sequenced, and expressed in heterologous cells.

By the beginning of the third decade, the genes encoding the EBV latent-infection nuclear and membrane proteins and the proteins themselves were identified in rapid succession. Six EBNAs, two LMPs, and two EBERs were involved in latency III and cell growth transformation. Studies of the biologic activity of these genes following single-gene transfer revealed EBNA-1 and oriP to be sufficient for plasmid episome maintenance, and LMP1 to have significant transforming activity in rodent fibroblasts. Gene transfer into BL cells and recombinant EBV molecular genetics emerged as important experimental paradigms. Activities of the EBNAs and LMPs were more evident in human B lymphocytes, a natural target of EBV infection.

In the last 5 years, knowledge of the roles of the EBNAs and LMPs in maintaining latent infection and growth transformation and of the various types of latent infection has rapidly advanced. The interaction of EBNAs and LMPs with B-lymphocyte proteins is offering increasing understanding of the mechanisms of EBV-induced and normal B-lymphocyte activation, proliferation, and survival. Molecular targets for inhibition of EBV-mediated growth transformation and cell survival have been identified. Immediate-early and early regulation of lytic EBV infection are better understood. New strategies for manipulating the transition to lytic infection and for genetic analyses have facilitated EBV research. These discoveries and those described in Chapter 75 on the molecular pathogenesis of EBV infection *in vivo* open new possibilities for understanding, preventing, diagnosing, and treating EBV infection and its associated malignancies.

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CHAPTER 75 Epstein-Barr Virus

Alan B. Rickinson and Elliott Kieff

HISTORY

The catalyst for the discovery of Epstein-Barr virus (EBV) was Denis Burkitt, a surgeon working in East Africa in the 1950s, who first draw attention to a novel childhood tumor, now known as Burkitt's lymphoma (BL), which he found to be unusually common throughout equatorial Africa. The apparent influence of climatic factors in determining those regions of Africa where BL was “endemic” led Burkitt to postulate that an infectious agent, possibly arthropod-borne, was involved in the tumor's etiology (60). This caught the attention of Tony Epstein who, with his Ph.D. student Yvonne Barr, succeeded in establishing a number of BL-derived cell lines in culture, of which the first to be examined in the electron microscope showed clear evidence of herpesvirus-like particles in a small proportion of the cells (124). The agent present in BL cells proved to be biologically and antigenically distinct from other members of the human herpesvirus

(HHV) family, thereby identifying EBV as the first candidate human tumor virus.

Contrary to expectations for an agent linked to an African tumor, seroepidemiologic studies showed that EBV was in fact widespread in all human populations with more than 90% of adults positive for serum antibodies to the virus (186). Furthermore, the virus could be rescued *in vitro* from the circulating lymphocytes and from the throat washings of seropositive individuals, indicating that EBV was able to persist in the serologically immune host (147,333). Most importantly, EBV was a B-lymphotropic agent that could transform its target cells *in vitro* into latently infected B-lymphoblastoid cell lines (LCLs) (188). Such behavior has since become the defining biologic characteristic of the lymphocryptoviruses (LCVs), a genus of the gamma herpesvirus subfamily whose other members are all homologs of EBV isolated from Old World, and more recently New World, primate hosts (88,484). The *in vitro* growth-transforming capacity of EBV provided strong circumstantial evidence that the association of the virus with BL, a tumor of B-cell origin, was more than just coincidental. Furthermore, experimental infection of certain species of New World primates led to the outgrowth of virus-positive B-cell lymphomas (125,421). These key observations did much to sustain interest in EBV over the ensuing years during which human tumor virology as a general field of investigation swung in and out of scientific fashion.

IN VITRO INFECTION

The extensive literature on EBV infection of target cells *in vitro,* much of it focusing on infections of human B cells, is reviewed in Chapter 74 (245). The essential points only are summarized here as a prelude to discussing what is known about EBV infection *in vivo*.

Growth-Transforming Latent Infection, Latency III

A wide range of human B cells, from pro-B to antigen-experienced memory cells (but excluding plasma cells), appear susceptible to EBV infection and transformation *in vitro* (227,333), broadly reflecting expression within the B-cell lineage of the principal EBV receptor CR2. The resultant LCLs all show a similar lymphoblastoid phenotype and a Latency III pattern of viral latent gene transcription, as illustrated in Figure 1. This involves expression of (a) the small nonpolyadenylated RNAs (or EBERs); (b) the BamHI A RNAs, a family of spliced polyadenylated transcripts whose protein products are still not well characterized; (c) six differentially spliced mRNAs expressed either from the BamHI W promoter (Wp) or more commonly from an adjacent BamHI C promoter (Cp) and encoding the viral nuclear antigens EBNAs 1, 2, 3A, 3B, 3C and -LP,; and (d) three spliced BamHI N RNAs encoding the latent membrane proteins LMP1, LMP2A, and LMP2B; note that LMP2B, which is expressed from the same bi-directional promoter as LMP1, represents an N-terminally truncated version of the independently expressed LMP2A protein. Most adult-origin, as opposed to fetal-origin, LCLs contain a small fraction of cells (less than 5%), which have switched from latency into virus productive (lytic) cycle, apparently coincident with differentiation toward a plasmacytoid phenotype (96). The inability to reproduce the Latency III form of infection in several other cell types (see later) implies that the growth-transforming program of EBV is indeed B-cell specific, one possible exception being CR2-positive follicular dendritic cells in which *in vitro* infection has been reported to induce the slow outgrowth of cells expressing the full range of EBV latent cycle proteins (281).

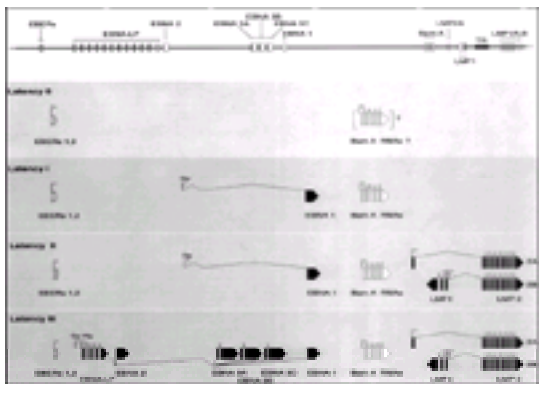

FIG. 1. Patterns of EBV latent gene transcription in four different forms of latency either postulated in the case of Latency 0 for an infection of noncycling cells or as observed in the cases of latency I, II, and III from *in vitro* model systems. Transcripts are drawn in correct orientation relative to one another, with promoters (*pennants*) and splicing patterns as shown; exons are illustrated for noncoding RNAs (*white*; including the BamHI A RNAs, whose coding capacity is still unresolved) and for mRNAs (*black*). The actual genomic positions of these exons are shown (*top*) on a linear map of the viral genome. Note that the genome is circularized in latently infected cells through fusion of the terminal repeats (TR), and the LMP2 transcripts are spliced across this fused TR region.

More Restricted Forms of *In Vitro* Latency, Latency II and I

The existence of more restricted forms of latent infection first became apparent from the study of EBV-positive malignancies but is best illustrated using experimentally generated *in vitro* models in which viral gene expression is homogeneous throughout the cell population and stably maintained with serial passage. It is important to note that Latency II and Latency I, defined using these *in vitro* models and presented as distinct entities in Figure 1, represent two ends of a spectrum with various intermediate possibilities depending on the relative activities of the LMP promoters.

Latency II can be established *in vitro* by fusing LCL cells with certain EBV-negative human epithelial, fibroblast, or hemopoietic cell lines (92,230). In the resultant cell hybrids where the cell phenotype resembles that of the EBV-negative parent rather than the LCL, there is a concomitant switch in the pattern of virus gene expression. This now involves (a) expression of the EBERs, (b) expression of the BamHI A RNAs, (c) silencing of the Cp/Wp promoters and selective

expression of a different EBNA1 mRNA from the BamHI Q promoter (Qp), and (d) expression of the LMP1, LMP2A, and LMP2B mRNAs, leading to a cell in which viral antigen expression is limited to EBNA1 and the LMPs (possibly plus BamHI A–encoded products).

Latency I can be established by fusing LCL cells with other human hemopoietic cell lines, such as HL60 (230). In this case, the resultant hybrids have again suppressed the LCL phenotype, and viral transcription is reduced to expression of (a) the EBER RNAs, (b) the BamHI A RNAs, and (c) the EBNA1 mRNA from Qp, with silencing of the Cp/Wp and LMP promoters. In this case, therefore, viral antigen expression is restricted to EBNA1 (possibly plus BamHI A–encoded products).

Various attempts have been made to establish these more restricted forms of latency by direct experimental infection of target cells rather than by manipulating the LCL phenotype. Infection of CR2-positive human B-cell lines (derived from EBV-negative lymphomas) leads to a transient Latency III pattern of gene expression but only rarely to stable conversion of cells to an EBV genome–positive state. In the absence of any selection system for EBV-positive converts other than enhanced survival under dense culture conditions, the rare converts detected tended to show a Latency III phenotype and pattern of viral gene expression (320), often carrying the EBV genome in an integrated rather than the expected episomal form (209). However, using a recombinant EBV with a drug selection marker has allowed the more efficient isolation of stable converts that carry the viral genome as an episome and display Latency I (483).

A number of studies have examined the infectability of human epithelial cells *in vitro,* prompted by the association of EBV with some nasopharyngeal and gastric carcinomas. Direct infection of primary skin or pharyngeal epithelium is detectable *in vitro* but very inefficient (429), whereas established skin keratinocyte lines, expressing CR2 from an introduced vector, are much more susceptible (279). By contrast, both primary gastric epithelium (334) and gastric carcinoma cell lines (512) can be infected by a CR2-independent route. In these susceptible epithelial systems, there is selective expression of Qp-initiated EBNA1 and of LMP2A transcripts, with little evidence of LMP1 expression either at the mRNA or protein level. This is one of several indications that forms of latency intermediate between Latency I and II can exist. A proportion of these epithelial cells, either in acutely infected cultures or in derived EBV-positive cell clones, are capable of entering a virus lytic cycle either spontaneously or after induction of terminal differentiation (254,279,334,429,512).

Somewhat similar findings have come from experimental infections of T-lymphoma cell lines, which EBV can infect by CR2-dependent (255) or CR2-independent (182) routes. These lines typically sustain latent infections characterized by EBNA1 and LMP2A expression with or without LMP1 (348). Conventional attempts to infect freshly isolated CD4+ or CD8+ T cells from peripheral blood have been unsuccessful. However, one study described the establishment of slow-growing EBV-positive T cells with a Latency I pattern of viral gene expression from cultures of virus-infected peripheral blood mononuclear cells (PBMCs) following electroporation with a drug resistance marker and drug selection (166). The mechanism underlying this rare T-cell outgrowth has yet to be identified.

Other Forms of Latency

All stable programs of latent gene transcription seen in EBV-infected cell lines *in vitro* or tumors *in vivo* must be consistent with maintenance of the viral genome in the proliferating cell population, that is, with continued expression of the virus genome maintenance protein EBNA1. This would not necessarily be the case were EBV to establish latency in a noncycling host cell. In this example, in principle, the virus might persist without expressing any viral antigens, as in latent alpha herpesvirus infections of neuronal cells *in vivo*. As illustrated in Figure 1, the analogous situation for EBV would therefore be a “Latency 0,” in which Cp/Wp, Qp, and the LMP promoters are silent and viral gene expression is limited to the noncoding EBERs, possibly accompanied by the BamHI A RNAs.

Lytic Infection

There are as yet no *in vitro* cell systems in which EBV infection leads directly into a fully productive lytic infection. Virus replication must always be studied by inducing latently infected cells into lytic cycle. Such induction is associated with a down-regulation of the latent cycle EBNA promoters (either Cp/Wp in Latency III or Qp in Latency I and II) and activation of a lytic promoter Fp immediately upstream of Qp. A minority of the Fp-initiated transcripts splice into the EBNA1 open reading frame (405) such that EBNA1 expression appears to be maintained in lytic cycle. Furthermore, in the Latency I BL cell line Akala, the LMP promoters are also activated after the induction of lytic cycle, such that the LMPs are expressed with the kinetics of early lytic cycle proteins (388).

There are, however, some *in vitro* systems in which EBV infection has been reported to enter an abortive lytic cycle without a detectable latent phase. These all involve atypical target cells, namely a CR2-positive subpopulation of immature fetal thymocytes (228), cultured syntiotrophoblasts again apparently expressing CR2 (469) and primary monocytes (401). The *in vivo* significance of such interactions is unknown, but at present none seems likely to provide a fully permissive *in vitro* system that is experimentally tractable.

INFECTION IN THE NATURAL HOST

Epidemiology of Primary Infection

Seroepidemiologic surveys of immunoglobulin G (IgG) antibodies to the viral capsid antigen (VCA) complex show that most children in the developing world become infected within the first 3 years of life and that antibody prevalence reaches 100% within the first decade (1,126). These early primary infections, which are almost always asymptomatic, probably reflect parent-to-child transmission by the oral route; indeed, there is now sound molecular evidence for the transmission of a single EBV strain between generations within families (158). By contrast, one consequence of lifestyle change in the more developed Western world is that up to half of children are still seronegative at the end of their first decade, many of whom subsequently become infected through intimate oral contact in adolescence or young adulthood. A serendipitous observation in the Henles' laboratory, that a young technician had seroconverted during the course of

infectious mononucleosis (IM) (187), prompted retrospective serologic studies that quickly confirmed EBV as the etiologic agent of this disease (127). Up to half of such delayed primary infections may be symptomatic, although only the more severe are likely to present clinically and be diagnosed as IM. About 5% of adults in Western societies remain EBV-uninfected, both by serologic criteria and by virus detection assays, and constitute a population from which rare cases of classic IM in older adults (200) and of iatrogenic “postperfusion” mononucleosis (148) are drawn.

Most of what is known about primary EBV infection comes from the study of IM patients, but it is not clear how closely the events occurring in IM reflect those occurring in an asymptomatic primary infection in childhood. It is worth remembering that, in evolutionary terms, IM is a novel disease that has arisen through a recent change in the epidemiology of virus transmission, as yet peculiar to the developed Western world.

Virus–Cell Interactions During Primary Infection

Lytic Infection at Oropharyngeal Sites

Little is known about the earliest events that follow oral transmission of EBV to the naive host because there is a 4- to 6-week incubation period before the clinical symptoms of IM appear. By this time, high titers of cell-free infectious virus are detectable in throat washings and saliva as measured by *in vitro* transformation using indicator cord blood B cells (147). Early evidence suggested that this virus was being produced from lytically infected oropharyngeal epithelium, with viral DNA or lytic cycle transcripts being detectable in desquamating epithelial cells by *in situ* hybridization (427). This issue is not fully resolved, however (225), particularly since histologic studies of IM tonsils could not identify any viral infection of tonsillar epithelium but did detect small numbers of lytically infected B-lymphoid cells, usually situated near crypt epithelium, expressing the immediate early BZLF1 protein and a component of early antigen (11,490). Interestingly, these cells had mostly lost expression of CD20 and had acquired an early plasmacytoid marker (328), consistent with an association first noted *in vitro* between lytic cycle entry and plasmacytoid differentiation (96).

One view is that epithelial cells, perhaps at a specialized oropharyngeal site, constitute the primary targets in which orally transmitted virus replicates and that progeny virions subsequently transfer into the B cell system. An alternative view is that, while there may be secondary spread of infectious virus to permissive epithelium, the virus primarily targets B cells infiltrating the oropharyngeal mucosa where the local milieu might favor lytic rather than latent infection. Circumstantial evidence for this latter view comes from X-linked agammaglobulinemia (XLA) patients, who lack circulating B cells and receive infusions of pooled Ig preparations; such patients do not harbor detectable EBV DNA either in throat washings or in peripheral blood and show no evidence of the virus-specific T-cell memory, which even a transient primary infection of oropharyngeal epithelium would have been expected to elicit (133).

Latent Infection: Colonization of the B-Cell System

In addition to virus replication in the oropharynx, acute IM patients also show a generalized infection of the circulating B-cell pool, apparent from the ease with which EBV-positive LCLs grow out spontaneously from cultures of PBMCs (358). Indeed, from studies of individuals fortuitously sampled in the incubation phase of IM, it is clear that virus colonization of the B-cell system is ongoing well before the onset of clinical symptoms (116,451). During the acute disease itself, immunofluorescence staining has shown that, in exceptional cases, 10% or more of the circulating B cells can be EBNA-positive (98,379); more usually, however, values lie in the 0.1% to 1% range (250). Similar frequencies are obtained when IM B-cell preparations are screened by *in situ* hybridization for cells expressing EBERs, a sensitive marker of latent infection (466). Even using the less sensitive assay of spontaneous LCL outgrowth, culture seedings of 10^4 to 10^5 PBMCs (equivalent to less than 10^3 to 10^4 B cells per well) are frequently sufficient to give growth (506). The inability to detect infectious virus in freeze-thawed extracts of freshly isolated IM B cells suggests that most of the EBV-positive cells are latently infected *in vivo*, although some lytic cycle gene transcription is detectable using highly sensitive nested reverse transcription–polymerase chain reaction (RT-PCR) assays (361). Interestingly, many of these latently infected cells appear to be rapidly triggered into lytic cycle when placed in culture. Spontaneous LCL outgrowth in fact largely depends on this process of virus replication, release, and infection and transformation of co-resident B cells rather than on the direct *in vitro* growth of *in vivo*–infected cells (375).

RT-PCR analysis of viral transcription in freshly isolated IM B cells (466) has detected many of the RNAs characteristic of a Latency III (LCL-like) infection, including the Cp/Wp-initiated EBNA mRNAs and the various LMP transcripts. This strongly suggests that at least some of the latently infected B cells generated during primary infection are growth-transformed and is consistent with the presence of rare EBNA-positive mitotic cells in IM blood (379). However, RT-PCR analysis also detected the presence of Qp-initiated (and not Fp-initiated) EBNA1 transcripts in the circulating B-cell pool (466), suggesting that cells sustaining alternative forms of latent infection may also be present.

The complexity of latent virus–cell interactions *in vivo* is graphically illustrated by screening IM tonsillar sections with a combination of RNA *in situ* hybridization for EBERs and monoclonal antibody staining for virus latent proteins or cell lineage markers. As illustrated in Figure 2, a substantial number of EBER-positive cells are present, mainly in the extrafollicular paracortical areas and only very rarely in the B-cell areas (follicles) or their constituent germinal centres (11,328,490). Most of the EBER+ cells are B lymphocytes, but some studies have also reported evidence of T-cell infection (11); this latter point remains controversial (328,474). The EBER-positive B-cell populations are heterogeneous both in morphology and viral antigen expression. They include (a) small cells expressing EBNA2 in the absence of LMP1, perhaps recently infected and progressing to the EBNA2-positive, LMP1-positive stage; (b) medium-sized lymphoblastoid cells expressing both EBNA2 and LMP1, consistent with a LCL-like Latency III infection and virus-driven B-cell growth; (c) large blast cells, morphologically similar to the malignant Hodgkin–Reed Sternberg (H-RS) cells of Hodgkin's disease (HD), which are EBNA2-negative, LMP1-positive; and (d) small cells, which are EBNA2-negative and LMP1-negative (260,328). How these different forms of infection interrelate in time is not known, but analysis of Ig-variable gene sequences in individual microdissected cells clearly shows that different cell phenotypes can be generated within the same EBV-infected B-cell clone (260). One possibility is that some cells leave the proliferating EBNA2-positive, LMP1-positive compartment and differentiate either to an EBNA2-negative, LMP1-positive, H-RS–like cell or to an

EBNA2-negative, LMP1-negative form of infection like that which characterizes EBV-infected B cells in the blood of virus carriers.

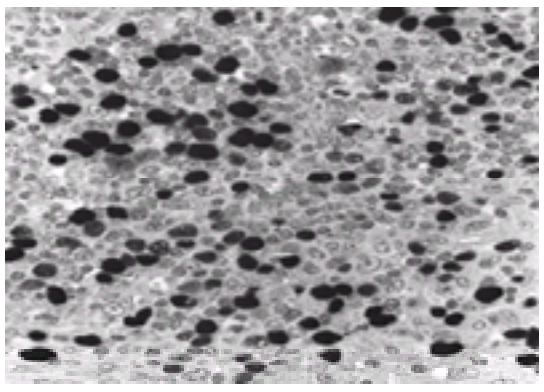

FIG. 2. *In situ* hybridization with digoxygenin-labeled probe demonstrating the high frequency of EBER-RNA–expressing lymphocytes (see dark nuclei) in tonsillar tissue taken from a patient with acute infectious mononucleosis. In separate experiments, double staining with a monoclonal antibody against the B-cell–associated membrane marker CD20 confirms the B-cell origin of most EBER-positive cells. (Courtesy of G. Niedobitek.)

An important lesson emerging from this work is that, although some individual EBV-positive cells in IM tonsils carry nonmutated *Ig* gene sequences characteristic of naive B cells, the expanding clones preferentially, if not exclusively, involve cells that already carry somatically mutated *Ig* genes (260). Such somatic mutations are diagnostic of an antigen-experienced (memory) B cell that has previously acquired these mutations during antigen-driven transit through a germinal centre (366). However, the EBV-driven expansion of such clones in IM tonsils occurs without further intraclonal hypermutation, consistent with the observation that these expansions are occurring outside germinal centers. Interestingly, the virus itself may determine the homing of these growth-transformed cells to extrafollicular sites. Thus, in a transgenic mouse model system, constitutive LMP1 expression in B cells prevents their involvement in germinal center reactions (471); furthermore, viral infection of human B cells induces expression of CCR7 (41), the receptor for a cytokine preferentially produced by dendritic cells in extrafollicular areas of lymphoid tissues (325). The preferential expansion or survival of EBV-infected memory, as opposed to naive, B cells in IM is as yet not understood; possibly, it reflects an increased ability of these more mature cells to differentiate from the proliferating EBNA2-positive LMP1-positive lymphoblastoid state to a resting EBNA2-negative, LMP1-negative resting state upon which their ability to persist in the immunocompetent host depends.

Virus–Cell Interactions During Persistent Infection

Lytic Infection at Mucosal Sites

As the acute symptoms of IM subside, virus replication in the oropharynx falls but is never completely eliminated. Even long-term virus carriers with no history of IM continue to secrete low levels of infectious virus, detectable in throat washings by the cord blood B-cell transformation assay (507). There is again debate over the source of this orally shed virus. EBV clearly can replicate in mucosal epithelium under some circumstances (163,478), but histologic screening of oropharyngeal and nasopharyngeal tissues suggests that lytic infection is restricted to small numbers of B cells, usually underlying mucosal surfaces, rather than to epithelium itself (330,460). Virus replication within sub-mucosal plasmacytoid B cells is again consistent with the recent finding that linear EBV DNA (diagnostic of lytic infection) is detectable only in a fraction of tonsillar lymphocytes that have lost the CD19 marker (23).

It is also clear that replication is not restricted to the oropharynx, having been detected also in the cervix (426) and in the lungs (118) of seropositive individuals. This supports the view that, once the virus has successfully colonized the B-lymphoid system, reactivation from latency can occur at any mucosal site with a B-cell infiltrate. Relevant in this context are iatrogenic primary infections in which the virus is acquired through latently infected B cells within a bone marrow graft (159) or blood transfusion (148). The fact that this virus can subsequently be isolated as infectious virions in a patient's throat washings clearly shows that a reservoir of latently infected cells can seed foci of virus replication at mucosal sites.

Latent Infection of the B-Cell System

EBV-infected B cells remain detectable in the peripheral blood and throughout all lymphoid tissues of asymptomatic carriers, albeit at much lower levels than seen in IM. This was again first recognized by spontaneous LCL outgrowth *in vitro* (333) and subsequently confirmed by direct PCR amplification of viral DNA (24,119,232,306). Most work has concentrated on the peripheral blood where seedings of 4×10^5 to 4×10^6 PBMCs (equivalent to less than 4×10^4 to 4×10^5 B cells) are usually sufficient to obtain LCL outgrowth, providing cyclosporine has been added to culture medium to prevent interference by immune T cells (505). The absolute frequency of circulating EBV-infected cells has been more accurately determined by PCR amplification on limiting PBMC numbers, giving a normal range of 1 to 50 per 10^6 B cells for healthy virus carriers (232,306). Both approaches have emphasized the stability of EBV-infected B-cell numbers in individual carriers when monitored over several years. In normal donors, there is no evidence of virus carriage in non-B leukocytes.

In contrast to the activated EBV-positive lymphoblasts seen during primary infection, infected cells in the blood of virus carriers all lie in the noncycling (G_0 phase) population, lacking activation markers such as CD23 or CD80 and the Ki-67 proliferation marker (305,306). Importantly, those infected cells all segregate within the memory subset of circulating B cells, a population defined by loss of surface IgD expression, and not within the naive IgD-positive, IgM-positive

population (24). Normally, exposure of a naive B cell to its cognate antigen induces clonal expansion of that cell within a germinal center; during this expansion, somatic hypermutation produces intraclonal diversification of antibody-coding (*Ig*) gene sequences, after which just a few progeny cells producing the highest-affinity antibody are positively selected into memory by virtue of their ability to compete for antigen binding (366). These events usually involve loss of IgD expression and are also frequently accompanied by Ig class switching; hence, the resultant IgD-negative memory pool contains some cells that have remained IgM-positive and others that have switched to become surface IgG-positive, IgA-positive, or IgE-positive. EBV-infected cells are detectable in all of these memory cell subsets (24,119), although apparently not (464) in another recently identified subset of memory B cells, which atypically remain IgD-positive and IgM-positive but are distinguished from the IgD-positive, IgM-positive naive population by their acquisition of the CD27 marker (252). The sequestration of EBV within the memory B-cell pool of virus carriers accords with the observation that during primary infection, clonal expansion of EBV-infected cells appears to favor those clones with a somatically mutated (i.e., memory) Ig genotype (260).

EBV latent gene expression in the circulating B cells of virus carriers is different from that seen in IM. Now only the EBERs (466), BamHI A RNAs (84), and LMP2A transcripts (305,363,466) are still detectable, in some cases accompanied by trace levels of Qp-initiated EBNA1 transcripts (305,466). This narrowing of viral gene expression between primary infection and the carrier state is consistent with the reversion of infected cells from an activated lymphoblast to a resting memory B-cell phenotype. Care must be taken in extrapolating from the results of RT-PCR assays on total cell populations to viral transcription in individual cells. Quantitative considerations suggest that most EBV-infected B cells in the blood are EBNA1 mRNA negative and that many could also be LMP2A mRNA negative; indeed where such transcripts are detectable, they may represent residual signals from cells that have recently left the lymphoid tissue environment (23). This implies the existence of a circulating pool of EBV-positive cells in which all viral antigen expression is suppressed and viral transcription is limited to the noncoding EBERs and possibly the BamHI A RNAs; this is equivalent to the Latency 0 form of infection postulated in Figure 1.

Analysis of lymphoid (mainly tonsillar) tissues from virus carriers suggests that the proportion of B cells that carry the virus here is again very low, similar to levels seen in the blood (11,24,330). *In situ* hybridization on tissue sections identifies occasional EBER-positive cells, again usually within extrafollicular areas, which have the appearance of small B lymphocytes and show no evidence of EBNA2 or LMP1 expression (17,330). A more complete histologic picture may be easier to discern, however, in the tonsils of virus carriers from areas where parasitic infection provides a chronic antigenic stimulus to the B-cell system. In such circumstances, recent work suggests that the extrafollicular load of EBER-positive small B cells is slightly elevated and that in some cases EBER-positive centroblasts, always EBNA2-negative but occasionally expressing LMP1, are detectable within germinal centres (17). Such findings would be consistent with a recent RT-PCR analysis of EBV gene transcription that has detected LMP1, LMP2A, and Qp-initiated EBNA1 mRNAs both in the germinal center B cell (CD19+, IgD–, CD77+) and in the memory B cell (CD19+, IgD–, CD77–) compartments of tonsillar lymphocytes (25,26). These appear to represent reactivations from the preexisting memory cell pool rather than *de novo* infections because such transcripts were consistently present in tonsils whether or not there was evidence of ongoing virus replication. Interestingly, Cp/Wp-initiated EBNA transcription, diagnostic of growth- transforming Latency III infection, was found only in tonsils in which virus replication was occurring to provide a source of progeny virus for *de novo* infections (25). Therefore, reactivations from the latently infected memory B-cell pool and *de novo* infections may be occurring simultaneously in the lymphoid tissues of virus carriers but are likely to involve different programs of virus gene expression and lead to different outcomes.

The spectrum of B-cell differentiation stages represented within lymphoid tissues is much broader than that represented in peripheral blood, and the resident forms of EBV infection are correspondingly more heterogeneous. EBV has evolved mechanisms to colonize, persist within, and reactivate from the human B-cell system that very probably exploit normal B-cell physiology, using differentiation-dependent cellular controls to activate or suppress different programs of viral gene expression at appropriate times in the life history of the infected cell. The full complexity of these viral strategies is only just being realized, and any current attempt to map the lineage, kinetic, and anatomic relationships between infected cells *in vivo* (see later overview section) must be regarded as preliminary.

ANTIBODY RESPONSES TO INFECTION

The seroepidemiology of EBV infection and of EBV-associated diseases relies heavily on a set of immunofluorescence assays developed in the laboratories of Werner Henle and George Klein within the first decade of EBV research (126). These measure antibody responses to the following serologically defined antigens: (a) the EBNA expressed in latently infected cells; (b) the early antigen (EA), divisible into diffuse (EA-D, methanol-resistant) and restricted (EA-R, methanol-sensitive) components and expressed within cells early in lytic cycle; (c) the virus capsid change (VCA) expressed within cells late in lytic cycle; and (d) the membrane antigen (MA) expressed on the surface of cells late in lytic cycle. As described in Chapter 74 (245), each of these “antigens” is a composite of several distinct viral proteins. Thus, EBNA is a complex of six distinct nuclear proteins, EBNA1, 2, 3A, 3B, 3C, and -LP, with antibodies to EBNA1 being the most frequent (but not the only) reactivity detected in the conventional anti-EBNA immunofluorescence test. EA is a plethora of immediate-early and early viral proteins, including the BZLF1 immediate early and the abundantly expressed BALF2, BHRF1, BMRF1, and BMLF1 early proteins. Much of what was termed VCA reactivity is diffuse cytoplasmic fluorescence and is directed against virus-encoded nucleocapsid components, including BcLF1, BFRF3, BLRF2, and the glycoprotein gp110, which are expressed in late lytically infected cells. On the other hand, most MA reactivity, detected by immunofluorescence staining of living cells, is directed against gp350, the most abundant viral protein present both on the surface of lytically-infected cells and on the viral envelope. Although less frequently assayed than anti-EA and anti-VCA, anti-MA levels in serum are of particular interest because they tend to correlate with virus neutralizing activity.

For clinical diagnostic work, attempts have been made to replace the above assays with enzyme-linked immunosorbent assay (ELISA)-based or other tests using as substrates either individual EBV proteins expressed in recombinant form or synthetic peptide mimics of immunodominant epitopes. Several such assays have been developed for the detection of primary EBV infection, based on IgM antibodies to the BALF2 and BMRF1 components of EA or to the low molecular weight VCA components BFRF3 and BLRF2. Screening on combinations of such antigens is still recommended for

diagnostic accuracy (57,450).

Responses in Primary Infection

IM patients again provide the bulk of information available on the primary antibody response to EBV; what little is known about asymptomatic primary infections in young children suggests that their pattern of response is broadly similar (353). By the onset of clinical symptoms, most IM patients already have substantial titers of IgM antibodies to VCA and rising IgG titers both to VCA and to EA (most frequently, to the D component). IgA responses to these antigens may also appear transiently at this time, but this has not been systematically studied. The IgM anti-VCA response subsequently disappears either during convalescence or over the next few months, whereas IgG anti-VCA titers rise to a peak then may fall slightly over the ensuing months to a stable steady-state level. The IgG anti-EA response usually falls slightly faster and further than IgG anti-VCA and either becomes undetectable or stabilizes at a very low level (185). Virus neutralizing antibodies in acute IM tend to be of low titer and complement-dependent, consistent with antibodies to the major neutralization target antigen gp350 being predominantly of IgM class at this time, with IgG titers rising relatively late in the disease. A transient IgA response to gp350 is also detectable (430). Another characteristic and interesting feature of IM serology is the pattern of anti-EBNA reactivities. Many, but not all, patients in the acute phase of the disease show an IgG response to the EBNA2 protein (and probably also to the EBNA3A, 3B, 3C family of proteins, although these are more difficult to assay), whereas an IgG response to EBNA1 is not usually detectable until convalescence (190). Antibodies capable of recognizing EBNA1 in immunoblots have been detected in the IgM fraction of acute IM serum, but these are thought to be part of the autoantibody response (see later), with cross-reactivity against the glycine-alanine repeat domain of EBNA1 (372). Antibody responses to the latent membrane proteins LMP1 or LMP2 have not been seen in IM patients and indeed are only detectable in a small proportion of healthy carriers even using the most sensitive assays (139,300).

What role the humoral response plays in the overall control of the primary infection remains uncertain. Clearly, the virus-neutralizing components of the response (predominantly anti-gp350 antibodies) have the capacity to prevent generalized spread of the virus as a cell-free viremia; even here, however, the possibility has been raised that the IgA anti-gp350 response may actually facilitate virus spread to epithelial compartments (430). The delayed antibody response to EBNA1 remains difficult to understand. The original view, that this reflected the delayed destruction of latently infected cells *in vivo* and hence delayed availability of the antigen, is hard to reconcile with the observation that responses to EBNA2, another latent protein, are detectable during the acute disease (190), although they may not persist in the longer term. Possibly, the change in anti-EBNA reactivities between primary and persistent infection may reflect the fact that different types of latently infected cells predominate at these different times.

In addition to the previously described EBV-specific antibody responses, the early phase of acute IM is associated with generally elevated levels of total serum IgM, IgG, and IgA, again consistent with a virus-driven polyclonal activation of the B-cell system (185). Concomitant with this general increase is the transient appearance of a range of heterophile and autoantibodies, mostly of IgM class, which may also be products of virus-infected B cells (145). Of these various reactivities, heterophile antibodies with the capacity to agglutinate sheep and horse erythrocytes form the basis of the Paul-Bunnell-Davidsohn test that, when positive, is diagnostic of EBV-associated IM. Not all primary EBV infections become heterophile antibody-positive, however, and thus distinguishing EBV-associated IM from the IM-like illnesses caused by other agents, such as cytomegalovirus or toxoplasma, requires EBV-specific serology (185). The overall patterns of antibody responses seen during the acute and convalescent phases of IM are illustrated diagrammatically in Figure 3 relative to the virologic events (virus shedding in the throat, virus-infected B cells in the blood) occurring over the same time frame.

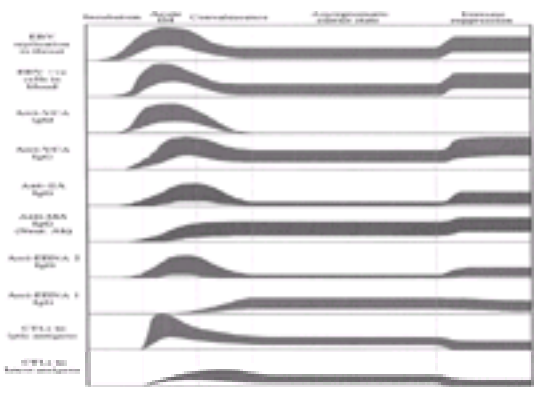

FIG. 3. Parameters of the EBV–host balance during the incubation, symptomatic and convalescent phases of acute infectious mononucleosis (i.e., primary EBV infection), during subsequent asymptomatic virus carriage and after the imposition of immunosuppressive therapy on an existing virus carrier state. For each parameter, the *shaded band* represents the broad range within which most infected individuals lie; where the band extends to the baseline, this indicates that the relevant responses are detectable in some, but not all, infected individuals.

Responses in Persistent Infection

Healthy virus carriers are consistently IgG anti-VCA positive, anti-gp350 neutralizing antibody positive, and anti-EBNA1 positive; the serologic picture in convalescent IM patients also gradually changes to assume this same profile (126,185). Titers can differ markedly among individuals, and such differences tend to be stable over time. A significant number of long-term virus carriers possess additional reactivities, for instance, to EA (most frequently to the R component) or to one or more of the other EBNA proteins, and these again are stably maintained. The basis for these individual differences is not known. Healthy individuals with anti-VCA, anti-EA titers at the top of the normal range are not necessarily those

showing the highest levels of virus replication, at least as measured by transforming virus titers in throat washings (507). Because antibodies to VCA, EA, and EBNA target intracellular antigen complexes, they seem unlikely to play a major effector role in recognizing and removing virus-infected cells *in vivo*. By contrast, anti-MA (anti-gp350) antibodies can bind to the surface of late lytically infected cells and also to virions, and they have the capacity to neutralize a broad spectrum of EBV strains. Such antibodies could therefore contribute to overall control of the established virus carrier state by preventing a recrudescence of viremia and, as discussed later, by helping to protect the immune host from superinfection with further strains of orally transmitted virus.

CELL-MEDIATED RESPONSES TO INFECTION

EBV has proved an important general model for studying the human cell-mediated response to virus infection. Key features in this regard are the ubiquity of the virus in human populations, its genetic stability within the infected host, its strong immunogenicity to the T-cell response, and the ability to follow both primary and memory phases of the response through prospective work on IM patients. Although many of the ground rules governing EBV-induced cellular responses were established in early work on healthy virus carriers (reviewed in 376), a more complete picture is now emerging that includes the events of primary infection (373).

Responses in Primary Infection

One of the key diagnostic features of IM is the presence of large numbers of “atypical mononuclear” cells in the blood. These are predominantly T lymphoblasts of the CD8+ subset but include some CD4+ T cells and also activated natural killer (NK)-like cells with the CD16 or CD56 markers.

The NK-like component, which displays broad-ranging killing of NK-sensitive targets in *ex vivo* cytotoxicity assays (441,468), may be the remnants of an innate immune response that is thought to act as a first line of defense against many types of virus infection. The paucity of studies of patients in the incubation period of IM means that little is known about the earliest cellular responses *in vivo*. However, challenging the PBMCs of uninfected individuals with large numbers of autologous LCL stimulators *in vitro* induces a potent activated NK-like response (477). Likewise, in experimentally infected PBMC cultures from nonimmune donors, a delay in the kinetics of B-cell outgrowth has been ascribed in some cases to a CD16+ NK cell–mediated response, and in others to the rapid induction of interferon-a (IFN-a) or IFN-g release (reviewed in 374). Only rarely, however, were such effects capable of preventing LCL outgrowth. Such findings imply that innate immune responses are of limited value, at least in the control of latent growth-transforming infections. The point is reinforced by observations on bone marrow transplant recipients following primary EBV infection when virus-associated lymphoproliferative lesions have appeared at times when NK cell function was intact but T-cell responses had not yet developed (13,289).

The nature of the highly expanded CD8+ T-cell response in IM has long been debated, with assays on LCL targets implying the presence of some latent antigen-specific cytotoxic T lymphocytes (CTLs) accompanied by other apparently irrelevant reactivities detectable on allogeneic LCL targets (441,468). A first indication of the possible magnitude of the virus-specific component was the markedly skewed Vb subset distribution seen within IM CD8+ T cells, with many of the preferentially expanded Vb subsets showing an oligoclonal pattern of T-cell receptor (TCR) usage (72). Such results were more consistent with an antigen-driven response than with “bystander” (441) or superantigen-driven (449) activations. Parallel studies focusing on the antigenic specificity of IM CD8+ T cells took advantage of the fact that several immunodominant EBV latent cycle epitopes (mostly drawn from the EBNA3A, 3B, 3C family of proteins; see Table 1, presented later) and their HLA class I restriction elements had already been identified from work with long-term virus carriers. Marked focusing of the EBV latent cycle-induced CTL response against the same limited number of viral epitopes was indeed apparent even at the height of primary infection (438). Studies of the CTL responses in young infants within months of primary infection led to a similar conclusion (456). However, when these EBV latent epitope-specific T cells in IM blood were visualized by the new technique of staining with HLA class I/peptide tetramers, responses to the most immunodominant EBNA3-derived latent cycle epitopes, such as B8/FLR, accounted for only 1% to 2% of the CD8+ T-cell pool (73); interestingly, limiting dilution assays with IM PBMC populations *in vitro* had given similar frequency estimates (438).

EBV antigen[a]	Epitope location	Epitope sequence[b]	HLA allele	EBV type specificity[c]
EBNA 1	407–417	HPVGEADYFEY	HLA-B35.01	1 + 2
EBNA 3A	458–466	RPPIFIRCH/QW	HLA-B35.01	1
	325–333	FLRGRAYGL	HLA-B8	1
	158–166	QAKWRLQTL	HLA-B8	1
EBNA 3B	416–424	IVTTSVIK	HLA-A11	1
	399–408	AVFDRKSDAK	HLA-A11	1
	244–254	RRARSLSAERY	HLA-B27.02	1
EBNA 3C	258–266	RRIYGLIEL	HLA-B27.02/45	1
	386–343	KEKVKQNAF	HLA-B44.02	1
	103–171	EEGVGSPQHW	HLA-B44.03	1 + 2
LMP2	426–434	CLGGLLTMV	HLA-A2.01	1 + 2
	419–427	TYGPVFMCL	HLA-A24.02	1 + 2
	200–208	IEDPPFNSL	HLA-B40.01	1 + 2
BZLF1	190–197	RAKFKQLL	HLA-B8	1 + 2
	256–217	SYNDRLRLL	HLA-B40.01	1 + 2
BRLF1	91–99	AENAGRDAC	HLA-B45	1 + 2
	109–117	YVLDHLIVV	HLA-A2.01	1 + 2
BMLF1	280–288	GLCTLVAML	HLA-A2.01	1 + 2
	397–405	DEVEFLGHY	HLA-B18	1 + 2

[a]Latent antigens are shown EBNAs 1, 3A, 3B, 3C, and LMP2; lytic antigens are BZLF1, BRLF1, and BMLF1.
[b]Epitopes are referred to in the text using the first three letters of the epitope sequence.
[c]For most of the lytic cycle antigens, conservation between type 1 and 2 strains is based on sequence data from limited numbers of virus isolates.

TABLE 1. *Examples of CD8+ T-cell epitopes in EBV latent and lytic antigens*

A different picture emerged when the analysis turned to include EBV lytic cycle antigens as potential targets. Memory T cells to a lytic cycle epitope (RAK from the immediate early protein BZLF1) had first been identified by peptide stimulation *in vitro* (45), but it was the study of IM T cells that revealed the presence of multiple CD8+ T-cell reactivities to epitopes from immediate early (BZLF1, BRLF1) and several early (BMLF1, BMRF1, BALF2) proteins of the lytic cycle. Examples of some of the best studied lytic cycle epitopes, their source antigens, and their HLA class I restriction elements are

shown in Table 1. Responses to such epitopes were clearly more abundant than latent epitope-specific responses whether detected in cytotoxicity assays using IM T-cell effectors *ex vivo* or by clonal outgrowth in *in vitro*–limiting dilution assays (437). When visualized by the appropriate HLA class I–peptide tetramers, the response in individual patients could show remarkable focusing on individual immunodominant epitopes. Thus, in HLA-B8–positive IM patients, regularly between 25% and 50% CD8+ T cells stain with the HLA-B8/RAK (BZLF1 epitope) tetramer (73). Likewise, in HLA-A2.01–positive patients, up to 25% CD8+ T cells are specific for one of two immunodominant A2.01 epitopes, YVL from the immediate early protein BRLF1 and GLC from the early protein BMLF1. Such findings strongly imply that EBV lytic antigen-specific reactivities make up the bulk of the highly expanded CD8+ T-cell pool in IM. Moreover, some of these immunodominant epitope-specific responses show highly restricted TCR usage (16) and clearly contribute to particular Vb expansions of the kind described earlier in IM blood (72). It is still too early to say whether immediate-early and early antigens of the virus lytic cycle are consistently immunodominant over late antigens as targets of the CD8+ CTL response because the analysis of potential late antigen targets has been mainly restricted to the viral glycoproteins; to date, only relatively weak reactivities to A2.01-restricted epitopes in gp350 and gp85 have been detected (242). The origin of the broad-ranging killing of LCL targets described in the earlier literature on IM T cells is now easier to explain. This lysis of HLA class I–mismatched targets proved to be through cross-reactive recognition of allo HLA–peptide complexes present on particular allogeneic LCLs and on EBV-negative lymphoblasts from the same donor (441). There are now several well-documented examples of such allo–cross-reactivity being mediated by EBV latent epitope-specific clones, including clonotypes that dominate responses *in vivo* (63,64,416), and a similar potential clearly exists for the even more highly expanded clonotypes recognizing immunodominant lytic epitopes (304).

In contrast to the wealth of information emerging on CD8+ T-cell responses in IM, still little is known about CD4+ responses. It is likely that such responses are present, although the only example to date of a defined HLA class II–restricted reactivity in IM involves an unusual CD4+, CD8+ T-cell clone specific for a BHRF1 epitope (493). From heteroduplex analysis of TCR usage in IM T cells, individual CD4+ reactivities are unlikely to show the massive expansion noted for dominant CD8+ clonotypes (295); however, a clearer picture should emerge as the identification of CD4+ T-cell epitopes proceeds and HLA class II–peptide tetramers are developed.

Resolution of the Primary Response

Most CD8+ T cells in IM blood, and indeed most cells within EBV-specific tetramer-positive populations, display a CD38+, HLA-DR+, CD45RO+ activated cell surface phenotype (73). These activated cells have low levels of bcl-2 and are highly susceptible to apoptotic cell death *in vitro* unless rescued by antigenic stimulation or cytokines of the interleukin-2 (IL-2), IL-7, IL-15 family (6,313). After acute IM, most of these CD8+ T cells die, presumably because antigen dose becomes limiting *in vivo*. The remaining tetramer-positive cells (operationally defined now as “memory”) mostly revert to a CD38–, HLA-DR– resting phenotype but show heterogeneous expression of CD45 isoforms, being found in both the RO+ and RA+ populations (73).

The controls that govern antigen-specific CD8+ T-cell selection into memory are poorly understood but, from prospective studies of IM patients, it is already clear that the composition of memory is not simply a scaled-down version of the primary response. Thus, the highly expanded EBV lytic cycle-specific populations in IM appear to be disproportionately reduced in number during memory selection compared with the less highly expanded latent cycle-specific populations (73). Indeed the percentage representation of some latent epitope-specific reactivities in the circulating $CD8^+$ Tcell pool may even go on rising during the first four months post-IM before stabilizing at a lower level; this is never the case for lytic epitope reactivities, which fail immediately post-IM. Interestingly, some of the TCR clonotypes that dominate the primary response to immunodominant lytic cycle epitopes such as A2.01/GLC or B8/RAK appear to be replaced by others in memory (16,422), whereas the clonotypic composition of the B8/FLR latent epitope-specific response appears not to change over time (71,423). These contrasts in the size and maturation kinetics of the lytic versus latent antigen-specific responses are illustrated in Figure 3 beneath the serologic profiles observed over the same time scale.

Responses in Persistent Infection

The existence of long-term T-cell memory to EBV latent cycle antigens was first apparent in experimentally infected PBMC cultures in which a complete regression of LCL outgrowth, mediated by *in vitro* reactivated CD8+ CTLs, occurred at 2 to 3 weeks but only in cultures from virus-immune (seropositive) donors (315). Subsequent analysis of LCL-stimulated bulk T-cell lines and derived clones showed that EBV latent antigen-specific memory is usually dominated by T cells reactive to epitopes derived from the EBNA3A, 3B, 3C family of proteins (236,319). Responses to LMP2-derived epitopes, although usually subdominant, are the next most frequent reactivity in such T-cell preparations, whereas responses to EBNA2, EBNA-LP, LMP1, and especially EBNA1 are rare. Very weak responses have also been detected to an epitope within one of the predicted BamHI A–encoded polypeptides BARF0 (246). This marked hierarchy of immunodominance among latent cycle proteins, seen in a wide range of donors with different HLA class I types (236,319), implies some form of differential access of the EBNA3 proteins to the proteasomal degradation through which most HLA class I epitope peptides are generated. Examples of some of the best characterized immunodominant latent cycle epitopes, their location within latent proteins, and their HLA restriction elements are shown in Table 1. Note that these epitopes were almost all identified through studies of European and North American donors infected with the type 1 virus strains that are prevalent in the Western world. Although such epitopes are conserved in most Western type 1 strains, sequence divergence between the EBNA3 family proteins of type 1 and type 2 viruses means that many of the immunodominant EBNA3-derived epitopes are not present in type 2 strains (314); still relatively little is known about the identity of type 2 virus epitopes (376).

What determines epitope immunodominance in the context of any one HLA class I allele is still not fully understood. In the case of the HLA-A11–restricted epitopes IVT and AVF, both of which are derived from EBNA3B, the consistent numeric dominance of IVT-reactive over AVF-reactive CTLs in A11-positive individuals appears to correlate with a higher representation of A11–IVT complexes at the LCL surface and with the induction of a clonotypically more diverse T-cell response to this more abundantly expressed epitope (106,276). However, not all immunodominant latent cycle epitopes are abundant at the cell surface (99) and not all induce clonotypically diverse responses (19). Indeed, the fact that T cells representing numerically dominant components of EBV-specific memory frequently kill LCL targets poorly in cytotoxicity

assays (197,416) implies that LCL-like cells may not be the antigen-presenting cell that primes these responses *in vivo*. Important in this context is the example of EBNA1, an antigen that was never recognized as a preferred target by LCL-restimulated CTL preparations. In its endogenously expressed form, EBNA1 is protected from presentation by the conventional HLA class I pathway by a large internal glycine-alanine repeat (GAr) domain (274). Recent work suggests that this protection, which can be transferred to any protein by GAr domain insertion, operates by preventing proteasomal degradation following the ubiquitination step that usually target proteins for breakdown (275,414). Although in chimeric proteins the strength of protection is influenced both by the nature of the recipient protein and the length of its GAr insert (101), the effect of the large native GAr domain in EBNA1 itself appears to be extremely robust and renders endogenously expressed EBNA1 immunologically silent as far as CD8+ T-cell responses are concerned. Yet CD8+ T cells specific for EBNA1 epitopes are nevertheless induced *in vivo* (43) and in some cases can be as abundant as responses to conventionally processed EBNA3 epitopes, both in primary infection and in memory (42). This strongly implies that antigen-presenting cells other than naturally infected B cells must prime the CD8+ T-cell response *in vivo*, possibly dendritic cells, which have the capacity to take up viral antigens (including EBNA1) released from infected cells and re-present their derived epitopes through the HLA class I pathway.

Latent epitope specificities appear to be stably maintained for life in the circulating T-cell pool of virus carriers. Staining with HLA class I–peptide tetramers indicates that T cells specific for the immunodominant B8/FLR or A11/IVT epitopes can account for 0.1% to 1% of CD8+ T cells in the blood. These values are on average fourfold higher than estimates of epitope-specific memory cell numbers obtained in ELISPOT assays of rapid peptide-induced IFN-g release, and 20-fold higher than apparent in limiting dilution assays requiring the *in vitro* outgrowth of clones with epitope-specific cytotoxic functions (458). Using similar approaches, one can show that virus carriers also maintain CTL memory to the immunodominant lytic cycle epitopes (16,45,73,437,458). Their frequencies often exceed those seen for latent epitope-specific memory and, by tetramer staining, can reach as high as 5% circulating CD8+ T cells in exceptional cases. Such a large commitment of the T-cell system to the control of a particular virus probably reflects the persistent nature of EBV infection and the fact that it is harbored within B lymphocytes. When responses to one of the more immunodominant EBV epitopes involves highly focused TCR usage (19), this can impact significantly on the TCR repertoire available within the circulating CD8+ T-cell pool (424); furthermore, when the epitope-specific receptor shows allo–cross-reactivity, as in the case of the dominant B8/FLR-specific TCR cross-reacting with HLA-B44.02, preexisting EBV-specific memory can profoundly influence the strength of allo-responsiveness to the HLA allele (63,64).

Information on CD4+ T-cell memory to EBV is still rudimentary by comparison, although such cells may well be important both for the maintenance of CD8+ T-cell memory and also as effectors in their own right. Until recently, reports of CD4+ responses to latent cycle antigens were limited to rare clones found within LCL-reactivated CTL preparations (376). A more systematic analysis stimulating CD8+ T-cell–depleted PBMCs with autologous dendritic cells expressing individual latent proteins from recombinant vaccinia vectors (318) or with peptide pools (269) is revealing a hierarchy of CD4+ memory T-cell responses with EBNA1, and to some extent EBNA3C (367), as rich sources of epitopes. Some of these reactivated cells have cytotoxic function in addition to their capacity for cytokine (IFN-g, tumor necrosis factor-a [TNF-a]) release. An important issue is the potential of such cells to recognize endogenously expressed (as opposed to exogenously acquired) latent cycle proteins (318) because this could offer a means of targeting EBV-positive tumors that is complementary to that begin pursued with CD8+ CTLs.

OVERVIEW OF THE VIRUS–HOST INTERACTION

Virus Infections and Their Immune Control

Figure 4 attempts to present the virus–cell interactions thought to be involved during primary infection, in the establishment of latency and in subsequent reactivation, alongside the cell-mediated immune responses to viral infection occurring at these different times.

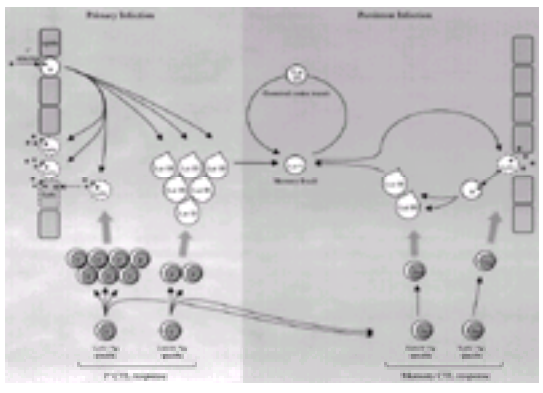

FIG. 4. A provisional model of EBV–cell interactions during primary infection and persistence. The model restricts itself to infections of the major target tissue, the B-lymphoid system and to potential infections of mucosal epithelium; lined arrows represent movements of cells, dotted arrows represent movements of infectious virions. Also shown is the cytotoxic T-lymphocytic arm of the immune response to lytic and latent cycle antigens; *broad shaded arrows* represent cytolytic effector function. Supporting data are cited in the text.

An initial focus of virus replication occurring at the site of transmission in the oropharynx may involve locally infiltrating B cells as the primary target. It is possible that virus-induced activation to a lymphoblastoid phenotype is a prerequisite for subsequent entry of these cells into lytic cycle within mucosa-associated lymphoid tissue. Certainly, proliferating LCL-like cells with a Latency III form of infection arise within tonsillar tissue during primary infection and are detectable in the

circulating B-cell pool.

Both lytic and latent infections induce CD8+ CTL responses, which, in the context of IM, are highly magnified and produce the exaggerated CD8+ T-cell lymphocytosis characteristic of this disease. The lytic cycle-induced response is an order of magnitude higher than that seen against latent antigens. This probably reflects the higher antigenic loads generated during virus replication and the ready accessibility of lytically infected B cells and their products for re-presentation to the CD8+ repertoire by dendritic cells; virus replicative events that are entirely restricted to mucosal epithelium (e.g., human papillomavirus infection) are unlikely to be so immunogenic. The relatively rapid reductions in oropharyngeal viral shedding and in the load of latently infected B cells in the blood seen in convalescent IM patients imply effective CTL-mediated lysis of the two previously mentioned target populations. As antigen load falls, activated CTL numbers are culled by apoptosis, especially those cells involved in the highly amplified lytic antigen response. The CD4+ T-cell response is probably also important at this time as a source either of effector cells or of cytokine help for the massive CD8+ T-cell expansion. A role for the antibody response during primary infection is less obvious because virus neutralizing antibodies, mainly directed against gp340, are slow to develop.

The establishment of a sustainable reservoir of latently infected B cells in the now immune host requires down-regulation of the growth-transforming Latency III program of viral gene expression, because a reservoir of LCL-like cells would almost certainly be cleared by CTL surveillance. The low numbers of infected cells detectable within the blood of virus carriers are in fact resting B cells in which viral latent antigen expression is largely, perhaps completely, extinguished; the fact that they carry the virus genome as a covalently closed episome (110) strongly suggests that such cells are the progeny of earlier viral transformation events. Evidence that cells can switch from the growth-transforming Latency III program to more restricted forms of viral gene expression, and that this coincides with a change in host cell phenotype, has come from *in situ* analysis of infected B cells in IM tonsils. However, the precise sequence of events remains to be determined. Interestingly, all EBV-infected cells in the blood of virus carriers lie within the memory (IgD–) B-cell subset, yet the initial viral infection in IM can presumably target both the naive and preexisting memory B-cell populations. Therefore, either transformants of memory B-cell origin are preferentially able to reenter the resting memory B-cell pool or viral transformation itself can impose a memory phenotype on any infected cell that subsequently enters the pool. The evidence strongly suggests that this pool of resting memory B cells is necessary and sufficient for virus persistence in the immune host. Thus, some bone marrow transplant recipients who undergo enough total body irradiation to destroy their lymphoid system (but leave other tissues such as mucosal epithelium relatively intact) are cleared of their resident EBV infection (161).

It must be remembered, however, that persistently infected individuals also show chronic virus replication at mucosal sites. The most likely source of this shed virus is through reactivation from the preexisting B-cell reservoir. The controls governing reactivation are not understood, but one clear possibility is that the virus exploits the natural events of the B-cell life cycle for this purpose. In this context, memory B cells, when reexposed to cognate antigen in lymphoid tissues, will take one of two routes (366). One option is to reenter a germinal center reaction and, through another round of somatic *Ig* gene mutation and selection, generate more memory cells. An EBV-infected cell taking this route has the potential to replenish the EBV-carrying memory B-cell pool if certain conditions are met; EBNA1 synthesis would be required if the virus genome is to be maintained during clonal expansion in the germinal center, whereas transient activation of LMP1 and LMP2 expression at the centroblast stage may be required to guarantee reselection of some infected cells back into memory. The second option for a memory B cell reexposed to cognate antigen is to differentiate to an antibody-producing plasma cell, some of which are known to locate at submucosal sites. An EBV-infected cell taking the pharmacyloid route of differentiation may activate lytic gene expression, thereby guaranteeing virus production at sites from which transmission of infectious virions to a new host can occur. These postulated events would be consistent with the observed patterns of viral gene transcription in different tonsillar cell subpopulations (25) and with the implication that chronic antigenic challenge to the B-cell system can influence the EBV–host balance in favor of the virus (17).

The relationship between this ongoing low-grade virus replication at lymphoepithelial sites and the circulating reservoir of latently infected resting B cells remains to be resolved. It is possible that new growth-transforming infections of host B cells, initiated at sites of virus replication, continually serve to replenish the memory cell pool in a recapitulation of the events of primary infection. Were that the case, long-term treatment with a drug such as acyclovir (ACV), which blocks virus replication, might be expected to lead to a slow reduction in the size of the latently infected cell reservoir. Experiments of this kind have so far not detected any such reduction over a 4-week period (505,506), but it will be necessary to follow patients receiving ACV for much longer times to examine the issue critically.

These multiple pathways of reactivation from latency are of course occurring in the immune host. It is likely that CTL surveillance continues to control reactivations into lytic cycle. Indeed, the strength of CTL memory to immediate-early and early lytic cycle antigens implies that many productive infections are contained before the cells can complete the cycle and release progeny virions. This could explain why RT-PCR assays of lytic gene transcription and immunofluorescence assays of lytic antigen expression on *ex vivo* samples so often only detect cells in the immediate-early and early stages of the cycle (330,361). *De novo* transformation events are also likely to be recognized by CTLs once the full spectrum of latent cycle proteins is expressed and the cells acquire a lymphoblastoid phenotype. In principle, cells in which a latency II form of infection (EBNA1, LMP1, LMP2+) has been activated would also be subject to T-cell control, but here it must be remembered that endogenously expressed EBNA1 is protected from recognition and that both LMP1 and LMP2 elicit at best only subdominant CD8+ responses. The capacity of such responses to recognize and kill target cells will be critically dependent on the level of expression of the target protein and the antigen-processing capacity of the infected cell.

Virus Evasion Strategies

Viruses have evolved multiple different strategies for evading host immune responses. For herpesviruses, which are persistent but genetically stable pathogens, these frequently involve an ability both to establish an antigenically silent form of latent infection and to impair the antigen-processing capacity of lytically infected cells (356). EBV has successfully achieved the former in that viral antigen expression is largely if not completely down-regulated in the

circulating B-cell pool. Whether EBV replication employs immune evasion mechanisms analogous to those shown by other herpesviruses in lytic cycle is still not known. There are, however, other strategies that appear unique to EBV.

The most obvious is the ability of the GAr domain to protect endogenously expressed EBNA1 from proteasomal processing and presentation to CD8+ T cells (101,274,275,414). This protection only operates in *cis* and has no effect on the presentation of coexpressed latent cycle proteins. It is in fact a moot point whether the acquisition of the GAr domain by EBNA1 is primarily an immune evasion strategy or a mechanism for ensuring the stability of a protein that is crucial for persistence of the viral genome in infected cells. The ability of EBNA1 to tether the viral episome to cell chromatin (see Chapter 74 [245]) may be important not just in proliferating cells but also arguably (using preformed EBNA1) in the long-lived resting memory B cell. Interestingly, the GAr-mediated protection from immune processing may be a recent evolutionary acquisition because the shorter (52 to 62 amino acid) glycine-serine-alanine repeats seen in the EBNA1 homologs of Old World primate LCVs apparently lack this function (44).

None of the other EBV latent cycle proteins have obvious immune evasion strategies; indeed, one of the many cellular changes induced by LMP1 in B cells, mirroring those induced in B cells by CD40 ligation (241), is to up-regulate expression of HLA class I molecules and of the TAP1/TAP2 transporter involved in furnishing these molecules with antigenic peptides (387). Such changes are to the evolutionary advantage of the virus. Thus, EBV's initial requirement for expansion of latently infected B cells in the naive host through growth transformation needs to be balanced against the threat of fatal lymphoproliferative disease were that expansion not to be recognized by the host's immune response.

The other novel routes whereby EBV might modulate immune responses all involve products of the lytic cycle. The first involves the gp42 glycoprotein, encoded late in the cycle by the BZLF2 gene and forming part of a tri-molecular complex with gp85 and gp25 on the surface of infected cells and on the viral envelope. Binding of gp42 to the b-chain of HLA-DR class II molecules appears to be involved in mediating viral entry into HLA class II–positive B cells after the initial gp350–CR2 attachment step (278). Recent work showed that gp42 complexes with nascent HLA-DR molecules in the endoplasmic reticulum of lytically infected B cells *in vitro* (46), raising the possibility of some interference with HLA class II maturation or epitope presentation to CD4+ T cells.

A second lytic cycle protein, BARF1, is naturally secreted from infected cells and functions as a soluble receptor for human colony stimulating factor-1 (CSF-1) (442). Both recombinant and naturally produced BARF1 are capable of interfering with at least one of the activities of CSF-1, namely the poly(I:C)-induced release of IFN-a from monocytes (91). Such interference could be important *in vivo* in view of the role that IFN-a may play in the early host response to EBV infection, either through its direct antiviral action or through activating NK cells.

Third, in common with certain other gamma herpesviruses, EBV encodes a viral homolog of cellular IL-10. This viral IL-10 (expressed by the late lytic gene *BCRF1*) retains the ability of the cellular cytokine to augment B-cell growth and differentiation and to inhibit antigen presentation by monocytes and dendritic cells, but apparently not its ability to directly impair T-cell function (282). At sites of virus replication and *de novo* infection, therefore, locally produced viral IL-10 could inhibit the priming of both lytic cycle-specific and latent cycle-specific T-cell responses, the latter effect having been seen experimentally in the *in vitro* regression assay (33). Other potential important effects of viral IL-10 may be mediated at the level of the *de novo*–infected B cell, both in transiently reducing the cell's antigen-processing capacity (516) and in enhancing the proliferative response to infection (443). Note that the *in vivo* relevance of all three potential immunomodulatory proteins is unknown and will become clear only when the appropriate gene knock-out viruses can be tested in an informative animal model.

Virus–Host Coevolution

EBV, like the LCVs of other primates, has a long history of coevolution with its host species. Evidence for such coevolution is apparent even within the 100,000 years since *Homo sapiens* radiated out of Africa. Thus, EBV isolates from modern-day European, Asian, and African populations are distinguishable at a number of polymorphic sites in the genome, reflecting the long period over which these host populations have been geographically isolated. Genetic comparisons between EBV and other LCVs (484), and to some extent between the geographically distinct EBV strains themselves (see later), indicate that the latent protein-coding sequences have evolved more rapidly than other regions of the genome; the reasons for this are not understood.

There are two broad types of EBV, types 1 and 2 (originally called A and B), distinguished by allelic polymorphism of their *EBNA2, 3A, 3B,* and *3C* genes (3,100,397). Interestingly, this may reflect an ancient subdivision within the LCV genus rather than a more recent branch of EBV evolution because rhesus LCV isolates also fall into two types based on polymorphism of their *EBNA2* homolog (87). The type 1 and type 2 versions of the EBNA2, 3A, 3B and 3C proteins, sharing 55%, 84%, 80% and 72% amino acid identity respectively, induce host antibody responses which for EBNA2 and 3A can be partially cross-reactive between types and which for EBNA3B and 3C are predominantly type-specific (391,411). As shown in Figure 5, immunoblotting of LCL extracts with the serum of a type 1 virus–infected individual (in this case with type 1–specific EBNA2, 3B, and 3C antibodies, weakly cross-reactive EBNA3A antibodies, and pan-EBNA1 antibodies) can clearly identify the type of the resident virus. Furthermore, individual type 1 strains can be distinguished through the different sizes of their EBNA1, 2, 3A, 3B, and 3C proteins, constituting a characteristic “EBNA print” for each individual strain (159,509). This size polymorphism largely reflects the facts that all the EBNA proteins contain repeat regions and that the precise number of repeats varies between virus strains (131). Less work has been done on the EBNA prints of type 2 virus isolates owing to the paucity of informative sera, but it appears that the type 2 EBNA2, 3A, 3B, and 3C proteins are less variable in size than their type 1 counterparts (391).

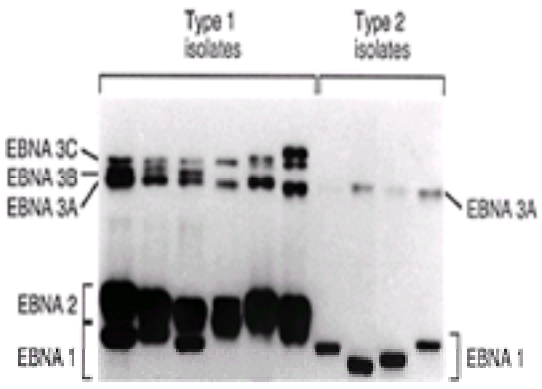

FIG. 5. EBNA printing of EBV isolates. Protein extracts of B-lymphoblastoid cell lines (LCLs) carrying individual (type 1 or 2) virus isolates were separated by gel electrophoresis and immunoblotted using a polyclonal human serum (from a type 1 virus–infected donor) with antibodies to EBNA 1, 2, 3A, 3B and 3C. Antibody cross-reactivity against the corresponding proteins in type 2 virus–carrying LCLs is restricted to EBNA1 and (weakly) to EBNA3A.

Based on virus isolates rescued as LCLs *in vitro*, about 90% of seropositive individuals in European populations carry type 1 and about 10% carry type 2 virus strains (157,160,509,518). Although the more potent *in vitro* growth-transforming activity of type 1 strains (377) could bias *in vitro* isolation in their favor, the balance of evidence from direct PCR amplification on *ex vivo* samples (428,472,475) still favors the greater prevalence of type 1 strains in most Western societies. In contrast, type 2 strains appear to have reached almost equal prevalence with type 1 in the general population in parts of equatorial Africa and in New Guinea (5,515,518). It may be that type 2 viruses, which appear slightly less efficient as B-cell–transforming agents, are better able to compete in circumstances in which high levels of parasitemia provide a preexisting chronic stimulus to the host B-cell system.

Several other polymorphic loci have now been identified in the EBV genome that are not necessarily linked to virus type. These lie within the *EBNA1* (39,171), *EBNA2* (5,408), *EBNA3* (243), *EBNA-LP* (135), *LMP1* (117,202,399), and *LMP2* (65,268) genes as well as at other sites (2,290). Many are markers of geographic origin, distinguishing for instance among European, Asian, and African strains, but in other cases, they can discriminate between strains from the same area (171,399,408). These polymorphisms are cited extensively in the context of two current debates in the EBV field. One concerns the possibility that particular virus strains have enhanced pathogenic and oncogenic activity; we shall return to this issue in the relevant disease sections of this chapter. The second concerns the possibility that immunologic pressure has influenced the long-term evolution of EBV within particular host populations. This idea was first raised by the observation that in Southeast Asian populations, in which the HLA-A11 allele is unusually common, the prevalent type 1 EBV strains show a variety of mutations in the *EBNA3B* gene that always destroy the immunodominant A11-restricted IVT epitope and frequently also destroy the subdominant AVF epitope (104,105). However, subsequent work has questioned the attribution of these changes to immune selection because similar mutations were also present among viruses from New Guinea Highland populations, which are entirely A11-negative, as well as in Lowland groups, in which A11 incidence is similar to that of other Southeast Asian populations (61,243). To date, no other potential examples of the population-driven selection of CTL epitope-loss variants have been found.

INFECTION IN THE T-CELL–IMMUNOCOMPROMISED HOST

Studies of transplant recipients and human immunodeficiency virus (HIV)-infected patients have helped to document the effects of T-cell impairment on the EBV–host interaction. In both cases, it is important to distinguish between the majority of patients (mainly adults) in whom iatrogenic or HIV-mediated immune impairment is imposed on a preexisting EBV carrier state, and the minority (mainly children) in whom T-cell competence is already impaired at the time of primary EBV infection. Effects on the virus–host interaction can be monitored using two parameters, described in the following sections.

Deregulation of the Virus–Host Balance

Early studies showed that the relatively low levels of immunosuppressive therapy used to maintain long-term renal allografts not only reduced EBV-specific CTL responses as measured in the *in vitro* regression assay but also increased viral shedding in the throat and the numbers of infected B cells in the blood (97,508). More recently, quantitative PCR assays have confirmed the increase in EBV genome load in the PBMCs of asymptomatic solid organ transplant recipients (27,29,248,386), with pediatric patients most likely to give values clearly above the normal range. Although such assays may prove useful in clinical monitoring, it is not clear to what extent higher virus genome loads reflect an increase in the frequency of latently infected cells in the blood and to what extent they reflect the detection of progeny genomes in lytically infected B cells. Recent work on adult patients soon after solid organ transplant reported frequencies of infected cells (1 to 1,670/10^6 B cells) that clearly extended beyond the normal range (1 to 50/10^6 B cells) but showed that, in some cases, although not necessarily those with the highest frequencies, both latently infected and lytically infected cells were present (23). Interestingly, the latently infected cells that accumulated in the blood after transplantation were not virus-transformed lymphoblasts with a Latency III form of infection, as might have been anticipated, but resting B cells that showed the same highly restricted form of latency as seen in healthy carriers (23). Arguably, the relaxation of T-cell control allows quantitative shifts in the virus–host balance in both blood and lymphoid tissues, but the resident forms of infection do not change.

Studies of HIV-infected patients with detectable T-cell impairment in the EBV-specific *in vitro* regression assay, but not yet with symptoms of acquired immunodeficiency syndrome (AIDS), have shown similar shifts in the EBV–host balance, although once again cohort values still overlap the normal range (115,231,504,511). Also, the analysis of lymphoid tissues from HIV-positive patients with persistent generalized lymphadenopathy has revealed only moderate increases in

the number of EBV-infected cells; these were again predominantly small EBER-positive B cells within extrafollicular areas, and only in a few cases could EBNA2-positive, LMP1-positive immunoblasts or evidence of virus-infected germinal center cells be detected (48,340). There is no obvious link, therefore, between EBV and the follicular hyperplasia characteristically seen early in HIV carriage.

The increased virus load seen in EBV carriers after iatrogenic or HIV-induced immune suppression is also reflected in serum antibody profiles. In both groups, existing IgG anti-VCA and anti-MA titers rise, and almost all patients become detectably anti-EA positive (508,511), presumably in response to the increased antigenic load, whereas anti-EBNA1 titers do not change. In more immunocompromised patients such as those with progressive HIV infection, antibodies to EBNA1 may fall and anti-EBNA2 antibodies increase, leading to an inversion of the usual anti-EBNA1–to–anti-EBNA2 ratio (190,498). These virologic and immunologic changes seen in asymptomatic virus carriers with onset of T-cell suppression are illustrated diagrammatically in Figure 3.

The effects of immune impairment are often more dramatic in bone marrow transplant recipients, where T-cell function is more strongly suppressed, and in seronegative transplant recipients (usually children), who often acquire their primary EBV infection iatrogenically either from the transplant itself or from blood transfusions (reviewed in 157). In bone marrow transplant recipients, EBV DNA loads in PBMCs assayed by PCR can reach very high values, especially in the first 3 to 6 months after transplantation (169,381,386) when there is a high risk for B-lymphoproliferative disease (see later). Primary EBV infection has been less well studied in HIV-positive children but appears to be more frequently symptomatic than in controls (353). It is also associated with higher rates of oral shedding, which is sustained in children who progress rapidly to AIDS (217), and in some cases, is characterized by an atypical antibody response. HIV-infected infants appear to be particularly susceptible to another EBV-associated B-lymphoproliferative condition, lymphocytic interstitial pneumonitis (15).

Multiplicity of Virus Strains

Isolations of EBV from bone marrow and cardiac transplant recipients and characterization of the derived LCLs by EBNA printing identified a significant proportion of patients carrying more than one viral strain (160), a situation seen only rarely among isolates from healthy immunocompetent individuals. It was not clear, however, whether this simply reflected the iatrogenic acquisition of new viral strains from donor B cells in the graft or in blood transfusions. Subsequently, studies of two other immunocompromised groups have suggested that T-cell impairment genuinely does sensitize the host to serial acquisition of EBV strains by the natural route. Thus, a group of young hemophilic patients, who had acquired HIV by contaminated factor VIII, and an HIV-positive male homosexual cohort both showed multiple co-resident EBV strains in 30% to 50% of individuals studied (504,511). Because these patients all had high serum antibody titers to EBV antigens (including neutralizing antibodies), the implication is that humoral immunity alone is insufficient to protect the host from additional serially acquired EBV infections. This is also the lesson drawn from clinical experience, where infusions of pooled human Ig were unable to prevent primary EBV infection (337).

In the above studies, all based in Western countries, both the transplant recipients and the HIV-positive hemophilia patients, whether singly or multiply infected, gave predominantly type 1 virus isolates; the overall incidence of detectable type 2 virus infection was no more than 10% (160,511), a figure broadly in line with that for the general population. By contrast, the HIV-positive homosexual cohort showed a more than 30% incidence of type 2 virus infection (504). This was consistent with earlier studies of HIV-positive patients, again mainly male homosexuals, which found an unexpectedly high frequency of type 2 virus-specific antibody responses (412), of type 2 virus isolations from the blood (410), and of type 2 viral DNA in oral lesions (479). Contrary to earlier suggestions, therefore, high type 2 virus prevalence is not associated with the immunocompromised state per se. Rather, type 2 strains have become unusually prevalent in homosexual communities in the West (472), from which most of the early HIV-positive study cohorts were drawn; this likely reflects the multiplicity of close contacts within these communities. One interesting consequence of the frequent co-infection of HIV-immunocompromised patients with type 1 and type 2 EBV strains is the greatly increased chance of intertypic recombination. Indeed, virus strains with a type 1 allele at the EBNA2 locus and type 2 alleles at the EBNA3A, 3B, 3C loci were isolated from 2 of 40 patients in an HIV-positive homosexual cohort (510). Such recombinants have never been seen in the general population in the West, although rare intertypic strains have been identified among isolates from New Guinea and China (62,301).

The frequency with which multiple infection occurs in immunocompetent EBV carriers remains to be resolved. It is clear that most individuals carry the same dominant strain over many years (509), in contrast to the changing patterns of dominance sometimes seen in prospective studies of HIV-positive patients (58). However, direct amplification of EBV DNA from *ex vivo* samples is a more sensitive assay than *in vitro* virus isolation and may yet reveal a higher incidence of co-resident strains in healthy carriers than is currently apparent (54,428).

DISEASES ASSOCIATED WITH EPSTEIN-BARR VIRUS

Infectious Mononucleosis

A proportion (in some studies up to 50%) of serologically confirmed primary infections in adolescence or early adult life manifest as IM. The virologic and immunologic events occurring in IM patients have already been discussed. Disease symptoms can range from mild transient fever to several weeks of pharyngitis, lymphadenopathy, and general malaise. The viral etiology of IM and the fact that EBV replication was detectable at high levels in the oropharynx of acute IM patients prompted clinical trials of ACV, a nucleoside analog that blocks lytic EBV infection *in vitro* through inhibition of the viral DNA polymerase. Although oral ACV clearly can block viral shedding in the oropharynx of IM patients, it did not reduce the load of latently infected B cells in the circulation as measured by *in vitro* outgrowth (506) and had little if any effect on the disease course (14). Disease symptoms are therefore unlikely to be the result of virus replication per se and indeed recent studies have indicated that subclinical primary infection in young adults may still involve high viral loads but are not accompanied by T cell expansion (424a). IM is therefore most likely to be an immunopathologic disease whose general symptoms are caused by proinflammatory cytokines, such as IL-1, IFN-g, and TNF-a, known to be

produced at high levels by the large numbers of reactive T cells seen infiltrating all tissues throughout the body (11,328,474). In addition, complications such as interstitial nephritis, hepatitis, and pneumonia have been linked to the combined effects of infiltration by EBV-infected B cells and reactive T cells.

It is not known why EBV causes IM in adolescence when primary infections in childhood are almost always asymptomatic. One possibility is that much higher doses of infectious virus are likely to be transmitted by an oral route to adolescents than to children. This may greatly increase the chances of the pool of infected B cells exceeding a threshold and driving the expansion of immune T cells to a point at which resultant cytokine levels are pathogenic. In this context, reported examples of postperfusion mononucleosis, a disease identical to classic IM but induced by the transfusion of seropositive donor blood to a seronegative recipient, frequently involve cases in which the donor happened to be in the incubation phase of IM, that is, in which an unusually large number of EBV-infected B cells are likely to have been transfused (148).

X-linked Lymphoproliferative Syndrome and Fatal Infectious Mononucleosis

Two papers appearing in the mid-1970s first drew attention to a rare familial condition, subsequently called the *X-linked lymphoproliferative syndrome* (XLP), affecting young boys and characterized by extreme sensitivity to EBV (30,362). The recent cloning of the *XLP* gene from the Xq24-25 locus (90,327,403) has given new impetus to unraveling the complex pathogenesis of this syndrome in its various clinical guises (reviewed in 448). Some 50% to 60% of cases present in childhood with hyperacute IM-like symptoms and a serologic profile consistent with primary EBV infection. Thereafter, the disease rapidly takes a fatal course, culminating in liver failure as a result of widespread lymphocytic infiltration and hepatic necrosis. Of the minority who survive the acute primary EBV infection, many subsequently develop marked immunodeficiency, hypogammaglobulinemia being the most common symptom; some 30% of XLP patients present in this way. A third aspect of the XLP phenotype, accounting for about 20% cases, is a high susceptibility to B-cell lymphoma. Perhaps surprisingly, these tumors, usually of Burkitt's or diffuse large cell type, are often EBV genome-negative and indeed have been seen to develop in boys who are still EBV-uninfected both by serologic criteria and by PCR screening for EBV DNA (52,440).

Early studies focusing on the fatal primary infection detected, as in IM itself, the coexistence of virus-infected B cells and reactive CD8+ CTLs in the circulation (448), although the virus specificity of the CD8 response has not yet been ascertained. From this point, the disease course follows one of two paths. In some cases, especially where immunosuppressive therapy has been attempted, EBV-transformed B lymphoblasts become dominant (130), and the patient dies of organ failure arising from lymphoblast infiltration. Similar B-cell proliferations have been observed in heavily immunosuppressed transplant recipients (see later) and also in very rare nonfamilial cases of fatal IM where, for unknown reasons, individuals fail to mount a CD8+ T-cell response to the virus (98,309,380). In many other cases of XLP, however, EBV-infected B cells are outnumbered by a massive proliferation of reactive T cells, culminating in widespread tissue necrosis and the repopulation of lymphoid tissues and bone marrow with phagocytic cells (histiocytes) (448); the result is a clinical and histologic picture essentially indistinguishable from virus-associated hemophagocytic syndrome (VAHS, see later). Furthermore, the lymphoid aplasia that characterizes the later stages of this process appears likely to underlie the hypogammaglobulinemia, which most survivors subsequently display.

It appears that the cell-mediated immune response to EBV is somehow deregulated or ineffective in XLP patients. The molecular mechanisms underlying these effects will no doubt soon emerge with the identification of the XLP gene product as a small SH2 domain-containing protein, currently called SH2D1A (90) or SAP (403), involved in lymphocyte signaling. This protein, which is preferentially if not exclusively expressed in T and NK cells, was first shown to bind to the intracytosolic domain of CD150 (SLAM), a cell surface molecule expressed on both B and T cells and mediating B-cell–T-cell co-stimulation through homotypic interactions (403). More recently, SH2D1A has also been shown to be involved in other lymphocyte-signaling pathways, in particular signaling through 2B4, a molecule expressed at the surface of NK cells and some CD8+ T cells. It appears that NK cell function, besides being controlled by the balance of activating and inhibitory receptors, in some circumstances also requires licensing through 2B4 signaling. In the absence of the SH2D1A regulator, ligation of 2B4 through its natural ligand CD48 on a target cell can inhibit rather than activate NK lysis (347). It seems possible that 2B4 and SH2D1A could also play a similar role in licensing CD8+ T-cell function and, if so, that the fatal IM syndrome stems from a functional impairment of both virus-specific CD8+ T cells and NK cell effectors responding to EBV infection. Importantly, the natural ligand for 2B4 is CD48, a molecule preferentially expressed on activated B cells and indeed induced on resting B cells after EBV infection (136). The apparently specific sensitivity of XLP patients to EBV and not to other common viruses may therefore reflect the fact that EBV preferentially infects and activates B cells, whereas the sensitivity to B-cell lymphoma may be a consequence of some more fundamental deregulation of B-cell–T-cell communication or B-cell–NK cell recognition.

Virus-Associated Hemophagocytic Syndrome

Hemophagocyte syndromes, grouped together on histologic grounds through their common features of prominent histiocytic proliferation in tissues and phagocytosis of blood cells, have multiple etiologies of which viral infection is only one. However, many childhood cases of VAHS are now firmly linked to EBV, with the patients showing serologic profiles (anti-VCA IgM, and IgG-positive, anti-EBNA1–negative) diagnostic of a primary infection (454). The disease, most commonly seen in Japanese and Chinese populations, is characterized by fever, lymphadenopathy, hepatosplenamegaly, hemophagocytosis in various tissues, and a tendency to pancytopenia, often leading to a fatal outcome. A similar condition, reported as Kawasaki-like disease, was shown to be associated with a rare EBV-infection of T cells (247). This prompted several studies, all of which confirmed the presence of oligoclonal or even monoclonal populations of EBV-infected T cells in the blood or tissues of VAHS patients (reviewed in 338 and 444). Both major T-cell subsets could be involved, and culturing the cells in IL-2–conditioned medium in some cases allowed the establishment of EBV-positive T-cell lines with a Latency II pattern of viral gene expression (212).

EBV-associated hemophagocytic syndrome is therefore a rare condition in which virus-driven T-cell proliferation arising in the setting of primary infection replaces the usual virus-driven B-cell and reactive T-cell proliferations seen in classic IM. The clinical symptoms again result either from the lymphocytic infiltrate per se or from the effects of proinflammatory

cytokine release. The latter is particularly relevant in the context of hemophagocytosis given the macrophage-activating capacity of cytokines such as TNF-a. Serum levels of TFN-a are markedly elevated in VAHS patients (214) and expression of the cytokine in T cell lines *in vitro* can be up-regulated by EBV infection (264). Two EBV-associated conditions, VAHS and XLP, can therefore lead to a common end point, hemophagocytosis, by different routes but with the inability to control T-cell proliferation as a common theme. Interestingly, some cases of familial hemophagocytic syndrome are now known to be caused by mutation of the perforin gene, again resulting in an inability to down-regulate CD8+ T-cell responses (436).

Chronic Active Epstein-Barr Virus Infection

Classic IM in young adults almost always resolves to an asymptomatic carrier state. Exceptions to this rule are extremely rare (409), and most young adult patients presenting with chronic fatigue-like symptoms show no authentic link to EBV infection. This contrasts with an increasing number of case reports of young children who suffer from recurrent fever, lymphadenopathy, and hepatosplenomegaly over several years dating from a proven or a presumed primary EBV infection (7,190,338). These all share a highly unusual serologic profile with extremely high titers of IgG anti-VCA (more than 1:5,120) and anti-EA (more than 1:640) antibodies, often with persistent IgM anti-VCA, low or undetectable IgG anti-EBNA1, and, where examined, high IgG anti-EBNA2 titers. This profile resembles that of an unresolved primary infection, and more recent analyses using semiquantitative PCR assays have confirmed the presence of very high genome loads in the PBMCs of these patients, greater than those seen in classic cases of acute IM (293,409).

A variety of immunologic investigations have so far failed to reveal any consistent abnormality in patients with such chronic active infection (338), although in most cases the possibility of an EBV infection of T cells has not yet been examined. Another potentially interesting observation comes from a subset of such patients in whom, despite high virus loads, it was either very difficult or impossible to isolate the resident strain by LCL establishment *in vitro* (7,216,409). These individuals all proved to carry a virus that, although replication-competent, was either impaired or completely defective in transforming ability, with clear evidence of genomic rearrangements in regard to standard EBV strains. To what extent such transformation-defective strains circulate in the wild and contribute to disease pathogenesis is not known.

Clinically Apparent Virus Replicative Lesions

The clearest example of an EBV replicative focus *in vivo* is oral hairy leukoplakia (OHL), a wartlike lesion on the lateral borders of the tongue. This was first recognized in immunocompromised HIV carriers (163) but now also reported in various transplantation settings and even occasionally in healthy individuals (478). Figure 6 shows a cross-section through an OHL lesion to illustrate the general thickening of the epithelium, consistent with a delayed or disturbed progress of cells through the normal squamous differentiation program. Cell replicating viral DNA (detectable by *in situ* hybridization, as illustrated) and expressing a range of lytic cycle antigens such as BZLF1 (detectable by monoclonal antibody staining) are reproducibly present in these lesions but restricted to the outer more differentiated half of the epithelium; late lytic cycle antigens are detectable once viral DNA replication has initiated. The delayed differentiation of epithelial cells within OHL lesions may reflect the influence of BHRF1, the viral Bcl-2 homolog, which is expressed as an early lytic cycle antigen and which has the capacity to prolong the lifetime of lytically infected cells either through anti-apoptotic effects (183) or through disturbance of the epithelial differentiation program (102). A second early lytic cycle protein, BALF1, may also mediate anti-apoptotic effects (296a). Clinically, OHL lesions respond well to high-dose oral ACV, with apparent clearance of virus replication and a restoration of normal epithelial architecture within 2 to 4 weeks (162). However, withdrawal of treatment usually leads to a recurrence of lesions within 1 to 4 months; it is not clear whether this reflects viral reactivation from an epithelial reservoir or reseeding of the infection from the lymphoid system.

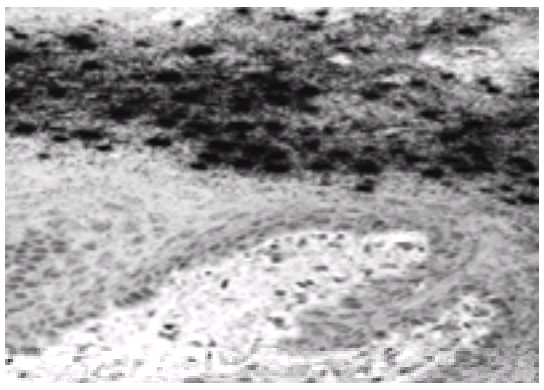

FIG. 6. EBV–epithelial cell interactions in oral hairy leukoplakia (OHL). A tissue section through an OHL lesion that was probed by *in situ* hybridization using a ^{35}S-labeled probe specific for the BamHI W repeat sequence of EBV DNA shows EBV genome–positive cells localized to the thickened outer layers of differentiating squamous epithelium; the less differentiated basal and parabasal layers show no significant labeling above background. (Courtesy of G. Niedobitek.)

Interestingly, OHL lesions show no evidence of either viral DNA or latent cycle markers (EBER RNAs or latent antigens) in cells of the basal and parabasal epithelial layers underlying the sites of virus replication (332,462). Surprisingly, however, a number of latent cycle antigens are expressed within the lytically infected upper layers, with EBNA1-positive cells consistently detectable by immunofluorescent staining (462) and, in some cases, smaller foci of EBNA2 or LMP1 staining (332,462). The corresponding latent cycle mRNAs are also detectable by RT-PCR analysis (480,487), whereas EBER expression is down-regulated (150). Although OHL lesions do produce transformation-competent virions (343), they also contain several defective forms of the EBV genome generated by illegitimate recombination or rearrangement.

These include various deletions or bizarre rearrangements of the *EBNA2* gene (479,480 and 481) and also rearrangements generating an aberrant BamHI W/Z junction fragment (351), which may itself promote BZLF1 expression and hence lytic virus replication. These OHL lesions also frequently harbor multiple virus strains, with evidence of interstrain and intrastrain recombination (478,479).

There is a more controversial literature describing EBV replication in lung epithelium and its possible association with a lung disease, idiopathic pulmonary fibrosis (IPF). Expression of VCA and gp350 has been reported in pulmonary epithelial cells in 70% of cases of IPF but only occasionally in control lung biopsies (118). However, the specificity of the staining and its correlation with the presence of PCR-amplifiable EBV DNA in biopsy specimens remains to be resolved (439,485). It is nevertheless clear that EBV-infected B cells do traffic to the lung, as witnessed by the instances of lymphocytic interstitial pneumonitis that occur in HIV-positive children after primary EBV infection (15). Such cells could well serve as a source of viral transmission to lung epithelium.

ASSOCIATED MALIGNANCIES OF THE IMMUNOCOMPROMISED HOST

The clearest evidence for the oncogenic potential of EBV in its natural host comes from the fatal B-cell lymphoproliferations, which are seen in three different groups of immunologically compromised patients.

Lymphomas in Congenitally Immunodeficient Patients

As described earlier, in some XLP patients, fatal IM presents as an EBV-positive B-lymphoproliferative disease arising within weeks of primary infection (448). These acute lesions generally appear to be oligoclonal by Ig markers or by size heterogeneity of the fused terminal repeat region of the EBV genome. In most cases, the proliferating cells display a lymphoblastoid phenotype and express several viral antigens, in particular EBNA1, EBNA2, and LMP1, consistent with a Latency III (LCL-like) form of infection (130,463). Children with other immune defects, such as common variable immunodeficiency or Wiskott-Aldrich syndrome, are also at risk for EBV-driven lymphoproliferative disease through an inability to mount effective T-cell control over the virus infection (reviewed in 120). Interestingly, Wiskott-Aldrich patients remain relatively susceptible to the disease even after total-body irradiation and transplantation with HLA-matched bone marrow (342), implying that the priming or maintenance of virus-specific T-cell responses *in vivo* may require a radio-resistant cell with antigen-presenting or helper function.

Posttransplantation Lymphomas

The link between sustained immunosuppression of transplant recipients and increased incidence of lymphoma, probably post-transplant lymphoproliferative disease (PTLD), has long been apparent and the association with EBV is now widely recognized (reviewed in 323). One major factor influencing PTLD risk, the intensity of T-cell suppression, underlies most of the differences seen in PTLD incidence in different transplantation settings. Thus, renal and liver transplantation programs have overall incidence rates of 1% to 2%, versus 3% to 8% for heart and lung transplantations where higher levels of immunosuppression are often given. Overall, rates of PTLD in allogeneic bone marrow transplantation programs are less than 1% but can rise dramatically to 12% to 24% among patients in whom donor T cells are depleted from the marrow graft. Because bone marrow transplantation frequently involves total ablation of host lymphoid cells (and can indeed clear a resident EBV infection [161]), the PTLDs that develop after transplantation often derive from EBV-infected donor B cells present within the bone marrow graft itself (159). Protocols that deplete the graft of both B and T lymphocytes, therefore, significantly reduce the risk for PTLD by reducing the incidence or effective dose of iatrogenic EBV infection (172).

A second major influence on PTLD risk is the patient's EBV status at the time of transplantation. A survey of PTLD cases arising in all solid organ transplant recipients cohorts at one major center showed a 20-fold higher incidence of the disease in EBV–seronegative recipients (198), explaining why young children are at particular risk. Subsequent studies of solid organ transplant recipients as a whole (476) and of recipients of renal (40,122), heart-lung (452), and liver (296) grafts have confirmed the age-independent predisposition to PTLD among patients who were EBV-seronegative at the time of transplantation. One of these studies also identified a history of cytomegalovirus-induced disease after transplantation as possibly a third independent variable predisposing to later PTLD development (296), and it will therefore be interesting to see how such an episode might affect the long-term position of the EBV–host balance.

Given the high EBV genome levels that develop in the PBMCs of immunosuppressed patients, it becomes important to know whether increasing loads might be used as a prognostic indicator of PTLD risk. Initial studies showing unusually high frequencies of EBV-infected cells in the blood of early-onset PTLD patients (402) have largely been confirmed by PCR assays of virus genome load, although most studies report some overlap with the wide range of virus loads shown by tumor-free transplant recipients (27,29,248,288,381,386). A recent prospective study of asymptomatic solid organ transplant recipients identified a small number with progressively increasing loads, one of whom became symptomatic (29). Virus load monitoring has also been used to identify bone marrow transplant recipients at high risk for PTLD for whom adoptive T-cell therapy is an option (249,381). To what extent ongoing virus replication might increase the risk for PTLD development through continual recruitment of new transformation events is another important but unresolved issue. Trials of prophylactic ACV in various transplantation settings have reported potential benefit in reducing PTLD incidence in some studies but not in others (reviewed in 352).

As interest in PTLD has grown, so has the variety of lymphomas reported to arise in transplant recipients (reviewed in 178). Nevertheless, most posttransplantation tumors still resemble the classic lymphoproliferative lesions first described in the literature (324). In this review, we use PTLD to refer specifically to these classic lesions, mostly arising within the first 1 to 2 years after transplantation. This disease is etiologically and histologically distinct from the range of other lymphomas (B-cell lymphomas of various kinds, and certain T-cell lymphomas), which are seen occasionally in transplant recipients, usually many years after transplantation. Although rare cases of BL are included among these late-onset malignancies, they are by no means all EBV genome positive, implying that PTLD and posttransplantation BL have

separate etiologies.

The classic PTLDs are themselves graded histologically into (a) early lesions involving plasmacytic hyperplasia and reactive elements similar to those seen in acute IM tissues, (b) polymorphic lesions described as B-cell hyperplasia or B-cell lymphoma, and (c) monomorphic lesions described as immunoblastic lymphoma. At least 90% of such lesions are EBV-associated (the exceptions being a recently identified subset of tumors of unknown etiology) and are either oligoclonal or monoclonal based on EBV terminal repeat and *Ig* gene analysis. Clonality does not necessarily correlate with polymorphic or monomorphic appearance and indeed there can be heterogeneity of histologic appearance both within a lesion and between different lesions arising in the same patient (reviewed in 178 and 323).

Most of these EBV-positive PTLDs arising in transplant programs in the Western world carry type 1 EBV strains typical of those prevalent in the general population (137,406). By immunostaining, the lesions are usually dominated by EBNA1-positive, EBNA2-positive, LMP1-positive cells (280,463,513), consistent with their being EBV-transformed LCL-like foci growing opportunistically in the absence of CTL surveillance. When the lesions show a T-cell infiltrate, this is mainly composed of nonactivated CD4+ T cells with little evidence of any ongoing CD8+ response (354). However, a minority of these tumors are EBNA1-positive, with low or undetectable levels of EBNA2 and LMP1 (76,111a,370), possibly reflecting the subsequent acquisition of cellular genetic changes, which have rendered some EBV-transforming functions redundant. In that context, a small proportion of PTLDs, usually among those with monomorphic histology, have acquired *p53* gene mutations (253), *c-myc* rearrangements detectable by DNA analysis (286), or identifiable cytogenetic changes (113,280). Some PTLDs with changes at the *c-myc* or *bcl-6* loci appear to remain both EBNA2 positive and LMP1 positive (280), whereas others, presenting with more complex chromosomal aberrations, display more restricted forms of latency (113). One of the earliest and most important clinical observations in this field was that many PTLDs, especially the early-onset lesions, regressed when immunosuppressive therapy was reduced (435). Indeed, a planned reduction of immunosuppression is now the first treatment option in many cases of PTLD (352), and its frequent success is thought to reflect reemergence of the patient's endogenous EBV-specific CD8+ CTL response (244).

Acquired Immunodeficiency Syndrome Lymphomas

HIV-infected individuals are at particularly high risk for developing B-cell lymphomas, with cumulative incidence rates estimated at up to 10% during the first decade of the AIDS epidemic (35). Not only is this higher than seen in most transplant recipients, but also the spectrum of tumor types is more diverse. Overall, about half of these AIDS lymphomas are EBV-associated, but the strength of the association differs between tumor types (reviewed in 143). Up to one third of the EBV-positive lymphomas of AIDS were found to carry a type 2 EBV strain in early surveys (49), reflecting the high prevalence of type 2 strains in the HIV-positive homosexual population that dominated AIDS cohorts at that time (511). In this same context, several studies (36,249) have reported that a high proportion of EBV-positive AIDS lymphomas carry virus strains with a small in-frame deletion at a mutational hot-spot in the *LMP1* gene. The argument that this is a marker of enhanced lymphomagenic potential remains controversial, however, because about 30% of type 1 and almost all of type 2 isolates from healthy individuals in Western countries also carry the same deletion (233).

One major category of AIDS lymphomas is essentially analogous to PTLD lesions and appears only in end-stage AIDS when patients are profoundly immunocompromised. These tumors present at a variety of extranodal sites as polymorphic B-cell hyperplasia or monomorphic immunoblastic lymphomas with varying degrees of plasmacytic differentiation; most of the AIDS lymphomas presenting in the central nervous system are of this type (292). Such PTLD-like lesions are again EBV-positive in at least 80% cases, the reported strength of the association varying between different studies because the histiologic boundary between these lesions and certain other AIDS lymphomas can be difficult to draw. When EBV positive, the tumors are largely composed of EBNA2-positive, LMP1-positive B lymphoblasts typical of an EBV-driven B-cell proliferation (143,174,175) and appear from *Ig* gene sequencing to be derived mostly from postgerminal center (memory) B cells (111). Outgrowth of these EBV-positive PTLD-like lesions is almost certainly linked to a failure of virus-specific T-cell surveillance. Thus, prospective studies of individual patients have shown a loss in EBV latent antigen-specific CTL function, and a concomitant rise in EBV-infected cell numbers in the blood, in patients who go on to develop lymphoma (231).

At least three other categories of AIDS non-Hodgkin's lymphomas appear to be etiologically distinct from the PTLD-like disease. Firstly, a small (noncleaved) B-cell lymphoma of Burkitt's lymphoma type (AIDS-BL) is unusually common and arises earlier than the PTLD-like lesions, in patients whose EBV-specific T-cell immunity is still intact (231). Such tumors show the characteristic *c-myc* translocations and a Bcl6–positive germinal center phenotype of classic BL but are EBV-positive in only 30% to 40% of cases; when positive, they lack EBNA2 and LMP1 expression completely or sometimes express LMP1 in a small percentage of cells (74,174,175). Secondly, some AIDS patients develop a large (noncleaved) B-cell lymphoma which may be difficult to distinguish histologically from PTLD-like disease (174) but differs from it in expressing the Bcl6 marker (74). As with AIDS-BL, these tumors are EBV positive in 30% to 40% cases and, when positive, tend to display an EBNA2-negative, LMP1-negative viral phenotype (74,143,175); this accords with *in vitro* studies of EBV-positive cell lines in which Bcl6 and LMP1 expression appear to be mutually exclusive (75). Thirdly, primary effusion lymphoma (originally called *body-cavity–based lymphoma*) is a rare tumor that arises in highly immunocompromised AIDS with HHV-8 infection. Such tumors are consistently HHV-8 genome positive and are thought to be pathogenetically linked to this virus (77); interestingly, most tumors analyzed to date also carry the EBV genome, with antigen expression restricted to EBNA1 and possibly trace levels of LMP1 (70,453). The contribution that EBV makes to the pathogenesis of these various AIDS lymphomas of non-PTLD type remains unknown.

Finally, there is evidence for a small but significant increase in the incidence of classic Hodgkin's disease (HD) in HIV-infected individuals (392). These tend to appear in more immunocompromised patients and in a more aggressive form. The histologic classification of AIDS-HD appears to be skewed toward those subtypes (mixed cellularity, lymphocyte-depleted) more frequently linked to EBV in classic HD (see later), and the tumors are EBV-positive in more than 90% cases, with a characteristic EBNA2-negative, LMP1-positive viral phenotype (22,34).

Smooth Muscle Cell Tumors

Leiomyosarcomas, an unexpected addition to the list of EBV-associated tumors of the immunocompromised (265,299,473), are especially seen in HIV-positive children, in whom they constitute the next most common malignancy after lymphomas. Similar EBV-positive tumors have also been seen in young transplant recipients (265) and, most recently, in a child with immunodeficiency (470). Like PTLDs, these tumors can be monoclonal or oligoclonal by EBV terminal repeat analysis, with examples of different clones at different sites within the same patient (299,473).

The consistency of the EBV association with such tumors and their unusually increased incidence in immunocompromised patients (compared with the general population, in whom smooth muscle tumors are very rare and EBV-negative [299]) strongly suggests an etiologic role for the virus in tumor development. It is interesting that these tumors frequently arise in sites (lung, gastrointestinal tract, liver) where EBV-infected B cells are known to accumulate in T-cell–compromised patients. However, the mechanism of viral entry into smooth muscle cells and the status of these cells with respect to CR2 expression are unresolved. Likewise, the form of latency established by EBV in the tumor cells has not been rigorously analyzed.

BURKITT'S LYMPHOMA

Endemic, Sporadic, and AIDS-Associated Forms

The endemic form of BL, first described by Denis Burkitt in Africa, had two remarkable epidemiologic features: (a) an unusually high incidence of 5 to 10 cases/10^5 per year over the first 15 years of life, making it the most common cancer of childhood in equatorial Africa, and (b) a marked geographic distribution, apparently determined by climatic factors and coincident with areas of holoendemic malarial infection (60,294). The same high-incidence tumor was subsequently identified in coastal New Guinea (359), the only other area of the world with such intense *Plasmodium falciparum* malaria. Endemic BL is classically a childhood malignancy (male-to-female ratio, 3:1) with an unusual pattern of presentation at extranodal sites, most frequently in the jaw in association with molar tooth development or otherwise in the orbit, in the central nervous system, or as an abdominal mass often involving the ovaries. All such tumors show the same homogeneous population of malignant cells, morphologically similar to germinal center B cells, interspersed with occasional macrophages which give the tumor its characteristic “starry sky” histology. The tumors are monoclonal and, more importantly, display one of three reciprocal translocations between chromosome 8 at or near the site of the *c-myc* locus at 8q24 and either the *Ig* heavy-chain locus on chromosome 14 (the common translocation seen in up to 80% tumors) or one of the light-chain loci on chromosomes 2 or 22 (the variant translocations). The coincidence of these clinical, histologic, and karyotypic markers firmly established endemic BL as a distinct pathogenetic entity (reviewed in 294).

Rare childhood lymphomas with the histologic appearance of BL and the same characteristic cytogenetic changes are now known to occur worldwide and are classified as *sporadic* BL. This tumor is best characterized in Europe and the United States, where its incidence is 50- to 100-fold lower than that of the endemic disease. Here, the tumor has a slightly later age peak and a different pattern of presentation, appearing much more frequently as an abdominal mass and occasionally in leukemic form (294). Studies of sporadic BL in other regions of the world are still at a relatively early stage. Nevertheless, it is already clear that incidence rates for histologically and cytogenetically confirmed BL may be higher in parts of South America and North Africa than in Europe and the United States (170,271).

As described earlier, there is a third form of BL, which appears in HIV-infected individuals often as one of the first symptoms of AIDS (74,143). Again, these tumors resemble classic BL on cytogenetic and histologic grounds, with sites of presentation following the sporadic rather than the endemic pattern. Because up to 10% of AIDS patients in Western societies develop B-cell malignancies of one form or another, the incidence of BL in this particular group is very much higher than either the endemic or sporadic BL of childhood. To what extent this susceptibility to lymphoma, and to BL in particular, is reproduced in HIV carriers in an African setting remains an important but unresolved question (93).

Association with Epstein-Barr Virus

The endemic form of BL shows the strongest and most consistent association with EBV. In virtually every endemic tumor analyzed to date, all the malignant cells are EBV genome–positive, and each tumor carries a monoclonal EBV episome (1,294). Interestingly, anti-VCA and anti-EA(R) antibody titers in the serum of endemic BL patients are considerably higher than in age-matched controls (186), and a large prospective survey of children in an endemic area showed that individuals who subsequently developed BL had significantly raised anti-VCA titers months or years before the clinical onset of the tumor (107,149).

The association between EBV and sporadic BL is less consistent but nevertheless still significant. In Europe and the United States, where sporadic BL occurs at very low incidence, only 15% to 25% of the tumors are EBV genome positive (1,271,294), although it is claimed that some EBV-negative tumors in fact harbor integrated viral sequences indicative of earlier virus involvement (369). In several other parts of the world, including the intermediate incidence areas of North Africa and equatorial South America, the proportion of EBV-positive cases reaches 50% to 85% (80,398). Such findings strengthen the view that wherever childhood BL exceeds the very low incidence rates seen in Western societies, most of the increase is EBV-associated (271). Set against this background, AIDS-BL again appears to be rather a special case. The incidence of this tumor among AIDS patients is unusually high, yet only a minority, some 30% to 40%, of AIDS-BL cases are EBV genome–positive (74,174). It is nevertheless important that, as with the endemic disease, in all EBV-positive cases of AIDS-BL and of sporadic BL so far analyzed, the EBV genome is detectable in every tumor cell; hence, the virus must have been present in the tumor progenitor cell before its expansion as a malignant clone.

There is to date no clear evidence that particular strains of EBV are preferentially linked with BL. Both type 1 and type 2 EBV strains have been found in association with the endemic tumor in Africa and New Guinea, whereas type 1 strains

are dominant among EBV-positive sporadic tumors in Europe (171,459,515,518), reflecting the relative prevalence of these two virus types within the general population in these different areas. A recent report that BL tumors tend to carry viruses with particular allelic polymorphisms in the *EBNA1* gene sequence (39) again seems more likely to reflect differences in geographic distribution than differences in pathogenicity between viral strains (171).

Cellular and Viral Aspects of the Burkitt's Lymphoma Phenotype

The B-lymphoid origin of Burkitt cells is clear from the regular expression of pan–B-cell antigens such as CD19 and CD20. Individual tumors are monoclonal in terms of *Ig* gene rearrangement and isotype expression; whereas most display surface IgM (μk or μl, usually without IgD), a minority appear to be derived from cells that have already switched to IgG or IgA production, again with appropriate light-chain restriction (294). In other respects, however, Burkitt's tumors display a remarkably consistent cell surface profile characterized (a) by the coexpression of two diagnostic markers, CD10 (CALLA) and the glycolipid moiety CD77 (BLA); (b) by the complete absence of B-cell activation antigens such as CD23, CD30, CD39, and CD70; and (c) by the absence or very low expression of molecules such as CD54 (ICAM1) and CD58 (LFA3), which normally play a role in cell adhesion to T lymphocytes, and CD80 (B7), which is a critical co-stimulatory molecule for T-cell activation (165,389). Cells with an essentially similar surface profile exist within the germinal centers of secondary lymphoid follicles (164); indeed, the similarity between the two cell types is reinforced by the consistent presence of the germinal center-associated protein Bcl-6 (75) and absence of the B-cell survival protein Bcl-2 (184,355) in BL cells.

Cell lines established from BL biopsies *in vitro* at first display the original biopsy cell phenotype and grow as a single cell suspension with no intercellular adhesions. On serial passage, some BL lines retain this group I (biopsy cell) phenotype, whereas other lines show marked phenotypic shift with the appearance in the culture of clumps of lymphoblastoid cells. These cells are clearly derived from the group I tumor cell population because they retain the relevant cytogenetic and Ig isotype markers but now express the full spectrum of B-cell activation antigens and adhesion molecules. As such cells come to dominate the culture, they frequently lose expression of the original CD10 and CD77 BL markers and thereby complete the move to a group III phenotype essentially indistinguishable from that of *in vitro*-transformed LCLs (390); other LCL-associated markers, such as cellular Bcl-2, have also been up-regulated (184). As illustrated in Figure 7, those cellular changes are associated with a change in the form of viral latency. Comparing isogenic but phenotypically distinct subclones of the BL line Mutu, group I cells express only the viral genome maintenance protein EBNA1, whereas group III cells express the full spectrum of viral antigens that are found in LCLs. Accordingly, group I cells, representative of BL *in vivo*, follow the Latency I pattern and express a uniquely spliced EBNA1 mRNA selectively transcribed from the Qp promoter region. By contrast, group III cells have moved to the Latency III pattern, involving a switch from Qp to Cp/Wp promoter usage, which leads to expression of all six EBNA species and hence to an EBNA2-mediated activation of the LMP promoters. Most of the cellular changes associated with the group I to group III transition can in fact be traced to activations of cellular genes induced by EBNA2 or LMP1 (184,482).

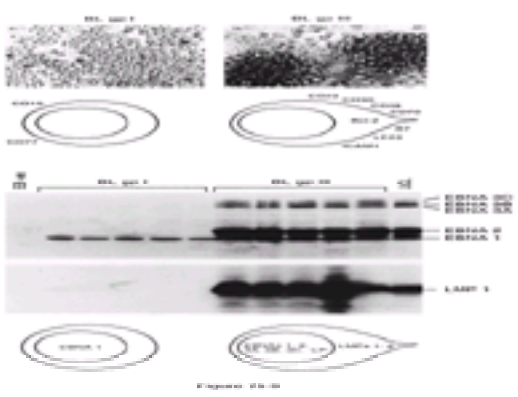

FIG. 7. Relationship between cellular phenotype and viral phenotype in EBV-positive Burkitt's lymphoma (BL) cells. Data are based on the analysis of phenotypically distinct subclones of the endemic BL cell line, Mutu. **Top:** Contrasting growth characteristics and cellular phenotypes of group I and group III subclones. **Bottom:** Different patterns of viral antigen expression in these same subclones. Protein extracts are probed with a polyclonal human serum reactive against EBNA1, 2, 3A, 3B, and 3C proteins (*upper gel*) or with the LMP1-specific monoclonal antibodies CS1–4 (*lower gel*). The negative-control track is loaded with protein from an EBV-negative sporadic BL line, the B-lymphoblastoid cell line control track with protein from a cord blood B-cell line transformed *in vitro* by virus rescued from Mutu cells.

It is not known why some EBV-positive BL lines stably retain the group I phenotype *in vitro* whereas others progress to group III. It may reflect differences in the degree to which extraneous viral gene expression is extinguished within the original tumor itself. Assays on biopsy cell preparations show that in some tumors, a small number of cells have spontaneously activated the expression of additional latent or lytic cycle genes (329,459).

The Burkitt's Lymphoma Phenotype and Immune T-Cell Recognition

EBV-positive group I BL cell lines appear to be “immunologically silent” in that they are not recognized by virus-specific CTL preparations with appropriate HLA class I restriction (382) and are extremely poor inducers not just of virus-specific but also of allo-specific CTL responses *in vitro* (297). Multiple features of the BL phenotype are now thought to contribute to this immunologic silencing.

First, the proteasome-dependent antigen-processing pathway that degrades endogenously synthesized proteins and delivers the resultant peptide fragments for cell surface presentation on HLA class I molecules is very much less active in BL cells than in LCLs. In fact, there appears to be a general impairment of proteasome function in the tumor cells, with

some of the proteasome's essential (nonimmunologic) functions being carried out by an alternative proteolytic system involving tripeptidyl peptidase II (146). Clear differences between BL and LCL cells are apparent in proteasome composition, with BL cells lacking the IFN-g–induced subunits found in immunoproteasomes of efficient antigen-presenting cells (32), and in the efficiency of peptide transport, with BL cells showing low-level expression of the TAP1/TAP2 transporter complex (387). In addition, group I BL cells show a general down-regulation of HLA class I antigen synthesis when compared with LCLs, with certain alleles particularly affected, leading to correspondingly lower steady-state levels of class I antigens at the cell surface (297,387). As a result of these multiple changes, group I BL cells are unable to present endogenously expressed target antigens to CTLs even when the target antigen is overexpressed in the cells from a recombinant vaccinia viral vector (235). These effects are reversed in BL lines that have switched to a group III LCL-like phenotype *in vitro*. In fact, antigen-processing function can be restored in group I BL lines by vector-mediated expression of LMP1 (387) or by ligation of CD40 (241); in both situations, there is a coordinate up-regulation of TAP1/TAP2 and of HLA class I antigen expression. Interestingly, despite deficiencies in the HLA class I pathway, group I BL cells retain the ability to process exogenous antigen through the HLA class II pathway for presentation to CD4+ T cells (239).

As a second feature of the immunologically silent BL phenotype, many of the ancillary molecules required for the efficient induction of T-cell responses are expressed weakly if at all at the cell surface. In particular, the very low level of CD80 expression is liable to be critical because CD80-mediated ligation of CD28 on the T-cell surface delivers an essential co-stimulatory signal for immune activation. In addition, BL cells lack adhesion molecules such as ICAM1 and LFA3, whose interaction with LFA1 and CD2, respectively, on the T-cell surface is thought normally to enhance the efficiency of immune stimulation (165). However, none of these ancillary pathways appears to be essential for recognition of the tumor by preactivated T cells because BL cells preincubated with an appropriate epitope peptide are killed by epitope-specific CTLs (238).

Third, and most obviously, the viral phenotype of BL cells is likely to be an important factor in reducing tumor susceptibility to EBV-specific CTL surveillance. Thus endogenous viral antigen expression is limited to EBNA1 (390), which is protected from CD8+ T-cell recognition, and possibly some BamHI A–encoded polypeptides that (even if expressed) appear to constitute very weak targets for the T-cell response (246).

It is tempting to conclude from the above that escape from virus-specific CTL surveillance has been a key selective step in the evolution of EBV-positive BL. However, EBV-negative BL also shows many of these same cellular features (165,235). Although it is possible that the presence of a different (as yet unidentified) virus in the EBV-negative tumor might likewise necessitate the selection of an immunologically silent tumor phenotype, a more plausible interpretation is that such a phenotype is either a characteristic of the normal cell from which BL is derived (164) or, as *in vitro* studies now suggest (146,357), has been imposed on the tumor progenitor cell by events that are essential for its malignant transformation but are unrelated to immune T-cell selection.

Pathogenesis, Chromosomal Translocation, and the Role of Epstein-Barr Virus

BL has a complex multistep pathogenesis with deregulation of *c-myc* expression and EBV infection being the best documented, but almost certainly not the only, factors involved. The relative contribution of these factors and their order of involvement remain to be determined.

The detailed structure of the *c-myc* gene translocations in BL has been extensively reviewed (294), the end result being to release the gene from its usual regulatory controls and to bring it under the control of long-range enhancer sequences within *Ig* loci. The common t(8:14) translocation tends to place the *c-myc* gene in one of two positions upstream of the IgH locus. In one case, a breakpoint within the body of the *c-myc* gene removes the 5'-regulatory exon and places the coding exons directly adjacent to IgH sequences; this is the pattern most often seen in (North American) sporadic and AIDS-associated BLs irrespective of their EBV genome status. In the other case, *c-myc* is some distance away from the breakpoint and so is translocated intact up to several hundred kilobases upstream of IgH sequences; this is the pattern seen in most endemic BLs. Variant t(2:8) and t(8:22) translocations, whether in endemic or sporadic tumors, produce an analogous situation in which *c-myc* is always positioned some distance upstream of the kappa or lambda light-chain loci. In addition, the translocated c-myc allele is frequently mutated both in and around the regulatory exon 1 (78) and in the protein-coding exons leading to key changes in the protein's N-terminal transcriptional activation domain (38). Both sets of mutations could have pathogenetic significance in addition to the effect of the translocation per se.

The position of the reciprocal breakpoints within the *Ig* gene loci is of particular interest because it could help to identify at what stage of B-cell differentiation the translocation occurred. Most work has focused on t(8:14) translocation where there are two main patterns of *IgH* gene breakage, again loosely correlating with tumor type. One group of (mostly sporadic) BLs show breakage within the Ig switch (S) region, usually involving Sμ but occasionally Sg or Sa; this suggests that translocation has occurred as an accident of the recombination events that normally occur in germinal center B cells during Ig class switching. Another group of (mostly endemic) BLs show breakage in the joining (J) region. However, the breakpoints do not target recombination signal sequences, as would have been expected if breakage had occurred as an accident of V-D-J joining in bone marrow pro-B cells during initial *IgH* gene rearrangement; instead, breakpoints are located within J region introns or rearranged V(D)J sequences (294). Recent work has shown that these areas are susceptible to breakage (leading to deletions and duplications) as a result of the process of somatic hypermutation occurring in germinal center B cells (154). This strengthens the possibility that all *c-myc/Ig* translocations in BL may originate as mistakes of physiologic processes occurring during the maturation of antibody responses in germinal centers. In this context, a germinal center origin for BL is supported not just by the tumor's phenotypic similarity to germinal centre B cells (164) but also by the fact that tumor-derived lines show evidence of ongoing hypermutation of their productively rearranged *Ig* genes (82,251,457).

It is also interesting that holoendemic malarial infection (12) and chronic HIV infection with its associated persistent generalized lymphadenopathy (213) both act as potent stimulators of lymphoid hyperplasia and produce greatly expanded germinal centers. These infectious agents may therefore be risk factors for BL development by virtue of their ability to act as chronic stimuli of germinal center cell proliferation rather than their frequently postulated role as

mediators of T-cell suppression. In this context, the immunosuppressive effects of malaria are generally restricted to short periods of acute disease (494), whereas those mediated by HIV do not become profound until late-stage AIDS, by which time the incidence of AIDS-BL is decreasing concomitant with a marked reduction in germinal center cell activity (213).

The mechanisms whereby EBV contributes to BL pathogenesis are still not fully understood. The potent growth-transforming ability of the virus suggests that it could act early in the lymphomagenic process by expanding the population of cells at risk for translocation or by increasing the survival and/or proliferation of a translocation-positive but still not fully malignant clone. These scenarios all envisage an important role for the Latency III growth transforming infection at some early or intermediate state of the lymphomagenic process, followed by a switch to Latency I as accumulated cellular changes render cell growth independent of viral-transforming proteins such as EBNA2 and LMP1. An important *in vitro* reconstruction of such a scenario has been achieved using as a starting point a recombinant EBV-transformed LCL in which EBNA2 is expressed as fusion protein with the estrogen receptor such that EBNA2 function, and consequently LMP1 expression and cell growth, is estrogen- dependent. When these cells were transfected with a *c-myc* expression construct cloned from a BL *c-myc/Ig* fusion locus, the constitutively active *c-myc* gene rendered cell growth estrogen-independent. Furthermore, the cells that grew out in the absence of estrogen had switched from an LCL-like to a group I BL-like phenotype and growth pattern (357). Enforcing high *c-myc* expression in an EBV-transformed B cell can therefore substitute for the growth-promoting effects of EBNA2 and LMP1 and can impose a BL-like phenotype on the resulting EBNA2-negative, LMP1-negative cell.

While EBV may play an initiating role in BL pathogenesis, it is also possible that the virus continues to contribute to the growth phenotype of the malignant clone. The evidence, however, is still circumstantial. Spontaneous loss of the viral genome has been noted in subclones of the Akata BL cell line (originally derived from an EBV-positive sporadic tumor) and was accompanied by a significant reduction in the cells' tolerance of suboptimal culture conditions, capacity for growth in soft agar and tumorigenicity in nude mice (419). Reinfection with virus reestablished the original latency I form of infection and restored the original growth phenotype. However, EBNA1, the anticipated mediator of this effect because of its reported lymphomagenic potential when expressed in the B cells of transgenic mice (495), was inactive in the Akata assay (257,393) whereas some restoration of the growth phenotype appeared to be mediated by *EBER* gene transfection (256). Further work is required to identify the viral contribution to the Akata cell phenotype and to determine how representative these results are of other BL cell backgrounds.

Whatever the mechanisms whereby EBV infection and chromosome translocation complement one another, it is unlikely that these are the only elements required for full malignant conversion. Thus, about 30% of BL biopsies, and an even higher proportion of BL cell lines, contain a mutated *p53* gene and frequently show loss of the second *p53* allele (132,142,497). The presence in a number of BL lines of karyotypic changes involving chromosome 1 (37) suggest that other relevant cellular genes remain to be discovered. The complex multistep nature of lymphomagenesis is further emphasized by the fact that BL, although expressing a germinal center-like phenotype, does not usually develop within lymphoid tissues. The tumor's unique pattern of presentation at extranodal sites, particularly in the jaw in association with molar tooth development, could therefore reflect additional clonal selection *in vivo* on the basis of migratory capacity or responsiveness to a locally available growth factor (294).

HODGKIN'S DISEASE

Epidemiology and Association with Epstein-Barr Virus

HD is an unusual lymphoma with a malignant population of mononuclear Hodgkin and multinuclear Reed-Sternberg (H-RS) cells within a reactive infiltrate of nonmalignant cells, which typically make up more than 98% of the tumor mass. Based on the H-RS–to–reactive cell ratio and the composition of the infiltrate, HD can be classified into four histologic subtypes, nodular sclerosing (ns), mixed cellularity (mc), lymphocyte depleted (ld), and lymphocyte predominant (lp) (179). Of these, ns, mc, and the (relatively rare) ld disease are now referred to as *classic HD,* whereas lp-HD is considered a separate clinical entity. Classic HD is seen worldwide and, although total incidence rates appear not to vary substantially with geography, there are interesting differences between countries in age incidence and subtype distribution (reviewed in 152). In Europe and the United States, HD incidence is low in childhood, shows a marked young adult peak dominated by ns disease, then appears to plateau through later life with equal contributions from both ns and mc subtypes. Patterns vary in other parts of the world, but generally, in less developed countries, childhood HD is more common, and the disease then shows a steady age-related increase with no young adult peak; the overall contribution of mc disease is therefore higher.

These differences suggested that the young adult disease seen in the West resulted from delayed exposure to a common infectious agent (291). The obvious epidemiologic parallel with IM raised the possibility of a link between EBV and HD; indeed, several studies have found that individuals with a history of IM have a roughly threefold subsequent increased risk for HD, the risk being greatest within the first 3 years after EBV infection (reviewed in 152). Furthermore, a retrospective analysis of banked serum samples showed significantly raised anti-VCA antibody titers in HD patients at least 3 years before development of the tumor, suggesting that deregulation of the EBV–host balance had preceded emergence of the malignant clone (317). However, the key findings that irrevocably linked EBV with HD were the detection of monoclonal viral genomes in some tumor biopsies by Southern blotting and terminal repeat analysis (492) and the localization of EBV DNA to the malignant H-RS cells of such biopsy specimens by *in situ* hybridization (9,491). In virus-positive cases, every malignant cell carries the viral genome, and in patients with multiple lesions, the same unique viral episome is present at every site (28).

The association between EBV and HD has subsequently been extensively investigated using archival HD material and the more sensitive screening methods of *in situ* hybridization for EBER RNAs (503) or immunohistochemical staining for LMP1 (192,344) in H-RS cells. Worldwide, some 60% to 80% of mc and ld subtype tumors are EBV genome-positive, compared with 20% to 40% of cases of nc subtype and very few of lp subtype (reviewed in 153). Thus, in the Western world, about half the cases of HD arising in children or in old age are EBV- positive, whereas the figure falls below 30%

for the young adult peak of disease; overall, some 35% of patients with classic HD carry the viral genome. Ironically, therefore, the young adult peak of predominantly ns-HD shows the weakest association with EBV. Even among young adult cases arising in patients with a proven history of IM, recent work indicates that many have EBV genome–negative tumors (431). By contrast, in other parts of the world (Africa, Asia, Latin America), more than 60% to 70% of all HD cases are EBV genome-positive, some of this increase being explained by the absence of the young adult peak. It is worth recalling that EBV infection occurs very early in life in these areas, and several reports now indicate that essentially every case of childhood HD from the above populations is EBV genome-positive (8,181,489).

As in several other tumor contexts, there is no clear evidence that particular strains of EBV might be linked with HD. Whether analyzed for virus type or for potentially interesting polymorphisms in EBV latent cycle genes, the spectrum of virus strains found in EBV-positive HD appears to reflect that seen in the general population as a whole (181,233). The extent to which HD incidence increases, if at all, in immunocompromised patients remains controversial. However, those cases of HD that do arise in transplant recipients (144), congenitally immunodeficient patients (467) and particularly AIDS patients (21) appear to be invariably EBV genome-positive.

Cellular and Viral Aspects of the Hodgkin's Disease Phenotype

The origin of the malignant cells in classic HD for many years remained unknown. Most H-RS populations (whether EBV genome-positive or -negative) do not express conventional B- or T-cell markers, although they are frequently positive for lymphocyte activation antigens such as CD25, CD30, and CD70, for a novel intermediate filament-associated protein restin, and for a carbohydrate antigen CD15 originally thought to be a myeloid-specific marker (reviewed in 345). However, PCR amplification of *Ig* gene loci from single micromanipulated H-RS cells has shown that, in all but very rare cases (321), classic HD is of monoclonal B-cell origin with all H-RS cells within a single tumor showing the same Ig heavy- and light-chain gene rearrangements (51,223,258,259). Furthermore, sequence analysis revealed that the cells carried heavily mutated *Ig* genes, often with stop codons in the reading frame or replacement mutations in the usually conserved framework regions (259,335). Such “crippling” mutations, which are normally inconsistent with B-cell survival *in vivo*, appear to be byproducts of somatic hypermutation in germinal centers (154). Their presence in H-RS cells, unaccompanied by any evidence of ongoing *Ig* gene mutation, therefore identified classic HD as a tumor of postgerminal center B-cell origin (258).

Whenever EBV is present in the H-RS cells of classic HD, the same pattern of viral gene expression is observed, described earlier as Latency II. This is characterized by expression of the EBER RNAs, the BamHI A transcripts, a Qp-driven EBNA1 mRNA, and the LMP1, LMP2A, and LMP2B transcripts (109). At the single-cell level, monoclonal antibody staining has confirmed the presence of EBNA1 (156), and unusually high levels of both LMP1 (192,344) (Fig. 8) and LMP2A (331) in the tumor cells. Only a few HD biopsy specimens showed any sign of lytic cycle antigen expression, and, when present, this appeared to be limited to the immediate early antigen BZLF1 seen in a small fraction of the tumor cells (345).

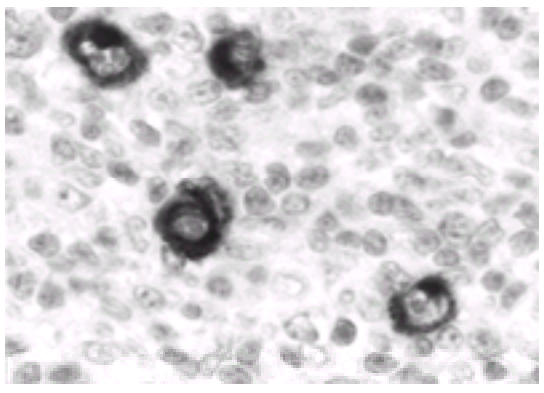

FIG. 8. Expression of LMP1 in the malignant cells of EBV-positive Hodgkin's disease (HD). A tissue section through an HD biopsy was stained with the LMP1-specific monoclonal antibodies CS1–4 and developed using APAAP immunohistochemistry shows strong cytoplasmic and membrane staining restricted to the malignant Hodgkin–Reed-Sternberg (H-RS) cell population. (Courtesy of G. Niedobitek).

The Hodgkin's Disease Phenotype and Immune T-Cell Recognition

The broader range of viral antigen expression in HD compared with that seen in BL raises the question of how these cells escape immune recognition by the EBV-specific T-cell response. Immunohistochemical staining has shown that in most EBV-positive tumors, the H-RS cells express both the TAP1/TAP2 peptide transporter and surface HLA class I and class II molecules; paradoxically, HLA class I expression is frequently down-regulated in EBV-negative cases of the disease (267,341). The implication is that antigen-processing function through the HLA class I pathway is intact in EBV-positive H-RS cells, as it is in small number of HD-derived cell lines available *in vitro* that, although from EBV-negative cases of the disease, have retained HLA class I expression (267,425).

A report that EBV-specific CTLs could be expanded from the T-cell infiltrate of virus-negative but not virus-positive HD (140) implied some local suppression of virus-specific responses by the tumor itself. Although subsequent work has in fact detected EBV-specific CD8+ T cells within EBV-positive HD samples, the range and frequency of these reactivities resembled those seen in the blood, and there was no selective recruitment of cells specific for viral antigens expressed in the tumor (81). Local evasion of T-cell control by the tumor, therefore, remains a possibility. In this context, H-RS cells appear to produce a range of cytokines, for example, transforming growth factor-b, IL-10, IL-13, and the thymus and activation-related chemokine (TARC) (reviewed in 360), which have the potential to suppress or divert cell-mediated

responses away from CTL induction. Of these, IL-10 has received most attention, being more commonly secreted by EBV-positive than by EBV-negative tumor cells (193); from *in vitro* studies, any effects of this cytokine on CTL responses appear more likely to be mediated at the induction rather than the effector stage (33,486).

Pathogenesis and the Role of Epstein-Barr Virus

In situ hybridization analysis has shown that individual tumors carry clonal chromosomal abnormalities but has not detected a common chromosomal marker that might provide a clue to tumor pathogenesis (reviewed in 258). A number of potentially interesting features of the H-RS cell phenotype have been reported, notably Bcl-2 expression, elevated levels of p53, and up-regulation of the IL-6 pathway of autocrine growth stimulation, but none of these is consistently present in all tumors or specifically linked to either EBV-positive or EBV-negative forms of the disease; furthermore, there is no evidence for *bcl-2* gene translocation or *p53* gene mutation in HD. By contrast, HRS cells do consistently stain strongly for components of the activated NF-Kb transcription factor complex, and in some HD-derived cell lines, the high NF-Kb activity appeared to be important both for continued cell growth and for the cells' resistance to apoptosis (31). Interestingly, such lines (all EBV-negative) frequently lacked the natural regulator of NF-Kb activity, IKba (501), and in some cases carried mutations in the *IK*ba gene (68,123). Recent extension of this work to fresh HD biopsies suggests that a significant fraction of EBV-negative tumors likewise carry mutant *IK*ba alleles (68,123,221).

The evidence that EBV, when present, plays a causative role in the development of HD is strong but circumstantial. Just as with BL, the most compelling argument for causality is the frequency with which the tumor cells in HD are EBV genome-positive. In the case of HD, however, a mechanism for the etiologic involvement of the virus is easier to conceive. As described previously, H-RS cells carry the genotype of a cell that has inappropriately escaped the apoptotic death to which most germinal center B cells succumb during the affinity maturation of antibody responses. Positive selection into the postgerminal center (memory) B-cell repertoire usually depends on the receipt of at least two cell surface signals, both of which may be mimicked by EBV. The first is normally delivered by the somatically mutated Ig molecule and requires high-affinity binding to cognate antigen. In this context, the intracytosolic N-terminus of LMP2 is capable of sequestering Src-family tyrosine kinases in a manner similar to that occurring at the cytosolic face of the surface Ig complex after antigen binding (245). LMP2 can therefore potentially substitute for a surface Ig-mediated signal, as it appears to do in a transgenic mouse system in which LMP2 expression in immature B cells allows such cells to populate the periphery even in the absence of *Ig* gene rearrangement and surface Ig expression (69). The second requirement for positive selection in germinal centers is normally delivered by the CD40 pathway and requires a cognate interaction between the B-cell and a CD40 ligand-expressing T cell. In this context, LMP1 is known to activate the CD40 pathway by constitutively binding members of the same TNF-receptor–associated factor (TRAF) family of signaling molecules as does CD40 following its ligand-induced aggregation in the membrane, leading to the activation of several transcription factors, including AP1 and NF-Kb (reviewed in 245).

A key aspect of the role of EBV in HD pathogenesis may therefore be to induce the inappropriate survival and possibly the proliferation of an otherwise sterile postgerminal center B cell through expression of the LMPs. When and where EBV acts relative to the cellular genetic events underlying tumor development can only be speculated on. However, cells with an H-RS–like appearance and a Latency II pattern of gene expression are generated within extrafollicular areas of lymphoid tissues during the course of primary infection. The fate of these cells is unknown, but they may form a persistent population from which HD might subsequently evolve by accumulated cellular changes; indeed, a proliferating clone of EBV-positive H-RS–like cells has been detected *in vivo* within 2 years of IM (224; see also next section). Whatever the role of the virus, the fact that all three histologic subtypes of classic HD can present in EBV genome–negative form indicates that the same end point can be reached by a different pathogenetic route. There are rare examples of EBV-negative HD that might have arisen from a preexisting virus-positive lesion (112), but generally tumors that are negative for the EBER and LMP1 markers lack any sign of integrated EBV genomic fragments (434), such as have been reported in some cases of EBER-negative sporadic BL (369). Indeed, there are well-documented cases of EBV genome–negative HD arising in patients with no serologic markers of prior EBV infection (273). It remains to be seen whether exposure to some other infectious agent is a factor in EBV-negative HD, particularly those cases of predominantly ns-HD arising in young adults in the Western world (152). However, the recent data on *IK*ba gene mutation in EBV-negative cases of the disease indicate that at least one aspect of EBV's likely pathogenetic role, the constitutive activation of NF-Kb, can be independently achieved by cellular genetic change.

OTHER B-CELL LYMPHOMAS

One of the findings that adds significance to the association of EBV with PTLD, BL, and classic HD is the rarity with which the virus is detected in other tumors of B-cell origin. Only a small percentage of non-Hodgkin's lymphomas arising in immunocompetent individuals show evidence of EBER expression—and then usually only in a small fraction of the tumor cells, suggestive of a passenger infection occurring after the malignant clone was established (reviewed in 173). There are, however, some rare B-cell lymphomas that do frequently carry the virus and that are potentially instructive in understanding some of EBV's better known lymphoma associations.

One is a B-cell lymphoma arising at certain sites of long-term inflammation, notably the pyothorax-associated lymphomas seen in patients sometimes 20 years or more after introduction of an artificial pneumothorax. These tumors are consistently EBV genome-positive (141,400), as are very rare examples of lymphomas arising from sites of chronic inflammation in bone and skin (94). Such tumors present as diffuse large cell lymphomas, sometimes with an immunoblastic appearance similar to PTLD and reportedly can display either full latent gene expression or more restricted forms of latency. It is thought that chronic inflammation might provide a local cytokine environment favoring EBV-induced B-cell proliferation at the expense of CTL control, predisposing over time to the evolution of lymphoma.

A second, unexpected association involves rare cases of chronic lymphocytic leukemia, a B-cell tumor not normally linked with EBV, which appear to transform to malignant cells with a H-RS–like phenotype. These H-RS–like cells, sometimes clonally related to the original leukemic population, are often EBV-positive and express LMP1 (224,308). There are indeed cases in which these lesions have progressed to classic EBV-positive HD (308), indicating the potential

value of such cases as a model for HD pathogenesis.

T-CELL LYMPHOMAS

The detection of EBV DNA in the circulating T cells of an infant with VAHS-like disease (247) and in three T-cell lymphomas arising in a setting of chronic active EBV infection (219) provided the first indication that EBV, long considered a strictly B-lymphotropic agent, could access the T-cell compartment. Recently, a wide range of T and NK cell tumors have been screened for EBV status by EBER *in situ* hybridization, and the following groups of virus-associated lymphomas have been identified (reviewed in 345 and 444).

Virus-Associated Hemophagocytic Syndrome T-Cell Lymphomas

The VAHS tumors are of mature CD4+ or CD8+ T-cell origin and are seen most frequently in Southeast Asian children either after acute primary EBV infection manifest as VAHS or in the setting of chronic active EBV infection with VAHS-like symptoms (219,445). The tumors are monoclonal by *TCR* gene rearrangement and EBV genomic analysis but often develop from a premalignant pool of EBV-infected T cells detectable in peripheral blood (222). When analyzed, they are reported to display a Latency II form of infection by EBV latent gene transcription but often with very low LMP1 transcription and only trace levels of LMP1 protein detectable by staining (83,222); such a pattern is better clarified as Latency I/II to distinguish from the classic Latency II seen in HD. The release of cytokines, particularly TNF-a, from the EBV-positive T lymphoma cells may induce macrophage activation and consequent histiocytic infiltration of the tumor (264), leading to its frequent reporting as malignant histiocytosis (445).

Nasal NK/T-Cell Lymphoma

This is a specific extranodal lymphoma of angiocentric type, again most common in Southeast Asia, which presents in the nasal cavity. The tumor causes progressive erosion of bone tissue, again probably cytokine-mediated; hence, its alternative description as lethal midline granuloma. A study of five cases in Japan first drew attention to the consistent presence of EBV in all tumor cells (177), and the strength of the association has been amply confirmed on similar tumors in Chinese and European patients (345). Some of these tumors display a CD3+, CD56+/– surface phenotype and carry TCR-b or -g chain rearrangements consistent with a T-cell origin, but most are CD3– and CD56+, have no TCR rearrangements, and appear to be derived from NK cells. They have, therefore, been jointly classified as *nasal NK/T-cell lymphomas* (215). EBV gene transcription again follows a Latency I/II pattern, with low LMP1 expression restricted to a minority of tumor cells (303).

Other T or NK Cell Tumors

A number of other uniformly EBER-positive T or NK cell tumors have been reported that do not fit easily into either of the above categories but that may share similar pathogenetic features with them (444). These include rare lymphomas presenting in the skin or gastrointestinal tract with either an NK/T-cell or distinct T-cell phenotype, other lymphomas of gd T-cell origin (20), and some NK cell tumors presenting as frank leukemias (180). Less consistent is the association between EBV and angioimmunoblastic lymphadenopathy (AILD)-like T-cell lymphomas (10,345), a heterogeneous group of node-based tumors that form a continuum with benign AILD lesions themselves. Often, the malignant T cells in these lesions are entirely EBER-negative, and EBV infection is restricted to small foci of B immunoblasts within the tumor mass. In contrast, EBER-positive T cells are detectable in about 40% of these tumors but, when present, constitute only a fraction (less than 5% to more than 50%) of the malignant population. When analyzed, such tumors are EBNA2-negative, LMP1-positive, but again, LMP1 expression is restricted to just a subset of the EBER-positive cells. Such situations, when EBV colonizes only a fraction of the malignant population, are most easily interpreted as a “passenger” infection, although in some cases, the infection might still confer a growth and survival advantage on the tumor cells *in vivo* (281,345).

EBV, therefore, appears etiologically linked to certain specialized groups of T-cell or NK cell lymphoma, of which the VAHS-associated T-cell lymphomas are potentially the most instructive in terms of tumor pathogenesis because of the speed of their development. Appearance of a malignant clone, often with defined chromosomal aberrations, can occur within 1 to 2 years of primary EBV infection and is frequently preceded by an EBV-positive T-cell lymphocytosis in peripheral blood (222,444). Because most surveys (see earlier) suggest that EBV rarely if ever accesses the T-cell system during the usual course of infection, the evidence from VAHS patients implies that EBV infection of T cells, if achieved, carries a high lymphomagenic risk. This could reflect the fact that EBV's latent genes have evolved for use in the B-cell system and that some of these genes, notably *EBNA1, LMP1,* and *LMP2,* could become inappropriately activated in a T-cell environment.

NASOPHARYNGEAL CARCINOMA

Carcinomas of nasopharyngeal epithelium are seen in all parts of the world but vary in incidence and histologic subtype among population groups (reviewed in 1). These tumors are now broadly classified into keratinizing carcinomas with clear evidence of squamous differentiation (originally designated World Health Organization [WHO] I), and nonkeratinizing carcinomas of poorly differentiated or undifferentiated epithelial cells (incorporating the original WHO II and III categories). The latter often show marked lymphocytic infiltration, hence their frequent description as *lymphoepitheliomas* of the nasopharynx. In many areas of the world, including Europe and North America, nasopharyngeal carcinoma (NPC) is a rare disease with an age-adjusted incidence below $0.5/10^5$ per year; both forms of the tumor are observed. By contrast, NPC is much more common among Southeast Asian populations, especially in Southern Chinese people, in whom incidence rates reach $25/10^5$ per year, as well as among Inuit people and in some North and East African populations. In all these high-incidence areas, most tumors are of the nonkeratinizing type and preferentially affect males (male-to-female ratio, 2:1 to 3:1), mostly in middle age.

Association with Epstein-Barr Virus

A link between EBV and NPC was first suggested on the basis of seroepidemiologic studies showing elevated antiviral antibody titers in NPC patients (339). Thereafter, DNA–DNA reassociation and *in situ* hybridization studies indicated the regular presence of the viral genome in NPC tumor cells, the first evidence for EBV infecting any epithelial target (499,519). Furthermore, analysis of the viral episome's terminal repeats showed that the tumor arose from a single EBV-positive progenitor cell (364). Essentially all cases of nonkeratinizing NPC analyzed worldwide are EBV genome-positive, and in most studies, this tumor type has been the sole focus of attention. However, EBV is now also linked to most cases of the rarer keratinizing form of NPC in Southeast Asia and to about 30% of such tumors seen in European populations (326,349).

The unexpected association of EBV with an epithelial tumor prompted speculation that particular viral strains might be preferentially epitheliotropic or have growth-transforming activity for epithelial cells. As described earlier, many of the unique polymorphic markers first identified in Chinese NPC-derived strains turned out to be characteristic of viruses prevalent in the general Chinese population. Nevertheless, the question of some form of preferential association of particular virus strains with this tumor remains open (290). Two recent studies, one based in Taiwan (502) and the other in North Africa (47), analyzed local EBV polymorphisms in the BamHI F and H regions, respectively, and found that the allelic distribution among NPC-derived virus strains is skewed compared with that seen in the general population and in EBV-positive lymphoid tumors from the same area.

An important development arising from EBV's association with NPC is the use of anti-EBV antibody responses as diagnostic and prognostic tools in disease monitoring. A key observation in this context was that most NPC patients have not only elevated IgG but also detectable IgA responses to VCA and EA(D) (189). The fact that IgA antibodies to EBV are only rarely present in healthy virus carriers prompted large-scale screening of Chinese populations in selected high NPC incidence areas. This identified 1% to 2% of the population who were persistently IgA anti-VCA positive, and many of the NPC cases arising over the next 3 years in the tested populations were drawn from this particular subset, indicating the value of IgA anti-VCA as a marker of elevated tumor risk (517). These approaches are not applicable to all populations, however, particularly in North Africa where a significant fraction of NPC arises in young adult males in the absence of a marked IgA response (108). Hence, work continues in an effort to identify individual EBV lytic cycle antigens, such as BZLF1, BRLF1, the viral thymidine kinase (BXLF1), or alkaline DNAase (BGLF), which when used as substrates in serologic assays, may offer greater specificity in identifying incipient NPC (1). An alternative and potentially more useful indicator of tumor status came with the finding that EBV DNA, presumably released from tumor cells, could be PCR amplified from the serum of NPC patients (322). This has led to the development of a real-time quantitative PCR assay for EBV DNA load in plasma and serum, which correlates with tumor burden and which can be used as a sensitive monitor of tumor recurrence (284,285).

Cellular and Viral Phenotype of Nasopharyngeal Carcinoma

Most work in this area has involved the direct analysis of biopsy material because the tumor has proved very difficult to grow both *in vitro* and in nude mice (66); indeed, the only NPC-derived *in vitro* line to retain the viral genome was established from tumor cells that had been first passaged in nude mice (86). Generally, the NPC cell phenotype resembles that induced in epithelial cells by cytokines such as IFN-g or TNF-a. Thus, the tumor cells express both HLA class I and class II antigens and have good antigen-processing function, at least in terms of presenting endogenously expressed proteins to CD8+ T cells by the HLA class I pathway (240,266). NPC cells also express cell surface molecules normally involved in epithelial–lymphoid interactions, namely CD40, CD54, CD70, CD80, CD86, and CD95 (4,404). It is not known to what extent this phenotype is imposed by EBV gene expression in the NPC cells themselves or is induced by cytokines derived from the largely CD4+ T-cell infiltrate. The concept of co-stimulation between the malignant and nonmalignant elements in NPC is frequently advanced, although which of the many candidate cytokine or co-stimulatory pathways might be important remains to be determined (4,207).

The pattern of viral gene transcription in NPC (55,67,226,432) is ostensibly similar to that seen in EBV genome–positive HD, but there are actually important quantitative differences that are also reflected at the level of antigen expression (129,514). Thus, the epithelial tumor cells are EBER-positive and appear to express unusually high levels of BamHI A transcripts; whether the latter encode any protein products in the tumor cells remains a controversial issue (246). The Qp-initiated BamHI Q/U/K–spliced EBNA1 transcript is readily amplifiable from tumor RNA, and this correlates with expression of the EBNA1 protein in the absence of other EBNAs. In contrast, LMP1 mRNA is frequently difficult to detect using the standard RT-PCR assay, in line with the absence of detectable LMP1 protein in up to 65% tumors. Although LMP2A and 2B transcripts are readily amplifiable, LMP2 protein expression has still not been unequivocally demonstrated in NPC using current reagents, although it is interesting that NPC patients are the only group who reproducibly show serum antibody responses to this protein (139,270). Given these results, NPC is best classified as displaying a Latency I/II form of infection, intermediate between those displayed by BL (Latency I) and HD (Latency II). Additionally, recent work has detected regular transcription of what was thought to be an exclusively lytic cycle gene, *BARF1,* in NPC, but not in other EBV-associated tumors such as HD (53). This could be pathogenetically significant since BARF1, if expressed at the protein level, has structural and functional homology with the cellular granulocyte CSF receptor (442) and has growth-transforming activity in some *in vitro* assays (488).

Finally, another puzzling aspect of the viral phenotype in NPC cells is the apparent absence by staining of lytically infected cells within the tumor population. The fact that antibody titers to lytic cycle antigens such as VCA and EA rise in proportion to tumor burden in NPC patients (191) suggests either that the antigenic stimulus comes from the tumor itself or that virus replication at some other site correlates with tumor evolution and progression. In this context, the analysis of viral DNA from NPC biopsy specimens showed that only episomal circular (latent) and not linear (lytic) forms were detectable (364). Likewise, screening tumor sections has in most cases either failed to find evidence of lytic antigen-positive cells or detected very occasional expression of the immediate early antigen BZLF1 only (89,168); sensitive RT-PCR assays have detected other lytic transcripts in NPC biopsy specimens, but it is difficult to know

whether these stem from tumor cells or from infiltrating B lymphocytes.

Nonviral Risk Factors

The high incidence of NPC in several Southeast Asian populations and in the anthropologically related Inuit people raise the possibility of genetic factors influencing NPC risk (reviewed in 1). Access to familial cases of NPC, reported in both high- and low-risk populations, should provide an important in-road into this question in the future. To date, most studies have focused on polymorphisms at the HLA locus, sibling pair data having suggested that a gene (or genes) closely linked to this locus is associated with a 20-fold increased risk for NPC (287). Although the susceptibility gene is unlikely to be an HLA allele itself, in Singapore Chinese, the HLA-A2, Bw46 and A19, B17 class I haplotypes and the DRB10803 class II allele are each associated with a more than twofold increased risk for NPC, whereas two other common class I alleles, HLA-A11 and B13, are each associated with a significant reduction in risk (1).

Environmental or dietary factors also appear to be important. Thus, in the immigrant Chinese population in North America, the NPC incidence is decreasing with time and is lower in the second generation, although still well above typical rates in whites (272). Most theories of dietary and environmental influence on NPC risk invoke as a mechanism chronic exposure of the nasopharyngeal mucosa to chemical carcinogens or physical irritants. Dietary exposure to salted fish (a source of volatile nitrosamines) in infancy has been implicated as a significant risk factor in Southern Chinese populations, whereas in some other Southeast Asian populations, occupational exposure to smoke or dust may be important (1). A number of environmental factors, dictated by diet or lifestyle, may therefore contribute to tumor risk. Furthermore, environmental and genetic influences may interact, as illustrated by the recent finding that individuals homozygous for a particular allele of cytochrome P450 2E1, an enzyme involved in nitrosamine activation, appear to be at increased risk for NPC (196).

Pathogenesis and the Role of Epstein-Barr Virus

Despite a current resurgence of interest, little is known at the molecular level about cellular genetic changes in the pathogenesis of NPC. Obvious candidate genes, such as *c-myc, ras, Rb,* and *p53,* are not detectably altered, although interestingly, some tumor cells do express high levels of the wild-type p53 protein (168). However, more recent work has detected frequent loss of heterozygosity at chromosomes 3p24, 9p21 (leading to deletion of the *p16* gene), 11q, 14q12-13, and 14q32 (203,204,208,283,433); such findings imply that a number of tumor suppressor genes may be targeted during NPC development.

The point at which EBV infection affects the process remains to be determined. However, several studies of tumor-free individuals in high NPC risk areas have been unable to find EBV-positive epithelial cells in nasopharyngeal biopsy specimens (85,396,461). NPC is therefore unlikely to evolve from a cell that naturally harbors EBV; rather, it must be a consequence of the rare entry of the virus into the target epithelium. How such entry is achieved is not known, but possibilities include direct CR2-independent transmission by B cell–epithelial cell contact, as has been observed *in vitro* (211,334,512), or entry of the virus as a virion–IgA complex by the polymeric IgA receptor (430). The initiating events of NPC pathogenesis may well be independent of EBV because the tumor usually presents in the fourth decade or later, yet the epidemiologic evidence suggests that exposure to environmental carcinogens early in life is an important factor. Inhaled particles of a particular size lodge in the fossa of Rosenmuller, the anatomic site from which NPC arises, and preneoplastic changes induced by such chronic exposure may even render cells more susceptible to latent EBV infection. Identifying preneoplastic lesions in the nasopharynx has been difficult, even in populations at high risk for NPC. One study of archival material identified two rare cases of EBER-positive NPC *in situ* (85), whereas another found six dysplastic and five carcinoma *in situ* lesions, all of which were EBV positive with a Latency II pattern of viral gene expression and uniformly expressing LMP1 by immunohistochemical staining (350). Most cases in the latter study developed frank NPC within 12 months of the original biopsy, indicating that the neoplastic process was well advanced at the time active EBV infection was detected. Whether EBV is involved at an earlier stage is not known, but other studies in Southern Chinese have detected low-grade dysplastic lesions in the nasopharynx with characteristic loss of heterozygosity at chromosomes 3p in the absence of detectable EBV infection; only higher-grade dysplastic lesions had acquired the virus (79).

The circumstantial evidence argues strongly for a role for EBV in NPC pathogenesis. Certainly, many of the tumor cell surface markers (CD40, CD54, CD70, CD80/86) are directly inducible in cultured epithelial cells by LMP1 expression (103,121), as are a number of other potentially interesting changes seen in LMP1 transfectants, notably up-regulation of the epidermal growth factor receptor (302) and of the matrix metalloproteinase MMP9 (455) as well as down-regulation of E-cadherin, leading to acquisition of a more invasive cell phenotype (128). Despite the pleiotropic nature of its effects *in vitro*, transgenic mouse studies indicate that LMP1 alone cannot push epithelial cells to full malignancy (496). Although not as well studied as yet, LMP2A could also have potentially important effects when expressed in epithelial cells, for example, through interaction with tyrosine kinase signaling pathways linked to cell adhesion molecules (407). Contributions from the other EBV proteins actually or putatively expressed in NPC cells, namely EBNA1, the BamHI A–encoded polypeptides (260a,432a), and BARF1 (488), remain possible but are as yet unsubstantiated.

OTHER LYMPHOEPITHELIOMAS AND GASTRIC CARCINOMA

Rare lymphoepitheliomas histologically similar to nonkeratinizing NPC (*undifferentiated carcinomas of nasopharyngeal type* [UCNT]) are seen at various anatomic sites. Very interestingly, when the target epithelium resembles the nasopharynx in being a foregut-derived tissue, the corresponding tumors are frequently EBER-positive. These include salivary gland carcinomas that are most common in Southern Chinese and Inuit people (205,394) as well as thymic (277), lung (500) and especially gastric (59,417) UCNT-type carcinomas. Surprisingly, in addition to these rare UCNT tumors of the stomach, about 5% to 15% of conventional gastric adenocarcinomas in human populations worldwide also carry the virus (418,454), whereas adenocarcinomas at other sites are consistently EBV-negative. Serologic assays have indicated a higher incidence of IgA anti-VCA antibodies among patients with EBV genome–positive disease than among healthy

controls or those with EBV-negative tumors, but the differences are much less marked than in the case of NPC (420).

The gastric tumors, whether of UCNT type or not, are uniformly EBER-positive throughout the malignant population and are slightly more restricted than NPC in their pattern of viral transcription; they express the Qp-initiated EBNA1 mRNA, the BamHI A RNAs, and usually also the LMP2A transcript but not LMP1 (210,447). A similar form of latency is established when EBV carrying a selectable marker is stably introduced into established virus-negative gastric carcinoma cell lines *in vitro* (512) or into primary gastric epithelial cell cultures (334). More importantly, EBV enhanced the doubling potential of these primary epithelial cells and in one case allowed the selection of clones that appear to be immortalized (334). Although the mechanism of this epithelial cell growth deregulation remains to be determined, these findings are again suggestive of an effector role for the virus in gastric carcinogenesis.

Interestingly, the EBV-positive gastric carcinomas as a group express both HLA class I and class II and show more consistent expression of CD54 and CD95 than their EBV-negative counterparts (395). EBV-positive tumors of UCNT type also show a greater infiltration of CD8+ T cells than is apparent in NPC (395), but the antigenic specificity of this infiltrate is as yet undefined (261).

TUMORS OF OTHER LINEAGES

A large range of other tumors and proliferative conditions have been examined for EBV association, usually by EBER *in situ* hybridization, with negative results in most cases. There are, however, rare exceptions worthy of mention. Apart from B cells, the only report of conventional EBV-induced cell transformation *in vitro*, albeit characterized by very slow growth, involves CR2-positive follicular dendritic cells (281). It is therefore interesting to note rare reports of EBV-positive follicular dendritic cell tumors (415) and of inflammatory “pseudotumors” composed of EBV-positive spindle-shaped cells that had markers of either follicular dendritic (413) or smooth muscle (18) origin. The latter lesions may represent earlier stages of a process that, in immunocompromised patients, culminates in EBV-positive leiomyosarcomas (265,299,473).

A second example of a situation in which EBV can be found in tumor cells involves hepatocellular carcinoma. Although hepatitis B and C viruses are much more likely to play an etiologic role in this context, a significant number of liver tumors in a recent study carried EBV DNA and expressed EBNA1 in a minority (7% to 13%) of cells in the tumor population (446). Surprisingly, these latently infected tumor cells were EBER negative, calling into question the validity of EBERs as a ubiquitous marker of latent EBV infection. As a result, the controversial question of an association between EBV and a subset of breast carcinomas, in which EBER *in situ* hybridization has given contrasting results in different laboratories (151,262), will need to be readdressed using alternative screening assays.

OVERVIEW OF MALIGNANCIES ASSOCIATED WITH EPSTEIN-BARR VIRUS

EBV is therefore now strongly linked to seven types of human malignancy, all of which satisfy two basic criteria: (a) that the viral genome is detectable as a monoclonal episome in a significant proportion of tumors of the relevant histologic subtype, and (b) that in virus-positive cases, the viral genome is present in every tumor cell. Table 2 summarizes the essential information regarding these seven tumor types, the length of the latency period between primary EBV infection (or initiation of immune suppression) and tumor development, the strength of the viral association, and the extent of viral antigen expression in tumor cells.

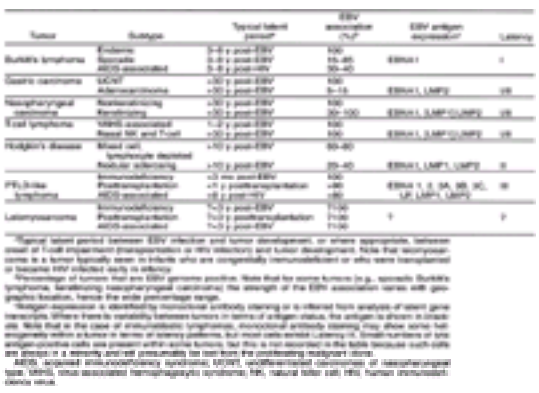

TABLE 2. *Overview of EBV-associated malignancies*

Note that the two tumor types arising in an immunocompromised setting, namely the PTLD-like lymphomas and the leiomyosarcomas, have relatively short latency periods. The lymphomas can arise in an immunodeficient patient within weeks to months of primary EBV infection and in a transplant recipient within the first year after transplantation when T-cell impairment is most marked. This reflects the fact that, in the special case of these PTLD-like lymphomas, EBV is both necessary and sufficient to induce tumor growth; the tumors are predominantly composed of EBV-transformed cells of the kind that appear transiently during all primary EBV infections but whose growth is usually curtailed by T-cell surveillance. The potential parallels between this situation and the pathogenesis of the EBV-positive leiomyosarcomas need to be explored; in that context, identification of the pattern of viral gene expression in the smooth muscle tumor cells is an important priority.

The only other virus-associated malignancy with a similarly rapid pathogenesis is the rare T-cell lymphoma that appears in young children either with an immediate history of VAHS linked to primary EBV infection or with chronic active EBV infection associated with VAHS-like symptoms. These children are not obviously immunodeficient before their EBV infection but arguably may become so as a result of the very event that underlies both VAHS and tumor development, namely an unusual level of virus entry into the T-cell system. The chronicity of VAHS-like symptoms in many patients

implies that when the virus does access the T-cell compartment, some viral antigen expression is maintained (as judged by resultant cytokine production), yet the infection is not cleared by immune surveillance. It will be important to understand how this occurs, what the early events of T-cell infection are, and what cellular genetic changes are subsequently involved in the rapid evolution of these lymphomas. Almost certainly, tumorigenesis does not involve a simple transfer of the virus' physiologic growth-transforming program from a B-cell to a T-cell environment. These T-cell lymphomas, and indeed all available *in vitro* models of T-cell infection, do not express the EBNA2 and EBNA3 family proteins characteristic of B-cell growth transformation but display a more restricted form of latency.

In contrast, BL, HD, the NK/T-cell lymphomas, and the gastric and nasopharyngeal carcinomas all develop over much longer latent periods, more typical of tumors with a complex multistage pathogenesis. In each case, we still know relatively little about the mechanism whereby EBV contributes to tumor development or about the timing of viral action. In at least four tumor types (BL, HD, and the two carcinomas), different subtypes of the tumor distinguishable by histologic or geographic criteria show different strengths of association with the virus. However, the existence of an EBV-negative subset of tumors need not weaken the case for an etiologic role for the virus when present. The example of HD is particularly instructive here in that a virus-induced effect that is postulated to be important for tumor development in EBV-positive disease (constitutive NFkb activation) is indeed also a feature of EBV-negative disease, although there it is produced by a quite different, virus-independent, mechanism.

The tumors with long latency periods are also distinct in that they all arise in nonimmunocompromised patients who (where studied) have virus-specific CTL memory still intact. The same tumors also display restricted forms of virus latency characterized by Qp rather than Cp/Wp usage and hence by down-regulation of the immunodominant EBNA3A, 3B, 3C proteins. This might be interpreted as indicating immunologic pressure for selection of a cell lacking the EBNA3 proteins, perhaps as a final rate-limiting step in tumor evolution. In fact, this is unlikely to be the case, not least because (with the possible exception of HD) these tumors show no evidence of increased incidence in T-cell–immunocompromised patients. It is much more likely that the resident form of virus latency is imposed by the tumor cell phenotype per se. Thus, the restricted forms of latent gene expression in B-cell malignancies such as BL and HD seem to reflect forms transiently adopted by EBV at particular times during the normal life history of an infected B cell. Likewise, the non–B-cell tumors appear to arise from the (relatively rare) entry of the virus into an atypical cell lineage in which Cp/Wp usage is not naturally activated or at least is not sustainable.

PREVENTION AND CONTROL OF DISEASE

The sheer range of EBV-associated diseases emphasizes the importance of developing either prophylactic vaccines to reduce disease burden or therapeutic strategies that specifically target virus-positive lesions. A number of approaches are currently being pursued towards these goals.

Prophylactic Vaccination to Modify Primary Infection

Identifying the major viral envelope glycoprotein gp350 as the dominant target of the neutralizing antibody response (199,465) was the initial spur to the development of a prophylactic EBV vaccine. However, it is now evident that neutralizing antibodies alone, or the combination of antibody and cell-mediated responses to a single envelope component, are unlikely to offer long-lasting sterile immunity against an orally transmitted agent such as EBV (504,511). Nevertheless, a gp350-based vaccine could reduce the dose of infectious virus acquired orally and, particularly if combined with other vaccine antigens eliciting appropriate CD8+ T-cell responses, could limit virus replication at the initial site of infection. The consequent reduction in virus load may therefore be sufficient to prevent primary infections leading either to the symptoms of acute IM or, in immunocompromised patients, to PTLD.

Prophylactic Vaccination in Animal Models

Certain species of New World primate, although carrying their own LCVs, do not have cross-reactive antibodies against EBV and remain susceptible to experimental infection. The common marmoset can be infected by submucosal injection of EBV at oropharyngeal sites, leading in some cases to mild mononucleosis-like symptoms followed by viral persistence in the lymphoid system (95). By contrast, cotton-top tamarins (421) and owl monkeys (125) are susceptible not just to EBV infection but also to an EBV-driven B-lymphoproliferative disease, which in histologic appearance, oligoclonality, and viral gene expression, mirrors PTLD. However, disease induction requires direct intraperitoneal injection of relatively large doses of virus rather than infection by the natural oral route. Despite these limitations, studies with cotton-top tamarins have clearly demonstrated disease protection both by subunit gp350-based vaccines in various adjuvant formulations and by recombinant vaccinia and adenovirus vectors expressing gp350 (310,311,365). Interestingly, protective immunity in these and other studies did not always correlate with the presence of virus neutralizing antibodies, implying a role for gp350-specific cell-mediated responses in disease protection (311).

Access to a more physiologic animal model of EBV infection has become important not just for testing vaccination strategies but also for studying more closely the biology of the virus *in vivo*. The LCVs of Old World primates are interesting in this regard because they share extensive genome technology with EBV, have similar B-cell–transforming activity *in vitro* and, as shown by the LCV of cynomologus macaques, can induce B-lymphoproliferative disease or lymphoma in their natural hosts when T-cell immunity is impaired (134). Recently, experimental infection of young rhesus macaques with rhesus LCV has been shown to induce a transient IM-like illness with oropharyngeal virus replication, lymphocytosis, and sequential antibody responses to lytic and then latent cycle antigens. This was followed by the establishment of a carrier state with apparent immunity to a second exogenous viral challenge (307). Such a model has a number of important potential applications.

Prophylactic Vaccination in Humans

The first clinical testing of a gp350-expressing recombinant viral vaccine, based on the Chinese Tien Tan strain of vaccinia, was carried out on small groups of EBV-seronegative and EBV-seropositive children in Southern China.

Antibody responses to gp350 were induced in the former group and elevated in the latter, and six of nine vaccinees who were EBV-seronegative at the beginning of the trial remained virus free for at least the following 3 years (167). More recently, a gp350 subunit vaccine preparation has been administered to young adults and neutralizing antibody responses induced; the efficacy and duration of vaccine-induced protection from natural EBV infection remains to be determined (114).

Recognition of the importance of CD8+ T-cell responses in controlling primary EBV infection opens up the possibility of an alternative vaccine strategy based around virally encoded T-cell epitopes. Thus, it may be possible to prime the CD8+ T-cell response in naive individuals by vaccinating either with the synthetic epitope peptides themselves or with vectors expressing polyepitope constructs carrying epitopes for different HLA alleles in linear sequence (316). Such a strategy would again be aiming to limit lytic virus replication or the proliferation of latently infected cells within the initial period after oropharyngeal EBV transmission, rather than to prevent infection per se. First-generation vaccines, using synthetic lytic and latent cycle epitope peptides restricted through the HLA-B8 and HLA-A2.01 alleles, are now being tested in seronegative young adults at risk for IM (316). It is possible that maintenance of CD8+ T-cell immunity requires virus-specific CD4+ T-cell help *in vivo*, and the identification of immunodominant CD4+ T-cell epitopes in EBV antigens of the lytic and latent cycles is an important focus of current research.

Therapeutic Intervention

The involvement of the virus in so many proliferative or frankly malignant diseases offers a number of novel therapeutic possibilities. One gene therapeutic approach is to exploit the presence within tumor cells of viral transactivators such as EBNA1 (220) or EBNA2 (138) in order to drive expression of a suicide gene. Alternatively, EBV-positive tumor cell growth might be reversed by targeting EBNA1 (206) or LMP1 (229,298) transcription using ribozymes or antisense oligonucleotides. Many of these strategies remain at the “proof of principle” stage, however, whereas considerably more progress has been made toward targeting tumors by immunotherapeutic approaches.

Immunotherapy of PTLD-like Lesions in SCID Mice

Attempts to reconstitute the human immune system by xenografting in the severe combined immunodeficient (SCID) mouse, which lacks mature B or T cells, provided an unexpected animal model of EBV-induced lymphomagenesis. Reconstitution with more than 5×10^7 PBMCs from EBV-seropositive donors frequently leads to the appearance within 8 to 16 weeks of EBV-positive human B lymphomas, which in terms of histologic appearance, oligoclonality, and EBV gene expression, closely resemble the early-onset PTLD lesions seen in transplant patients (312; reviewed 218). Interestingly, tumor development requires the presence of autologous T cells (preferentially the CD4+ subset) in the original inoculum. This suggests that T-cell–derived cytokines enhance the early survival and growth of EBV-infected B cells in the SCID mouse environment at a time when autocrine B-cell growth factors are limiting. Thereafter, established tumors can be cured, or their growth severely impaired, by infusions of EBV-specific CTLs generated from the autologous donor by LCL stimulation *in vitro* (50,56,263), whereas lymphokine-activated killer cells and NK cell clones had little if any effect in the same assay (56,336).

Immunotherapy of PTLD in Humans

While the above work was in progress, an exactly analogous therapeutic strategy was being developed and tested in the clinic (reviewed in 195 and 336). This involved bone marrow transplant recipients, who can be at high risk of PTLD immediately after transplantation and in whom the disease arises in bone marrow-derived donor B cells. A first report indicated that PTLD growth could be reversed by a single infusion of small numbers of fresh PBMCs from the healthy EBV-seropositive bone marrow donor (346). This was associated with a dramatic and sustained increase in the EBV-specific CTL precursor frequencies in the patient's blood over several weeks after infusion (288), but was also accompanied by a high incidence of graft-versus-host disease (336,346). A second report showed that polyclonal preparations of EBV latent antigen-specific CTLs, reactivated from donor PBMCs by LCL stimulation *in vitro*, achieved equally effective tumor control without graft-versus-host disease and also could be used prophylactically to reduce high EBV genome loads in the blood of patients at risk for PTLD (383). Again, in many patients, there was a marked increase in EBV-specific CTL precursor frequency in the blood after a few weeks of infusion, and gene marking showed persistence of the infused cells for at least the next 3 years, during which time all infused patients remained tumor free (194,195,384). Several observations imply an effector role for EBV-specific T cells in this situation: first, the inability of nonspecific T-cell preparations to reduce viral load (169); second, the specific infiltration of infused cells at the site of the tumor (384); and third, the finding that a rare tumor that grew out in the face of T-cell therapy carried an EBV strain that had lost the two CD8+ epitopes against which the infused T cells were predominantly directed (155). Although CD8+ T cells recognizing latent cycle epitopes are thought to be the major effectors against PTLD, CD4+ T cells are present within all infused preparations (usually as a minor but occasionally as the major component) and may be important either in facilitating the CD8+ T-cell expansion *in vivo* or as effectors in their own right.

Extending this approach to the treatment of PTLD in solid organ transplant recipients is logistically more difficult because these tumors are usually derived from host rather than donor B cells. In most cases, therefore, preparations of autologous EBV-specific T cells have to be made from posttransplantation bleeds when the patient is already immunocompromised. A recent study has nevertheless shown that active CTL preparations can indeed by generated from such individuals by a modification of the *in vitro* LCL stimulation protocol, and that infusing such T cells can efficiently target PTLD lesions (234). Nevertheless, the above logistic difficulties have prompted some laboratories to attempt adoptive therapy using EBV-specific T-cell preparations from allogenic but partially HLA-matched donors (176,201,336). Although such effectors are unlikely to persist *in vivo*, they may nevertheless provide an immediate means of targeting PTLD lesions, especially in patients who have undergone primary EBV infection at the time of transplantation and in whom the virus-specific T-cell response has not yet developed.

Immunotherapy for Other EBV-associated Tumors

The fact that most EBV-positive malignancies other than PTLD show highly restricted latent antigen expression, with loss in particular of the immunodominant EBNA3A, 3B, 3C proteins, emphasizes the difficulties inherent in trying to target these tumors by immunologic means. Unless it proves possible to activate expression of these immunodominant EBNA3 targets in tumor cells, for instance, by using a demethylating agent such as 5'-azacytidine to rescue Cp transcription (378), immunotherapeutic approaches are likely to have to rely on subdominant components of the CD8+ T-cell response. Most interest to date has focused on HD, in which two subdominant target antigens, LMP1 and LMP2, are expressed in the malignant cells. Conventional LCL-stimulated CTL preparations can be generated *in vitro* from the blood of HD patients and, although predominantly reactive to EBNA3 antigens, sometimes contain weak reactivities to LMP2 or, rarely, to LMP1 (81,140, 385,425). Infusing such T-cell preparations back into the patients has so far shown persistence of the cells for several months and a corresponding reduction in EBV genome load in PBMCs (385); more studies will be required to determine whether there is any effect on tumor growth.

A further step will be to generate CTL preparations that are enriched for LMP reactivities at the expense of the usually dominant EBNA3 response. Selective *in vitro* reactivation of LMP-specific CTLs from memory can be achieved by stimulating PBMCs with epitope peptides when these sequences have been identified (237,371) or with monocytes and dendritic cells expressing whole antigen from recombinant viral vectors (368,425). It is encouraging in the context of HD therapy that both LMP1 (237) and LMP2 (268) epitopes are known that are weakly immunogenic and restricted through HLA-A2.01, a frequent allele in Western populations. Similarly, in the context of NPC, a tumor in which LMP2 appears to be more frequently expressed than LMP1, there are LMP2 epitopes that are restricted through the HLA-A11.01, HLA-A24.02, and HLA-B40.01 alleles, all of which are relatively common in Southeast Asian populations (268). Their existence offers the chance of targeting these EBV-positive malignancies either with combinations of epitope-specific CTL clones reactivated and expanded *in vitro* before reinfusion, or through boosting the patient's endogenous response to these epitopes by vaccinating with vectors expressing whole antigen or polyepitope constructs.

SUMMARY

Considerable progress has been made in our understanding of the biology of EBV infections and in our appreciation of the full extent of EBV-associated disease. A series of experiments on isolated B-cell subpopulations, now being complemented by the analysis of individual microdissected cells, is finally opening up the study of EBV infections within the human B-cell system *in vivo*. This is showing that EBV has evolved sophisticated mechanisms first for colonizing the B-cell system of the naive host, then for persisting within that system in the face of immune surveillance, and finally for rescuing infectious virus from latently infected cells within the persistent pool. Progress has also been made in identifying the full range of CD8+ T-cell responses that control EBV infection, in the process revealing the unexpectedly large size of the lytic cycle-specific response, particularly in IM patients. An equally thorough analysis of CD4+ T-cell, and perhaps also NK cell, responses is now required. An improved understanding of the immune response to EBV infection, combined with the lessons being learned from primary infections in XLP and VAHS patients, has the potential to unravel the pathogenesis of IM. There is, however, still much to learn about the biology of EBV infections, and an animal model that faithfully reproduces this biology will be essential for hypothesis testing, in particular for the analysis of individual viral gene functions *in vivo*. The recently developed rhesus LCV model holds considerable potential in that regard.

The existence of atypical primary infections, leading in the case of immunocompromised patients to PTLD lesions and in the case of VAHS patients to T-cell lymphoma, provides an important opportunity to study accelerated examples of EBV-positive tumor development at the molecular level. At the same time, the range of other EBV-associated tumors goes on increasing, the most significant recent additions being the two histologic subtypes of gastric carcinoma and the smooth muscle cell tumors of immunosuppressed patients. The number of tumors now linked to EBV is unexpectedly large, but in each case, the consistency and specificity of the association are impressive, and the circumstantial evidence for a viral involvement in etiology is correspondingly strong. This presents students of this virus with two challenges. The first is to explain at a mechanistic level how EBV contributes to the development of so many distinct malignancies. The pace of recent work analyzing the biochemistry and effector functions of EBV latent proteins (reviewed in Chapter 74 [245]) suggests that this is an achievable goal in the near future, especially as host cell pathways of growth control are characterized in ever-greater detail. The seminal work identifying HD as a tumor of B-cell origin illustrates how advances outside the immediate EBV field can profoundly affect our understanding of the virus' role in tumor pathogenesis. The second major challenge is to exploit our increased understanding of EBV biology in designing new ways to prevent or treat virus-associated disease. It should be possible to develop an effective prophylactic vaccine that, although not protective against infection per se, nevertheless reduces the chances of primary infection leading to acute disease (acute IM, VAHS, or PTLD). Whether such a vaccine would alter the long-term risk for malignancy is debatable. However, with respect to established EBV-positive malignancies, the continued expression of viral proteins in tumor cells offers the prospect of at least two therapeutic interventions: pharmacologic targeting to interfere with the viral effector mechanisms that promote tumor growth, and immunologic targeting exploiting the immune system's capacity to recognize viral antigens as foreign. In this latter regard, the success of adoptive T-cell therapy for PTLD represents an important proof of principle and illustrates how effective an immunotherapeutic approach can be in the right circumstances. The extension of this strategy to other EBV-associated malignancies is awaited with interest and not a little uncertainty. EBV, the first human tumor virus to be discovered, therefore continues to provide a fascinating series of challenges to both basic and clinical scientists working in an area of human medicine in which virology, immunology, and oncology meet.

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CHAPTER 76 Cytomegaloviruses and Their Replication

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The cytomegaloviruses (CMVs) are a distinct, widely distributed subgroup of herpesviruses that share common growth characteristics and induce a cytopathology involving characteristic nuclear and cytoplasmic inclusions (432,499,500). Viruses in this group were generally known as salivary gland viruses until the common name cytomegalovirus was proposed by Weller et al. (595) to reflect both virus-induced cytopathic effects and the virus's role in congenitally acquired cytomegalic inclusion disease.

Cytomegaloviruses are the principal members of the betaherpesvirus subgroup, and they share several characteristics with other herpesviruses, including virion structure and the ability to establish persistent and latent infections. The CMVs exhibit a number of distinguishing biologic characteristics common to the betaherpesviruses, including salivary gland tropism, strict species specificity, and slow growth in cultured cells, all of which have been reviewed (216,378,461). Mammals each have a characteristic CMV (216,432,522). Based on genome nucleotide sequence comparisons of human CMV (92) and murine CMV (446), or human CMV and human betaherpesvirus HHV-6 (188), members of the betaherpesviruses have diverged markedly in different animal species as well as within the same animal species. Despite this divergence, significant predicted amino acid sequence homology in a core set of 70 to 80 betaherpesvirus-common open reading frames (ORFs) permits all of these viruses to be classified in the same subfamily (92,188,402,446,577) (see Chapter 80 and Chapter 81). In a similar way, amino acid sequence homology in as many as 46 viral ORFs controlling DNA replication, genome packaging, and virion morphogenesis shows that betaherpesviruses are related to all mammalian herpesviruses (see Chapter 71). Such standard sequence comparisons as well as more elaborate approaches (251) provide evidence that betaherpesviruses are more closely related to each other than they are to other herpesviruses.

In most areas of the world, human CMV spreads at an early age and infects a large majority of the population. This pattern has been altered by increased hygiene in developed countries, where this virus may reach only 40% to 60% of the population. Thus, in the developed world, a large proportion of the adult population remains susceptible to primary infection, increasing the risk of congenital transmission and subsequent disease (see Chapter 77). The importance of human CMV as a pathogen has also risen over the past three decades with the increase in organ allografting and immunosuppressive posttransplant therapies and the increase in acquired immunodeficiency syndrome (AIDS). These conditions predispose individuals to a primary CMV infection or to reactivation of latent infection, which may lead to fulminant, life-threatening disease that can be difficult to treat despite available antiviral drugs (141,379) (see Chapter 77).

VIRUS STRUCTURE AND GENOME ORGANIZATION

The virion of human CMV consists of an icosahedral capsid encasing a 235-kbp linear genome, surrounded by a tegument or matrix (354,617) and enveloped in a lipid bilayer carrying a large number of virus-encoded glycoproteins (66,511). By cryoelectron microscopy, the capsid appears similar to, though larger than, the herpes simplex virus (HSV)-1 capsid (95). In thin sections of infected cells (Fig. 1A,Fig. 1B) or by negative staining, the CMV envelope appears more pleiomorphic than the envelope of other herpesviruses (617), allowing intact CMV virions to be distinguished from other herpesviruses based on morphology. The genomes of the characterized CMVs are all linear DNA molecules ranging in size from 200 to 240 kbp (Table 1), which is significantly larger than those of other herpesviruses. The human CMV genome also exhibits a pattern of terminal and inverted repeats that vary in size depending on the virus strain and passage history (see Fig. 1C).

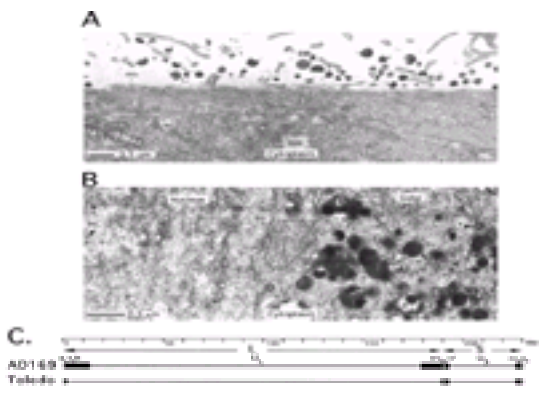

FIG. 1. Virus particles, replication, and genome structure. **A:** Surface of a cell onto which virions (*V*) and dense bodies (*DB*) of human CMV (Towne strain) have attached. This illustrates the size and approximate ratio of these two types of particles in a virus preparation, and the ability of both to attach to the cell surface. **B:** Productively infected cell with nucleocapsids (*NC*) in the nucleus and maturing virus (*V*) and dense bodies (*DB*) in the enlarged Golgi region of the cytoplasm, which forms the characteristic cytoplasmic inclusion of CMV. Many capsids lacking a dense core of nucleic acid can be observed in abundance in the nucleus, where, along with nucleocapsids, they form a characteristic nuclear inclusion of CMV (giving an “owl's eye” appearance to cells) (216). Envelopment of nucleocapsids (and capsids) occurs at the inner nuclear membrane, where thickened patches of modified membrane develop, as well as in the cytoplasm, whereas dense body envelopment occurs exclusively at cytoplasmic membranes. The *bar* represents 0.5 µm. (Micrographs similar to these were first published in ref. 387.) **C:** Human CMV genome structure. The *top line* is a size scale in kbp. The *second line* delineates the L and S components of the genome by *arrows.* The completely sequenced AD169*var*UK strain genome structure (92,124,386) is shown on the *third line* with unique sequences (*thin lines*) flanked by inverted repeats (*boxed areas*). The predicted genome structure of a low-passage level strain is depicted on the *fourth line* based on limited sequence analysis (82). Note that the leftward *b* sequence is present as a unique sequence, and the rightward *b* sequence repeat is truncated in clinical strains and is replaced by an additional unique (U_L) sequence . The lettering above the genome depicts the following features: The L-terminal *a* sequence repeat (a_L), zero to several (*n*) additional copies of the *a* sequence (a_n), the L-terminal *b* sequence repeat of the L component, the unique sequences of the L component (U_L), the L-S junction *b* sequence inverted repeat (*b*'), one to several (*m*) additional copies of the *a* sequence inverted repeats at the L-S junction (a'_m), the L-S junction *c* sequence inverted repeat (*c*'), the unique sequences of the S component (U_S), the S-terminal *c* sequence repeat, and the S-terminal *a* sequence repeat (a_S) with a variable number of additional copies of the *a* sequence (a_n). The *b* and *b*' repeats are also called TR_L and IR_L, respectively, and *cs*' and *c* repeats are also called IR_S and TR_S, respectively (92). In different strains of CMVs, including clinical isolates, the *a* sequence ranges in size from 700 to 900 bp (506).

Virus	Size (kbp)	Isomers (N)	Class[a]	Refs.
Human	229–235	4	E	92,170,200,536
Simian	208–220	1	F	206, Hayward (unpublished)
Rat (Wistar/ATCC)	230	1	F	35,266,577
Rat (Maryland)	230	1	F	74
Murine	230	1	F	144,302,360,362,445
Guinea pig	230	1	F	164,359

[a] E, genome with two components with direct and inverted repeat elements that isomerize to give four sequence arrangements; F, genome with small or no repeat elements. (See Chapter 7.)

TABLE 1. *Size and isomerization of cytomegalovirus genomes*

Simian and human CMV-infected cells contain several capsid forms (called A, B, and C capsids) that are characteristic of herpesvirus replication and represent stages in virion morphogenesis (176,228,315,565). Type A capsids lack DNA and accumulate because of a failure to stably package the viral genome; type B capsids are found predominantly in the nucleus as precursors of mature capsids that lack DNA but contain the viral scaffolding/assembly protein (75,315); and type C capsids are fully mature nucleocapsids. B capsids and virion nucleocapsids are composed of 12 pentons, hexons, and triplexes linking adjacent capsomeres at threefold symmetry positions in a T=16 icosahedral lattice (75,95,565). In addition, cryoelectron microscopy suggests that the overlying tegument in CMV virions is highly ordered in the region immediately proximal to the capsid, distinct from HSV-1 (95). CMV also exhibits a more compact interhelix DNA packing distance than HSV-1 (26 Å versus 30 Å), which explains how its larger genome is accommodated (40).

Three types of virus particles are produced in human CMV-infected cells: virions, noninfectious enveloped particles, and dense bodies; these three types can be separated based on physical differences (176,228,477). Virions contain a 130-nm nucleocapsid that is 5% larger than HSV (HSV) and results in a 17% increase in capsid volume (75,95,56). The noninfectious enveloped particles are readily differentiated from virions in electron micrographs because they lack an electron-dense DNA core (176,228,498,617). They appear to be an enveloped B capsid precursor. Together with dense bodies, they contribute to the relatively high particle-to-PFU (plaque-forming units) ratios (ª100) observed in virus stocks (536). Similar detailed observations have been made in ultrastructural studies of African green monkey (Colburn strain) CMV (565). Dense bodies (see Fig. 1A), which are present in virus preparations in numbers approximately equal to those of virions, are composed of several tegument proteins, predominantly pp65 (ppUL83), enclosed in an envelope derived from cytoplasmic me5)mbranes (117,477). These abundant particles completely lack both a nucleocapsid and viral DNA

(228,271,307,477).

Virion Proteins

Purified virus particles of human CMV have been estimated to contain 30 to 40 polypeptides with molecular weights ranging in size from 20 to over 300 kd (26,175,268,531). The capsid is composed of seven proteins, all of which are homologous to those identified in HSV (see Chapter 72). The major capsid protein (MCP) is encoded by UL86 and forms both hexons and pentons. Minor capsid protein (mCP) encoded by UL85, minor capsid binding protein (mC-BP) encoded by UL46, the smallest capsid protein (SCP) encoded by UL48.5 (also called UL48/49), and three distinct assemblin/assembly protein (AP)-related proteins encoded by UL80, UL80a, and UL80.5 associate with capsids (26,95,176,177 and 178,565). The genes for these proteins are distributed on the viral genome within the herpesvirus-common set of genes (Table 2).

TABLE 2. *"Composite" human CMV genome ORFs, proteins, and characteristics*

The virion envelope carries two prominent herpesvirus-conserved glycoprotein complexes (66,511). One is composed of covalently linked, proteolytically processed dimers of glycoprotein B (gB) encoded by UL55 (118,420,515), and the other is composed of the products of the UL75, UL115, and UL74 genes—gH, gL, and gO, respectively (224,514). These glycoprotein complexes play critical, but as yet incompletely understood, roles in entry that may be common to all herpesviruses. Glycoprotein B is one of the most highly conserved herpesvirus-common proteins (91). Unlike the alpha- and gammaherpesviruses, which have subgroup-specific envelope glycoproteins such as gD and gp220/350 that are central to entry, the only prominent envelope glycoproteins on CMV are members of the herpesvirus-common set. The CMV genome also carries a large number of ORFs with characteristics of integral membrane glycoproteins (see Table 2) that have not been studied in any detail but may encode minor envelope constituents that play roles in attachment and entry.

Between the capsid shell and envelope is a tegument that contains as many as 25 proteins, many of which are phosphorylated as denoted by the prefix *pp.* The most prominent are ppUL83 (pp65), ppUL32 (pp150), ppUL99 (pp28), ppUL82 (pp71), and ppUL48 (huge tegument protein) (63,175,176). Although several transcriptional transactivators (ppUL82, ppUL69, pTRS1, pIRS1) have been localized to the tegument (323,462,609), the functions of most tegument proteins remain uncharacterized. Tegument proteins are conserved among betaherpesviruses but are not herpesvirus-common.

Capsid Proteins

Initial characterization of the protein constituents of different types of CMV capsids (176) highlighted the overall similarity to other herpesviruses; however, the CMV capsid is larger than most and accommodates a significantly larger genome (75,95,565). The MCP in human CMV is a 1,370-amino-acid protein (pUL86) that is one of the most highly conserved herpesvirus-common proteins (91) and forms the major constituent of capsid pentamers and hexamers. Two smaller, less abundant capsid proteins, the 306-amino-acid mCP (pUL85) and the 290-amino-acid mC-BP (pUL46), associate in a 2:1 ratio to form triplexes, major surface structures that interdigitate hexons and pentons (75,95,565). The 75-amino-acid SCP (pUL48.5 or pUL48/49) has not been resolved in cryoelectron microscopic analyses (75), but it is thought to decorate hexon tips and direct tegumentation in a manner similar to its HSV homolog, VP35 (558).

A 708-amino-acid protein encoded by UL80 gives rise to at least three different proteins that carry out distinct functions in capsid assembly. These multiple UL80-derived capsid proteins generate two different-sized scaffolding proteins by posttranslational processing. The most abundant is denoted assembly protein (AP) and is derived from the carboxyl portion (UL80.5) by autocatalytic cleavage events mediated by a 30-kd serine-like maturational protease, assemblin (176,594). Assemblin is itself embedded in the amino-terminal portion of this ORF (30,179,592). The detailed structure of the protease (96,442,487,563) reveals a unique fold and novel type of catalytic triad composed of Ser-132, His-63, and His-157 in contrast with the Ser-His-Asp triads of other serine proteases. In addition, a fourth residue, Arg-166 (320), and dimerization of the protease seem to be necessary for activity. These characteristics make this protein a candidate target for antiviral agents (176).

Assemblin (UL80a) cleaves UL80 products made from a family of 3' co-terminal transcripts encoded in this region (592) into AP (176). The largest UL80 precursor is autocatalytically cleaved a minimum of three times. The first two cleavage events are involved in the maturation and release of the 45-kd human CMV AP, and the third appears to inactivate the protease (30,593). The AP is a major constituent of capsid intermediates and forms a recognizable electron-dense core ring in B capsids that accumulate prior to encapsidation of DNA (75,95,565). This protein directs formation of the capsid shell, a function similar to that of bacteriophage scaffolding protein (176). In addition to its role in maturation, the CMV

assembly protein remains associated with an alternative capsid form that matures as noninfectious enveloped particles (176,228) but appears to be absent from mature virions (565).

Envelope Glycoproteins

CMV is unusual among herpesviruses because it encodes as many as 60 putative glycoproteins, some or all of which could be present on the virion envelope. The envelope of human CMV contains viral proteins embedded in a host lipid bilayer and is derived from intracellular membranes. The acquisition of the envelope and its associated glycoproteins occurs at both nuclear and cytoplasmic sites. Envelope glycoproteins are important targets of the host antibody-mediated immune clearance mechanisms, and many reviews have been written about the synthesis, mapping, function, and immunogenicity of the more prominent envelope glycoproteins (66,378,511). Early studies revealed approximately eight major glycoprotein species in the human CMV envelope (175,531), with one, gB (gpUL55), being recognized as the major envelope constituent (66,175).

As the most highly conserved glycoprotein in mammalian and avian herpesviruses, gB is a type I integral membrane protein that influences virus binding to and entry into cells, cell-to-cell transmission, fusion of adjacent cells, and targeting of progeny virus to apical membranes for release from polarized cells (52,114,398,423,567,568 and 569). Glycoprotein B is the major heparan sulfate proteoglycan-binding glycoprotein (114), and it binds to a 30- to 36-kd cellular protein that is a suspected receptor (7,551). As homologs of gB play an essential role in attachment and penetration in other herpesviruses, it is likely that CMV gB is required for CMV entry (66).

Human CMV gB exhibits important distinguishing characteristics compared to gB from other herpesviruses (reviewed in refs. 66 and 511). Like homologs in most other herpesviruses (but unlike gB from HSV-1), the 906- to 907-amino-acid (150 kd) precursor of human CMV gB is cleaved between amino acids 460 and 461 by a host cell Golgi-localized furin protease (515,575) into two cleavage products of 93 and 55 kd. These proteins remain complexed and appear as a heterodimer on the virion envelope (68). Both N- and O-linked carbohydrates are added to gB, and at least a proportion of this protein is phosphorylated (405) in a manner that may regulate cellular distribution (154). Glycoprotein B is found on all membranes of infected cells and becomes incorporated into virions as they undergo envelopment at different sites (473). Because it is abundant, highly conserved, and the most immunogenic and best studied envelope glycoprotein, gB has been the principle candidate of subunit vaccine initiatives (6,415,515).

As the principal target of neutralizing antibodies detected in human sera (67,120,397), gB has been the subject of many monoclonal antibody epitope mapping studies (28,66,397,515,573). The 55-kd C-terminal cleavage product is immunodominant and contains many neutralizing epitopes (28,515,573); the 93-kd N-terminal cleavage product has fewer neutralizing determinants, and these vary among strains (28,397,515). Polyclonal antisera to the 55-kd component, as well as antisera raised against the intact gB heterodimeric complex, are generally capable of neutralizing viral infectivity (66,420). Although overall strain-to-strain sequence variation in gB is quite low (118,286,515), DNA sequence analysis has revealed that four distinct gB genotypes are in circulation (105). The existence of these genotypes has proven useful in epidemiologic studies (159).

Human CMV gH, or gpUL75, is a 742- to 743-amino-acid envelope glycoprotein (411) forming part of a second, relatively abundant glycoprotein complex to which neutralizing antibodies may be directed (445). Although highly divergent, gH is considered to be a herpesvirus-common glycoprotein because homologs share a pattern of cysteine residues, the presence of a conserved asparagine-glycine-threonine-valine glycosylation signal adjacent to a transmembrane region, and an unusually short carboxyl-terminal cytoplasmic domain (119). The gH complexes with at least one other herpesvirus-common glycoprotein, denoted gL, which facilitates transport to the cell surface (253,514). CMV gL is encoded by UL115 (see Table 2) and is a 278-amino-acid (32-kd) glycoprotein modified by N-linked and possibly O-linked carbohydrates. In CMV, a 145-kd protein (192), the likely product of the UL74 gene, called gpUL74 or gO, also joins with gH/gL to form a heterotrimeric envelope glycoprotein complex (gH/gL/gO) (224). In the gammaherpesvirus Epstein-Barr virus (EBV), viral proteins that complex with gH/gL influence cell tropism (586), and CMV gO may function in a similar role.

CMV gH is a target for complement-independent neutralizing antibodies (445), and it exhibits even less overall variation than gB (101), suggesting that evolutionary constraints have preserved protein conformation (514). Anti-gH antibody can prevent cell-to-cell transmission of virus (445), consistent with a role for this protein in membrane fusion. Antibodies that mimic the gH/gL complex block virus penetration of cells (254,256) and have implicated a cellular 92-kd phosphorylated glycoprotein as a possible receptor (255,256). Thus all three components of the gH/gL/gO complex are likely to be essential for viral entry.

A third herpesvirus-conserved glycoprotein complex is likely to be associated with the CMV envelope. One protein, encoded by UL100, initially referred to as the integral membrane protein (317), is part of this complex (250). UL100 and UL73 are the positional homologs of alphaherpesvirus UL10 (gM) and UL49.5 (gN), respectively. CMV UL73 also exhibits sequence homology with EBV BLRF1 (gN) (92). A gM/gN glycoprotein complex has been characterized in EBV and alphaherpesviruses (see Chapter 72 and Chapter 74), and, by analogy, a similar complex between human CMV pUL73 and pUL100 has been observed (Britt and Mach, personal communication, 2000). In fact, this gM/gN complex is probably the CMV envelope glycoprotein gcII complex (250). In other herpesviruses, both gM and gN are dispensable for growth in cell culture.

Several additional viral glycoproteins are likely to be minor envelope constituents but are not apparently associated with any major glycoprotein complexes. The glycoprotein gp48, or gpUL4 (86), is synthesized at both early and late times, with protein accumulating to highest levels at late times via complex transcriptional and translational controls. The 48-kd size of gpUL4 is the result of heavy modification of the 152-amino-acid polypeptide by both N-linked and O-linked carbohydrates. Glycoprotein UL4 is dispensable for growth in cell culture (455,547).

The products of one (UL33) of the four ORFs (UL33, UL78, US27, US28) that exhibit amino acid sequence similarity to

seven-transmembrane-spanning (7TM) receptors (94,188) have been detected in purified virions (347), suggesting that this class of protein is likely to be carried in the virion envelope. The presence of over 60 ORFs with sequence characteristics of integral membrane proteins (82,92) strongly suggests that additional functions will continue to emerge for less abundant envelope glycoprotein constituents.

Tegument Proteins

At least 25 proteins are located in the tegument layer between the virion capsid and envelope (176). The products of 11 ORFs (UL25, UL26, UL32, UL47, UL48, UL48.5, UL82, UL83, UL85, UL88, UL99) have been detected when virion/dense body polypeptides are electrophoretically separated (26,176), and all appear to be both phosphorylated (175,458) and highly immunogenic (303). These ORFs are all conserved in the betaherpesviruses (188,402,446,577), although only a small number are herpesvirus-common (see Table 2). Two tegument proteins, pp150 (ppUL32 or basic phosphoprotein), a 1,048-amino-acid (150- to 155-kd) protein, and pp65 (ppUL83 or lower matrix protein), a 561-amino-acid (65- to 68-kd) protein, are the most abundant proteins made during replication (175,531).

The large amount of pp65 produced by laboratory strains of virus is associated with abundant dense body production. Less pp65 is present in virions made from fresh clinical isolates (271). The target antigen in antigenemia assays that are used for rapid diagnosis of CMV clinical infection is pp65 (see Chapter 77). Although all tegument proteins are phosphorylated, pp65 is a major phosphate acceptor in infected cells as well as a primary target for phosphorylation *in vitro* by the virion-associated protein kinase (458,508). Protein kinase activity attributed to pp65 itself is probably caused by the interaction of pp65 with a host-derived kinase (162,458). Given its abundance, it is surprising that pp65 is completely dispensable for replication in cell culture (481).

The other major tegument phosphoprotein, pp150 (ppUL32 or basic phosphoprotein) (175,458), is the product of UL32 (234). It is incorporated preferentially into virions rather than dense bodies, and it is estimated to make up 20% of the virion mass (37). The pp150 is also a target for phosphorylation by the virion-associated protein kinase (458). This protein contains 19% serine or threonine and has a single O-linked N-acetylglucosamine addition (37), a modification that has also been observed on cellular proteins localizing to nuclear membranes. Also, pp150 is a broadly recognized viral antigen that is detected by 85% to 90% of CMV-seropositive human sera (303), resulting in its inclusion in recombinant protein-based serologic assays (456).

A number of additional proteins have been identified as known or likely tegument constituents. One abundant phosphoprotein, called pp71 (ppUL82 or upper matrix protein), functions as a transactivator of gene expression (323). A second, ppUL69, is also a virion tegument-associated transactivator (610). This protein exhibits limited sequence similarity to HSV ICP27, a nonstructural a gene product with a role in regulating late posttranscriptional events in replication. Mutations disrupting the UL82 or UL69 genes have shown that both are dispensable for high multiplicity of infection (MOI) of cultured fibroblasts (Greaves, personal communication, 1999) (204). Additional viral proteins have been localized to the tegument of partially purified virus particles using resistance to protease digestion as a criterion for being contained within enveloped particles. These include the viral DNA polymerase (345), the UL97 kinase (574,611), a late protein encoded by UL94 (607), the transactivator pTRS1/IRS1 (462), and pUL36 (417); however, none is sufficiently abundant to be directly detected as a protein species in purified virus particles (26,176).

The tegument also contains a number of functionally uncharacterized proteins, including one relatively abundant phosphoprotein denoted pp28 or ppUL99, a 190-amino-acid (28-kd), highly immunogenic glycoprotein (303) that localizes near the capsid surface in virions (307). A protein implicated in genome encapsidation, pp130 (ppUL56) is virion associated (51,63). Based on drug resistance studies (290), pp130 may complex with the CMV terminase (ppUL89) to package viral DNA during virion maturation. One additional protein, pp67, which includes some UL65 sequence (125), has not been consistently observed in characterized virus strains. The association of UL25, UL26, UL47, and UL88 products with the tegument has been determined by sequencing proteins directly isolated from virus particles (26).

Additional tegument proteins may be predicted from the nucleotide sequence of the human CMV genome because they are arranged in blocks with positional similarity to known or suspected herpesvirus tegument proteins (378). The human CMV UL47–UL53 cluster may serve functions similar to that of HSV UL37–UL31; CMV UL56 is likely to serve the same function as HSV UL28; CMV UL76 and UL77 may be analogous to HSV UL24 and UL25; CMV UL87 may be analogous to HSV-1 UL21; and the CMV UL89–UL104 cluster contains functions similar to those of HSV-1 UL15–UL6 (see Table 2). Discussions of the various homologs is presented in reports that deal with betaherpesvirus genome sequence analyses (92,188,402,446,577).

Host Proteins

A number of abundant host cell proteins, including b $_2$-microglobulin, actin, and various cellular enzymes, have been observed to associate with purified virions (26,162,194,371). The association of these host proteins with virus particles raises several interesting implications. Reports have identified proteins with size characteristics (183,371) of candidate cellular receptors for this virus (7,256,502,551), suggesting that the virus may mature carrying away receptors bound to its surface. Adherence of b $_2$-microglobulin suggests a way that CMV attaches to cells (194), and a report of a host cell actin-related protein suggests a means of cytoplasmic transport (26). Kinase activity associated with pp65 in virions (458) appears to be a host cell enzyme (162).

Other Virion Constituents

The virion of CMV, unlike that of other DNA viruses, contains two classes of RNA in addition to the DNA. One type of RNA forms hybrid structures within the origin of replication (*ori*Lyt) in packaged genomes (440), and the other type of RNA appears to be within the tegument and to be expressed after entry into cells (65). Except for a single copy of the tightly packed DNA with RNA at *ori*Lyt (40), the core of the human CMV virion contains only spermine and spermidine, at a ratio of approximately 2:1 (181), which probably counteracts the negative charge of packaged DNA. CMV replication is

sensitive to chemical agents that disrupt polyamine synthesis (181,570).

Viral DNA

The genome of human CMV (235 kbp) contains internal repeats in a class E genome structure (see Table 1 and Fig. 1C) and undergoes inversion of two genome components similar to HSV-1 (see Chapter 71). The genomes of all other characterized betaherpesviruses, including all the animal CMVs, are linear and lack internal repeats (see Table 1). The human CMV genome has two unique components, U_L and U_S, flanked by inverted repeats *b* (TR_L/IR_L) and *c* (IR_S/TR_S) (see Fig. 1C). A directly repeated *a* sequence is found at the genome termini (383) and is also present in inverted orientation at the L-S junction. This arrangement of *a* sequences promotes genome inversion. The *a* sequence carries cis-signals, called *pac*-1 and *pac*-2, for cleavage and packaging of the viral genome (260,360,383,512). The *pac*-1 and *pac*-2 sequence elements are conserved in all herpesvirus genomes (361,383); however, their location relative to the cleavage site varies. In human CMV, cleavage occurs to one side of the *pac*-1 and *pac*-2 elements (260), whereas in other herpesvirus genomes that have been examined, including rodent and guinea pig CMVs (349,359,578), cleavage occurs between these elements. DNA cleavage varies by a few nucleotides in some strains but always leaves ends with 3' overhanging nucleotides (383,550).

The human CMV genome is G+C rich and contains a significant number of direct and inverted repeats, exhibiting a greater density of genomic repeats than other herpesviruses. Aside from the *a* sequence that contains a large array of short degenerate repeats (512), three regions show an extremely high density of closely spaced inverted and direct repeats—the DNA replication origin, *ori*Lyt, and two transcriptional enhancers, *ie*1/*ie*2 and US3 (350).

Following serial undiluted passage, human CMV generates defective viruses carrying DNA molecules that are shorter than genome length (444,536). These defective DNA molecules are approximately one-third smaller than intact viral genomes, or roughly 150 kbp rather than 235 kbp, which is the size packaged in other herpesviruses. The structure of defective DNA has not been evaluated further except to show that CMV does not generate the head-to-tail concatemeric repeats observed for other herpesviruses. Novel restriction fragments are found in defective CMV DNA, suggesting that specific deletions or rearrangements occur relative to standard viral DNA.

Genome Organization

The only human CMV strain that has been completely sequenced is a laboratory-adapted strain, AD169 (465). This 230,283-bp sequence (accession number X17403) with 208 nonoverlapping ORFs of greater than 80 codons originally reported (92) is among the largest and most complex animal virus genomes. ORFs are numbered sequentially and designated by their location within the unique (U_L, U_S) and repeated (TRL/IRL, TRS/IRS) regions of the viral genome, and a nomenclature for naming viral genes and proteins has evolved (511). The ORFs initially identified in strain AD169 did not represent a full set of viral genes, however; thus all known human CMV ORFs are listed in Table 2. Gene functions of the 213 known genes are assigned based on direct study as well as on information from sequence homologs in other herpesviruses. As in all herpesviruses, the genes encoding different human CMV temporal classes and functions are interspersed across the genome. For example, the earliest genes to be expressed during viral replication, the a or immediate-early (IE) genes, map to diverse locations in both the L and S components.

The AD169 strain of human CMV chosen for genome sequence analysis (92) is a variant (AD169*var*UK) that was propagated under growth conditions to attenuate virulence for its use as a live attenuated vaccine (146). Its genome was plasmid cloned following 25 years of serial passage in various laboratories (409). The reported sequence contains errors requiring correction (497) as well as omissions that complicate its use as a prototype. For example, the report misses a 929-bp sequence in the UL42–UL43 region that is carried by all strains of CMV, including AD169 (124,386) (accession number AF01963), and a large, approximately 15-kbp region with 19 ORFs that is absent from AD169 but present in the Toledo strain and clinical isolates of CMV (82). Furthermore, when portions of clonal isolates of Towne (passage 125) and Toledo (passage 9) (accession number U33331) strains were subjected to nucleotide sequence analysis, a large U_L-*b*8' region that is absent from AD169 but present in fresh clinical isolates of CMV was identified (82). By combining all of this information, Table 2 approximates the expected genome organization of a wild strain of CMV.

Families of ORFs that result from gene duplication events during evolution are present in all betaherpesviruses (92,188,378,402,446,577). DNA sequence comparisons of the genomes of various animal betaherpesviruses to human betaherpesviruses reveal a remarkable level of sequence divergence, such that the viral DNAs do not cross-hybridize to any extent, and only short stretches of nucleotide sequence are conserved. These regions include the *pac* sequences (for cleavage and packaging of viral genomes, as discussed in the preceding section), as well as some transcription factor binding sites, most notably in the major immediate-early enhancer (61,140). In contrast to the relatively low level of DNA sequence conservation, betaherpesvirus genomes retain significant amino acid sequence homology in approximately 80 ORFs in the UL24 to UL116 region (188,402,446,577) (see Table 2). By an adopted convention, nomenclature of animal CMV ORFs follows that set in place for human CMV ORFs, so murine or rat CMV ORFs that retain sequence similarity to human CMV ORFs are designated with upper-case letters (e.g., M44, R44) whereas those not conserved in human CMV are represented by lower-case letters (m144, r144). ORFs found in U_S or the repeated regions of the human CMV genome are not well conserved in other betaherpesviruses.

Although genome sequence analysis confirms the relationship of all betaherpesviruses, this subgroup is the most divergent and ancient of the herpesvirus subgroups (187,251,355). The presence of as many as 46 herpesvirus-common ORFs provides strong evidence of a common origin for all herpesviruses (see Table 2). Using information largely from studies characterizing HSV-1 mutants, approximately one quarter of these ORFs appear to encode DNA replication and metabolism functions, and three quarters are predicted to play roles in virion maturation and structure (see Table 2) (92,378). All betaherpesviruses exhibit a characteristic genome organization distinct from either alpha- or gammaherpesviruses. If the human CMV U_L ORFs are arranged into seven conserved blocks denoted ABCDEFG, the arrangement in HSV-1 and EBV would be depicted GF'EDBA'C' and A'CGF'EDB, respectively. A prime (') indicates an inverted orientation. Thus, these conserved sequence blocks have a characteristic order in each of the herpesvirus

subgroups. These sequence features suggest that general characteristics of DNA replication, virion maturation, and virion organization are likely to be conserved in all mammalian herpesviruses.

Although analysis of individual gene functions in human or other animal CMVs is still incomplete, nearly 70 ORFs are known to be dispensable for productive replication in cultured fibroblasts, indicating that a large proportion of the genetic complexity of CMV is committed to optimizing growth, dissemination, tissue tropism, immune evasion, and pathogenesis in the host. A total of 46 genes are known to be dispensable for growth as a result of spontaneous deletions arising during passage in cell culture (82,278,389,546), including UL1–UL10, UL36, UL42–UL43, UL133–UL153, US1–US15, and one copy of the TRL4/IRL4–TRL14/IRL14 gene cluster. In addition, targeted mutagenesis shows that UL16, UL18, UL20, UL33, UL36, UL83, UL124–UL128, US27, US28, TRL4/IRL4, IRS1 and US2–US11 are all dispensable for growth (49,71,190,243,252,347,365,417,513,576) (Browne and Minson, personal communication, 1994; Duke and Mocarski, unpublished). Consistent with their nonessential role for replication in cell culture, four of these genes (US2, US3, US6, US11) have been shown to function in immune evasion (429). Though difficult to assess in human CMV, several gene products needed for infection in the host animal have been identified in murine CMV (127,152,155,343,468). Finally, several regulatory gene mutants (UL69, UL82, UL123) (190,204,382) (Greaves, personal communication, 1999) and an *ie*1*ie*2 enhancer utant (363) replicate to normal levels after passage at high MOI but are unable to replicate at low MOIs. In addition, two DNA replication/metabolism gene mutants (UL97, UL114) grow slowly at any MOI (438,439).

Strain Polymorphism

Various lines of evidence suggest that all human CMV strains are closely related. DNA:DNA reassociation reveals an overall 90% to 95% DNA sequence homology (220,441). Electrophoretic patterns of virion or infected-cell proteins from different strains are similar (197), to the extent that variation in electrophoretic mobility of viral proteins is too rare to differentiate strains (103,174). Antibodies raised against one strain of CMV generally react with other strains, although several strain-specific epitopes have been identified on different viral proteins (66,421). Restriction site polymorphism of viral DNA (84,142) or sequence analyses of polymerase chain reaction (PCR) amplified DNA fragments (104) of laboratory-adapted and clinical strains are used to differentiate strains. DNA sequence-based analyses are, of course, the most sensitive means to differentiate strains, and they demonstrate that a large number of different CMV strains are in circulation worldwide.

One type of genome variation is the deletion of U_L sequences and the expansion of the *b* sequence repeats that have arisen independently within viral strains following high MOI serial passage in cell culture. All currently propagated AD169 strain variants, including AD169*var*UK and AD169*var*ATCC have replaced approximately 15,000 bp of nucleotide sequence and 19 to 21 ORFs (UL133–UL153) with a large, 11-kbp *b*' region (82), as depicted in Figure 1C. AD169*var*UK differs from other AD169 variants (e.g., AD169*var*ATCC) in growth properties (69) as well as in genome sequence. The 929-bp deletion that removes portions of UL42 and UL43 in AD169*var*UK is retained in AD169*var*ATCC and all other strains of CMV examined (124,386). AD169*var*ATCC also exhibits distinct properties, such as a UL36 gene product with altered localization and stability relative to other strains (417), suggesting that this variant also carries distinct mutations relative to wild strains. Although the AD169 strain was isolated almost half a century ago (465) and became a widely used strain because of its efficient replication in cultured cells, little care has been taken to ensure that a single, uniform stock was distributed to laboratories interested in studying CMV, or to ensure use of low MOI passage. The existence of large deletions relative to clinical strains and multiple strain variants call into question its de facto role as a prototype. Towne (passage 125), a vaccine candidate that has become a common laboratory strain (430), also has variants generated as a result of passage, and it carries a deletion affecting the U_L-*b*' region that is different from that observed in strain AD169*var*UK (82). In the case of Towne, however, an earlier passage was preserved that exhibits patterns of gene expression and a restriction map that is distinguishable from Towne (125,184). Some preparations of the Towne strain, including those available from ATCC, are a mixture of at least two variants, one of which carries a U_L-*b*' deletion (82) and the other of which appears to be intact based on sequence analysis (Kemble and Duke, personal communication, 1999), even though all of these variants show an attenuated phenotype when looked at as vaccine candidates (5). As yet, there has been only limited study of CMV genome stability during long-term serial passage in cell culture or in different cell types; however, the genome of a particular strain appears to be stable during limited passage at low MOI in cell culture (69,103,105), and biologic properties of low-passage strains are not readily lost during limited passage or plaque purification (431). Virus strains that are propagated on a particular cell type, such as endothelial cells or fibroblasts, retain tropism for that cell type and often fail to replicate efficiently on other cell types (583), suggesting that adaptive mutations may accumulate with prolonged passage. The significance of adaption in cell culture to the biology of CMV in the host remains unclear, although it seems likely that high-MOI serial passage carried out in an attempt to attenuate virulence (146,430) may have contributed to deletions similar to what is suggested to have occurred in defective virus genomes (536), as well as in spontaneous mutants that carry large deletions in other regions of the genome (278).

VIRUS GROWTH

General Considerations

Human CMV exhibits a highly restricted host range in cell culture. Primary differentiated human fibroblasts (HFs) from skin or lung show the greatest susceptibility to viral infection. During natural infection, fibroblastic, epithelial, macrophage, smooth muscle, and endothelial cell types support productive replication (491). Although HFs are commonly used to isolate and propagate CMV in culture, studies have clearly shown that freshly isolated human CMV replicates preferentially on endothelial cell types (454,493,583,613). The genetic alterations that underlie virus adaptation to different cell types remain unknown. Undifferentiated, transformed, or aneuploid human cells, including almost all cell lines, are nonpermissive (297,465). Fibroblasts from chimpanzees are permissive (422), but cells from more distantly related primate species are not. The host cell restriction to human CMV replication observed in nonpermissive cells is the result of a postpenetration block in viral gene expression and not to a failure to enter cells; virus has never been adapted to replicate in any completely nonpermissive cell type. CMV replication in an epithelial cell line (CaCo-2) has been associated with this host's undifferentiated state (235), and virus strain differences or other factors may also contribute (149). Certain astrocytoma cell lines, such as U373MG, although fully permissive (143), do not support viral replication to

the high levels observed with fibroblasts. HFs that have been immortalized with the human papillomavirus type 16 E6/E7 genes are just as permissive as primary HFs (112,189), and such cells have proved to be invaluable as a basis for the construction of complementing cell lines to isolate viral mutants (190,382).

The initial events in the replication cycle are only partially understood. Attachment and penetration at the cell surface (113) is rapid and efficient in permissive as well as in nonpermissive cell types, suggesting that cellular receptors for CMV are widely distributed. Addition of exogenous heparin, or treatment of cells with heparinase, blocks viral attachment and implicates the major cell surface proteoglycan heparan sulfate in the initial interaction between virus and cell (114,270,401), as is the case for other herpesviruses. Initial interaction with heparan sulfate is followed quickly by heparin-independent adsorption and penetration steps that employ widely distributed but poorly characterized receptors (7,256,551). The efficiency of penetration depends on the presence of these receptors (407). Initial interest in a 30- to 36-kd gB-binding cell surface protein (7,551), appearing to be annexin II, has been tempered by the observation that annexin II does not have to be present on cells for entry (423). Interest in a gH-binding phosphoprotein (255,256), which may be important in penetration (254), has led to the partial cloning of a CMV fusion receptor (CMVFR) (27), although its full characterization remains to be completed.

A number of mechanisms for entry have been proposed for cellular and viral functions that may influence the efficiency of entry into cells (46,71,576). A b_2-microglobulin bridge model (194,356) suggests an interaction between major histocompatibility complex (MHC) class I on target cells and the viral MHC class I homolog pUL18, but UL18-deficient virus replicates normally (71). CMV US28 chemokine receptor is a coreceptor for human immunodeficiency virus (HIV) entry (428) and increases fusion activity of other viral envelope proteins (427), but US28 itself is completely dispensable to CMV (46,576). Cellular CD13 influences entry (183), but cells devoid of this protein are nonetheless susceptible. Thus, many proposed CMV entry mechanisms may influence the behavior of virus in specific tissues or cell types, but none has been assigned a central role in entry.

Attachment to the cell surface is followed by penetration, mediated by fusion of the virion envelope and the cell surface in a pH-independent manner (113), a process that probably involves the gH/gL complex (254). Viral nucleocapsids rapidly make their way to the nucleus, so the tegument protein ppUL83 (122) can be detected within 20 to 30 minutes following exposure to a virus inoculum, and expression of *ie*1/*ie*2 gene products follows shortly thereafter (370). Several studies have shown that CMV (or CMV glycoprotein) binding to cells is sufficient to initiate a signalling cascade even in the absence of viral penetration (54,62,627,633). Viral attachment may therefore stimulate specific cellular processes that facilitate viral replication, or they may be part of some other host cell response to viral infection. Low-speed centrifugation (centrifugal enhancement) increases the efficiency of adsorption and penetration of murine or human CMV (8,410,614) by stabilizing weak interactions or encouraging fusion (217).

Productive replication in permissive HFs follows a pattern of coordinate expression with viral genes assigned to different kinetic classes depending on time of expression and sensitivity to inhibition of protein synthesis or viral DNA synthesis. These classes have been referred to as a or immediate early (IE), b or early, and g or late. Expression of a genes immediately follows entry and does not depend on the expression of any viral genes. Expression of b genes depends on the expression of functional a gene products. The b genes may be further divided into b_1 and b_2, which differ in timing and shutoff characteristics. Expression of g genes occurs last and may be divided into g_1 and g_2 based on the timing and dependence on viral DNA replication (378). The relative lack of dependence of g gene expression on viral DNA replication, and the existence of subclasses of b and g genes are major reasons for employing a, b, and g terminology in defining gene classes (see Chapter 72). Although the replication cycle of human CMV is slow, requiring 48 to 72 hours to begin the release of progeny (534), the expression of a gene products starts immediately after viral entry. In ways that are not well understood, virion tegument proteins influence this earliest phase of infection, and viral functions expressed early play regulatory roles later in infection. The switch from early phases to late phases is delayed until 24 to 36 hours postinfection (hpi), with maximal levels of virus release starting 72 to 96 hpi. Once maturation starts, infected fibroblasts continue to produce virus at peak levels for several days.

During productive infection in HFs, a gradual increase in human CMV DNA synthesis is observed starting as early as 14 to 16 hpi and is accompanied by a stimulation of host cell metabolism (378). Viral replication and maturation parallel accumulation of viral DNA replication functions, features suggesting that the prolonged replication cycle of this virus may result from this slow accumulation of essential gene products. CMV infection does not inhibit general cellular metabolism but rather stimulates both RNA and protein synthesis (378). Early work suggested that stimulation of host cell DNA synthesis might be a prerequisite to CMV DNA replication, but more recent studies have established that resting (G_0) HFs do not progress into the S phase of the cell cycle but rather give the appearance of a G_1/S block when productively infected with CMV (64,138). HFs already in S or G_2 may proceed to a later, G_2/M block (327), although expression of viral gene products is either delayed (470) or aborted (131) in such cells. These observations, all carried out at relatively uniform, high MOIs (>3), do not explain why CMV replicates best in serum-stimulated, actively dividing fibroblast cultures when infection is carried out at low (<0.1) MOIs (532).

Regulation of Gene Expression

Host cell RNA polymerase II and associated basal transcription machinery direct the transcription of CMV genes, which have promoters and may be spliced in ways that resemble cellular genes (377,535). Host cell transcription factors are influenced by virus-encoded transactivators that modulate expression of viral as well as host genes during infection. The cellular basis of permissiveness for human CMV replication depends more on the efficiency of early viral gene expression and subsequent DNA replication and less on entry into cells. The transcriptional enhancer controlling *ie*1/*ie*2 expression, in particular, functions in a cell type– and differentiation state–dependent fashion (364). It is also activated by signalling through cell surface receptors (62) and may be modulated by virion and other viral regulatory proteins (109,364,489,516,529).

P>Control of expression within the viral genome is complex and relies on promoters that produce 3' co-terminal mRNA transcripts encoding different gene products from a single region. For example, the *ie*1/*ie*2 region encodes productive

phase transcripts immediately after infection that differ from those produced late in productive infection (529) or during latent infection (281). Many regions of the viral genome produce more than one class of productive phase transcript and more than one species of protein through selective promoter usage as well as by alternative mRNA splicing.

Transcriptional Enhancers and Modulators

There are two loci in the human CMV genome whose expression is controlled by prominent transcriptional enhancer-modulators with many functional similarities. One is the *ie*1/*ie*2 gene, which encodes a family of MIE regulatory proteins from differentially spliced transcripts (516,529) using one of the strongest transcriptional enhancers yet identified in mammalian biology [mapping at 174,000 nucleotides (nt)] (61). The other is the US3 gene (mapping at 195,000 nt), which encodes an immunomodulatory protein (12,245) using a second viral enhancer (597).

The MIE (*ie*1/*ie*2) promoter-enhancer was initially identified by its ability to promote high level *in vitro* transcription (559) and to rescue replication of enhancerless simian virus 40 (61). Its impact on transcription is attributed to a dense collection of host transcription factor binding sites (403,404), including sites for CREB/ATF, NF-kB/Rel, Sp1, AP1, NF-1, retinoic acid receptor, serum response factor, ELK-1, C/EBP, p53, and Gfi-1, that span a genomic region approximately 500 bp upstream of the promoter (173,364). Shortly after infection, several transcription factors, such as NF-kB, AP-1, Sp1, and CREB/ATF are induced and activate expression from the *ie*1/*ie*2 promoter-enhancer by binding to repetitious elements, referred to by their sizes as 16-, 18-, 19-, and 21-bp repeats (61,559). As infection proceeds, expression is repressed by an IE2-dependent process (337) that acts via a specific signal, the cis-repression sequence (*crs*) near the transcription start site (529). Expression and repression are completely independent of the upstream modulator region (363,365).

The 19-bp repeats contain a cAMP response element (CRE, also called an ATF-binding site) (88,403,524) that contributes in a major way to strong basal enhancer activity (226) and that is also responsive to the virion transactivator ppUL82 (pp71) (323). The 18-bp repeats, which contain consensus binding sites for NF-kB (99,472), confer responsiveness to signal transduction resulting from virus binding (62,627,633), to autoregulation by the viral IE1 regulatory protein (99), and to inducers of *rel* family transcription factors such as phorbol esters (472) and cytokines (295). The 21-bp repeats carry both Sp1 and YY1 binding sites that may be involved in activation or repression of the enhancer (324), and one report suggests that the 16-bp repeats bind NF-1 (404).

An analogous enhancer, which conserves both 18- and 19-bp elements of the *ie*1/*ie*2 promoter-enhancer but little other nucleotide sequence identity, has been found upstream of the major regulatory gene locus in both murine and simian CMVs (87,140). The human CMV enhancer has been used in place of its murine CMV counterpart to support full replication in cell culture (21) as well as in lethally irradiated mice (195), suggesting that these diverse enhancers function in a similar fashion, although each probably contributes to cell tropism differences.

The enhancer-promoter shows extreme tissue and cell type specificity in expression, as illustrated by the activity of various promoter-enhancer constructs in cultured cells (364) and by the dramatic cell type specificity of expression in transgenic mice (29,274). Its activity depends on the presence of appropriate transcription factors in cells and is influenced by the adjacent modulator region that confers cell type and differentiation state dependence (173,364). This distal regulatory region extends from approximately 500 bp to approximately 1,000 bp upstream of the *ie*1/*ie*2 start site and contains cellular factor binding sites (285,490), including YY1 sites, which may repress the promoter-enhancer in undifferentiated cells (324).

Considerable experimental evidence from transient expression systems suggests that the modulator is dispensable for maximal expression of *ie*1/*ie*2 in many different common cell lines (173), but that it represses expression in undifferentiated teratocarcinoma NTera-2 cells and in myelomonocytic THP-1 and U937 cells. In these settings, efficient expression depends on cell differentiation state (173). Regions overlapping with or upstream of the enhancer appear to bind proteins that affect activation of enhancer function in differentiated cells (223,324,489). However, when assayed in recombinant virus constructs, the modulator has little role in cell type or differentiation state–dependent expression (363,365). Viruses with mutations that remove only the modulator region replicate and express the *ie*1/*ie*2 products as well as parental viruses, whether assayed in differentiated or nondifferentiated cells (365). The differences between results obtained in transient expression assays and infected cells draw into question the value of the transient assays to evaluate such processes.

The region upstream of US3 has also been shown to have the characteristics of a complex transcriptional enhancer (597), and it carries two types of repetitious sequences that bind host transcription factors. One (R_2) contains NF-kB sites and is very likely involved in enhancer responsiveness to phorbol esters, cytokines, virus binding, and the viral IE1 $_{491aa}$ regulatory protein. The other (R_1) is not yet fully characterized but is probably involved in modulating the enhancer (83,560). This promoter-enhancer is most likely regulated in a manner similar to that observed for the better-studied MIE promoter-enhancer (42,310). Unlike the regulatory proteins encoded from the *ie*1/*ie*2 locus, whose expression is necessary for efficient replication (190,363,382), the US3 gene and associated enhancer is dispensable for replication in cultured cells (243,278). The US3 enhancer contains a modulator (or silencer) region upstream of the enhancer, as well as a *crs* element between the TATA element and the start site that is analogous to the *ie*1/*ie*2 promoter-enhancer (83,310). Evaluation of the US3 regulatory elements within the context of the viral genome (310) reveals the importance of IE2-mediated repression via the *crs* element. Expression of the US3 enhancer is also subject to negative regulation in permissive fibroblasts because of a distal silencer region consisting of seven copies of the R_1 repeat, as first demonstrated by transient assays (83,560) and confirmed directly using viral mutants (310). Thus, US3 expression is down-modulated by both a silencer and a *crs* element, so that maximal levels of this gene product are never fully achieved in permissive cells. Although IE2-mediated repression of US3 expression has been best characterized, CMV mutants that accumulate lower levels of *ie*1/*ie*2 gene products as a result of an enhancer mutation have also been observed to accumulate reduced amounts of US3 (363), suggesting that activation by *ie*1/*ie*2 products may dominate over repression in a way that still needs to be clarified.

Virion Transactivators

The virion carries several proteins localized to the tegument that carry out regulatory activities. One of the abundant tegument proteins, ppUL82 (also called pp71 or upper matrix protein), is able to activate the *ie*1/*ie*2 promoter-enhancer in transient transfection assays (323). Its effect is mediated through the binding sites for the cellular transcription factors CREB/ATF or AP-1 found in the 19-bp enhancer repeats. UL82 expression starts early but peaks at late times in the infection (175). Deletion of UL82 from the viral genome results in a multiplicity-dependent growth phenotype correlated with poor expression of the *ie*1/*ie*2 locus (Greaves, personal communication, 1999), suggesting that optimal activation of MIE gene expression depends on adequate ppUL82. When expressed in an HSV recombinant devoid of its own transactivators (VP16, ICP0, and ICP4), ppUL82 transactivates HSV gene expression as well as expression of a human CMV *ie*1/*ie*2 indicator gene (218). In addition, transiently expressed ppUL82 increases the infectivity of transfected viral DNA in a manner that appears independent of a role for *ie*1/*ie*2 expression (25). Taken together, these studies suggest that ppUL82 functions as a more general transactivator of gene expression rather than as a specific activator of *ie*1/*ie*2 gene expression.

Additional transactivators of viral gene expression that are present in the virion as minor tegument proteins may play regulatory roles following entry. One phosphorylated subform of the viral protein ppUL69 associates with the virion tegument (610) and transactivates expression from the *ie*1/*ie*2 promoter-enhancer, particularly when used in combination with ppUL82 (608). Unexpectedly, UL69 mutant virus growth is delayed under high MOI conditions due to an impact on DNA replication rather than on *ie*1/*ie*2 gene expression (204). At low MOI, growth of this mutant is severely compromised, although the point of inhibition has not been established. The viral pIRS1 and pTRS1 transactivators, a gene products that cooperate with other viral regulatory proteins (231,463,527), are also associated with virions and dense bodies (462), suggesting that they may contribute to virion transactivation. In addition to regulatory proteins, the virion may carry host protein kinases (162) and phosphatases (372) that might modulate the activity of viral regulatory proteins following entry. Finally, virus particles also contain mRNAs that may contribute to regulatory processes following entry (65).

a (Immediate-Early) Gene Expression and Gene Products

Four regions of a gene expression have been mapped on the human CMV genome, UL36–38, *ie*1/*ie*2 (UL122–123), TRS1–IRS1, and US3 (109,516,529), with one additional region (UL115–119) that may also fit into this class (109,538). The expression of a genes begins within one hpi and peaks between 4 and 8 hpi, independent of *de novo* protein synthesis.

The overwhelming majority of expression immediately after viral entry arises from a single major locus, the so-called MIE locus, located between 169 and 175 kbp on the human CMV genome (see Table 2) (92,535). Transcription from the *ie*1/*ie*2 promoter-enhancer gives rise to differentially spliced and polyadenylated transcripts encoding the two major products $IE1_{491aa}$ and $IE2_{579aa}$, which share a common 85-amino-acid amino-terminal domain but splice either to UL123 for $IE1_{491aa}$, or to UL122 for $IE2_{579aa}$ (516,529). In addition to an important role for the MIE products in viral replication, ancillary a genes play roles in regulation (TRS1/IRS1) (231,527), inhibition of apoptosis (UL36 and UL37) (185,186), and modulation of host cell MHC class I expression (US3) (12,245). All animal CMVs examined have an analogous MIE locus, but they vary with respect to the presence of ancillary a genes.

$IE1_{491aa}$ (or IE1p72) and $IE2_{579aa}$ (or IE2p86) are nuclear phosphoproteins that have apparent sizes of 68 to 72 kd and 80 to 86, kd respectively. $IE2_{579aa}$ is likely to be the major regulatory protein encoded by this virus (516,529,530). In addition to the abundant 579-amino-acid *ie*2 product, a less abundant rotein, called $IE2_{425aa}$ (or IE2p55), shares the same 85 amino-terminal amino acid with $IE1_{491aa}$ and $IE2_{579aa}$. $IE2_{425aa}$ is encoded from an mRNA with an additional spliced intron, resulting in an in-frame deletion within UL122 (265,516,529). Even more complex UL122 splice variants arise in granulocyte-monocyte-macrophage lineage cells (265,281). The *ie*1/*ie*2 gene products accumulate differentially during infection, depending on infection conditions and host cell type. $IE1_{491aa}$ expression continues throughout infection, whereas $IE2_{579aa}$ expression decreases during the early phase of infection and rises again late in infection (525,530). Whereas $IE2_{579aa}$ and several additional minor IE2-related proteins are made from splice variants immediately after infection, the most abundant *ie*1/*ie*2 gene product is a true late (g2) protein called $gIE2_{338aa}$, encoded from a distinct, unspliced late transcript expressed from a promoter within UL122 (238,529).

Evidence from a wide range of transient assay experiments using promoter targets from both heterologous sources and CMV genes shows that the *ie*1/*ie*2 gene products are positive and negative regulators of viral as well as host cell gene expression (516,529). The major a gene products, $IE1_{491aa}$ and $IE2_{579aa}$, autoregulate *ie*1/*ie*2 and US3 expression by activating via NF-kB binding sites ($IE1_{491aa}$) and repressing via IE2 cis-repression signals (*crs*) in these promoter-enhancers. The major role of $IE2_{579aa}$ discerned from transient assays appears to be activation of viral b and g gene expression, with $IE1_{491aa}$ functioning as an accessory protein in this process. The MIE gene locus in other animal CMV genomes retains a remarkable level of mRNA splicing-pattern similarity but encodes proteins that share limited amino acid sequence identity with those of human CMV. Where assayed, these have thus far all functioned similarly.

Regulatory Activities of Human CMV IE1

Two predicted roles of $IE1_{491aa}$ have been substantiated by studies using UL123 null ($IE1_{491aa}$-deficient) viruses, including a role in activation of *ie*1/*ie*2 promoter-enhancer expression (382) and a role in efficient expression of viral b and g genes (190). In transient assays, transactivation by $IE2_{579aa}$ alone or in combination with ancillary regulators such as pTRS1/pIRS1 is influenced by the presence of $IE1_{491aa}$ (231). Consistent with this role, viral mutants lacking the ability to express any $IE1_{491aa}$ because of a complete deletion of UL123 replicate well at high MOIs but exhibit growth deficiency at low MOIs (190,382). Given the fact that $IE1_{491aa}$-null mutants do not exhibit single-hit infectivity (190), some or all of the

known $IE1_{491aa}$ activities must be crucial to the viral replication cycle under conditions where a single virion initiates infection.

In addition to its ability to cooperate with $IE2_{579aa}$, $IE1_{491aa}$ has been observed under idealized transfection conditions to directly influence gene expression via a number of different transcription factors, including NF-kB (44,380,584), CCAAT box–binding protein (205), and E2F (23). $IE1_{491aa}$ appears to increase the levels of NF-kB activity without engaging in a stable association with this transcription factor or with DNA (289,380). The impact of $IE1_{491aa}$ on a TATA-less promoter with E2F sites (23) and the presence of this viral protein in complexes with E2F family members (346) led to studies showing that $IE1_{491aa}$ binds to the amino terminus of retinoblastoma (Rb) family member p107, in a region distinct from the "pocket" normally associated with E2F binding. $IE1_{491aa}$ binding is sufficient to overcome p107-mediated cell growth suppression (240,436). $IE1_{491aa}$ may also induce AP1-dependent gene expression via a protein kinase cascade involving mitogen-activated protein kinase/ extracellular signal-regulated kinase pathway (269).

Like the ancillary regulatory functions of other herpesviruses (ICP0) and adenoviruses (E4) (229,352), $IE1_{491aa}$ associates with nuclear domain 10 structures (PML bodies) and disrupts their integrity (10,230,257,282,603). Interaction between $IE1_{491aa}$ and PML bodies is direct and relies on $IE1_{491aa}$ domains distinct from those involved in transactivation and association with chromatin (9,603). SUMO-1-modification of PML protein itself is blocked while $IE1_{491aa}$ becomes SUMOylated (394). PML body reorganization does not appear to contribute to viral replication in cultured cells because $IE1_{491aa}$-deficient virus fails to rearrange PML bodies under high MOI conditions where this virus replicates as well as parental virus (9). Thus, disruption of PML nuclear bodies is not required for these domains to function as sites of organization for viral transcription (230) and DNA replication (11,353), at least under conditions of high MOI.

An additional activity long associated with $IE1_{491aa}$ is the ability to bind to cellular chromatin (249,298) although this protein fails to bind DNA directly. The extremely acidic carboxyl terminus is necessary for this association (603). Chromatin binding is not a component of activation of gene expression, even though the same acidic carboxyl terminus appears to be required for $IE1_{491aa}$ to function as a transactivator (Greaves, personal communication, 1999). Finally, $IE1_{491aa}$ has been expressed in a variety of bacterial and mammalian cells, including a line of HFs used for propagation of $IE1_{491aa}$-deficient mutants (190), without any evidence that expression interferes with cell growth. One report suggests that this protein is mutagenic under certain conditions (486), although it is not responsible for chromosome breaks observed during infection of human cells (157).

Regulatory Activities of Human CMV IE2

$IE2_{579aa}$ is the predominant viral regulatory protein with two established core functions: (a) controlling the switch from a to b and g gene expression during productive infection, and (b) as the infection proceeds to the late phase, ensuring down-modulation of the two a genes, *ie*1/*ie*2, and possibly US3, via binding to a *crs* located near the start site of transcription of either gene. Viral $IE2_{579aa}$-deficient mutants have not yet been used for studies on this protein, so that functional evaluation has relied on introduction of $IE2_{579aa}$ into cells to reveal functions implicated in the control of replication (364,516,529). This protein has been introduced either transiently or stably into established human as well as nonhuman cells where many of its broad activation and repression characteristics are preserved. Evidence has accumulated that stable $IE2_{579aa}$ expression is toxic to cells. Experimental observations comparing stable versus transient expression systems include inconsistent reports showing failure (59) or success (600) of an IE2-mediated block to G_1 progression, as well as either success (634) or failure (185) in demonstrating an IE2-mediated block in apoptosis. These contradictory results may be explained by analysis of $IE2_{579aa}$ cDNA in stable cell lines used to derive some of these phenotypes, which reveals that nonfunctional mutants are selected because they are less cytotoxic than wild-type $IE2_{579aa}$ (Greaves, personal communication, 2000). Although most attention has been focused on the prominent a gene product, alternative forms of $IE2_{579aa}$ that arise as a splice variant, $IE2_{425aa}$, or as the highly abundant product of a late transcript, $gIE2_{338aa}$, retain different subsets of activation and repression functions (238,529).

$IE2_{579aa}$ and $gIE2_{338aa}$ function directly in the shutoff of a gene expression by binding to at least two specific sites between the TATA box and the transcription initiation sites of the *ie*1/*ie*2 gene (98,322,424) denoted the cis-repression signal, or *crs*. A similar mechanism has been suggested for regulation of the US3 gene (310). The *crs*-specific repression requires only the carboxyl-terminal dimerization (amino acids 346 to 579), metal-binding and *crs*-specific DNA binding domains (160,338,581) of these proteins. Given that $IE2_{579aa}$ is expressed early as well as late, and $gIE2_{338aa}$ is expressed abundantly at late times, a reduction in a gene expression at late times of infection appears to be the reason for this function. The *crs* is a 14- to 15-bp DNA sequence element with a twofold axis of symmetry (the underlined portions) just upstream of the transcription start sites ($^{+1}$) in the *ie*1/*ie*2 (CGTTTAGTGAACCG^{+1}) and US3 [CGT(G/T)CAGTCCACACG^{+1} (41,310) genes. The *crs* functions in either orientation, can repress basal or activated promoter-enhancer expression, and will confer IE2-dependent repression on heterologous promoters independent of a complex upstream enhancer as long as it is positioned near the transcription start site (98,322,424). Recombinant IE2 species purified from prokaryotic expression clones have long been known to bind to *crs* (308,338) and to block *in vitro* transcription by altering RNA polymerase II preinitiation complex formation (338,618) when assays are carried out with high protein levels. Recombinant IE2 (309) or dephosphorylated mammalian cell–derived $IE2_{579aa}$ binds to *crs* as a dimer using minor groove contacts (581). The phosphorylation state of $IE2_{579aa}$ dictates its ability to bind DNA such that the mammalian cell–derived native protein is inactive as a result of phosphorylation at Ser or Thr (581). Unfortunately, these data are not entirely consistent with direct mapping of major phosphorylation sites on $IE2_{579aa}$ from infected cells (202), which suggested that host serum–stimulated MAP kinases phosphorylate this protein in regions distinct from the protein kinase A sites controlling DNA binding activity (581). These data are most consistent with a model of different phosphorylation pathways controlling transactivation and DNA binding properties of this protein. Thus, $IE2_{579aa}$ appears to inhibit transcription of *crs*-bearing a genes as a dimeric or oligomeric Ser/Thr phosphorylation-sensitive DNA-binding protein that physically blocks recruitment of RNA polymerase (pol) II to the preinitiation complex without impacting on the

binding of other basal transcription factors (314).

$IE2_{579aa}$ is a promiscuous transactivator that acts more efficiently in the presence of $IE1_{491aa}$ (reviewed in refs. 516 and 529). $IE2_{579aa}$ alone or with $IE1_{491aa}$ regulates different promoters via a variety of unrelated cellular transcription factors that include a potential common effect on the basal transcription factor $TF_{II}D$ (330,331). $IE2_{579aa}$-mediated transactivation has a long-recognized similarity to adenovirus E1A (see Chapter 67), in that no single common target sequence or transcription factor is involved in transactivation (331,516,529). CMV $IE2_{579aa}$ plus $IE1_{491aa}$ are able to complement an E1A mutant (518,555). $IE2_{579aa}$ contains two transactivation domains (an amino-terminal 51-amino-acid domain and a carboxyl-terminal 33-amino-acid domain) and two nuclear localization signals (529). A range of mutations has been introduced into $IE2_{579aa}$ that interfere with transactivation (80,100,529,624), supporting a role for most of the protein in this process.

An appealing model for $IE2_{579aa}$-mediated transactivation suggests that its role is that of an adapter-like protein stabilizing $TF_{II}D$-associated factors on the promoter (330). The model is supported by the ability of this protein and $IE1_{491aa}$ to contact a subset of $TF_{II}D$-associated factors, including $TAF_{II}130$ and $TAF_{II}110$ (266,330), possibly with $IE1_{491aa}$ as a coadapter with $IE2_{579aa}$ binding simultaneously to the transcription machinery via $TAF_{II}130$ (330). Consistent with this model, three regions of $IE2_{579aa}$ interact with the carboxyl terminus of TBP and with $TF_{II}B$ (80,248,331,507). Bridging interactions between $IE1_{491aa}$ and $IE2_{579aa}$ are also consistent with the observations that $IE1_{491aa}$ can be replaced by host cell Sp1 transcription factor in transactivation assays (267).

Regulatory Activities of Other a Gene Products

Over the past couple of years, a significant number of mechanistic studies have clarified the role of ancillary a gene products, extending most of them beyond a role in transactivation of gene expression, although some may certainly be involved at this level as reviewed in (109). The US3 region encodes a number of related, differentially spliced products that have characteristics of integral membrane glycoproteins (554). These are glycosylated proteins dispensable for viral growth in HFs (243,278) and epithelial cells (189). US3 encodes endoplasmic reticulum (ER)-targeted proteins that block egress of peptide-loaded MHC class I proteins and therefore represents the earliest of several viral gene products that down-modulate MHC class I expression (12,241,245). This property is discussed in the section, Immune Evasion and Piracy.

Differential splicing in the UL36 to UL38 region of the viral genome gives rise to a number of mRNAs capable of expressing proteins whose individual functions have not yet been fully characterized (109). The predominant UL37 product in CMV strain AD169-infected cells is gpUL37, which, like US3, attracted significant attention as a glycoprotein expressed from a spliced transcript immediately after infection. Both gpUL37 and pUL37x1, a nonglycosylated amino-terminal form of this protein expressed from an unspliced transcript (554), are localized to the cytoplasm of infected cells. Despite their localization pattern, these proteins are suspected of having regulatory functions because the genomic region (UL36 to UL38) cooperates in transactivation (109,231) in ways that implicate one or more of these functions in the regulatory cascade leading to DNA replication (231,413). Recently, UL37 cDNAs were isolated when CMV-encoded functions that block apoptosis were sought by expression-cloning strategies (185). This gene encodes a novel viral mitochondria-localized inhibitor of apoptosis (vMIA) distinct from Bcl-2 family members (185). Three related proteins, pUL37x1, gpUL37, and another multiply spliced form denoted $gpUL37_{M}$, have been expression-cloned and shown to be functional. The pUL37x1 (vMIA), which is the smallest fully active UL37 gene product and which resides at the mitochondrial surface in association with the adenine nucleoside transporter, is the only UL37 gene product expressed during infections with the Towne strain, suggesting that this gene product is sufficient for UL37 function. Despite the complete lack of any structural hallmarks of known Bcl-2 family members, vMIA blocks apoptosis at a point downstream of caspase 8 but prior to cytochrome c release from mitochondria in a Bcl-2-like manner. This work suggests that the long-established role of UL37 as a transactivator (109) in transient gene expression assays may result from its ability to block apoptosis.

UL36 encodes a singly spliced transcript that encodes a nonglycosylated cytoplasmic protein (417). pUL36, the only other known a gene in the UL36-38 cluster implicated in regulation (109,231), is also antiapoptotic, inhibiting at a central point in the caspase cascade. This inhibitor can block at a pre-caspase 8 step despite the absence of sequence hallmarks of known signalling or inhibitory molecules, including the Fas-associated death domain (FADD) or FADD-like interleukin (IL)-1b-converting enzyme inhibitory protein (FLIP) functions (186). Expression patterns of UL36 suggest unusual strain variability in function (417), consistent with observations that this protein is already mutated in many commonly used laboratory strains (186) and is inactive as a result of a point mutation (Cys-131) in AD169 *var*ATCC used for many experiments (417). Laboratory strains of virus may rely more directly on pUL37x1 than on either gpUL37 or pUL36 to prevent apoptosis during productive replication in cultured cells.

TRS1 and IRS1, two members of the US22 family (92), transactivate in cooperation with $IE1_{491aa}$ plus $IE2_{579aa}$ (231,463,527) by a poorly understood mechanism. TRS1 and IRS1 appear to be distributed in both the cytoplasm and the nucleus of infected cells. Both are expressed immediately after infection and are detected in virion preparations (462), although a truncated form representing the carboxyl terminus of IRS1 ($IRS1_{263aa}$) is not virion associated. TRS1 consistently displays stronger transactivation than IRS1, and IRS1 is dispensable for replication so long as TRS1 is retained (243). The presence of TRS1 or IRS1 together with ppUL82 and/or pUL69 dramatically increases the transactivation of the TRS1 or IRS1 promoters themselves, and the truncated form, $IRS1_{263aa}$, blocks TRS1- or IRS1-enhanced transactivation (463). TRS1 or IRS1 cooperates with $IE1_{491aa}$ and $IE2_{579aa}$ to transactivate b genes that have been tested (231,463,527), which suggests that their role may be to increase expression of DNA replication functions (413).

b and g (Delayed Early and Late) Gene Expression: General Considerations

The designation of viral genes into the b or g class depends on timing of expression as well as sensitivity to inhibitors of viral DNA synthesis, such as phosphonoformate (582), or 9-(1,3-dihydroxy-2-propoxymethyl)guanine (DHPG, ganciclovir) (344). A conditional DNA polymerase mutant has also been employed to evaluate sensitivity of gene expression to viral DNA synthesis (134). Both classes of genes are believed to be activated by a ene products targeting the TATA and initiator elements as well as upstream promoter elements located within 100 to 200 bp of transcription start sites. Many nonstructural CMV genes expressed early during infection are transcribed to their highest levels at late times in infection, and transcription of structural genes begins early in the infection even in the presence of viral DNA synthesis inhibitors. Although a genes are active immediately after entry, b and g gene expression is delayed and parallels the very slow synthesis of viral DNA, structural proteins, and progeny. Transcription of b (delayed early) genes starts by 24 hpi (most starting at 4 to 12 hpi) and can be divided into b_1 (early) and b_2 (early late) transcript classes based on the timing of peak protein expression, impact of viral DNA synthesis inhibitors, and changes in expression levels at late times. g (late) gene transcription starts later than 24 hpi and can also be divided into two classes, g_1 (leaky late), where transcripts are made at reduced levels but are not blocked by inhibition of viral DNA synthesis, and g_2 (true late) transcription, which is completely blocked by inhibition of viral DNA synthesis. Both b and g gene expression requires prior expression of a gene products (533). The timing and expression of transcriptionally active viral genes are further modulated by (a) translational and other posttranscriptional controls that dictate appearance of proteins (17,77,262,377), (b) sensitivity of viral replication to the physiologic state of the host cell, and (c) other events, such as activation of host cell protein degradation machinery, that influence the accumulation of b and g gene products during infection.

Although the kinetic class of each gene is dictated by transcriptional activation events and activation of transcription is a major regulatory step, most b and g transcripts have a polycistronic structure. This correlates with the relatively few polyadenylation signals on the viral genome, such that many 3'-co-terminal families of transcripts are generated from a series of promoters, each controlling a separate gene. In addition, many regions of the genome, including the *ie*1/*ie*2 locus, the UL36-38 locus, and the UL115-119 locus, produce both a and g transcripts, whereas other regions such as UL4, UL112-113, US6, and UL44 produce both b and g transcripts from multiple promoters. Thus, the same or related gene products are encoded at different times through the differential activation of transcription in concert with mRNA splicing signals and choice of polyadenylation sites. Once made, transcripts are subject to regulation of transport and translation. At early times, mRNA from selected regions of the viral genome is preferentially associated with polysomes. Most polyadenylated RNA is retained in the nucleus, to be found on polysomes only at late times (377). Two specific types of posttranscriptional regulation have been studied: (a) alterations in the timing or accumulation of cytoplasmic transcripts, possibly caused by differential splicing or 3'-end processing, either one controlling the expression of alternative proteins (313,530,615), and (b) delay in translation (166), one type of which has been ascribed to short ORFs in the 5' leader region of certain transcripts (17,76,77 and 78,167,168).

b (Delayed Early) Gene Expression and Gene Products

Two abundantly expressed b genes, encoding 1.2-kb ($b_{1.2}$) and 2.7-kb ($b_{2.7}$) transcripts, remain prototypes for studies on gene regulation despite the fact that their translation products have not been studied (516). Additional genes whose regulation and gene products have been studied in some detail are involved in DNA replication (UL112–UL113, UL54/DNA polymerase, UL44/DNA processivity factor), immunomodulation (US11), and an early glycoprotein expressed from multiple promoters employing transcriptional and posttranscriptional controls, UL4 (gp48).

$b_{1.2}$ and $b_{2.7}$ map to adjacent positions near the L-component terminus and have been studied solely because they represent 20% to 40% of total viral transcription from early times during infection (378,516). These are located within the *b* repeats of popular laboratory strains such as AD169 (191); however, only single copies of these genes are present in low-passage strains (82) (see section, Strain Polymorphism). The 1.2-kb gene has a biphasic pattern of expression starting very early during infection, and transient assays suggest that the upstream transcription factor binding sites for early activation are less important late in infection so long as plasmids used in transient assays contain a CMV DNA replication origin (516). The $b_{2.7}$ gene encodes the single most abundant viral b or g transcript in infected cells (191,589). This transcript steadily accumulates during infection and accounts for more than 20% of the viral mRNA. When used to drive indicator gene expression, the $b_{2.7}$ promoter confers abundant expression throughout the infection, which is not influenced by the site of insertion in the viral genome (388). Although difficult to detect in infected cells, the conserved TRL4 ORF on this transcript encodes a nucleolar-targeted protein (38) whose expression is subject to translational down-modulation (38,43,167,168,273). Both copies of this abundantly expressed $b_{2.7}$ gene have been deleted from a virus without altering its ability to replicate in cultured cells (Duke and Mocarski, unpublished).

US11 is a b_1 gene encoding an ER-resident protein that directs degradation of MHC class I protein by the proteasome and helps the virus evade immune detection (12,212,485,601) (see section, Immune Evasion and Piracy). Its expression peaks within the first 24 hpi and depends on two separate upstream CRE and ATF binding sites that together contribute to maximal activation in both transient assays and in the context of the viral genome (90). Thus, for this b_1 gene, regulation in transient assays mirrors that observed in the context of the viral genome and implicates specific host transcription factors known to be responsive to viral transactivators.

The UL112-113 (also called 2.2-kb) gene encodes a family of early phosphoproteins (233,616) that regulate expression of core DNA replication genes (231,413) and organizes viral DNA replication compartments as they begin to form during infection (11,419) (see section, DNA Replication). Differentially spliced transcripts of 2.1, 2.2, 2.5, and 2.65 kb in size arise from a single promoter and represent splice variants that express four phosphoproteins of 50, 43, 84, and 34 kd, respectively (516). Although these transcripts are all made very early in infection, the proteins appear in cells as if they represent two temporal classes. The 34-, 43-, and 84-kd species exhibit b_1 kinetics and accumulate from 8 hpi, whereas the 50-kd species exhibits b_2 kinetics and appears later in infection. The transcription start site used early in infection shuts down and shifts 62 nt upstream at late times, although this switch in transcription start site does not drive the late expression of the 50-kd protein. Transient assay analysis of the UL112-113 promoter has indicated that both the TATA box and an upstream CRE/ATF site contribute toward responsiveness to the viral transactivator $IE2_{579aa}$, but that

activation is independent of four IE2 binding sites in the promoter (22,483,484). When studied in the context of the viral genome, expression at early times shows dependence on a CRE/ATF site, but this site is unnecessary for maximal expression at late times (459). The most proximal $IE2_{579aa}$ binding site contributes to full activation at late times.

Transcript from the DNA polymerase (UL54) gene is first detected at 8 hpi but is classified as a b_2-class gene, because levels of transcript and enzyme increase dramatically to maximal levels late in infection. In transient assays, this promoter exhibits much greater response to $IE1_{491aa}$ acting in concert with $IE2_{579aa}$ than to $IE2_{579aa}$ alone, even when introduced into cells with accessory transactivators TRS1 and UL112-113 (261,264). Maximal expression in transient assays is most strongly affected by an upstream inverted repeat element (IR1) (261,264)—that is, a nonconsensus Sp1 site (333)—and binds a complex that includes both $IE2_{579aa}$ and Sp1 in infected cells (619). When evaluated in recombinant viruses, UL54 regulatory elements that are important for early activation are the same as those identified in transient assays, and they implicate IR1 in the activation. In contrast, late expression is not significantly affected by the presence or absence of this site (261), reminiscent of the UL112-113 promoter (459). When the UL54 promoter is evaluated in the context of the viral genome, mutations in the CRE/ATF binding site severely reduce overall expression levels early as well as late during infection (263), arguing strongly that this transcription factor is important for authentic regulation at late times. Together, these experiments suggest that CREB/ATF may provide distinct roles for different genes involving mechanisms that will need further analysis in virus-infected cells.

The polymerase processivity factor (UL44) gene, often referred to as a late gene because its accumulation in cells is to such strikingly high levels at late times of infection (166,175,533), may be operationally considered in the b_2 gene class even though it has three spatially distinct transcription start sites that are activated at different times during infection (312). Two of the three are b promoters that are active at 8 hpi, and the third is a g promoter that is not active until 36 hpi. These three promoters are independently regulated in transient assays (527). Although initially characterized as needing both $IE1_{491aa}$ and $IE2_{579aa}$ to impact on UL44 expression, promoter substitutions demonstrated that TRS1 gives maximal stimulation together with $IE2_{579aa}$, without any requirement for other viral regulatory proteins (463). Although the mechanism of TRS1 function remains to be determined, preliminary analysis suggests that regions bracketing any of the three UL44 gene TATA boxes may be sufficient for TRS1-enhanced $IE1_{491aa}$ plus $IE2_{579aa}$ transactivation (Stasiak and Mocarski, unpublished), which would be consistent with a co-modulator role much like that discussed for $IE1_{491aa}$.

The gpUL4 gene, which encodes an early structural glycoprotein (gp48), appears to be directly responsive to $IE2_{579aa}$ without a requirement for $IE1_{491aa}$ in transient assays (342). UL4 has three transcription start sites that are differentially regulated during viral infection (85,86). Two of the three are b promoters and one of the three is a g promoter. All three promoters are independently responsive to viral infection, but only one of the b promoters is responsive to $IE1_{491aa}$ plus $IE2_{579aa}$ or $IE2_{579aa}$ alone when tested in transient assays (342). UL4 provides the best-characterized example of regulation by posttranscriptional mechanisms, here manifested as translational control by short ORFs in the 5' leader of an mRNA that block progression of ribosomes to downstream ORFs (17,43,76,77 and 78,129,479). The mRNA encoding the UL4 ORF contains three additional short ORFs upstream of UL4, the second of which, uORF2, encodes a 22-amino-acid nascent peptide that stalls the translation machinery by blocking the release step of the last tRNA ($tRNA^{Pro}$). The extent to which other CMV genes are subject to such regulation remains to be determined. A recent evaluation of uORF2 from UL4 genes of a few clinical isolates showed variable ability to stall ribosomes (17).

The ultimate understanding of the regulatory cascade leading to expression of the array of b and g gene products during CMV replication will require dissection of cis-acting as well as trans-acting viral functions acting at many levels. An understanding of the importance of transcriptional regulation by *ie*1/*ie*2 gene products, acting either alone or in combination as transactivators and repressors, will require further detailed study of a range of individual a and b promoters. Transient assay analysis that provides mechanistic insights must be accompanied by studies in the context of the viral genome and viral infection. Only through such studies will the physiologic significance of transcriptional and posttranscriptional regulatory activities become clear.

g (Late) Gene Expression and Gene Products

Even from the limited number of investigations in this area, regulation of g_1 (leaky late) and g_2 (true late) genes appears to differ markedly. From the little that is known, g_1 genes are regulated in a fashion analogous to the b_2 class described earlier, although less detailed characterization of promoter elements is available, and very little has been undertaken in the context of the viral genome. UL83, a g_1 gene encoding the major tegument protein pp65, is transactivated in transient assays by the combination of $IE1_{491aa}$ plus $IE2_{579aa}$, and an upstream octamer sequence contributes to responsiveness (134). When introduced into the viral genome, however, the level of activation of this promoter at early times and dependence on the octamer were not dramatic, suggesting that other regulatory events provide a more dominant influence on expression (277). Additional studies are needed to address g_1 gene class regulation.

The only major g_2 gene whose regulation has been dissected in some detail is UL99, which encodes the tegument protein pp28 (135) from either of two g_2 class transcripts under separate promoter control. The more abundant of these transcripts contains a portion of the UL98 ORF upstream of UL99 (2,135,369). This UL99 promoter is not very responsive to transient transactivation and does not rely on particular upstream elements (135). Indeed, when examined in the context of the virus, sequences upstream of –40 relative to the transcription start site are not required for authentic expression (277). Maximal authentic expression occurs when a small promoter fragment containing little more than a TATA element (–40 to +6 relative to the start site) is placed in the context of the virus, although expression is a balance between slightly reduced transcript levels and dramatically increased indicator gene expression, because of the disruption of a G:C-rich hairpin structure involving the first 25 nt of the transcript (262). Evaluation of the adjacent UL94 gene by transient assay has suggested that full activation can be achieved with a similar promoter fragment containing little more than a TATA element (–45 to +1 relative to the start site) (606). The balance of transcriptional and translational controls appears important, but additional studies need to be completed on the less-abundant UL99 transcript as well as

more detailed studies on other g_2 genes before a complete picture of regulation can emerge.

Effects on the Host Cell

Host cell transcription and translation increase during CMV infection (533). Virus binding and/or entry results in the elaboration of cellular signalling responses (15) as well as the induction of host immediate and interferon response genes (53,55,56,399,626,628,632,633). This behavior indicates that activation via cell surface receptors, delivery of tegument proteins, or some other virus particle-mediated process plays a predominant role in stimulating host cell gene expression. Binding of ultraviolet-inactivated virions to cellular receptors initiates cellular signalling cascades (53,56,626,628,632,633), with soluble forms of gB or gH able to stimulate cells on their own, presumably via their interaction with cellular receptors (62,627). As yet there is no experimental distinction between the stimulation by virus binding and the introduction of particular virion transactivators as a result of viral entry; however, the combined impact of these events on cellular gene expression is broad under experimental conditions (587,632). A majority of host genes whose expression is stimulated within the first 24 hours after infection appear to respond to virion-mediated events preceding expression of viral regulatory genes.

Viral regulatory proteins expressed after penetration further regulate cellular signalling pathways and gene expression as infection proceeds (14,232,460,475,532). The viral $IE1_{491aa}$ gene product, in particular, contributes to both stimulation and suppression patterns of host cell gene expression (587). Optimal virus replication depends on host gene expression (161,305), possibly of genes that are specifically stimulated, such as host DNA metabolism enzymes, DNA polymerase a and proliferating cell nuclear antigen (PCNA) (138,215); cell cycle functions cyclin E, cyclin B, pRB, and p53 (138,236,470,488); and transcription factors (53,472,629), some or all of which require viral gene expression to be adequately induced.

Co-infection studies with other viruses also reveal the striking impact of human CMV infection on cellular metabolism. HSV-1, adenovirus, adeno-associated virus, human T-cell lymphoma virus (HTLV)-1, and papovavirus replication have been shown to be activated by human CMV infection (110,357,414,518). Both activation and suppression of HIV and HTLV-1 have been observed, apparently dependent on cell type (237,288,311,545).

Despite a pattern of cellular gene expression consistent with cell cycle progression (236,470), productive infection of permissive HF cells does not induce either cellular DNA synthesis or cell division (14,64,131,132,138,327). When serum-stimulated at the time of or just prior to infection, resting HFs fail to synthesize host cell DNA and exhibit a profile consistent with a G_1/S arrest (64,138). Permissive HFs that have already entered S or later phases at the time of infection may arrest at different points (236,327) and exhibit a delay in viral gene expression as well as replication (470). A very different series of events may occur in abortively infected cell types, where virus-induced stimulation of host cell DNA synthesis is readily observed (16) and stimulation of DNA synthesis may prevail under certain conditions in particular differentiated cell types that are permissive for viral replication (488). Although the response varies considerably, depending on many different experimental factors, the reasons and implications for such contrasts in the cell response to viral infection remain to be fully elucidated.

Two viral functions may contribute to the G_1/S arrest phenotype in HFs, the virion tegument protein ppUL69 and the major regulatory protein $IE2_{579aa}$. Virion-associated ppUL69 has been shown to contribute to cell cycle dysregulation during CMV infection (328) because deletion of this gene from the viral genome results in reduced G_1/S arrest that can be reversed when ppUL69 is incorporated into mutant virions (204). $IE2_{579aa}$ is able to induce cell cycle arrest following transient expression (600), and it interacts with a number of cell cycle regulated proteins, including p53 (393,521,566), pRB (199,507), and $p21^{cip1}$ (488), suggesting mechanisms of cell cycle regulation that may include physical interactions with cell cycle regulatory functions.

The observation that human CMV infection stimulates host cell metabolism underlies possible involvement in chronic proliferative diseases, ranging from cancers, adenocarcinoma, or Kaposi's sarcoma (464,517), to vascular diseases such as atherosclerosis and restenosis after angioplasty (147). Although epidemiologic associations are complicated by the ubiquitous distribution of human CMV in the adult population, the difficulty of using molecular detection methods to unambiguously associate small amounts of viral nucleic acids with a chronic disease and controversy regarding the epidemiologic methods used, these areas remain of active interest. Three “morphologic transforming regions” on the human CMV genome, called *mtr*I, *mtr*II, and *mtr*III, mapped near 156,000 bp, 159,000 bp, and 174,000 bp, respectively, have been characterized for their ability to growth-transform rodent fibroblasts (139). CMV-induced growth transformation of rodent cells follows a hit-and-run paradigm (339), complicating assignment of a role for any CMV genetic element in transformation. A 79-amino-acid ORF related to UL111A within *mtr*II (395,557) that appears to encode the amino terminus of a CMV IL-10 homolog (284,326) is the only region that is retained in transformed cells. The ability of human CMV $IE1_{491aa}$ or $IE2_{579aa}$ to increase adenovirus E1A-mediated transformation (486) can be related to a potential antiapoptotic activity (634), but neither of the CMV gene products is retained in outgrowing E1A-transformed cells and neither exhibits transforming activity on its own. This area of research is complicated by several contrasting observations, such as reports that constitutive expression of $IE1_{491aa}$ does not alter either growth or permissiveness of HFs (190,382), whereas expression of $IE2_{579aa}$ is toxic and induces cell cycle arrest (600).

The mutagenic effects of human CMV virion binding/penetration events are readily scored (1). Recent evidence for breaks introduced specifically into chromosome 1 when permissive HFs are exposed to virus particles (157) opens a host of questions about how much of a role is played by signalling cascades induced by virion binding or by structural proteins delivered to cells through the entry process.

DNA Replication

CMV replication is believed to follow a productive replication scheme that is common to other herpesviruses. The definition of CMV DNA replication has been derived predominantly from transient DNA replication assays employing viral

genes (20), with only limited investigations using viral mutants (134,438). The roles of some viral gene products are relatively well understood by analogy to other herpesviruses, whereas others are still poorly understood because they appear to be betaherpesvirus specific. Six herpesvirus-conserved CMV replication-fork proteins—a DNA polymerase (pUL54) and its associated processivity factor (ppUL44), a single-stranded DNA binding protein (ppUL57), and a three-subunit helicase-primase complex (pUL70, pUL102, and pUL105)—are required for viral origin (*ori*Lyt)-dependent DNA synthesis. In some assays, the products of additional CMV genes (UL84, UL112-113, UL114) also appear necessary for optimal replication (20,438,476). Importantly, UL84 may play a key function in initiation of *ori*Lyt-specific replication (476).

Human CMV DNA replication begins at approximately 16 hpi and peaks at 60 to 80 hpi (533) with the production of several thousand genome copies per infected cell (536) that mature and are released from cells over a 24- to 48-hour period (419). The CMV genome circularizes in the nucleus of permissive cells within 4 hpi, and replication produces concatemers late in infection. Most replicating viral DNA is larger than unit genome length and lacks terminal fragments. This is consistent with a directional rolling-circle mode of replication (296,358) that may require cellular replication proteins in addition to viral functions. Host cells are stimulated in such a way that they have the appearance of transiting the S phase (236,391) but are actually blocked from S phase progression when supporting viral replication (64,132,133,138,327,470). These conditions appear to favor viral DNA synthesis, although this remains a slow process even under optimal conditions.

Origin/Replicator

Human, murine, and simian CMV genomes contain a single *ori*Lyt located within the U_L region and adjacent to the ORF encoding the single stranded DNA binding protein, ppUL57 (19,201,350,351). This relative position is preserved in all betaherpesviruses and is analogous to the arrangement observed for HSV-1 ori_L (510). Human CMV DNA replication proceeds bi-directionally from *ori*Lyt, based on chain termination assays (201), which fulfills functional requirements of an authentic productive DNA synthesis initiation site. Although CMV enters latency with retention of the viral genome as an extrachromosomal circle (57) in cells with the potential to divide, a genome maintenance origin has not yet been identified. In addition, genes corresponding to the origin-binding proteins of alphaherpesviruses (see Chapter 72) or the related functions in the betaherpesviruses HHV-6 and HHV-7 (see Chapter 80) are not encoded by any CMV, limiting understanding of the initiation of CMV DNA synthesis.

The minimal *ori*Lyt maps to a large region between 91,751 and 93,299 nt on the AD169 genome (19,350,635); however, an even larger region spanning 90,500 to 93,930 nt is required for full replicator function. The minimal *ori*Lyt sequence contains clustered and dispersed repeats and binding sites for various transcription factors (19,201,350,588), the precise function of which awaits further study. Human CMV encodes a number of short transcripts within the *ori*Lyt region (222,440) and virions carry stable *ori*Lyt-specific RNA–DNA hybrid species (440), suggesting that transcription may play some role in the initiation of viral DNA synthesis. A 189-bp repeat sequence present in varying copy numbers in the origins of human CMV strains AD169 (93,337 to 93,525 nt) and Towne has been shown to be dispensable for DNA replication (97,258). Introduction of heterologous DNA at this location abolishes DNA replication, implicating a role for at least one copy of this sequence in CMV DNA synthesis. One origin sequence element, a 31-nt homopyrimidine (Y block), has been shown to be absolutely essential for replication (222). In addition, scanning mutagenesis of this entire region revealed that many mutations within and adjacent to the minimal origin reduce replication efficiency from 50- to 100-fold to undetectable levels (635). Two absolutely essential regions were confirmed in this screen, one overlapping the Y block between 92,209 and 92,573 bp and the other, between 92,979 and 93,145 bp, overlapping an RNA species arising within the *ori*Lyt region.

DNA Replication Enzymes and Sites of CMV Replication

Human CMV DNA polymerase (pol) enzymatic activity is readily measured (219). A DNA pol mutant that fails to make viral DNA has been described (134) but has not been subjected to detailed study. Conservation of a high level of sequence similarity and enzymatic properties of CMV DNA pol with similar enzymes encoded by other herpesviruses indicates that this protein functions as a key replication-fork protein responsible for resistance to high salt concentrations and sensitivity to particular deoxyribonucleoside and pyrophosphate analog antiviral agents (158,219,582). Conserved structural domains present in other cellular and viral DNA polymerases are also conserved in the CMV DNA polymerase (107). CMV DNA polymerase is dependent on an associated polymerase processivity factor, ppUL44 in human CMV (148,591). This protein is conserved in function, but not sequence, in all herpesviruses.

The product of UL44 was initially referred to as the major late DNA binding protein (175,180) because it binds to double-stranded DNA (175,180,385), an activity that underlies its role as the DNA polymerase processivity factor that prevents dissociation of the polymerase from the DNA template (148,591). Thus, ppUL44 shares biochemical characteristics with the processivity factor encoded by HSV-1 UL42, but it does not exhibit significant sequence conservation (92,446). The other DNA binding protein is encoded by UL57 and has similar size and biochemical activities to the HSV-1 single-stranded DNA binding protein (18,259). By analogy to HSV-1, the product of UL57 is predicted to prevent reannealing of DNA strands following unwinding by the putative human CMV helicase–primase complex, UL105–UL102–UL70, which is analogous to the HSV-1 UL5–UL8–UL52 helicase–primase (see Chapter 72). This process has received little direct study in CMV.

Current antiviral therapies, including ganciclovir (158), cidofovir (620), and foscarnet (582), target the CMV DNA polymerase. Though CMV does not encode a specific deoxynucleoside (or thymidine) kinase, the phosphorylated active form of ganciclovir accumulates within infected cells through the action of the CMV gene UL97 (48,543). UL97 appears to encode a kinase (93) that is the homolog of a herpesvirus-conserved serine/threonine protein kinase responsible for autophosphorylation as well as for phosphorylation of other viral proteins (208). UL97 is unusual in appearing to use the nucleoside ganciclovir as a substrate (321,543). One recent study suggests that UL97 is also able to phosphorylate acyclovir but to a lesser extent (548), which may explain this drug's lower activity against CMV compared to HSV-1. Point mutations in either the CMV DNA polymerase (24,106,542) or UL97 (102,495) confer resistance to these therapies. Although CMV does not encode a gene for thymidylate synthase, it appears to require this cellular enzyme for efficient

DNA replication. Treatment of human or murine CMV–infected cells with inhibitors of thymidylate synthase results in inhibition of viral DNA synthesis (108,193).

In addition to these core replication proteins, four viral gene loci are known to influence replication as presumed regulatory proteins—*ie*1/*ie*2, TRS1/IRS1, UL112-113, and UL36-38 (231,412,413). The *ie*1/*ie*2 and TRS1/IRS1 genes may function to activate gene expression (see section, Regulation of Gene Expression), but the key functions of the UL36 and UL37 genes appear to be prevention of apoptosis (see section, Regulatory Activities of Other a Gene Products) (185,186). Furthermore, two loci (UL84, UL112-113) needed for viral DNA synthesis (412,413) may be more directly involved in the process. The ppUL84, a 75-kd protein, stably interacts with IE2 $_{579aa}$ in infected cells (471,519); however, it is still unclear how this property relates to the evidence that this is an origin-specific initiator protein, which was based on the ability of this protein to facilitate human CMV *ori*Lyt-dependent replication using the six EBV replication-fork proteins (476). The ppUL84 is unable to initiate *ori*Lyt-dependent replication with the human CMV replication-fork proteins in the same assay. The ppUL84 interaction with IE2$_{579aa}$ inhibits transient transactivation (169), but the impact of this complex on replication requires further analysis. Finally, the four phosphoproteins encoded by the UL112-113 region (233,616) appear to orchestrate recruitment of replication-fork proteins to nuclear sites associated with viral transcription and DNA synthesis (11,419).

Human CMV DNA synthesis proceeds in distinct subnuclear regions within the infected cell. The viral replication functions, ppUL44 and ppUL57, follow an ordered progression of localization patterns, leading eventually to the formation of large globular intranuclear structures that bear resemblance to HSV-1 replication compartments (419,572). These subnuclear domains are sites of human CMV DNA replication based on bromodeoxyuridine incorporation (419). The products of UL112-113 localize into replication compartment precursors earlier than any of the known replication-fork proteins and appear to function as recruiting proteins for these enzymes (11). Based on bromodeoxyuridine pulse-chase experiments, the early precursors of CMV replication compartments are sites of both cellular and viral DNA synthesis (419). Interestingly, small amounts of viral DNA are still synthesized in the presence of viral DNA polymerase inhibitors (391,419). This, together with the localization of viral DNA replication to known sites of cellular DNA synthesis, suggests that early phase viral DNA amplification may depend on cellular replication enzymes.

The human CMV genome also carries a number of enzymes involved in nucleotide metabolism and DNA repair. These include the large subunit of ribonucleotide reductase, deoxyuridine triphosphatase, deoxyribonuclease, and uracil DNA glycosylase (92). At least one of these proteins, the human CMV uracil DNA glycosylase encoded by UL114, is required for efficient DNA replication, based on analysis of a viral mutant (438). UL114 does not score as necessary in transient replication assays, but a human CMV mutant in UL114 exhibits a prolonged growth cycle corresponding to a restriction prior to viral DNA synthesis. Further analysis of this mutant has revealed that it is able to synthesize DNA to 10% of the level of wild-type virus, suggesting that UL114 is required for the transition to high-level, late-phase DNA replication (Courcelle and Mocarski, unpublished data).

Genome Inversion and Maturation

Genome inversion and maturation occur in the nucleus of infected cells, with most newly replicated DNA maturing into virions that are released over a period of 2 days after synthesis (419). Inversion results in progeny genomes appearing as any one of four isomers differing only with respect to the orientation of L and S components (see Fig. 1C). This process occurs at late times in infection, during or following replication, and possibly within concatemeric intermediates found prior to DNA cleavage and packaging (358). Human CMV is the only member of the betaherpesviruses that undergoes inversion of two genome components. As is the case in other herpesviruses, inversion of the human CMV genome is dispensable for replication in cultured cells (Duke and Mocarski, unpublished observation). The recombination that drives inversion requires inverted repeat sequences located at the ends of each genomic component. The genomic *a* sequence is necessary and sufficient for inversion, because deletion of the L-S junction *a* sequence without disruption of either *b* or *c* repeats freezes the viral genome in one isomer (Duke and Mocarski, unpublished observation), and duplicating an *a* sequence segment results in additional inversion events (260). When murine CMV is engineered to carry an additional copy of the joined termini in inverted orientation, the viral genome uses these sequences to support inversion as well as cleavage/packaging progeny genomes in a new register (362). When the same fragment is inserted in direct orientation, only packaging occurs (360). The biologic role of inversion in those herpesviruses that carry out this process remains a mystery.

Like other herpesviruses, human CMV packages its genome into preformed B capsids composed of MCP, mCP, mC-BP, and SCP together with the AP precursor (pUL80.5) (176). The functions that are likely to play some role in this process are all tegument proteins, and they include UL56, UL89, and UL104, as indicated by antiviral resistance mapping (290,571) (Rubsamen-Waigmann, personal communication, 2000), as well as UL51, UL52, UL77, UL47, and UL48 based on sequence similarity to most functions known to be important in HSV genome maturation (see Chapter 72). Resistance to 2-bromo-5,6-dichloro-1-(b-D-ribofuranosyl)-benzimidazole (BDCRB) and 2,5,6-trichloro-1-(b-D-ribofuranosyl)-benzimidazole (TCRB) maps to UL89 and UL56 (290,571), and resistance to BAY 38-4766 maps to UL89 and UL104 (Rubsamen-Waigmann, personal communication, 2000), directly implicating these genes in the cleavage/packaging process inhibited by these different compounds. The importance of UL89, a homolog of the terminase, and UL56, a DNA binding protein, is also suggested by studies that show that these proteins associate into a complex (50) (Bogner, personal communication, 2000) in a manner similar to their HSV homologs (see Chapter 72).

The signals used to direct cleavage and packaging of CMV genomes, denoted *pac*1 and *pac*2, are highly conserved short sequence elements found near the genomic termini in all mammalian herpesviruses. Human, murine, and rat CMVs contain elements that are homologous to both *pac*1 and *pac*2 elements (348,349,383,512,549,578). These elements are essential cleavage/packaging signals (260,360) and can allow HSV-1 to recognize and package plasmids bearing a CMV *a* sequence (512). In most herpesviruses that have been examined, including murine and rat CMVs that lack an *a* sequence, *pac*1 and *pac*2 are positioned on opposite sides on the cleavage site, whereas in human CMV both appear to be carried on the S-component proximal side of the *a* sequence (260). Cleavage leaves a *pac*2 element near a free

S-terminal fragment that is present on concatemers. The packaging machinery preferentially inserts termini derived from the S rather than the L component into maturing capsids (358). This *pac*2-leading directional packaging from concatemers appears to be a common theme and occurs in murine CMV and guinea pig CMV, as well as in HSV-1 (361). Although the precise cleavage site appears to vary slightly from strain to strain in human CMV, the two strains that have been examined (Towne and AD169) have a single 3' nucleotide extension on both termini (383,550). Even when the terminal direct repeat is extremely small, such as in murine CMV where *pac*1 and *pac*2 lie outside the 30-bp terminal repeat, both elements are essential for the genome maturation process (360). The cleavage/packaging process results in a variable number of *a* sequences at human CMV genomic termini and the L-S junction (383), with a single *a* sequence predominating at all positions. A poorly studied process of *a* sequence duplication and amplification occurs at some step during replication. Studies in murine CMV suggest that the duplication of an inserted terminal repeat only occurs at the same time the additional junction is recognized for cleavage. Anywhere from one to eight directly repeated *a* sequence copies may be detected at the L or S terminus and L-S junction, and a small proportion of genomes lack an *a* sequence altogether at the S terminus (260,383,550). The sequence within a 220-bp region that carries both *pac*1 and *pac*2 is sufficient to convey a site for cleavage/packaging as well as inversion on a recombinant human CMV (260).

Little is known about the cellular proteins that might contribute to genome inversion or maturation events. A cellular protein has been detected, partially purified, and shown to bind to the human CMV *pac*-2 sequence elements (260), but characterization of its role in replication, genome inversion, or genome maturation has yet to be completed. Human CMV expresses several functions with similarity to suspected HSV-1 maturation functions. Based on direct studies of protein function, the human CMV UL46 product (mCP) appears to be a homolog of HSV-1 19C (92,175), a protein involved in anchoring DNA into HSV-1 nucleocapsids. Human CMV UL48, the homolog of the huge virion protein (VP1) of HSV-1, and CMV UL98, the homolog of the HSV-1 deoxyribonuclease, are conserved genes and may play roles in genome maturation.

Virion Morphogenesis

Ultrastructural studies (354,498) show nucleocapsids accumulate in the nucleus (see Fig. 1B) and form a nuclear inclusion that is readily observed by light microscopy as the typical “owl's eye” cytopathic effect. Maturation starts in uclear inclusions, where nucleocapsids mature within a nuclear fibrillar network consisting of viral structural proteins adjacent to a DNA replication compartment (156,419). Particles with and without electron-dense DNA cores mature (see Fig. 1B). Formation of capsids is controlled by MCP–AP (1:1) precursor complexes that initially associate via an AP precursor conserved carboxyl-terminal domain following synthesis at cytoplasmic sites (408,612). This interaction is responsible for both translocating the MCP to the nucleus (426) and initiating capsid assembly (176,306). In the nucleus, a conserved amino-terminal AP precursor domain facilitates oligomerization that drives the formation of hexons, pentons, and higher-order structures. A triplex of mCP–mc-bp (2:1) complexes interdigitate hexons and pentons on oligomerized AP precursors as they form an internal scaffold, which is structurally observed as a dense core ring in B capsids (75,95,565). The association of SCP on the outer capsid surface and a series of assemblin-mediated protease cleavage steps separate AP from the MCP and inactivate the protease itself. This completes the formation of B capsids, into which viral DNA is packaged. The insertion of DNA displaces AP. The process is dependent on the viral UL97 kinase (610a). Progeny nucleocapsids locate to thickened concave patches in the inner nuclear membrane, where they bud into the perinuclear cisternae (176). The nuclear membrane is modified with viral glycoproteins that are also present on the mature viral envelope (443,537). Thus, the initial site of virion envelopment is at the inner nuclear membrane (378), but the site of final envelopment has not been unambiguously determined and may occur at cytoplasmic membranes through a process of deenvelopment/reenvelopment best characterized in HSV-1 (599). Evidence that the human CMV envelope has the lipid composition of early endosomal membranes (564) has been complemented by studies that suggest cytoplasmic maturation sites are where some tegument proteins exclusively reside (473,474). Electron micrographs support the removal and re-acquisition of the envelope during egress (498). Dense bodies, consisting solely of tegument material surrounded by an envelope, mature exclusively at cytoplasmic sites, and nonenveloped nucleocapsids have been observed free in the cytoplasm as well as budding into cytoplasmic vesicles. The virion is transported in vesicles via the Golgi apparatus to the cell surface, suggesting that egress from cells involves an exocytic pathway sensitive to brefeldin A (145). This observation reinforces the need for glycosylation and transport of the virion through the Golgi apparatus prior to release. The characteristic cytoplasmic inclusion that develops in CMV-infected cells represents an expanded Golgi complex in which nucleocapsids and dense bodies accumulate (see Fig. 1B). This inclusion distorts the cell nucleus, giving it a characteristic kidney-bean appearance. Progeny virus is efficiently released from cells, with approximately half of the progeny virus at very late times after infection found in the culture fluid and half remaining associated with the cell.

PATHOGENESIS, PERSISTENCE, AND LATENCY

Human CMV exhibits a ubiquitous pattern of distribution and efficient transmission via direct contact in all areas of the world regardless of socioeconomic conditions (216) (see Chapter 77). The usually benign primary infection by this virus is controlled by a combination of innate and adaptive immune control functions, after which latent infection continues for life. Primary infection, lifelong latency, and intermittent shedding resulting from reactivation commonly occur without any marked disease consequences. These characteristics underscore the remarkable balance this pathogen has developed with its host.

Primary infection typically starts with replication in mucosal epithelium as a result of direct contact with infectious secretions from an infected individual. A high incidence of infection in the general population and accumulating evidence that hand-washing prevents acquisition of CMV from infants who shed high levels of virus in urine and saliva provide excellent evidence that this virus reaches a large proportion of the population because it is efficiently transmitted (3,4,523). A systemic phase of infection disseminates virus in the host via a leukocyte-associated viremia that may last for several months as assessed using assays for viral DNA or viral antigen (450,631). Cell-free infectious virus is not found in blood, although viral DNA, somehow protected from degradation, is detected in plasma from immunocompromised individuals (520). Peripheral blood (PB) neutrophils, monocytes, and endothelial cells have all been observed to carry infectious virus in immunocompromised hosts (171), but the role of particular leukocyte subsets as vehicles for dissemination during primary infection in the immunocompetent host is still unclear. The suggestion that

neutrophils act as vehicles to disseminate infectious virus and viral products has been supported by cell culture studies on transmission of clinical strains in the absence of full replication (172). PB monocytes are vehicles for dissemination of murine CMV (111,539), and this interaction is controlled by viral tropism determinants (468). The systemic phase of primary infection in adults is accompanied by persistent viral shedding in urine, saliva, breast milk, and genital secretions, which may be important sources for transmission between hosts. The distribution of virus during acute infection is best understood from studies in the immunocompromised host, but a range of endothelial, epithelial, and hematopoietic cells in tissues are also believed to be susceptible in immunocompetent individuals (425). Systemic replication leads to a well-documented (216) seeding of the ductal epithelia where replication releases virus into secretions. Myeloid-lineage hematopoietic cells become important targets for lifelong latency. Viremia continues for a long period of time after an adaptive immune response can first be detected, typically at least 6 months in adults (450,631) and several years in young children (416). This prolonged period of primary infection is surprising given the intensity and quality of the host immune response, although this pattern reinforces the balance that characterizes all aspects of CMV pathogenesis (216). Although an initial persistent infection is often observed, clearance of acute infection is the norm in all immunocompetent individuals (416,523), and it is correlated with a slow rise in cell-mediated immunity (631). The relatively long period of persistent shedding observed in infants correlates with their immune status and allows the virus an efficient means of transmission to uninfected individuals (4).

CMV is also transmitted as a result of transplantation of solid organs or bone marrow and transfusion of whole blood, where blood leukocytes are an important reservoir for transmission (128,623). PB mononuclear cells in the myeloid lineage support latent infection (281,489,503,505). Latently infected cells most likely originate from the bone marrow in healthy seropositive individuals (280,281,375) and virus has occasionally been recovered from PB leukocytes subjected to long-term co-culture (137,503). Latent virus is strongly suspected to play an important role as a source for transmission with transplanted solid organs or transfused blood products, although studies that correlate latent viral load with risk of transmission are still needed.

Pathogenesis of CMV disease is directly linked to the immune status of the host. Primary infection, as well as reactivation from latency, contributes to pathogenesis (see Chapter 77). Innate and adaptive immune mechanisms control acute virus infection, maintain latent infection, and modulate virus during reactivation. Clearance mechanisms are subjected to a range of viral immunomodulation that both thwart the effectiveness of the immune response and use the immune system to ends that benefit the virus. CMV infection directly perturbs immune functions, leaving the host open to disease, such as during transplant procedures, bone marrow or stem cell allografting (300), and PB stem cell autografting (45). This virus has been shown to down-regulate MHC class I protein expression to reduce the impact of cytotoxic T-lymphocyte (CTL) immunity (211,429).

Because of its strict species specificity, human CMV has never been successfully introduced into another animal, although human tissue implants carried in severe combined immunodeficient (SCID-hu) mice are permissive (69,381). Epithelial cells are prominent targets for replication in SCID-hu (thy-liv) mice, where variation in replication efficiency of different virus strains (69) has provided insights into genome variation (82). Murine, guinea pig, rat, and rhesus macaque CMVs have been employed as surrogate models of human CMV infection, latency, and disease (73,126,225,325,522,544), providing insights into CMV pathogenesis, including host immune control (283,452), mechanisms of dissemination, and tissue tropism (58,81,468,539,544), disease in the immunocompromised host (433,448,528), contribution to chronic disease (437,580), and mechanisms of congenital disease (325), latency (294), and immunomodulation (151,210). All of these areas are important contributors to viral pathogenesis.

Immunity

A central importance of cell mediated immunity to the control of CMV infection is reinforced by the observation that individuals with deficiencies in cellular immunity exhibit the highest risk for CMV disease. Resistance to CMV infection depends on successful collaboration between the innate and adaptive immune systems. Both natural killer (NK) cells (60) and CTLs (452), whose presence correlates with protection from CMV disease (47,319), play prominent roles in immune clearance. The humoral antibody response is critical in some settings such as neonatal disease (623) and solid organ transplant settings (501), although it is less clearly important in other settings such as bone marrow allografting. Adoptive transfer of CMV-specific CTL clones provides a benefit to the host, above and beyond antiviral therapy in bone marrow allograft recipients (452,585), and demonstrates that MHC class I restricted CTL response to a small set of structural protein determinants on ppUL83 and ppUL32 is sufficient for adoptive immunotherapy (585). CTL responses to CMV ppUL83 are evident over the life of the individual and remain a remarkably consistent percentage of the total immune repertoire (239,590).

Immune clearance in humans has focused on MHC-linked control, although in mice, resistance to CMV disease maps to MHC-linked as well as nonlinked determinants. These include one locus controlling the permissiveness of cells (203) and another, called *cmv*-1, influencing NK cell function (70,151,478). MHC-linked resistance in mice extends beyond CTL immunity and includes differences in interferon levels, macrophage function, and other cytokine-mediated immune response elements that control CD4+ as well as CD8+ T-cell clearance mechanisms (283,489). NK responses are important in human CMV infections (47), and studies using murine CMV reveal their central role in modulating virus in the earliest stages of infection. Specific adaptive immunity is responsible for clearance and survival (151,596). In murine CMV, in fact, the adaptive cellular immune response by CTLs is sufficient to clear infection from most tissues and is sufficient for survival (283). Clearance from particular organs, such as from salivary glands, is dependent on a CD4+ T-helper-cell response rather than on CTLs (329). It is likely that specific adaptive cellular immune mechanisms are most important for control of acute infection and maintenance of latency.

Immune Evasion and Piracy

Viral functions committed to evading the host immune response, optimizing viral growth, and promoting viral dissemination are encoded by many genes that are dispensable for productive replication in cultured cells. Initial insights into the roles of such genes came from murine CMV, where a viral gene (m133), denoted *sgg*1, was shown to be a determinant of replication in salivary glands (299,343) and from human CMV, where a cluster of dispensable genes

(US2–US11) has been shown to encode a function (gpUS9) that influences cell-to-cell spread of virus in polarized cells (341), as well as four functions (gpUS2, gpUS3, gpUS6, gpUS11) that down-modulate MHC class I expression to reduce the effectiveness of CTL immunity (241,429). MHC class I down-regulation is a theme common to human CMV (33,211,622) and murine CMV (130), although the modulation is carried out by a distinct, unrelated set of viral genes in each virus (210,429). In human CMV, MHC class I down-regulation occurs as a result of expression immediately after entry (12,212,241,601) of gpUS3, an a gene product that resides in the ER and prevents MHC class I transport to the cell surface (12,245). Subsequent down-modulation is by gpUS2 and gpUS11, two b_1 gene products residing in the ER (12,241,244,601) that direct the proteasomal degradation of newly transported MHC class I proteins by binding to MHC protein ectodomains while signalling degradation from the carboxyl-terminal, cytoplasmic tail (485,540). Even later in infection, MHC class I translocation is prevented by gpUS6, a g_1 gene product that targets the ER and binds to the lumenal side of the transporter of antigen processing (TAP) complex, inhibiting peptide translocation (13,213,316). One additional specialized mechanism may reduce MHC class I–dependent presentation of the nuclear protein IE1 $_{491aa}$ via a phosphorylation event controlled by tegument protein ppUL83 (182). In the host, these immunomodulatory processes probably reduce effectiveness of the CTL response enough to slow clearance and encourage persistence. Despite the elaboration of this myriad of immunomodulators, CTL-mediated immune clearance remains the most important component of the immune response contributing to viral clearance (452), providing evidence of the delicate balance between the virus and host. To avoid the impact of down-modulation, CTLs may recognize input structural proteins presented before viral immunomodulatory gene products have a chance to alter class I levels (453,604). Although MHC class I synthesis and processing are reduced by viral infection, sufficient levels of class I remain on the surface of infected cells to allow killing, but with altered sensitivity to CTLs (211). It remains unclear whether down-modulation of MHC class I plays a more important role during the induction phase of the immune response than during the elaboration of effector functions. In this regard, mutations in the murine CMV immunomodulatory functions (152,155,210) may play different roles in various phases of the immune response or infection.

CMV infection also modulates IFNg-induced MHC class II expression, with a likely impact on antigen presentation during either induction or effector phases of the CD4+ T-lymphocyte response (374). Human CMV infection inhibits cytokine-induced MHC class II expression by blocking the induction of transcription activator, CTA_{II}, a target of the Janus kinase 1 (Jak1) pathway (373). Although not yet as well studied as MHC class I down-modulation, multiple mechanisms appear to control MHC class II modulation during human CMV infection, with a US2-dependent degradation system also contributing to reduction in MHC class II levels (562). In murine CMV, down-regulation of IFNg-induced MHC class II expression reduces antigen presentation on infected macrophages (209). Induction of host IL-10 that occurs during murine CMV infection of macrophage-lineage cells suppresses even constitutive MHC class II expression (449). The discovery of a functional viral IL-10 in human CMV (284,326) suggests that both viral and cellular IL-10 may contribute to MHC class II down-regulation in addition to the impact of other viral gene products. Human and other primate CMVs encode a homolog of IL-10 from spliced mRNA that retains a subset of introns found in host IL-10 (326), including, in human CMV, an amino terminus derived from UL111A (92). The human CMV IL-10 signals via IL-10 receptors (284), indicating an antiinflammatory role first suggested for the EBV IL-10 homolog (390).

A number of other human CMV genes encode homologs of cellular gene products that probably play a role in pathogenesis. An MHC class I heavy-chain homolog (UL18) binds b_2 microglobulin (72) and peptides (150) as does host MHC class I (89), but it is dispensable for replication in cell culture (71). The pUL18 has been thought to prevent an effective NK cell response by replacing host cell MHC class I on the infected cell surface. The cellular receptor for pUL18 is related to killer cell inhibitory receptors (KIRs) found on NK cells, and it is denoted lymphocyte IR-1 (LIR-1) because of more prevalent distribution on lymphocytes and monocytes (115,116). The small populations of LIR-1-positive NK cells bind pUL18 as well as different MHC class I alleles, which is different from the pattern of allelic recognition in KIRs (579), and they function by contacting common structural features in pUL18 or MHC immunoglobulin domains (89). A proposed NK inhibitory role for pUL18 (451) has not been sustained in settings where expression of the viral protein can be followed (318); however, a comparison of LIR-1^+ and LIR-1^- NK cells may shed further light on the function of this protein. In mouse CMV, deletion of the MHC class I homolog (m144), which does not interact with LIRs, nonetheless specifically enhances virulence through a decrease in NK-mediated clearance (152). One additional event in human CMV–infected cells may be related to NK killing. Expression of HLA E, which is an inhibitor of NK cells in certain assays, is enhanced during infection (632), apparently because a leader peptide from CMV gpUL40 can enhance expression of this nonclassical MHC protein at the cell surface (561).

Four human CMV genes (UL33, UL78, US27, US28) encoding homologs of 7TM receptors (94) are expressed at early and late times during infection. Some encode virion envelope proteins (347). The best-studied and only known functional member of this family (US28) encodes a C-C chemokine G-protein-coupled receptor (GCR) (400), which binds both CC (163,291) and CX_3C chemokines (272), and signals in response to some. The pUS28 may function as a chemokine sink (49), mediate intracellular signalling (46), and control the level of host cell activation through inducing chemokinesis or chemotaxis (541). The outcome in any of these scenarios would be that the behavior of infected cells is altered. Chemokine receptors normally control migration of cells in chemokine gradients (i.e., chemotaxis); however, evidence for US28-mediated chemotaxis has been difficult to obtain. Recent studies using adenovirus vectors to efficiently direct expression in vascular smooth muscle cells suggest that this receptor might direct cell movement (541). Although any relationship to the pathogenesis of AIDS remains unclear, human CMV pUS28 also provides coreceptor function for HIV entry (428,466). Neither mouse nor rat CMV has a US28 homolog, but deletion of other 7TM homologs, including murine CMV m33 (127) and rat CMV r78 (34), results in altered growth patterns in the natural host. US28, US27, and UL33 are dispensable for replication in cell culture (49,347,576).

Virus-encoded chemokines also influence the behavior of leukocytes during infection. Two CMV genes (UL146, UL147) exhibit cysteine spacing and weak amino acid homology to CXC chemokines, with UL146 encoding a fully functional IL-8-like chemokine denoted vCXC-1 (418). The vCXC-1 is exported and found in a medium of infected cells. vCXC-1 induces chemotaxis of neutrophils but no other PB cell type via the human chemokine receptor CXCR2. Thus, vCXC-1 may play a role in attracting neutrophils or possibly other CXCR2-positive myeloid cells to sites of infection, where they may acquire virus or contribute to dissemination (172). This viral chemokine does not bind to the US28 chemokine receptor. Although neutrophils are attracted to sites of CMV replication, it is unclear whether vCXC-1 is

immunomodulatory or acts in some other way to orchestrate host leukocytes. A role for positively acting chemokines in viral infection can be gleaned from studies on the murine CMV CC chemokine, MCK (335,336), which signals to monocyte-macrophages (468) and influences dissemination in the host (155,468). MCK expression increases the level of monocyte-associated viremia during infection (468) by increasing the size and composition of the inflammatory response at sites of infection, attracting host monocytes that carry virus into the bloodstream.

Several additional viral functions have received less study but may also be immunomodulatory. A tissue necrosis factor (TNF) receptor sequence homolog is carried in the Toledo strain genome (UL144) (82) and recognized through its similarity to one of the cellular proteins that mediate HSV-1 entry, HveA (see Chapter 72). The gpUL144 itself does not bind any known TNF ligands (36) but may interfere with TNFa or Fas ligand–mediated killing of infected cells (Leong, Phillips, and Lanier, personal communication, 1999). The CMV T-cell gamma chain receptor sequence homolog (UL20) exhibits similarity to the variable and constant domains (31), but nothing more is known about its function. An IgG Fc receptor is induced in cells infected with human CMV (334) and is likely to be a virion envelope constituent (621), although a gene assignment has not yet been made. Murine CMV m138 encodes an Fc receptor (556), but this function plays an unexpected and unexplained antibody-response-independent role in virus infection (121).

Latent Infection

Evidence has accumulated that human CMV remains latent in lineage-committed myeloid cells, including progenitors that give rise to granulocytes, macrophages, and dendritic cells (200,489,505), and possibly endothelial cells (492). Viral DNA is present in mononuclear cells derived from PB or bone marrow of healthy seropositive individuals (39,367,494,526,553), but infectious virus is not present. Viral gene expression in latently infected progenitor cells is restricted to latency-associated transcripts that have been observed in both natural and experimental infection in cells with cell surface marker CD33 expressed together with lineage-specific markers (CD14, CD15, CD1a, or CD10) (200,280,281). The pattern of latent gene expression changes as these cells mature into monocytes or dendritic cells (200) and may become productive as a result of differentiation under proinflammatory conditions. Earlier investigations employing a variety of cell lines revealed a now well-accepted role for cell differentiation state in controlling permissiveness (173). Differentiation of primary PB monocytes to monocyte-derived macrophages in the presence of activated T cells promotes cell differentiation and supports reactivation of latent virus (503), although the precise factors that control this process are not yet clear (505).

Latently infected cells are very difficult to detect in PB or bone marrow–derived monocytes from latently infected individuals. Despite ready detection in PB leukocytes of immunocompromised individuals (123,198) and one report suggesting easy detection of viral IE transcripts in lymphocytes of healthy seropositive individuals (482), only a very small percentage (0.01%) of latently infected cells are present in bone marrow–derived mononuclear cell preparations. These are largely CMV IE transcript negative even when very sensitive, PCR-driven *in situ* hybridization is employed (494). Lymphocytes and other PB leukocyte subsets remain uniformly negative during lifelong latency (552,553).

Viral Genome and Gene Expression

The CMV genome is maintained as a covalently closed circle in the nucleus of CD14+ PB mononuclear cells (57). CMV latency-associated transcripts (CLTs) originating from both DNA strands in the *ie*1/*ie*2 region are the only viral gene products that have been associated with latent infection, with sense CLTs transcribed from the same strand as MIE gene products and antisense CLTs transcribed from the opposite DNA strand (281). Sense CLTs are oriented in the same direction as productive MIE transcripts, initiate at two unique transcription start sites that map in the MIE enhancer region, and are present in a subset of naturally or experimentally infected CD33+ myeloid progenitors (200,281,494). Four ORFs (ORF55, ORF42, ORF45, and ORF94) are predicted to be uniquely encoded by sense CLTs, and three ORFs (ORF59, ORF154, and ORF152/UL124) are present on antisense CLTs. Antibodies to these ORFs are recognized by sera from heathy CMV seropositive individuals (281,304), suggesting that they are expressed during natural infection. Only one, a 94-amino-acid basic protein encoded from sense CLTs, exhibits a nuclear localization pattern when expressed in mammalian cells, and only this ORF has been subjected to detailed study. Genetic analysis of a virus carrying an ORF94-specific mutation has not revealed a role for this gene product in either productive or latent infection (598). Mutations in the MIE modulator or enhancer region (363,365) or in other predicted sense or antisense gene products have not yet been evaluated for impact on latency. Thus, it is not yet clear that establishment or maintenance of latency requires specific viral gene expression.

Reactivation

Reactivation is believed to be more important than primary infection as a source of virus-causing disease in the immunocompromised host, and it is thought to occur as a result of at least three collaborating events: (a) cytokine stimulation of latently infected cells causing differentiation into a permissive cell type that supports viral replication (200,275,280,503,505), (b) viral amplification caused by reduced immune surveillance that leads to a widespread systemic infection with a readily detectable viremia (153,469,480) and virus in many tissues (396), and (c) disease states developing as a result of a poorly understood collaboration of immune-, organ-, and virus-specific determinants (see Chapter 72). These three stages of reactivation help illuminate an important interplay of the immune response in reactivation and pathogenesis. Immunocompetent individuals who support persistent infection or who frequently reactivate and shed virus may be predisposed to more rapid amplification and clinical disease because the first event is unnecessary. Most clinical manifestations of reactivation result from the second event, after virus replication reaches readily detectable levels. The consistency with which CMV reaches high systemic levels prior to and in the absence of disease is the basis of preemptive antiviral (630) or adoptive CMV-specific CTL therapies (452), as well as being the basis for the dramatic reduction in incidence of CMV retinitis and gastroenteritis resulting from immune reconstitution that follows highly active retroviral therapy in AIDS patients (136,279).

The earliest event in reactivation (the first event) is a fascinating area of virus biology. Cytokine-mediated differentiation of PB monocytes into macrophages under strongly proinflammatory conditions is undoubtedly key to this process

(364,503,505). Experimentally infected myeloid progenitor cells reactivate latent virus when cultured in the presence of IFNg or TNFa, which are part of a natural inflammatory response (200), but this signal is insufficient to reactivate naturally infected bone marrow–derived cells. Naturally infected PB monocyte-derived macrophages require the presence of allogeneically stimulated T cells to reactivate latent virus, and IFNg contributes important signals to this process (503,504). The accumulated data suggest that latency is preserved as most monocytes differentiate and that specific proinflammatory signals must be delivered to latently infected cells for reactivation to ensue (505). Such proinflammatory signals are likely to be generated during allograft transplant rejection, reinforcing a clear connection between inflammatory signals, allogeneic rejection, and reactivation. A low level of reactivation and replication of CMV itself may have an amplifying effect on inflammation that drives further reactivation. Investigation of murine CMV latency and reactivation suggests that latent virus is distributed widely in both endothelial and myeloid cells (276,292,376,434,435). Reactivation with immunosuppression alone is very difficult to achieve (79,246,447). Proinflammatory cytokines play a critical role in reactivation aside from the viral amplification that results in an immunocompromised host (275).

Given the prolonged, persistent nature of primary infection (450,631) and the sporadic shedding that occurs throughout life (509), persistent infection very likely contributes to transmission as well as pathogenesis. Low-level persistent infection, kept in check by effective immune surveillance, would amplify rapidly in the immunocompromised host and facilitate susceptibility to clinical disease. The relative importance of such amplification still needs to be addressed. In terms of the molecular basis of CMV disease, persistence and latency may exist side-by-side in different cell types or tissue, or as parts of a continuum in a particular lineage. The widely held view that latency and reactivation depend on the level of permissiveness of myeloid lineage cells (200,280,503,505) illustrates the difficulty in separating these phenomena in natural infection.

Latency is operationally defined as the ability to reactivate virus from cells of infected tissues following co-cultivation with permissive cells in culture without being able to detect virus directly in disrupted tissues (247). Using these criteria, murine CMV remains latent in epithelial, endothelial, and hematopoietic cells in a wide variety of tissues, including spleen, salivary gland, blood, skin, prostate, and testes (216,247,275,276,292,293,376,434,435). The tendency for persistent infection to depend on the age, immune status, and strain of mouse, and the evidence that latency extends to a number of cell types in different tissues rather than a single target, continue to complicate interpretation of studies on murine CMV latency. Because of ready access and reproducible reactivation, the most commonly studied sites of latency have been the spleen and lungs; however, the importance of bone marrow and salivary glands as sites of persistence and latency remains poorly characterized (380). Indeed, studies in this model have been complicated by a failure to recognize that latency cannot be studied as a cellular or organ-specific phenomenon while ignoring persistent infection at other sites within the same host. *In situ* methods of detecting latent viral genomes and distinguishing latent and persistent infection help (275,276,293), but the range of results that have been reported cannot yet be reconciled.

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CHAPTER 77 Cytomegalovirus

Robert F. Pass

HISTORY

The medical and public health impact of cytomegalovirus (CMV) results largely from its role as the leading cause of congenital infection. The history of the discovery of CMV is closely linked to early 20th century studies of the pathology of fetuses and newborns who died from a multisystem disease characterized by prominent central nervous system, hepatic, and hematologic abnormalities. In 1904, Jesionek reported on the appearance of abnormal cells in the lungs, kidney, and liver of a premature fetus thought to have died from syphilis (147). Features consistent with CMV cytopathology were described, including an intranuclear inclusion surrounded by a clear zone, although the abnormal appearance of cells was considered suggestive of protozoal infection. In the same year, Ribbert reported having seen similar cells in 1881 in the kidneys of a stillborn infant (241). Within 3 years of these initial descriptions of distinctive pathology, similar cellular changes were reported in salivary glands of older infants and children (179). Other pathologists also reported finding intranuclear inclusions in salivary glands, liver, lungs, or kidneys from young infants (77,115). The similarity between this cytopathology and the inclusions seen in lesions produced by other herpesvirus infections such as varicella zoster and herpes genitalis was noted (115,174,297). Goodpasture and Talbot used the term *cytomegalia* in 1921 (115). Mueller suggested that the presence of this cytopathology in stillbirths implied a prenatal insult (192). Thus in addition to their descriptive contributions, early investigators provided evidence that the agent responsible for these cytomegalic cells could be acquired prenatally, affected multiple organs, was present in the salivary glands of children without systemic disease, and produced cytopathology similar to that produced by infectious diseases due to herpesviruses.

Continued study of infants with cytomegalic disease and of salivary gland pathology provided evidence that the fetal and infant disease was due to a common viral agent. Farber and Wolbach reported on cells with intranuclear and intracytoplasmic inclusions in 14% of salivary glands from 183 infant postmortem examinations (92), which indicated that the agent responsible for the cytopathology and organ dysfunction noted in stillbirths and infant deaths was more common than might be inferred from the occurrence of the multiorgan disease. In 1926, experiments with guinea pigs had shown that a filterable agent that was inactivated by heating at 54°C for 1 hour was transmissible and produced cytomegalic cells in salivary glands (71). In the early 1950s, the term *generalized cytomegalic inclusion disease* was used to describe the infants with multiorgan involvement (313). Fetterman described premortem detection of cytomegalic cells in the urine of a newborn who died shortly thereafter and was found on autopsy to have cytomegalic cells with inclusions in multiple organs (94). Not long after, the detection of inclusion-containing cells in urine was described as a diagnostic technique, and developmental delay and cerebral palsy were reported in a surviving infant (181,186). Using electron microscopy, Minder described apparent viral particles in the cytoplasm and in the clear zone around the intranuclear

inclusion in cytomegalic cells in the pancreas in 1953 (190).

However, tissue culture isolation of human CMV was not achieved until the mid 1950s, when three laboratories were successful at roughly the same time (244,260,302). Weller has reviewed the circumstances and chronology of the initial culture isolation of CMV (303). Margaret Smith isolated a mouse salivary gland virus in tissue culture in 1954 and at about the same time isolated and serially propagated human salivary gland virus and an isolate from the kidney of an infant who died from cytomegalic inclusion disease (259,260,303). The first isolation of CMV from tissues of a living human was made serendipitously in 1955 when Weller's laboratory was attempting to isolate *Toxoplasma* from a liver biopsy taken from an infant with hepatosplenomegaly, cerebral calcification, and chorioretinitis. The tissue culture (embryonic skin-muscle) developed foci of swollen cells with the cytopathology now associated with CMV. Continued study of this isolate, the Davis strain, led Weller and colleagues to conclude that they had recovered the agent of cytomegalic inclusion disease (302,303). Rowe and colleagues, studying adenoviruses from adenoids of children, came across cytopathology that differed from that expected from adenoviruses (303). Collaborating with Weller's laboratory, they were able to determine that their agent was similar to that isolated from cases of cytomegalic inclusion disease by Weller's group and by Margaret Smith (303). Rowe's group was able to prepare a complement fixation antigen, and their initial paper reporting isolation of a salivary gland virus-like cytopathogenic agent from human adenoids included data indicating that the majority of adults were infected by this agent (244). The ability to propagate CMV in tissue culture provided investigators with the means to use virus isolation diagnostically, to prepare antigens for serology, and to begin to study the virus *in vitro*.

INFECTIOUS AGENT

Propagation and Cytopathic Effect in Cell Culture

Although a variety of cell culture systems are permissive, human CMV is propagated best in fibroblasts of human origin. The growth of human CMV is characterized by a relatively prolonged replication cycle and distinctive focal cytopathic effect (CPE) with cell enlargement and rounding. Human foreskin fibroblasts and the human embryonic fibroblasts, WI-38 and MRC-5, are commonly used for propagation and isolation of CMV. Cell rounding and early cytoplasmic inclusions are visible by 5 hours postinfection (11). Nucleoli become distinctly defined in the centrally placed nucleus by 24 hours. The characteristic eosinophilic perinuclear inclusion appears and, with progression of the replication cycle, becomes more distinctly defined. By 48 to 72 hours postinfection, CPE is well developed with prominent cell enlargement, a basophilic nuclear inclusion, and a large, well-defined, eosinophilic perinuclear inclusion. The nuclear inclusion is an accumulation of nucleocapsids and the perinuclear inclusion is a complex of nucleocapsids, dense bodies, and Golgi apparatus. Progeny virus accumulates in the cytoplasm of infected cells, and about 96 to 120 hours postinfection, infectious virus appears in tissue culture fluids. Inoculum size, cell culture lineage, and CMV strain influence the appearance of CPE in a tissue culture monolayer. Spread of wild isolates of human CMV in the fibroblast monolayer appears to be principally by cell-to-cell contact, as they usually (unless the inoculum is very large) produce only scattered foci of CPE that often take 10 to 14 days to become easily seen (Fig. 1). In addition, wild isolates usually yield very little extracellular virus unless they have been adapted to cell culture growth by serial passage. In contrast, laboratory-adapted strains, such as AD169 or Towne, yield extracellular virus readily and can produce generalized CPE involving the entire cell monolayer in around 7 to 10 days postinoculation. Distinct patterns of CPE are expressed respectively by the two prototypic laboratory-adapted strains of human CMV, AD169 and Davis (12,240). Davis strain CPE is characterized by rounded cells with an eccentric, kidney-shaped nucleus. Fibroblasts infected by the AD169 strain recover from early cell rounding and retain their elongated shape. In addition, nuclear morphology is less distorted and perinuclear inclusions are less distinct (11). Clinical isolates usually exhibit the Davis-type CPE.

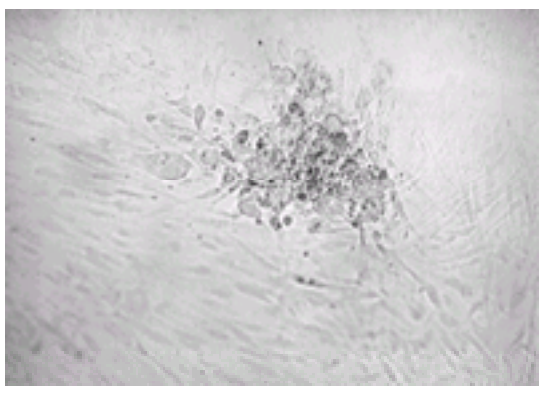

FIG. 1. Focal cytopathic effect of a clinical isolate of human cytomegalovirus in unstained fibroblast monolayer 14 days after inoculation.

In addition to fibroblasts, a number of other cell types of human origin are permissive, although CMV does not replicate as well in them; these include macrophages, smooth muscle cells, vascular endothelial cells, astrocytes, epithelial cells, and trophoblasts (131,132,141,219,226,257,299). Permissiveness of vascular endothelial cells for laboratory strains of CMV varies according to the origin of the endothelial cells and the virus. Infection of human umbilical artery endothelial cell cultures by laboratory strains of CMV propagated in fibroblasts (AD169 or Towne) is nonproductive, whereas low-passage, recent clinical isolates replicate in cultured endothelial cells (136,299). Transformed cell lines derived from fibroblasts, epithelial cells, glioblastomas, and malignancies of myelomonocytic origin can also be productively infected *in vitro*, although virus yields are generally much reduced compared with infection of fibroblasts (219).

Assay in Cell Culture

Quantitation of human CMV in the laboratory is best accomplished in monolayers of human fibroblasts. Assays typically use log_{10} dilutions of virus stock to estimate quantity of infectious virus in terms of tissue culture infective dose ($TCID_{50}$) or by enumeration of plaques. CMV is particularly well suited to the latter because CPE typically develops slowly and focally. Rapid plaque assays have been developed using centrifugation enhancement of infectivity and monoclonal antibodies to immediate-early proteins to identify infected cells (214).

PATHOGENESIS AND PATHOLOGY

Host Entry and Initial Replication

The cellular pathways that are used by CMV to gain entry into the host have not been proven experimentally, nor has it been possible to perform the type of observational or serial histopathologic studies that might delineate entry pathways during naturally acquired infection in humans. Epidemiologic evidence suggests that the entry of CMV follows contact between infectious virus and a mucosal surface. The sites of entry for CMV appear to be epithelium of the genitourinary tract, upper alimentary tract, or respiratory tract. Virus is often isolated from these sites and histopathologic and immunochemical studies on tissues obtained at autopsy have shown evidence of CMV in epithelial cells in each of these locations (257). However, initial infection of mucosal epithelial cells is not required, as CMV infection is readily established by blood transfusion and transplanted organs. For fetal CMV infection, virus most likely enters via the hematogenous route. Studies of placental cells have shown that the cytotrophoblasts, cells that form the barrier between maternal and fetal circulation, can be readily infected *in vitro* (122,132).

Spread Within the Host

Leukocytes and vascular endothelial cells appear to be important in spread of CMV within the host. The importance of leukocytes in dissemination of virus is suggested by early studies of viremia and of transfusion-acquired CMV infection. Among patients with CMV mononucleosis and in symptomatic immunocompromised patients, virus is frequently isolated from buffy coat but not from plasma (172,312). Transmission of CMV through blood products from seropositive donors can be prevented by removal of leukocytes (110). Virus-encoded chemokines could facilitate dissemination of CMV from the site of initial replication by attracting neutrophils and monocytes. Recently, CMV UL146 was shown to encode a late gene product with a chemokine activity; this viral CXC-1 was shown to be fully functional in neutrophil degranulation and chemotaxis (217). A murine CMV encoded b-chemokine was found with ability to promote trafficking of leukocytes with appropriate surface receptors to virus-infected cells, and viruses with deletions in the gene for this b-chemokine had significantly reduced levels of viremia (245). Although the key pathways for spread of human CMV from the initial site of replication are not clearly proven, circulating virus is usually found in leukocytes, and CMV appears to have evolved mechanisms for promoting leukocytic dissemination of virus.

When viewed in terms of the whole organism, human CMV infection in immunocompromised patients is a dynamic process, characterized by rapid turnover of virus in blood. Emery and colleagues assessed the dynamics of human CMV replication by serial quantitative polymerase chain reaction (PCR) for DNA in whole blood (90). They used the slope of the decline in viral DNA levels in ganciclovir-treated AIDS and marrow transplant patients to calculate the rate of CMV clearance, as others had done previously for human immunodeficiency virus (HIV). They found that the doubling time or half-life of CMV in blood was around 1 day, and that the rate of increase in viral load is an independent predictor of disease in immunocompromised patients (89).

Tissue Tropism

Studies of tissue obtained at autopsy show that CMV infects a wide range of tissues and often multiple cell types within an involved organ (reviewed in refs. 137,219, and 257). Cells exhibiting typical CMV cytopathology have been identified in salivary glands, lung, liver, pancreas, kidney, eye, ear, placenta, alimentary tract, heart, ovaries, pituitary, adrenals, thyroid, brain, and skin. The most common cell type exhibiting CMV cytopathology is the ductal epithelial cell. Cells with cytopathology typical of CMV have been described in the epithelium of salivary glands, bile duct epithelium, bronchial epithelium, islet cells, renal tubular epithelium, capillary endothelium, astrocytes, neurons, and epithelial cells of the inner ear (cochlea, stria vascularis, Reissner's membrane, limbus spiralis, saccule, utricle, and semicircular canals) (137). Studies using monoclonal antibody to immediate-early antigen and DNA hybridization techniques have expanded the number of cell types that are known to harbor CMV and reveal that normal-appearing cells are often positive for immediate-early antigen or viral DNA (137,196,257,292). Not surprisingly, most histopathologic studies have used tissue obtained from patients, usually infants with fatal cytomegalic inclusion disease or immunocompromised patients. However, tissues from subjects who died from trauma or other causes not linked to CMV infection have also been studied. Ho summarized the results of nine studies of salivary glands from children from 1907 to 1957 and noted that cytomegalic cells were reported in from 8% to 32% of salivary glands (137). Toorkey and Carrigan found CMV immediate-early antigens in apparently normal cells of kidney, lung, liver, spleen, and brain from seropositive patients who died from trauma (292).

The medical significance of congenital CMV infection is the ability of the virus to infect and damage the central nervous system (CNS). Animal experiments as well as classical pathologic study of brains indicate that CMV can infect a wide variety of cells within the CNS. In cases of fatal human congenital CMV infection, inclusion-bearing cells have been found in neurons, glia, ependyma, choroid plexus, meninges, and vascular endothelium (31). Lesions in the CNS are usually multiple, small, necrotic foci surrounded by an inflammatory infiltrate. Inclusion-bearing cells have also been detected in multiple locations within the ear, including epithelial cells in the semicircular canals, vestibular membrane, and cochleae (Fig. 2). Pathologic changes in the inner ear include inflammatory cell reaction and edema. In the eye, areas of focal retinal necrosis and inclusion-bearing cells have been described. Using immunofluorescence, CMV antigens have been found in the organ of Corti and in neurons of the 8th cranial nerve (278). Using a murine model, van den Pol et al. used recombinant murine CMV to introduce reporter genes into a variety of CNS cell types, including glia, neurons, endothelial cells, radial glia, ependymal cells, microglia, meningeal, and choroid cells (294). Their results

suggested that CMV infection could impair the developing brain by a number of mechanisms, including lytic infection of cells, interference with neuronal migration, loss of neuronal stem cells, and vascular compromise.

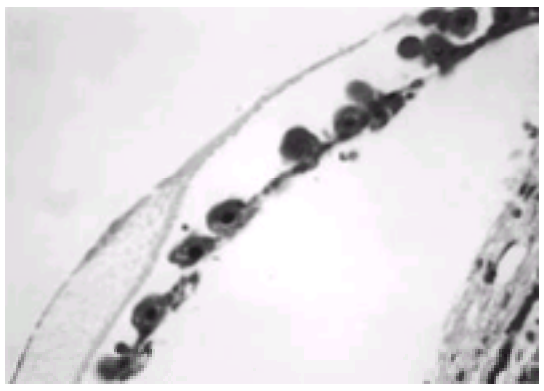

FIG. 2. Cytomegalovirus infection of the inner ear. Epithelial cells of Reissner's membrane show typical cytomegaly and inclusions.

Fatal cases of congenital CMV infection usually have evidence of damage to multiple organs and tissues outside the CNS as well (31). Inclusion-bearing cells in lungs of fatal cases of congenital CMV infection have been described, although inflammatory reaction is usually absent. The liver may exhibit inclusion-bearing cells in bile ducts, necrosis of parenchymal cells with giant cell transformation, evidence of bile stasis, and periportal lymphoid infiltration. Inclusion-bearing cells are also found in the kidney, especially in distal tubules; glomerular involvement is uncommon, as is evidence of renal damage. Cytomegalic cells are usually not seen at autopsy in the bone marrow or spleen, although congenital CMV infection clearly affects marrow function. Anemia and thrombocytopenia are common in symptomatic infections and leukopenia is occasionally seen. Extra-medullary hematopoiesis is sometimes seen in the skin, liver, spleen, and kidneys. Glandular epithelial cells of salivary glands, pancreas, and endocrine glands often have typical inclusions.

Benirschke et al. reported a wide spectrum of placental pathology in congenital CMV infection, ranging from no abnormalities to massive villous necrosis (34). Placental villitis was the most typical lesion, described as focal necrosis of villi with surrounding lymphocytic and plasma cellular infiltrate accompanied by capillary and venous thrombosis, with or without inclusion-bearing cells.

Immune Response

Natural Killer Cells

Evidence from animal experiments and from investigation of human infections shows that natural killer (NK) cells and specific cellular and humoral immunity all play important roles in control of CMV disease. A link between resistance to murine CMV infection and NK cell function was established by studies that showed that beige mice (genetically deficient in functional NK cells) have increased susceptibility to murine CMV (252), and that depletion of NK cells from mice led to more serious infections after challenge (56). It was also shown that spleen cells with NK cell phenotype conferred protection from a lethal murine CMV challenge (56,57). A case report of a patient with deficient NK cell responses and severe infection by CMV and other herpesviruses provides clinical evidence for a role for NK cells in initial control of CMV infection in humans (36). Borysiewicz et al. studied human NK responses to CMV *in vitro* and found that they were mediated by large granular lymphocytes, were independent of interferon, and were manifest at the time early viral proteins were expressed (50). Indirect evidence for the importance of NK cells in defense against CMV infection comes from the observation that the viral gene products that appear to be conserved can protect against NK cell–mediated lysis (290).

Antibody

Experimental and clinical evidence indicate that humoral immunity to CMV plays a key role in protecting the host from disease. In a murine CMV model, passive immunization and active immunization against the envelope glycoprotein B protected mice from lethal challenge (233). In the guinea pig model, immunization of pregnant dams with affinity purified guinea pig CMV envelope glycoprotein preparation decreased the rate of fetal loss and fetal CMV infection after challenge (125). Among solid organ transplant patients, CMV disease is more frequent and more severe when the transplant recipient is seronegative and the donor is seropositive. The severity of disease due to primary CMV infection in renal transplant patients was significantly reduced by pretransplant immunization with Towne strain live CMV vaccine (222) and by administration of a high-titer CMV immune globulin (261). Among very low-birth-weight newborns who acquire CMV from blood products, the principal determinant of protection from disease is the presence of maternal antibody to CMV prior to transfusion (315).

Antibodies to approximately two dozen immunogenic human CMV proteins can be detected in sera from seropositive humans (reviewed in refs. 53 and 163). Studies of infected cell lysates, purified virions, and mixtures of recombinant proteins have shown that immune human sera vary in their reactivity with these immunogenic proteins. The proteins to which immune human sera most consistently react are listed in Table 1. Nearly all immune human sera have antibody to envelope glycoproteins gB (UL55) and gH (UL75), to the tegument phosphoprotein pp150 (UL32), and to a nonstructural DNA binding phosphoprotein, pp52 (UL44) (119,250). Neutralizing antibody is directed principally against envelope glycoproteins gB and gH but also against other envelope glycoproteins (53). Experimental and clinical evidence indicates neutralizing antibody is associated with protection from infection and disease, but protective levels of neutralizing

antibody have not been defined (8,233,251). Although humoral immunity alone as provided by passive immunization cannot prevent CMV infection (in contrast with disease), the extent to which humoral immunity from naturally acquired infection or from active immunization contributes to the host's ability to prevent or limit reinfection is unknown.

Name (ORF)	Location	Immune response	Application
gB (UL55)	Envelope	Major target of neutralizing antibody	Subunit vaccines and serologic assays
gH (UL75)	Envelope	Major target of neutralizing antibody	Therapeutic hu-Mabs and serologic assays
pp150 (UL32)	Tegument	Nonneutralizing antibody and CD8+ CTL	Serologic assays
pp28 (UL99)	Tegument	Nonneutralizing antibody	Serologic assays
pp71 (UL82)	Matrix	Nonneutralizing antibody	Serologic assays
pp65 (UL83)	Matrix	Main target of CD8+ CTL and target of antibody	Component of recombinant vaccines and of serologic assays; the antigen of "antigenemia" assays for viremia
pp52 (UL44)	NS, DNA binding	Nonneutralizing antibody	Serologic assays
pp72 (UL123)	IE, NS, regulatory	Target of nonneutralizing antibody and CTL	IE antigen detected in rapid culture assays ("shell vial")

Note: These proteins were selected because of their current or investigational clinical applications. They are important targets of the immune response to CMV infection, and their immunogenicity is the basis for investigational immunotherapy and novel serologic assays. ORF, open reading frame; NS, nonstructural; IE, immediate-early; CTL, cytotoxic T lymphocyte; hu-Mabs, humanized monoclonal antibody.

TABLE 1. *Immunogenic proteins of human cytomegalovirus (CMV)*

Cell-Mediated Immunity

Although there is ample evidence of the importance of cellular immunity in control of CMV infection, perhaps none is more persuasive than the observation that severe CMV disease (excepting congenital infection) is seen almost exclusively in patients with profoundly impaired cell-mediated immunity, such as patients who are undergoing bone marrow transplantation or have AIDS. In fact, the severity of disease due to CMV infection parallels the degree of impairment of T-lymphocyte responses. Understanding the immune response to CMV infection in terms of the role of specific components of the immune response as well as specific viral proteins has been improved by the study of animal models and by the ability to assess human responses to recombinant CMV proteins. Early investigations using murine models showed that suppression of T-lymphocyte function led to reactivation and dissemination of natural infection (107), and the importance of murine CMV specific cytotoxic T-lymphocyte (CTL) responses in recovery from infection was demonstrated (229). Further studies with the murine model showed that adoptive transfer of CD8+ cytotoxic T cells protected mice from lethal challenge, independent of CD4+ helper T cells; murine CMV specific CD8+ T-cell clones specific for both structural and nonstructural proteins were identified (235,236). An important role for CD4+ T lymphocytes in g-interferon production was suggested (134).

In humans, lymphocyte proliferative responses to CMV preparations are consistently present in seropositive adults, but they appear to be markedly suppressed after CMV mononucleosis, in young children with congenital infection, in immunocompromised transplant patients with CMV disease, and even in normal adolescents after asymptomatic primary infection (172,207,227,319). In humans with naturally acquired CMV infection, CD4+ helper T-lymphocyte responses to multiple structural and nonstructural CMV proteins have been demonstrated. Although CD4+ T-lymphocyte responses to envelope glycoproteins gB and gH, to the lower matrix protein pp65, and to immediate-early proteins are most commonly detected, responses to other proteins have also been reported (14,33,76,98,126,175).

Quinnan et al. studied both lymphoproliferative responses and human leukocytic antigen (HLA) class I restricted CTL responses of peripheral blood lymphocytes from bone marrow transplant patients. CTL response to CMV was associated with clinical recovery from CMV disease (230). In humans, CD8+ CTL responses to a number of CMV proteins have been described also, including pp65 (UL83), pp150 (UL32), gB (UL55), and IE1 (UL123) (35,47,51,113,242). Comparison of precursor frequencies of CTL specific for gB (UL55) and the 72-kd IE protein (UL123) using vaccinia recombinants to infect target cells revealed a low frequency of gB-specific CTLs (£6% of CTL), whereas 18% to 58% of CTLs recognized vaccinia IE–infected targets (51). An assessment of CTL precursor frequencies to five CMV proteins, pp65 (UL83), gB (UL55), gH (UL75), pp50 (UL44), and pp28 (UL99), found that pp65 was immunodominant (47). Kern et al. used multiple, overlapping pentadecamer peptides spanning the entire 491 amino acid sequence of the 72-kd IE1 protein to identify CD8+ T-cell epitopes and to measure CTL frequencies (153). They found that the frequencies of IE1-specific and pp65-specific CTLs were similar in most donors; however, some donors had responses to IE1 or to pp65, but not to both. Although it is likely that more targets for CTL will be identified among CMV proteins, at the present time the pp65 (UL83) and IE1 (UL123) appear to be the major targets.

Based on the observed association between recovery of CTL responses to CMV and recovery from CMV pneumonia in bone marrow transplant patients (237), adoptive transfer of CMV-specific clones of CTL was evaluated as a therapeutic intervention (300). Marrow donor lymphocytes were stimulated *in vitro* by autologous, CMV-infected fibroblasts and clones of CD3+CD8+CD4– CTL were harvested and infused into the bone marrow recipients at intervals after transplant. The recipients had increasingly vigorous CMV-specific CTL responses with serial infusions of CTL. In addition, none of the 14 treated transplant patients had viremia or CMV disease, although nine were seronegative recipients of marrow from seropositive donors and the other five were seropositive before the transplant. Although the clinical role of this approach to adoptive immunotherapy in control of CMV disease is still being determined, this study provided *in vivo* evidence for the importance of CTLs in controlling human CMV infection, and it also showed that persistence of the CD8+ CTLs required CMV-specific CD4+ T-helper-cell function.

Immune Evasion

A characteristic feature of CMV infection in the normal host is the persistence of productive infection, viremia, and virus excretion for months or even years in the face of the host immune response. The ability of the virus to avoid elimination or termination of active infection by the host's immune system may be due at least in part to CMV gene products that

have the potential to interfere with the immune response (reviewed in refs. 135 and 305). CMV has evolved strategies for immune evasion that involve multiple gene products that can interfere with the host immune response via multiple mechanisms, and at different times in the viral replication cycle (Table 2). For example, escape from CTL responses could be mediated by retention of major histocompatibility complex (MHC) class I heavy chain/b-2 microglobulin complexes in the endoplasmic reticulum (US3) (10), through destruction of MHC class I heavy chain molecules (US2 and US11) (148,306), by interference with antigen presentation (US6) (169), and by phosphorylation of a potentially important CTL target, the 72-kd immediate-early protein, preventing antigen processing (111). In addition to these mechanisms for interfering with MHC class I presentation, US2 can cause degradation of MHC class II proteins, preventing their presentation of antigen and the host CD4+ T-lymphocyte response to CMV (291). The extent to which some putative mechanisms of immune evasion are responsible for the persistence or virulence of human CMV infection is not yet clear. In some cases, the apparent mechanism of action of an immune evasion molecule is controversial. For example, the CMV-encoded MHC class I homolog (UL18) was nitially thought to block NK cell activity through binding with the killer inhibitory receptor as endogenous MHC class I would (150,238). However, Leong et al. found that UL18 was associated with enhanced NK cell killing of CMV-infected fibroblasts and did not appear to bind to the killer inhibitory receptor (171). A more convincing mechanism for escape from NK cell attack was offered by Tomasec and colleagues (290). They found that human CMV glycoprotein UL40 (gpUL40) contains a nine-amino-acid sequence homologous to the class I MHC sequence that up-regulates surface expression of HLA-E. HLA-E protects potential target cells from NK cell-mediated lysis by binding to inhibitory receptors on the surface of NK cells. The fact that the gpUL40 sequence was conserved in multiple isolates is consistent with a key role for NK cells in control of CMV, and with a significant role of gpUL40 in evading this control mechanism. In addition to the mechanisms listed in Table 2, CMV exhibits a number of other activities that could impede viral host defense. These include binding and sequestration of b-chemokines (US27, UL28, UL33) (39), competition with the TNF receptor superfamily through a homolog (UL144) (32), interference with apoptosis (322), down-regulation of MHC class II expression on the surface of macrophages (95), and protection of infected cells from complement-mediated lysis (267,276).

Host response	CMV gene	Evasion mechanism
NK cell killing	UL40	↑ Cell surface expression of HLA-E, which binds inhibitory receptors on NK cells
MHC I–antigen presentation/processing	US3	Retention of MHC I complexes in endoplasmic reticulum
	US11	Dislocation of MHC I heavy chains into cytosol
	US2	Export of MHC I heavy chains from endoplasmic reticulum via Sec61
	US6	Inhibition of TAP-mediated peptide translocation into the endoplasmic reticulum
	UL83	Inhibition of presentation of 72-kd IE antigen to CD8+ T cells
MHC II–presentation of antigen	US2	Degradation of MHC class II proteins, preventing presentation of antigens to CD4+ T cells

NK, natural killer; MHC, major histocompatibility complex; HLA, human leukocyte antigen; TAP, transporter associated with antigen processing; IE, immediate-early.
Source: Adapted from ref. 135, with permission.

TABLE 2. *Human cytomegalovirus (CMV) genes associated with evasion of NK cell, CD8 CTL, and CD4 immune responses*

Release from the Host

After initial infection, CMV is shed in multiple body fluids, including saliva, urine, tears, semen, cervicovaginal fluid, and breast milk. In each of these sites, viral excretion is probably the result of infection of glandular epithelial cells, with release of progeny virus into excretions. Shedding of virus from multiple sites after primary infection implies dissemination of virus to multiple organs, although it must be noted that shedding from sites in the genitourinary tract and upper alimentary/respiratory tract could represent continued replication of virus at the original site of inoculation. During the first year after asymptomatic primary infection, CMV shedding is more common and more persistent in urine than in saliva or cervicovaginal secretions (319). However, in seropositive subjects with past infections, CMV excretion is most commonly found in the female and male genital tracts (84,157,239). One could hypothesize (based on the model of latency described later) that reactivation of CMV in these sites is the result of stimulation of latently infected monocytes to differentiate to permissive tissue macrophages by inflammation from intercurrent infections due to other pathogens.

Virulence

Although a number of host factors or clinical variables (see later section, Clinical Features) related to virulence of CMV infection have been identified, the contributions of specific viral genes and gene products in determining virulence of CMV infection are poorly understood. Evidence that CMV strains differ in ability to cause disease was suggested by challenge experiments with Towne vaccine strain and a low-passage isolate, Toledo. Healthy adults injected with 10 or 100 plaque-forming units (pfu) of Toledo developed a systemic, mononucleosis-like illness and shed virus. In contrast, vaccine recipients had only local reactions, with neither systemic illness nor virus shedding, and they were protected from disease when challenged with Toledo (221,223). To gain insight into the genetic basis for the virulence of Toledo (or attenuation of Towne), Cha et al. compared nucleotide sequences from a heterogeneous region near the U_L and *b*-repeat boundaries of Towne, Toledo, AD169, and five clinical isolates (58). Toledo and the clinical isolates had 13 to 15 kbp of DNA, approximately 22 open reading frames, not present in the highly laboratory-adapted Towne and AD169 viruses. Some of these open reading frames appear to encode glycoproteins. The function and biologic role of these genes remains to be defined. Experiments with severe combined immunodeficiency (SCID)-hu mice confirmed the ability of the Toledo strain to grow better in human tissue than Towne or AD169 CMV and demonstrated significant variability among wild human isolates in growth in this model (55).

A role for the upper tegument protein of CMV, pp71 (UL82), as a determinant of infectivity was suggested by experiments in which pp71 expression was shown to enhance infectivity of viral DNA, increase late gene expression, and increase

cell-to-cell spread of virus (25). Differences in ability to replicate could be an important marker of CMV virulence, as lytic infection of host cells is a likely pathogenic mechanism. Furthermore, observations in immunocompromised patients have linked viral load in various body fluids to severity of CMV disease.

The envelope glycoprotein gB has been proposed as a determinant of virulence based on reported associations between gB genotype and disease in immunocompromised patients. Human CMV was subdivided into four genotypes based on sequence analysis of a variable region within the gB gene by Chou and Dennison (68); a fifth genotype was later identified by others (254). Some studies of gB genotype distribution among immunocompromised patients have linked specific genotypes to clinical outcome, such as protection from severe or fatal CMV disease among bone marrow transplant patients (gB1), development of fatal bone marrow suppression (gB3 and gB4), acute rejection episodes in liver transplant patients (gB1), and retinitis in AIDS patients (gB2) (105,243,293,295). However, the biologic mechanism through which gB genotypes affect virulence is a matter of conjecture at this time. In addition, variations in genotype have been related to geographic origin and route of infection; different genotypes have been recovered simultaneously from the same immunocompromised patient and the reproducibility of the clinical associations has not been consistent between studies (44,234,324).

The different mechanisms through which CMV evades host defenses offer a number of possible virulence determinants. For example, in the mouse model, deletion of the murine CMV gene for the MHC class I homolog results in virus with diminished ability to infect *in vivo*, and this attenuation is linked to NK cell activity (93). However, the interplay between the evasion strategies and the host immune response is complex, and virulence of human CMV infection has not been linked to alterations in these strategies to date.

Persistence

Human CMV persists within its host indefinitely. There is growing evidence that cells in bone marrow and peripheral blood are a key reservoir for CMV through latent infection. A small percentage of peripheral blood monocytes from subjects with past (presumably latent) infection harbor CMV DNA, but viral gene expression is limited to early genes (75,141,289). However, tissue macrophages (a differentiated form of circulating monocytes) express early and late CMV antigens (256). These observations led investigators to propose a model for human CMV persistence and reactivation in which bone marrow precursors of monocytic peripheral blood cells are a key site of latency; peripheral blood monocytes provide a means to disseminate virus, and differentiation of latently infected monocytes into macrophages leads to reactivation and productive infection. Kondo et al. showed that granulocyte–macrophage progenitors from human fetal liver and bone marrow could be latently infected *in vitro*; these cells had a CD14+, CD15+, CD33+ phenotype and expressed only IE1 transcripts (158). Söderberg-Nauclér et al. reported reactivation of CMV *in vitro* by allogeneic stimulation of peripheral blood mononuclear cells from seven out of seven healthy, seropositive donors (263). They demonstrated recovery of CMV from macrophages expressing dendritic cell markers CD1a and CD83, as well as monocyte/macrophage markers CD14 and CD64. They estimated the frequency of CMV latently infected peripheral blood mononuclear cells at 0.01% to 0.12%. Hahn et al. reported that between 0.001% and 0.01% of CD33+/CD14+ or CD33+/CD15+ bone marrow mononuclear cells from seropositive human donors express transcripts associated with latency, whereas these latency-associated transcripts could not be found in T cells, B cells, or CD33– mature granulocytes and macrophages (121). The experimental evidence supports a model of CMV persistence in which myelomonocytic stem cells resident in bone marrow maintain latent infection, and latently infected CD14+ monocytes circulate in peripheral blood. Differentiation of latently infected monocytes into tissue macrophages as a result of allogeneic stimulation (transplantation or transfusion), or triggering of proinflammatory cytokines as occurs with intercurrent infections, leads to productive CMV infection.

Using traditional virus isolation techniques, infectious virus can be recovered from various body sites for many months after primary infection. It is likely that application of more sensitive techniques for detection of CMV in body fluids will reveal that the duration of viral shedding with primary infection is longer and the frequency of episodes of shedding (reactivation of latent virus) years after primary infection is greater than current knowledge suggests. The possibility remains that some tissues maintain productive CMV infection over many years.

EPIDEMIOLOGY OF HUMAN CMV INFECTION

Distribution, Age, and Demographics

Seroepidemiologic studies show that CMV is universally distributed among human populations from developed, industrial societies to isolated aboriginal groups (13,209). Although prevalence of CMV infection increases with age in every group that has been studied, the overall prevalence of infection and the age at initial acquisition of virus vary greatly according to living circumstances. In general, prevalence is greater and CMV is acquired earlier in life in developing countries and in the lower socioeconomic strata of developed countries. Although in the United States and Europe, CMV infection rates are higher in nonwhites than in whites, racial differences appear to reflect differences associated with socioeconomic status and living circumstances. Seasonal variation in incidence of CMV infection has not been recognized, and epidemics have not been described, aside from reports of very high infection rates in institutional populations such as day care centers.

Transmission

Although CMV is predictably transmitted in settings where susceptibles have frequent contact with body fluids from persons excreting virus, CMV is not highly contagious and transmission appears to require direct contact with infectious material. Following initial acquisition of CMV, infectious virus is present in urine, saliva, tears, semen, and cervical secretions for months to years. Not surprisingly, high rates of CMV infection occur in settings where close contact with body fluids is expected, such as between sex partners, among children in day care centers, and between preschool-aged children and their care givers.

Transmission of CMV through sexual contact is an important means of spread. The prevalence of CMV infection is higher among populations with other markers of sexual activity, such as greater number of partners and sexually transmitted diseases (59,218,264). High rates of incident CMV infection have been reported in clinics for sexually transmitted diseases and among sexually active adolescents (59,197). Restriction enzyme analysis of DNA from CMV strains from sex partners has demonstrated identical restriction fragment patterns, confirming transmission of virus (123). Spread of CMV also occurs frequently in child care centers. Cohort studies and restriction enzyme analysis of CMV strains indicate that children in day care centers frequently transmit virus to each other (3,208). Those who are infected, either from a maternal source or in the day care center, shed virus in saliva and urine for years, providing the opportunity for continued spread to classmates or day care workers. Transmission in this setting is undoubtedly enhanced by frequent mouthing of hands and toys by preschoolers and by the almost unavoidable contact with body fluids that occurs in the care of children this age. Surveillance cultures in day care centers detected CMV on toys, surfaces, and the hands of workers (140). Day care workers have markedly increased rates of CMV infection, ranging from 7% to 20% per year, compared with an expected rate in U.S. adults of around 2% per year (4,193,213). Parents of young children who attend day care centers are also at increased risk for CMV infection, as are parents of premature babies who acquired CMV nosocomially while in the hospital nursery (210,316). Among the former group, risk of CMV infection is clearly related to shedding of virus by the child; a seroconversion rate of 45% per year was noted in parents of toddlers who shed CMV, compared to 0% among parents whose child did not shed CMV (210). Restriction enzyme analysis of viral DNA has provided laboratory confirmation of transmission of CMV from child to parent and from child to day care worker (3,211). Table 3 lists rates of CMV seroconversion for various groups, including sexually transmitted disease clinic patients, child care workers, and parents.

Group	Rate*	Ref.
Blood donors	1.57	28
Hospital employees		
Richmond, VA	2	4
Birmingham, AL	2.2	24
Pregnant women		
Middle income	2.5	283
Low income	6.8	
Women in STD clinic	37	60
Parents of CMV-shedding child		
0–12 months	47	316
≤18 months	32	210
19 months–6 years	13	210
Day care workers		
Iowa	7.9	193
Richmond, VA	11	4
Toronto	12.5	97
Birmingham, AL	20	213

*Rate is percent per year who seroconverted from antibody negative to antibody positive.
STD, sexually transmitted diseases.

TABLE 3. *Incidence of CMV infection in various groups*

Vertical Transmission

Cytomegalovirus is unique among the human herpesviruses in that transmission from mother to fetus or newborn not only occurs but is common and plays an important role in maintaining CMV infection in the population. Human CMV is spread from mother to baby by three routes: transplacental, intrapartum, and human milk (Table 4). Transplacental infections occur in women who were infected long before conception (recurrent infection) as well as in those who have primary CMV infection during pregnancy. The role of recurrent maternal infection as a source of fetal CMV infection was initially suggested by the occurrence of congenital infection in consecutive offspring to the same mother (277,314). In addition, a high rate of congenital CMV infection was reported in an African population with near-universal acquisition of CMV prior to sexual maturity (249). The observation that congenital CMV infection rates are higher in populations with high rates of maternal seropositivity has been offered as evidence that in many populations most congenital CMV infection is caused by reactivation of endogenous, latent virus (284). Confirmation of the role of recurrent maternal infections was provided by a cohort study in a low income population by Stagno and colleagues (279); the rate of congenital infection in offspring of women with CMV infection more than a year prior to conception (documented by virus shedding or serum antibody) was 2.2%. Congenital CMV infection in offspring of women who were immune prior to conception has usually been considered the result of reactivation of latent virus, but these infections could also be caused by reinfection or by persistent infection due to primary infections within the past few years. Although CNS damage in congenital CMV infection is usually the result of primary gestational infection, it has also been reported after recurrent maternal infection (49,99,282,283).

Route	Maternal situation	Rate of CMV in infant
Transplacental	Seropositive before conception	0.2%–2.2%
	Primary gestational infection	20%–40%
Intrapartum	Seropositive	5%
	Virus in genital tract at term	50%
Breast milk	Seropositive mother	25%
	Virolactia	65%–70%

TABLE 4. *Vertical transmission of human CMV infection*

The rate of primary maternal infection during pregnancy varies from 0.7% to 4.1%, with higher rates noted in young, unmarried women of low socioeconomic status (215). The rate of transplacental transmission of CMV to the fetus with primary maternal infection during pregnancy is around 20% to 40% (215). There is no clear evidence of a gestational age effect on the rate of transmission.

Intrapartum transmission of CMV is related to local shedding of virus. Approximately 10% of women shed CMV from the vagina or cervix; near the time of delivery, rates of 2% to 28% have been reported (281). Genital tract shedding is more common among younger women and distinctly uncommon after age 30. If virus is present in the maternal genital tract at the time of delivery, the rate of transmission to the newborn is around 50% (239). Newborns who acquire CMV during birth are virus negative during the first 3 weeks of life and usually begin to shed virus at around 6 weeks of age.

The most common route for mother-to-infant transmission of CMV is human milk. Transmission of CMV to nursing infants is related to duration of nursing prior to weaning to formula and the presence of virus in milk. Dworsky et al. followed a cohort of infants nursed by CMV seropositive mothers (86). They found that no infants of seropositive mothers nursed less than 1 month were infected, compared with CMV infection in 39% of those nursed for more than 1 month. With repeated sampling, CMV was recovered from the milk of 30% of seropositive women. Overall, 25% of infants nursed by seropositive mothers acquired CMV by 1 year of age. When CMV could be detected in mother's milk by virus isolation, 69% of infants were infected, compared with 10% of infants nursed by seropositive mothers whose milk was negative for CMV. A Swedish study of serial milk specimens from seropositive women reported isolation of CMV from 70% of samples collected from 9 days to 3 months postpartum; all of the samples collected prior to or after this interval were negative (9). More recent studies using PCR to detect CMV DNA in milk reported similar rates of transmission of CMV from seropositive mothers and a strong relationship between the presence of viral DNA in milk and transmission of CMV to the infant (22,296).

Although intrapartum and breast milk transmission of CMV are not associated with the morbidity seen in congenital CMV infection (except in very small premature newborn infants), they play an important role in the population epidemiology of CMV infection. Young infants who acquire CMV from a maternal source usually shed CMV for years and are an important source of virus that can be transmitted to other children and to adults. The proportion of infants who acquire CMV during the first year of life is directly related to the prevalence of maternal infection and the proportion of mothers who nurse their infants. In countries where almost all mothers are seropositive and breast-feeding is nearly universal, more than 50% of infants acquire CMV before 1 year of age. In the United States, it has been estimated that around 10% of infants acquire CMV from a maternal source (209).

Molecular Epidemiology

Molecular tools have improved knowledge of the epidemiology of CMV infection, mainly by providing laboratory evidence for suspected sources of infection and routes of transmission. A number of approaches have been used (Table 5). All are based on the general premise that epidemiologically linked isolates of human CMV share extensive nucleotide sequence homology, and furthermore that isolates that are not epidemiologically related have sequence diversity that is readily detectable. Early investigations compared CMV isolates based on restriction enzyme analysis of genomic DNA using multiple restriction enzymes and radioisotope or ethidium bromide labeling for identification of restriction fragments separated in agarose gels (139,268). This approach is slow and expensive because it requires laboratory propagation of CMV isolates to acquire sufficient viral DNA. In addition, serial passage *in vitro* could select genetic variants. Techniques that are more rapid and require much smaller amounts of DNA were developed by focusing on the variable junctional region of the CMV genome and by using PCR to amplify selected segments of the genome (65,270,318). More recently, envelope glycoprotein genes from clinical isolates have been sequenced and compared. Chou and Dennison found that clinical strains and laboratory strains Towne and AD169 could be grouped into one of four gB genotypes (68). The distribution of gB genotypes has been studied in immunocompromised patients, but the epidemiologic and clinical significance of gB genotype remains unclear. Although gH sequence is more highly conserved, peptide variation in the first 37 amino acids of gH segregated clinical isolates into two groups, with laboratory strains Towne and AD169 falling into different groups (66).

Method	Ref.
RFLP	139
Junctional hybridization	270
PCR + RFLP	65
α-Sequence PCR	318
Nucleotide sequencing, selected genes	66, 68

RFLP, restriction fragment length polymorphism; PCR, polymerase chain reaction.

TABLE 5. *Approaches to molecular epidemiology of CMV infection*

Molecular investigations of CMV isolates have been tremendously valuable in increasing our understanding of the epidemiology of CMV infection. They have provided laboratory evidence for the following: vertical transmission of reactivated endogenous virus (139), transmission of CMV from child to child and child to parent (2), spread of virus from children to day care workers (4), spread of virus from child to mother to fetus (194,211), nosocomial spread of CMV in a hospital nursery (269), acquisition of donor strains of CMV by transplant patients (64), and reinfection by new strains in

immunocompromised patients, children, and sexually active women (27,60,84,168). In some cases, molecular tools have failed to confirm homology between CMV isolates thought to be epidemiologically linked, thus providing powerful evidence against the spread of CMV from a suspected source in the hospital (5,317).

CLINICAL FEATURES

CMV Mononucleosis

It has been estimated that CMV is responsible for 20% to 50% of heterophile-negative mononucleosis and that it accounts for approximately 8% of all cases of mononucleosis (156). As a rule, acquired CMV infection in the normal host is clinically silent. Studies of CMV infection in pregnant women show that less than 5% of acquired infections are symptomatic, and an even smaller percentage are associated with a mononucleosis syndrome (215). Symptomatic infection is also uncommon in infants who acquire CMV during birth or from mother's milk and in preschool-aged children who acquire CMV in day care centers. Even among nonimmunocompromised patients with transfusion-acquired CMV, most infections are without clinical manifestations (70).

Mononucleosis due to CMV is very similar in clinical manifestations to the more common Epstein-Barr virus (EBV)-induced disease (149,197,204). Clinical manifestations encountered in patients with CMV mononucleosis are listed in Table 6. Malaise, headache, and fatigue are commonly present and may persist for weeks. Fever is present in 98% of cases of CMV mononucleosis. The mean duration of fever is 2 weeks, and temperatures as high as 39° to 40°C are common. Lymphadenopathy, pharyngitis, and splenomegaly are each seen in around 30% of patients and thus are more constant findings in the EBV-induced disease. Rash is also present in around 30% of CMV patients. Over 90% of patients have mild elevation of hepatic transaminases. Slight elevation of serum bilirubin and jaundice have been reported but are uncommon. Lymphocytosis with atypical lymphocytes in peripheral blood is common. A variety of other clinical abnormalities have been associated with acquired CMV infection in the normal host, including Guillain-Barré syndrome, peripheral neuropathy, meningoencephalitis, Rasmussen encephalitis, myocarditis, hemolytic anemia, thrombocytopenia, retinitis, gastrointestinal ulceration, hepatitis, and pneumonitis (138,197). These complications are sufficiently unusual that even when their occurrence is temporally related to CMV infection, other possible etiologies should be carefully sought and the immune competence of the patient should be assessed.

Feature	Frequency (%)
Fever >10 days	94–95
>18 days	41–53
Cervical adenopathy	6–56
Sore throat	56
Exudative pharyngitis	0
Splenomegaly	33–53
Hepatomegaly	0–53
Rash	0–44
Elevated serum ALT or AST	78–100
Lymphocytosis (>5000/μL)	78–95
Atypical lymphocytes (>1000/μL)	71–95

Note: Table was compiled from three published reports covering a total of 45 adult patients (149,156,204). These patients typically had malaise, fatigue, and myalgia, as well as the features listed.
ALT, alanine transaminase; AST, aspartate transaminase.

TABLE 6. *Clinical findings in CMV mononucleosis*

Congenital CMV Infection

When congenital CMV infection is clinically evident (customarily termed symptomatic), it is typically manifest by signs and symptoms that indicate *in utero* involvement of multiple organ systems (45). The chief clinical findings are summarized in Table 7. These abnormalities are nonspecific and are very similar to those produced by other viral and nonviral causes of congenital infection. Laboratory evaluation of newborns with symptomatic congenital CMV infection confirms the multisystem nature of the disease. Hematologic abnormalities are common; approximately 75% of patients have thrombocytopenia and 50% have evidence of hemolytic anemia. Modest elevations of hepatic transaminases are present in over 80%, and a similar proportion have elevated conjugated bilirubin. Cerebrospinal fluid is usually normal except for elevation of protein to above 120 mg/dL in around 50%. Approximately two thirds of newborns with symptomatic congenital CMV infection have evidence of CNS involvement such as microcephaly, elevation of cerebrospinal fluid protein, or neurologic abnormalities such as poor feeding, lethargy, or generalized hypotonia (45). Cranial computed tomography (CT) scans are abnormal in 75% of symptomatic newborns; periventricular calcification, a hallmark of congenital CMV infection, is the most common abnormality (Fig. 3) (48). Other abnormalities include cortical atrophy, lissencephaly, and ventriculomegaly. Although the abnormalities caused by involvement of the reticuloendothelial system (jaundice, hepatitis, hepatosplenomegaly, petechiae, thrombocytopenia) usually clear spontaneously within a few weeks of birth, newborn disease can be severe, leading to prolonged hospitalization for supportive care and to neonatal death in around 10% of symptomatic newborns (45). However, only around 5% to 10% of newborns with congenital CMV infection are symptomatic as newborns.

Feature	Frequency (%)
Physical signs	
Petechiae	76
Jaundice	67
Hepatosplenomegaly	60
Abnormal hearing	56
Microcephaly	53
Lethargy/hypotonia	27
Chorioretinitis or optic atrophy	10
Premature birth (<38 weeks)	34
SGA[a]	50
Laboratory abnormalities	
Thrombocytopenia (<100,000/μL)	77
Conjugated hyperbilirubinemia (>4 mg/dL)	69
Elevated CSF protein (>120 mg/dL)	46

[a]SGA, small for gestational age, birth weight less than 10th percentile; CSF, cerebrospinal fluid.
Source: Adapted from ref. 45, with permission.

TABLE 7. *Clinical findings in 106 newborns with symptomatic congenital CMV infection*

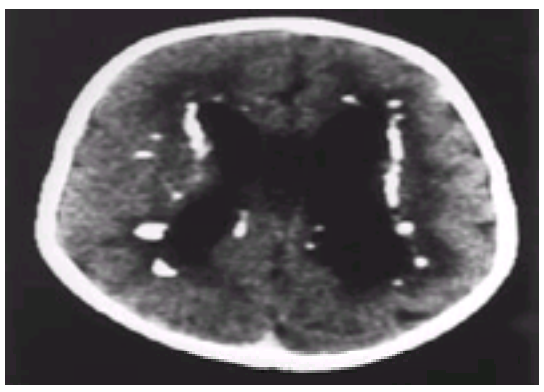

FIG. 3. Computed tomographic scan of the brain of an infant with symptomatic congenital CMV infection, demonstrating periventricular calcification.

Congenital CMV infection is an important medical and public health problem because it causes damage to the CNS and organs of perception. The majority (50% to 90%) of symptomatic newborns will have some combination of deficits, including mental retardation, cerebral palsy, sensorineural hearing loss, and impaired vision (26,205,232). Newborn clinical abnormalities associated with increased risk of CNS sequelae in symptomatic congenital CMV infection include chorioretinitis (Fig. 4), microcephaly, an abnormal neurologic examination, and an abnormal cranial CT scan (48,73). CNS sequelae also occur among the over 90% of newborns with congenital CMV infection who are asymptomatic at birth, but much less frequently than in the symptomatic group. Approximately 7% to 25% of infants with asymptomatic congenital CMV infection will have CNS sequelae, mainly sensorineural hearing loss (100,143,160,246,308). Type of maternal infection (primary or recurrent) is also an important determinant of outcome. Primary maternal infection is more likely to result in symptomatic congenital infection and significant CNS sequelae in infected infants (99,283). However, symptomatic congenital infection and CNS damage have been reported after recurrent maternal infection. In a low income population where most mothers of babies with congenital CMV infection were young, single African-American women, symptomatic congenital CMV infection was as common in babies born to mothers who were immune prior to conception as in those born to mothers with primary infection (49). Considering both the frequency and outcome of congenital CMV infection, it has been estimated to be the leading cause of sensorineural deafness and the leading infectious cause of brain damage in children (100).

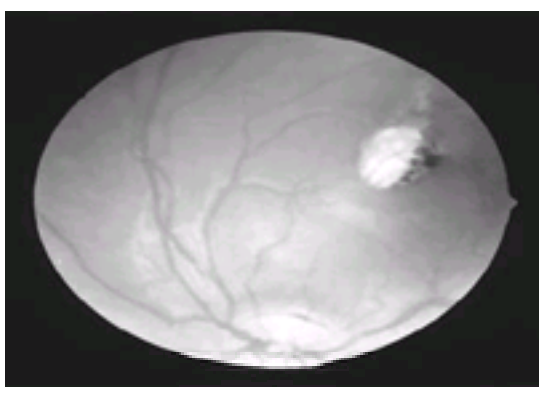

FIG. 4. Retinal lesions caused by congenital CMV infection. (Courtesy of Dr. Catherine Amos, Birmingham, AL.)

CMV Infection in Infants and Children

Although CMV is frequently spread from mother to infant during birth or through milk, clinical evidence of infection is rare in the healthy, full-term neonate infected intrapartum or postnatally from a maternal source. Associations between CMV infection in young infants and hepatomegaly, mild elevations of hepatic transaminases, and pneumonitis have been reported (63,161,280). Others have found little or no evidence of clinical abnormalities associated with perinatal CMV infection in term infants (117,278). However, among very low birth weight premature infants, acquired CMV infection is a significant cause of morbidity. Risk of disease is particularly great for newborns with birth weight less than 1,500 g who

are born to CMV-seronegative mothers and are infected via receipt of red blood cell transfusions from seropositive donors (316). Even infections acquired from mother's milk can cause disease in small premature newborns. A recent report described CMV excretion in mother's milk and transmission of virus to 59% of preterm infants fed CMV-positive (by culture or PCR) milk (296). Abnormalities associated with the onset of CMV excretion included thrombocytopenia, neutropenia, apnea, and sepsis syndrome. As in adults, mononucleosis-like illness can accompany acquired CMV infection in children. A comparison of adults and children with CMV mononucleosis showed that children were less likely to be febrile (43% versus 94%) but were more likely to have hepatomegaly (100% versus 53%) and splenomegaly (86% versus 53%) (204).

CMV Infection in the Immunocompromised Host

Cytomegalovirus is one of the most common and difficult of the opportunistic pathogens that complicate the care of immunocompromised patients. Infection is common because it can occur (a) by reactivation of latent virus, (b) by reinfection in patients who have had past infection, and (c) by primary infection. Procedures that are part of the patient's care, such as transfusions or organ transplant, can transmit CMV at a time when the patient is maximally immunosuppressed. CMV infection in the immunocompromised patient is difficult because diagnosis requires not only detection of virus but also determining whether CMV is causing disease: CMV shedding and viremia are common in patients with impaired cellular immunity even when disease due to CMV is not present. In addition, preventive and therapeutic options are few and not completely effective. The severity of CMV infection roughly parallels the degree of immunosuppression. The most severe infections are seen in recipients of allogeneic bone marrow transplants and in patients with acquired immunodeficiency disease (AIDS) with very low CD4 counts; CMV disease is also seen in solid organ transplant patients, in patients receiving immunosuppressive chemotherapy for cancer or collagen vascular disease, and in congenital immunodeficiencies. Infection is often clinically silent even in the immunocompromised, and when disease occurs, its severity ranges from a brief, self-limited febrile illness to multisystem disease that can be life-threatening or debilitating.

Observations of CMV infections in immunocompromised patients have resulted in improved understanding of the pathogenesis of human CMV infection. For example, evidence of reinfection with new strains of virus was convincingly demonstrated in immunocompromised patients. The importance of antibody was supported by observations and clinical trials in transplant patients (261). The key role of CTLs in limiting CMV infection and preventing disease was confirmed *in vivo* by adoptive transfer of autologous CMV-specific CD8 cells in bone marrow transplant patients (300). The only evidence for clinical efficacy of a CMV vaccine came from studies of Towne vaccine in renal transplant patients (222). The development of antivirals for CMV infection resulted primarily from the need to manage infections in immunocompromised patients, and studies that led to definition of a molecular basis for antiviral resistance were performed in these patients as well.

Solid Organ Transplantation

The potential for CMV infection must be considered for every patient undergoing solid organ transplantation. Risk factors for disease and the manifestations of disease can vary with different organs, and a wide variety of diagnostic tools and management strategies are employed by transplant centers. Recent reviews provide detailed coverage of this subject (128,176,228). The onset of CMV infection, as indicated by detection of virus in blood or shedding of CMV is around 4 to 8 weeks after solid organ transplant. However, the time of onset is variable; it may be shorter with profound immunosuppression or delayed if antiviral prophylaxis is used during the immediate posttransplant period. The sources of these CMV infections include reactivation of the recipient's latent virus or virus from cells in the transplanted organ or blood products. Primary infection acquired from the transplanted organ is generally more severe than reactivation or reinfection, although this varies with the specific organ and with the degree of immunosuppression. Table 8 compares the rates of CMV infection and disease according to donor and recipient serology in kidney and liver transplant patients.

Pretransplant serostatus of donor/recipient	Type of infection	Kidney		Liver	
		Infection (%)	Disease (%)	Infection (%)	Disease (%)
−/+	Reactivation or reinfection	51	19	66	23
+/+	Primary	67	50	77	61
−/+	Reactivation	59	12	45	18
+/−	Primary, not from graft	0	0	10	10

Source: Prince et al. 1992, [illegible], with permission.

TABLE 8. *Rates of CMV infection and disease in kidney and liver transplant patients, according to type of infection*

The most common manifestations of CMV disease are fever, leukopenia, malaise, arthralgias, and macular rash. Studies performed prior to the widespread use of prophylactic or preemptive antiviral treatment showed that the majority of patients with primary infection after transplant, experience a febrile illness related to the onset of CMV excretion, and most of these resolve spontaneously, although some patients develop life-threatening disease such as pneumonitis, gastrointestinal ulceration, severe hepatic dysfunction, opportunistic fungal infection, or impaired graft function. Primary CMV infection in solid organ transplant patients has been consistently linked with dysfunction of the transplanted organ—renal impairment in kidney recipients, hepatitis after liver transplantation, coronary stenosis or rejection in heart transplant patients, and pneumonitis after lung or heart-lung transplant. Febrile illnesses and even the more severe forms of disease can also be seen in patients who were seropositive prior to transplantation, but reactivation infections are

generally less likely to be symptomatic or to be associated with life-threatening abnormalities.

Cytomegalovirus can be isolated from urine, saliva, or white blood cells (WBC) from patients with primary infection after transplant. The majority of patients with reactivation infection in the posttransplant period shed CMV in urine or saliva, and a smaller percentage have viremia detectable by virus isolation. Although viruria is not strongly predictive of disease, viremia is associated with increased risk of clinically significant CMV disease. Quantitation of viremia using PCR, DNA hybridization, or detection of CMV pp65 antigen in WBC has been used to establish threshold levels above which risk of CMV disease is high and antiviral treatment justified (127,129).

Bone Marrow Transplantation

Among bone marrow transplant patients, as among solid organ transplant patients, the main sources of CMV infection are reactivation of endogenous virus in seropositive patients and transplanted cells or blood products in seronegative and seropositive patients. Pneumonitis is the most common clinically significant CMV disease in bone marrow transplant patients. For allogeneic transplants, it has been reported that 0% to 15% of seronegative recipients of marrow from a positive donor and 6% to 36% of seropositive patients have CMV pneumonia (41). In contrast, CMV pneumonia has been reported in 2.1% to 8.5% of autologous marrow transplant patients (41). In a review of CMV infection and disease in allogeneic marrow recipients in a Swedish hospital, there were 384 transplants in which donor, recipient, or both were seropositive for CMV over a 20-year period, and 33 (8.6%) developed CMV disease (176). Among patients with CMV disease, 21 (63.6%) had pneumonitis, three (9.1%) had pneumonia plus gastrointestinal disease, six (18.2%) had gastrointestinal disease alone, and there was one case each of hepatitis, encephalitis, and retinitis. Ljungman noted that rates of CMV disease by day 100 after transplant and death due to CMV infection decreased respectively from 13% and 13%, to 2.2% and 0%, comparing 1975–1985 with 1992–1994 (176). Similar results have been reported from a major U.S. transplant center, where the incidence of CMV disease in allogeneic marrow transplant patients during the first 100 days after transplant fell from 38% to 5% over an approximately 10-year interval (41). Reasons for the decline in CMV disease include prevention of transfusion-acquired CMV infection in seronegative patients, decreased frequency of graft-versus-host disease, improved methods for rapid diagnosis and prediction of patients at risk, the availability of effective antiviral agents, and improved knowledge of how to use antiviral agents to prevent CMV disease.

In addition to the pretransplant serostatus of recipient and donor, allogeneic reactivity is a significant risk factor for CMV disease. Excretion of CMV is common after autologous or identical twin marrow transplant, but CMV disease is not. Among allogeneic marrow recipients, degree of donor–recipient mismatch has been identified as a risk factor for CMV disease. However, graft-versus-host disease and the treatment it necessitates are more common when there is significant donor–recipient histoincompatability. Ljungman performed multivariate analysis of risk factors for CMV disease, including the following variables in the model: prophylactic antiviral treatment, preemptive antiviral treatment, high-dose immune globulin, intravenous (IGIV), HLA match, acute graft-versus-host disease, and total-body irradiation (176). Only graft-versus-host disease emerged as a statistically significant risk factor for CMV disease and death due to CMV. Although the approach to diagnosis, prevention, and treatment of CMV infection varies considerably among marrow transplant centers, there is general consensus that successful management of CMV infection requires pretransplant donor and recipient screening, preventing blood product transmission of CMV, virologic and clinical screening for CMV infection and disease after transplant, and appropriate use of antivirals. Because peripheral shedding of CMV is not very predictive of disease in marrow transplant patients, laboratories have focused on detection of CMV in blood and bronchoalveolar lavage fluid, and on relating the quantity of virus in blood to the risk of disease. Antiviral therapy can be administered prophylactically to all patients at risk for CMV infection, preemptively to those in whom weekly posttransplant virologic surveillance indicates impending disease, or as treatment when disease develops. Prophylactic or preemptive antiviral therapy are currently favored, but specific regimens vary from center to center (130,228).

CMV Infection and HIV/AIDS

Cytomegalovirus infection is important in patients with HIV infection because it is arguably the leading opportunistic infection among adults with advanced AIDS, and some investigators have found that CMV infection is associated with clinical progression of HIV infection. Almost all adults and approximately 50% of children with HIV infection have serologic evidence of CMV infection. Among children born to HIV-infected women, the rate of congenital CMV infection is not increased, although the rate of acquisition of CMV during birth and infancy is increased in HIV-infected infants compared to the uninfected (159,195). Prior to the use of combination regimens of highly active antiretroviral agents, it was estimated that around 40% of adults and 9% of children with AIDS would develop disease due to CMV. Among adults with advanced HIV-1 infection, approximately 10% to 30% per year develop CMV disease (102,253). Risk of CMV disease is linked closely with immune impairment as reflected by low CD4 T-cell counts. A cohort study of over 1,000 adults with CD4 counts below 250/mm^3 who were on zidovudine found that 109 developed CMV disease (106). Among patients with under 100 CD4 cells/mm^3 at enrollment, 21.4% developed CMV disease within 2 years, compared with 10.3% for patients with above 100 CD4 cells/mm^3. The distribution of clinical manifestations among the 109 patients with CMV disease was retinitis, 85.3%; esophagitis, 9.2%; colitis, 7.3%; and both retinitis and esophagitis, 2.7%. Less common clinical presentations of CMV disease in AIDS patients include encephalitis, peripheral neuropathy, polyradiculoneuritis, pneumonitis, gastritis, and hepatitis (62). Retinitis due to CMV infection in HIV-1-infected children is less common than in adults, although this difference is at least in part the result of the lower prevalence of CMV infection in the children (78). Among 40 infants with HIV-1/CMV co-infection who were followed to 18 months of age, only three developed characteristic CMV diseases (one each: disseminated disease, colitis, pneumonitis) (159).

Cytomegalovirus has been suspected of being a cofactor in the development of AIDS because clinical studies link CMV and progression of HIV-1 infection, and laboratory studies provide a biologic framework for CMV enhancement of HIV-1 infection. Proposed mechanisms through which CMV could enhance HIV-1 replication or reactivation from latently infected cells include transactivation of the promoter in the HIV-1 long terminal repeat (30), induction of Fc receptor expression (184), and immune activation with production of proinflammatory cytokines (201). Although some clinical studies in adults have linked markers of active CMV infection (such as viremia or quantitative plasma CMV load

determined by PCR) with progression of HIV infection, others have not found CMV to be a risk factor for progression of HIV-1 disease (253,274,301). Among children with HIV-1 infection, CMV infection has been associated with progression to AIDS, encephalopathy, and death (103,159). The frequency of development of progressive CNS disease (loss of developmental milestones, impaired brain growth, and symmetric motor deficits) in infants co-infected with HIV-1 and CMV, compared with those with only HIV-1 infection, was particularly striking in a recent cohort study (159). Whether co-infection with CMV promotes progression of HIV-1 infection, or the occurrence of CMV infection and disease are simply markers for immune dysfunction remains a point of controversy. It is, however, quite clear that treatment with combinations of highly active antiretroviral agents that reduce HIV-1 viral load is associated with a reduction in CMV viremia and disease (79,109,202).

CMV and Atherosclerosis

Epidemiologic data and pathologic studies from the 1980s suggested a link between CMV infection and atherosclerosis (1,133,185). These associations between CMV and vascular disease have been given biologic plausibility by data from cohort studies and animal experiments, and from the identification of CMV gene products with the potential for facilitating development of atherosclerotic lesions. Zhou et al. measured serum IgG antibody to CMV in patients undergoing atherectomy for symptomatic coronary disease, and they performed coronary angiography 6 months later to assess restenosis (320). They found that seropositive patients had more coronary artery narrowing 6 months after the procedure, and that CMV seropositivity and the amount of CMV IgG antibody were each independently associated with restenosis in a multivariate model, achieving odds ratios of 12.9 and 8.1, respectively. Two studies in cardiac transplant patients reported that CMV infection was significantly associated with the development of posttransplant coronary artery restenosis (118,183). Grattan et al. reported that the CMV-infected cardiac transplant patients had higher rates of graft rejection, more severe coronary artery atherosclerosis, and higher death rates (118). McDonald et al. examined seven putative risk factors in a multivariate model and found that only CMV infection was significantly associated with risk of posttransplant coronary artery disease (183). Experimental CMV infection in rats and mice has been shown to increase the accumulation of lipids in the vascular wall (266). In the rat model, CMV infection was shown to enhance smooth muscle proliferation and neointimal formation even when virus could not be detected in the vessel wall, suggesting a general proinflammatory effect (170,173). *In vitro* studies of the cell biology of CMV infection have suggested several mechanisms through which CMV could influence the development of atherosclerotic vascular abnormalities (Table 9). These mechanisms provide a pathogenic connection between CMV infection and vascular inflammation. Considering all of the available evidence, one could postulate a role for CMV in the pathogenesis of atherosclerosis that involves reactivation of latent CMV followed by virus-induced enhancement of vascular inflammation and damage. Circulating monocytes will be drawn to developing atherosclerotic lesions because of the inflammatory process. In seropositive patients, latent CMV will be reactivated in these monocytes by inflammatory cytokines and by contact with vascular endothelial cells, smooth muscle cells, and oxidized low-density lipoproteins (120). Reactivation of CMV would then promote further vascular changes through the mechanisms listed in Table 9, leading to smooth cell proliferation, uptake of low-density lipoproteins by smooth cells, neointimal formation, and narrowing of the vessel lumen. Although the role of CMV in atherosclerosis remains uncertain, it is clear that CMV is neither a necessary nor a sufficient factor for the development of atherosclerosis. Substantial numbers of patients who have never had CMV infection have coronary artery disease, and atherosclerotic vascular disease is uncommon in many populations in which there is near-universal acquisition of CMV at an early age. However, atherosclerosis is a multifactorial disease process of which inflammation is a key component. There is growing evidence that CMV can contribute to vascular damage, which might eventually make it possible to change the course of vascular disease by antiviral treatment.

Type of study	Results	Ref.
In vitro		
Human SMC	CMV infection of SMC ↑ p53; IE86 binds to and inhibits p53	311
Promonocytic cell line	Endothelial cells, SMC and exposure to LDL ↑ reactivation of CMV	120
SMC	IE84 antagonizes p53-mediated apoptosis	288
Rat SMC	HCMV infection → ↑ SMC proliferation; ↑ SMC migration toward PDGF; ↑ PDGF β receptor expression	321
SMC	CMV US28 promotes migration in presence of CC chemokines	287
In vivo		
Rat, with and without hypercholesterolemia	CMV infection increases lipid accumulation in proximal aorta	266
Rat, aortic allografts	CMV infection → ↑ SMC proliferation; ↑ intimal arteriosclerotic changes	170
Rat, aortic allografts	CMV infection → ↑ neointimal proliferation in MCH mismatched grafts	173
Rats, carotid injury	CMV infection ↑ neointimal response	321
Cardiac transplant patients	CMV infection associated with ↑ graft atherosclerosis, ↑ graft rejection, and ↓ patient survival	118
Cardiac transplant patients	CMV infection in 83% of patients with posttransplant CAD; vs. 35% in patients free of CAD	183
Patients with postangioplasty restenosis	CMV in lesions associated with ↑ p53	311
Postatherectomy patients	CMV infection associated with restenosis in 43% vs. 8% in patients free of CMV	320

SMC, smooth muscle cells; HCMV, human CMV; PDGF, platelet-derived growth factor; LDL, low density lipoprotein; CAD, coronary artery disease.

TABLE 9. *Experimental and clinical evidence that CMV infection plays a role in development of atherosclerosis and coronary artery restenosis*

DIAGNOSIS

Differential Diagnosis

The most common signs of congenital CMV infection in the newborn, jaundice, petechiae hepatosplenomegaly, intrauterine growth retardation, and microcephaly, are nonspecific. Congenital CMV infection cannot be reliably distinguished on clinical grounds from congenital infection caused by other agents such as *Toxoplasma gondii*, syphilis, and rubella virus, and patients with noninfectious diseases such as erythroblastosis fetalis, osteopetrosis, and metabolic diseases can have jaundice, petechiae, and organomegaly that suggest congenital infection. Specific laboratory tests for isolation or detection of CMV resolve the diagnosis in newborns quickly and reliably. In immunocompetent children and adults with mononucleosis-like illnesses, CMV infections must be distinguished from acute infections caused by EBV, *Toxoplasma gondii*, human immunodeficiency virus, and hepatitis viruses (A, B, and C).

In transplant patients, the signs of CMV infection are also nonspecific. A variety of viral (notably EBV and human herpesvirus-6, in addition to CMV) and nonviral infections, as well as rejection episodes or graft-versus-host disease, can cause fever, leukopenia, malaise, hepatitis, pneumonitis, enteritis, or impaired graft function. The role of CMV infection is sorted out by knowledge of the CMV serostatus of donor and recipient and by specific laboratory tests for CMV infection. Disease caused by CMV in AIDS patients typically presents as retinitis, enteritis, or esophagitis. Hepatitis, encephalitis, pneumonitis, peripheral neuropathy, or polyradiculopathy are less frequently encountered. Although retinitis caused by CMV infection can be identified with reasonable accuracy by ophthalmologic examination, there are myriad other possible viral, bacterial, and fungal causes for the other clinical syndromes in AIDS patients. Sorting out the role of CMV infection may require tissue diagnosis, exhaustive search for other pathogens, and assessment of response to antiviral therapy aimed at CMV.

Laboratory Diagnosis

Serology

Serologic tests for antibody to CMV are useful for determining whether a patient had CMV infection in the past, a determination of great clinical importance for organ and blood donors, and in the pretransplant evaluation of prospective transplant recipients. In addition, serologic tests for seroconversion and for IgM antibody to CMV are commonly used to establish whether or not infection in the normal host occurred recently. The laboratory diagnosis of recent primary infection is useful in patients with mononucleosis and in pregnant women who, based on clinical signs or exposure, might have primary CMV infection. A variety of different laboratory methods have been used successfully to measure antibody to CMV (214).

Traditionally, serologic evidence of recent primary CMV infection depended on the demonstration of conversion from IgG antibody negative to positive or demonstration of IgM antibody. Tests for IgM antibody to CMV often lack specificity for primary infection because of false-positive tests or because patients with past infection may have IgM antibody to CMV. The reliability of the serologic diagnosis of primary CMV infection in pregnant women is reportedly improved by testing for low-avidity antibodies in women who were positive for IgM antibody when screened (167). Antibody tests are not useful in the diagnosis of CMV disease in the immunocompromised host. Although detection of IgM antibody to CMV has been used to diagnose congenital CMV infection, viral isolation techniques are more accurate and are preferred.

Studies of the kinetics and isotype specificity of the antibody response to CMV reveal patterns that can be exploited for diagnostic purposes. Peak levels of neutralizing antibody are achieved later than antibody to virion after primary infection in renal transplant patients and in normal adolescents (206,319). Schoppel et al. reported that appearance of antibody to gB and gH was delayed by 50 to 100 days compared with antibody to other structural and nonstructural proteins, pp28 (UL 99), pp71 (UL82), pp65 (UL83), p52 (UL44), and pp150 (UL32), after primary infection in normal hosts and solid organ transplant recipients (250). Others have reported delayed appearance but indefinite persistence of antibody to pp150 in contrast to antibody to pp65 and to p52, which appeared early after infection and then frequently declined to undetectable levels with passage of time (119,163,199). Schoppel reported that among healthy adults with past CMV infection, the percent with antibody to pp150, gB, and gH was respectively 98%, 97%, and 82%, compared with only 33% with antibody to pp65 (250). Some differences in isotype specificity of responses to CMV proteins have also been noted. Preferential binding of IgM antibody to p52 (UL44), pp65 (UL83), p38, and pUL57 have been reported (164,298). In the future, use of specified combinations of CMV proteins in serologic assays in place of infected cell lysates or virions may improve ability to establish timing of infection and allow preparation of consistent, standardized lots of antigen.

Virus Detection

The time-honored method for detection of CMV is inoculation of human embryonic lung or foreskin fibroblast monolayers with clinical material and identification of the slowly developing, focal CPE that is characteristic of CMV. However, this approach is slow, sometimes taking more than 2 weeks for recognition of CPE, and it is expensive because of the cost of maintaining tissue and holding cultures for 4 to 6 weeks. In addition, viral isolation is less sensitive than more modern methods. Rapid CMV isolation in tissue culture based on enhancement of infection of the monolayer by low-speed centrifugation and detection of immediate-early antigens by monoclonal antibodies allows detection of virus within 24 to 48 hours, but it is no more sensitive than traditional virus isolation (46,112,231).

Widely used nonculture assays for detection of CMV are detection of pp65 antigen in WBC and PCR detection of CMV DNA. These methods are clearly more sensitive than viral isolation for detection of CMV in blood, and both have been extensively evaluated in immunocompromised patients. A number of other nonculture methods for detection of CMV have been studied, including detection of antigens by enzyme immunoassay, DNA and RNA hybridization techniques, RNA amplification, and cyto-hybridization techniques. These are discussed in recent reviews and will not be discussed further here (129,166,214). Both pp65 antigenemia and CMV DNA PCR provide results that are semiquantitative, and quantity of pp65 antigenemia or CMV DNAemia has correlated with risk and severity of CMV disease in immunocompromised patients (23,40,88,101,102,108,312). Detection of pp65 antigenemia has the advantage that it yields results rapidly and is commercially available, allowing standardization between centers; a major disadvantage to detection of pp65 antigenemia is that samples must be tested within a few hours of collection. PCR has the advantage that it can be used to detect CMV DNA in samples other than WBC, such as whole blood, plasma, cerebrospinal fluid, or bronchoalveolar lavage fluid (23,69,271,311). The major disadvantage to PCR is that its great sensitivity can detect low levels of CMV that are not predictive of disease (80,154,323).

PREVENTION OF CMV INFECTION AND DISEASE

Community-Acquired Infection

Although prevention of maternal CMV infections that lead to congenital infection is an important public health goal in the

United States, an effective means of achieving this goal is not currently available. In the United States and Europe, 25% to 85% of women are seronegative to CMV during their reproductive years (281). CMV infection is very common in the community; it is almost always clinically inapparent, and it results in shedding of virus for months to years. These features of CMV infection make it difficult to plan strategies for prevention based on limiting exposure to the virus. For most community-acquired CMV infections, the timing of infection and the source of virus are not known. Although exposure to young children and sexual activity have been linked with increased risk of CMV infection, it is unlikely that avoidance of either of these activities will be a practical means of preventing CMV infection for women of childbearing age. Use of good hand washing and careful hygiene have been recommended as a means to prevent CMV infection in day care workers and parents of young children (16, 200). A more rigorous approach, involving formal instruction in hygiene and prevention of contact with body fluids and use of gloves, has been evaluated in a controlled trial in parents of young children who were shedding CMV (6). A statistically significant advantage (reduced rate of maternal infection or time to onset of infection) for the intervention group was not demonstrated, although the study was limited by small sample size. Prevention of disease due to congenital CMV infection in the United States and in other countries where primary infection is common during the childbearing years will probably require wide use of a vaccine.

Hospital-Acquired/Nosocomial Infection

Hospital Workers

Hospital workers are potentially at risk for acquisition of CMV infection through contact with body fluids from patients who are shedding CMV, such as immunocompromised patients, infants, and children. Studies of hospitalized patients show that from approximately 1% to 30% of hospitalized patients shed CMV in urine or saliva (212). However, in the era of universal precautions, hospital workers do not appear to have an increased risk of CMV infection. The incidence of CMV infection in dialysis and newborn nursery personnel was found to be similar to that of blood donors or the general population (28, 85a). A study in a children's hospital that included employees with and without patient contact found no statistically significant difference in incidence of CMV infection among workers with patient contact (24). The lack of evidence of increased risk of CMV infection for hospital workers suggests that infection control procedures such as hand washing and avoiding contact with body fluids that are a routine part of care of every patient are effective in preventing transmission of CMV.

Blood Transfusion

Transmission of CMV to immunocompromised patients and premature newborns through blood products should be avoided. Screening blood donors so that blood products from CMV seronegative donors can be provided for seronegative recipients is an effective means of preventing transmission, although this screening and selection process adds significantly to the cost of preparing blood products and may limit the availability of donors in populations where almost all donors are seropositive (189, 247). WBCs are the main source of CMV in peripheral blood, and procedures that remove them from blood products have been shown to reduce the risk of CMV transmission. Filtration of whole blood using cotton-wool filters has been shown to be very effective in removing WBCs and platelets and in preventing transmission of CMV infection (110, 247).

Organ Transplantation

The most common source of CMV infection in organ transplant patients is reactivation of the transplant recipient's latent virus, and there is no means to prevent these infections. Although selection of seronegative donors for seronegative recipients will prevent primary CMV infection in solid organ transplant patients, this approach is not practical because of the restrictions that it places on availability of donors. Prophylactic and preemptive antiviral regimens have been used to prevent disease from primary and reactivated CMV infection, as described later in the section on Antiviral Treatment. It is common practice to provide only irradiated, CMV seronegative, or filtered blood products for transplant patients.

Other Routes of Nosocomial Infection

Cross-infection with CMV in a hospital nursery has been documented by restriction enzyme analysis of viral isolates (269). The presumed mechanism of spread is through hands of hospital workers. These events appear to be rare based on restriction enzyme study of DNA from CMV isolates collected from patients in a newborn nursery (5). Human milk is another potential vehicle for nosocomial transmission of CMV, where banked human milk is provided to hospitalized newborns without consideration of donor and recipient serostatus. Milk from seropositive donors should not be given to seronegative newborns, especially those who are premature or hospitalized.

Excretion of CMV in semen is common (72, 165). Virus is shed in higher concentration and for a longer period in semen compared with other body fluids. Shedding of CMV in semen is more common in younger men and is particularly common and persistent among homosexual men (72, 191). Testing of cryopreserved semen from sperm banks by culture and PCR detected CMV in samples from 2.0% of donors (3.9% of seropositive donors) (180). Thus, there is the potential that CMV could be transmitted during artificial insemination, causing maternal and even fetal infection. Although there are no reports in the medical literature of congenital CMV infection attributed to donor insemination, it is reasonable to take steps to avoid this complication. The American Fertility Society has recommended serologic screening of semen donors for antibody to CMV (15). Whether semen from seropositive donors should be screened for virus is a matter for further consideration. Seronegative patients receiving donor insemination should be limited to semen from seronegative donors. Whether it is reasonable to take similar precautions in donor oocyte programs is uncertain. A study of oocytes from 71 women undergoing infertility evaluations (62% seropositive) reported no detection of CMV by PCR in any donated oocytes, although 25% of semen samples from 60 partners were positive (310).

Passive Immunization

Cytomegalovirus immune globulin is used in organ transplant patients as prophylaxis against CMV disease. A regimen

using a high-titer CMV immune globulin prior to kidney transplant and weekly for 3 weeks after transplant was shown to decrease the severity of CMV disease significantly in seronegative recipients of kidneys from seropositive donors (261). Not surprisingly, passive immunization did not prevent infection. CMV immune globulins have been evaluated in transplant patients receiving other solid organs and in bone marrow transplant patients (52,74,187,262,309). In addition, there are numerous studies of CMV immune globulin or immune serum globulin in combination with various antiviral agents. Results have been mixed and there continues to be difference of opinion concerning the role of immune globulins in prevention of CMV disease in transplant patients (41,155,176).

CMV Vaccines

Although attempts to develop a vaccine for prevention of maternal and congenital CMV have been made for over 25 years, no vaccine is currently licensed or near licensure. However, improved understanding of the role of specific viral proteins in eliciting protective immune responses and recent recognition of the potential benefit to society and cost-effectiveness of CMV vaccines will probably stimulate development of new vaccines (286). Investigational vaccines employing different formats have been evaluated in clinical trials; these include attenuated live virus, a recombinant protein in a fowl pox vector, and recombinant protein given with a new adjuvant. The Towne strain of CMV, originally isolated from a child with congenital CMV infection, was passaged more than 125 times *in vitro* to achieve attenuation (220). Subsequent study showed that the Towne strain was attenuated in that it did not produce a febrile illness in challenge studies, whereas a low-passage Toledo strain did (223). In addition, Towne strain of CMV could not be recovered from vaccine recipients even after immunosuppression (222). Immunization with the Towne CMV vaccine induced both antibody responses and lymphocyte proliferative responses to CMV (285). An efficacy trial in renal transplant patients showed that pretransplant immunization of seronegative patients with Towne vaccine protected them from severe disease although it did not prevent infection (222). The Towne CMV vaccine was also evaluated in a placebo-controlled trial in seronegative parents of preschool age, CMV-infected children (8). There was no evidence of vaccine efficacy in this group of normal hosts; the CMV infection rate was nearly identical in vaccine and placebo groups. Although the Towne vaccine is immunogenic, the infection that it produces is quite different from that induced by wild-type viruses, which results in prolonged viral shedding and induces stronger immune responses; it does not appear to confer the protection from reinfection that comes from naturally acquired infection. Genomic comparisons of the Towne CMV strain and Toledo, a low-passage strain that retains the ability to cause disease and can be isolated from body fluids when injected into volunteers, revealed differences in their DNA sequences (58). In an attempt to produce a live virus vaccine that would be less attenuated and more immunogenic, chimeric viruses that include portions of Towne genome and portions of the Toledo genome that appeared to have been lost from Towne have been produced as potential vaccine candidates; these are in phase I clinical trials (151).

A subunit vaccine composed of a modified gB molecule expressed in Chinese hamster ovary cell culture combined with a novel oil-in-water adjuvant has been developed (265). Initial clinical trials with this subunit vaccine established safety, immunogenicity, and optimal antigen dose (104,216). Immunization of healthy adults with three doses of vaccine on a 0-, 1-, and 6-month schedule resulted in serum antibody to gB, neutralizing antibody, and lymphocyte proliferative responses to gB similar to those from naturally acquired infection. This subunit vaccine is currently being evaluated in an expanded phase II placebo-controlled clinical trial that will test its ability to prevent infection in women of childbearing age.

Canary pox has been used a vector for CMV gB (224). This format has potential advantages in that it undergoes a single round of replication in vaccinees, thereby allowing optimal, intracellular presentation of antigens for stimulation of CTL responses and avoiding concerns over persistence and reactivation. Preclinical trials with the canary pox vector in mice and guinea pigs showed that it could be used to stimulate antibody and CTLs (114). It has also been used in phase I clinical trials to prime vaccinees for boost immunization with Towne vaccine (7). Multiple other approaches to producing a CMV vaccine have been investigated, including viral vectors for CMV proteins and DNA vaccines (225).

ANTIVIRAL TREATMENT

Although antiviral agents currently available for CMV are far from ideal, their use has decreased the burden of CMV disease in transplant patients and improved survival and quality of life of AIDS patients with retinitis and other severe diseases due to CMV. The three antiviral agents currently approved by the U.S. Food and Drug Administration for treatment of CMV disease in immunocompromised patients are listed, along with key pharmacologic properties, in Table 10; their pharmacology is reviewed in Chapter 15. Each of the available antivirals for treatment of CMV disease has the potential for significant toxicity. Because CMV infections in the normal host resolve spontaneously, antiviral treatment is not indicated. A role for antiviral treatment of the extremely rare patient with a presumably normal immune system and severe CMV infection has been suggested and is probably justified if the disease is life-threatening or likely to lead to disability (87).

Agent (refs.)	Route	Plasma half-life (hr)	Usual dose and frequency for treatment: Induction	Usual dose and frequency for treatment: Maintenance	Major toxicity
Ganciclovir (18,20,41,271)	Intravenous (i.v.) Oral (prophylaxis only)	i.v. 3.5 ± 0.8 p.o. 4.8 ± 0.9	5 mg/kg, q12 hr	5 mg/kg/d or 6 mg/kg/d, 5 days per week	Hematologic
Foscarnet (20,142,200)	i.v.	4.0 ± 2.0	90 mg/kg, q12 hr	90–120 mg/kg, q24 hr	Renal
Cidofovir (20,152,182)	i.v.	2.6[a]	5 mg/kg per week,[b] for 2 weeks	5 mg/kg, alternate weeks[b]	Renal, neutropenia

Note: Recommended dosages vary considerably depending on renal function, other organ dysfunction, concomitant medications, and indication for treatment. Acyclovir and valacyclovir, although not appropriate for treatment of CMV disease, have been used prophylactically to prevent disease in organ transplant patients.

[a] Intracellular half-life is much longer, allowing weekly administration.

[b] Dosage must be individualized based on patient's renal function, and probenecid is given prior to dose to minimize renal excretion of drug.

TABLE 10. *Antiviral agents used for treatment and prevention of CMV infections in immunocompromised patients*

Treatment of CMV Disease in AIDS Patients

Nearly all adult AIDS patients have antibody to CMV. With progression of AIDS and decline in the immune system, virologic evidence (viral shedding, viremia) of CMV infection are common. Prior to the use of highly active antiretroviral therapy (HAART), based on combinations of agents, CMV disease occurred in around 40% of adults with advanced AIDS, and a number of clinical trials evaluated antiviral therapy for prevention and treatment of CMV disease (62). The risk of CMV disease is closely associated with severity of immune system damage; the majority of patients with CMV disease have CD4 counts of less than 100. Not surprisingly, the ability of HAART to prevent (or delay) the onset of profound immune impairment in AIDS patients has resulted in a dramatic reduction in the frequency of CMV disease (83).

The association between immune impairment and CMV disease suggests a role for antiviral prophylaxis in AIDS patients at high risk for CMV disease. Two randomized, placebo-controlled clinical trials of oral ganciclovir for prevention of CMV retinitis were performed and yielded disparate results. One found a statistically significant reduction in occurrence of retinitis or disseminated disease in patients receiving ganciclovir (273); the other did not (54). The benefit of prophylaxis must be weighed against the disadvantages of possible drug-induced neutropenia and anemia, increased risk for development of antiviral resistance, and high cost. Currently, oral ganciclovir is the only agent approved for prevention of CMV in AIDS patients. The U.S. Public Health Service/Infectious Diseases Society of America Guidelines for the Prevention of Opportunistic Infections in Persons Infected with Human Immunodeficiency Virus recommend consideration of oral ganciclovir for prophylaxis in adults and adolescents who are CMV seropositive and have CD4+ T-lymphocyte counts of less than 50/µL (19). However, prophylaxis with oral ganciclovir is not completely effective in preventing retinitis, and it is not clear that it will result in improved survival or preservation of sight compared with clinical screening and treatment at the onset of disease (182). No other regimens are currently approved or recommended for consideration as prophylaxis.

Clinical trials have provided evidence of the ability of intravenous treatment with ganciclovir, foscarnet, or cidofovir to bring acute retinitis into remission and to slow the progression of retinitis (61,162,203,272). In untreated patients, retinitis usually progresses within 2 to 3 weeks; antiviral treatment lengthens this interval to approximately 2 to 3 months. In addition, a trial comparing ganciclovir to foscarnet found a survival advantage for the latter (17). Usual intravenous dosages for ganciclovir, foscarnet, and cidofovir for induction and maintenance therapy are listed in Table 10. Local treatment for retinitis with intravitreal injection of an antiviral or use of an implant, which allows slow release of drug, are also options for treatment of retinitis (182). Successful treatment of gastrointestinal, neurologic, and respiratory disease caused by CMV in AIDS patients has been reported, although the evidence for a beneficial effect of anti-CMV therapy with disease in these organ systems is less clear (62). Antiviral therapy for esophagitis, enterocolitis, encephalitis, peripheral neuropathy, polyradiculoneuropathy, and pneumonitis has usually been accomplished with intravenous ganciclovir or foscarnet regimens similar to those used for retinitis (38,62,81). Combination therapy with foscarnet and ganciclovir has been studied in patients with advanced AIDS and severe CMV disease. In a three-arm study in retinitis patients, active disease recurred at around 4 months in those assigned to combination therapy with both drugs, compared with 2 months or less for those on monotherapy with either ganciclovir or foscarnet (18,82). Patients with CNS or neurologic disease often respond poorly to monotherapy, and combination therapy has been recommended for them (21). Combination therapy should be considered for patients who show poor clinical response to monotherapy or develop new manifestations of CMV disease while on monotherapy.

Maintenance therapy for CMV retinitis is an essential part of treatment in patients with advanced AIDS, and it is often employed for other CMV disease syndromes, especially CNS or neurologic disease. Whether the immune restoration that accompanies HAART is sufficient to allow discontinuation of maintenance therapy for CMV disease in retinitis patients has not yet been determined. In addition to the intravenous route, oral and local approaches to maintenance anti-CMV chemotherapy are available (85). Oral ganciclovir, 1 to 2 g three times per day, has advantages over the intravenous maintenance regimen; it is more convenient for patients and avoids the potential risks of intravenous lines. However, oral maintenance is not as effective as intravenous in preventing disease in a contralateral eye that is initially disease free, and there is concern that it may not be as effective in preventing extension of scars near the fovea or optic nerve (62). Local treatment of CMV retinitis has been accomplished using intraocular injections and implantable devices. Intraocular regimens with weekly intravitreal injections or with intraocular implants are similar in efficacy to intravenous maintenance therapy (145). Although intraocular maintenance avoids the inconvenience and risk of intravenous treatment, it appears to be less effective in preventing disease in the contralateral eye, and it increases risk of ocular complications such as infection, retinal detachment, and hemorrhage.

Treatment and Prevention of CMV Disease in Transplant Patients

In transplant patients, the considerable body of knowledge of the sources of CMV infection, timing of onset of infection and disease, expected frequency and severity of CMV disease, and risk factors for severe disease, provides a foundation for selecting an approach to prevention. In seropositive recipients of allogeneic bone marrow, studies have shown that prophylactic treatment with ganciclovir will reduce the incidence of CMV disease during the first 100 days after transplant, although overall mortality and graft survival are not improved (41). Compared with early regimens employing intravenous or oral acyclovir, ganciclovir is more effective in reducing the rate of CMV infection and disease (41). Ganciclovir regimens typically involve an induction phase of around 1 week before the transplant, with treatment doses (5 mg/kg twice a day), followed by a maintenance dose (half the treatment dose), given once a day for 5 or 7 days per week up to 120 days after transplant. Disadvantages of prophylactic ganciclovir treatment include neutropenia in 30% or more of patients, selection of antiviral resistance mutations, and the occurrence of late-onset CMV disease in around 5% of patients after prophylaxis is discontinued (41). Foscarnet has been studied for prophylaxis in marrow transplant patients as a means to avoid ganciclovir's marrow suppression; active CMV infection occurred in 15%, compared with 3% to 20% with ganciclovir regimens (142,228). Regular prolonged administration of antiviral agents is clearly able to reduce the amount of CMV disease in marrow transplant patients at risk (either donor or recipient is seropositive). However, use of prophylactic antiviral treatment means that all patients are subject to the notable disadvantages of this approach (marrow suppression or renal toxicity, development of antiviral resistance, and risk of late-onset disease) to prevent the CMV

disease that occurs in about one third of allogeneic marrow recipients.

An alternative approach to preventing CMV disease involves sentinel laboratory screening, using a positive result on a test for CMV as the indication for initiation of antiviral therapy. The ability to detect CMV prior to the onset of disease is clearly critical to this approach, as is a detection method for which a positive result is reasonably predictive of disease. Early studies of preemptive antiviral therapy used culture of multiple body fluids (116) or detection of CMV in bronchoalveolar lavage fluid at day 35 after transplant (248). More recently, in an effort to increase the sensitivity of early detection of CMV, investigators have focused on viremia, using detection of pp65 antigenemia in WBCs and PCR detection of CMV in blood. Because of the extreme sensitivity of the latter, methods to provide an estimate of quantity of input DNA have been studied (130). Comparison of prophylactic ganciclovir with preemptive therapy based on pp65 antigenemia found that CMV disease rates at day 100 after transplant were higher in the latter group (42). Einsele et al. studied PCR screening of blood for CMV as a basis for initiation of preemptive treatment with ganciclovir, and negative PCR results as the basis for withholding or stopping antiviral treatment; they reported a significant reduction in CMV disease, reduced antiviral use, and improved survival when PCR was used for screening (88). Foscarnet appears to be similar to ganciclovir in effectiveness in preemptive therapy (177).

Among autologous marrow and stem-cell recipients, the rate of CMV disease is lower than among allogeneic marrow recipients, and prophylaxis with antivirals for CMV is generally considered unnecessary. Preemptive antiviral treatment has been investigated in autologous marrow and peripheral blood stem-cell recipients (43,127). This approach is complicated by the fact that approximately 30% of these patients do not have detectable viremia prior to the onset of disease (41). Improved, more sensitive and specific approaches for identifying autologous and peripheral blood stem-cell marrow recipients at risk for CMV disease are needed. Frequent PCR screening of blood is promising for this purpose (127).

Immune globulin preparations (including CMV hyperimmune globulin) have also been evaluated in prophylactic regimens for CMV in marrow transplant patients. Whether their prophylactic use is able to prevent CMV disease is a controversial point (41). Their use for prevention of CMV disease in bone marrow transplant patients does not appear to be justified at this time, although immune globulin is of benefit in treatment of interstitial pneumonitis associated with graft-versus-host disease and CMV (41).

In summary, the frequency and severity of CMV disease in bone marrow transplant patients is influenced by multiple factors. There is no universally accepted approach to prevention and treatment with antivirals at the present time. Continued improvement in the therapeutic index and ease of administration of antiviral treatment for CMV and refinement of methods for early detection of infection are needed and will lead to improved control of CMV disease in these patients.

Among solid organ transplant patients, the issues with regard to antiviral prevention and treatment of CMV disease are similar to those encountered in marrow transplantation. In the era prior to availability of antivirals, mortality due to CMV was low among solid organ transplant patients, in contrast with the situation in bone marrow transplantation. However, morbidity associated with CMV disease in solid organ transplant patients results in prolonged hospital stay, substantially increases the cost of care, increases the risk of opportunistic fungal infection, and is associated with impaired graft function. Strategies to control this morbidity have included antiviral prophylaxis, preemptive treatment based on laboratory evidence of active infection, and use of immune globulin preparations. A recent meta-analysis aimed at developing practice guidelines for renal transplant patients using evidence-based medicine, recommended use of prophylactic antiviral therapy when donor or recipient is seropositive if antilymphocyte treatment is included in the immunosuppressive regimen, and for seronegative recipients of grafts from seropositive donors (146). Among renal transplant patients, prophylaxis with high-dose oral acyclovir has been shown to reduce the incidence of CMV disease in seronegative recipients of a kidney from a seropositive donor (29), and prophylaxis with high-dose oral valacyclovir has been shown reduce the rate of CMV disease in seropositive recipients as well as seronegative patients with seropositive donors (178). Oral ganciclovir was found to be superior to oral acyclovir in preventing CMV disease in a comparative trial in renal transplant patients (96). Morbidity due to CMV is generally greater among heart, lung, heart-lung, and liver transplant patients than among renal transplant patients. In these patients, regimens based on intravenous ganciclovir, oral ganciclovir, and oral acyclovir have been evaluated and considered effective (128). The preemptive approach to prevention of CMV disease was also found to be effective in liver transplant patients (255). The availability of laboratory techniques for early detection and quantitation of CMV in blood will potentially allow greater use of preemptive rather than prophylactic antiviral therapy, thus decreasing the numbers of patients exposed to antiviral toxicity and decreasing the risk of development of resistance. Prophylactic treatment with CMV immune globulin has been shown to reduce the incidence of severe CMV disease in seronegative renal transplant patients with seropositive donors (261). A similar study in liver transplant patients found that prophylaxis with CMV immune globulin reduced the rate of CMV disease, but curiously, not in seronegative patients with seropositive donors (262). The approach to prevention of CMV disease among solid organ transplant patients will differ among programs based on assessment of the risks and consequences of CMV disease in patients and the costs, benefits, and risks of the various approaches.

Antiviral Treatment of Congenital CMV Infection

At present, there are no antiviral agents approved for use in treating congenital CMV infection. A number of case reports, a small series comparing two different administration regimens (198), and a phase II clinical trial (304) describe the use of ganciclovir in newborns with symptomatic congenital CMV infection. In sum, these reports indicate that ganciclovir is relatively safe in newborns, based on acute toxicity. Although the authors were encouraged by clinical improvement in study patients, there is currently no evidence that ganciclovir treatment of the newborn with symptomatic congenital CMV infection decreases mortality or will improve the long-term outcome. Caution is recommended in concluding, on the basis of uncontrolled studies, that treatment improves newborn clinical abnormalities (thrombocytopenia, jaundice, abnormal liver function), given the fact that these abnormalities resolve spontaneously in surviving patients (45). Acute toxicity of ganciclovir required withholding or reducing the dose of medication in 19% of patients in the phase II clinical trial because of neutropenia (304). Compared with experience with ganciclovir in adult immunocompromised patients, the drug appears to have less impact on CMV shedding in newborns with congenital infection. Although the proportion of patients with viruria decreased steadily over the 6-week period of treatment, a number of patients were viruric for more

than 4 weeks (304). Viruria resumed within 1 week of cessation of ganciclovir in all patients. A randomized, placebo-controlled phase III clinical trial of ganciclovir in newborns with severe symptomatic congenital CMV infection was recently completed. Participants all had evidence of CNS disease prior to enrollment; they were randomized to receive 12/mg/kg/day of glanciclovir or no treatment for six weeks. Although results of this clinical trial have not been published as of this writing, treatment was associated with significant reduction in risk of worsening of hearing loss at 6 months of age and at one year or older (Personal communication, D.W. Kimberlin, UAB School of Medicine). Over half of treated patients experienced neutropenia which cleared when drug was discontinued or dosage was reduced. The study did not demonstrate a beneficial effect of treatment on newborn disease, and it was not designed to assess the effect of treatment on other sequelae such as mental retardation or cerebral palsy. Ganciclovir treatment of newborns with symptomatic congenital CMV infection and evidence of CNS disease may improve outcome by preserving hearing, but this benefit must be considered in relation to possible risk of late complications of therapy such as reproductive toxicity or carcinogenicity. Unfortunately there is insufficient data on the risk of these potential late complications of ganciclovir therapy in new borns to allow an informed risk:benefit analysis. However, evidence that antiviral treatment of newborns with symptomatic congenital CMV infection improved outcome is a very encouraging finding that should spur further development and evaluation of antiviral drugs for this indication.

Antiviral Drug Resistance

Chronic administration of antiviral drugs in immunocompromised patients is associated with the appearance of mutations in CMV genes for the UL97 phosphotransferase and UL54 DNA polymerase. Specific mutations have been associated with phenotypic resistance to antiviral drugs both *in vitro* and *in vivo* (see Chapter 15 for a discussion of the mechanisms and genetics of antiviral drug resistance). Clinically, development of antiviral drug resistance is associated with treatment failure, although it is important to recognize that breakthrough disease or treatment failure are common in severely immunocompromised patients with CMV disease, independent of the appearance of mutations associated with resistance. Genetic mutations associated with resistance are uncommon in patients prior to antiviral treatment, but they are quite common after 3 or more months of antiviral therapy (37,144). Patients are often infected with multiple strains of CMV that may differ with regard to antiviral susceptibility, and resistant strains can persist for months after antivirals have been discontinued (67,91). Recognizing and managing antiviral drug resistance in the clinical setting is a complex problem because *in vitro* susceptibility testing is not widely available and the relationship between *in vitro* susceptibility or genotypic changes and clinical effectiveness of ganciclovir, foscarnet, or cidofovir for CMV infection is not well defined. The problem is further compounded by the fact that the isolate selected for study may not contain the same strain or mix of CMV strains present at the site of disease. In addition, cross-resistance to cidofovir occurs in patients with high-level ganciclovir resistance, and patients with multiply resistant viruses have been reported (67,124,258). Clinically significant antiviral drug resistance should be considered a possibility for patients who are responding poorly to treatment and have a history of exposure to antivirals. If phenotypic or genotypic testing of viral isolates is available, it can be used to guide selection of an alternative regimen. However, choices are limited at this time. Changing to a drug to which the patient's virus is susceptible, optimizing the dose of antiviral based on serum levels within the limits of toxicity, and use of combination therapy may prove beneficial.

FUTURE DIRECTIONS

Control of CMV disease, whether the result of congenital infection or immune impairment, is proving to be a difficult goal. Current antiviral therapy has limited efficacy and significant toxicity, and an effective vaccine is more a dream than reality. Improved understanding of two broad areas in the biology of CMV infection is needed. We need to know more about the role of specific CMV genes in determining the virulence, persistence, and establishment of latency in humans. This knowledge might help explain the wide range in severity of CMV disease among patients. More important, it could form the basis for rational design of attenuated live vaccines that lack the ability to cause disease or establish latent infection. In addition, a more complete view of the complex interaction between host defense mechanisms and the virus is needed. This view should encompass knowledge of how CMV immune evasion mechanisms interact with host defense mechanisms. This knowledge could be exploited to lead to new strategies for control of CMV in hosts with immune impairments and for design of attenuated vaccine strains that are more readily controlled by the host immune system.

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CHAPTER 78 Varicella-Zoster Virus and Its Replication

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CLASSIFICATION

Based on its morphology, varicella-zoster virus (VZV) is classified as a member of the family *Herpesviridae* (210). It is further classified together with the herpes simplex viruses (HSV), the prototypical herpesviruses, in the subfamily *Alphaherpesvirinae* by virtue of their common abilities to replicate and spread efficiently, and to destroy their host cells in the process. Although HSV replicates in tissues or cells of numerous animal species, VZV has a narrow host range extending almost exclusively to selected cell types of human and simian origin. In common with other alphaherpesviruses, VZV establishes a life-long latent infection of sensory nerve ganglia, from which it can later reactivate on one or more occasions.

VZV, pseudorabiesvirus (suid herpesvirus 1), and equine herpesvirus 1 (equid herpesvirus 1) are subgrouped together as members of the genus *Varicellovirus*, as is the closely related simian varicella virus (cercopithecine herpesvirus 9). This simian virus exhibits marked genome organizational and sequence homology to VZV and is emerging as a useful model for the study of VZV biology and disease pathogenesis (93,145,257). All of the alphaherpesviruses of this genus share an ability to disseminate widely through their natural hosts to produce infection of the skin, mucous membranes, viscera, and nervous system. They also share a genomic arrangement in which two isomeric forms predominate by virtue of inversion of the shorter unique DNA segment and its flanking repeat elements.

To complete its classification, VZV is the formal species name for the only *Varicellovirus* known to infect humans. Archaic names for this virus, such as herpes varicellae, herpes zoster virus, varicella virus, or zoster virus, reflect earlier uncertainties as to whether the causative agents of varicella and zoster were one and the same organism (20,252,255).

VIRUS STRUCTURE

Morphology

A full investigation of the VZV virion and its structural elements has not been conducted. One formidable obstacle to any such undertaking is the inability to emulate in cell culture that which VZV achieves in the course of natural infection—namely, the efficient assembly and release of infectious progeny.

The VZV particle was first identified by light microscopy of vesicle fluids recovered from patients with varicella or zoster (8). Van Rooyen and Illingworth (251) described the VZV “elementary bodies” as being both less numerous and smaller (only 125 to 175 nm in diameter) than those seen in smallpox lesion fluids. Despite this observation, there remained confusion for a number of years as to whether VZV particles were inherently different from those of smallpox; questions regarding the relationship between the viruses of varicella and zoster persisted even longer. Ruska (212) discerned that the VZV elementary bodies are distinct, spherical, pleomorphic particles. More refined electron microscopic studies showed the viruses of varicella and zoster to be indistinguishable in appearance (75,243).

Before the electron microscopic studies, clinical observations had already suggested that the agents that cause varicella and zoster were identical. In 1888, von Bokay (252) observed varicella arising in children exposed to family members with zoster. In 1925, Kundratitz (134) induced varicella in susceptible children by inoculating them with zoster vesicle fluids.

Serologic studies were also fully concordant with identity, if not merely a close antigenic relationship, between the viruses of varicella and zoster. Netter and Urbain (187) and Brain (20) used vesicle fluids as the source of viral antigens in complement-fixation assays. Convalescent sera from patients with varicella and zoster responded comparably.

With the development by Weller (255) of procedures to propagate VZV in cultured cells, further analyses were undertaken with the more plentiful and reliable source of virus. The cytopathic effects of each virus were

indistinguishable, as were the complement-fixing and neutralizing antibody responses (256). Molecular proof that the viruses of varicella and zoster were identical, however, awaited electrophoretic analyses of their proteins and genomes and the ultimate demonstration that a single electropherotype of virus was responsible for both the primary varicella episode and the subsequent reactivation zoster in the same person (238). The VZV particle, like that of all herpesviruses, is now appreciated as one comprising four major elements: the core, the nucleocapsid, the tegument, and the envelope (Fig. 1).

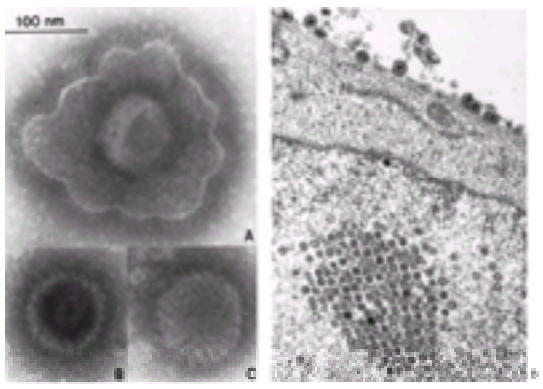

FIG. 1. Electron microscopic appearance of the VZV particle and infected cells. **A:** Enveloped particle. **B:** Purified nucleocapsid. **C:** Negatively stained nucleocapsid preparation. **D:** Infected Mewo (melanoma) cell. (A, B, and C from ref. 236, with permission, and courtesy of W. Ruyechan.)

The Core

One copy of the viral linear, double-stranded DNA genome is incorporated into each virion. Existing data regarding VZV structure and morphology are consistent with the models developed for HSV. Initial suggestions that the genome is wound tightly as a toroid around a dense protein rod have been questioned by recent findings. Particles fixed for electron microscopy with nondehydrating agents show a loose fibrillar cage of strands surrounding a dense cylindrical core of DNA fibers (202). Zhou et al. (266) examined herpes simplex virus by electron cryomicroscopy and found that the viral DNA was organized in regularly spaced layers within the nucleocapsid.

The Nucleocapsid

The VZV nucleocapsid is indistinguishable in appearance from that of other herpesviruses. Electron microscopic studies verify an assemblage of 162 capsomeres with a 5:3:2 axial symmetry in which pentameric proteins form the vertices of an 80- to 120-nm icosahedron, and hexameric elements comprise its facets (6). Whereas HSV nucleocapsids have electron-dense cores, VZV nucleocapsids obtained from virus grown in cell culture often lack dense cores, perhaps reflecting the low infectivity of VZV released from cultured cells (96,103). The specific viral gene products that assume these structural roles have not been verified but should include the products of VZV open reading frames (ORFs) 20, 23, 33, 33.5, 40, and 41, the analogs of HSV-1 capsid proteins VP19C (UL38), VP26 (UL35), VP24 (UL26), VP22 (UL26.5), VP5 (UL19), and VP23 (UL18), respectively.

The Tegument

The prototypical tegument component of the herpesviruses is the VP16 protein of HSV-1 and -2, and it was no surprise when the VZV homolog of VP16, the ORF10 gene product, was found in its tegument. Kinchington et al. showed that the VZV tegument also includes immediate-early (IE) proteins encoded by ORFs 4, 62, and 63 (124,126), and Stevenson et al. (228) showed that ORF47 is very likely a tegument protein.

The Envelope

The nucleocapsid assembles within patches of host cellular membranes that have been modified to display virally encoded glycoproteins. Modifications of the particle occur while it is transported along various membranous networks extending from the nuclear envelope to the cell surface, where infectious virions accumulate and bud outward to infect neighboring cells. Opportunities exist for the evolving particle to capture membranous elements of the nucleus, Golgi, rough endoplasmic reticulum, cytoplasmic vesicles, and the cell surface so that the “true” or singular source of the viral envelope is not known (3,41,72). The enveloped particle is pleomorphic to spherical, and 180 to 200 nm in diameter.

Physical and Chemical Properties

The VZV particle is fragile. Its lipid envelope renders VZV susceptible to disinfection by brief treatments with organic solvents (216). The particle is also subject to degradation by multiple other physical and chemical treatments, including detergents, proteases, heating to 60°C, prolonged storage at temperatures of –10°C or above, extremes of pH (<6.2 or >7.8), or ultrasonic disruption (23,91). Ironically, this latter physical procedure is traditionally used to release infectious cell-free virus from cells (23).

For analytic studies, VZV particles have been concentrated from stocks of cell-free virus using a variety of precipitation and centrifugation techniques (60,140). Enveloped particles band at a density of 1.274 g/cm^3 in cesium chloride; VZV DNA exhibits a buoyant density in cesium chloride of 1.705 g/cm^3 (140).

GENOMIC STRUCTURE AND ORGANIZATION

The complete nucleotide sequence of the VZV genome (strain Dumas) was determined in 1986 by Davison and Scott (50). The genome is a linear double-stranded DNA molecule, about 125,000 bp in length (Fig. 2). It consists of a unique long region (UL, about 105,000 bp) flanked by inverted repeat regions [terminal repeat long (TRL) and internal repeat long (IRL), each 88.5 bp], and a unique short region (US, 5,232 bp) flanked by internal repeat regions [terminal repeat short (TRS) and inverted repeat short (IRS), each 7,319.5 bp]. The inverted repeat structure of the VZV genome has been verified using electron microscopy of the VZV DNA molecule (66,90,237). The genome has a G+C content of 46%. The G+C content of the UL and US regions is 43% to 44%, whereas the G+C content of the TRS and IRS regions is 59% and of the TRL and IRL regions is 68%.

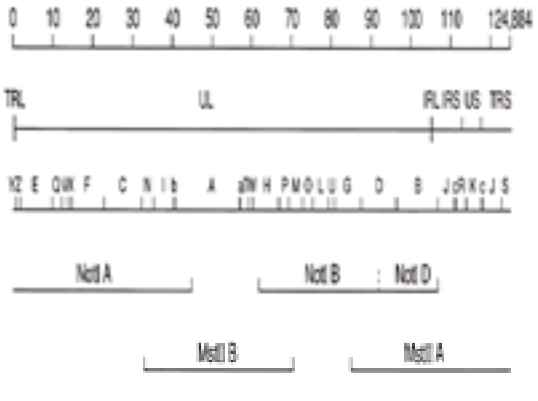

FIG. 2. Structure of the VZV genome. The prototype VZV genome (50) consists of 124,884 bp of double-stranded DNA (*line 2*) arranged into unique long (*UL*) and unique short (*US*) regions bounded by terminal repeats (*TR*) and internal repeats (*IR*) (*second line*). A *Bam*HI restriction map (*line 3*) is shown. *Not*I and *Mst*II restriction fragments are used to produce recombinant VZV by transfection (*lines 4 and 5*).

Although the HSV-1 genome is similarly organized, the length of the US region of VZV is about 40% the size of the HSV-1 US, whereas the length of the VZV TRL/IRL region is about 1% of the size of the corresponding region in HSV-1. In addition, the HSV-1 genome, unlike that of VZV, exists as four isomers in equal proportions.

The VZV genome is usually present as a linear molecule in virions; however, a circular, supercoiled form has been detected in rare virions (129,234). The VZV genome exists predominantly in two different isomeric forms (61,66,234). The US region is directed in either orientation in 50% of genomes; however, the UL is directed in one orientation in 95% of genomes and in the opposite orientation in only 5% of genomes (47,129). Thus, VZV, like HSV, can exist in four isomeric forms; however, in VZV two isomeric forms predominate. The ends of the VZV genome have a single unpaired nucleotide at each 3' terminus; these residues, C at the left end and G at the right end, may base-pair to form a circular molecule (47). In latently infected cells, the termini are adjacent, suggesting a circular molecule (27).

The genome consists of five regions with direct sequence repeats, termed R1 to R5 (50). R1 to R4 are GC-rich, like the GC-rich repeats present in HSV-1. The R1, R2, and R3 regions are located in the UL segment of the genome and each is in the coding sequence of a gene. The R1 region, located within ORF11, consists of four separate repeated elements (132). The R2 region, present within the glycoprotein C (gC) gene, consists of 42 bp repeats, the number of which differs among different strains of VZV (130). The largest repeat region, R3, is about 1,000 bp in length and is located in ORF22 (50). The R4 region is located in both IRS/TRS regions between ORF62 and ORF63 (and ORF70 and ORF71) and consists of multiple 27-bp elements (22). The R5 region is located between ORF60 and ORF61, consists of 88-bp and 24-bp repeats, and is AT-rich (109). The Dumas strain of VZV has only one copy of the R5 repeats, whereas other strains of VZV have multiple R5 repeats. The R1 to R5 repeats differ in length and have been used to distinguish different strains of VZV by restriction endonuclease analysis (109,235). The variability in the number of repeats implies that the genomes of the different strains of VZV have different overall lengths.

Sequencing of different isolates of VZV shows many minor nucleotide changes. For example, comparison of the nucleotide sequence of 34 kb of VZV strain Dumas with the Oka vaccine strain showed more than 80 nucleotide differences, resulting in 24 predicted amino acid differences (9). Moreover, comparison of the sequence of the Oka vaccine strain with its wild-type parental strain showed 15 nucleotide differences in ORF62 alone (92). The predicted amino-acid sequences of five proteins were identical in the vaccine and parental virus; however, ORF62 showed eight amino acid differences. The vaccine and parental Oka viruses could be distinguished by restriction fragment-length polymorphisms at some of these sites. Sequencing of glycoprotein genes from 10 VSV strains showed multiple sequence polymorphisms on gH amd gE (74a).

The genome contains at least 70 unique genes, three of which are present in two copies in the IRS and TRS regions (Table 1). Although some of the genes have putative polyadenylation signals shortly after the end of their ORFs, many are arranged in unidirectional families of up to four genes with a single putative polyadenylation signal at the 3' end of the family. Eleven VZV genes are thought to have overlapping reading frames. Two ORFs (ORF42, ORF45) are thought to be spliced segments of the same gene (50); one ORF (S/L) is spliced in the 31 noncoding region (119a).

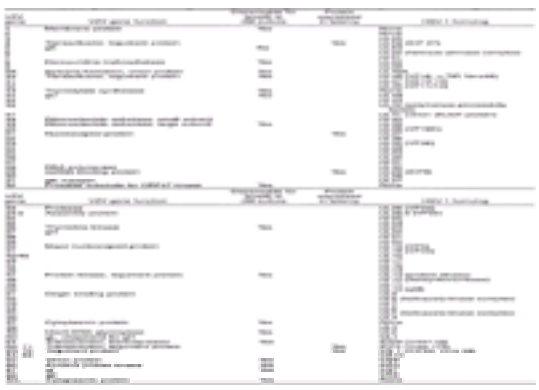

TABLE 1. *VZV genes with their functions and HSV-1 homologs*

Large portions of the VZV genome are colinear with the HSV-1 genome (51). Comparison of the predicted amino acid sequences of VZV and HSV-1 proteins indicates that the organization of homologous genes is similar in both genomes. Within the UL region of VZV, 56 of the 62 genes have HSV homologs, and 55 are located in a similar region of the genome. One gene in the UL region, ORF61, has its HSV-1 homolog, ICP0, located in the IRL/TRL region of HSV-1.

In the US region of VZV, all four of the VZV genes have homologs in the US region of HSV-1, although the positions of the genes are rearranged relative to those of HSV-1 (49). The three genes in the IRS/TRS regions of VZV (ORF62, ORF63, ORF64) each have HSV-1 homologs; ORF62 has its HSV-1 homolog ICP4 located in the IRS/TRS repeat region of HSV-1, whereas ORF63 and ORF64 have their HSV-1 homologs located in the US region of HSV-1.

Six genes in VZV (ORF1, ORF2, ORF13, ORF32, ORF57, and ORF S/L) do not have homologs in HSV-1. Five of these genes (ORF1, ORF13, ORF32, ORF57, and ORF S/L) are dispensable for growth of VZV in cell culture (33,36,42,119a,205). Four of them (ORF1, ORF2, ORF32, and ORF57) have homologs in equine herpesvirus types 1 and 4, which also are members of the alphaherpesvirus subfamily. A fifth gene, ORF13, has no homolog in the alphaherpesvirus subfamily, but it does have homologs in gammaherpesviruses (human herpesvirus 8, herpesvirus saimiri, and herpesvirus aeteles).

Comparison of the VZV genome with that of the seven other human herpesviruses indicates that VZV has at least 40 conserved genes shared with them, all of which are located in the UL region of VZV (46). These core genes are located in several blocks that have been rearranged during evolution of the viruses. Many of the genes are colinear between viruses within a given block. These conserved genes include an IE gene (ORF4), viral enzymes, and structural proteins (major nucleocapsid protein, glycoproteins gB, gH, gL).

There are only three regions of the VZV genome that apparently do not contain ORFs. These are located at the 3' end of UL between ORFs 60 and 61, and between ORFs 62 and 63 (and in its corresponding repeat ORFs 70 and 71). This last region contains an origin for VZV DNA replication, whereas other regions may contain sites important for cleaving and packaging viral DNA.

STAGES OF REPLICATION

Overall Pattern of Replication

Like other herpesviruses, VZV is thought to attach to cells by fusion of the viral envelope with the cytoplasmic membrane (Fig. 3). VZV proteins encoded by ORF4, ORF10, and ORF62 (124,126), located in the viral tegument, may be released and transported with the nucleocapsid to the nucleus, where they may serve to initiate transcription of viral genes. Other tegument proteins, including ORF47 and ORF63, may also be important early in infection. VZV IE messenger RNAs (mRNAs) are transcribed, transported to the cytoplasm, and translated, and the proteins are transported back to the nucleus. VZV early mRNAs may then be transcribed in the nucleus, translated in the cytoplasm, and transported back to the nucleus, where they facilitate viral DNA replication. VZV late mRNAs are then transcribed, they are translated in the cytoplasm, and capsid proteins are transported back to the nucleus for assembly of capsids. Newly replicated VZV DNA is then packaged into capsids and transported out of the nucleus. The capsids are enveloped and carried to the cytoplasmic membrane, where the virions are released.

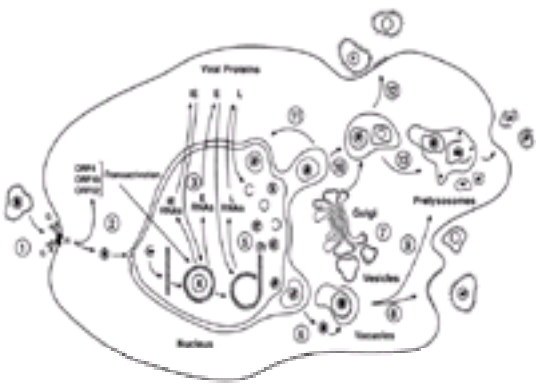

FIG. 3. Replication of VZV. The schema shown accommodates observations from multiple sources and relies as well on analogies drawn from studies of HSV replication. (*1*) Infectious virions attach to cell surface glycosaminoglycans (*G*) and engage specific, but still unidentified, receptors (*R*), and nucleocapsids penetrate into the cell. (*2*) The nucleocapsid enters the nucleus where, with the aid of regulatory tegument proteins encoded by ORFs 4, 10, and 62, (*3*) a cascade of

viral immediate-early (*IE*), early (*E*), and late (*L*) transcripts and proteins are synthesized. (*4*) Viral DNA replication follows a rolling-circle model. (*5*) Nucleocapsids are assembled and package newly synthesized DNA. Controversy exists regarding the mode of nucleocapsid egress from the nucleus, the site of envelopment, and the pathway leading to release of particles from the infected cell. (*6*) In one view by Gershon et al. (88), nucleocapsids are transiently enveloped as they bud through the inner nuclear lamellae. These nucleocapsids lose their envelopes when they fuse with the rough endoplasmic reticulum, resulting in the release of naked nucleocapsids in the cytoplasm. (*7*) Virion glycoproteins are synthesized in the rough endoplasmic reticulum, mature in the Golgi, and line the inner surfaces of cytoplasmic vesicles. Viral tegument proteins are synthesized by free ribosomes and line the outer surfaces of the cytoplasmic vesicles. (*8*) Nascent particles are drawn into these vesicles and intact, enveloped virions coalesce within them. These vacuoles may fuse with the cell membrane to release infectious progeny. (*9*) Many of the vacuoles, however, fuse with pre-lysosomes in which the particle is partially degraded before release, leading to an abundance of defective progeny VZV. (*10*) Another model by Harson and Grose (103) proposes that nucleocapsids retain their envelopes as they egress the nucleus. Their envelopes are glycosylated by fusion with Golgi-derived vesicles. Late in infection some particles reenter the nucleus (*11*), although most are transported to the cell surface (*12*) for release or (*13*) to pre-lysosomes. Pathways that avoid the pre-lysosomes (*8* and *12*) must exist in humans for efficient spread of infection

Studies using VZV-infected cells to inoculate uninfected cells indicate that viral proteins are expressed 4 to 6 hours after infection, and virus spreads to neighboring cells as early as 8 to 16 hours after infection (11,204).

Attachment, Entry, and Uncoating

Specific cell surface receptors have been identified for some alphaherpesviruses but not VZV. For all alphaherpesviruses, including VZV, the initial attachment to the cell surface appears to entail nonspecific electrostatic interactions between viral envelope glycoproteins and cellular surface glycosaminoglycans like heparan sulfate, but unlike chondroitin sulfate (267). In HSV, glycoproteins gB and gC participate in electrostatic attachment to cells. Work to date verifies that gB binds to heparin sulfate (115), but it is uncertain that gC does so (R. Williams and S. Straus, unpublished observations). Zhu et al. (267) reported that VZV glycoproteins bearing mannose-6-phosphate residues bind to another low-affinity receptor, facilitating entry.

Following low-affinity interactions with the cell surface, HSV gD binds to one of a family of cell surface receptor molecules known as the herpesvirus entry mediators. VZV, however, possesses no obvious gD homolog, and the virus may not utilize any of the known herpesvirus entry mediators.

Following the interaction of gD with its receptor, HSV gB, gH, and gL are required for fusion of the viral envelope with the cell membrane. VZV possesses homologs of all three of these glycoproteins, but all of their roles in fusion of the viral envelope and subsequent penetration of the nucleocapsid into the cell are unproven. Rodriquez et al. (209) have shown, however, that VZV gH facilitates fusion of the VZV envelope with the cell membrane. Monoclonal antibody to gH inhibits syncytia formation and cell-to-cell spread of VZV in culture.

Because antibodies directed against VZV gB, gE, gH, and gI all neutralize infectivity (79,153,261), these four proteins must participate in one or more aspects of attachment and penetration of the particle. Once penetration has occurred, the nucleocapsid is transported to the nuclear surface, through which it penetrates and releases its genomic contents. It is not known whether cellular cytoskeletal elements facilitate centripetal movement to the nucleus or in what form the viral core arrives there. Because various preformed tegument proteins (ORFs 4, 10, and 62 products) transactivate IE gene expression, they must accompany the viral core into the nucleoplasm.

Viral Transcripts and Their Products

All evidence suggests that the process of VZV transcription is a coordinated one that follows a temporal program, as does HSV transcription. Nonetheless, the inability to obtain high-titered, cell-free VZV has made it difficult to achieve synchronous infection of cells in culture required to define the temporal classes of individual VZV genes. Infection of cells with relatively high-titered VZV in the presence of cycloheximide followed by treatment with actinomycin-D has identified ORF4 (53), ORF62 (81,218), and ORF63 (52) as IE proteins. Although each of the HSV-1 IE genes contains an upstream TAATGARAT sequence element, only VZV ORF62 encodes an upstream TAATGARAT-like element (131,173) that the other VZV IE genes lack.

Transcription Map

The most extensive transcription mapping of VZV used double-stranded DNA and single-stranded RNA probes to map the polarity of transcripts in VZV-infected cells (207). There were 78 abundant transcripts of 6.8 kb or less, and the orientation of 77 of the transcripts was determined. Thirty-five of these RNAs were transcribed from left to right, and 42 were transcribed from right to left. Many of the transcripts were located in regions corresponding to the ORFs predicted by Davison and Scott (50) (Fig. 4), but most transcripts could not be assigned with certainty to a given gene.

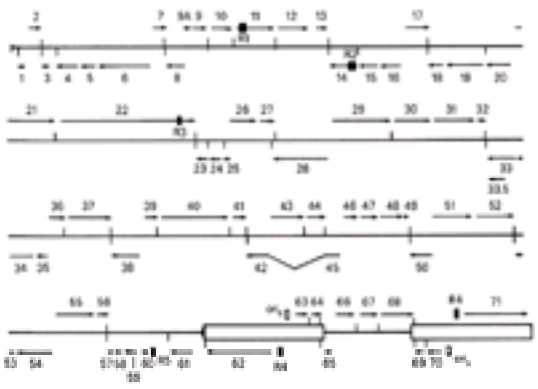

FIG. 4. VZV ORFs. The genome contains 69 unique ORFs, including ORF9A and ORF33.5 (an in-frame truncation of ORF33), but not counting ORFs 69 through 71, which are duplications of ORFs 62 through 64. The genome consists of unique regions (*horizontal lines*), terminal and internal repeats flanking the unique short region (*open boxes*), and terminal and internal long repeats flanking the unique long region (*short vertical lines*). ORF transcripts are numbered and shown by *arrows*, and potential polyadenylation sites are indicated by *light vertical lines.* Repeats (R1 to R5) are indicated (*filled rectangles*). The origins of DNA replication (oris) are shown. ORF S/L, which begins at the right end of the circularized genome and extends across the terminal repeat into the unique long region at the left end of the genome, is not shown. (Adapted from ref. 50, with permission.)

Transcripts for 12 viral genes (ORFs 4, 14, 21, 28, 29, 36, 37, 61, 62, 63, 67, and 68) have been formally mapped, and several features of VZV transcription can be gleaned from these studies. First, a single VZV ORF can be encoded from multiple colinear and coterminal transcripts. For example, three transcripts (1.65, 2.9, and 3.6 kb) map to gI (ORF67); the most abundant transcript (1.65 kb) is thought to be the primary transcript used for expression of the gene (117,138). Second, VZV uses TATA elements whose sequences may differ substantially from the standard consensus sequence. Although some transcripts are generated from standard TATA sequences [ORF61 (229)], other transcripts arise from TTTTAA [ORF62 (156)], ATAAAA [ORF67 (138)], or ATTTAAATT [ORF14 (137)] sequences located 25 to 33 bases upstream from the transcription start site. Third, VZV polyadenylation signals may deviate from the typical AATAAA or ATTAAA elements. Although some transcripts have a typical polyadenylation signal [ORF67 (138)], other transcripts use AGTAAA [ORF36 (50)], ATAAA, ATAATT, or ACGTAAAA [ORF14 (137)]. Fourth, VZV, like HSV-1, contains GU-rich sequences GTCTGTGT and TGGTGGTA [ORF14 (137)], downstream of polyadenylation sites that may be important for termination of transcription. Fifth, VZV genes that are homologous to HSV-1 genes may manifest different patterns of transcription. VZV ORF14 (137) and VZV ORF61 (229) are thought to be expressed from unspliced transcripts, whereas their HSV-1 homologs gC and ICP0, respectively, are encoded by spliced transcripts. The VZV ORF14 transcripts are derived from coterminal 5' ends, whereas the HSV-1 gC transcripts have coterminal 3' ends (137). Sixth, different strains of VZV may vary in the level of transcription of a given gene. VZV strain Scott expresses about 20-fold more gC mRNA than the amount of gC mRNA expressed from a particular strain of VZV Oka (137).

Another feature of VZV transcription was revealed in studies of the VZV polymerase (ORF28) and major DNA-binding protein (ORF29) genes. These two genes are transcribed in opposing directions from overlapping promoters. The ORF28 and ORF29 promoters are responsive to VZV transactivators, and the activity of one promoter cannot be separated from the other (161). VZV ORF62 protein interacts with the transcription factor USF to activate the bi-directional VZV ORF28 and ORF29 promoter (159) and the ORF4 promoter (163). In VZV-infected cells, ORF28 is transcribed as a 4.1-kb RNA, and ORF29 may arise from either of two RNAs of 4.1 and 4.2 kb. In contrast, in human trigeminal ganglia, ORF29 is expressed only from the 4.2 kb transcript, whereas ORF28 is apparently not expressed (158).

Regulators of Transcription

ORF62

VZV ORF62 encodes a 175-kd phosphoprotein (76,81) that is incorporated into the viral tegument (126). The protein is primarily localized to the nucleus of infected cells. Although ORF62 contains a nuclear localization signal (14), coexpression of ORF66 with ORF62 results in cytoplasmic accumulation of ORF62 (125). ORF62 is the homolog of HSV-1 ICP4 and has been shown to complement HSV-1 ICP4 mutants (76). An HSV-1 mutant in which both copies of ICP4 have been replaced by VZV ORF62, can replicate efficiently, further confirming that ORF62 is the functional homolog of ICP4 (57). Replacement of the DNA binding domain of ICP4 with that of VZV ORF62 resulted in an HSV mutant that cannot appropriately regulate expression of ICP4 (247). VZV ORF62 increases the infectivity of transfected VZV DNA (182).

VZV ORF62 and HSV-1 ICP4 share extensive homology in conserved regions 2 and 4 (155), including a conserved DNA binding domain that recognizes similar sites in their corresponding promoters (260). HSV ICP4 and ORF62 proteins can bind to the same consensus sequence in the ICP4 DNA binding site (15). A single amino acid change in the DNA binding domain of ORF62 results in loss of its transactivating function (244). While the DNA binding domain of ORF62 can dimerize and form a heterodimer with the corresponding domain of ICP4 (245), the DNA binding domain of ORF62 is less sequence-specific and does not induce DNA bending upon binding its own promoter, when compared with HSV-1 ICP4 (246).

VZV ORF62 transactivates VZV putative IE (ORF4, ORF61), early (ORF36), and late gene promoters in transient expression assays (111,196). VZV ORF62 activates its own promoter in lymphocytes but represses its own promoter in simian cells (58,76,198). The differential activation of a broad spectrum of promoters by ORF62 reflects its capacity to interact with cellular transcription factors and depends on the sequence of the TATA motif in the targeted promoter (194).

Some activities of VZV ORF62 are different from those of HSV-1 ICP4 in transient expression assays. For example, HSV-1 ICP4 represses the expression of some HSV-1 IE genes, such as ICP0 and ICP4. Furthermore, although ICP4 activates the HSV-1 thymidine kinase (TK) promoter in transient expression assays, it does not activate the VZV ORF36 promoter (112). VZV ORF62 contains a transcriptional activation domain at its amino terminus that is not conserved with HSV-1 ICP4 (30,197).

Only 45 bases upstream of the ORF62 cap site, or 15 bases upstream of the TATA box, are necessary to mediate activation of the ORF62 promoter by ORF62 in lymphocytes. In contrast, 124 bases upstream of the ORF62 cap site are necessary to mediate activation by HSV-1 ICP4; therefore, activation of the ORF62 promoter by ORF62 and HSV-1 ICP4 appears to occur through different mechanisms (198). HSV-1 infection stimulates expression of ORF62 (76). The upstream region of ORF62 contains three TAATGARAT-like elements. These elements contribute to the transactivation of the ORF62 promoter by the VZV ORF10 protein (173). The ORF62 promoter also contains a cyclic AMP-responsive element and a GA-rich element that are important for transactivation of the promoter. The ORF10 protein forms a complex with two of the TAATGARAT-like elements in the ORF62 promoter that lack an overlapping octamer-binding motif, but not with the TAATGARAT-like element with the octamer motif. Two cellular proteins, Oct1 and host cell factor (HCF) form a complex with the ORF10 protein and at least one of the TAATGARAT-like elements on the ORF62 promoter. Oct2 also binds to the TAATGARAT-like elements on the ORF62 promoter and inhibits ORF10-mediated transactivation. Two additional cellular proteins, the NF-Y CCAAT box-binding protein (175) and a member of the ATF/AP-1 family, have also been shown to bind to the ORF62 promoter (156).

ORF61

ORF61 encodes a 62- to 65-kd phosphoprotein that localizes to the nucleus of infected cells (229,230). Unlike the other VZV IE genes, ORF61 is not present in the viral tegument (124). VZV ORF61 protein is homologous to HSV ICP0 and can functionally complement an HSV-1 ICP0 deletion mutant (179). The amino terminus of ORF61 can substitute for the corresponding region of ICP0 in transient expression assays (174).

VZV ORF61 protein transactivates putative VZV IE, early, and late promoters in transient expression assays (181). When transfected into lymphocytes, it cannot transactivate diverse VZV promoters (196). VZV ORF61 can either repress or enhance the activation of other VZV genes by ORF4 and ORF62, depending on the transfection conditions and cell lines used (181,185,196). The amino terminus of ORF61 contains a RING-finger domain that binds zinc (74) and is required for the transactivating activity of the full-length protein (174). Transient expression of VZV ORF61 enhances the infectivity of VZV and HSV-1 DNAs (181). Carboxyl-terminal truncation mutants of ORF61 that retain the RING-finger domain act as dominant-negative transrepressors and reduce the infectivity of viral DNA.

HSV-1 ICP0, the homolog of ORF61, shows different patterns of activities in transient expression assays. Whereas ICP0 activates the HSV-1 TK promoter in transient expression assays, ICP0 does not activate the VZV ORF36 promoter (112). Unlike ORF61, ICP0 does not show repressing activity in transient expression assays.

Deletion mutants of VZV ORF61 can replicate, but they are impaired for syncytia formation and growth in cell culture (32). Cells infected with these mutants show reduced expression of gE but normal levels of ORF62 protein.

ORF4

VZV ORF4 encodes a 52-kd protein that is predominantly localized to the cytoplasm; however, when coexpressed with ORF62, ORF4 protein is primarily located in the nucleus (54). ORF4 protein is associated with viral particles, most likely in the tegument (124). ORF4 has amino acid sequence homology to HSV-1 ICP27; however, infection of cultured cells with VZV does not effectively complement infection with an HSV-1 ICP27 mutant (77), and a cell line expressing ORF4 does not complement infection with HSV-1 ICP27 mutants (178). The region of greatest homology of ORF4 with ICP27, the carboxyl portion, contains a putative zinc-finger domain; however, mutagenesis of this domain indicates that several cysteine residues are not required for functional activity of ORF4 (195). The amino terminus of ORF4 protein is required for transactivation and can functionally replace the corresponding domain of ICP27 (176).

In transient expression assays, ORF4 protein transactivates expression of some, but not all, VZV putative IE promoters, early promoters, and late promoters. For example, ORF4 does not transactivate expression of the ORF63 IE or ORF28 putative early gene promoter. In transient expression assays, VZV ORF4 synergizes with VZV ORF62 to transactivate expression of putative IE, early, and late promoters. ORF4 activation of target genes occurs at the level of transcription (195) and probably also at a posttranscriptional level (53). Gene activation by ORF4 requires its dimerization, and several portions of the protein are required for its transactivation function (13). ORF4 protein has been shown to directly interact with TATA-binding protein, transcription factor IIB, and the nuclear factor (NF)-kB p50 and p65 subunits *in vitro* (56). ORF4 protein also binds to an underphosphorylated form of ORF62 (226). Unlike HSV ICP27, ORF4 has not been shown to have transrepressing activity (55,111,178,196). The ORF4 promoter was recently mapped, and a region between 59 and 69 bases upstream of the transcription start site was shown to be critical for its activity (163).

ORF63

ORF63 encodes a protein of 45 kd; additional smaller proteins of 38 and 28 kd have also been reported in infected cells (52,124,189). ORF63 is phosphorylated predominantly in the third quarter of the protein (231). ORF63 protein is located primarily in the nucleus of infected cells, although early in infection the protein is present in both the cytoplasm and the nucleus. The protein is present in virions, most likely in the tegument. ORF63 is the homolog of HSV-1 ICP22. Although ORF63 has been reported to repress the ORF62 promoter, another study suggested that ORF63 has little transregulatory activity (114,133).

Replication of Viral DNA

Replication Proteins

VZV encodes homologs (see Table 1) of each of the HSV-1 genes that are essential for origin (ori)-dependent replication of viral DNA; however, studies of VZV have not yet been performed to determine the minimum repertoire of genes needed to do so.

DNA Polymerase

The large subunit of the VZV DNA polymerase is encoded by ORF28 (50,73). The VZV DNA polymerase has been partially purified from VZV-infected cells and requires magnesium for maximal activity. Unlike the cellular polymerase, the VZV enzyme is sensitive to phosphonoacetic acid, and its activity is enhanced in the presence of ammonium sulfate (151,164). Comparison of the VZV and HSV-1 DNA polymerases indicates that the VZV enzyme has a different elution profile during phosphocellulose chromatography, is less sensitive to inhibition by phosphonoacetic acid, is less active in the presence of ammonium sulfate, and is more thermolabile (150). The large subunit of the VZV DNA polymerase gene has been inserted into baculovirus, and the recombinant 130-kd protein has enzymatic properties and inhibition profiles similar to those of the native protein from VZV-infected cells (73). The small subunit of the VZV polymerase is thought to be encoded by ORF16.

DNA Binding Proteins

VZV ORF29 encodes the 130-kd single-stranded DNA binding protein that localizes to the nucleus of VZV-infected cells (50,127). About 50% of the solubilized ORF29 protein extracted from VZV-infected cells binds to single-stranded DNA, and the protein can be eluted with 0.5M sodium chloride. Both the VZV ORF29 and the homologous HSV-1 ICP8 proteins bind single-stranded DNA, although the VZV protein binds with lower efficiency (127) and cannot substitute for its HSV homolog with other HSV proteins in a DNA replication assay (254). The ORF29 protein binds to the VZV gI promoter *in vitro* (19). Whereas the ORF29 protein does not affect transcription when expressed alone in transient expression assays, the ORF29 protein increases or decreases transactivation of a reporter gene by the ORF62 protein, depending on the cell line used (19). ORF29 protein is secreted from VZV-infected cells *in vitro* and the extracellular protein can be endocytosed by neurons (7).

VZV ORF51 encodes the ori binding protein (233). The carboxyl portion of ORF51 binds to three DNA sequence motifs in the VZV origin of replication *in vitro* with similar affinities (24). As in its HSV-1 homolog UL9, a CGC triplet in the VZV origin is required for binding of ORF51 protein. VZV ORF51 can substitute for UL9 with other HSV-1 proteins in an origin-dependent HSV DNA replication assay (254). ORF51 can partially complement the growth of an HSV-1 UL9-null mutant *in vitro* (25). Five additional DNA binding proteins have been identified, ranging from 175 to 21 kd (208).

Replication of the Genome

VZV DNA replication is thought to occur in four stages, based on evidence from replication of the HSV genome (47). First, the linear DNA molecule in the virion circularizes shortly after it enters the cell. VZV has an unpaired nucleotide at both 3' ends of the genome (C at the left end, G at the right end). These ends could ligate together to form a circle; the presence of a fused UL-US joint, demonstrated by cloning of VZV genomic DNA, supports this concept. Second, the circular DNA molecule begins to replicate, and isomerization may occur by homologous recombination between the inverted repeats. Third, more extensive replication of the circular molecule generates head-to-tail concatemers, probably by a rolling-circle mechanism (see Fig. 3). Fourth, the concatemers of VZV DNA are cleaved by sequence-specific nucleases to generate linear DNA for packaging into virions. The finding of concatemeric VZV DNA molecules supports the view that VZV, like HSV-1, replicates by a rolling-circle mechanism (232).

Both the VZV and the HSV-1 genomes have an AT-rich sequence near the left end of the genome and an AT-rich sequence surrounded by a GC-rich sequence at the right end of the genome. The HSV-1 sequences have been implicated in cleavage and packaging, and thus the VZV sequences may have a similar role (48). Because VZV exists predominantly as two of four isomers, cleavage presumably occurs at one UL-US joint 95% of the time, but occasionally (5%) at the other UL-US joint to yield the minor two isomers (129).

The VZV genome contains an origin of replication in the IRS/TRS regions—termed ori $_S$—located between ORFs 62 and 63 [and between ORFs 70 and 71 (50,232)]. It does not contain a replication origin within the UL region, ori $_L$, as HSV-1 and HSV-2 do. Transfection of cells with a plasmid containing ori $_S$, followed by infection of the cells with VZV, results in replication of the plasmid containing ori $_S$. The ori$_S$ contains a 45-bp, almost-perfect palindrome with the sequence $(TA)_{17}$ at its center. Mutation of the palindrome results in loss of replicative activity. HSV-1 has the sequence $(AT)_5$ and $(AT)_6$ in its ori$_L$ and ori$_S$, respectively. Adjacent to the VZV ori$_S$ palindrome is the sequence CGTTCGCACTT, which is present in both the ori$_S$ and the ori$_L$ of HSV-1 (232). Further upstream from the ori$_S$ palindrome is the sequence CATTCGCACTT. Transfection experiments using the VZV replication assay indicate that the 45-bp palindrome and the sequence CGTTCGCACTT are essential for replication activity, whereas the sequence CATTCGCACTT contributes additional activity but is not essential. A second CGTTCGCACTT sequence further upstream from the other two sequences does not contribute to replication activity (233).

The VZV ori-binding protein (encoded by ORF51) recognizes three sites adjacent to the palindrome at the sequences CGTTCGCACTT (located at two sites) and CATTCGCACTT (24). The former sequence conforms to, whereas the latter sequence contains a single base mismatch of, the consensus sequence YGYTCGCACT used by the HSV-1 ori-binding protein.

As indicated, VZV does not have an analog of the HSV-1 ori $_L$ sequence. Transfection with a plasmid containing the VZV region corresponding to HSV ori $_L$, between ORFs 28 and 29, followed by infection with VZV, does not result in replication of the plasmid. Furthermore, the ORF28-to-ORF29 region does not contain a palindrome similar to that seen in HSV-1 ori $_L$. VZV is not able support the replication of a plasmid containing HSV-1 ori $_S$; however, HSV-1 is able to partially support the replication of a plasmid containing VZV ori $_S$ (232).

Other Viral Enzymes

Thymidine Kinase

VZV ORF36 encodes the viral TK gene (215). The VZV enzyme is a 70-kd homodimer composed of two identical 35-kd subunits. The viral enzyme has optimal activity at a higher pH and it has a broader substrate specificity than its cellular homolog (26,98). The VZV enzyme has greater deoxycytidine kinase than TK activity, unlike its HSV-1 counterpart (59,84,190); therefore, it has been referred to as a deoxypyrimidine kinase. In addition, the VZV enzyme is more thermostable and more susceptible to inhibition by deoxythymidine triphosphate than its HSV-1 homolog (190). The enzyme is active when expressed in *Escherichia coli* (215) or when translated *in vitro* (143). The VZV TK phosphorylates and thereby activates acyclovir and the similar guanosine analog, penciclovir. Most acyclovir- (and penciclovir-) resistant strains of VZV have point mutations in the TK gene near the adenosine triphosphate (ATP) or thymidine binding site, or that result in a truncated protein (172,214). Mutagenesis of the VZV deoxypyrimidine kinase gene followed by phenotypic selection in *E. coli* has been used to define the ATP and substrate binding sites for the enzyme (239). Replacement of the HSV TK with its VZV homolog resulted in an HSV mutant with *in vitro* sensitivities to nucleoside analogs that resembled VZV rather than HSV (17). The VZV TK gene is dispensable for replication of the virus *in vitro*.

Ribonucleotide Reductase

VZV ORF18 and 19 are homologous to the small and large subunits of HSV-1 ribonucleotide reductase, respectively (50). The ribonucleotide reductase enzyme has been partially purified from VZV-infected cells and has similar enzymatic activities to its HSV-1 homolog. Both the VZV and HSV-1 enzymes differ from the cellular enzyme in that they are not inhibited by increased intracellular levels of deoxyribonucleotides; however, the activity of the VZV enzyme, unlike that of its HSV-1 counterpart, is inhibited by ATP and magnesium (225). Chemical inhibition of the VZV ribonucleotide reductase potentiates the antiviral activity of acyclovir, presumably by decreasing the intracellular pool of deoxyribonucleoside triphosphates and increasing the level of acyclovir triphosphate (224). Antibody to a synthetic peptide corresponding to the small subunit of the HSV-1 ribonucleotide reductase immunoprecipitates the small subunit of the VZV enzyme (63). VZV ORF19 is not essential for growth of the virus in cell culture. Deletion of VZV ORF19 from the viral genome abolishes viral ribonucleotide reductase activity, impairs the growth of the virus *in vitro*, and potentiates its inhibition by acyclovir (105).

Protein Kinases

VZV ORF47 encodes a 54-kd serine-threonine protein kinase, a capsid/tegument component, whose putative catalytic domain is homologous to that of the HSV-1 enzyme encoded by UL13 (221,228). The activity of VZV ORF47 protein kinase is enhanced by manganese, and the enzyme phosphorylates both acidic and basic proteins. Like its UL13 HSV-1 homolog, ORF47 protein uses both ATP and guanosine triphosphate (GTP) as phosphate donors and phosphorylates exogenous casein (188). This protein phosphorylates both itself and ORF62 protein in protein kinase assays *in vitro* (189); however, ORF47 is not required for phosphorylation of ORF62 in VZV-infected cells *in vitro* (106). VZV ORF47 protein is responsible for posttranslational modification of VZV ORF32 protein associated with its phosphorylation (205). Although ORF47 is dispensable for VZV replication in cell lines (106), it is important for infection of, and spread from, primary lymphocytes *in vitro* (223). In addition, ORF47 is required for growth of VZV in human fetal lymphocytes and skin in the severe combined immunodeficiency (SCID)-hu mouse (168).

VZV ORF66 encodes a 48-kd phosphoprotein located in the cytoplasm of infected cells (228). ORF66 is thought to encode a serine–threonine protein kinase whose putative catalytic domain is homologous to the corresponding region of the HSV-1 US3 protein kinase (154). Cells infected with VZV unable to express both ORF47 and ORF66 lack a 48-kd phosphoprotein that is present in cells infected with VZV unable to express either of the VZV proteins alone (108). ORF66 is dispensable for replication *in vitro* and in human fetal skin in the SCID-hu mouse; however, VZV unable to express ORF66 is impaired for growth in human fetal lymphocytes in the mouse (168).

Miscellaneous Viral Enzymes

VZV ORF8 encodes a dUTPase, which hydrolyzes dUTP to dUMP and pyrophosphate (211). VZV unable to express this enzyme is unimpaired for growth *in vitro*.

VZV ORF13 encodes the viral thymidylate synthetase, which is dispensable for replication of virus *in vitro* (33). *E. coli* expressing the VZV enzyme or virus-infected cells contain a protein that binds to 5-fluoro-2-deoxyuridylate, a ligand specific for the enzyme (242).

VZV ORF59 encodes the VZV uracil DNA glycosylase that removes uracil residues from DNA (206). VZV unable to express this enzyme, as well as virus expressing an inhibitory protein that inhibits both the viral and cellular enzyme, are unimpaired for replication in cell culture.

VZV-infected cells express a DNase activity (26) thought to be encoded by ORF48, the homolog of HSV-1 UL12.

Late Gene Products

ORF10

VZV ORF10 encodes a 50-kd protein that is incorporated into the viral tegument (126). ORF10 protein is expressed diffusely in the cytoplasm after transfection into cells; however, ORF10 protein is localized to the trans-Golgi network after cotransfection of ORF10 and gI or after the virus infection (252a). The VZV protein is the homolog of the HSV-1 a-transinducing factor VP16. ORF10 complements an HSV VP16 mutant that lacks the transactivating function of VP16, and it enhances the infectivity of transfected VZV virion DNA (180).

VZV ORF10 transactivates the VZV ORF62 but not the putative IE ORF61 and ORF4 promoters. ORF10 forms a complex with TAATGARAT-like sequences and Oct1 and HCF to transactivate the IE ORF62 promoter (173). VP16 is more active than ORF10 in transactivating a VZV IE gene promoter in transient expression assays (180). Whereas the transactivating domain of VP16 is located at its carboxyl terminus, the activation domain of ORF10, which has a similar sequence, is located near the amino terminus of the protein (177). Surprisingly, although VP16 is essential for replication of HSV, ORF10 is dispensable for replication of VZV in cell culture (35).

ORF9A and ORF65

VZV ORF9A encodes a 7-kd virion protein located in the membranes of infected cells (211; Sato and Cohen, unpublished data). Although the protein is not required for replication in cell culture, VZV unable to express the protein is impaired for syncytia formation *in vitro*.

VZV ORF65 encodes a 16-kd virion protein that localizes to the Golgi apparatus of virus-infected cells (32a).

Nucleocapsid Proteins

ORF33 (Protease)

VZV ORF33 encodes the precursor of the viral serine protease. Expression of ORF33 in baculovirus results in a protein that is autocatalytically cleaved at two sites (86,157). The mature 31-kd protease is the amino-terminal cleavage product. The VZV protease has been expressed in a functional form in bacteria and the crystal structure has been determined (203). The active form of the VZV protease is predicted to be a dimer. The structure has an overall protein fold similar to that of the cytomegalovirus protease. A serine and two histidines comprise the catalytic site of the VZV protease; mutation of one of these histidines in the protease precursor protein prevents self-cleavage.

ORF33.5 (Assembly Protein)

The 40-kd precursor of the viral assembly protein is encoded by VZV ORF33.5, which is located in the 3' half of the ORF33 gene. The mature 37-kd assembly protein is obtained after cleavage of its precursor by the viral protease (157,201). The assembly protein is presumed to act as a scaffolding protein for assembly of the nucleocapsid, and it can function as a scaffolding protein for HSV capsid shell proteins. When expressed *in vitro*, the ORF33.5 protein forms long, hollow rods, predominantly in the nucleus of cells (201).

Three nucleocapsid forms have been detected in VZV-infected cells (102). Immature B capsids lack viral DNA and contain 32- and 36-kd proteins, presumed to be the mature viral protease and assembly proteins. Mature C capsids contain viral DNA but lack the 32- and 36-kd proteins. Intermediate B/C capsids are thought to contain viral DNA and the 32- and 36-kd proteins. These proteins are probably identical to the 32- and 36-kd proteins that bind DNA and are present in the nuclear matrix of VZV-infected cells (82).

Other Nucleocapsid Proteins

VZV ORF40 encodes the 155-kd major nucleocapsid protein, which is present in the nucleus of VZV-infected cells. Monoclonal antibody to the VZV major nucleocapsid protein cross-reacts with the corresponding protein (UL19) from HSV-1; conversely, monoclonal antibody to the HSV-1 UL19 protein cross-reacts with VZV ORF40 protein (249).

VZV ORF21 encodes a 115-kd protein located in the nucleocapsid (144). The protein localizes to both the nucleus and the cytoplasm of infected cells.

VZV Glycoproteins

VZV Glycoprotein B

Glycoprotein B (gB, gpII), encoded by VZV ORF31, has a higher degree of amino acid identity (49%) with its HSV-1 homolog than the other VZV glycoproteins (which range from 24% to 27% (139)]. Polyclonal or certain monoclonal antibodies raised against HSV gB detect both HSV and VZV gB (69). Glycoprotein B is the second most abundant VZV glycoprotein. It is cleaved in the cell into two polypeptides that are held together by disulfide bonds. Thus, gB appears as a 140-kd protein under nonreducing conditions; under reducing conditions, two proteins of 60- and 70-kd are detected. Two regions in the cytoplasmic domain of gB are essential for its transport from the endoplasmic reticulum to the Golgi (107a). Glycoprotein B contains both N- and O-linked sugars and is sialated, sulfated, and palmitylated (67,94,118,169). Recombinant gB binds to cellular heparan sulfate proteoglycans, and the recombinant protein delays VZV adsorption of virus to cells (115). Like its HSV homolog, gB may also be important for entry of VZV into the cell (250).

VZV Glycoprotein C

Glycoprotein C (gC, gpV), encoded by ORF14, contains N-linked sugars. Different strains of VZV contain different numbers of the R2 repeat unit, which is located in the coding sequence of gC; therefore, the size of gC varies from 80- to 170-kd, depending on the strain of virus. The amount of gC expressed in infected cells also varies depending on the strain of VZV and on the level of transcription of gC RNA (128,130,137). Glycoprotein C is dispensable for growth in cell culture (34), but gC-negative mutants are impaired for replication in fetal human skin in the SCID mouse (167).

VZV Glycoprotein E

VZV glycoprotein E (gE, gpI), the product of VZV ORF68, is an 85- to 100-kd protein that contains both N- and O-linked sugars and is heavily sialated (68,70,94,171). Glycoprotein E is the most abundant VZV glycoprotein on the surface of virus-infected cells (171). It is modified by myristylation, palmitylation, and sulfation (67,100,186). Its monomeric form is phosphorylated at serine and threonine residues by casein kinase II, whereas the dimeric form is phosphorylated by a tyrosine kinase (191,264). Glycoprotein E forms a heterodimer with VZV gI that requires the amino terminus of gI for its interaction (123,263). Glycoprotein E binds the Fc receptor of human immunoglobulin (139). A portion of gE is homologous to the cadherins (165a). Expression of gE in epithelial cells induces formation of tight junctions and enhances establishment of trans-epithelial resistance, resulting in increased cell-cell contact. A naturally occurring isolate of VZV that lacks a B cell epitope of gE has enhanced cell-to-cell spread both *in vitro* and in human fetal skin implants in the SCID mouse (213a).

After synthesis and maturation in the Golgi apparatus, gE is transported to the cell membrane either directly or by the trans-Golgi network (TGN). This transport requires a tyrosine motif (AYRV) and an acidic amino acid–rich patch located in the cytoplasmic tail (268,269). At the cell membrane, gE can undergo endocytosis to the TGN and subsequently be recycled through endosomes back to the cell membrane (5,193). A tyrosine localization motif (YAGL) in the cytoplasmic tail of gE is required for its endocytosis (193). Although gI is also endocytosed from the cell surface and has a methionine-leucine internalization motif in its cytoplasmic tail, the gE–gI complex undergoes endocytosis more efficiently than either protein alone (4,192).

VZV Glycoprotein H

Glycoprotein H (gH, gpIII), the product of VZV ORF37, is a 118-kd glycoprotein that contains N-linked but not O-linked sugars, and it is sialated and sulfated (67,94,119,170) and secreted into the medium of cells in culture (219). Glycoprotein H is the third most abundant VZV glycoprotein. It acts as a fusogen that facilitates cell-to-cell spread of virus because monoclonal antibody to gH blocks the entry, cell-to-cell spread, and egress of VZV from infected cells (113,209). Glycoprotein H requires gL for processing in the Golgi and transport to the cell surface (65,80,152). Coexpression of gH with either gE or gI allows an immature form of gH to be transported to the cell surface but it is not fusogenic (64).

VZV Glycoprotein I

Glycoprotein I (gI, gpIV), encoded by VZV ORF67, is a 58- to 62-kd glycoprotein that contains N- and O-linked sugars (94,171,262). Glycoprotein I is phosphorylated on its cytoplasmic tail by a cyclin-dependent kinase (265). It is a component of the gE–gI Fc receptor (139). It is not required for replication of VZV in human cells, but it is required for replication in Vero cells (31,149). VZV gI mutants are impaired for efficient cell-to-cell spread of virus, adsorption to cells, and syncytia formation *in vitro*. A threonine in the endodomain of gI targets the glycoprotein to the TGN when expressed alone in cells. When gI is expressed with gE, however, gI is more rapidly localized to the TGN (4,253). The carboxyl terminal domain of gI is required for envelopment of the virus in the TGN (252a). VZV gI is required for normal maturation of gE and appropriate distribution of gE on the cell surface.

VZV Glycoprotein L

Glycoprotein L (gL, gpVI), encoded by ORF60, is a 20-kd glycoprotein that forms a complex with gH. Glycoprotein L is thought to have an N-linked oligosaccharide (65). Unlike gH, which requires gL for its maturation, gL is fully processed in the absence of gH. Expressed alone or with gH, gL accumulates in the cytoplasm, but not on the surface, of transfected cells (64). It acts as a chaperone that associates with the immature but not the mature form of gH (65). Glycoprotein L contains an endoplasmic reticulum–targeting sequence and is recycled between the endoplasmic reticulum and the Golgi (64). Coexpression of gH and gL leads to syncytia formation; expression of either protein alone does not (65).

VZV Glycoprotein K

Glycoprotein K (gK), encoded by ORF5, is a 40-kd glycoprotein containing N-linked sugars (166). It is required for replication of VZV; HSV gK cannot complement a VZV gK-minus virus. Overexpression of VZV gK in a cell line inhibits syncytia formation by ild-type virus.

Assembly and Release

Few studies of human tissues illuminate the assembly and release of VZV *in vivo*, but infectious progeny are liberated efficiently, accounting for the infectiousness of virus-laden aerosols and vesicle fluids. Weller first noted that VZV is highly cell-associated in culture (255). In electron microscopic studies, Cook and Stevens (40) showed that most cytoplasmic particles develop dense cores and fragmented membranes, supporting a degradative insult. The implication of this is that the replication of VZV in cultured cells is different from that in cells in the human body, and that any model of how VZV replicates *in vitro* or *in vivo* must account for these properties of virus growth. Figure 3 is a schematic effort to depict various aspects of the competing notions about VZV assembly, maturation, degradation, and release

(40,85,87,88,103,116).

Two similar models of VZV replication *in vitro* have been proffered. One model stipulates that nucleocapsids are enveloped as they exit and traverse the nuclear membrane and then become incorporated into large cytoplasmic vesicles (103,116). Virus-specific glycoproteins are released from the TGN borne in microvesicles. These fuse with large cytoplasmic vesicles so that their glycoprotein content is available to decorate the envelopes of the newly arriving particles. The vesicles migrate to the cell surface, where progeny are released by reverse endocytosis. In this model, it is presumed that only a tiny proportion of particles are formed properly (see Fig. 3, pathway 12), but the probability that they do so is greater in cell lines derived from melanocytes than in human diploid lung cells.

Gershon et al. (87,88) propose a slightly different scenario for VZV maturation and release. In their view, nucleocapsids first acquire and then lose their envelopes as they traverse the nuclear membrane. “Naked” nucleocapsids in the cytosol are enveloped by cisternae of the TGN. The cisternae are decorated with VZV-encoded glycoproteins with their cytoplasmic tails in the cytosol. Tegument proteins adhere to the cytosolic face of the TGN. The naked nucleocapsids attach to the tegument and invaginate into the cisternae. In cultured cells, these vesicles are usually diverted to pre-lysosomes, where they are subjected to degradative enzymes (see Fig. 3, pathway 9). The particles enter pre-lysosomes because some of their envelope proteins are glycosylated with mannose-6-phosphate residues (85). Mannose-6-phosphate side chains render the evolving particles susceptible to binding and uptake by lysosomes bearing mannose-6-phosphate receptors. This latter feature of the model requires that an alternative secretory pathway must be operative in some cell types *in vivo* to allow virus egress without lysosomal degradation (see Fig. 3, pathway 8).

Fate of the Host Cell: Apoptosis, Effects on Host Proteins

Productive infection of cells with VZV results in lysis of the cell. The nuclei become enlarged, the nucleoli are fragmented and contain dense granular aggregates, and the chromatin is marginated (41). Basophilic granules, followed by eosinophilic inclusions, form in the nucleus of infected cells.

Light microscopy of infected cells initially shows increased refractility followed by formation of syncytia. In melanoma cells, virions cluster on the surface of the infected cells in elongated patterns termed viral highways (Fig. 5) (103). The syncytia gradually enlarge and the cells detach from the monolayer. At least five VZV proteins, ORF9A (211), ORF61 (32), gI (149), and gH–gL (64) have been shown to contribute to syncytia formation. Syncytia formation is less prominent *in vivo*.

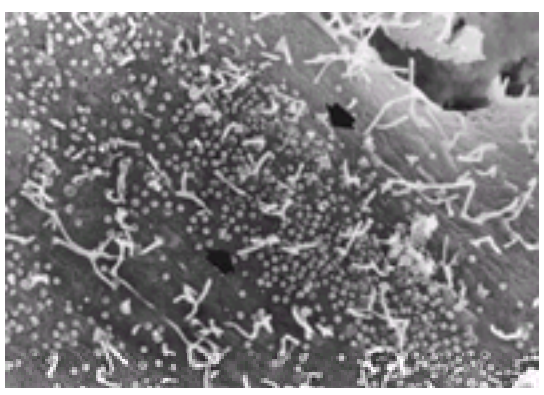

FIG. 5. Scanning electron micrograph of VZV viral highways. In infected cell monolayers, VZV emerges in distinctive linear patterns that have been termed viral highways. The highways consist of thousands of viral particles, as demarcated between the *solid arrowheads* (×10,000). (Micrograph provided by J. Padilla and C. Grose.)

Unlike HSV, VZV does not rapidly shut off host protein synthesis (11). Host protein synthesis gradually diminishes over a period of 48 hours after infection. Although VZV ORF17 encodes the homolog of the HSV virion host shutoff (vhs) protein, the VZV protein has not been shown to shut off host protein synthesis. The predicted sequence of the VZV vhs protein shows greater homology to the equine herpesvirus 1 vhs than to the HSV-1 vhs protein. Unlike the HSV vhs, the equine herpesvirus vhs does not induce degradation of cellular mRNA, and no virion-associated inhibition of protein synthesis is seen with the equine herpesvirus (78).

VZV induces apoptosis in Vero cells, with DNA fragmentation and chromosomal condensation (213). In more permissive cells, such as human fibroblasts and melanoma cells, however, VZV infection does not induce apoptosis (Turner and Cohen, unpublished data).

Like other alphaherpesviruses, VZV down-regulates expression of major histocompatibility complex (MHC) class I molecules on the surface of infected cells (1,28). The total amount and level of nascent synthesis of class I molecules, however, is unchanged in VZV-infected cells. VZV also inhibits interferon-gamma induction of MHC class II molecules on the surface of infected cells (2). VZV inhibits the expression of Stat 1-alpha and JAK2 proteins, and it inhibits the transcription of interferon regulatory factor I and the MHC class II transactivator that mediate interferon-gamma signal transduction. VZV also down-regulates expression of glial fibrillary acidic protein on human astrocytes *in vitro* (122).

Latency

The nature of VZV latency has not been clarified as well as that of HSV (see Chapter 73). Latency is established during varicella, a primary viremic infection. Its persistence in sensory nerve ganglia was predicted by the seminal studies of Head and Campbell in 1900 (104), who mapped all cutaneous dermatomes according to the distribution of lesions in 392

cases of zoster. VZV may access neural tissues either hematogenously or by centripetal neural transport from mucocutaneous lesions. Histopathologic and *in situ* hybridization studies of human ganglia recovered after fatal chickenpox showed widespread infection, favoring the hematogenous mechanism of spread (43,184). That the distribution of zoster lesions closely mimics that of primary varicella, and that the Oka vaccine virus tends to cause zoster outbreaks at sites of initial inoculation, both argue that VZV can access the ganglia by anterograde neural transport (99,227).

Using the polymerase chain reaction (PCR), latent VZV DNA is readily detected in most human trigeminal ganglia and many dorsal root ganglia (147,148). VZV DNA also has been found in human geniculate ganglia and olfactory bulbs (83,136). The cellular localization of the persistent virus, the quantity of latent virus, and the pattern of latent gene expression, however, are less well established.

Initial reports by Mahalingam et al. (147) suggested that only 6 to 31 copies of the VZV genome can be detected in DNA equivalent to 100,000 human ganglion cells. In more recent studies using quantitative real-time PCR techniques, Pevenstein et al. (200) reported, on average, 258 genome copies per 100,000 ganglion cells—a level about 1/10th that for latent HSV-1 in human trigeminal ganglia. Similar studies by Cohrs et al. detected 577 to 55,543 VZV genomes, but fewer HSV-1 genomes, per 100,000 ganglionic cells in older persons (38a).

Using *in situ* hybridization, Hyman et al. (110) and Gilden et al. (89) showed that latent VZV resides in neurons. Croen et al. (43) and Meier et al. (158), however, showed that VZV persists in nonneuronal cells (Fig. 6A). Several more recent studies established that VZV can persist in both neuronal and nonneuronal cells of sensory ganglia. Lungu et al. (141) used an enzyme-amplified *in situ* hybridization method to detect VZV DNA in 5% to 30% of neurons and nearly as great a percentage of nonneuronal cells. Using different *in situ* hybridization and PCR-*in situ* techniques, Kennedy et al. (120) showed VZV DNA in 2% of human dorsal root neurons and some 0.1% of nonneuronal cells. LaGuardia et al. (135), using PCR to analyze dissociated human neurons and nonneuronal cells, found that VZV was present almost exclusively in neurons at a frequency of two to five copies per neuron.

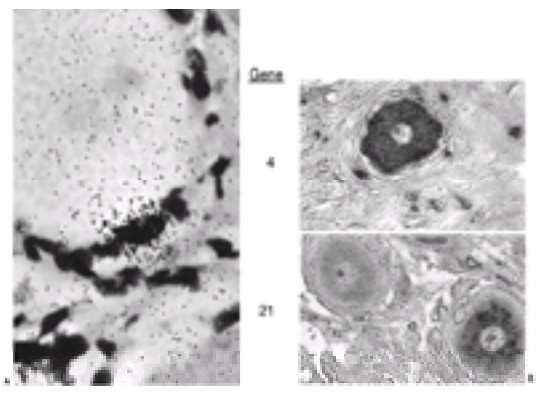

FIG. 6. A: *In situ* hybridization of an adult human trigeminal ganglion demonstrating expression of VZV gene 29 transcripts. Concentrations of silver grains indicates the specific accumulation of VZV RNA, primarily over a satellite cell (×498). (Reproduced from ref. 158, with permission.) **B:** Immunohistochemical detection of VZV proteins encoded by genes 4 and 21 in latently infected human dorsal root ganglia. The proteins were detected using purified rabbit antibodies raised to specific GST-fusion proteins and then an alkaline phosphatase-conjugated goat anti-rabbit secondary antibody. The *upper panel* displays reactivity with antisera to VZV gene 4 protein over the cytoplasm of one neuron and several nonneuronal cells. The *lower panel* shows reactivity over the cytoplasm of one of two neurons. (Modified from ref. 220, with permission.)

Unlike HSV latency, in which only one gene is efficiently transcribed, VZV latency is characterized by the transcription of multiple genes. Meier et al. detected polyadenylated RNAs homologous to ORF29 and ORF62 but not to ORF28 or ORF61 (158). Through a series of studies of cDNAs prepared from human ganglia, Cohrs et al. identified sequences encoded by ORFs 21, 29, 62, and 63 but not by ORFs 28, 40, or 51 (37,38 and 39). Using *in situ* RT-PCR, Kennedy et al. (119b,121) showed that ORFs 4, 21, 29, 62 and 63 RNA are expressed in human ganglia. The VZV genome possesses no obvious homolog of the HSV latency associated transcript (LAT)-encoding sequences, and nothing analogous to the LATs has been detected in VZV latency.

Studies in VZV-infected animals suggest another major difference between VZV and HSV latency in addition to the expression of multiple VZV transcripts. Although animal models have, in general, not been very helpful adjuncts to clarifying VZV pathogenesis because the virus does not replicate efficiently and cause disease in them, they have shed light on the mechanisms of VZV latency. Debrus et al. (52) reported the detection by immunohistochemical methods of VZV ORF63 protein in dorsal root ganglionic neurons of rats that had previously been inoculated in their footpads with virus-infected cells. Similar methods were then applied to human ganglia by Mahalingam et al. (146) and Kennedy et al. (119b), who demonstrated ORF63 protein in neurons. Lungu et al. (142) reported that proteins encoded by ORFs 4, 21, 29, 62, and 63 are expressed in human neurons (see Fig. 6B).

Persistence in both neuronal and nonneuronal cells suggests a unique mode of latency and reactivation of VZV that could explain diverse features of zoster that distinguish themselves from recurrent HSV infection (43,160).

YIELD OF CELL-FREE AND CELL-ASSOCIATED VIRUS

VZV is highly cell-associated when grown in cell culture. Despite its growth and passage in numerous cell types, no primary cells or cell lines in culture that support VZV replication yield substantial amounts of cell-free virus (255).

Because of this limitation, virus is usually propagated by treating VZV-infected cells with trypsin and passaging them onto uninfected cells.

Cell-free virus is usually prepared by sonicating VZV-infected cells. Titers of 10^3 to 10^5 plaque-forming units per milliliter have been obtained by mechanically dislodging VZV-infected thyroid, human melanoma, or human embryo lung fibroblast cells (97,101). Electron microscopic examination of cell-free VZV prepared in this manner often shows virus particles attached to fragments of cell membranes. Precipitation of cell-free virus with polyethylene glycol allows an up-to-50-fold concentration of the virus with preservation of infectivity. Concentration by other methods, including ultracentrifugation and dialysis against hydrophilic compounds, typically leads to a marked decrease in virus titer. VZV has been further purified with retention of infectivity by centrifugation in 10% to 50% linear potassium tartrate or potassium tartrate and glycerol viscosity–density gradients; however, centrifugation in cesium chloride or metrizamide gradients is associated with loss of infectivity (95,97).

Analysis of VZV excreted from human embryonic lung cells in culture shows 1 to 2×10^9 viral particles per milliliter of medium, and 30% to 80% of the particles are enveloped; however, no infectivity could be detected. A similar analysis of particles from sonicated cells showed 1 to 2×10^9 particles per milliliter, and 30% to 50% of the particles had envelopes. The particle-to-infectivity ratio was 10^6:1. In contrast, HSV-2 obtained from sonicated cells had a particle-to-infectivity ratio of 60:1 (219). Cell culture–derived VZV often has an incomplete coat and may lack a central core, unlike cell culture–derived HSV-1, which is enveloped and has a dense, central core (40). Unlike VZV grown in cell culture, vesicle fluid contains substantial amounts of infectious cell-free virus, which is less labile than virus obtained from cultured cells (23,241).

INHIBITORS OF REPLICATION

Effective agents are available to treat VZV infections (29). The clinical application of these agents is reviewed more fully in Chapter 15 and Chapter 79. The first proven treatment, human leukocyte interferon, benefited immunocompromised patients with varicella (10) or zoster (162). Greater antiviral activity is exhibited by nucleoside and pyrophosphate analogs. The first effective analog was vidarabine (258). Vidarabine is a modestly active, but selective, competitive inhibitor of the VZV-encoded DNA polymerase.

Acyclovir proved more versatile, effective, and safe than vidarabine for treatment of varicella and zoster in the immunocompromised (12,217,259) and in the normal host (62,183). Acyclovir is an analog of deoxyguanosine, bearing an acyclic side chain in the 9 position in place of deoxyribose. VZV replication is inhibited *in vitro* at concentrations of 1 to 5 µg/mL, whereas cellular growth and metabolism are not impaired until concentrations exceeding 50 µg/mL are achieved (18,45). Acyclovir is selectively monophosphorylated by the VZV-encoded TK and further modified to acyclovir di- and triphosphates by cellular kinases (165). Acyclovir triphosphate not only accumulates preferentially in VZV-infected cells, but it serves as a selective substrate and inhibitor of the VZV DNA polymerase. Incorporation of acyclovir terminates chain growth by virtue of its lack of a 3'-OH group to which the subsequent nucleotide would link (71).

Molecular proof of the pivotal role of the VZV TK in the action of acyclovir and penciclovir (another guanosine analog) was derived both from the cloning and stable expression of the gene in TK-deficient cells, and from the subsequent demonstration by sequencing of the VZV gene in acyclovir-resistant viral mutants (214,240). Missense mutations in the ATP-binding site and the nucleoside-binding site, and mutations clustered between these two sites, confer acyclovir resistance. Nonsense mutations leading to expression of grossly truncated VZV TK protein also result in drug-resistant viruses. Acyclovir resistance also can be conferred by mutations in the VZV DNA polymerase, but these are rare and less well characterized. VZV strains that are resistant to both acyclovir and penciclovir, because of mutations in the TK gene, retain sensitivity to the pyrophosphate analog, foscarnet. Foscarnet is effective for acyclovir-resistant VZV infection (21). Cidofovir is a novel acyclic nucleoside phosphonate that retains activity against acyclovir-resistant strains of HSV and VZV. It may be useful as a topical or systemic alternative to foscarnet (222).

Two nucleoside analog prodrugs have been approved in the United States for treatment of zoster (16,248). Valaciclovir is a six-valine ester derivative of acyclovir that is deesterified by hepatic enzymes to yield acyclovir (199). Famciclovir is a diacetyl derivative of penciclovir that is enzymatically converted to penciclovir by intestinal and hepatic enzymes (44). The advantage of these prodrugs is that they are better absorbed from the gastrointestinal tract than their parent compounds acyclovir and penciclovir, allowing higher serum levels of drug. Penciclovir has an added pharmacologic advantage in achieving and sustaining higher intracellular triphosphate concentrations, yet, on balance, this advantage is offset by the poorer ability of penciclovir triphosphate to inhibit VZV DNA polymerase compared with acyclovir triphosphate (44).

PERSPECTIVE

The study of VZV has long lagged behind that of the other human alphaherpesviruses HSV-1 and HSV-2. This was a result of the low titers of cell-free VZV that could be obtained *in vitro* and, until recently, the lack of a genetic system to produce viral mutants. Two groups have now independently generated sets of cosmids that have been used to produce VZV mutants. The ability to manipulate the VZV genome and the availability of an effective VZV vaccine may allow VZV to be used as a vaccine vector for expression of additional antigens. For example, VZV expressing HSV-2 gD partially protects animals from genital herpes (107).

Although the VZV and HSV genomes have a large number of genes in common, many of their genes have strikingly different properties. For example, whereas HSV VP16 is essential for virus replication, the homologous VZV ORF10 is dispensable for growth *in vitro*. Continued elaboration of the function of VZV genes will undoubtedly show further differences in homologs shared between these viruses.

VZV also has a large number of biologic properties that are different from those of HSV. Unlike HSV, VZV disseminates throughout the body during primary infection. Some of the genes that contribute to this aspect of pathogenesis have only

recently been defined. The ORF47 protein kinase is important for virus replication in lymphocytes, which may be important for its dissemination, whereas gC is needed for VZV penetration into the dermis.

It is striking that VZV usually reactivates only once, if at all, whereas HSV usually reactivates multiple times. While HSV expresses only one family of LATs, VZV expresses several genes, including their protein products, during latent infection. The mechanism for VZV latency, and especially the role of the large number of latency-associated gene products, remains a conundrum. The ability of these latently infected cells, which express a number of viral proteins, to escape destruction by the immune system, raises a number of questions for future research.

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CHAPTER 79 Varicella-Zoster Virus

Ann M. Arvin

Varicella-zoster virus (VZV) is a human alphaherpesvirus that causes varicella, commonly called *chickenpox,* during primary infection of the host. Varicella is a febrile illness characterized by a generalized, pruritic vesicular rash and is most prevalent in childhood. Primary VZV infection is highly contagious, producing annual epidemics among susceptible individuals during winter and spring in temperate climates. VZV is unique among the alphaherpesviruses in its tropism for T lymphocytes, which allows dissemination of the virus to skin. The virus establishes latency in cells of the dorsal root ganglia during primary infection. Its reactivation from latency produces herpes zoster, which is commonly referred to as *shingles,* and is usually observed in elderly or immunocompromised patients. Herpes zoster is a vesicular rash restricted to a dermatomal distribution. Acute VZV reactivation produces severe acute pain, which may progress to the chronic syndrome called *postherpetic neuralgia* (PHN). The symptoms of varicella and herpes zoster are known throughout the world because VZV infection is ubiquitous in the human population. Ordinary names for varicella in other languages are *varicelle* (French), *varicela* or, in older usage, *viruela* (Spanish), and *windpocken* (German); the terms for herpes zoster are *zone, zona,* and *gurtel rose.* Speculations about the origin of the colloquial English term, *chickenpox,* include an association between the appearance of varicella lesions and that of the chickpea, which is called chiche in French and a corrupted form of the Old English word for itching, which is *gican* (131). The term *zoster* derives from the Greek word for girdle, while shingles originates from the equivalent Latin term, *cingere,* reflecting the partial encircling of the abdomen when the rash involves the lower thoracic or lumbosacral dermatomes.

HISTORY

Varicella and herpes zoster were described in the earliest medical literature, although the similarities between the exanthems of smallpox and varicella delayed the definitive recognition of varicella as a separate clinical entity. Heberden distinguished these two diseases in 1867, but since smallpox has been eradicated, its differentiation from varicella is of historical interest only. Galen referred to clusters of vesicular skin lesions as herpes but did not distinguish lesions now associated with herpes simplex from herpes zoster. The dermatomal eruption of herpes zoster and the associated neuralgia were recognized later as differentiating characteristics.

Von Bokay suggested the infectious etiology and the probable pathogenic relationship between the causative agents of varicella and herpes zoster in 1892, based on the occurrence of varicella in children after household contact with herpes zoster (46). Varicella was then observed in susceptible children after the inoculation of vesicular fluid recovered from herpes zoster lesions; episodes of secondary transmission resulted in typical varicella (323). The cutaneous lesions of varicella and herpes zoster were similar in early histopathologic studies (78,211). “Elementary bodies” in vesicular fluid differed from those in smallpox vesicles but suggested that the causative agent was a virus (7), and virions from varicella and herpes zoster were shown to be identical by electron microscopy (5). That herpes zoster was caused by reactivation of latent virus acquired during varicella was considered by Garland in 1943 and further elucidated by Hope-Simpson (128,177,178).

In 1944, Goodpasture and Anderson observed multinucleated cells and cells containing intranuclear inclusions when human skin cells, maintained by grafting to chick embryo chorioallantoic membranes, were inoculated with vesicular fluid from a herpes zoster lesion (147). The first isolation of VZV in tissue culture was reported by Weller and Stoddard in

1953 (366). Focal cytopathic effects (CPE) and virion morphology were indistinguishable in cells inoculated with vesicular fluid recovered from cutaneous lesions of patients with varicella or herpes zoster (62,367,368,369 and 370). Comparable antigenic profiles were documented using immune sera (211). VZV was first classified as a DNA virus because its replication in tissue culture was inhibited by iododeoxyuridine (281). Restriction endonuclease analysis of VZV DNA from viruses isolated from an immunocompromised patient during varicella and herpes zoster confirmed that herpes zoster resulted from the reactivation of the same VZV strain (325).

Much progress has been made recently toward understanding the molecular biology of VZV. Recent milestones include the sequencing of the complete VZV genome and the production of infectious virus from cosmids (71,87,235). Generating mutants from VZV cosmids has overcome the difficulty of plaque purifying VZV that had hampered the functional analysis of VZV genes. Critically important medical advances have been made in parallel, including the demonstration that VZV infections, whether primary or recurrent, can be treated with antiviral agents selected for their capacity to inhibit VZV replication *in vitro* (374). VZV is also the first human herpesvirus for which a vaccine has been developed (134,330,335). Live attenuated varicella vaccine protects susceptible individuals against primary VZV infection and is recommended for universal immunization in the United States (6).

INFECTIOUS AGENT

The virion structure of VZV and its replication are described in Chapter 78. Knowledge about the unique characteristics of VZV as an infectious agent, particularly those that distinguish VZV from other alphaherpesviruses, is beginning to emerge. Nevertheless, information about how specific viral gene products mediate VZV infectivity in cell culture or, most importantly, within differentiated cells that compose intact host tissues, remains limited.

Propagation and Assay in Cell Culture

VZV can be isolated from cutaneous lesions or other infected tissues using primary, diploid, or transformed human cells (137,152,367). Human cell lines are the most sensitive for initial recovery of the virus, but VZV can be propagated in nonhuman cells, including primary rhesus or African Green monkey kidney cells, Vero cells, and guinea pig embryo fibroblasts (137). CPEs usually appear within 2 to 7 days after inoculation of vesicle fluid, which contains cell-free VZV, but may require up 14 days (137). VZV replication is highly cell associated, with no virus release into supernatant; efficient propagation of the virus requires the use of intact infected cells or an infected cell inoculum disrupted by trypsin, sonication, or freeze-thawing. The infectious virus yield from VZV-infected monolayers is low (131). Infected cells are dispersed with trypsin at 50% to 90% CPE, transferred to a fresh monolayer at ratios of 1 infected cell to 2 to 10 uninfected cells, and incubated for 24 to 72 hours. Centrifugation, as well as blind passage, can enhance initial replication when viral titers are low (362).

Even keratinocytes, which release cell-free virus into cutaneous vesicle fluid *in vivo,* do not allow viral egress *in vitro* (392). Sonication and gradient purification permits the recovery of low quantities of cell-free VZV from various cell types, but the best ratios of plaque-forming units (PFUs) to uninfected cells that can be achieved range from 1:5 to 1:10 (131).

Plaque assays of VZV infectivity can be performed using human fetal diploid cells, melanoma cells, Vero cells, or primary African green monkey kidney cells (137). Trypsinized infected cells or sonicated preparations of infected cells are inoculated in serial dilutions, adsorbed for 30 to 60 minutes, incubated for 5 to 7 days, and stained with neutral red or crystal violet to enhance plaque visibility. VZV plaque assays may be performed without an overlay because the virus is so cell associated, but detachment of infected cells can lead to secondary foci. Early detection of infected cell foci can be accomplished with immunohistochemical stains (355,382). By using a standard inoculum of infected cells, VZV plaque titration methods can be adapted to assess neutralizing antibody titers or the activity of antiviral drugs (307).

Biologic Characteristics in Cell Culture

VZV infectivity requires the preservation of an intact virion envelope (96) and is also relatively temperature dependent; optimal growth of VZV in melanoma cells occurs at 32°C (169). Complete viral inactivation is observed after short intervals of exposure to temperatures of 56° to 60°C, or storage at temperatures above –70°C. Rapid freezing with 5% to 15% glycerol helps to maintain the viability of VZV-infected cell preparations (150). Lyophilization preserves the infectivity of cell-free virions as well as VZV-infected cells (37).

VZV-infected cells are visible by immunohistochemical staining methods within 4 to 10 hours after inoculation (62,387). When CPE appears, it is characterized by syncytial formation; the presence of syncytia is a hallmark of VZV infection in cell culture and contrasts with the CPE caused by most herpes simplex virus (HSV) isolates. CPE appears about 24 hours after infection of melanoma cells; syncytial formation involving about 75% of the monolayer is observed by 48 hours, and at 60 hours, the fused cells detach from the surface. VZV induces large foci of rounded, refractile cells in uman diploid cells (Fig. 1). Multinucleated giant cells appear with enlarged nuclei, abnormal nucleoli, and marginated chromatin (see Fig. 1C). VZV virions are usually enclosed in membrane-lined vacuoles within the cytoplasm (123,152). By electron microscopy, the virions appear to deteriorate as the particles move through cytoplasm into extracellular spaces, explaining the failure to detect infectious virus in supernatants (81,82,284). At late times, exposure to protein-precipitating fixatives reveals eosinophilic intranuclear inclusion bodies that do not appear to contain viral DNA. By scanning electron microscopy, both complete and light particles, lacking a nucleocapsid, are visible, and VZV CPE is associated with the formation of “viral highways”; viral particles are visible on the surface of cells, clustered along the syncytia (284).

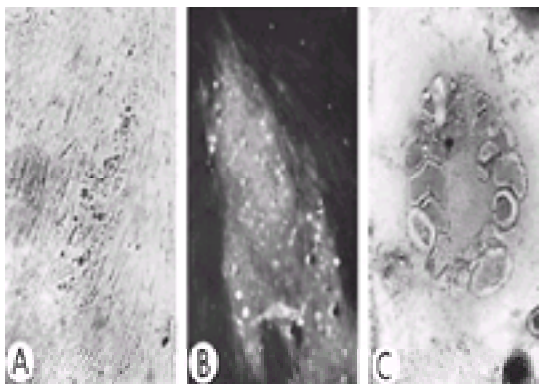

FIG. 1. Varicella-zoster virus (VZV) infection of human fibroblasts *in vitro.* **A:** An early focus of VZV infection in a monolayer of human embryonic fibroblasts. (H&E, ×240) **B:** Immunofluorescence in a focus of VZV-infected human embryonic lung fibroblasts after reaction with human convalescent zoster serum and fluorescein isothyocyanate–conjugated goat antihuman immunoglobulin G. (H&E, ×2,100) **C:** An isolated multinucleated giant T lymphocyte containing intranuclear inclusion bodies from a monolayer of VZV-infected human fibroblasts. The cells were fixed in Bouinís solution (H&E, ¥2,600). (From ref. 127, with permission.)

VZV exhibits a high degree of genetic stability during *in vitro* propagation, at the level of assessment possible by restriction digest analysis of viral DNA using several enzymes (92). Although VZV isolates have variable restriction enzyme profiles, altered patterns of CPE have not been associated with spontaneously occurring genetic variations. The only exception is a newly identified VZV strain with a mutation in glycoprotein E (gE), which exhibits accelerated cell-to-cell spread and increased numbers of cell surface virions (298).

Several genes have been shown to be dispensable for VZV replication in tissue culture, without changing VZV CPE, including thymidine kinase (TK), dUTPase, thymidylate synthetase, uracil DNA glycosylase, open reading frames 47 and 66 (ORF47 and ORF66), serine-threonine protein kinases, ORF10 virion-associated transactivator, the gene products of ORF32, ORF57, and ORF61 (72) and gC (see Chapter 78). However, when gI (ORF67) is deleted, or its expression is blocked, VZV mutants exhibit a small plaque phenotype with distortion of polykaryocyte formation in melanoma cells, and no growth is detected in Vero cells (76,235). Syncytial formation is also reduced if the products of both ORF8 and ORF9A are not expressed (287).

VZV infection of cells in culture is lytic, rather than transforming (375). Early experiments suggested that VZV could transform primary hamster embryo cells; these nonpermissive cells, which were infected with a clinical isolate of VZV and passaged blindly, exhibited abnormal morphology, showed transient expression of VZV proteins, and induced fibrosarcomas in inbred hamsters, but no VZV DNA was detected in the transformed cells (133,388).

Replication in Specialized Cell Types in Culture

VZV replication has been studied *in vitro* using differentiated primary human cells or cell lines selected to reflect the tropisms of the virus *in vivo.* When VZV interactions with peripheral blood mononuclear cells (PBMCs) were examined by *in situ* hybridization, only 0.01% of T lymphocytes showed nuclear hybridization (207). Infectious VZV was recovered from cultures of activated T lymphocytes (207), and VZV infects cord blood T lymphocytes *in vitro* (318a). The major immediate early protein IE62 has the potential to transactivate all classes of VZV genes *in vitro* in a human T-cell line (264), and abortive replication occurs in a CD4+ T-cell hybridoma line (II23 cells) (392). The nonpermissiveness of most PBMCs, including resting T lymphocytes, for VZV replication *in vitro* parallels the low frequency of VZV-infected mononuclear cells that can be detected during acute varicella (207,234); this limited replication, despite virion uptake and functional activity of the IE62 protein, benefits the host, but its mechanisms have not been explained. VZV also replicates in EBV-transformed human B lymphocytes *in vitro* (207); VZV gE and IE62 protein are expressed on the surface of these cells. Infection of keratinocytes was demonstrated, but infectious virus yields were low (310).

VZV infects cells cultured from nervous system tissue, including both neuronal and nonneuronal cells (Schwann cells and astrocytes) (30,143,240,318,376). When cells derived from fetal dorsal root ganglia were infected with VZV, the supporting nonneuronal cells were more susceptible to CPE than were the neurons (376). VZV replication was more rapid in astrocytes than in Schwann cells and neurons, although CPE was seen in all cell types. In astrocytes and fibroblasts, a VZV IE protein was detected before a putative early protein, whereas in Schwann cells and neurons, a putative early protein was detected before an IE protein (28). Infection of cultures containing both human fetal neurons and satellite cells in the presence of bromovinylarauracil (BVaraU) followed by withdrawal of the antiviral resulted in recovery of infectious VZV, but persistence did not occur when neurons or satellite cells were cultured separately (318). Infection of human fetal neurons with a wild-type isolate of VZV resulted in sevenfold greater virus replication compared with the vaccine Oka strain. VZV inoculation of a human neuroblastoma cell line (IMR-32) also yields infectious virus (53).

Effects on Cell Functions

In addition to the effects on cellular morphology that are evident as syncytial formation and typical CPE, VZV alters other characteristics of mammalian cells in culture. VZV elicits down-regulation of the constitutive expression of class I major histocompatibility (MHC) molecules on the surface of infected cells (1,73). Because class I MHC molecules present peptides to CD8+ T lymphocytes, interference with their transport to the cell surface makes the infected cells less visible to this component of the antiviral immune response. This effect is observed on VZV-infected T lymphocytes, which may facilitate the transport of VZV to cutaneous sites of replication during the incubation period, without evoking adaptive immune responses (1). Class I MHC molecules are also decreased on fibroblasts infected with VZV, which may delay

clearance of the virus at cutaneous sites (1,73). In contrast to HSV and cytomegalovirus (CMV), class I MHC molecules transit the endoplasmic reticulum (ER) but are retained in the Golgi compartment of VZV-infected cells (1).

VZV infection also interferes with the up-regulation of class II MHC expression that is usually triggered by interferon-g (IFN-g); IFN-g–induced up-regulation of class II MHC expression is blocked at the level of host cell gene transcription through the Jak-Stat pathway (2). Because viral peptides complexed with class II MHC molecules elicit antigen-specific CD4+ T lymphocytes and support their clonal expansion, this effect may restrict the initial CD4 T-lymphocyte response to VZV. Examination of skin biopsy specimens taken early in the course of varicella or herpes zoster lesion formation demonstrate no class II MHC expression on VZV-infected cells *in vivo,* suggesting transient protection of VZV-infected cells from immune surveillance by CD4 T lymphocytes. In contrast, VZV does not down-regulate class II MHC expression on cells that are exposed to IFN-g before infection. Therefore, VZV-specific CD4+ T lymphocytes that produce IFN-g at the site of VZV replication can limit virus spread to adjacent uninfected cells because local release of IFN-g should make these secondarily infected cells subject to immune clearance.

Other examples of effects of VZV on cell function include interference with apoptosis mediated by Fas ligand (166) (see Chapter 78) and the capacity of VZV gE to act as a Ca^{2+}-independent adhesion molecule, triggering rapid confluence of polarized epithelial cells *in vitro* (244).

Description of Major Antigenic Proteins

As described in Chapter 78, the VZV genome consists of at least 69 unique ORFs (87). No systematic analysis has been made, but sera from human subjects who have had recent varicella or herpes zoster contain immunoglobulin G (IgG) antibodies to many of these gene products, and many have been shown to elicit antibody production in animals (94,395). The antigenicity of viral proteins was first defined by antibody reactivity, but major antigens can now be characterized in terms of their recognition by VZV-specific memory T lymphocytes as well. The broad spectrum of reactivity observed by serologic methods is also documented by T-lymphocyte recognition (23). Whether antibody or T-cell responses are used as the measure, no single protein or class of VZV proteins has emerged as the major antigen or antigens of the virus. The comparison of epidemiologically distinct VZV strains using polyclonal or monoclonal antibody reagents indicates that there is little antigenic diversity among VZV isolates. Japanese isolates have been shown to lack an epitope within the ORF10 gene product when compared with VZV strains from the United States and Europe, as assessed by reactivity to an antiserum generated to an ORF10 synthetic peptide (204). The ORF10 mutation and the gE ectodomain mutant identified in the United States (298) are the only examples of VZV antigenic variants that have been reported.

Initial analyses of the antigenic determinants of VZV focused on the glycoproteins. VZV produces several mature glycoproteins—gB, gC, gE, gH, gI, gL, and gK; other ORFs for putative glycoproteins (e.g., gM) are also present in the genome (86,101,117,148,151,199,203,243,244,253,342) (see Chapter 78). The VZV glycoproteins are important antigens because they mediate the binding of complement and Fc-receptor formation and are presumed to be involved in virus entry and cell fusion (148). The gE protein (Gp I [ORF68]), which is the glycoprotein produced most abundantly in VZV-infected cells, binds the Fc fragment of IgG; it induces antibodies that have neutralizing activity in the presence of complement, as does gI (gp IV [ORF67]), which is noncovalently linked to gE in infected cells (101,224,389). Dominant antibody-binding epitopes of gE have been localized to residues 86 to 105, 116 to 135, and 56 to 75 using human sera (120). Human monoclonal antibodies that bind to gE have been generated (116,326). The gB protein (Gp II [(ORF31]) elicits antibodies that have complement-independent neutralizing activity against VZV and probably has a role in virus entry, by analogy with HSV (148). The gH (Gp III [ORF 37]) and gC proteins (gpV [ORF14]) also induce antibodies with complement-independent neutralizing function (117,199,200 and 201). A monoclonal antibody to gH (gp III) prevented syncytial formation, suggesting that anti-gH (gp III) antibodies can modulate viral pathogenesis even after infection of the target cell (284). Although gC is antigenic, in contrast to the other glycoproteins, gC (gp V) is not always produced by VZV after passage in cell culture (201,246). VZV gK illustrates the discordance between viral protein antigenicity and the role of the protein in replication; gK is not a major antigenic protein, as defined by immunologic criteria, but it is an essential protein for VZV replication (244). All of the major glycoproteins are equivalent and prominent antigens as defined by T-lymphocyte recognition (22). VZV proteins other than the glycoproteins are also important antigens. For example, an increase in antibodies to the viral thymidine kinase follows VZV reactivation; and the IE62 and IE63 proteins are equivalent to the major viral glycoproteins in inducing T-cell responses (14,20,202,294).

Early studies of the antigenic relationships between VZV and HSV demonstrated heterologous antibody responses, especially in individuals who were already immune to HSV when primary VZV infection was acquired (307,380). Comparisons of the DNA sequences and predicted proteins of VZV and HSV, as well as direct experiments using purified VZV glycoproteins and monoclonal antibodies, have accounted for these antigenic relationships, with cross-reactivity due to homology between gB (gp II) and HSV-1 gB being particularly prominent (148). ORFs with homologies to VZV are also present in the pseudorabies virus and simian varicella genomes, but the extent to which the corresponding viral proteins share antigenic determinants is not known (276).

Infection in Experimental Animals

The host range of VZV is highly restricted compared with that of other herpesviruses (250a). VZV infects some nonhuman primates, but the animals have remained asymptomatic, except for varicella in an infant gorilla and transient lesions after experimental inoculation of two chimpanzees (74,251). VZV DNA was detected in PBMC of the chimpanzees after inoculation with VZV, and an erythematous, papular rash appeared in the dermatome near the site of inoculation. Experiments inoculating primates with VZV have also been done in African green monkeys, *Erythrocebus patas,* and pygmy marmosets (248). Infectious virus was not recovered from these animals, but VZV IgG antibodies were elicited; the administration of VZV antiserum before challenge blocked seroconversions in African green monkeys, suggesting that viral replication was required to induce the host response. Protection experiments cannot be done with VZV in primates, except possibly in chimpanzees, because of the absence of symptoms and the failure to recover infectious virus. Simian varicella viruses are antigenically related to human VZV, but the disease caused by these viruses in primates is usually severe (112,115). African green monkeys infected with simian varicella virus expressing green

fluorescent protein developed necrotizing pneumonia (233). Simian varicella infection has been used to evaluate antiviral activity of nucleoside analogues (317). Inoculation of patas monkeys with VZV protects against challenge with delta herpesvirus (112). Human leukocyte interferon prophylaxis modified the interval to onset of disease in an outbreak of varicella-like disease in patas monkeys (19). Simian varicella viruses establish persistent infection of dorsal root ganglia in primates, but symptomatic recurrent disease has not been observed (317). Subclinical reactivation probably occurs because outbreaks of simian varicella have been described in primate colonies housing colony-borne and wild-borne animals (19).

Mice, including suckling mice, are resistant to any signs of disease after infectious VZV challenge, but viral DNA can be detected in ganglia (58). The guinea pig model reproduces some critical events in VZV pathogenesis (231,248,300a). Viremia was demonstrated in strain 2, Hartley, and euthymic hairless guinea pigs after subcutaneous inoculation of VZV-infected cells (231,300a) and nasopharyngeal shedding with animal-to-animal transmission was documented. Strain 2 guinea pigs kept at low temperatures developed a mild exanthem, and euthymic hairless guinea pigs, which are immunologically intact but lack essential genes for hair growth, also have a transient cutaneous eruption that is blocked by passive antibody prophylaxis or antiviral drugs. Limitations of the model include difficulties with achieving adequate inocula of infectious virus and variations in the percentage of animals that develop cutaneous lesions. Administering immunosuppressive drugs to Hartley guinea pigs did not enhance VZV pathogenicity (231). Guinea pigs and rabbits have been used to investigate the pathogenesis of VZV-induced ocular keratitis, and pneumonitis has been described after intrabronchial inoculation of guinea pigs (263,350). VZV reaches the dorsal root ganglia, as shown by *in situ* hybridization (336); VZV was detected in trigeminal ganglia tissue from 63% of infected guinea pigs by polymerase chain reaction (PCR), and thoracic dorsal root ganglia tissue was positive in 75% of the animals (231). Intravitreal inoculation of guinea pigs with a VZV recombinant that expressed *lacZ* caused chronic uveitis, with viral persistence in the ciliary body, iris, and retinal pigmented epithelial cells in some animals (75).

Guinea pigs can be used to evaluate the host response to VZV (232,238,299). Guinea pigs inoculated with VZV develop neutralizing antibodies, antibodies to glycoproteins, delayed-type hypersensitivity (DTH), and helper and cytotoxic T lymphocytes (21,170,232,238). Priming of the T-lymphocyte response was observed in animals immunized with gE, gI, gC, or IE62 protein and exposed to VZV. In protection experiments, the IE62 protein was highly effective, whereas the major DNA-binding protein (ORF29), a nonstructural DNA replication protein, was not (291).

Transfer of virus to dorsal root ganglia cells has been documented after subcutaneous inoculation of adult rats and intraperitoneal inoculation of suckling mice and in mice after ocular infection (10,203,293,384). In adult rats, ganglia harvested after 1 to 9 months showed VZV persistence by immunoperoxidase and *in situ* hybridization; blind passage of cells cultured from ganglia of some animals yielded infectious virus. VZV DNA and antigen were detected in rat neurons and satellite cells 2 weeks after subcutaneous inoculation (10). VZV was also detectable in dorsal root ganglia and other tissue sites for 33 days after ocular inoculation in a mouse model (384).

The SCID-hu Mouse Model

The SCID-hu mouse model of VZV infection differs from other animal systems that assess VZV infectivity across species barriers or evaluate viruses related to VZV in their natural hosts. SCID-hu mice carry differentiated human tissues, which contain the expected cellular subtypes (248). The immunodeficient state allows maintenance of allografts of human thymus-liver, containing CD4+, CD8+, and dual positive T cells, or intact human skin with no murine cells in the allografts. In contrast to HSV, VZV does not escape into murine cells. The SCID-hu model allows the analysis of VZV tropism for these critical cell types (245,246 and 247). Because the mice are immunodeficient, no immune-mediated effects on viral pathogenesis are observed; conversely, the host response to VZV cannot be evaluated in this model. VZV infectivity for T lymphocytes was demonstrated by infectious focus assay and by flow cytometry analysis using VZV-specific antibodies and dual staining with reagents that bind T-lymphocyte markers, such as CD3, CD4, and CD8. Maximum virus titers were observed from 7 and 14 days after inoculation of thymus-liver implants with low passage VZV strains, and cell-free virus is released by VZV-infected T lymphocytes. Infection of skin implants resulted in epidermal lesions that progressed to large vesicles; cell destruction penetrates the basement membrane, extending into the dermis. The histopathologic changes were typical of those observed in varicella lesions; many intact virions were observed by electron microscopy and release of infectious virus was documented (Fig. 2).

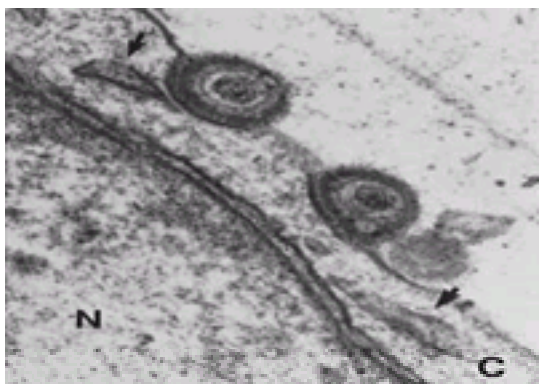

FIG. 2. Varicella-zoster virus (VZV) release from skin cells. Transmission electron micrograph of skin cells 21 days after inoculation with VZV-S. **Left:** Enveloped virons containing dense cores have dispersed from the cell (*arrows*). Vacuoles from which particles egressed are visible adjacent to the cell membrane. Nucleocapsids are not visible in the nucleus (*N*) by this staining method (×24,000). **Right:** Higher magnification view of virions with intact lipid bilayer on cell surface. Remnants of cytoplasmic vacuoles which carried virions to plasma membrane are indicated by *arrows* (×108,000), N, nucleus; C, cytoplasm. (From ref. 246, with permission.)

PATHOGENESIS AND PATHOLOGY

Varicella

Entry into the Host

Primary VZV infection is presumed to be initiated by inoculation of the mucous membranes of the respiratory tract, with infectious virus transferred in respiratory droplets or by contact with infectious vesicular fluid from an infected individual (149). However, documentation of the inoculation stage of VZV pathogenesis by isolation of infectious virus from respiratory sites has not been accomplished.

Site of Primary Replication and Viral Spread

The pathogenesis of primary VZV infection appears to fit the Fenner model for exanthematous illnesses in studies of mousepox, as suggested by Grose (149). Although several of the stages of pathogenesis have not been proved, infectious VZV probably moves to regional lymph nodes after inoculation of mucous membranes, and may undergo a phase of replication at this site (Fig. 3). This event is thought to be followed by a primary viremia during which the virus is transported to the liver and other cells of the reticuloendothelial system. These stages occur during an incubation period of 10 to 21 days. The occurrence of a secondary viremia in the last 4 or 5 days of the incubation period or during the first 24 hours of acute varicella has been documented by isolation of the virus from PBMCs, by *in situ* hybridization, and by PCR (25,206,234,260,344) (Fig. 4). This secondary viremic phase allows the dissemination of the virus to cutaneous epithelial cells. Cell-associated viremia continues after the initial skin lesions develop, as shown by the recovery of VZV from 11% to 24% of PBMC samples taken within 24 hours after the appearance of the varicella exanthem and its detection by *in situ* hybridization or PCR in 67% to 74% of otherwise healthy individuals who were tested immediately after the appearance of the rash (206,302). The frequency of circulating PBMCs that carry the virus is quite low in the immunocompetent host; VZV was detected by *in situ* hybridization in only 0.01% to 0.001% of PBMC during acute varicella (206). Infected, circulating PBMCs are usually cleared within 24 to 72 hours after the appearance of the rash in the immunocompetent host. The phenotype of the PBMC subpopulations that are infected with VZV during the secondary viremic phase has not been determined precisely in children with varicella because of the low frequency of positive cells; B cells and monocytes from acutely infected individuals bind antibodies to VZV proteins, but whether these cells have surface VZV antigens only or are infected is not certain (234). That differentiated human CD4+ as well as CD8+ T lymphocytes are permissive for VZV *in vivo* was demonstrated in the SCID-hu model (245).

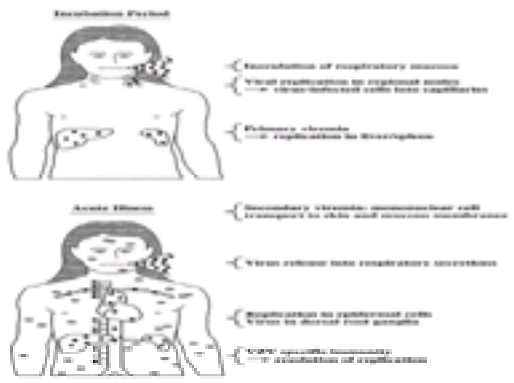

FIG. 3. The pathogenesis of primary infection with varicella-zoster virus (VZV). Primary infection with VZV is initiated by inoculation of the respiratory mucosa. During the incubation period, which lasts from 10 to 21 days, the virus probably spreads to regional lymph nodes and then causes a primary viremia with associated viral replication in the liver and spleen. A secondary cell-associated viremic phase begins at 24 to 96 hours and results in the transport of the virus to skin and respiratory mucosal sites. Replication in epidermal cells causes the characteristic rash of varicella, often referred to as *chickenpox.* The induction of VZV-specific immunity is required to terminate viral replication. The virus gains access to cells of the trigeminal and dorsal root ganglia during primary infection and established latency.

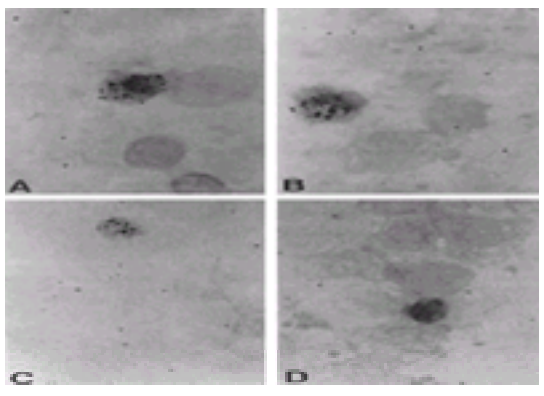

FIG. 4. Detection of varicella-zoser virus (VZV) in peripheral blood mononuclear cells (PBMC) during primary VZV infection by *in situ* hybridization. PMBC obtained from subjects 24 hours after the onset of acute varicella (**A** and **B**) and from a guinea pig 5 days after inoculation with VZV, 9 $\times 10^5$ PFU, showed VZV DNA within the cells after hybridization with a VZV DNA probe (From ref. 207, with permission.)

During the late incubation period, VZV must also be transported back to respiratory mucosal sites because it can be transmitted to susceptible contacts who are exposed within 24 to 48 hours before the appearance of cutaneous lesions. Nevertheless, infectious virus has rarely been recovered from nasopharyngeal secretions during the preeruptive phase and was found in only 4.2% of children with acute varicella; viral DNA has been detected by PCR in oropharyngeal specimens obtained before and after the appearance of the cutaneous rash (302).

Cellular and Tissue Tropism

The essential cellular tropisms of VZV during primary infection include T lymphocytes, skin cells, and cells of the dorsal root ganglia. Although T-lymphocyte tropism is a necessary component of primary VZV infection, the site where T lymphocytes become infected and the molecular mechanisms by which virus entry occurs have not been defined. After the virus has been transported to skin during primary VZV infection, the cells of the epidermis become the major target cells for further replication (Fig. 5; see Fig. 2). The virus may gain access to cutaneous epithelial cells by migration of intact, infected T lymphocytes out of the capillaries into cutaneous tissue, followed by release of virions (245). Alternatively, infectious virus may move from T lymphocytes into endothelial cells forming the capillary walls, followed by replication and spread to adjacent epithelial cells. Viral inclusions and viral proteins have been observed in capillary endothelial cells and in adjacent fibroblasts as well as in epithelial cells from VZV lesion biopsies (9,255). The lymphatics of the superficial dermis are also involved in cutaneous infection, showing dilated lining cells with intranuclear inclusions (220). The pathogenesis of the characteristic varicella lesion was described by Tyzzer in 1906 (340), who noted that the first changes consisted of vasculitis involving the endothelium of small blood vessels and that enlarged, multinucleated epithelial cells with intranuclear eosinophilic inclusions were typical of the second, maculopapular stage of the lesions. These intranuclear inclusions are identical to those that appear in tissue culture cells during VZV replication, indicating direct viral infection of the affected cell type. Progressive “ballooning” degeneration of epithelial cells associated with formation of multinucleated cells, coalescence of fluid-filled vacuoles between cells, and increased numbers of infected cells at the base of the lesion are noted as the maculopapular lesions evolve into vesicles; destruction of the germinal layer of the epithelium is observed in larger lesions (131,220) (Fig. 6).

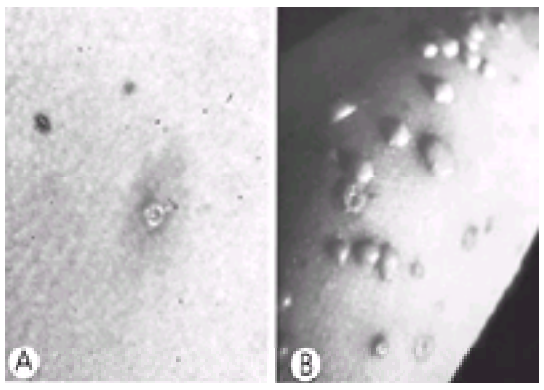

FIG. 5. Varicella skin lesions. **A:** Umbilicated, somewhat elliptical early lesion. **B:** Multiple stages or “crops” of lesions appearing simultaneously. (From ref. 127, with permission.)

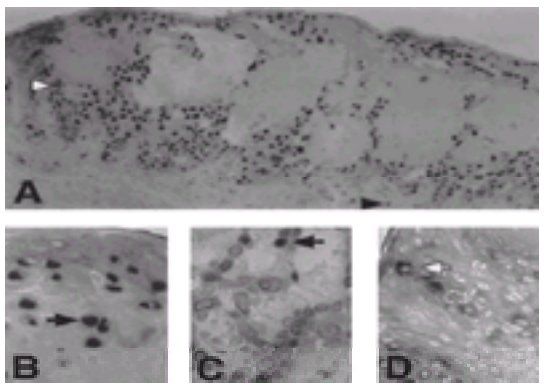

FIG. 6. Punch biopsy specimen of a varicella-zoster virus (VZV) skin lesion analysed for VZV proteins by immunohistochemistry. **A:** ORF29p in the nuclei of VZV-infected cells underlying an intact vesicle. The *open arrow* indicates an infected epithelial cell; the *closed arrow* indicates an inflammatory cell. **B:** Higher magnification of **A**, showing the nuclear localization of ORF29p. **C:** IE63p, which is located predominantly in the nucleus (*closed arrow*). **D:** gC in the cytoplasm of epithelial cells (*open arrow*). (Courtesy of Paula Annunziato.)

Events in the initial stages of primary VZV infection occur with little or no sensitization of the host immune system, as measured by antibody or T-cell responses to VZV antigens. Infiltration of the involved skin sites by inflammatory cells is minimal in the early vesicular phase. Later, cutaneous lesions progress to ulceration and are characterized by necrosis into the dermis. Using electron microscopy, virions can be seen in the capillary endothelial cells of varicella lesions, in the keratinocytes of the epidermis, and as cell-free virus in vesicular fluid. Extensive aggregates of virus particles, including complete and hollow capsids, are visible in the nuclei of some keratinocytes (220). Virions are also detected in lysosomal compartments of macrophages within late-stage lesions. In skin biopsy specimens analyzed by immunohistochemical or *in situ* hybridization methods, early lesions showed IE63 in keratinocytes; later, gE, gB, and IE63 were detected in keratinocytes, sebocytes, Langerhans cells, dermal dendrocytes, monocyte-macrophages, and

endothelial cells. Viral proteins were also expressed in dermal nerves and in perineural type I dendrocytes (9,257).

VZV exhibits tropism for other tissues when cell-associated viremia and viral replication are not controlled adequately by the host response to primary infection; under these circumstances, VZV produces disseminated infection that may involve the lungs, liver, central nervous system, and other organs (250). Lung cells are the most important secondary target of VZV from a clinical perspective because pneumonia is the most common complication of varicella and has a high mortality rate. The pathologic changes of varicella pneumonia are associated with active infection of the epithelial cells of the pulmonary alveoli (13). Infection induces mononuclear cell infiltration of the alveolar septa and edema of alveolar septal cells, along with the accumulation of exudate, formation of hyaline membranes, and desquamation of alveolar cells in the alveolar spaces. Desquamated septal cells in the alveoli and bronchiolar epithelial cells contain eosinophilic intranuclear inclusions, and dense patterns of virions are visible in these cells by electron microscopy. Alveolar cell desquamation and interstitial inflammation interfere with oxygen transfer from the alveoli to the pulmonary capillaries, resulting in severe hypoxemia and respiratory failure. Focal necrosis involving capillaries, alveolar cells, and arterioles is characteristic of fatal varicella pneumonia. Transient replication in the liver is probably a usual component of the pathogenesis of primary VZV infection (149). This process differs from the extensive viral replication in the liver associated with progressive varicella, which causes widespread lysis of hepatocytes and clinical signs of fulminant hepatic failure (107).

The pathogenesis of central nervous system involvement, manifest as meningoencephalitis or cerebellar ataxia, during primary VZV infection is not well defined. The pathologic mechanism of cerebellar ataxia is particularly intriguing because it is associated so uniquely with varicella. Varicella encephalitis is observed in compromised patients who have persistent cell-associated viremia, suggesting a role for direct viral invasion in its pathogenesis. VZV was isolated from the brain tissue of a leukemic child with fatal varicella (332). The detection of herpesvirus particles in cerebrospinal fluid (CSF) by electron microscopy and of viral DNA by PCR support the possibility that varicella encephalitis involves active viral replication in the central nervous system in some cases. That VZV has the potential for invading and replicating in brain tissue is also evident in patients with a form of multifocal leukoencephalitis; VZV has been isolated, detected by electron microscopy, or demonstrated by staining cells for the expression of late viral proteins in these lesions (61). The histopathology of brain tissue from rare cases of fatal varicella encephalitis demonstrates demyelination, axonal damage, perivascular infiltration with mononuclear cells, microglial proliferation, and neuronal degeneration; however, histologic abnormalities are often minimal (332). Vasculitis involving large and small vessels in the central nervous system is an important pathogenic event in these disorders (8,205).

Some neurologic syndromes of varicella have been attributed to an immune-mediated process. The usual complete resolution of neurologic disorders associated with varicella contrasts with the necrotizing encephalitis caused by HSV and is consistent with a vasculitic or immune-mediated pathogenesis. These mechanisms of pathogenesis could account for the common failure to detect VZV in neural tissue and for the predominance of perivenous demyelination, mononuclear cell infiltration, and microglial proliferation, as opposed to the focal necrosis with neuronal destruction that would be expected with direct viral invasion and cell-to-cell spread of infectious virus. Histopathologic correlates of cerebellar ataxia have not been described because this complication is not fatal. It may be an immune-mediated disorder, but ataxia and encephalitis have been described during the preeruptive phase of varicella (225,345).

Thrombocytopenia, a marked decline in the number of platelets in the circulation, can be a pathologic event during primary VZV infection, causing coagulopathy and bleeding disorders. Interference with platelet production by direct viral infection is suggested by the detection of herpesvirus particles within bone marrow megakaryocytes by electron microscopy. Megakaryocytes have toxic changes, including vacuolization and degeneration of the nuclei in cases of hemorrhagic varicella. Platelet survival may be reduced as a result of endothelial cell damage caused by vasculitis or transient hypersplenism, or as part of the syndrome of intravascular coagulopathy. Thrombocytopenia is also a postinfectious complication of varicella, associated with increased production but shortened platelet survival and detection of antiplatelet antibodies (383a).

Adrenal infection, characterized by focal necrosis, is not uncommon in fatal, disseminated varicella. Renal pathology may occur, with hypercellularity of the glomeruli, epithelial proliferation, focal necrosis, and an interstitial inflammatory infiltrate; whether these changes are due to immune-mediated mechanisms or to direct viral invasion is not known. The virus can also cause arthritis, uveitis, myocarditis, pancreatitis, and orchitis, but these inflammatory processes are unusual in the pathogenesis of primary VZV infection.

Placental infection with chronic villitis has been described after maternal varicella in pregnancy (283). VZV can be transmitted to the fetus, probably secondary to maternal viremia, and in rare instances, it causes the congenital varicella syndrome. Varicella embryopathy exemplifies the consequences of VZV infection of cells of the developing nervous system. The unusual characteristic limb hypoplasia of congenital varicella syndrome is attributed to denervation due to infection of spinal ganglia cells, whereas damage to sensory neurons accounts for the unusual cicatricial skin scars, cutaneous defects, and hypopigmented skin areas. Esophageal dysfunction and hydroureter result from damage to the developing autonomic nervous system. Severe microcephaly with cortical atrophy and calcification follows intrauterine encephalitis and necrosis of brain parenchyma.

Release from the Host

An important difference between VZV and the other human herpesviruses is that infectious VZV is released from the infected host in respiratory secretions, allowing its airborne transmission. Isolation of infectious virus from respiratory mucosal sites has rarely been accomplished, despite intensive efforts, but viral DNA can be detected by PCR in specimens obtained from these sites in the late stage of the incubation period and during the first few days of rash (24,302). These observations confirm the epidemiologic evidence that VZV is transmissible for 24 to 48 hours before the exanthem begins (56). VZV DNA has also been detected on the hands of cases and household contacts as well as on environmental surfaces (28). Because many VZV virions are released into vesicular fluid, direct contact with cutaneous lesions provides another mechanism for VZV transmission and probably accounts for the high rate of secondary spread

among close contacts.

Virulence

Naturally circulating strains of VZV do not exhibit intrinsic differences in virulence, as judged by the similar patterns of clinical illness that occur during the annual epidemics of primary VZV infection. VZV isolates that have different geographic or temporal origins vary in their restriction enzyme profiles, but associations with virulence have not been identified. No systematic analyses have been undertaken, but animal experiments done with different VZV strains do not suggest variations in pathogenic potential. Knowledge about the effects of genetic mutations on VZV virulence is limited. In addition to the spontaneously occurring gE mutant, gC (gp V)-negative viruses can be plaque purified after serial passage of VZV in cell culture (201,203,246), and TK-negative variants can be recovered from wild-type virus by selective pressure with antiviral agents (333). These mutations do not affect replication *in vitro,* but VZV gC-negative mutants have limitations in infectivity for human skin cells in the SCID-hu mouse model (246); VZV TK-negative viruses have been isolated from patients given prolonged acyclovir therapy, but no altered pathogenicity has been described (374).

Based on analysis of VZV tropisms in the SCID-hu model, the ORF47 gene product is a virulence determinant for T lymphocytes and skin cells, the ORF66 kinase contributes to infectivity for T lymphocytes, and gC is required for effective replication in skin (245,246 and 247). Eliminating the expression of ORF66, a second serine-threonine protein kinase unique to the alphaherpesviruses caused a partial reduction of VZV infectivity for T lymphocytes but did not impair replication in skin (247). These experiments provided the first evidence that specific VZV gene products have essential roles for viral pathogenesis in differentiated human cells within intact tissues. Despite the fact that the viral proteins are dispensable for VZV replication in cell culture, certain gene products are necessary for efficient T-cell replication (ORF66), and others are required in both skin and T cells (ORF47).

Persistence

Current information about the cellular sites of VZV latency is reviewed in Chapter 78. Briefly, although pathologic documentation is incomplete, latent infection of cells in the dorsal root ganglia appears to follow primary VZV infection invariably (121,143,183,325). How the virus reaches the site of latency is not known. By analogy with HSV, VZV may travel from mucocutaneous sites of replication along the neuronal cell axon to the dorsal root ganglia. Alternatively, as indicated by *in situ* hybridization testing of tissue from a child with fatal varicella, the virus may be carried to sensory ganglia by infected PBMCs before any cutaneous lesions appear.

The characteristics of VZV persistence have been considered to differ significantly from the other human herpesviruses, in which frequent episodes of asymptomatic reactivation are demonstrated by recovery of infectious virus from mucocutaneous sites, or the virus persists in circulating peripheral blood cells. It is true that reactivation of VZV has been difficult to document virologically in the absence of cutaneous lesions. However, periodic episodes of subclinical VZV reactivation probably occur, unless mechanisms for restricting the replication of the latent virus are extraordinarily efficient compared with those governing other herpesviruses. In support of this hypothesis, subclinical VZV viremia was demonstrated by PCR in 19% of bone marrow transplant recipients who had no clinical symptoms of herpes zoster, and elderly adults have episodes of transient, asymptomatic VZV viremia (90,308,377).

Recurrent Infection: Herpes Zoster

The pathologic consequence of VZV reactivation is a vesicular rash that usually involves the dermatomal distribution of a single sensory nerve. The pattern of unilateral dermatomal involvement is so consistent that early mapping of the sensory dermatomes was based in part on observations of the cutaneous areas involved during episodes of herpes zoster (190). The histopathology and cellular expression of viral proteins in recurrent cutaneous lesions generally resemble varicella lesions (9,255,361) (see Fig. 6). By immunohistochemistry, VZV proteins were detected in the erythematous stage only in ballooning cells of the lower epidermis or follicular epithelium; biopsies of vesicular lesions showed viral proteins in cells within the intraepidermal vesicles and in histiocytes of the dermis; and in the late pustular stage, viral antigens were detected primarily in necrotic keratinocytes (249). In addition, viral proteins and inclusion bodies have been described within the small nerves in the dermis beneath the vesicles, which is consistent with transmission of the virus to the epidermis along cutaneous nerve pathways instead of by infected PBMCs, as occurs during primary VZV infection (9,220).

As described in Chapter 78, the pathologic changes of acute VZV reactivation in dorsal root ganglia have been documented in a few patients who died of other causes during an episode of herpes zoster. VZV has been identified in both neuronal and nonneuronal cells of dorsal root ganglia by the histopathologic detection of inclusion-containing cells, immunofluorescence staining to demonstrate cells expressing viral proteins, viral culture, and *in situ* hybridization (36,106,143). The pathologic changes include inflammatory necrosis of dorsal root ganglia, motor and sensory root degeneration, neuritis and segmental myelitis, and localized leptomeningitis. In contrast to HSV, recovery of infectious VZV from explant cultures of human ganglia has been accomplished only when the patient had acute infection at death (270,314).

Cell-to-cell spread of VZV within ganglia, or reactivation in many cells, allows access of the virus to sensory neural axons for transport to skin. The appearance of clusters of lesions in scattered areas of the involved dermatome suggests infectious virus transport along multiple axons. Subsequent cell-to-cell spread probably occurs between skin cells in the involved dermatome. Inflammation and necrosis sometimes extend into the anterior horn cells, producing deficits of motor function. VZV has been isolated from the spinal cord of a patient with transverse myelitis, and herpes particles were observed in the glial cells of the spinal cord by electron microscopy; extensive necrosis of the affected segments of spinal cord was observed with destruction of gray and white matter, mononuclear cell infiltration, and replacement of neural tissue with fibrin (194). VZV-infected cells have been detected in subependymal microvessels in fatal cases of VZV reactivation (305). VZV reactivation in the absence of cutaneous rash was documented using histochemical stains to

demonstrate VZV-infected cells in the meninges and nerve roots of an immunosuppressed patient who had acute radiculoneuropathy (95). Three pathologic patterns of VZV encephalitis have been described, including cases associated with infarction of large or medium vessels; those characterized by deep white matter, ovoid mixed necrotic, and demyelinative lesions due to small vessel vasculopathy; or cases with ependymal and periventricular necrosis associated with vasculopathy of subependymal vessels and secondary infection of ependymal and other glial cells in the periventricular region. Many cases have features of two or three of these patterns (205).

The pathogenesis of VZV reactivation in the immunocompromised host differs in that the dermatomal rash is usually more extensive, and cutaneous replication is often accompanied by cell-associated viremia (109,341). Unusual, chronic, verrucous lesions have been described in patients with acquired immunodeficiency syndrome (AIDS) who had recurrent VZV infection (Fig. 7); these lesions were characterized histologically by epidermal hyperplasia and massive hyperkeratosis with multinucleated giant cells, necrotic acantholytic keratinocytes, and the detection of VZV by viral culture and PCR (3,219,256). In compromised patients, VZV can disseminate hematogenously to cause the same pathologic consequences that occur in patients with progressive varicella, including infection of lung, liver, central nervous system, and other organs. In some cases, VZV reactivation in immunocompromised patients produces visceral dissemination without clinical signs of localized dermatomal disease; these cases suggest that the virus can be taken up by T lymphocytes trafficking through ganglia, so that cell-associated viremia occurs without initial axonal transport of the virus to skin and cutaneous replication (229). VZV has been found by PCR in CSF from patients who have human immunodeficiency virus (HIV) infection, with or without signs of VZV encephalitis or meningoencephalomyelitis (68). Multiple cutaneous isolates or cutaneous and blood isolates from individual patients with herpes zoster and viremia have indistinguishable restriction endonuclease patterns when viral DNA is analyzed, indicating that no alteration of the virus is required for progressive or invasive disease.

FIG. 7. Recurrent varicella-zoster virus (VZV) infection in a patient with human immunodeficiency virus–related acquired immunodeficiency syndrome (AIDS). This photograph illustrates the unusual hyperkeratotic cutaneous lesions observed in patients with AIDS who have VZV activation and who fail to establish latency after varicella. A chronic course with continued or intermittent formation of new lesions is common. (Courtesy of S. Straus.)

Reinfection

Reinfection with VZV appears to be a very rare event. Second episodes of varicella are described, but confirming reinfection with VZV is difficult because vesicular rashes in childhood have other etiologies and laboratory documentation is usually lacking. One adult known to have antibodies to VZV before exposure developed varicella 15 days later; clinical varicella has also been described in three children with leukemia despite serologic evidence of past infection with VZV (136). In a cohort of adults who developed varicella despite a past history, none had VZV antibodies in sera that had been obtained before the acute illness (349). Most immunocompromised patients who have had primary VZV infection and develop clinical signs of varicella probably have generalized, atypical reactivation without dermatomal involvement, rather than reinfection. Differentiating generalized reactivation of endogenous latent virus and reinfection requires comparing restriction endonuclease patterns of the DNAs of the VZV isolates from the first and second episodes, which is seldom possible. Immunologic evidence of reinfection is indirect; whether the increases in antibody and cellular immunity to VZV that occur when immune subjects have close contact with the virus mean that the virus reaches any of the sites of replication that are characteristic of primary VZV infection is not known. Antigen presentation with enhancement of adaptive immunity may occur without any viral replication, as is observed when immune individuals are given inactivated varicella vaccine (320).

The Host Response

Primary Infection: Varicella

Although primary VZV infection almost always causes disease, the host response terminates its progression within a few days. VZV has evolved to infect cells of the host immune system directly, exhibiting tropism for T lymphocytes. Although the virus has mechanisms for transient immune evasion of CD4 and CD8 T-lymphocyte responses, replication is controlled when virus-specific immunity is induced (22). Unless the host is compromised, this pattern of virus–host interaction favors the virus first because cutaneous viral replication allows VZV transmission, and then the host, because the acquisition of memory immunity protects against illness upon subsequent exposures and preserves latency, or at least blocks disease owing to VZV reactivation.

Innate Immunity

Natural killer (NK) cells from nonimmune individuals have the capacity to lyse VZV-infected fibroblasts, which can be

enhanced by incubating the effector cells with interleukin-2 (IL-2); this cytotoxic activity is eliminated when PBMCs are incubated with anti-CD16 antibodies in the presence of complement (47,185,186). IFN-a is detected in serum at the onset of varicella (16,18,348) and may limit early viral replication, as suggested by its clinical efficacy in modifying the severity of varicella in immunocompromised children and the association of more severe varicella in adults with reduced serum IFN-a. Granulysin is a novel cytolytic protein made by NK cells as well as by antigen-specific CD8+ T lymphocytes. Granulysin enhances the death of VZV-infected cells *in vitro* and inactivates intracellular virus (166), reversing viral inhibition of apoptosis. IFN-g, NK cell cytotoxicity, and granulysin represent innate mechanisms for controlling spread of the virus before adaptive immunity is elicited.

Adaptive Immunity

Primary VZV infection is associated with the production of IgG, IgM, and IgA antibodies directed against many viral proteins (13,15,45,94,137,214,327). Antibodies are not present in serum during most of the incubation period but may be measured in low concentrations at the time of onset of the varicella exanthem and become detectable within 3 days in most individuals. Antibodies to VZV proteins have neutralizing activity, either directly or in the presence of complement, and function in antibody-mediated cellular cytotoxicity (118,185,307). Neutralizing activity by antibodies to VZV gE is mediated through three distinct domains as mapped using monoclonal antibodies (385). That virus-specific antibodies can interfere with the initial phases of VZV replication *in vivo* is evident from studies showing that the attack rate for varicella is reduced by VZV immune globulin prophylaxis (390). Even if infection occurs, the severity of the cutaneous disease and the risk for varicella pneumonia are modified by passive antibody administration, indicating that passive antibodies present at the beginning of the incubation period can restrict the pathogenic potential of the virus. The modulation of viral replication *in vitro* by monoclonal antibody to gH (gp III) suggests that anti-gH (gp III) antibodies may mediate this effect (284). Whether antibodies limit replication at initial sites, such as mucous membranes or lymphoid tissue, or block or diminish primary viremia is not known. Transplacentally acquired IgG antibodies to VZV also protect infants from primary VZV infection, or modify the severity of varicella. In contrast, passive antibodies administered after the appearance of the varicella rash have no effect on disease progression. The role of the active humoral immune response in limiting the progression of primary VZV infection also appears to be limited. Children with agammaglobulinemia had uncomplicated varicella, whereas those with primary immunodeficiency diseases affecting cell-mediated immunity often died (13). Among healthy children, the early detection of high IgG or IgM antibody titers against VZV does not predict milder infection, and immunocompromised children may develop progressive varicella despite adequate production of VZV antibodies (16). Whether failure to produce antibodies to particular viral proteins is important in the host response is not known, although some immunocompromised children with progressive varicella lack VZV IgG antibodies to some major viral proteins (360).

The critical role of virus-specific cell-mediated immunity has been shown in studies correlating the induction of virus-specific T-cell recognition with the outcome of primary VZV infection (reviewed in 22 and 23) (Table 1). Intact cellular immunity appears to be important for terminating cell-associated viremia as well as limiting virus replication at localized cutaneous sites. Healthy children who have T lymphocytes that recognize VZV antigens within 24 to 72 hours have milder primary VZV infection, with fewer cutaneous lesions (16). An absolute lymphopenia of less than 500 mm^3 at the onset of illness and a failure to acquire VZV-specific T-cell proliferation correlates with persistent viremia and dissemination in high-risk children (16,210). T-lymphocyte proliferation to VZV antigen is accompanied by the production of cytokines of the helper T-cell subtype 1 (T_H1) type, including IL-2 and IFN-g (38,190,394). IFN-g, which potentiates the clonal expansion of virus-specific T cells, is detected in serum during acute varicella (16). By comparison with children, adult subjects were less likely to have detectable IFN-g at the onset of primary VZV infection, but there was no correlation between relative serum tumor necrosis factor-a (TNF-a, IL-2, or IFN-g concentrations and disease severity in adults with varicella (348).

Host	Clinical/VZV T-cell proliferation Correlations		
Healthy susceptible	Varicella	Early acquisition	Mild infection
Immunodeficient susceptible	Varicella	Delayed or no acquisition	High risk for dissemination
Healthy immune	Varicella exposure	Enhanced response	No disease
Healthy immune	Age >60 y	Low or absent response	High risk for herpes zoster
Immunodeficient immune	Low or absent response	High risk for herpes zoster and dissemination	

From Arvin AM, Cell-mediated immunity to varicella-zoster virus. J Infect Dis 1992;165(Suppl 1):S62–S67.

TABLE 1. *Correlations between cell-mediated immunity and varicella-zoster virus disease*

T lymphocytes from healthy immune subjects recognize gB, gC, gE, gH, gI, and the immediate early gene products of ORF62 and ORF63 (22,23,169,294). T-cell responses to gE, gH, and the IE62 protein are elicited during primary VZV infection, although the kinetics of acquisition of T-cell immunity to each of these VZV proteins varies; VZV infection also induces T lymphocytes that recognize gB and gC (169). Cell-mediated immunity to IE62 and IE63 proteins, gE, gB, gH, and gC persists for years after primary VZV infection in the immunocompetent host (14,23) (Table 2). When responses to synthetic peptides corresponding to residues of the IE62 protein and gE were evaluated, immune donors had circulating T lymphocytes that recognized an average of 7 of 10 IE62 peptides and 6 of 10 gE peptides (39). Dendritic cells acted as potent antigen-presenting cells to induce recognition of VZV peptides by naive donor T lymphocytes, and positive response to a peptide was predictive of T-cell sensitization against the same epitopes after *in vivo* exposure to VZV (191). T-cell clones that recognize amphipathic regions of gB and gI, some of which exhibit B-cell helper function, are also present in CD4+ T-cell populations from immune individuals (169). The memory helper T-cell response is associated

with the persistence of IgG antibodies that bind to all of the glycoproteins and to many other VZV polypeptides (57,94,151,360).

Assay	T-cell subset	Viral proteins
T-cell proliferation		
Proteins		gEgp I, gBgp II, gHgp III, gVgp VI, IE62
Peptides		gEgp I, gBgp II, gVgp VI, IE62
T-cell cytotoxicity		
Induction of clonal expansion by secondary stimulation	CD4+	gEgp I, gBgp II, gVgp VI
Recognition of target cells expressing VZV proteins	CD4+	gEgp I, gVgp VI, gCgp II, IE62
	CD8+	gEgp I, gVgp VI, gCgp II, IE62

From Arvin AM. Cell-mediated immunity to varicella-zoster virus. J Infect Dis 1992;165(Suppl 1):S95–S41.

TABLE 2. *Varicella-zoster virus proteins and peptides recognized by T lymphocytes from immune individuals*

T lymphocytes that exhibit class I and class II MHC-restricted lysis of target cells expressing VZV antigens can also be demonstrated from immune individuals (92,175,179). VZV-specific CD4+ T cells, which are class II MHC restricted, have cytotoxic function against autologous lymphoblastoid cells expressing VZV proteins (92). CD8+-mediated cytotoxic T-lymphocyte (CTL) activity has also been demonstrated using VZV-infected fibroblasts as targets, which express only MHC class I antigen, and by testing highly purified subpopulations of CD8+ T lymphocytes as effectors against lymphoblastoid cells expressing VZV proteins (20,175). CTLs that recognize the IE62 protein or gE are present in both the CD4+ and CD8+ memory T-cell populations, at equivalent precursor frequencies (22). VZV gC or gI is also recognized by CTL from immune donors, as are the structural-regulatory proteins encoded by ORF4 and ORF10 (22,179,312). The ORF33 gene product, a virion assembly protein, is immunogenic in animals but has not been evaluated for recognition by human T lymphocytes (127).

Memory immunity to VZV is required to preserve latency. VZV memory T lymphocytes are maintained at precursor frequencies of about 1 per 20,000 to 40,000 PBMCs in immune adults (168,221). Memory CD4+ and CD8+ CTLs specific for particular VZV proteins are present at frequencies of about 1:150,000 PBMCs. By intracellular cytokine assay, the mean percentage of VZV-specific CD4 T lymphocytes was 0.12% by IFN-g synthesis and 0.34% using TNF-a as the marker (29). Immune subjects also have persistent DTH responses to VZV skin test antigens (195). Cellular immunity is likely to be enhanced periodically in immune individuals because of exogenous reexposures. Enhanced VZV T-cell proliferation responses, as well as increases in IgG antibodies, occur in immune adults after household contact (17). Immune children who are reexposed to varicella also have a significant enhancement of DTH to VZV. Although symptomatic reinfection with VZV is rare, these immunologic changes indicate that reexposure to the virus may result in subclinical reinfection, or at least in a sufficient uptake of viral antigens by antigen-presenting cells to boost immunity. VZV DNA was detected by PCR in nasopharyngeal secretions from immune household contacts of children with varicella (28,80). The potential for endogenous reexposure to increase the frequency of VZV specific T lymphocytes is also suggested by the enhanced responses of elderly immune adults given varicella vaccine (171,259). The gene products of ORF4, ORF21, ORF29, ORF62, and ORF63 may be expressed in neuronal or satellite cells during latency, but peptides can only be detected in MHC complexes, and class I MHC requires IFN-g exposure to be up-regulated on neurons; because satellite cells express class I MHC, and class II can be induced, these cells could function for antigen presentation. Whether co-stimulatory molecules necessary for signaling CD4 and CD8 T-lymphocyte expansion are expressed by neurons or satellite cells harboring VZV is not known. However, if conditions for antigen presentation occur, VZV immunity could be maintained by repeated antigenic stimulation due to subclinical VZV reactivations, producing endogenous reexposure to viral antigens. This possibility is supported by the recovery of cell-mediated immunity to VZV in bone marrow transplant recipients who have not had any clinical signs of herpes zoster but in whom subclinical reactivations can be detected by PCR (377).

Recurrent Infection: Herpes Zoster

The high incidence of herpes zoster in elderly and immunocompromised patients suggests that host factors have a significant effect on whether VZV reactivation occurs, or at least on whether reactivation produces symptomatic disease. That cellular immunity is essential for maintaining the balance between the host and the virus is indicated by correlations between diminished *in vitro* T-lymphocyte proliferation to VZV antigens in elderly patients and in patients receiving immunosuppressive therapy, and their susceptibility to herpes zoster (13). Among these high-risk populations, low or absent cellular immune responses to VZV represent a necessary but not a sufficient condition for the occurrence of symptomatic recurrent VZV. Patients who have the most severe and prolonged suppression of VZV-specific cellular immunity are at highest risk for herpes zoster, viremia, and dissemination. The age-related increase in the risk for herpes zoster among elderly subjects is attributed to immunosenescence and has been correlated with a decrease in DTH responses to VZV skin test antigen as well as a decrease in the frequencies of circulating T lymphocytes that recognize VZV antigen (29,195).

When reactivation occurs, T-lymphocyte responses increase immediately (168). The resolution of the rash is accompanied by local production of IFN-a in lesions (324). Unless further immunosuppression intervenes, enhanced immunity persists for a prolonged period and may explain why second episodes of herpes zoster are rare. Herpes zoster in young children after intrauterine or early postnatal varicella and the short interval between primary and recurrent VZV infections in children with HIV infection probably represent inadequate cell-mediated immunity (31,89). Among children with HIV infection, 70% of those with fewer than 15% CD4 T lymphocytes, when they acquired varicella, developed

zoster (89).

The susceptibility of elderly and immunocompromised individuals to VZV reactivation is not associated with decreasing titers of VZV IgG antibodies (360). No effect on viral replication at local cutaneous sites was demonstrated when high-titer VZV immune globulin was given to immunocompromised individuals with herpes zoster (324), but low VZV IgG titers may enhance their susceptibility to viremia (360).

EPIDEMIOLOGY

Incidence and Prevalence

Varicella

VZV is a ubiquitous human pathogen with a worldwide geographic distribution. The annual incidence of varicella has been 4 million cases a year in the United States, equivalent to the annual birth rate, but this incidence is expected to fall with the introduction of the varicella vaccine (161). Without immunization, early childhood is the peak age for primary VZV infection. In the 1970s to 1980s, the highest annual incidence occurred in U.S. children who were 5 to 9 years old (89 to 90 cases per 1,000), followed by those aged 1 to 4 years (79 to 82 per 1,000); in the early 1990s, the incidence was highest in preschool-aged children, suggesting a change in the epidemic pattern, perhaps related to day care center exposures (371). By 20 to 29 years of age, only about 5% of individuals remain susceptible in the United States and other geographic areas with temperate climates (88,108); the susceptibility rates are higher among African Americans in each age cohort up to 40 years. Although the percentage of adults who give a clinical history of varicella is as low as 50%, the discrepancy reflects failure to identify or recall mild cases of varicella or incorrect diagnoses (226). In tropical regions, only about half of individuals who are younger than 24 years old have had varicella (129,236,259). In these regions, rural populations have lower seroprevalence than adults living in cities (236). Only 42% of rural adults had been infected with VZV; higher susceptibility rates were also observed in early studies in rural Massachusetts, suggesting that living conditions rather than climate may account in part for the lower incidence of varicella in some areas. The varicella attack rate is very high when susceptible adults from tropical areas move to temperate zones.

Restriction digest patterns demonstrate the identity of epidemiologically related VZV isolates and variability in unrelated isolates (49). For example, profiles obtained using a few restriction enzymes differentiate many VZV isolates recovered in the United States from children with varicella from the Oka strain, a Japanese clinical isolate used to produce the live attenuated varicella vaccine (3,213). However, these differences are not known to reflect clinically significant strain differences that could account for variations in prevalence (331). Further analysis of VZV isolates from diverse geographic locations may lead to new understanding of these patterns of high and low prevalence. Although incidence and prevalence rates must be multifactorial, differences in incidence may also have some as yet undefined relationship to host susceptibility to primary VZV infection.

Herpes Zoster

Because herpes zoster is caused by the reactivation of latent virus, susceptibility requires previous primary VZV infection. Given the high incidence of varicella, most adults are at risk for VZV reactivation. Early studies documented the incidence of herpes zoster as 3.4 cases per 1,000 people per year in the United Kingdom (177). Herpes zoster was very rare in children younger than 10 years old, occurring at a rate of 0.74 per 1,000 persons per year. Although herpes zoster is rare in childhood (155), varicella during the first year of life, or VZV infection acquired by intrauterine transmission, is associated with an increased risk; the incidence of herpes zoster was 2.8 times higher compared with that in children who had varicella after the first year (31). Hope-Simpson observed that the incidence rose to 7.8 cases per 1,000 people older than 60 years of age, compared with 2.5 per 1,000 people 20 to 50 years of age (177). The incidence continues to rise after age 60 years, such that the lifetime risk is as high as 50% among those who survive to 85 years (304). The disease burden of herpes zoster in the United States is about 600,000 cases per year (1.7 per 1,000 population) and will increase predictably as the population ages (304). Incidence rates are equivalent between men and women. Herpes zoster is significantly less common in black Americans than in whites (303). A comparison of white Hutterite and non-Hutterite populations in Manitoba revealed a lower rate of herpes zoster among the Hutterites as well as a lower incidence of varicella before age 10 years and lower seroprevalence at all ages, suggesting that later age at acquisition of primary VZV infection may correlate with a reduced risk (288). Analysis of stressful life events did not reveal a definite relationship to risk of herpes zoster (304). Second episodes of herpes zoster are rare among healthy individuals; the frequency was 4% in the Hope-Simpson study and 1.7% to 5.2% in U.S. studies (125,279).

Herpes zoster is particularly frequent among patients with leukemia, Hodgkin's and non-Hodgkin's lymphoma, and oat cell carcinoma of the lung. Patients with Hodgkin's disease often undergo splenectomy, but splenectomy alone does not appear to predispose patients without underlying malignancy to VZV reactivation. The incidence of herpes zoster after bone marrow transplantation is very high, with attack rates of 25% to 50% reported during the first year. Among renal transplant recipients, 15% had had herpes zoster at a mean of 5 years after transplantation. Patients with systemic lupus erythematosus are also more likely to develop herpes zoster, with an estimated incidence of 22 cases per 1,000 person years (237). Clinical experience suggests that steroid therapy enhances the risk in patients with collagen vascular diseases, but prospective studies have not been reported. The incidence of herpes zoster is estimated to be 15 to 25 times higher in individuals with HIV infection than the general population, and 3 to 7 times higher in those with HIV than among healthy elderly adults (59,140,176). HIV infection increases the risk for herpes zoster regardless of age. Among HIV-infected children, 27% had herpes zoster within a mean of 1.9 years after varicella (138).

Although cancer is correlated with a higher risk for herpes zoster, herpes zoster in healthy individuals is not associated with an increased risk for malignancy (280). Herpes zoster associated with risk factors for HIV infection suggests this underlying diagnosis, but herpes zoster is not a marker for enhanced risk for HIV progression.

Epidemic Patterns

Episodes of herpes zoster provide a continuous source for transmission of VZV to susceptible individuals, who then develop varicella. VZV causes annual epidemics of varicella in temperate regions, particularly during the late winter and spring. Epidemic years characterized by a high incidence of varicella are followed by years in which fewer cases occur (67,371). Varicella epidemics probably begin from sporadic cases in children caused by exposures to recurrent VZV in adults. Amplification to involve large numbers of susceptible children results from the relative efficiency of VZV transmission by the respiratory route. The annual pattern of varicella epidemics may be related to the return of children to school, which heightens close contact among newly infected and susceptible children. A clinical experience reported from the Shetland Islands illustrated the origin of a varicella outbreak in an isolated population from a single adult case of herpes zoster. The attack rate for previously uninfected household contacts exposed to varicella is about 90%, whereas the transmission rate from more casual exposures, such as those associated with school classrooms or hospitals, is estimated at 12% to 33%. In a day care center outbreak, the cumulative attack rate was 88% over a 15-week period, indicating a much lower secondary attack rate from index cases than occurs in households (99a). The attack rate for primary VZV infection in children is not affected by sex or by ethnic or socioeconomic factors. Varicella epidemics may have been less well sustained in rural communities when the demographics of the United States was characterized by a more scattered distribution of the population. Why varicella epidemics are less extensive in tropical areas is difficult to explain because the overcrowded living conditions that are common in many of these regions of lower prevalence should enhance the spread of the virus. These effects may be countered by more dispersed population in rural conditions.

New cases of herpes zoster are diagnosed with the same frequency throughout the year, exhibiting no seasonal pattern (177,279). The epidemiology of herpes zoster reflects the fact that this infection originates from the reactivation of endogenous, latent virus rather than exogenous acquisition. Some clinical case reports have suggested that exposures to varicella resulted in herpes zoster, but the epidemiologic data do not support a mechanism of transmission in which reinfection results in localized disease, and there is no known mechanism by which reactivation of endogenous VZV would be triggered by a new exposure. The probable effect of these exposures is to enhance VZV immunity and, potentially, to reduce the risk for recurrent VZV disease (17).

Morbidity and Mortality

The high prevalence of symptomatic primary and recurrent VZV infections in the general population means that almost all individuals experience some morbidity due to VZV in the course of their lives (64,65,271). Morbidity in otherwise healthy individuals is usually limited to the symptoms caused by the acute illness and the inability to pursue usual activities for a period of 1 to 2 weeks. The risk for life-threatening morbidity or mortality during primary or recurrent VZV infection depends on host factors rather than virulence characteristics of the virus.

Varicella

In 1935, Bullowa and Wishik (60) observed that varicella is “the disease of childhood which is taken least seriously by the laity and by the medical profession.” Nevertheless, varicella complications result in annual hospitalization rates of 11,000 per year in the United States (2.2 to 4.3 per 1,000 cases). Serious complications are least common among children 1 to 9 years old and are 6 to 15 times higher among adults. Case fatality rates are 10 and 24 times higher in infants (6.7 per 100,000 cases) and adults (17.1 per 100,000 cases), respectively, than in children 1 to 4 years of age (0.7 per 100,000) (311). Secondary bacterial infections, usually caused by *Staphylococcus aureus* or *Streptococcus pyogenes,* constitute the most common cause of varicella-related morbidity and mortality in children (114,173,174,213). Mortality from sepsis or necrotizing fasciitis remains significant despite antibiotic therapy (114). One third of invasive group A streptococcal disease in children is associated with varicella (154). Most of the increased morbidity and mortality in adults is due to varicella pneumonia (67,156,157,258,271).

Neurologic complications are the second most common indication for hospitalization of immunocompetent children with varicella (114,157). Primary VZV infection accounted for 13% of encephalitis cases of known etiology from 1972 to 1977 (271). Central nervous system morbidity is higher among patients younger than 5 years and older than 20 years of age (272). The mortality rate due to neurologic complications of varicella among otherwise healthy children and adults is difficult to estimate because compromised patients are included in some reports, and VZV encephalitis is not easily distinguished from Reye's syndrome. Reye's syndrome was estimated to affect healthy children with varicella in 2.5 per 10,000 cases before the association with salicylates was reported (182). Estimates of mortality due to central nervous system complications have ranged from 5% to 18%; when encephalitis was distinguished from cerebellar ataxia, mortality rates were 35% and 0%, respectively (192). No precise data are available concerning the risk for long-term sequelae; most patients recover fully, but some have recurrent seizures and neurologic deficits (271). Although arterial ischemic strokes are rare in children, one case-control study showed a significant association with VZV infection (309).

Data concerning varicella morbidity and mortality among children with lymphoproliferative malignancy or solid tumors, accumulated before effective antiviral drugs were available, showed that 32% to 50% of patients developed visceral dissemination; varicella pneumonia occurred in 20% of cases; and the mortality rate was 7% to 17% (111). Children with acute lymphoblastoid leukemia or lymphoma were at highest risk, and disease severity was enhanced if chemotherapy had been given during the incubation period—especially within 5 days before the appearance of the rash—and if the patient had an absolute lymphopenia of less than 500 cells.

Varicella during pregnancy may be fatal, or serious for the mother and fetus or infant. Most women of childbearing age who were born in geographic regions where epidemics occur annually have had varicella. In the United States, the incidence of maternal varicella was 0.7 per 1,000 women; a recent prospective study in Germany showed an incidence of 2 per 1,000 pregnancies (103). Although some retrospective reports indicate maternal mortality rates as high as 41%, Siegel and colleagues (316) found one maternal death due to varicella pneumonia among 150 pregnancies (0.7%) complicated by varicella; surveillance of 1,373 cases showed a predicted maternal mortality rate of 1 per 2,000 cases

(103). Varicella pneumonia is the cause of most maternal deaths and may be more common when varicella occurs during the third trimester. The outcome of pregnancy may be affected adversely by spontaneous abortion, fetal demise, and premature delivery, although the frequency of these complications is low (32).

The congenital varicella syndrome (CVS) is a rare consequence of maternal varicella during pregnancy (103,275). The highest risk for varicella embryopathy follows primary VZV infection during the first 20 weeks of gestation and is estimated to be 1% to 2% (32,103). Among 1,373 pregnancies, CVS occurred in 0.4% when maternal varicella occurred from 0 to 12 weeks' gestation, compared with 2% for infection from 13 to 20 weeks' gestation (103). Maternal varicella later in pregnancy was associated with cutaneous scarring, limb deformities, or unilateral ocular defects (241,296). VZV-specific immunity in asymptomatic infants and herpes zoster in infancy, without prior varicella, indicate that the virus is transmitted to the fetus during the second and third trimester of maternal varicella with about the same frequency as in the first trimester (262); in a large prospective study, no infants had CVS or other morbidity at birth if the mother had varicella between 25 and 36 weeks' gestation, but the incidence of herpes zoster in early childhood, a marker for intrauterine infection, was 1.7% (103). When maternal varicella occurs at the end of gestation, infants are at risk for neonatal varicella, with an attack rate of about 20% (272). Without passive antibody prophylaxis, the mortality rate was about 30% in infants whose mothers developed varicella from 4 days before to 2 days after delivery, whereas none of the infants whose mothers had varicella from 21 to 5 days before delivery had serious disease.

Herpes Zoster

Herpes zoster is not commonly fatal, even in compromised patients. Postherpetic neuralgia (PHN) is the most common and potentially disabling morbidity due to herpes zoster. PHN lasting from 4 weeks to more than 10 years occurs in about 10% of all patients with herpes zoster (279,280). The frequency of PHN is higher in older patients, increasing from 3% to 4% in those 30 to 49 years of age, to 21%, 29%, and 34% in patients 60 to 69, 70 to 79, and more than 80 years of age, respectively (177). Children are not at risk for PHN (172). Among older patients, symptoms persisted for more than 1 year in 22%. The average age of patients with PHN was 67 years, compared with 46 years among those without PHN (279). The prevalence of pain at 30 days after the onset of rash was 15-fold higher among those older than 50 years of age. In an analysis of 2,367 subjects who participated in controlled trials of antiviral drugs, age over 50 years, prodromal symptoms, and the severity of acute pain at diagnosis showed the highest correlation with risk for PHN. The risk for prolonged pain was higher when two or more of these factors were identified (375). The risk for PHN is not predictable from the severity of the cutaneous eruption. Immunocompromised patients appear to be at higher risk for PHN.

The incidence of zoster-associated encephalitis ranges from 0.2% to 0.5%. Risk factors include older age and cranial nerve involvement. The mortality rate of cerebral angiitis following herpes zoster is much higher, with a case-fatality rate as high as 20%. Immunosuppressive therapy for malignancy increases the morbidity and mortality associated with herpes zoster, due primarily to a higher risk for VZV pneumonia, although the overall risk for fatal VZV infection after reactivation of the virus in immunocompromised patients remains less than 1%. VZV reactivation as atypical, nonlocalized zoster in immunocompromised patients has a much worse prognosis than localized herpes zoster; the risk for visceral dissemination was 45% in bone marrow transplant recipients, and mortality was 28% without antiviral treatment (229). HIV infection predisposes patients to a higher risk for progressive or chronic cutaneous lesions, meningoencephalitis, and retinitis (4,59,70,79).

Motor deficits occur in an estimated 1% of cases of herpes zoster and are prominent when the facial nerve is involved (279). Significant residual loss of function has been described in 10% to 15% of patients. Transverse myelitis is an extremely rare complication of herpes zoster, but the mortality rate for this complication was 22 of 41 cases.

Herpes zoster is not unusual in pregnancy, but it does not cause significant maternal or fetal morbidity. Enders and colleagues identified no intrauterine infections in 366 pregnancies complicated by maternal herpes zoster (103). The only case of congenital varicella syndrome after maternal herpes zoster was associated with disseminated infection, indicating viremia. No cases of neonatal varicella have been associated with maternal herpes zoster at term.

CLINICAL FEATURES

Varicella

Varicella follows an incubation period that ranges from 10 to 21 days, with symptoms most often beginning between 14 and 16 days (386). Prodromal symptoms of fever, malaise, headache, and abdominal pain precede the appearance of the rash by 24 to 48 hours in about 50% of patients. Fever, irritability, listlessness, and anorexia are prominent during the 24 to 72 hours after the first cutaneous lesions are noted; respiratory symptoms and vomiting are unusual. The temperature elevation is usually less than 101.5°F but may be as high as 106°F. The varicella exanthem begins on the scalp, face, or trunk (see Fig. 5). The cutaneous lesions consist of erythematous macules that evolve rapidly (within several hours) to form a clear, fluid-filled vesicle surrounded by an irregular erythema margin, the classic “dew drop on a rose petal.” Lesions in the maculopapular or vesicular stages are usually intensely pruritic. After 24 to 48 hours, the vesicular fluid becomes cloudy in appearance, and the lesions umbilicate as crusting begins. As the first cutaneous lesions are progressing through these stages, new crops of lesions form on the trunk and then the extremities. Lesions that develop late in the course of the illness are more widely distributed and may resolve without progressing to form vesicles. Lesions of the mucous membranes of the oropharynx, conjunctivae, and vagina are common. In the final phase, the crusted lesions are sloughed as new epithelial cells are generated at the lesion site.

The duration of varicella in otherwise healthy children, judged by the number of days of new lesion formation, ranges from 1 to 7 days. In a large clinical study, more than half of the children developed new lesions for 3 to 6 days, whereas only 1 of 521 children had new lesions for 7 days (286). The total number of varicella lesions ranged from 12 to 1,968, with most children having fewer than 300 lesions. Although the illness may be so mild that it escapes diagnosis, subclinical varicella is documented rarely when susceptible children are examined carefully during the period of risk. Preexisting skin damage such as eczema or sunburn acquired during the incubation period exacerbates the severity of

the exanthem (48). Children who develop varicella after household exposure are more likely to have more extensive varicella (97,286). Hypopigmentation of the skin often persists for several weeks, but extensive scarring is unusual. However, a single, shallow scar on the forehead is a common consequence of varicella because the initial lesions, which often occur along the hairline or eyebrow, involve deeper layers of the skin.

Abnormal laboratory values are to be expected during primary VZV infection, including a decrease in the total white blood cell count, reflecting lower polymorphonuclear leukocyte and lymphocyte counts during the first 72 hours of rash, which is then followed by a relative and absolute lymphocytosis (60). Lymphoblasts and prolymphocytes may be present in the peripheral blood during the acute and convalescent phases of varicella. Slight elevations of liver function tests are also common (107).

Complications

S. aureus and *S. pyogenes* (group A b-hemolytic streptococcus) cause secondary bacterial infection of skin lesions (60,114,189,379). “Impetigo” may be difficult to differentiate from bullous varicella caused by VZV (13). Cellulitis, bacterial lymphadenitis, or subcutaneous abscesses usually appear by 3 to 6 days but may appear up to 2 weeks after exposure (85,154,266,378). In a case-control analysis of group A streptococcal complications, cases were more likely to be secondary household cases, to have fever after day 2, and to have asthma (266). Varicella gangrenosa or necrotizing fasciitis is usually due to *S. pyogenes* (54,393). In its classic presentation, a single varicella lesion, usually on the trunk or an extremity, manifests an unusual appearance early in the course of the varicella eruption. The surrounding skin becomes erythematous, warm, and painful. The erythematous area enlarges rapidly over a few hours, often turning a dusky, dark-red color, and extensive edema develops in the soft tissue around the lesion. Bacteremia, usually caused by *S. aureus* or *S. pyogenes,* may lead to acute bacterial sepsis, with high temperature, hypotension, and disseminated intravascular coagulopathy. Alternatively, the hematogenous spread of the bacterial pathogen may not be detected until signs of a focal infection appear. Staphylococcal or streptococcal pneumonia, arthritis, and osteomyelitis following varicella exemplify this process.

Varicella pneumonia is a serious concern in healthy adults and particularly in the third trimester of pregnancy (258,278). In one early series, varicella pneumonia was diagnosed in 9% of adults (364), but more recent studies indicate that it is somewhat less common (278,346,347). Cough and dyspnea begin about 3 days (1 to 6 days) after the onset of the rash and may be associated with cyanosis, pleuritic chest pain, or hemoptysis (364). Although rales and rhonchi may be detected, the abnormal physical findings are often restricted to fever and tachypnea. Hypoxemia is often much more severe than is suggested by the physical examination or by the chest radiograph. The chest radiograph may be normal but more often demonstrates diffuse bilateral infiltrates with small nodular densities, especially in the perihilar area. Varicella pneumonia is often transient, resolving completely within 24 to 72 hours, although calcified nodules may persist for weeks or months. In severe cases, interstitial pneumonitis progresses rapidly to cause respiratory failure.

Varicella hepatitis can occur subclinically, although some children with the highest elevations of SGOT (range 200 to 800 IU/L) have severe vomiting (107), which must be differentiated from Reye's syndrome. Aspirin is contraindicated in children with varicella because it predisposes to this syndrome of fatty degeneration of the liver and encephalopathy. Varicella encephalitis or cerebellar ataxia have been described during the incubation period, but neurologic symptoms usually occur within 2 to 8 days (13,100,192,205,267,345). Ataxia may be accompanied by nystagmus and slurred speech; meningoencephalitis presents with vomiting, seizures, diminished level of consciousness, nuchal rigidity, and extensor plantar reflexes. Patients with encephalitis usually have the sudden onset of seizures and altered sensorium, whereas those with cerebellar disease show a gradual progression of irritability, nystagmus, and gait and speech disturbances. Some patients have fever, headache, and meningismus only, without altered consciousness or seizures. The CSF usually shows a mild lymphocytic pleocytosis (less than 100 cells/mL) and a slight to moderate protein level (less than 200 mg) with normal glucose. Cell counts and CSF protein are higher with encephalitis than with cerebellar disease, but CSF is often normal. Encephalitic symptoms usually resolve rapidly (within 24 to 72 hours); sequelae are rare. Ataxia may persist for days to weeks, but resolution is almost always complete. A few cases of transverse myelitis have been associated with varicella; paraplegia and sensory loss occurred 5 to 16 days after onset of lesions (144,192). Optic neuritis may occur alone or with transverse myelitis; a few cases of Guillain-Barré syndrome have been reported.

Acute thrombocytopenia causes petechiae and purpuric skin lesions, hemorrhage into the varicella vesicles, epistaxis, hematuria, and gastrointestinal bleeding. Hemorrhagic complications are usually transient, but platelet counts may remain low for days to weeks. Postinfectious thrombocytopenia occurs from 1 to 2 weeks or longer after varicella and causes bleeding complications for an average of 5 weeks. Purpura fulminans, due to arterial thrombosis and hemorrhagic gangrene, is a rare but life-threatening complication. The symptoms of varicella nephritis are hematuria and diffuse edema with or without hypertension and appear within about 3 weeks (13). Laboratory abnormalities include proteinuria, microscopic hematuria, elevated blood urea nitrogen and creatinine, and decreased creatinine clearance. A few cases of nephrotic syndrome and hemolytic-uremic syndrome have been reported in children with varicella. Varicella arthritis is rare; it resolves spontaneously within 3 to 5 days with no residual joint disease. Other rare complications of varicella include myocarditis, pericarditis, pancreatitis, and orchitis. Varicella vesicles are common on eyelids and conjunctivae, but eye disease is unusual (102); if present, unilateral anterior uveitis or corneal lesions usually resolve spontaneously.

Varicella in High-Risk Populations

Immunocompromised children are susceptible to prolonged new lesion formation and visceral dissemination manifesting as varicella pneumonia, hepatitis, encephalitis, and disseminated intravascular coagulopathy (13,33). Varicella vesicles in these patients are usually larger and more numerous, and more than half of the immunocompromised children have new lesion formation for at least 7 days, with an average time to lesion crusting of 14 days (374). They may develop diffuse interstitial nodular pneumonitis within 3 to 7 days after the onset of skin lesions, with rapid progression to respiratory failure. All of the deaths from varicella in children with leukemia occurred within 3 days after the diagnosis of varicella pneumonia (111). Varicella hepatitis is accompanied by jaundice and elevated serum transaminase levels. Thrombocytopenia and disseminated intravascular coagulopathy are common; hemorrhage into lesions is a sign of severe varicella, as is severe abdominal or back pain. Encephalitis occurs but is rarely the immediate cause of death; the

syndrome of inappropriate antidiuretic hormone secretion may accompany disseminated varicella with or without clinical encephalitis. Myocarditis and severe hypertension, possibly related to nephritis, are less frequent complications. Disseminated VZV infection in children with cancer has also produced pancreatitis, necrotizing splenitis, esophagitis, and enterocolitis. Neutropenia due to chemotherapy and radiation enhances susceptibility to secondary bacterial infections.

Children who acquire varicella after organ transplantation are also at risk for progressive VZV infection (122). Thrombocytopenia and hepatitis, rather than pneumonitis, were the major clinical complications in renal transplant recipients. Among 2,320 pediatric patients who received renal transplants between 1987 and 1993, 44 children required hospitalization for varicella during the first year; varicella was not fatal and was not associated with transplant rejection. Children on long-term, low-dose steroid therapy for asthma are not usually at risk for serious varicella (321). However, fatal varicella has been described in patients receiving higher doses of prednisone during the incubation period. Severe cutaneous lesions occur in children receiving steroid therapy for other diseases, such as rheumatoid arthritis, nephrotic syndrome, and ulcerative colitis, but visceral dissemination is unusual.

Untreated varicella is severe or fatal in children with severe combined immunodeficiency or T-cell deficiencies, including adenosine deaminase or nucleoside phosphorylase deficiency and cartilage hair hypoplasia. Varicella can also be severe in children with Wiskott-Aldrich syndrome or ataxia telangiectasia. Fatal varicella in a previously healthy child does not suggest that siblings are at risk for severe VZV in the absence of an identifiable inherited immunodeficiency disorder. Unusual clinical findings of varicella, including lesions that develop a unique hyperkeratotic appearance, and chronic new lesion formation for weeks or months have been described in children with HIV infection (89,138,194,200,219,328).

Maternal varicella may result in premature labor or, rarely, the congenital varicella syndrome (32,103,254,262,273,274,300). Affected infants have unusual cutaneous defects, with cicatricial skin scars, atrophy of an extremity, microcephaly, seizures, mental retardation, intrauterine growth retardation, and other sequelae, including chorioretinitis, microophthalmia, and cataracts. Dysfunction of the autonomic nervous system produces neurogenic bladder, hydroureter, hydronephrosis, and severe gastroesophageal reflux with recurrent aspiration pneumonia. Limb anomalies and other sequelae of intrauterine varicella may be apparent by ultrasonographic evaluation of the fetus (165). Varicella is common in infants born to women with acute varicella beginning just before or after delivery; transmission of the virus may occur prenatally, but signs of disseminated intrauterine infection are not evident. Some infants with neonatal varicella are born with the typical varicella exanthem in various stages, including vesicles, crusted lesions, or hypopigmented skin areas; other infants develop symptoms during the first few weeks of life. Infants whose lesions are present at birth or within the first 5 days of life are not at risk for severe varicella, probably because the interval between maternal infection and delivery permits transplacental transfer of maternal IgG antibodies to VZV along with the virus. Those who are born within 4 days after or 2 days before the onset of maternal varicella may develop progressive varicella. Most infants are protected from exposure to nonmaternal contacts by passively acquired VZV antibodies.

Herpes Zoster

The classic clinical manifestation of recurrent VZV infection is a cluster of vesicular lesions surrounded by erythema, localized unilaterally in the dermatomal distribution of one or more adjacent sensory nerves (Fig. 8). The initial cutaneous lesions usually appear in groups at one or several sites anteriorly and posteriorly along the affected dermatome; discrete vesicles resembling varicella lesions often merge to form larger, fluid-filled lesions as the exanthem evolves. The cutaneous lesions are often preceded by and almost always accompanied by the pain and hyperesthesias of acute neuritis and by pruritus. About half of the cases involve the thoracic dermatomes, particularly T5 to T12; the eruption is in the distribution of one of the cranial nerves in 14% to 20% of patients (177,279). Cranial nerve V was involved in 14% of the cases described in the classic study by Hope-Simpson (177). Lumbosacral dermatome disease was observed in 16% of these patients, predominantly in the L1 to L2 distribution. The localized dermatomal infection is occasionally accompanied by disseminated cutaneous lesions in the normal host. The cutaneous eruption usually subsides rapidly, with new lesion formation in the primary dermatome ceasing within 3 to 7 days. However, the cutaneous rash may progress to confluent involvement of the whole dermatome. Crusting followed by complete healing of the cutaneous process is expected within 2 weeks but may require as long as 4 to 6 weeks. When herpes zoster occurs in children, the cutaneous eruption is mild, and symptoms of acute neuritis are unusual. Patients who have neuropathic pain but no cutaneous eruption may have the syndrome of *zoster sine herpete* (141,144,145). Idiopathic facial palsy without obvious cutaneous herpes zoster has also been attributed to VZV reactivation (110).

FIG. 8. Herpes zoster in the distribution of the ventral division of the right eighth and ninth thoracic nerves. **B:** Herpes zoster involving the ophthalmic division of the trigeminal nerve with associated conjunctivitis and involvement of the side of the nose. (Courtesy of A. A. Gershon.)

Many patients with uncomplicated herpes zoster have CSF pleocytosis, consisting predominantly of mononuclear cells, as well as elevated protein. VZV can be isolated from CSF and is detected by PCR (146,160,277). Abnormal CSF does not predict chronic sequelae, but spinal cord abnormalities seen by magnetic resonance imaging were associated with prolonged pain (160).

Complications

The most common complication of herpes zoster is PHN, a severe, debilitating, chronic pain (356). Hypersensitivity of the skin to touch or temperature changes persists for several months among 5% to 10% of patients (279,375). When meningoencephalitis occurs, it produces an altered state of consciousness, headache, photophobia, or meningismus at an average of 9 days after rash, with the range up to 6 weeks (205). The electroencephalogram may be abnormal, with diffuse slowing or epileptiform activity. The mean duration of encephalitic symptoms is 16 days, or longer among patients with associated paresis. Neurologic sequelae are unusual. VZV was isolated from brain tissue of a patient who had subacute encephalitis and AIDS, but progressive panencephalitis is rare (142,205). Syndromes attributed to encephalitis, particularly hemiplegia, in patients with herpes zoster may often represent vasculopathy involving the large or small arteries of the central nervous system (6,205). Cerebral angiitis, or granulomatous arteritis, is associated with contralateral herpes zoster involving the trigeminal nerve in elderly patients. Cerebral angiography demonstrates segmental constriction of the ipsilateral cerebral arteries, and computed tomographic brain scans usually show cerebral artery infarcts.

VZV reactivation in the trigeminal ganglion can produce ophthalmic disease, including conjunctivitis, dendritic keratitis, anterior uveitis, iridocyclitis with secondary glaucoma, and panophthalmitis (197,381). Blindness following herpes zoster ophthalmicus is very uncommon. Loss of vision associated with herpes zoster is predominantly caused by retrobulbar neuritis and optic atrophy. Some episodes of retinitis in patients with AIDS are due to VZV rather than human CMV (130,174,222). Herpes zoster of the seventh cranial nerve produces facial palsy on the same side as the cutaneous lesions. Facial palsy may accompany VZV reactivation, affecting other cranial nerves, including the third, fourth, and sixth cranial nerves. Herpes zoster oticus and facial palsy, referred to as the Ramsey-Hunt syndrome, is associated with VZV reactivation in the geniculate ganglion of the seventh cranial nerve and the eighth nerve (110). Herpes zoster of the second branch of the fifth cranial nerve produces oral lesions, primarily on the palate, that can be difficult to diagnose clinically. Lesions on the tongue indicate seventh nerve involvement and are associated with loss of taste.

Neurogenic bladder dysfunction or ileus with intestinal obstruction can accompany lumbosacral herpes zoster (358). Transverse myelitis, with ascending paralysis and sensory deficits, occurs (173), but Guillain-Barré syndrome is rare. Chronic paralysis of an extremity can follow herpes zoster.

Herpes Zoster in High-Risk Populations

Herpes zoster in compromised patients is characterized by a more severe local dermatomal infection (33,163,290,338). Before antiviral therapy was available, the duration of cutaneous disease in these patients was about a week longer than the disease in healthy patients (374). Occasionally, immunocompromised patients, particularly patients with AIDS, develop a chronic reactivation of VZV, which can persist for months (4,124). Cutaneous dissemination occurs in 10% to 40% of immunocompromised patients with herpes zoster. Cutaneous lesions outside of the primary dermatome indicate that the patient has VZV viremia and provide an important marker for potential visceral dissemination (109). Hematogenous spread can produce pneumonia, hepatitis, encephalitis, and disseminated intravascular coagulopathy. Atypical nonlocalized herpes zoster is a syndrome seen in immunocompromised patients, especially bone marrow transplant recipients (229); these patients have widely scattered vesicular lesions that cannot be distinguished from varicella. Neurologic complications are more common in immunocompromised patients (205).

Patients with HIV infection have unusual complications, including chronic cutaneous disease, retinitis, and central nervous system infections that occur without any associated cutaneous disease or are progressive after resolution of the herpes zoster rash (4,59,70,79,153,174,217).

DIAGNOSIS

Differential Diagnosis

Historically, the most important differential diagnosis for varicella was smallpox, or generalized vaccinia after smallpox immunization. Now, varicella must be differentiated from vesicular rashes associated with infections caused by other common pathogens, such as enterovirus or *S. aureus,* or with rashes due to scabies, drug reactions, contact dermatitis or rarely, rickettsial pox, or disseminated HSV infection.

The clinical diagnosis of herpes zoster is most difficult when acute pain and paresthesias precede the cutaneous eruption; the pain is severe enough to mimic myocardial infarction, cholecystitis, appendicitis, and other conditions. Localized contact dermatitis is the most common alternative cause of the cutaneous signs. A localized vesicular rash involving the lumbosacral dermatomes must be distinguished from HSV; a history of recurrent symptoms at the same site suggests HSV-2 disease. Generalized atypical VZV reactivation, such as is seen in severely immunodeficient patients, can be differentiated from primary VZV infection only if the patient has VZV IgG antibodies before symptoms appear.

Laboratory Diagnosis

Laboratory diagnosis is not necessary for the clinical management of most VZV infections in the immunocompetent host. Rapid diagnostic techniques are often useful to document suspected cases of varicella or herpes zoster in high-risk

patients so that informed decisions can be made about antiviral treatment.

Virologic Methods

Virologic methods for diagnosis include the detection of viral proteins using immunologic reagents, viral isolation in tissue culture, and detection of viral DNA by *in situ* hybridization or PCR (84,119,137). Rapid diagnosis of cutaneous VZV infection is accomplished by obtaining epithelial cells from the base of a lesion in the vesicular stage and staining with immunohistochemical methods, including an HSV control (55). Enzyme immunoassay methods can be used to test solubilized preparations of cells and vesicle fluid from cutaneous VZV lesions. Immunohistochemical stains are also valuable to demonstrate VZV-infected cells in lung, liver, and other tissues when disseminated VZV infection is suspected. Cytologic methods can be used to detect multinucleated giant cells, but false-negative results are common, and VZV and HSV infections are not differentiated. VZV virions cannot be distinguished morphologically from other herpesviruses by electron microscopy.

A definitive diagnosis requires the recovery of infectious virus using cell culture methods (51,137). Because the pathogenesis of cutaneous VZV infection involves the release of virus into the vesicle, viral isolation is enhanced by obtaining vesicular fluid and infected cells from the base. VZV is most often recovered from early, clear vesicles. Immunologic assays for viral proteins may be positive when cultures are negative. Infectious VZV is usually recoverable from varicella lesions for 3 days, and for 7 days or longer from herpes zoster lesions. Specimens should be kept on dry ice or frozen at –70°C; storing the specimen at –20°C for 24 hours or longer usually inactivates VZV (137). Viral cultures usually do not influence clinical decisions but rather confirm diagnoses made using rapid viral antigen detection methods. The shell vial culture method may improve the sensitivity of VZV isolation and permit earlier identification of positive specimens, within 1 to 3 days (51,52,306). Even with improved methods, a significant percentage of viral cultures remain negative despite VZV detection by direct immunofluorescence stain of lesion material (69). The similar plaque morphology produced by VZV, HSV, and human CMV requires identification with virus-specific antisera.

Other clinical specimens from which infectious VZV may be recovered include peripheral blood mononuclear cells, joint fluid, CSF from patients with herpes zoster, and bronchial washings from patients with varicella pneumonia. The lungs are the most common autopsy tissue from which VZV has been isolated, but the virus has been recovered from many sites, including heart, liver, pancreas, gastrointestinal tract, brain, and eyes.

VZV can be detected in clinical specimens from patients with primary or recurrent VZV infection using radiolabeled or biotinylated nucleic acid probes that hybridize to VZV DNA or RNA in Southern blot or *in situ* hybridization procedures or by PCR (84,277,302). VZV PCR methods have been shown to detect the virus in PBMC during acute varicella and in some cases of herpes zoster. CSF from patients with central nervous system syndromes related to VZV and from patients with uncomplicated herpes zoster may be positive by PCR. PCR methods are usually more sensitive than viral culture, but positive results do not indicate whether infectious virus is present. Because of risk for contamination, PCR assays may lack specificity; therefore, relying on PCR for diagnosis of VZV as an etiology in clinical circumstances in which cutaneous signs of disease are absent is problematic. VZV detection in CSF by PCR has not been evaluated in enough cases to allow its use for prognostic purposes. Assessing fetal risk after maternal varicella during pregnancy by PCR is complicated by the fact that infection often occurs without damaging the fetus. The relatedness of VZV isolates from clinical specimens can be established by restriction endonuclease digestion of viral DNA, but this information is rarely needed for clinical purposes. The virologic diagnosis of intrauterine VZV infection is difficult because methods, including PCR, lack sensitivity, and detection of VZV does not mean that the fetus has been damaged.

Serologic Methods

The characteristics of the humoral immune response to VZV limit serologic approaches to the diagnosis of acute varicella or herpes zoster. Serologic diagnosis by VZV IgG titers is retrospective, and methods to detect VZV IgM antibodies lack sensitivity and specificity; false-positive results are common. Because VZV reactivation also induces IgM antibodies to VZV in many patients, their presence in serum does not differentiate primary from recurrent infection (15). Similar problems are encountered with assays for VZV IgA antibodies. Fetal VZV infection does not elicit an IgM response consistently, and VZV IgG antibodies in fetal blood reflect transplacentally transferred maternal antibodies. Local antibody production in CSF has been documented in some patients with VZV-related encephalitis (137).

Assays for VZV IgG antibodies are most useful for determining the immune status of individuals whose clinical history of varicella is unknown or equivocal (208). Defining VZV immune status is important to assess the risk for primary VZV infection in exposed individuals, the risk for reactivation in patients who are receiving immunosuppressive therapy, and the need for varicella immunization. The most sensitive methods detect binding of antibodies in sera to membranes of unfixed VZV-infected cells, referred to as the *fluorescent-antibody membrane antigen assay* (FAMA) or to purified VZV protein preparations by enzyme immunoassay (137); neutralization and radioimmunoassay are sensitive. Most commercial enzyme immunoassay methods have high specificity, generating few false-positive results, but 10% to 15% of immune individuals may be identified as susceptible. Using cell-mediated immunity to VZV as the marker of past infection, VZV enzyme immunoassay had a sensitivity of 87%, specificity of 91%, positive predictive value of 87%; latex agglutination had a sensitivity, specificity, and positive predictive value of 96%, 91%, and 97%, respectively, compared with enzyme immunoassay (363). Latex agglutination methods provide a useful alternative to FAMA (322).

TREATMENT

The severe complications of varicella and herpes zoster in patients with malignant diseases created an urgent need for antiviral agents. Initial studies of antiviral therapy were done using vidarabine (adenine arabinoside) or human leukocyte IFN-a; both agents reduced the duration of viral replication and the risk for disseminated illness (18,239,319,373). These agents were replaced by the nucleoside analog, acyclovir (9,2-hydroxyethoxymethylguanine), because of its higher specific activity against VZV, better clinical efficacy, and few adverse effects (43). Famciclovir and valaciclovir are nucleoside analogs that have improved oral absorption and are licensed for treatment of herpes zoster (99,126,339).

Dihydroxypropoxymethylguanine (ganciclovir) has *in vitro* activity against VZV equivalent to acyclovir, but efficacy studies have not been done because of its toxicity. Sorivudine [1-b-D-arabinofuranosyl-E-(2-bromovinyl)uracil] is an oral drug with high *in vitro* activity against VZV and is effective *in vivo* in patients with HIV infection complicated by herpes zoster (126); however, toxicity from interactions with other drugs, particularly 5-fluorouracil, has blocked its licensure in the United States. Netivudine [1-(beta-D-arabinofuranosyl)-5-(1-propynyl)uracil] is another new agent that inhibits VZV *in vitro* (113).

Acyclovir, famciclovir, and valaciclovir are metabolized to the triphosphate form within infected cells by the viral TK; the phosphorylated compound functions as a competitive inhibitor and chain terminator of viral DNA polymerase. The antiviral activity of acyclovir is significantly lower against VZV than HSV-1 and HSV-2, which are usually inhibited by 0.125 µg/mL and 0.215 µg/mL of acyclovir, respectively, in contrast to 0.82-4.64 µg/mL for VZV, with variations from as low as 0.3 to as high as 10.8 µg/mL. Ensuring that plasma concentrations are several-fold above the inhibitory concentrations for most VZV isolates requires the intravenous administration of the drug at doses of 10 mg/kg or 500 mg/m^2; these doses achieve plasma drug concentrations of 15 to 25 µg/mL (373). Only about 20% of acyclovir administered orally is absorbed; even high doses yield peak plasma concentrations of only about 1.0 to 1.5 µg/mL.

Prolonged exposure of VZV isolates to acyclovir *in vitro* permits the emergence of TK-negative mutants (188,223,295); selection of these variants *in vivo* is rare, but it has been observed in severely immunocompromised patients, especially AIDS patients, who are treated with acyclovir for repeated or chronic episodes of VZV infection. Foscarnet, which acts directly on the viral DNA polymerase, is an alternative to the nucleoside analog drugs for the treatment of resistant VZV infection; recombinant interferon may also be useful (239,295,343). Resistance to foscarnet can also emerge, probably owing to a mutation in the viral DNA polymerase (343). The VZV polymerase, ribonucleotide reductase, and other genes are targets for new antiviral drug development.

Varicella

Acyclovir therapy diminishes the clinical severity of varicella in immunocompromised children by ensuring that cell-associated viremia is terminated efficiently despite the impaired host response. Early antiviral therapy prevents progressive varicella and visceral dissemination (309). The dosage of intravenous acyclovir for varicella in high-risk patients is 500 mg/m^2 every 8 hours, with administration continuing for 7 days, or in milder cases until no new lesions have appeared for 48 hours. When intravenous acyclovir was given to children with malignancy in a placebo-controlled trial, the incidence of varicella pneumonia was decreased from 45% to none (342). At one pediatric cancer center, none of 16 children given acyclovir developed pneumonitis, compared with 6 of 21 (29%) treated with vidarabine (111). None of 37 children with varicella who received vidarabine or acyclovir had fatal infection, compared with a case-fatality rate of 7% before antiviral drugs were available.

The optimal use of acyclovir requires beginning treatment within the first 24 to 72 hours after the onset of the rash. Waiting to initiate treatment until prolonged new lesion formation has been documented is not an option because visceral dissemination occurs during the same time period. Most high-risk children do not have clinical signs of VZV dissemination immediately, but the interval during which therapy to prevent viral spread can be initiated is only a few days. In addition to preventing life-threatening dissemination, early acyclovir therapy minimizes cutaneous disease, which may reduce the risk for secondary bacterial infections. Immunocompromised patients who have pneumonia, hepatitis, thrombocytopenia, or encephalitis should receive immediate treatment with intravenous acyclovir (374). In addition to antiviral treatment, these patients require supportive therapy with assisted ventilation and other intensive care measures.

Oral acyclovir can be used to diminish varicella symptoms in healthy children, adolescents, and adults when administered within 24 hours after the appearance of the initial cutaneous lesions (6,34,35,88). In children 2 to 12 years of age, acyclovir given at 20 mg/kg four times a day for 5 days reduced fever, pruritus, days of new lesion formation, and total lesions. Acyclovir recipients also had fewer lesions that progressed from the maculopapular to the vesicular stage. These effects suggest that the plasma and tissue drug concentrations were sufficient to decrease viremia, resulting in diminished transport of the virus to epithelial cells, and to inhibit replication when the virus reached skin sites. Although the clinical benefit was modest because varicella is usually less severe in the youngest children, immediate acyclovir therapy converted all cases to an illness in which fever and lesion formation continued for 3 days or less and total lesions were fewer than 300 (365). Children 5 to 12 years of age and secondary household cases may be judged more likely to benefit because the duration of symptoms was longer and the rash was more extensive in untreated children in these groups. No effect on the risk for secondary bacterial infection or other serious sequelae of varicella was documented given the size of the study cohorts.

The benefit of oral acyclovir for varicella in adolescents aged 13 to 18 years and in adults has more clinical relevance because of the age-related increase in severity of varicella (35,346). Adolescents given acyclovir were significantly less likely to have residual hypopigmented skin lesions at 4 weeks, suggesting that drug therapy minimized the spread of the virus to cells deeper in the dermis. Although the study groups were not large enough to document it, the evidence of diminished viremia and cutaneous viral replication supports the assumption that oral acyclovir therapy reduces the risk for varicella pneumonia. In adults, as in young children, acyclovir must be given within 24 hours after the first lesions appear to have demonstrable clinical efficacy. Adults with varicella pneumonia require immediate treatment with intravenous acyclovir (159). Acyclovir did not have any detrimental effect on the host response to VZV when measured by assays for humoral as well as cell-mediated immunity (88,98,104,289). This finding supports the concept that limited viral replication provides sufficient antigenic stimulation to induce persistent immunity to VZV and is consistent with evidence that protective immunity is elicited even when varicella is mild.

Herpes Zoster

Acyclovir is effective for the treatment of recurrent VZV infection in healthy and immunocompromised patients (Table 3). Among healthy individuals, the period of continued new lesions in the involved dermatome was shortened, as was the

time to complete healing (374,382,383). Acyclovir treatment is important for ophthalmic zoster because of the risk for acute uveitis and chronic keratitis. Acute neuropathic pain was reduced, but effects on PHN have been less predictable (181). The definitive effect of antiviral therapy on acute pain suggests that different mechanisms may contribute to these two components of zoster-related pain (375). There is no evidence that longer courses of treatment alleviate PHN (373).

No. subjects		Interval to treatment	Viral replication	Measures of drug effect		Postherpetic neuralgia	Reference
Acyclovir[a]	Placebo			resolution of cutaneous disease	Acute pain[b]		
93	94	≤72 hr	2.3 d vs. 3.1 d ($p < .0001$)	7.1 d vs. 9.6 d[c] ($p < .0001$)	7.8 d vs. 9.5 d (NS) 4-6 mos (NS)	1-3 mos (.01)	199
43	43	≤72 hr	ND	21.5 d vs. 27.5 d[d] ($p < .027$)	1-4 wk (NS)	2 mos (.01)	282a
102	105	≤48 hr	ND	7.8 d vs. 10.0 d[e] (.032)	Decrease at day 6 (.0268)		281

[a]The dose of acyclovir was 800 mg five times per day in all studies.
[b]Pain was evaluated using scoring systems as described for each study.
[c]50% healing in the affected dermatome.
[d]Full healing in the affected dermatome.
[e]100% crusting in the affected dermatome.
ND, not done.

TABLE 3. *Efficacy of early oral acyclovir therapy for herpes zoster in immunocompetent adults*

The therapeutic benefit of oral famciclovir against cutaneous disease was comparable to acyclovir, and resolution of PHN was accelerated (98,339). Valaciclovir, the l-valyl ester of acyclovir, is metabolized to acyclovir *in vivo* but is absorbed threefold to fivefold more efficiently than acyclovir. Valaciclovir treatment was associated with faster resolution of herpes zoster–associated pain compared with acyclovir and with a decrease in the proportion of patients with pain persisting for 6 months (42,383). Thus, both famciclovir and valaciclovir, in addition to the simplified three-times-daily dosage regimen, have better therapeutic effects against zoster-associated pain. Whether the addition of steroid therapy as an antiinflammatory drug to initial antiviral treatment reduces chronic pain has been difficult to document (374).

Acyclovir is given intravenously to immunocompromised patients who are at high risk for disseminated disease, at a dose of 500 mg/m^2, or 10 mg/kg given every 8 hours. Acyclovir is given for 7 days or for 2 days after the cessation of new lesion formation, whichever provides the longer treatment course. This dosage regimen resulted in improvement of the cutaneous symptoms within 24 hours, cessation of new skin lesions after 24 hours, and prevention of visceral dissemination. Among bone marrow transplant recipients (198,229), acyclovir compensation for the deficient immunity produces earlier cessation of new lesions, more rapid healing, and prevention of dissemination when compared with vidarabine (313). Acyclovir initiated within 72 hours after the onset of VZV reactivation reduces new lesion formation to about 3 days, cessation of acute pain within 4 days, crusting by 7 days, and complete healing by 2 to 3 weeks. Some benefit is observed even when therapy is delayed for more than 3 days because the pathologic process is more extensive (373). Among HIV-infected patients, sorivudine was more effective than acyclovir for rapid cutaneous healing, but no differences were seen effects on pain (126).

Although acyclovir therapy usually eliminates the risk for visceral dissemination, relapse of herpes zoster occurs in some immunocompromised patients. In one series, 5 of 40 bone marrow transplant recipients had recurrent herpes zoster (12%); 3 of the 5 patients developed new lesions within 4 days after treatment was stopped (228). Nevertheless, most patients respond to treatment with a second course of acyclovir. Zoster recurrences and new episodes were experienced by 11% of HIV-infected patients given sorivudine, compared with 26% of those who received acyclovir (126). Oral acyclovir is reserved for patients considered at lower risk for dissemination (228). Foscarnet is an option for rare cases of VZV resistant to acyclovir (50).

The treatment of PHN is often difficult. Mild pain is managed with nonopioid analgesics or nonsteroidal antiinflammatory drugs. These agents can be given in combination with adjuvant analgesics, including tricyclic antidepressants or anticonvulsants. Opioids are used when these combination drug regimens fail. Short-term pain relief may result with lidocaine or other topical analgesics. In the most severe cases, anesthetic interventions such as nerve block are used, but their efficacy may be limited.

PREVENTION AND CONTROL

In most circumstances, preventing the transmission of VZV to susceptible individuals is difficult because infected individuals are contagious for 24 to 48 hours before the onset of varicella. As a consequence, restrictions on attendance failed to alter the course of varicella epidemics in schools (56). Infection-control practices, including caring for infected patients in isolation rooms with filtered air systems, are essential in hospitals that treat patients for whom varicella might be life-threatening. VZV transmission by air-flow systems has been documented, and VZV has been detected in air samples by PCR (218,301). Susceptible health care workers who have had a close exposure to varicella are not permitted to care for high-risk patients during the incubation period or acute illness (6,158).

Passive Antibody Prophylaxis

Varicella zoster immune globulin (VZIG) is a passive antibody preparation containing VZV IgG antibodies made from high-titer immune human serum. VZIG is distributed by the American Red Cross Blood Services; the dosage is 1 vial per 10 kg given intramuscularly. VZIG is indicated for susceptible individuals who have had a close exposure to an individual with varicella or herpes zoster and who are at risk for serious varicella (6,63). Its efficacy depends on administration within 96 hours and preferably within 48 hours after exposure. VZIG prophylaxis is recommended for compromised children, pregnant women, and newborn infants exposed to maternal varicella (282). Adults should be tested for VZV IgG

antibodies because many with no history of varicella are immune.

Giving VZIG prophylaxis does not eliminate the possibility that primary VZV infection will occur (242,390). VZIG administration can prolong the incubation period to 28 days. The incidence of varicella despite VZIG prophylaxis is significantly higher after household exposures and is related to the VZV IgG titer of the preparation. Passive antibody prophylaxis lowered the risk for varicella pneumonia, but 11% of children developed pneumonia (111). Some immunocompromised patients, particularly bone marrow transplant recipients, are given high-dose intravenous immune globulin (100 to 400 mg/kg) for other indications; if these preparations have been given within 3 weeks before the exposure, it is not necessary to administer VZIG. Because passive antibody titers decline over 2 to 4 weeks, a second dose of VZIG should be given if a new exposure occurs after 2 weeks. Although the risk for VZV transmission from an individual with herpes zoster is low, close contact between a susceptible, high-risk patient and a patient with recurrent VZV lesions also justifies the administration of VZIG.

Passive antibody prophylaxis dose not reduce the risk for VZV reactivation in high-risk populations, and passive antibody administration after the onset of illness does not alter the severity of varicella or herpes zoster (324).

Antiviral Prophylaxis

Although acyclovir has been given as prophylaxis during the incubation period, but the benefit, compared with treatment if and when acute illness occurs, is not well defined (180,329). Acyclovir prophylaxis has been evaluated for the prevention of recurrent VZV infection in bone marrow transplant recipients; in one series, the incidence of herpes zoster was 15% in the placebo group and none in the acyclovir recipients. However, VZV reactivation occurred when acyclovir was discontinued, and prophylaxis did not result in an overall reduction in episodes by 1 year. Acyclovir prophylaxis is not a universal practice because the prompt initiation of acyclovir for the treatment of recurrent VZV infections is very effective (265). Avoiding prolonged, low-dose administration of acyclovir may help to minimize the emergence of TK-negative VZV strains that are resistant to the drug.

Varicella Vaccine

The live attenuated varicella vaccine is the first human herpesvirus vaccine licensed for clinical use in several countries (66,134,209,335). Its development and initial clinical evaluation was first reported by Takahashi in 1974 (330). The vaccine virus was derived from a clinical isolate of VZV, the Oka strain, and is propagated in guinea pig embryo fibroblasts and WI38 cells. In contrast to experiments in which susceptible children were inoculated with infectious VZV from vesicular fluid (46), tissue culture propagation appears to attenuate the virus so that vaccine containing as much as 9,000 PFU per dose of infectious virus induces immunity to VZV but does not produce clinical symptoms (12).

The molecular mechanisms of attenuation of the Oka strain have not been identified. The attenuation of the Oka vaccine strain is not related to known restriction enzyme profile differences because it resembles other Japanese isolates that cause varicella. The comparative analysis of the vaccine virus in the SCID-hu mouse model demonstrated that vaccine Oka has a diminished capacity to replicate in human skin but remains infectious for T lymphocytes (246). The pathogenicity of vaccine Oka was compared with that of its parent, another low-passage clinical isolate, and VZV-Ellen, a standard laboratory strain. All of these VZV strains replicated well in tissue culture, but only the low-passage clinical isolates, including parent Oka, were fully virulent in skin, whereas prolonged growth of VZV in tissue culture cells was associated with reduced infectivity. Analysis of a variant of vaccine Oka showed that infectivity of the vaccine strain was further diminished in the absence of gC expression, but the vaccine strain itself was not diminished for gC expression *in vivo.* The clinical attenuation of V-Oka in healthy children can be explained by its impaired virulence for human skin. The fact that the vaccine Oka strain retained infectivity for T lymphocytes explains its capacity to cause scattered lesions in some vaccinees and to cause varicella-like symptoms in some immunocompromised children. Sequence analyses of vaccine Oka strain, its parent, and other clinical isolates have identified some differences in vaccine Oka, but their relationship to attenuation has not been established.

Clinical Experience

The live attenuated varicella vaccine (Oka-Merck strain) was administered to more than 7,000 children and more than 1,600 healthy, susceptible adults during clinical trials in the United States. It is now licensed and recommended as a routine childhood vaccination and for immunization of susceptible older children and adults (282). From a societal perspective, an analysis based on medical and other costs associated with the annual epidemics of varicella indicates that universal immunization programs are cost-effective (83,187,226). In prelicensure trials, the vaccine-induced protection against household exposure with an efficacy rate of more than 95% in an initial placebo-controlled study; subsequently, vaccine efficacy has been judged in open trials based on the predicated attack rate of 90% following household contact (212,359,372). Seroconversion rates are more than 95%, with complete protection against disease in 85% to 95% of exposures. Mild breakthrough infections have been observed in some children, but the disease is modified to fewer than 50 cutaneous lesions without associated fever (134,227,354). In the experience with 2,163 vaccinees evaluated at one center, 114 children developed varicella a median of 44 months after immunization; the median number of skin lesions was 18; the incidence of transmission to other vaccinated children in the household from these cases was 12% (354). Achieving seroconversion rates of more than 95% in susceptible adults has required the administration of two doses of the vaccine, separated by at least 4 weeks, which is consistent with the evidence of a diminished host response to natural varicella among adults (12,135,252). Adults are also more likely to have adverse effects, particularly transient local reactions and rash, and to have waning VZV antibody titers. However, immunizing susceptible adults has important clinical value because of their susceptibility to serious varicella, and protection or modified illness is documented despite lower antibody titers. Health care workers and others with frequent exposures to children benefit from immunization (357).

Varicella vaccine is immunogenic when administered concurrently with measles, mumps, and rubella vaccines (105). A reformulation of live attenuated varicella vaccine allows its storage at +4°C to +8°C without loss of infectious virus titer.

The safety and immunogenicity of this preparation has been established in healthy children aged 9 to 24 months (335).

The vaccine Oka strain of VZV can be transmitted from individuals when the vaccine virus causes rash, but transmission is unusual because most healthy children do not have vaccine-related rash. In one experience, a healthy adult developed a rash 12 days after vaccination; 16 days later, two children who were household contacts had lesions due to vaccine virus, as shown by restriction endonuclease analysis (132,215). The vaccine virus was also transmitted from a healthy child to a pregnant contact; there was no laboratory evidence of transplacental transmission, which is consistent with the low risk for wild-type VZV spread across the placenta (296).

The Oka-Merck varicella vaccine has been given to children with acute leukemia in remission, reducing the attack rate after household exposure to 13% (135,139). Seroconversion in these patients is associated with a high degree of protection, but most children required two doses of vaccine. Varicella vaccine must be given to these children with careful attention to the status of their underlying disease and immunosuppressive therapy regimens because it contains infectious virus (11,184). Vaccine-related rashes occurred at about 1 month after immunization in about 50% of children with leukemia in remission. Rashes occurred at the site of inoculation but also as widely scattered vesicles, indicating that the vaccine virus has not lost the capacity to cause cell-associated viremia. The vaccine virus was transmitted from about 15% of leukemic children to their healthy, susceptible siblings, but the contacts had mild clinical illness or asymptomatic seroconversion; this clinical experience provides further evidence that the vaccine virus is attenuated (134). Vaccine virus was not transmitted unless the immunocompromised child had rash, indicating that there was no significant replication of the vaccine at respiratory sites (337). Immunizing susceptible, healthy household contacts may reduce the risk for household exposure (297). No transmission of vaccine virus was observed in this clinical setting despite the heightened susceptibility of immunocompromised children to varicella, indicating that the vaccine virus does not replicate effectively in respiratory mucosa, in contrast to wild-type VZV (91,196). VZV was detected by PCR in oropharyngeal secretions from 7% of children given varicella vaccine in Japan, but infectious virus was not recovered (261).

VZV reactivation has been described in a few healthy vaccine recipients (269). When the incidence of herpes zoster was assessed in children with leukemia who had vaccine-induced immunity versus past natural infection, the vaccine virus was found to reactivate less often than wild-type VZV (164). Whether this observation indicates that the vaccine virus is less likely to establish latency or has less potential for reactivation is not known. If symptomatic reactivation occurs, the vaccine virus is susceptible to inhibition by acyclovir. In one instructive case, an otherwise healthy adult vaccine recipient had household exposure to varicella but did not develop symptoms of primary VZV infection; a subsequent episode of herpes zoster was shown to be caused by wild-type VZV, indicating that superinfection with the wild-type virus had occurred despite vaccine-induced immunity (162). Assuming that introduction of the varicella vaccine will eliminate or modify the annual varicella epidemics, exposures should be reduced significantly, and opportunities for superinfection with wild-type VZV will be minimal.

In addition to vaccination to induce primary VZV immunity, the live attenuated varicella vaccine and a heat-inactivated preparation are being evaluated for capacity to enhance immunity in seropositive individuals. Studies are in progress to determine whether the vaccine might be used to prevent or reduce the risk for VZV reactivation associated with age (221,320). The safety of the varicella vaccine also suggests that VZV may be useful as a live virus vector, and the expression of Epstein-Barr virus gp350 and hepatitis B surface antigen by recombinant VZV strains has been demonstrated *in vitro* (230,315).

Vaccine-Induced Immunity

Clinical studies of the Oka-Merck live attenuated varicella vaccine demonstrated seroconversion in more than 90% of children. The varicella vaccine also elicits VZV-specific cellular immunity (38,93,252,351), which is likely to be essential to its long-term protective efficacy. T-lymphocyte responses to VZV are detected in 98% to 100% of healthy children given the varicella vaccine by 2 to 6 weeks after immunization, as would be expected among healthy children who were convalescent from natural varicella (38). Some more immunogenic vaccine preparations had higher relative antigen content, suggesting a role for antigen as well as infectious virus in inducing the optimal host response (38,353). Vaccination elicited neutralizing antibodies and antibodies to gB and gE in titers that were equivalent to those detected after natural infection (167). In addition to requiring two doses of vaccine to achieve high levels of seroconversion, cellular immunity to VZV was significantly lower in adults given two doses compared with children who received one dose of vaccine, and lower T-lymphocyte proliferation correlated with lower IFN-g responses (190,252).

The kinetics of the induction of VZV-specific T-lymphocyte proliferation and cytotoxicity indicate rapid acquisition of these effector cells after immunization (38,92,93,208,312,352). This early T-lymphocyte response may account for the efficacy of vaccine given immediately after exposure (26). VZV-specific T lymphocytes were detected within 10 to 14 days in most individuals, whereas IgG antibodies to VZV were detected in only 40% of children tested at 2 weeks. Immunization elicits T lymphocytes that proliferate and produce lymphokines in response to stimulation with the IE62 protein and the viral glycoproteins, generating responses that are comparable to those of subjects who have naturally acquired immunity to VZV (38). Immunization with the varicella vaccine also induces memory cytotoxic T lymphocytes that can lyse cells expressing VZV proteins, with responder cell frequencies that are comparable to those elicited by natural VZV infection (221,312). When the persistence of cytotoxic T-cell responses to IE62 protein, gE, gI, and gC was examined in vaccinees about 4 years after immunization, the mean CTL frequencies were comparable in vaccinees and in adults with natural immunity (312). By intracellular cytokine assay, VZV-specific CD4+ T-cell frequencies in young adults with vaccine-induced immunity were comparable to frequencies in those with natural immunity, representing about 0.1% of peripheral blood T cells (29). The induction of VZV-specific T cells and IgG antibodies was associated with transient increases in IL-10 and IL-12 production (190).

Persistence of immunity has been documented using assays for IgG antibodies and T-cell proliferation in 94% to 100% of vaccine recipients; sustained protective efficacy of 95% was shown among vaccine recipients from the original placebo-controlled trial who were followed for 7 years (246). At a mean of 5 years after vaccination, 93% of children and 94% of adults had VZV IgG antibodies to VZV, with titers significantly higher at 5 years than at 1 year after immunization

(391). Cell-mediated immunity to VZV was detected in 87% of children and 94% of adults and was also significantly higher at 5 years. Initial differences between cell-mediated responses in children and adults diminished over time; by 5 years, cytokine responses to VZV, including IL-2, IFN-g, and IL-10, were equivalent between children and adults. In a 6- to 10-year study, 2% to 3% of children developed varicella (193). The frequency of breakthrough varicella and severity of symptoms did not change with time after vaccination; those with low or undetectable FAMA titers 6 weeks after immunization were four times more likely to have varicella. A two-dose regimen has been associated with higher immune responses in children evaluated after 1 year, but whether protective efficacy is more complete has not been determined (252,355). Evaluation of Japanese vaccine showed persistence of humoral and cellular immunity for as long as 20 years (27).

Cell-mediated immunity is elicited less reliably in leukemic children given the varicella vaccine, which is consistent with the finding that immunocompromised children often have diminished T-lymphocyte proliferation to VZV antigens after natural varicella (139). VZV-specific T-lymphocyte responses were higher in leukemic children who had been vaccinated but who did not experience recurrent VZV compared with children who developed herpes zoster (216).

The availability of the varicella vaccine has made it possible to assess the effects of exogenous reexposure to VZV antigens upon virus-specific, cell-mediated immune responses and to consider its immunogenicity among older individuals with waning responses to VZV. VZV-specific responder cell frequencies increased from 1 per 68,000 to 1 per 40,000 healthy adults older than 55 years of age who received varicella vaccine, which was equivalent to the numbers of VZV responder cells in naturally immune individuals aged 35 to 40 years; the higher responder cell frequencies persisted for at least 2 years after immunization (221). Increased production of IFN-g by T lymphocytes incubated with VZV antigen was also documented, and administration of the vaccine to elderly individuals was associated with the recovery of skin test reactivity to VZV antigen in most vaccinees. Vaccination might help to reduce the risk for herpes zoster associated with waning immunity in the elderly (40,221,320). When the immunogenicity of vaccine with concentrations of infectious virus of 3,200, 8,500, and 41,650 PFU/dose was evaluated in healthy older adults, vaccination enhanced humoral and cellular immunity regardless of the infectious virus content (41). Live attenuated vaccine may be most effective for boosting immunity because it was more likely to increase class I MHC-restricted CTL function than inactivated VZV vaccine (171).

PERSPECTIVES

New knowledge about VZV pathogenesis and immunity have been accompanied by significant advances in antiviral drug therapies and vaccine prevention of VZV disease. Further progress depends on a better understanding of VZV replication, the genes that determine VZV tropisms for lymphocytes, skin and cells of the dorsal root ganglia, and mechanisms of immune evasion. These efforts can be expected to bring further improvements in the clinical efficacy of antiviral agents against VZV and in the design of varicella vaccines.

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CHAPTER 80 Human Herpesviruses 6A, 6B, and 7 and Their Replication

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Human herpesvirus 6 variants A and B (HHV-6A and HHV-6B) and human herpesvirus 7 (HHV-7) are relatively recently discovered herpesviruses. They share many properties with other herpesviruses, including virion structure, some features of genomic and genetic architecture, high prevalence in their natural host, and the ability to establish latent infections in the host. The outline of the lytic replication cycle shown in Figure 1 is similar in its general features to that for other herpesviruses, but as described later, it differs in its details and in essence describes viruses that share a number of properties that make them molecularly and biologically distinct from their relatives elsewhere in the herpesvirus family.

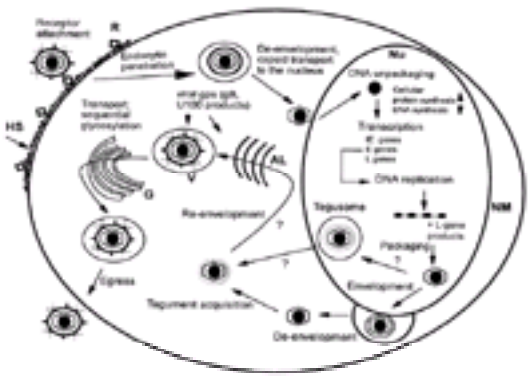


FIG. 1. Stages of replication. The replication scheme is based on published data (23,50,108,139,147). A single receptor is shown, although it is likely that the virus employs multiple receptors to enable entry into a wide range of cell types. AL, annulate lamellae; G, Golgi complex; HS, heparan sulfate; Nu, nucleus; NM, nuclear membrane; R, receptor (CD46 for HHV-6A and HHV-6B, CD4 for HHV-7); V, vacuole.

CLASSIFICATION

HHV-6A, HHV-6B, and HHV-7 compose the *Roseolovirus* genus of the betaherpesvirus subfamily. Members of this genus are characterized by growth in T lymphocytes (although they can also infect other cell types), high prevalence, and association with febrile rash illness. As betaherpesviruses, the roseoloviruses share several properties with the more extensively studied human cytomegalovirus (HCMV), including having genetically colinear genomes, encoding several genes found only in members of this subfamily, and having generally protracted replication cycles.

The HHV-6 variants are closely related, yet molecularly and biologically distinct viruses that differ with respect to their cell tropisms, interactions with cells and signaling pathways of the immune system, DNA sequences, and epidemiology (2,9,113; reviewed in 1,18,21,37, and 67). *In vivo* recombinants have not been detected that would indicate a genetic gradient between the variants. These viruses satisfy the requirements for recognition as separate herpesvirus species (18,21,37), although they have not been formally designated as such; thus, the variant designations.

VIRION STRUCTURE

Members of the *Roseolovirus* genus share the standard features of herpesvirus virion structure: an icosahedral capsid 90 to 110 nm in diameter that contains the 145- to 170-kb double-stranded DNA genome, a tegument that surrounds the capsid, and a lipid bilayer envelope that is studded with viral specified integral membrane proteins and glycoproteins; enveloped extracellular virions are 160 to 200 nm in diameter (12,13,75,147) (Fig. 2). Under some conditions of fixation, by thin-section electron microscopy, HHV-6 teguments are smooth and fill the space between the capsid and envelope, whereas the HHV-7 tegument has an electron lucent space between the tegument and envelope (13,75). Immunoelectron microscopy with a monoclonal antibody (MAb) against a tegument protein encoded by the HHV-7 gene *U14* showed

reactivity primarily at the outer periphery of the tegument (126), indicating that although the tegument can appear to be amorphous by electron microscopy, it is an ordered structure.

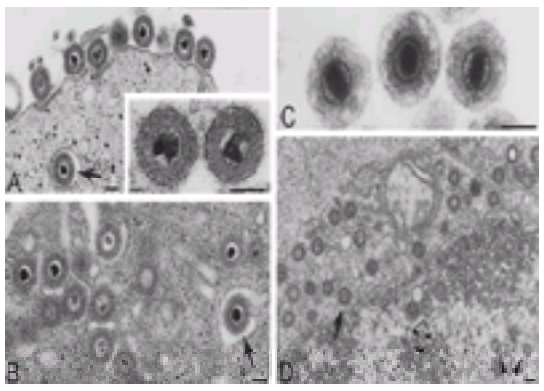


FIG. 2. Ultrastructural characteristics of HHV-6B(Z29) and HHV-7(SB) grown in human umbilical cord blood lymphocytes. **A:** HHV-6B virus particles accumulated along the plasma membrane and within a vesicle (*arrow*) of an infected cell. **Inset:** Detail of HHV-6B virions. **B:** Tegument-coated nucleocapsids of HHV-6 within the cytoplasm and apparently budding into the cisternae of the Golgi apparatus (*arrow*). **C:** Extracellular HHV-7 virions. **D:** HHV-7 nucleocapsids within the nucleus (*arrowheads*), accumulated within the perinuclear space (*open arrow*), and surrounded by tegument within the cytoplasm (*arrow*). Bars, 100 nm. (Courtesy of C. S. Goldsmith, C. Lopez, P.E.P., and J. B. Black, Centers for Disease Control and Prevention.)

GENOME STRUCTURE AND ORGANIZATION

Genomic Architecture

Genomic architecture is similar among the roseoloviruses: genomes are composed of a central unique segment (U) bracketed by a pair of direct repeat structures, DR_L and DR_R (77,86) (Fig. 3; Table 1). The overall genetic arrangement of HHV-7 is more compact than that of HHV-6. HHV-6 DR consists of 25 to 29 base pairs (bp) of unique sequence, a conserved *pac1* (cleavage and packaging) element, an array of heterogeneous repeats (L-TRS) based on the sequence TA(A/G)(C/G)(C/T)C that span about 300 bp in laboratory-passaged strains and up to 3 kb in low passage viruses, about 7 kb of unique sequence that encodes a handful of genes, an array of sequences similar to those present at the telomeres of mammalian chromosomes [R-TRS, (TAACCC)n], a conserved *pac2* element, and 16 to 18 bp of unique sequence (32,37,56,74,77,132). HHV-7 DRs follow this basic pattern but differ in some details, including R-TRS being more heterogeneous than for HHV-6 (36,109,118). Despite the differences, HHV-7 cleavage and packaging signals are recognized and processed during HHV-6 infections, and vice versa (109). Intriguingly, the HHV-6 telomeric repeat element (TAACCC) is also sparsely dispersed throughout the U segment in a palindromically symmetric manner that is centered at the origin of lytic replication (*ori*Lyt), with TAACCC to the left of the origin and GGGTTA to the right (37,57). Other repeat arrays (R0 to R3) are located in the U segment of each of the viruses (37,57,67,88,96) (see Table 1 and Fig. 3). Compared with other herpesviruses, roseoloviruses have low G+C content (see Table 1).

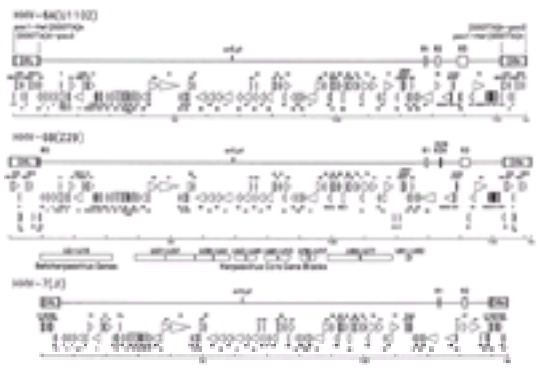
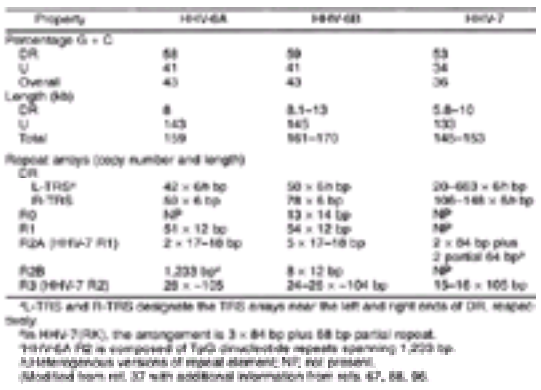


FIG. 3. HHV-6A, HHV-6B, and HHV-7 genomic and genetic architecture. The diagrams are based on annotations of published genomic sequences (37,57,88,96). The positions and arrangements of the major internal repeat elements, the origin of replication (*ori*Lyt), and the structure of the DR termini are shown for each virus. Open reading frame (ORF) sizes, orientation, and location are indicated. HHV-6A genes *DR4, DR5, DR8, U1, U61, U78, U88, U92,* and *U93* do not have counterparts in the other viruses; ORFs found in HHV-6B and not in HHV-6A are designated B1 through B9, and those present in HHV-7 and not in HHV-6A or HHV-6B as H1 through H8. The orientation and order of the seven conserved herpesvirus core gene blocks are indicated, as is the block (*U2* to *U19*) found only in the betaherpesviruses. Genes unique and common to the roseoloviruses are indicated by horizontal lines between the forward and reverse strand genes.



Property	HHV-6A	HHV-6B	HHV-7
Percentage G + C			
DR	58	59	53
U	41	41	34
Overall	43	43	36
Length (kb)			
DR	8	8.1–13	5.8–10
U	143	145	133
Total	159	161–170	145–153
Repeat arrays (copy number and length)			
DR			
L-TRS[a]	42 × 6/7 bp	50 × 6/7 bp	20–663 × 6/7 bp
R-TRS	50 × 6 bp	78 × 6 bp	106–148 × 6/7 bp
R0	NP	13 × 14 bp	NP
R1	51 × 12 bp	54 × 12 bp	NP
R2A (HHV-7 R1)	2 × 17–18 bp	5 × 17–18 bp	2 × 84 bp plus 2 partial 64 bp[b]
R2B	1,233 bp[c]	8 × 12 bp	NP
R3 (HHV-7 R2)	28 × ~125	24–28 × ~104 bp	15–16 × 105 bp

[a] L-TRS and R-TRS designate the TRS arrays near the left and right ends of DR, respectively.
[b] As HHV-7 (RK), the arrangement is 3 × 84 bp plus 68 bp partial repeat.
[c] HHV-6A R2 is composed of TpG dinucleotide repeats spanning 1,233 bp.
NP, heterogenous versions of repeat element; NP, not present.
(Modified from ref. 37 with additional information from refs. 67, 88, 96).

TABLE 1. *Properties of roseolovirus genomes*

The function of the mammalian telomere-like sequences is not known. They appear to have no role in either nuclear retention of viral DNA, efficiency of DNA replication, or packaging-associated DNA cleavage (19,32). A possible function is mediation of the site-specific integrations of HHV-6 genomes that have been observed in the vicinity of host chromosome telomeres (31,32,138).

Genetic Structure and Genetic Relationships

Protein-encoding open reading frames (ORFs) are encoded on both strands, with little overlap among adjacent ORFs (see Fig. 3). In addition to most of the repeat arrays, there are two genomic regions of no obvious protein coding capacity: the region between *U41* and *U42* that harbors the origin of lytic DNA replication (*ori*Lyt), and the region between *U77* and *U79.*

Many of the genes encoded in the central portion of roseolovirus genomes are conserved among the alpha-, beta-, and gammaherpesviruses (37,57,67,88,96) (herpesvirus core genes; Table 2; see Fig. 3 and Chapter 71). A group of genes conserved among the betaherpesviruses is located to the left of the core genes. Clusters of genes unique to the roseoloviruses are located to the left and right of the core genes.

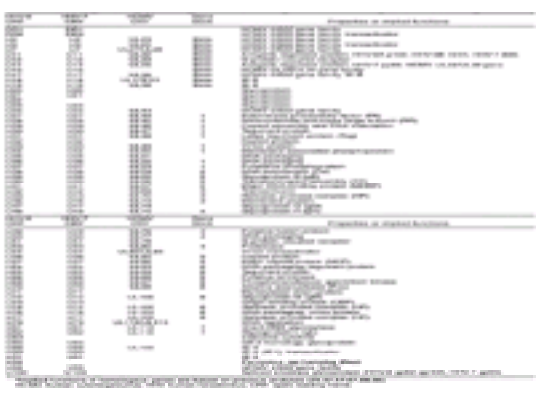


TABLE 2. *HHV-6 and HHV-7 genes of known or implied functions and their HCMV homologs*[a]

In comparisons with *Cytomegalovirinae* and *Muromegalovirinae,* the roseoloviruses are genetically colinear over the conserved domains, although the cytomegaloviruses encode many genes that have no counterparts in roseoloviruses. Among the roseoloviruses, HHV-7 is about equally related to the HHV-6 variants, with amino acid sequence identities ranging from 22% to 75%, most being in the vicinity of 50% (37,67,96). HHV-6A and HHV-6B are closely related viruses, with overall nucleotide sequence identity of 90%; the portions of the genomes that span *U32* to *U77* are highly conserved (95% identity) (37,67). The segment spanning U86 to U100 is 72% identical, with only one gene in this region having amino acid sequence identity over 90% (*U94,* 97.4% identity).

Roseolovirus DNA sequences vary less from strain to strain within a species than do many other herpesviruses, such as herpes simplex virus (HSV) or HCMV. Across segments spanning several kilobases, HHV-6A strains differed by less than 0.5%, although there were short segments of greater variability (54,55,130); HHV-6B strains HST and Z29 differ by less than 1% across their entire genomes (G.D. and P.E. Pellett, unpublished analysis); other than repeat copy number differences, HHV-7 strains JI and RK differ by only 179 bp (0.1%) over their genomes (88). Conservation can be even higher at the amino acid sequence level, with U94 amino acid sequences being identical among 13 HHV-6B isolates from Africa, Japan, and the United States (105), and nearly as high conservation in HHV-7 glycoprotein B (gB) (48). The biologic forces that restrict sequence variation this tightly are not understood but must in some way be linked to the low G+C content of these viruses, which precludes easy variation at third-position “wobble” codons.

There is evidence for subgrouping within the HHV-6B variant at some genomic loci (27,54) and among HHV-7 isolates (48), but there is no indication that this is anything other than intraspecies allelic variation.

Roseoloviruses differ somewhat from each other in their genetic complement. According to a recent analysis, nine HHV-6A ORFs do not have HHV-6B counterparts, and nine HHV-6B ORFs have no HHV-6A counterpart (37). Interpretation of these differences is difficult because somewhat different criteria have been employed to predict functional ORFs of the sequenced viruses. In addition, in the absence of clear similarity with genes from other

herpesviruses or in the sequence repositories, such predictions must be considered provisional pending experimental study. Megaw and colleagues (88) compiled a list of 16 HHV-6A ORFs considered unlikely to encode proteins, one of which was subsequently shown to encode a functional cytokine (151). It remains to be seen whether other of these or the other small unique ORFs are also functional. *DR3, U6, U22, U83,* and *U94* are encoded by both HHV-6A and HHV-6B but not HHV-7; interestingly, a *U94*–parvovirus *ns1–rep* homolog is encoded adjacent to a U95 homolog in rat cytomegalovirus, but in neither human nor murine cytomegalovirus (141). HHV-7 encodes a duplicated gene (*U55A* and *U55B*) that is present in a single copy in HHV-6A and HHV-6B. Among the betaherpesviruses, roseolovirus-specific genes include *U20, U21, U23, U24, U24A, U26,* and *U100.*

In addition to their overall genetic colinearity with the cytomegaloviruses, two aspects of similarity among the betaherpesviruses warrant specific mention. First, although the amino acid sequences have diverged to the point of no detected similarity, the general layout and splicing patterns of two cytomegalovirus immediate-early (IE) regions are conserved in the roseoloviruses. Thus the roseolovirus IE-B domain that spans *U17* to *U19* corresponds to the HCMV *UL36* to *U38* IE locus, and the IE-A domain spanning *U86* to *U90* corresponds to the HCMV major IE locus (85,96). Second, every betaherpesvirus encodes several members of a family of duplicated and diverged genes that are related to HCMV *US22* and are referred to as the *US22* gene family. *US22* homologs encode at least one of four conserved motifs (96). For HCMV, some are transcribed as IE genes and encode transactivating functions. In the roseoloviruses, *DR2, DR6/7, U2, U3, U5, U7* (*U5/7* in HHV-7), *U8, U17/16, U25,* and *U95* are all members of this gene family. Although their transcription has not been studied extensively, as discussed later, at least four of the HHV-6A US22 homologs encode transactivators (*DR6, U3, U16,* and *U25*).

STAGES OF REPLICATION

Entry

CD46 was recently identified as a cellular receptor for HHV-6 (111). CD46 is a member of the regulator of complement fixation family and is expressed on the surface of all nucleated human cells; it is a cellular receptor for measles virus. Evidence for the CD46 receptor activity included (a) down-modulation of CD46 expression in activated primary human CD4+ lymphocytes infected with HHV-6A or HHV-6B, (b) inhibition of HHV-6 infections and associated cell fusion by MAbs against CD46 and by soluble CD46, and (c) nonhuman cells being rendered susceptible to HHV-6–mediated membrane fusion and HHV-6 entry by expression of CD46. Nonetheless, expression of CD46 was not sufficient for HHV-6 fusion and infection in all human cell types, suggesting either that a specific co-receptor is needed or that some cells express an inhibitor of the CD46 receptor activity. In previous work, Lusso and colleagues, as well as others, also showed that HHV-7, but not HHV-6, uses CD4 as one of its receptors (84,144,146), although the virus can infect cells that do not express detectable CD4 (72). It is likely that other host molecules act as receptors; the need for multiple sequential receptors to enable cell-to-cell migration of the virus in tissues is a well-documented phenomenon in other herpesviruses (see Chapter 72).

The human immunodeficiency virus type 1 (HIV-1) co-receptors, CXCR4 and CCR5, have been studied to determine whether they serve similar functions for the roseoloviruses. Cells from individuals lacking CCR5 were able to support growth of all three viruses, and these individuals were seropositive for the viruses, indicating that this molecule is not essential for viral replication (143). Data for CXCR4 are conflicting. An MAb against CXCR4 did not inhibit infection by any of the viruses (120,143,149), nor did a specific CXCR4 antagonist that can inhibit HIV-1 infectivity (149). Nonetheless, the natural CXCR4 ligand, SDF-1a, inhibited HHV-7 infection at 2.5 µg/mL (120) but not at 1.0 µg/mL (149).

On the virus side, two HHV-7 glycoproteins have been identified as being able to bind the cell surface proteoglycans heparan and heparan sulfate: the virion glycoprotein, gB, and the spliced glycoprotein encoded by *U100* (102,119,123). It is likely that other virion proteins are involved in receptor interactions or entry, such as the gH–gL complex (4,45,78,128), which along with products of *U100* (102), are targets for complement-independent neutralization. Once attached, viral entry appears to be through an endocytic pathway rather than by fusion at the plasma membrane (29).

Transcription

Herpesvirus genes generally belong to one of two main categories: latent or lytic. As described for Epstein-Barr virus, latent gene expression can follow more than one program. There are three kinetic classes of lytic genes: IE, or genes that provide functions for regulation of gene expression; early (E), or genes that encode proteins needed for DNA replication; and late (L), or genes that encode virion proteins. IE gene transcription does not require *de novo* protein synthesis and begins at very early times after infection. Transcription of E genes is dependent on IE gene activity but not on viral DNA replication, and transcription of L genes is either partially or entirely dependent on viral DNA replication.

The kinetics of roseolovirus gene expression have not been extensively studied but appear to follow the general program of other herpesviruses. Studies of gene expression in these viruses have been complicated by the lack of methods for preparing high-titered cell-free virus stocks; the low-multiplicity infections used thus far have limited the sensitivity of detecting transcripts and proteins. For HHV-6A and HHV-6B, *U83* and *U89/90* encode experimentally proven IE genes (49,68,105,112). In both cases, multiple differently regulated transcripts are expressed. A protein encoded within *U89/90* has transcriptional activation activity, but its role in HHV-6 infections has not been defined. The IE transcript of *U83* is spliced and would express a truncated version of the chemokine encoded by *U83,* whereas the full-length transcript is expressed as a late gene (49,151; K. Yamanishi, personal communication). Genes *U16* through *U19* have been designated as IE locus B based on their HCMV homologs, but their transcriptional kinetics have not been studied experimentally. *U42* encodes a homolog of the HSV IE gene a*27* and HCMV *UL69,* which is regulated as an E gene; HHV-6B *U42* is not transcribed in the absence of *de novo* protein synthesis; thus, its regulation is similar to that of its HCMV counterpart (105).

Several genes encoding proteins involved in replication or structure of both HHV-6 and HHV-7 were identified as IE genes in studies that involved reverse transcription coupled with 35 cycles of polymerase chain reaction (PCR) (89,91); it

seems likely that the sensitivity of the assay led to detection of leaky E transcription under IE conditions and misassignment of the kinetic classes. In experiments that involved quantitative comparisons of transcript levels, some of these previously identified IE genes were not expressed in the absence of *de novo* protein synthesis (105). In studies of temporal kinetics, expression of the *U90* IE gene rose to more than 10 copies per cell within 8 hours, whereas a structural gene transcript (*U11*) took 16 hours to reach comparable levels; at 72 hours after infection, *U11* was expressed at several hundred copies per cell. Transcripts of the gene needed for initiation of DNA replication (*U73*) and the parvovirus *ns1–rep* homolog (*U94*) were expressed at much lower levels (less than 10 copies/cell).

Most transcripts expressed by these viruses are not likely to be spliced, although several are, some more than once. Among them are the major IE locus and *U100* (103,123). In addition, the use of some splice sites is kinetically regulated (89,91,112,151); others use noncanonical donor and acceptor sequences (49,151). On the basis of HHV-6A and HHV-7 genomic sequences, Megaw and colleagues provided detailed predictions of splicing patterns that will be useful in guiding and interpreting future experiments (88).

The cis-acting sites important for regulating the expression of roseolovirus genes have not been well-characterized. The locations of the 5' ends have been determined for a number of transcripts, which will enable study of their regulatory elements. The HHV-6 R3 repeat element (R2 in HHV-7) is a potential IE enhancer (134). R3 is present about 300 bp upstream of the *U89/90* IE ORF and is present in more than 25 copies per genome (see Table 1). The elements contain potential transcription factor–binding sites (NF-B and AP2 in HHV-6A, PEA3 and AP2 in HHV-6B, and PEA3 in HHV-7). The regulatory function of this region has not been studied experimentally. The two HHV-6A promoters that have been studied experimentally are from the genes encoding the DNA polymerase (*U38*) and its processivity factor (*U27*). Both promoters were active in infected, but not in uninfected, cells; thus, they have virally responsive promoter elements (3,131). The *U38* promoter is TATA-less, and its activity is dependent on a palindromic ATF/CREB-binding site (3). *U27* expression is dependent on a sequence that is located 52 to 73 bp upstream of the transcription initiation site and may be controlled by both positive and negative regulators.

Of the genes examined, the only transcript detected in primary peripheral blood mononuclear cells (PBMCs) that were PCR-positive for HHV-6 DNA was derived from *U94* (110), raising the possibility that this gene, or one whose transcript spans it, plays a role in establishment or maintenance of latency. If these results are confirmed and a role for *U94* in HHV-6 latency is identified, this would constitute a significant biologic difference from HHV-7 because HHV-7 does not encode a *U94* homolog. Hints to *U94* function may come from studies of rat cytomegalovirus, which encodes a *U94* homolog (141) and is amenable to genetic manipulation and then study in its natural host species. In a different study, none of three HHV-7 lytic cycle transcripts studied were detected in HHV-7 DNA-positive PBMCs (89), suggesting that the virus is truly latent in these cells.

Translation and Transport

Studies of roseolovirus translation have been limited by (a) the low multiplicities of infection described previously, (b) background due to stimulation of host cell protein synthesis by infections with these viruses, and (c) lack of extensive panels of MAbs to viral proteins. Nonetheless, progress has been made, and some interesting areas have been identified.

Infected cell proteins have been studied *en masse* by immunoprecipitation of radiolabeled HHV-6A proteins with high-titered human serum (10,11,122). Twenty to 25 polypeptides were identified, ranging in size from 26 to 280 kd, including at least 10 glycoprotein species. In one set of studies, a prominent species of 135 kd was detected by all sera tested (10,11). For HHV-7, human serum and serum from mice immunized with infected cells recognized 16 to 20 proteins, with sizes ranging from 26 to 210 kd, and including at least seven glycoproteins (44,94).

MAbs have been developed against proteins encoded by HHV-6A (10,20,107), HHV-6B (43,99,100,142), and HHV-7 (44,94). Some MAbs are cross-reactive with all three viruses, some cross-react only between HHV-6A and HHV-6B, and some are virus specific (94; summarized in 15,43, and 44).

HHV-6 *U94* is expressed as a low-copy-number spliced transcript that encodes several small AUG-initiated ORFs upstream of the *U94* ORF (105). None of the initiation codons, including that for *U94,* has the hallmarks of being efficiently translated. This suggests that in addition to tight control of its transcription, *U94* is translationally regulated to prevent high expression.

U51 encodes a G-protein–coupled receptor homolog that is expressed on the surfaces of transfected T-cell lines but in other cell lines accumulates intracellularly without being transported to the cell surface (90), suggesting the presence of a cell-specific transport or processing function.

Virally Encoded Transcription Regulators

As would be expected for herpesviruses, roseoloviruses encode genes that are predicted to regulate transcription. In addition, the broad stimulation of host cell protein synthesis on infection is evidence for viral modulation of cellular promoter activity (10,13,14). Thus far, HHV-6 genes have been studied only for their ability to regulate cellular promoters or promoters from other viruses. This provides evidence of the ability of the roseoloviruses to interact with and modulate cellular transcriptional machinery, which is likely to be important both during lytic growth and to maintain a persistent infection in the host. Little attention has been focused on the role of roseolovirus gene products in regulating expression from their native virus.

The regulators that have been studied are encoded by the HHV-6A IE-A and IE-B loci, *DR6, U3, U27,* and *U94.* Positive regulatory effects have been seen by transfection or infection in a variety of cell types on heterologous viral promoters, including the HIV-1 and HTLV-1 LTR (22,40,52,53,71,85,87,92,150), Epstein-Barr virus LMP-1 and EBNA-2 (30), HSV gD (22), human papillomaviruses 16 and 18 (26), and the adenovirus *E3* and *E4* genes (85). In addition, negative regulatory

effects have been observed on cellular promoters for p53, H-ras, and CXCR4 (6,70,143), along with mixed effects on the CD4 promoter (positive by HHV-6A and negative by HHV-7) (42,117). Up-regulation of the HIV-1 LTR is variously dependent on the presence of the NF-kB, AP-1, TAR, and SP1 sites, whereas its suppression by *U94* is independent of NF-kB and SP1 but dependent on the TAR element.

Functions of Other Virally Encoded Gene Products

Functional studies have been done on several genes in addition to those described in detail elsewhere in this chapter.

Structure and Morphogenesis

U11

U11 encodes p100 and 101K, the HHV-6A and HHV-6B homologs of HCMV pp150 (95,101). The protein is a tegument protein and is the dominant antigen recognized by HHV-6–specific IgG in immunoblots. The HHV-7 *U11*-encoded counterpart, 89K, is one of the major HHV-7 antigens (125; and E. Balada and P.E Pellett, unpublished data).

U14

HHV-7 *U14* encodes pp85, an 85-kd tegument-associated phosphoprotein that is its major antigen (46,125). As mentioned previously, an MAb against pp85 reacted with the outer surface of the tegument by immunoelectron microscopy, providing the first evidence that herpesvirus teguments are ordered structures (126).

gB

The glycoprotein gB is encoded by *U39.* It is a conserved herpesviruses glycoprotein that is found on virions of most herpesviruses, including roseoloviruses (28). It has roles in attachment and penetration and is a target for complement-independent neutralization (129). The primary HHV-6A gB translation product of about 112 kd is proteolytically cleaved to form disulfide-bond–linked subunits of 64 kd and 58 kd (39,47); for HHV-6B and HHV-7, the corresponding products were about 102, 59, and 50 kd, and 112, 63, and 51 kd, respectively (20,47,59). Similar to gBs of other herpesviruses, the extracellular domain of HHV-7 gB binds to cell surface heparan sulfate proteoglycans, suggesting a role in adsorption of the virion to cells (119).

gH and gL

As for other herpesviruses, the conserved virion glycoproteins gH and gL, encoded in roseolovirus by ORFs *U48* and *U82,* respectively, form heterodimers with each other (78,79,93), and together have roles in membrane fusion associated with both virus entry and cell-to-cell spread (45,104).

U53

The proteinase required for capsid maturation, DNA packaging, and virus assembly is encoded by *U53.* The HHV-6 proteinase, like that from other herpesviruses, is translated as a precursor molecule that is autolytically processed at two sites (the release and maturation sites) to produce the mature proteinase and assembly proteins. As in the other herpesviruses, the processing sites consist of an Ala-Ser sequence. In contrast to the other herpesviruses, roseoloviruses lack an internal autocatalytic cleavage site (Ala-Ala instead of Ala-Ser), which results in their proteinase being a single-chain enzyme (136,137).

Gene Regulation

U3

U3 is a member of the HCMV *US22* gene family. In HHV-6A, it is expressed as two unspliced transcripts of 2.0 and 3.5 kb. *U3* is expressed as early as 24 hours after infection and localizes to cytoplasmic granules associated with the endoplasmic reticulum. The U3 protein can transactivate the HIV-1 LTR (92).

U94

HHV-6A and HHV-6B *U94* encodes a homolog of the parvovirus *ns1* or *rep* gene (133), which is essential for parvovirus DNA replication (see Chapter 69). The Rep68 protein of adeno-associated virus type 2 (AAV-2) is a site-specific adenosine triphosphate–dependent endonuclease and helicase and is involved in site-specific integration of the viral genome into the host genome. HHV-6A *U94* can serve as a helper for AAV-2 replication, and *U94* can complement a point mutant of AAV-2 *rep* (135). In addition, *U94* suppressed H-*ras* and BPV-1 transformation (7) and transcription from the H-*ras* and HIV-1 LTR promoters (6). Cell lines expressing HHV-6B *U94* were refractory to infection by HHV-6A (110). As mentioned previously, *U94* is the only HHV-6 transcript detected during latency in PBMC (110), and HHV-7 does not encode a *U94* homolog, although rat cytomegalovirus does.

Replication of Genome

The three major problems these viruses need to solve to replicate their genomes lytically are ensuring adequate pools of nucleotides, initiation and chain elongation at a replication fork, and packaging and cleavage of nascent molecules.

Nucleotide Metabolism

As do the other herpesviruses, roseoloviruses encode several enzymes that have roles in nucleotide metabolism and provide the precursors needed for nucleic acid synthesis: ribonucleotide reductase, a phosphotransferase, alkaline exonuclease, and uracil-DNA glycosylase. In contrast to many ribonucleotide reductases, the one encoded by roseoloviruses consists of a single subunit that is homologous to the large subunit of two-subunit enzymes (97). Unlike some other herpesviruses, the roseoloviruses do not encode a thymidylate synthase or thymidine kinase.

Initiation and Elongation

Lytic DNA replication is initiated at an *ori*Lyt that is similar to alphaherpesvirus *ori*Lyts and markedly different from the *ori*Lyts of other betaherpesviruses and the gammaherpesviruses. As defined in transient replication assays, HHV-6 and HHV-7 *ori*Lyts map between the 5' end of *U41* and the 3' end of *U42* (33,109,140). *U41* encodes the major DNA-binding protein; *ori*Lyts for other herpesviruses also map in the region upstream of their homolog of this gene. The minimal *ori*Lyt segment is about 300 bp long, and a fully functional *ori*Lyt is about 800 bp long (33). The precise role of much of this sequence is not understood, but a critical element near the center of *ori*Lyt consists of two palindromically arranged sequences, OBP-1 and OBP-2, that are recognized by the origin-binding protein (OBP) encoded in roseoloviruses by *U73* (a homolog of HSV-1 *UL9*) (64). The core OBP sites flank an AT-rich sequence of about 30 bp (Fig. 4). Several positions within the OBP-binding sites are conserved among the viruses that encode OBP homologs, whereas others differ in a manner that provides viral specificity (65); mutations in these sequences can reduce or eliminate OBP binding and origin activity (34,64,65,140). Replication efficiency is also affected by the sequence and composition of the AT-rich spacer (38). By analogy with HSV, replication initiation is likely to initiate through a process that involves OBP molecules binding to the two OBP sites, dimerization through an amino-terminal domain (64) between OBP molecules bound to adjacent sites, bending- and helicase-induced strand separation in the AT-rich region between the OBP-binding sites, and then assembly of the replication fork complex on the OBP-anchored complex (17).

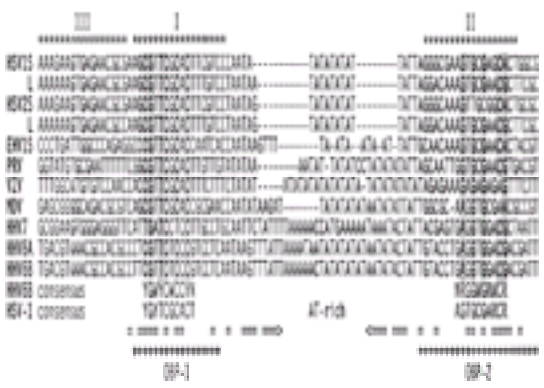


FIG. 4. OBP-binding regions of *ori*Lyts of alphaherpesviruses and roseoloviruses. HSV1 L, herpes simplex virus type 1 oriL; HSV1 S, herpes simplex virus type 1 oriS; HSV2 L, herpes simplex virus type 2 oriL; HSV2 S, herpes simplex virus type 2 oriS; EHV1 S, equine herpesvirus 1 oriS; PRV, pseudorabiesvirus; VZV, varicella-zoster virus; MDV, Marek's disease virus. Residues identical to the HHV-6 consensus are in *bold type. Horizontal arrows* indicate a structure with dyad symmetry in the HHV-6B sequence. I, II, III: Box I, II, III of HSV-1 oriS. (Modified from refs. 64 and 65; Courtesy of Naoki Inoue, Centers for Disease Control and Prevention.)

HHV-6A and HHV-6B are able to support replication of each other's *ori*Lyt (34,109), an indication of their close relationship. In contrast, plasmids containing the HHV-6 *ori*Lyt were replicated in HHV-7–infected cells, but not vice versa, suggesting that *ori*Lyt sequences and binding specificities differ between the viruses (140).

In some passage lineages of HHV-6B, an 856-bp segment that contains the OBP-binding sites and corresponds almost exactly to the minimal efficient origin region defined in transient replication assays was amplified to as many as 12 tandem copies per genome (124). In addition to the amplified segment conferring a growth advantage on cultured virus, a similar tandemly reiterated segment enabled more efficient replication of an origin-containing plasmid (34). The structure of the repeated element and its flanking region suggested that HHV-6B or a progenitor virus acquired *ori*Lyt by transposition from an unknown exogenous source.

In addition to OBP, roseoloviruses encode homologs of the six other virally encoded proteins needed for replication of plasmids containing an HSV *ori*Lyt. By analogy with HSV, these proteins are expected to operate at the replication fork; they include the major DNA-binding protein, the DNA polymerase and its processivity factor, and three gene products that compose the helicase–primase complex. In an *in vitro* DNA synthesis system that employed the single-stranded M13 genome as a template, the HHV-6A DNA polymerase alone synthesized molecules shorter than 100 nucleotides (76). Upon the addition of the 41-kd polymerase processivity factor homolog encoded by *U27* (p41), molecules greater than 7,200 nucleotides in length were synthesized, similar to the processivity increase produced by its homologs in other herpesviruses. The two proteins could be reciprocally immunoprecipitated from infected cells, an interaction mediated by domains from both the N- and C-terminal regions of the polymerase. The N-terminal portion of the processivity factor was required for the processivity-enhancing activity.

Genome Packaging and Cleavage

About 5% of genomes contained in nucleocapsids are circularized, creating a juxtaposition of DRR and DRL similar to that in concatemerized DNA (77); by analogy with HSV, it is possible that additional molecules are circularized when nucleocapsid DNA is released into the nucleus. Circularized molecules provide the template for rolling-circle replication, which leads to production of long concatemers of nascent DNA for packaging into capsids (109). In addition, the

juxtaposition of the DR elements results in assembly of a complete cleavage and packaging signal. The structure of an HHV-6 concatemeric or circular junction is as follows: DR_R-(TRS)n-*pac2*-$N_{16–18}$-junction-$N_{25–2ϱ}$-*pac1*-het(TRS)n-DR_L (32,37,56,109,118,132) There is some heterogeneity in the sequences of concatemeric junctions, with 1 to 8 bp of additional, possibly untemplated, DNA being inserted into some molecules (37,118). In the model of Frenkel and Roffman (50), concatemers are cleaved between properly juxtaposed *pac1* and *pac2* sequences after a headful threshold has been reached during packaging, leading to unit-length packaged genomes.

Identification of the *ori*Lyt and cleavage and packaging signals allowed development of HHV-6– and HHV-7–based amplicon systems (32,109) that feature T-cell replication, *ori*Lyt, and cleavage-packaging signals and can be used for expression of foreign genes. They have potential use as gene therapy vectors.

Assembly and Release

There appear to be two main pathways for herpesvirus virion maturation (see Chapter 71). One, used by HSV and other viruses, involves acquisition of the envelope during passage of the nascent virion from the nucleus through the inner nuclear membrane. Current evidence suggests that this envelope is retained throughout the subsequent maturation process; thus, this is the envelope worn by the virus when it leaves the cell. The other pathway, used by VZV and the roseoloviruses (among others), appears to involve a process of successive envelopment, de-envelopment, and re-envelopment as the virion moves from one membrane-bounded compartment to another. A model of roseolovirus virion maturation that is consistent with current information is illustrated in Figure 1. DNA replication and capsid assembly take place in the nucleus; nascent capsids that contain DNA appear about 3 days after infection (13,16). A feature that is likely to be a result of the envelopment-development process is the presence of abundant nonenveloped, tegumented nucleocapsids in the cytoplasm (12,13,98,108,139,147). In some studies, particles with neither tegument nor envelope were observed in the cytoplasm (13,139). In these studies, teguments appeared to be acquired cytoplasmically in pools of electron-dense material. The virion glycoproteins gB, gH–gL, and gp82–gp105 (encoded by *U100*) are absent from both the nuclear and plasma membranes but are concentrated in cytoplasmic structures known as annulate lamellae (23,28,139), which appear to be related to membranes of the endoplasmic reticulum and are possible sites for envelopment and glycoprotein maturation. Some authors have observed membrane-bound nuclear structures, in which capsids appear to have acquired their tegument. These structures, termed *tegusomes,* have been proposed as the site of tegument acquisition (50,98,108), but given the relatively low frequency with which they are observed, it seems unlikely that this is the major pathway. The absence of viral glycoproteins from plasma membranes precludes these membranes from being a site for envelopment or egress by budding directly from the cytoplasm. Thus, after passage through the Golgi, mature virions are released by exocytosis or cell lysis (28,139).

Fate of Host Cell

Roseolovirus infection has profound effects on host cells, including margination of chromatin (98), shutoff of host cell DNA synthesis (35), and a generalized stimulation of host cell protein synthesis (10,13,14). This leads to the development of the classic cytopathic effect of ballooning, refractile, multinucleated giant cells. For at least HHV-7, the multinucleated cells develop, not by fusion of cells into syncytia, but by polyploidization that is linked to dysregulation of cdc2 and cyclin B. This leads to an accumulation of cells in the G_2 to M phase of the cell cycle, with nuclei continuing to reproduce in the absence of cell division (114). Cytokines can be induced, including interferon-a, interleukin 1b (by HHV-6A but not HHV-6B), and tumor necrosis factor (41,63,73), as is the G-protein–coupled peptide receptor, EB1 (ligand unknown) (58). In addition, HHV-6A and HHV-6B can inhibit the proliferative responses of PBMCs to antigens and mitogens (61).

HHV-6A infection induces cell-surface expression of CD4, which then allows infection by HIV-1 of cells such as natural killer (NK) cells that were previously refractile to infection (80,81,83). HHV-6A, but neither HHV-6B nor HHV-7, down-regulates cell surface expression of CD3; HHV-7 sharply down-regulates CD4 (51,82,117).

Down-regulation of expression of the HIV-1 co-receptor, CXCR4, and a coupled reduction in Ca^{2+} flux in response to SDF-1a was observed in two studies (120,143); in contrast, no effect of HHV-7 infection on CXCR4 expression was seen in another study (149).

HHV-6A and HHV-6B modestly (twofold) induced several T-cell adhesion markers, including CD2, CD4, CD11a, CD44, CD49d, and HLA-DR, whereas HHV-7 had little effect on these or other tested markers. HHV-6B infection had little effect on CD4+ cytotoxic lymphocytes, but HHV-6A and HHV-7 decreased their activity; the inhibition by HHV-7 was overcome by lectin stimulation (51). Thus, the three viruses differ in their effects on T cells.

HHV-6A, HHV-6B, and HHV-7 were evaluated for their effects on *in vitro* colony formation of hemopoietic progenitor cells derived from human umbilical cord blood lymphocytes (69); HHV-6A suppressed erythroid colonies, HHV-6B suppressed erythroid and granulocyte-macrophage colonies, and HHV-7 had no effect on either lineage. In contrast, in bone marrow-derived cells, both HHV-6A and HHV-6B suppressed erythroid, granulocyte-macrophage, and multipotential precursors of the granulocyte, erythrocyte, monocyte, and megakaryocyte lineages, with HHV-6A being the more potent inhibitor (24).

Some parallels have emerged in studies of apoptosis induction during infections with HHV-6 and HHV-7. In fresh umbilical cord blood lymphocytes infected cells are apoptotic (13,62), whereas in cell lines, infected cells die by necrotic lysis and apoptosis is triggered in apparently uninfected or nonproductively infected cells (66,116). The latter observation suggested that the virus may be able to inhibit apoptosis in at least some cells and that its replication might be enhanced by suppression of apoptosis. This was borne out experimentally for HHV-7 in cells expressing high levels of the apoptosis suppressor, *bcl-2* (115). During *in vivo* primary HHV-6B infections, apoptosis was detected in 15% to 20% of circulating PBMCs (145). On the basis of other studies, it seems unlikely that such a high percentage of cells would be infected; thus, HHV-6B appears able to induce apoptosis in bystander cells. This is a possible cause of the transient lymphocytosis associated with primary HHV-6B infection and may contribute to controlling the viremic stage by reducing

the number of infected circulating lymphocytes.

The molecular mechanisms are just beginning to be understood for the many effects these viruses have on cells. *U83* encodes a cytokine that can induce transient calcium mobilization and serve as a chemoattractant for mononuclear cells (151), possibly to attract susceptible cells to sites of viral replication. At least one of the two homologs of cellular G-protein–coupled receptors encoded by HHV-6, *U12,* encodes a functional b-chemokine receptor, which has a selectivity that differs somewhat from its cellular homologs in its higher relative affinity for RANTES relative to macrophage inhibitory proteins 1a and 1b or monocyte chemoattractant protein 1 (68).

Growth Properties

Roseolovirus replication cycles are about 3 days in activated primary T lymphocytes grown in the presence of interleukin-2 (IL-2). Infectious yields are relatively low, with the concentration of virus in culture supernatants or in stocks prepared from infected cell lysates commonly ranging from 10^3 to 10^5 infectious units per milliliter; centrifugal concentration can be used to increase the infectious titer. Particle-to-infectious unit ratios of 10^3 and 10^4 were observed for HHV-6B in the culture supernatant and in sonicated infected cells, respectively (121).

In vivo, these viruses have been detected in a variety of cell types, but *in vitro,* they replicate most efficiently in activated primary lymphocytes, although both HHV-6A and HHV-6B can also replicate in primary fetal astrocytes and dendritic cells (8,60). Both HHV-6A and HHV-6B can produce infected foci in fibroblasts, but only HHV-6A produced infectious progeny (at low yield) (106). Laboratory strains have been adapted for growth in continuous T-cell lines that include HSB-2 and JJHAN for HHV-6A, Molt-3 for HHV-6B, and SupT1 for HHV-7 (reviewed in 15 and 18). Other than the use of CD46 and CD4 as the HHV-6 and HHV-7 receptors, respectively, the molecular restrictions on host cell range have not been identified.

INHIBITORS OF REPLICATION

Ganciclovir and phosphonoformate (foscarnet) and cidofovir are potent inhibitors of roseolovirus replication *in vitro;* acyclovir and other thymidine kinase–dependent drugs are only marginally effective (13,127,148; reviewed in 18). Ganciclovir is probably activated by the phosphotransferase encoded by gene *U69,* which can confer ganciclovir susceptibility on recombinant baculoviruses (5).

PERSPECTIVES

Studies of the roseoloviruses have been hampered by several factors, including the inability to prepare high-titered cell-free virus, the availability of relatively few MAbs, and lack of either a useful animal model system or a system for genetic manipulation of the viruses. Nonetheless, several interesting features of these viruses have been identified, including the presence near the viral genomic termini of arrays of sequences (TRS) that are similar to sequences found at mammalian telomeres, the presence of an OBP homolog and cognate-binding sequences that are similar to those previously found only in the alphaherpesviruses, a broad stimulation of host cell protein synthesis in infected cells that is unique with respect to effects on host cell metabolism described for other herpesviruses, and the presence in HHV-6A and HHV-6B genomes of a homolog of a parvovirus replication and regulatory protein. We anticipate that the implications of these observations will become apparent and that other novel aspects of the biology and molecular biology of these viruses will be identified in future studies.

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CHAPTER 81 Human Herpesvirus 6 and Human Herpesvirus 7

Koichi Yamanishi

HISTORY

Human herpesvirus 6 (HHV-6) was first isolated from patients with lymphoproliferative disorders in 1986 and was initially named *human B-lymphotropic virus* (131). Several reports subsequently described the isolation of similar viruses that have been isolated mainly from patients with acquired immunodeficiency syndrome (AIDS), but later it was found mainly to infect and replicate in lymphocytes of T-cell lineage. HHV-6 isolates are classified into two groups as variants A (HHV-6A) and B (HHV-6B). The two variants are closely related but show consistent differences in biologic, immunologic, epidemiologic, and molecular properties. HHV-6B is the major causative agent of exanthem subitum (ES) (173), but no clear disease has yet been associated with HHV-6A.

Human herpesvirus 7 (HHV-7) was isolated in 1990 from a healthy individual whose cells were stimulated with antibody against CD3 and then incubated with interleukin-2 (IL-2) (62). This virus is one of the causative agents of ES (153). HHV-6 and HHV-7 are very ubiquitous, and more than 90% of adults have antibody to both viruses. These viruses have strong homology to each other and belong to the betaherpesvirus subfamily, as does human cytomegalovirus (HCMV).

INFECTIOUS AGENT

Propagation and Cell Tropism

HHV-6 infects a variety of human cells, such as T cells, B cells, natural killer (NK) cells, g/d T lymphocytes, and monocytes of peripheral blood (107,108 and 109). HHV-6 is, however, mostly T-cell tropic in the course of the *in vitro* infection of normal mononuclear cells, and especially HHV-6 infects the predominant CD4 mature T-lymphocyte of peripheral blood *in vitro* (107,151). Fresh isolates can be adapted to grow efficiently in continuous T-cell lines, such as HSB-2 and J JHAN cells for HHV-6A, Molt-3, and MT-4 T cells for HHV-6B. Although T cells are most widely used for propagation of HHV-6A and HHV-6B, cell lines of megakaryocytes and glioblastoma, neural, epithelial, and fibroblastic cells are also sensitive for HHV-6 infection, but their replication is generally poor (3,39,101,136). Both HHV-6A and HHV-6B can also infect highly purified primary fetal astrocytes *in vitro,* and infected cells showed cytopathic effects, forming giant syncytia (68). Recently, CD46 was proposed as a receptor of HHV-6 (132).

On the other hand, HHV-7 has a narrow cell tropism of CD4+ T cells, such as PHA-stimulated human cord and peripheral blood mononuclear cells (PBMCs), and in an immature continuous T-lymphoblastoid cell line SupT-1 (2,25,35). Both HHV-6 and HHV-7 induce a cytopathic effect in infected cells, which is characterized as a ballooning feature.

The *in vivo* cell tropism of HHV-6 is wider than *in vitro.* HHV-6 genomes or antigens are detectable in lymph nodes (96), PBMCs (1,74), tubular epitherial cells, endothelial cells and histiocytes in kidney (93), salivary glands (1,61,74,92), and central nervous system (CNS) tissues, where viral gene products have been localized to neurons and oligodendrocytes (36,103,104). HHV-6 was isolated from CD4+/CD8– and CD3+/CD4+ mature T lymphocytes but could not be isolated from CD4–/CD8+, CD4–/CD8–, and CD3– T cells in PBMCs of ES patients (151). Furthermore, it was reported that there was a relationship between high CD4+ cell numbers before HHV-6 infection and high HHV-6 infection rate (91). HHV-6 is also present in lesions of Langerhans cell histiocytosis in Langerhans cell histiocytosis (95).

HHV-7 infects CD4+ T lymphocytes *in vitro.* The glycoprotein CD4, a member of the immunoglobulin superfamily, is a critical component of the receptor for HHV-7 (112). A selective and progressive down-regulation of the surface membrane expression of CD4 was observed in human CD4+ T cells in the course of HHV-7 infection. Various murine monoclonal antibodies (MAbs) to CD4 and the recombinant soluble form of human CD4 caused a dose-dependent inhibition of HHV-7 infection in primary CD4+ T lymphocytes. Moreover, radiolabeled HHV-7 bound specifically to cervical carcinoma

cells (HeLa) expressing human CD4.

So far, two cell types were recognized as sites of HHV-7 infection *in vivo:* CD4+ T lymphocytes, believed to be the site of latent infection; and epithelial cells of salivary glands, the site of productive infection and viral shedding (23,62,164). Recent study showed that cells expressing the HHV-7 structural antigen were also detectable in lungs, skin, and mammary glands. Liver, kidney, and tonsils were also positive, although the number of HHV-7–positive cells was low. Large intestine, spleen, and brain were negative for infection (79).

Antigens

More than 20 proteins and 6 glycoproteins specific for HHV-6–infected cells were identified in total-cell extracts, and rabbit antibodies immunoprecipitated 33 HHV-6–specific polypeptides with approximate molecular weights ranging from 180 to 31 kd (21). Virion was found to have at least 29 polypeptides, and 6 polypeptides were identified in the envelope fraction (22,134,135). MAbs to HHV-6–infected cells reacted with single and multiple polypeptides specific for virus-infected cells and immunoprecipitated three distinct sets of glycoproteins, which were designated gp105k and gp82k; gp116k, gp64k, and gp54k; and gp102k (22). The sera and the MAbs reacted strongly with a 101-kd protein in the immunoblot, and human sera lacking HHV-6–specific antibodies and seropositive for one or more of the other human herpesviruses failed to react with this protein, indicating that it is a specific serologic marker for HHV-6 infection (171).

Hyperimmune rabbit and mouse sera raised to HHV-7–infected cells and an immune human serum identified 20 proteins, including 7 glycoproteins specific for HHV-7–infected cord blood mononuclear cells, ranging in apparent size from 136 to 30 kd (59). Human antibodies raised in response to HHV-7 infection are directed predominantly to one or more HHV-7–infected cell proteins with apparent molecular weight of about 85 (phosphoprotein 85 = pp85) to 89 kd. The pp85 is considered a major determinant of the human immune response to HHV-7, discriminating HHV-6 from HHV-7 infection, and human sera recognize additional epitopes of pp85 that are required for their full reactivity (60,142). It was also reported that the most commonly observed antigenic protein had an apparent molecular mass of 52 kd using hyperimmune human sera (117). Some MAbs reacted in immunofluorescence assays with HHV-6 antigens to the same degree as to HHV-7 protein (59,117).

Animal Model

Only PBMCs from chimpanzees were found permissive to productive infection by HHV-6 *in vitro,* indicating that the host range of HHV-6 may not be restricted to humans (111). A serologic survey of monkeys was conducted to determine the prevalence of antibody to HHV-6, and the antibody was found in species of monkeys, suggesting the existence of HHV-6 or an HHV-6–related virus in monkeys (71). Furthermore, when cynomolgus and African green monkeys were inoculated with HHV-6, an antibody response was observed, and HHV-6 DNA was detected in the lymph nodes and spleen of monkeys. The infection was asymptomatic, except in one monkey with rash. The results suggest that HHV-6 infects these species of monkey and that this system could be useful as an animal model of HHV-6 infection (170).

PATHOGENESIS AND PATHOLOGY

Transmission

Both HHV-6 and HHV-7 are ubiquitous viruses, and infection occurs during infancy. The mode of transmission of HHV-6 and HHV-7 to children is, however, not fully clear yet. In general, the route of viral infection during infancy is horizontal from people living closely with the infants, such as their parents and siblings, or doctors and nurses at the time of delivery or transplacental infection. Local spread and seasonal outbreak of ES are rare, although occasionally outbreaks of HHV-6 infection are observed in institutions for children (120). HHV-6 DNA has been detected in saliva or throat swab of children and healthy adults including their mothers by polymerase chain reaction (PCR) (65,74), suggesting the horizontal infection through saliva, although it is extremely difficult to isolate HHV-6 (K. Yamanishi, personal data). HHV-6 is also detectable in the cervix of pregnant women in the late stages of their pregnancies (121) and in the female genital tract by PCR (94), suggesting that infectious virus is also transmissible to newborn infants by perinatal spread through transvaginal delivery. No difference was found in the prevalence rates of HHV-6 infection between breast- and bottle-fed infants or between babies born by cesarean section and transvaginal delivery.

The next possible route of infection is intrauterine transmission. It was reported that viral DNA could be detectable in fetus or blood of new born babies (18,67,91). Furthermore, HHV-6 DNA was detected in 41% to 44% of the samples during months 3 to 8 of pregnancy, which is significantly higher than control (24% of age-matched controls) (47), and specific DNA could be detected in the specimens of 1% to 1.6% of cord blood of babies born to ostensibly healthy mothers (5,47). Thus, HHV-6 reactivation appears common during pregnancy, and transfer of HHV-6 to the fetus may occur. More interestingly, HHV-6 was found in chromosomes of peripheral blood as an integrated form (48,106), suggesting a possible transplacental route of transmission as an integrated form in chromosomes, if virus is reactivated in a baby.

Finally, other routes of transmission have been reported, such as during transplantations. HHV-6 latently infects early bone marrow progenitor cells, and HHV-6 may be transmitted longitudinally to cells that differentiate along the committed pathways. HHV-6B was also isolated from recipients about 2 weeks after liver transplantation, and a significant rise in HHV-6 antibody titers was observed in all recipients (177).

HHV-7 can frequently be isolated from saliva of the healthy adults (23,164). The possible transmission of HHV-7 from grandparents to parents and to children through close contact within a household was also reported by using molecular epidemiologic technique (152). It is also speculated that breast-feeding may be one of the transmission routes of HHV-7, although this is not the main route (63). Thus, a mode of transmission similar to that in HHV-6 can be speculated in HHV-7.

Immune Response During the Primary Infection

Serologic studies on the association between ES and HHV-6 infection have been reported mainly in Japan. When serum samples obtained from ES patients during the acute and convalescent phases were assayed for IgG and IgM antibodies, IgM antibodies were detected on day 5, and persisted for 3 weeks, but were not detectable in most sera 1 month after the onset of disease. As for IgG antibody, antibody was first detected at 7 days after the onset of illness, increasing until 3 weeks and lasted at least 2 months (88) (Fig. 1). The viremia observed during primary infection subsides with the appearance of neutralizing antibodies of the infected child (13). Interestingly, antibody titers to HHV-6 are boosted during infection with other viruses, such as HHV-7 (77,155).

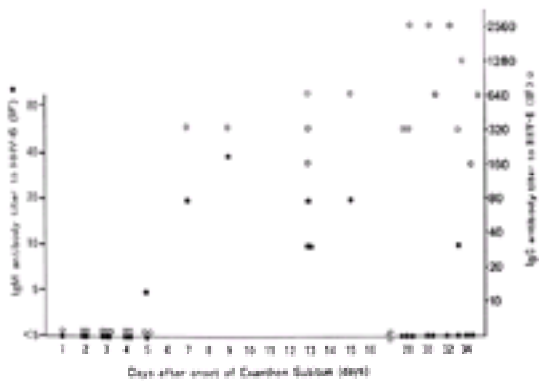

FIG. 1. Immunoglobulin M (IgM; *closed circle*) and IgG (*open circle*) antibody response in exanthem subitum patients. IgM and IgG antibody titers were tested by immunofluorescence antibody..

Clinical data indicate that a cellular immune function is important for control of the primary HHV-6 infection. The proliferative response of PBMCs from healthy adults to HHV-6 was examined, and a T-cell immune response against HHV-6 infection is generally present in healthy adult populations (162,168). *In vitro* study, T-cell clones with activity against HHV-6 have been described (169), and some T-cell clones responded not only to HHV-6 but also to HHV-7 and HCMV, indicating that the presence of epitopes common to HHV-6 and HHV-7 and to HHV-6, HHV-7, and HCMV, which are recognized by CD4+ T cells (174).

Only interferon-a (IFN-a) showed a significant increase in the plasma of patients during the acute febrile phase of ES compared with the convalescent period (150). NK cell activity was also significantly augmented in the acute phase, probably through IL-15 induction (58), especially in the exanthem period, rather than in the convalescent phase (150). These results suggest that the enhanced IFN-a response and NK cell activity in the acute early phase of the disease may play pivotal roles in the recovery from ES.

In *in vitro* studies, IFN-a activity was detected in PBMCs from healthy seropositive adults as early as 12 hours after exposure to HHV-6, plateaued at days 2 to 5, and gradually decreased thereafter. The response of cord blood mononuclear cells was lower than that of the cells from healthy adults (82). HHV-6 also induces IL-1b and tumor necrosis factor-a, but not IL-6 in PBMCs (56). These results indicate that HHV-6 can exert a strong immunomodulatory effect by stimulating the cells of myeloid lineage to produce these cytokines. IFN-g regulates the production of IL-10 and IL-12 at a transcriptional level mainly through inhibiting endogenous IL-10 production in HHV-6–infected monocyte-macrophage lineage (99).

Reports on the immune response during HHV-7 primary infection are few because HHV-7 primary infection appears to be mostly unapparent. Tanaka and colleagues (153) reported that HHV-7 was isolated from PBMCs of two infants with typical ES. During the convalescent period (16 days after onset of the disease) of the first patient, the titer of antibody to HHV-7 rose from less than 1:10 to 1:320 by an immunofluorescence antibody (IFA) test, whereas the titer of antibody to HHV-6 remained less than 1:10. In the second patient, who had two independent episodes of ES during 2 months, both HHV-6 and HHV-7 were sequentially isolated; seroconversion to HHV-7 during the second episode was observed at 4 days after the onset of the disease.

Host also responds to HHV-7 infection by up-regulating IL-15 production, which then results in an enhancement of NK cell activity; this, in turn, may play a major role in the control of the viral infection (17).

Immune Suppression

HHV-6 infects various kinds of immune cells as described previously and may immunomodulate *in vivo* and *in vitro.* In fact, infection of PBMCs or T-cell–enriched cultures by HHV-6A suppressed IL-2 synthesis after mitogen stimulation, and levels of IL-2 transcripts were diminished in experiments with enriched T-cell cultures or Jurkat cells (57).

Suppression of bone marrow function may be one of the most serious effects of HHV-6 infection in bone marrow transplant recipients. When normal bone marrow mononuclear cells were infected *in vitro* with HHV-6, the outgrowth of granulocyte-macrophage colony-forming units (GM-CFUs) was decreased by about 43%, that growth of granulocyte-erythrocyte-macrophage-megakaryocyte colony-forming units (GEMM-CFUs) was inhibited by an average of 71%, and that the erythroid burst-forming unit was decreased by approximately 73%. Further, outgrowth of the marrow stromal layer was reduced 74% (85). It was also reported that HHV-6B could inhibit GM-CFU and erythroid burst-forming unit from human bone marrow (51). Although HHV-6A suppressed the formation of erythrocyte burst-forming units colonies as efficiently as HHV-6B, the former did not exhibit significant suppressive effect on the formation of GM-CFU colonies.

HHV-6 either encodes or induces a soluble mediator or mediators that can interfere with the responses of bone marrow to growth factors and possibly block the normal differentiation of macrophages from marrow precursors (30). These studies implicate HHV-6 as a cause of bone marrow suppression in bone marrow transplant recipients.

Gobbi and associates (64) recently attempted to develop an *in vivo* model for immune suppression by HHV-6 using SCID-hu Thy/Liv mice. HHV-6A efficiently infected the human thymic tissue implanted in these mice, leading to the destruction of the graft. Viral DNA was detected in Thy/Liv implants by quantitative PCR as early as 4 days after inoculation and peaked at day 14. HHV-6 replication was associated with severe, progressive thymocyte depletion involving all major cellular subsets. However, intrathymic T progenitor cells appeared to be more severely depleted than the other subpopulations, and a preferred tropism of HHV-6 for intrathymic T progenitor cells was demonstrated by quantitative PCR on purified thymocyte subsets. These findings suggest that thymocyte depletion by HHV-6 may be due to infection and destruction of these immature T-cell precursors. Similar results were also obtained with a primary isolate belonging to HHV-6B. The severity of the lesions observed in this animal model underscores the possibility that HHV-6 may indeed be immunosuppressive in humans.

HHV-7 had no effect on hematopoietic colony formation (72) and few effects on immunosuppression by HHV-7 infection.

Persistence and Reactivation

In general, in virus infection, the persistent infection can tentatively be classified into latent infection and chronic infection. During latent infection, infectious progeny are not produced, and viral gene expression is limited to the genes required for maintenance of latency, but during chronic infection, the progeny is produced to be a source of transmission. The previously identified human herpesviruses all persistently infect their hosts after primary infection and reactivate late in life. Therefore, it is expected that HHV-6 and HHV-7 also persistently infect humans after primary acquisition.

Because HHV-6 is isolated from immunosuppressed patients, it is speculated that virus could be reactivated in immunosuppressive patients, such as those with AIDS, leukemia, lymphoma, organ transplants, chronic fatigue syndrome, or collagen vascular diseases. HHV-6 DNA or its antigen has also been detected in tissues of some lymphomas, kidneys, and salivary glands, as described previously. Viral antigens or genomes were shown to be located in glomerular cells of the kidney, histiocytes, and lymphocytes in rejected kidney (93) and lymph nodes of patients with necrotizing lymphadenopathy (147). These findings suggest that after the primary infection, this virus infects the body and can be reactivated in some conditions, such as the immunosuppressed state. HHV-6 is reactivated not only in immunosuppressive diseases as described previously but also in infections by Dengue virus (20) and measles virus (146).

The site of latent infection with HHV-6 is not completely clear. The amount of HHV-6 DNA that was detectable in PBMCs of healthy blood donors ranged among a variety of groups, possibly owing to the sensitivity of PCR. Although the virus was not isolated from PBMCs of ES patients during the convalescent phase or from healthy adults, HHV-6 DNA was detected in a fraction of them, mainly in monocytes (89). Because monocytes do not live long in a normal condition *in vivo,* it can be speculated that HHV-6 may be frequently reactivated in the monocyte-macrophages of the peripheral organs and infect other monocyte-macrophages. It was also reported that HHV-6 latently infects early bone marrow progenitors *in vivo* (105). These results suggest that HHV-6 may infect monocyte-macrophages and early bone marrow progenitors in a latent state, although it is still not clear whether these cells are the main site of its latent infection *in vivo.*

HHV-6 is also detectable by PCR from saliva, and HHV-6 antigen has been detected in salivary glands, as described previously. A high proportion of children with a history of HHV-6 infection retain HHV-6 DNA in cerebrospinal fluid (CSF) or viral antigens in CNS, suggesting that the CNS may also be a reservoir for persistent infection (33,36,37). HHV-6 DNA was detectable in biopsy samples of kidneys, lungs (45), cervix of pregnant women, and vaginal secretions of women.

In *in vitro* systems, HHV-6 infects latently in monocyte-macrophages (89); in novel cell lines, Philadelphia chromosome–positive myeloid cell lines from a patient with blast crisis of chronic myelogenous leukemia were infected latently with HHV-6 (175), indicating that these systems will be useful for studying the mechanisms of HHV-6 latency and reactivation.

It has been reported that HHV-7 infects latently, similar to HHV-6, after the primary infection in CD4+ cells (62), CD4+ T lymphocytes, epithelial cells of salivary glands, and other organs, such as lungs, skin, and mammary glands, liver, kidney, and tonsils, suggesting that a variety of tissues harbor cells at a late stage of infection (79).

HHV-7 is reactivated from latently infected PBMCs by T-cell activation, although HHV-6B could not be reactivated under similar conditions; however, the latent HHV-6B could be recovered after the cells were infected with HHV-7 (77,155). Therefore, HHV-7 can provide a transacting function, mediating HHV-6 reactivation from latency.

EPIDEMIOLOGY

A number of factors, including different methods and the cutoff value for enzyme-linked immunoabsorbent assay (ELISA), may account for differences in reported HHV-6 seroprevalence. Unfortunately, there have been few attempts to distinguish between HHV-6A and HHV-6B in serologic studies, and in addition, there is significant cross-reactivity between both HHV-6 and HHV-7, as described later.

When the study on the seroprevalence for HHV-6 infection was attempted in children under 2 years of age, children 0 to 5 months old had antibody, and their antibody titers gradually decreased with age in this period. Then, from 6 months after birth, the number of children having antibody gradually increased again, and almost all children older than 13 months of age had antibody (122). These results clearly suggest that almost all children are exposed to HHV-6 in the latter half of the first year of life. Because almost all pregnant women have antibody to HHV-6, immunoglobulin G (IgG) antibody is transferred from mother to child through the placenta, and the antibody is detectable in infants during the

early months after birth, it is believed that infants are protected against HHV-6 infection by antibody from their mother. A number of investigators have reported a decline in seropositivity with age among adults (28,54,98), but others have found no significant differences (27).

Serologic studies of HHV-6 infection have been undertaken throughout the world. The incidence of antibody to HHV-6 is high in almost all areas, but seroprevalence varied from about 39% to nearly 100% among ethnically diverse adult populations (97,128,166).

The infection by HHV-7 appears to occur later than that by HHV-6 (165). Antibody to HHV-7 was detected in 6 of 12 children (50.0%) at 0 to 5 months of age, in 4 of 12 (33.3%) at 6 to 11 months of age, in 12 of 16 (75.0%) at 12 to 23 months of age, and in 13 of 14 (92.9%) at 2 to 8 years of age (154). Another group reported that the positivity rate of antibody was 40% in the first 2 months of life, declined during the first 6 months, then gradually increased and was 45% at 1 to 4 years of age. It reached the highest level (60%) at 11 to 13 years of age and was maintained until the end of the third decade, then decreased thereafter (179).

CLINICAL FEATURES

Primary Infection

The first report of the HHV-6 infection and ES appeared in 1988 (173). ES was first described in 1910 by Zahorsky (182) and is a common disease of infants all over the world. Normally, an infant gets sudden fever, which lasts for a few days, and rash appears on the trunk and face and spreads to lower extremities during subsiding fever. A virus was thought to cause this disease because there was evidence: a long incubation time and leukopenia, lack of response to antimicrobial agents, and bacteria that have never been recovered from various body fluids of the patients. Furthermore, human transmission of the infection by experimental inoculation of blood of ES patients into other infants was reported by Kempe and associates (78) and by Hellstrom and Vahlquist (69). For a long time, efforts to isolate the causative agent of ES were unsuccessful. Isolating HHV-6 was finally successful at high frequency from ES patients during the acute phase of this disease. For isolation of HHV-6 from ES patients, PBMCs of ES patients were collected during the febrile period of the disease and were cultured in medium supplemented with IL-2 and phytohemagglutinin. About 10 days after cultivation, a cytopathic effect (CPE) could be observed, which showed balloon-like cells. When cells were fixed and stained with sera during the acute and convalescent phases of ES, positive staining was observed only using convalescent sera (Fig. 2). The typical herpesvirus particles could be found in the nucleus and cytoplasm by electron microscopy. This virus was serologically identified as HHV-6 using Z29 strain, which was already isolated by Lopez and associates (100), and antigen was reacted with paired of acute and convalescent phases sera from ES patients. The rate of virus isolation from PBMCs of ES patients was higher at the febrile phase than the exanthem phase of ES (13). Furthermore, it was reported that HHV-6 infection without clinical symptoms of rash or fever, or even without any clinical symptoms, could be seen in patients as the primary infection of HHV-6 (15,126,144). ES is caused by HHV-6B, and the magnitude of the virus replication in infants with ES is reflected in the severity of the disease (12). In most cases, ES is benign and is associated with other symptoms, including diarrhea, cough, lymph node swelling, and bulging fontanels. In adults, primary infections can cause mononucleosis-like disease and hemophagocytic syndrome (7,141). Symptomatic HHV-6A infection is rather rare, but reported manifestations appear to be similar to those of HHV-6B infection (70).

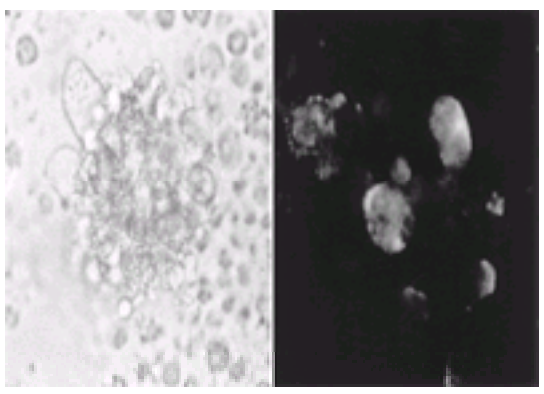

FIG. 2. Cytopathic effect by human herpesvirus 6. **A:** Balloon-like cells surround clumps of lymphocytes with positive staining by immunofluorescence antibody (IFA). **B:** Balloon-like cells stained by IFA using convalescent serum from exanthem subitum patient.

Although the rate appears to be low, primary infection of HHV-7 is similar to that of HHV-6 and causes ES or high fever (34). HHV-7 was isolated from PBMCs of an infant with typical ES. During the convalescent period of the patient, the titer of antibody to HHV-7 rose significantly, whereas the titer of antibody to HHV-6 remained negative. Some infants suffer a second episode of ES. Sera from children who had episodes of ES were tested for antibodies to HHV-6 and HHV-7, and five of seven patients had seroconversion to HHV-7 just after having typical symptoms of ES. The typical clinical case of ES caused by HHV-6 and then HHV-7 is shown in Figure 3. These results suggest that HHV-7 is one of the causative agents of ES. To define the clinical and virologic characteristics of primary HHV-7 infection and to compare these characteristics with those of primary HHV-6 infection, a prospective convenience sample study of 496 children aged less than 3 years old was recently attempted in more detail (34). Children with primary HHV-7 infection were identified and compared with children with primary HHV-6 infection detected during the same time period. All children were febrile, with no difference in the degree of fever, frequency of rash, or gastrointestinal complications between the groups. The median age of children with primary HHV-7 infection was 26 months, significantly older than that of children with primary HHV-6 infection (median, 9 months of age).

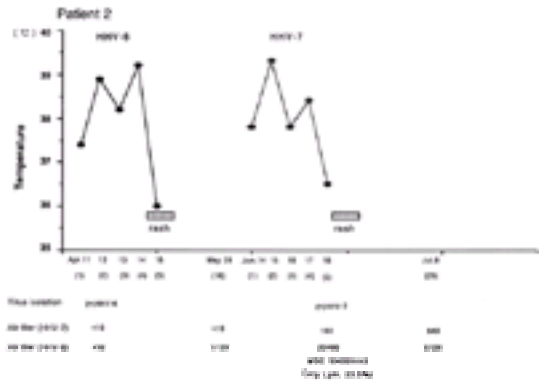

FIG. 3. The clinical course of an infant who had two episodes of exanthem subitum (ES). Human herpesvirus 6 and 7 were isolated from this infant at the first and second episodes of ES, respectively. Antibody to human herpesvirus 6 and 7 increased significantly after the first and second episodes of ES, respectively.

Associated Symptoms with Primary Infection

The most commonly reported severe symptom associated with ES is febrile convulsion, but its prognosis is not bad. The exact incidence of seizures occurring with ES is difficult to estimate. In a large study, Hall and colleagues (67) found that of the 160 infants and young children with acute HHV-6 infection, 21 (13%) were hospitalized, and 21 had seizures. The seizures often appeared late and were prolonged or recurrent. HHV-6 infections accounted for one third of all febrile seizures in children up to the age of 2 years. In this study, 7 (24%) of 29 CSF specimens were positive for HHV-6 by PCR, including 2 of 7 from children with convulsions. The detections of HHV-6 DNA were also reported in CSF from patients with neurologic manifestations at the time of primary infection of HHV-6 (33,145,178). HHV-6 persists within the CNS and has been reported to increase the risk for recurrence of febrile convulsions (90). However, a recently reported prospective study suggested that a first febrile seizure associated with an acute HHV-6 infection may be associated with a reduced risk for recurrent seizures (75). The seizures are mostly self-limited and benign; however, meningitis and encephalitis can happen, and residual deficits have been associated with HHV-6 infection. Cases of possible HHV-6–associated encephalitis in young children were also reported (14,114,178). Knox and associates (87) described an HIV-infected infant who developed fulminant encephalitis as a complication of HHV-6 infection and demonstrated that the virus present in the CNS tissue was predominantly the HHV-6A. Tang and colleagues (156) reported that a total of 60 CSF specimens from patients manifesting symptoms resembling viral CNS disease were examined for the presence of five human herpesviruses, including HHV-6, and the presence of DNA specific for two distinct herpesviruses in the same CSF specimen, providing molecular evidence that co-infection with this group of viruses may occur in the CNS.

Liver dysfunction is also associated with HHV-6 infection. Usually, liver dysfunction is mild, but fatal hepatitis and chronic hepatitis have been also reported. The first case reported was a fatal case of fulminant hepatitis, probably owing to HHV-6 (16). In another case, liver dysfunction was followed by ES, and HHV-6 was isolated from the peripheral blood of both patients (149). There have been several reports about idiopathic thrombocytopenic purpura, associated with primary infection of HHV-6 (83,138). The pathogenesis of the primary infection by HHV-6 is illustrated in Figure 4.

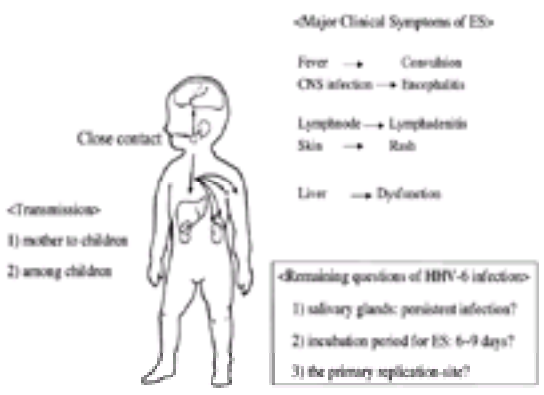

FIG. 4. Pathogenesis of human herpesvirus 6 infection.

The primary infection by HHV-7 can cause a highly febrile illness in childhood, complicated by seizures (34,159). It was reported in two ES children associated with CNS manifestations, such as by acute hemiplegia (159), suggesting that HHV-7 also infects the CNS.

Reactivation and Its Clinical Symptoms

Because herpesviruses persist in hosts after primary infection, they are important pathogens in immunocompromised hosts. Reactivation of HHV-6 occurs after bone marrow transplantation (BMT); solid organ transplantation, such as renal, liver, and heart transplantation; and AIDS.

Bone Marrow Transplantation

Asymptomatic HHV-6 reactivation appears to be common after allogeneic BMT (46), but HHV-6 reactivation with

symptoms such as bone marrow suppression, encephalitis, pneumonitis and acute graft-versus-host disease (GVHD) in bone marrow transplant recipients have also been recognized. Sixteen adults were studied for the first 100 days after allogeneic BMT to assess the pathogenic role of HHV-6 infection in patients with unexplained febrile illnesses. HHV-6 was directly isolated from the blood of six patients, and idiopathic marrow suppression occurred frequently in patients with concurrent HHV-6 viremia (52). Yoshikawa and colleagues (180) studied 25 pediatric patients who received BMT prospectively to determine the relationship between BMT and HHV-6 infection by the virus isolation from peripheral blood or bone marrow and by determining neutralizing antibodies to HHV-6 during the 2 months after BMT. HHV-6 was isolated from peripheral blood or bone marrow mononuclear cells in 40% of the 25 recipients between day 14 and day 22 of BMT, but not from any other day. Two additional recipients showed a significant increase in the antibody titer. Thus, infection with HHV-6 was confirmed in 12 (48%) of the 25 recipients. Four of the 12 developed skin rashes; 3 of these 4 had a febrile episode when the virus was isolated, whereas none of the remaining 13 developed the skin rash. These results suggest a frequent infection with HHV-6 only a few weeks after BMT and a close association between the infection with the virus and the development of skin rashes. Appleton and associates (9) recently investigated skin and rectal biopsy specimens from 34 allogeneic BMT recipients and 23 comparative autologous recipients. Twenty-eight (90%) of the 31 patients who engrafted developed clinical GVHD, which was mild in 5, moderately severe in 9, and severe in 14. Overall, HHV-6 DNA was detected in PBMCs in 74% of autologous recipients and 76% of allogeneic recipients, and in biopsy tissue in 48% of autologous recipients and 71% of allogeneic recipients. However, HHV-6 DNA was detected in skin or rectal biopsy specimens more frequently in allogeneic recipients with severe GVHD (92%) than in those with either moderate (55%) or mild GVHD (22%), suggesting that the presence of HHV-6 DNA in the skin or rectum may be a factor in determining GVHD severity.

The association of HHV-6 infection and pneumonitis was also reported, and the infected cells were primarily intraalveolar macrophages, although infected lymphocytes were seen (32). Cone and colleagues (45) also reported the possible role of HHV-6 infection in pneumonitis in immunocompromised patients by using quantitative HHV-6 PCR to study lung biopsy specimens from 15 patients with pneumonitis after BMT. In 15 immunocompetent subjects without pneumonitis, HHV-6 DNA was detected in lung tissue from all 15 patients, in 14 seropositive control subjects, and in none of the 7 seronegative control subjects. Six patients had levels of HHV-6 DNA in lung tissue that were 10 to 500 times higher than those in any of the other patients or control subjects. Increased levels of HHV-6 DNA correlated significantly with a decreased risk for death from pneumonitis, an increased severity of GVHD, and the presence of idiopathic pneumonitis. Their results suggested that the concentrations of HHV-6 genome in lung tissue and their relation to changes in serologic titers support an association between HHV-6 infection and idiopathic pneumonitis in immunocompromised hosts.

HHV-6 may also be a causative agent of encephalitis (52) and transient erythroblastopenia in immunocompetent children (125) because it can suppress the formation of erythroid burst-forming units and GM-CFUs *in vitro* and *in vivo* in HHV-6–infected patients who undergo BMT.

Recent studies have shown more rapid immune reconstitution after allogeneic peripheral blood stem cell transplantation (PBSCT) compared with allogeneic BMT. HHV-6 DNA was detected most frequently 3 weeks after transplantation, whereas HCMV and EBV DNA were detected later (2 to 3 months). Detection rates of HHV-6 DNA at 3 and 4 weeks after allogeneic BMT were significantly higher than those after allogeneic PBSCT. Furthermore, detection of HHV-6 DNA within the first 4 weeks was associated with delayed platelet engraftment after both allogeneic BMT and allogeneic PBSCT. These results suggest an advantage for allogeneic PBSCT over allogeneic BMT in terms of suppression of HHV-6 reactivation and prevention of subsequent complications (113).

Solid Organ Transplantation

Wade and associates (161) reported that children having no antibody to HHV-6 before transplantation rejected their kidneys early after transplantation. A relationship between rejection episode of kidneys and HHV-6 infection was reported (4,119). About 30% of renal transplant recipients showed a significant increase of serum antibody titer against HHV-6 after transplantation, and all these recipients suffered severe kidney rejection. Biopsy specimens of rejected kidneys were examined for the presence of HHV-6 antigens, and HHV-6 antigens were detected in the tubular epithelium as well as in infiltrating histiocytes and lymphocytes. These results suggested that HHV-6 can infect renal tissues and that the infection may be correlated with rejection or with immunosuppressive therapy (119). Another survey was attempted (181), and the infection with HHV-6 was confirmed in 55% of the recipients. The results indicate that the virus is activated in many cases in the early posttransplantation period and that HHV-6 establishes latency in the kidney tissue. However, there was no correlation between HHV-6 infection and acute rejection or the antirejection prophylaxis.

Singh and associates (137,138) reported the relationship of clinical features and HHV-6 infection in liver transplant recipients. HHV-6 infection occurred in four liver transplant recipients at a median of 50 days after transplantation. Severe cytopenia was observed in all patients. One of the four patients had interstitial pneumonitis due to HHV-6. These authors suggest that HHV-6 can be a pathogen in liver transplant recipients; idiopathic bone marrow suppression is its predominant clinical sequelae. They also described acute febrile illness characterized by life-threatening thrombocytopenia, progressive encephalopathy, and skin rash developed in association with invasive HHV-6B infection in a liver transplant recipient.

Because other herpesviruses can be also reactivated during the course of transplantation, a potential association between HHV-6 and HCMV after renal transplantation was explored by testing serial serum specimens for HHV-6 and DNA (49,129). HHV-6 reactivation occurred in 35 (66%) of 53 transplant recipients. HHV-6 reactivation was significantly associated with primary HCMV infection and HCMV syndrome and with trends for HCMV-related hepatitis, HCMV-related neutropenia, and serious HCMV disease. However, Osman and associates (123) also studied prospectively for 3 months or longer after transplantation by PCR for HCMV, HHV-6, and HHV-7. The relative risk for HCMV disease for patients with concurrent HCMV and HHV-7 was increased, whereas the association between HCMV and HHV-6 was inconclusive. HCMV disease was associated with rising antibody titers to HHV-7, and weakly so with HHV-6. Therefore, they concluded that in patients with HCMV DNAemia, concurrent infection and reactivation of HHV-7 is associated with an increased risk for progression to HCMV disease. Griffiths and colleagues (66) conducted a prospective study to see the possible relationship of HHV-6 and HHV-7 infection with the the clinical symptoms after liver transplantation. After

transplantation, 536 blood samples were tested by PCR, and active infection with HCMV was detected in 28 of 60 (47%), HHV-6 in 19 of 60 (32%), and HHV-7 in 29 of 60 (48%) patients. Although the virus load for HCMV was significantly greater than that for HHV-6 or HHV-7, HHV-6 and HHV-7 may be the cause of some episodes of hepatitis and pyrexia. From these results, HHV-6, HHV-7, and HCMV may cause some symptoms by reactivating doubly and triply.

Acquired Immunodeficiency Syndrome

Reactivation of HHV-6 is possibly associated with interstitial pneumonia, encephalitis, and retinal disorder in AIDS patients. The possible role of HHV-6 as a cofactor in HIV infection has been discussed because both viruses can infect CD4+ cells, and HHV-6 can transactivate the long terminal repeat of human immunodeficiency virus type 1 (HIV-1). Furthermore, the observation that HHV-6 can induce CD4 gene transcription and expression in CD4– cells was reported, and then HIV can infect those CD4+ cells. Lusso and Gallo (110) described the most important issue with respect to HHV-6–related disease in HIV-positive people as being not so much a direct effect on specific target organs as the putative cofactor role of HHV-6 in contributing to immune deficiency of the host.

Studies of serologic responses to HHV-6 in HIV-infected patients have shown different results. Comparisons between HIV-infected patients and uninfected controls have shown either no difference in seroprevalence and titer or higher or lower prevalence among those infected with HIV. The detection rate of HHV-6 DNA in PBMCs and saliva is also variable, depending on the investigators. One study found no correlation of HHV-6 seroprevalence with progression of AIDS (140), whereas another found that slow progressors had decreased HHV-6 seroprevalence compared with those whose disease advanced rapidly (38).

HHV-6–infected cells were detected in all lung, lymph node, spleen, liver, and kidney tissues obtained at necropsy from an unselected series of 9 patients with AIDS. Lung infection with HHV-6 was extensive enough in one patient to account for fatal pneumonitis. In other tissues, increased numbers of HHV-6–infected cells were related to the presence of lymphocyte infiltrates or residual lymphoid tissue, with lymphocytes being predominantly infected (84). The retinitis associated with AIDS and HHV-6 was also reported (127).

Because HHV-6 has been shown to be neuroinvasive, as described previously, HHV-6 may be important in the neuropathogenesis of AIDS in children. HHV-6 was more extensively disseminated in neural cells of brains of HIV patients, suggesting a contribution to the pathogenesis of AIDS encephalopathy (130). It was also reported that adult patients with AIDS had areas of demyelination in their CNS tissue at the time of death (86).

Recently, two reports appeared on the progression to AIDS by HHV-6 infection. The presence of HHV-6 in an organ is significantly associated with an elevated HIV-1 proviral load (53). Another study was done in Thailand in a total of 227 children born to HIV-seropositive mothers. When 22 HIV-carriers were studied for association of HHV-6 infection with progression of HIV disease, 5 of 12 patients (42%) without HHV-6 infection had no progression of HIV disease by 1 year of age, whereas all 10 (100%) children with HHV-6 infection progressed to HIV disease (91). These results suggest that there is an association between HHV-6 infection and progression of HIV disease and that HHV-6 may play a role as a cofactor in the HIV disease process.

Pityriasis Rosea

In 1998, Drago and colleagues (50) reported the association of pityriasis rosea (PR) with HHV-7 infection and proposed that PR is a clinical presentation of HHV-7 reactivation. PBMCs from PR patients showed ballooning cells and syncytia after 7 days in culture, whereas PBMCs from controls and recovered PR patients did not. PCR identified HHV-7 DNA in PBMCs, plasma, and skin from all patients with active PR, and only in the PBMCs 10 to 14 months later. However, controversial results were also reported. Watanabe and associates (163) reported that there were no significant differences in DNA and antibody tests between patients and control groups. Further experiments are necessary.

Possible Associated Diseases

Neoplasia

It has been reported that HHV-6 has a transforming capacity in mouse cells, but the oncogenic role of HHV-6 in human cancer has not been established.

HHV-6 has been demonstrated by detection of DNA in non-Hodgkin's lymphoma (73,76), Hodgkin's disease (158), and other neoplasias (40,102). The prevalence of HHV-6A and HHV-6B in carcinoma biopsy specimens from the mouth, salivary gland, larynx, breast, and cervix using variant-specific oligonucleotide probes was studied (10), and high prevalence was found in oral carcinoma tissue and salivary gland carcinoma tissue (167). The frequency of HHV-6 variants in certain tumors suggests a cofactorial role in multistep carcinogenesis. A possible specific association of HHV-6 with the unusual disorder S100-positive, T-cell chronic lymphoproliferative disease was also reported (26). As yet, there has been no conclusive demonstration that either HHV-6A or HHV-6B is involved in a causative way with any malignancy.

Chronic Fatigue Syndrome

There have been some reports on an association of chronic fatigue syndrome (CFS) and HHV-6 infection. CFS is an illness characterized by disabling fatigue associated with complaints of fevers, sore throat, myalgia, lymphadenopathy, sleep disturbances, neurocognitive difficulties, and depression.

More CFS patients than controls had elevated levels of HHV-6 early-antigen–specific IgM, perhaps indicating active replication of HHV-6 in CFS (124). However, there have been controversial studies (29), and further work is required to

determine whether HHV-6 contributes to the clinical manifestations.

Multiple Sclerosis and Other Neurologic Disorders

Multiple sclerosis (MS) is characterized clinically by neurologic signs and symptoms that wax and wane over time and involve multiple nerve pathways. Several studies have suggested an association between HHV-6 and MS. A DNA fragment that was the major DNA-binding protein gene of HHV-6 was detected in MS brains by representational difference analysis. Examination of 86 brain specimens by PCR demonstrated that HHV-6B was present in more than 70% of MS cases and controls and is thus a commensal virus of the human brain. Nuclear staining of oligodendrocytes using MAbs against HHV-6 virion protein 101K and DNA-binding protein p41 was observed in MS cases but not in controls; in MS cases, it was observed around plaques more frequently than in uninvolved white matter. MS cases showed prominent cytoplasmic staining of neurons in gray matter adjacent to plaques, although neurons expressing HHV-6 were also found in certain controls (36). Next, Soldan and colleagues (139) demonstrated increased IgM serum antibody responses to HHV-6 early antigen (p41/38) in patients with relapsing-remitting MS, compared with patients with chronic progressive MS patients with other neurologic disease, patients with other autoimmune disease, and controls.

Negative results, however, were seen in other reports. Fillet and associates (55) reported that there was no significant difference in detection of HHV-6 DNA in sera and CSF in groups of 32 MS patients, 21 patients with other neurologic diseases, and 13 controls. Furthermore, Coates and Bell (44) reported that there was no significant difference between MS patients and non-MS patients by staining brain immunocytochemically. Therefore, further experiments are needed.

Progressive multifocal leukoencephalopathy (PML) is a primary demyelinating disease of the CNS occurring almost exclusively in individuals with impaired cell-mediated immunity. The JC polyoma virus has been accepted as the etiologic agent of PML. Using a two-step *in situ* PCR procedure to amplify and detect genomic DNA of HHV-6 in formalin-fixed paraffin-embedded archival brain tissues, a high frequency of infected cells was consistently detected in PML white matter both within and surrounding demyelinative lesions, and HHV-6 genome was found mainly within oligodendrocytes. Lesser amounts of HHV-6 genome were detected in most tissues of patients with AIDS, patients with other neurologic disease, and controls. Immunocytochemistry for HHV-6 antigens showed actively infected nuclei of swollen oligodendrocytic morphology within the demyelinative lesions of PML but not in adjacent uninvolved tissue. In addition, no HHV-6 antigens were detectable in control tissues, including brain of patients with HIV-1 encephalopathy but without PML. Double immunohistochemical staining for JC virus large T antigen and HHV-6 antigens demonstrated co-labeling of many swollen intralesional oligodendrocytes in the PML cases. The evidence suggests that HHV-6 activation in conjunction with JC virus infection is associated with the demyelinative lesions of PML (116).

Drug Hypersensitivity

Drug-induced hypersensitivity syndrome is characterized by a severe, potentially fatal, multiorgan hypersensitivity reaction that usually appears after prolonged exposure to certain drugs. Clinical signs include a maculopapular rash progressing to exfoliate erythroderma, fever, and lymphadenopathy. Leukocytosis, atypical lymphocytes, liver dysfunction, and renal disturbance were also observed. Its delayed onset and clinical resemblance to infectious mononucleosis suggest that underlying viral infections may trigger and activate the disease in susceptible individuals receiving these drugs. Reactivation of HHV-6B, possibly in concert with HHV-7, can contribute to the development of a severe drug-induced hypersensitivity syndrome (148,157).

DIAGNOSIS

Virus Isolation and Assay

HHV-6 is easily recovered from PBMCs of ES patients during acute phase. The method of virus isolation from peripheral blood was described previously (173). Lymphocyte activation with either phytohemagglutinin (PHA) or antibody to CD3 and maintenance in IL-2 are required. CPE can be seen 7 to 10 days after cultivation, as shown in Figure 2. The refractile giant cells usually contain one or two nuclei, and after the occurrence of CPE, lytic degeneration of the cells takes place. Mononuclear cell–associated viremia caused by HHV-6 was detected in 66% of blood samples from ES children between days 0 and 7 of the disease. The rate of virus isolation from mononuclear cells was 100% on days 0 to 2 (just before appearance of skin rash), 82% on day 3, 20% on day 4, 7% on days 5 to 7, and 0% on day 8 and thereafter. The cell-free virus was also detected in blood in blood samples during the same period (13).

HHV-7 is sometime isolated from peripheral blood of ES patients as for HHV-6 (154), and frequently from saliva of individuals who have antibody to HHV-7 by using cord blood mononuclear cells, which are stimulated with PHA and IL-2 (23,164). In contrast, the isolation of HHV-6 from saliva is uncommon, although DNA is detectable there. The reason of the difference in virus isolation in these viruses is unclear.

Serologic Assays

Several serologic assays are available for HHV-6 studies, including IFA, ELISA, neutralization (NT), radioimmunoprecipitation, and immunoblot. Indirect IFA is the most commonly applied method for HHV-6 viral antigen and antibody detection. The HHV-6–infected cells are used as antigens for IFA. For detection of HHV-6–specific IgM, separation of serum IgM from IgG and IgA significantly increases the specificity. To remove interfering substances, density gradient columns, absorption by protein A from *Staphylococcus aureus* and anti-IgG treatment techniques can be applied. Another IFA test is anticomplement IFA (100). This assay is useful to reduce the nonspecific binding of fluorescein isothiocyanate–conjugated antibody. The NT test using chemically attached MT-4 cells (11) and by reading CPE (13) is used only in limited laboratories. The dot-blot neutralization assay was recently developed, and the neutralizing antibody titers appeared to correlate with the antibody titers determined by the indirect IFA test (160).

An enzyme-linked immunosorbent assay, immunoblot, and indirect IFA were developed for detection of HHV-7 antibodies

in human serum. Cross-absorption studies with ELISA or IFA using HHV-7 and HHV-6 antigens indicated that most human sera contain cross-reactive HHV-6 and HHV-7 antibodies and that the degree of cross-reactivity varies between individual serum specimens. Inhibition of homologous antibody activity by absorption with heterologous virus ranged from 0% to 57% by ELISA. However, for every sample tested, absorption with homologous virus removed more activity than did heterologous virus. An 85- to 89-kd protein was identified as an HHV-7–specific serologic marker by immunoblot (142). To detect antibody to HHV-7 specifically and sensitively, the p89/85 protein, encoded by open reading frames *U14* (85 kd) and *U11* (86 kd) of HHV-7, was expressed as recombinant protein in bacteria and developed for immunoblot kit. Activity to this protein was not removed by absorption with HHV-6 antigen. Of the three assays, the ELISA was the most sensitive, whereas the immunoblot was the most specific (24).

Genomic Detection by Polymerase Chain Reaction

HHV-6 DNA can be detected by hybridization and by PCR. Southern blot hybridizations can be useful for rapid screening of large numbers of specimens, but it is generally a less sensitive technique than PCR. Numerous PCR primer sets, which are sensitive and specific, have been described for HHV-6, and some of these sets allow easy discrimination of the variants (19,42,133,172). Variant-specific oligonucleotide hybridization is based on the amplification of two distinct regions of the HHV-6 genome, followed by hybridization of amplimers with variant-specific oligonucleotide probes. Variant-specific primer PCR is based on the amplification of each variant by using variant-specific primers (19). The putative coding region of immediate-early genes of HHV-6A was 2,517 nucleotides long, and two large additive regions of 108 and 228 base pairs were found in HHV-6B. PCR amplification using primers covering one of these regions results in PCR products with different molecular weights. This method is useful to differentiate HHV-6A and HHV-6B by PCR followed by staining with ethidium bromide (172). HHV-6 DNA is easily detectable by PCR in peripheral blood of ES patients during he acute phase, but appropriate precaution should be taken for contamination from saliva in laboratory for PCR. Detection of cell-free virus in serum or plasma by PCR offers the possibility of diagnosing active HHV-6 infections. Detection of HHV-6 DNA in plasma and a high virus load in whole blood had sensitivity rates of 90% and 100%, respectively, in diagnosing primary HHV-6 infection. However, both were occasionally found in patients with other infections, possibly associated with HHV-6 reactivation (41).

A new reverse transcription–PCR (RT-PCR) assay for HHV-6 was recently developed and validated that can determine the presence of HHV-6 in clinical specimens and differentiate between latent and replicating virus. The primers for amplification of messenger RNA (mRNA) of a major structural gene (*gp105*), which is expressed as a late gene with its mRNA having a spliced structure, were used. Therefore, the amplification of this gene has an advantage in detecting a replication of virus and readily distinguishing mRNA and residual DNA contamination. This method showed a low rate of false-positive results (1.2%) and a specificity of 98.8%. This RT-PCR assay can reliably differentiate between latent and actively replicating HHV-6 and should allow insight into the pathogenesis of this ubiquitous virus (118).

A quantitative competitive PCR assay for HHV-6 was also developed and demonstrated the persistence of a high HHV-6 load in the absence of apparent disease (43). It was recently reported that the primer sequences based on consensus sequences in the DNA polymerase gene of herpesvirus to detect herpesviruses by PCR were valuable in testing for six different herpesviruses simultaneously by a sensitive and straightforward technique rather than screening for only those viruses causing infection as suggested by clinical signs (115).

Qualitative and competitive-quantitative nested PCR assays were also developed for HHV-7. These assays amplify a DNA sequence encoding part of the HHV-7 *U42* gene (80). A multiplex PCR method was recently developed for the simultaneous detection of HHV-6 and HHV-7 in clinical samples, using primers that amplify a section of the HHV-6 *U67* gene and the HHV-7 *U42* gene. Comparison of the multiplex assay with the respective single PCR assays, using cloned HHV-6 and HHV-7 sequences as targets for amplification, showed equivalent sensitivity and specificity for the assays. It is proposed that this multiplex assay, in which HHV-6–positive samples may be typed directly from the reaction products, is an efficient and cost-effective approach to the analysis of large numbers of samples to determine the epidemiologic importance of HHV-6 and HHV-7 (81).

PREVENTION AND CONTROL

Few reports have been made concerning the response of HHV-6 to antiviral agents (6,8,31,143). Recently, the effect of several antiviral drugs against HHV-6 was evaluated, including phosphonoformic acid (PFA), 9-(2-hydroxyethoxymethyl)guanine (ACV), 9-[(1,3-dihydroxy-2-propoxy)methyl]guanine (DHPG), and (S)-1-[(3-hydroxy-2-phosphonylmethoxy)propyl]cytosine [(S)-HPMPC]. DHPG and (S)-HPMPC were more effective than the others. The sensitivity of HHV-7 to the guanine analogs was different from HHV-6, suggesting a difference in selectivity of specific viral enzymes (176). Prophylaxis with ACV did not prevent the occurrence of HHV-6–associated CNS disease after allogeneic BMT. The inhibitory effects of IFN-a and IFN-b on HHV-6 replication was also demonstrated *in vitro,* as described previously.

PERSPECTIVES

Although HHV-6 causes ES as an acute infection, its pathogenesis is still unclear. Although the main routes of transmission of HHV-6 and HHV-7 appear to be close contacts between infants and parents or siblings through saliva, it was reported that HHV-6 DNA was detectable in cord blood and PBMCs of infants at the early periods after the birth, suggesting possible vertical transmission in some cases. More interestingly, recent reports suggested that HHV-6 may integrate in the chromosome and transfer from mother to baby. The mechanism of integration into chromosome should be solved in future. Next, the detection rate of HHV-7 in saliva of adults is much higher than that of HHV-6, and even the isolation of HHV-7 is relatively easy from saliva, suggesting that the chance of transmission to infants should be high. However, the seroepidemiologic studies have indicated that the primary infection of HHV-7 also occurs during childhood, as for HHV-6, but the age of infection to HHV-7 appears to be later than that of HHV-6. HHV-7 has a narrow cell tropism for infection *in vitro,* such as in CD4+ cells, indicating the low chance of infection, but the real mechanism remains

unclear.

As for other herpesviruses, HHV-6 and HHV-7 infect latently after the primary infection. The possible sites of the latency of HHV-6 and HHV-7 are monocyte-macrophages and CD4+ T lymphocyte, respectively. However, the mechanism of the latency and its reactivation is not clear yet. It is extremely difficult to analyze the pathogenesis of HHV-6 and HHV-7 infection because the only known host for HHV-6 and HHV-7 is humans. We do not have a good animal model so far, and even the HHV-6– and HHV-7–related herpesviruses have not been found in animals. Animal models are also prerequisite for understanding the functions of the viral genes *in vivo.* For example, ES is a common disease among infants who show typical rash, but it is not clear whether virus exists in the skin.

The CNS infection by HHV-6 is one of the most serious problems among infants. Furthermore, HHV-6 and HHV-7 reactivate during immunosuppressive states, such as AIDS and transplantation, and the various clinical symptoms related to their reactivation are reported. Although the relationship between these clinical manifestations and their reactivation should be clearer, the prevention and treatment against these viruses should be developed in future.

Recently, the entire sequences of both viruses have been determined, accelerating the research of the molecular biology of these viruses. The functions of most viral gene products of HHV-6 and HHV-7 in the lytic and latent infections have not yet been elucidated. The development of recombinant virus systems is awaited for the detailed examination of functions of the viral genes in the context of the viral genomes.

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CHAPTER 82 Kaposi's Sarcoma–Associated Herpesvirus

Patrick S. Moore and Yuan Chang

Kaposi's sarcoma-associated herpesvirus (KSHV), or human herpesvirus 8 (HHV-8), is the most recently discovered human tumor virus. There are currently only three proliferative diseases unambiguously associated with KSHV infection: Kaposi's sarcoma (KS), body cavity–based or primary effusion lymphoma (BCBL or PEL), and some plasma cell forms of multicentric Castleman's disease (MCD). KS is a tumor of endothelial cell origin, whereas PEL is a B-cell lymphoma, and MCD is a B-cell lymphoproliferative disorder. These tumors are most frequently found in immunosuppressed patients, particularly patients with acquired immunodeficiency syndrome (AIDS). KSHV also causes at least one non-plastic disease, acute bone marrow failure in transplant patients (186a).

KS was first described by Moritz Kaposi in the 1870s as an aggressive tumor affecting a younger age group than is commonly seen today, suggesting viral evolution in pathogenicity during the past century. Careful reading of Kaposi's initial description, however, shows that the clinical manifestations in Kaposi's time are not markedly different from those seen today (153). KS is frequently an indolent tumor affecting elderly men, occurring most commonly in Mediterranean and African populations. With the onset of the AIDS epidemic, a particularly aggressive form of KS appeared that can disseminate and cause death (6,147). KS in severely immunosuppressed people, such as transplant recipients, and in young children can also progress rapidly.

KSHV is one of several pathogens discovered by molecular biologic techniques (259). It was identified from an AIDS-KS skin lesion in 1993 using representational difference analysis (181), a polymerase chain reaction (PCR)-based subtractive hybridization procedure that isolated two small fragments of the viral genome (52). DNA-based detection of viral DNA allowed rapid extension of these results to HIV-unrelated forms of KS (3,34,60,70,87,170,211,276,297) as well as to PELs and MCD (47,289). Identification of infected cell lines derived from PEL tumors (48,165,262) in turn led to the development of first-generation serologic assays used in the characterization of the biology and epidemiology of this virus (115,116,159,173,203,212,262). Within 2 years of the initial discovery of the virus, the 145-kb coding region of the KSHV genome was sequenced from a PEL-derived cell line (269) and from a KS lesion (220).

One critical feature of KSHV is that it, like other rhadinoviruses (2,221,281,311), has captured a number of recognizable cellular cyclic DNAs (cDNAs) into its genome (“molecular piracy”). These genes play important regulatory roles in maintaining and propagating KSHV in the hostile environment of the host cell. Remarkably, although most of these pirated genes are unique to KSHV, close inspection reveals that they affect the same cellular signaling pathways modulated by viral proteins from other herpesviruses, particularly Epstein-Barr virus (EBV, or HHV-4) (210).

Knowledge about KSHV epidemiology and its involvement in immunosuppression-associated tumors has rapidly grown since its discovery (272). KSHV fulfills Hill's criteria (106,138) for causing KS (272), and the molecular basis for its pathogenicity is currently under active investigation. Humans are the only known host, and serologic assays now available can detect most cases of infection. Unlike most herpesviruses, KSHV is not ubiquitous, and only a small minority of people in most developed countries are KSHV positive. In some regions of the world, including large portions of Africa, however, most of the population has serologic evidence of past KSHV exposure. KSHV infection is characterized by a prolonged viral and clinical latency that is presumed to be lifelong. Thus, previously healthy, symptom-free carriers may develop KSHV-related tumors years after primary infection, particularly with onset of acquired immunodeficiency from human immunodeficiency virus (HIV) infection or immunosuppressive treatment to prevent transplant rejection. Although a role for KSHV in KS, PEL, and MCD is firmly established, reports of its involvement in other diseases, such as multiple myeloma, sarcoidosis, and posttransplantation skin tumors, have not been confirmed to date.

Despite rapid progress in the molecular biology and epidemiology of this virus, virologic characterization of KSHV has been hampered by difficulties in directly culturing KSHV *in vitro.* This has precluded performing reverse genetics experiments with deletion viruses, which have been successfully used to define the biology of other herpesviruses, such as HSV-1 and EBV, and little is known about interactions between multiple KSHV genes *in vivo.* Further, knowledge of cell-mediated immune responses critical for understanding viral pathogenesis and vaccine development is at a rudimentary stage. Despite these obstacles, KSHV provides an important new model for human tumor virology.

BASIC SCIENCE

Classification of the Virus

KSHV is a large, enveloped, double-stranded DNA virus sharing many characteristics of other herpesviruses (see Chapter 71). It belongs to the *Rhadinovirus,* or gamma-2 herpesvirus, genus within the subfamily *Gammaherpesvirinae* (Fig. 1). KSHV is closely related to herpesvirus saimiri (HVS) (saimirine herpesvirus 2) of squirrel monkeys, the prototypical and most extensively studied rhadinovirus before KSHV (2,22,83). It is also the only human rhadinovirus identified to date. The typical rhadinovirus genome consists of a single long unique region flanked by variable numbers of direct terminal repeats. Coding regions of rhadinoviruses contain conserved genome segments of structural and metabolic protein genes common to all herpesviruses interspersed with nonconserved clusters of genes related to latency and host cell regulation. These nonconserved regions diverge between even closely related species and frequently contain pirated genes originating from cellular cDNA. Rhadinoviruses do not encode a reverse transcriptase; hence, the mechanisms by which these viruses captured cellular cDNAs remain enigmatic (221).

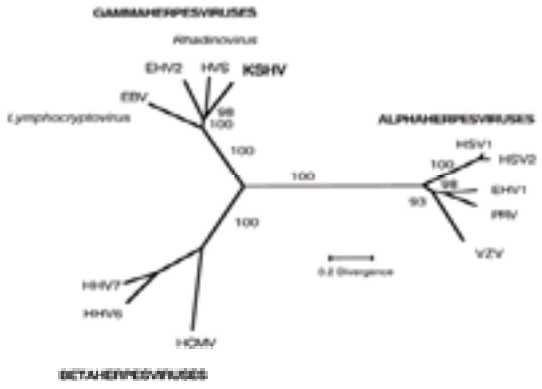

FIG. 1. Unrooted phylogenetic tree based on comparisons of nine conserved proteins showing the relationships between members of the alpha, beta, and gamma subfamilies of herpesviruses. Kaposi's sarcoma-associated herpesvirus (KSHV), like the Epstein-Barr virus (EBV), is a gammaherpesvirus but is the first rhadinovirus known to infect humans. It branches with H. saimiri (HVS), the prototypical rhadinovirus. A number of newly discovered rhadinoviruses (not shown) from Old World primates cluster together with KSHV. [Reprinted with permission (212).]

Related Rhadinoviruses

Since the discovery of KSHV, the list of newly discovered primate rhadinoviruses has rapidly expanded. Rhesus rhadinovirus (RRV) is a common infection of rhesus macaques that can be readily cultured in owl monkey kidney (OMK) cells like HVS (73). DNA fragments from two other rhadinoviruses associated with retroperitoneal fibromatosis of rhesus macaques (267) and two rhadinoviruses from African green monkeys (279) have been isolated, but these viruses have not been propagated *in vitro.* These discoveries suggest that the Old World primate progenitor was infected with two separate lineages of rhadinoviruses, which have subsequently evolved with their primate hosts (267,278). More recently, KSHV-like viruses have been identified in chimpanzees and gorillas (128b,165a). One primate virus lineage has genetic relatedness and *in vitro* culture properties similar to KSHV, whereas the other is RRV-like. For these nonhuman primate viruses, a nomenclature is being designed that will take into account the host monkey species and the viral lineage (KSHV-like versus RRV-like). Because the human RRV counterpart has not been discovered, there may be at least one more human rhadinovirus that has not yet been found and characterized.

The presence of rhadinovirus homologs to cellular proteins makes members of this genus uniquely suited for studies of virus–cell interactions. For example, scrutiny of rhadinoviral genomes (307) has led to the discovery of the corresponding cellular pathway proteins, such as FLICE-inhibitory proteins (FLIPs) (143). Murine MHV-68 is an important experimental rhadinovirus because both the virus and the host can be genetically manipulated, allowing a rigorous identification of host–virus determinants of pathogenicity (316). Further, many rhadinoviruses induce lymphoproliferative disorders when introduced into nonnative species, and rhadinoviruses serve as important but underexploited models for viral tumorigenesis (151). Herpesvirus saimiri and Herpesvirus ateles cause fulminant polyclonal T-cell lymphomas in nonnative host monkeys, and H. saimiri immortalizes human T cells.

KSHV Evolution and Strain Variability

The origin of KSHV and whether or not it recently entered into the human population are scientific questions that are being explored actively. Comparisons between conserved herpesvirus genes suggest that the KSHV-RRV lineage separated from the *H. saimiri* lineage about 35 My, corresponding to separation between their respective Old and New World monkey hosts (198,212). Phylogenetic examination of homolog genes allows a unique evolutionary analysis because both viral and host genes can be directly compared. For example, *vIL-6* genes are present in KSHV and RRV but not in *H. saimiri,* and sequence analysis lends support to the notion that the ancestral KSHV-RRV virus captured this gene after the split between New and Old World monkeys (198). Evolutionary assignments based on other homolog genes are less clear, in part because of the limited numbers of primate sequences available for comparison.

Phylogenetic and molecular epidemiologic studies of KSHV isolates have been difficult because the nucleotide sequence tends to be highly conserved. KSHV sequences from the BC-1 PEL cell line (269) and from a KS lesion (220) show that the central genomic regions have less than 0.4% nucleotide variation between these two isolates. Regions of higher variability are present at internal direct repeat regions, where repeats may be gained or lost (114a). Marked sequence variation, however, is also present at the right and left ends of the long unique region (LUR) adjacent to the terminal repeat sequences. Despite these regions of variability, the overall genomic integrity of KSHV appears to be high, and no clear genotypic strain variation has been found for different types of clinical isolates (333).

At least two major variants of KSHV can be distinguished based on sequence differences at the right end of the genome in the region between open reading frame 75 (*ORF75*) and the terminal repeat region. The two well-characterized variants are named the P and M alleles (246), and additional alleles (N and O) have been described (G. Hayward, unpublished observation) (246). Both P and M variants contain a *K15* gene encoding the highly spliced latency-associated membrane protein (LAMP), which is variable between isolates (124,246). M and P variants are found among widely dispersed KSHV clades and do not follow phylogenetic clusters, suggesting homologous recombination between viral strains after their dispersal into separate clades (333).

The left end of the genome also has a hot spot of genetic variability in the *ORF K1* gene locus (Fig. 2). *ORF K1* encodes a transmembrane protein with structural similarity to the immunoglobulin receptor family of proteins. The cytoplasmic region of K1 protein is relatively uniform, but the extracellular domain is highly variable between isolates, and multiple synonymous and nonsynonymous amino acid substitutions are common (166,172,226). Hypermutability in the K1 ectodomain probably reflects immunologic pressure exerted on infected cells expressing this protein. Viral sequences from different patients generally have different K1 sequences, whereas samples taken from the same patient over time (or from multiple sites) have identical K1 sequences (186a,333).

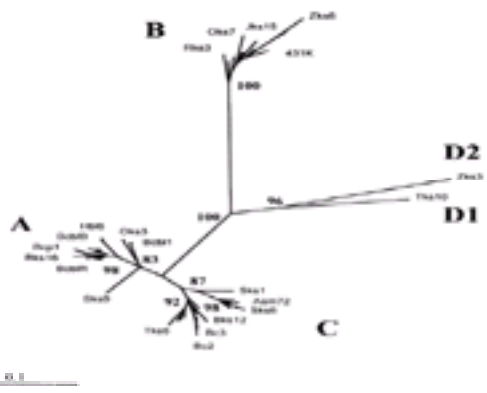

FIG. 2. Genotypic variation in ORF K1 protein sequences between 63 different geographic isolates shows that KSHV strains cluster into three distinct clades. Isolates from Africa and Asia predominate in the B and D clades respectively, while North American and European isolates are predominantly from the A and C clades (reprinted with permission (333)).

Based on variability at the K1 locus, KSHV can be divided into four clades (A to D) (333). African isolates frequently belong to the B clade, whereas the D clade is most common among Asian isolates (135,333). From these findings, it has been suggested that KSHV entered the human population before out-migration from Africa 100,000 years ago and is therefore a relatively ancient human pathogen (135). An alternative explanation, based on conserved sequences and the higher rates of infection in countries adjacent to Central Africa, is that KSHV entered the human population from a monkey host within the past several thousand years and has slowly spread locally to neighboring areas. Extreme sequence variability of K1 may reflect recent adaptations of KSHV to its new host (198). In either case, understanding the spread and evolution of KSHV requires taking into account its recent intercontinental dissemination before and during the AIDS epidemic (8,20,200).

Virion Structure and Replication

Virus Structure

KSHV virions have a target-like ultrastructural appearance (262), with an electron-dense nucleocapsid core surrounded by an amorphous proteinaceous tegument and a lipid bilayer envelope (Fig. 3). Three-dimensional reconstruction of the KSHV capsid imaged by electron cryomicroscopy shows size and structural organization similar to previously studied herpesviruses (309a,320a). Herpes-virus-like particles were found by electron microscopy in AIDS-KS lesions as early as 1984 (314). Recently, rare cells displaying intranuclear inclusions by light microscopy and containing KSHV virions have been documented in KS lesions (232). Little is known about the mode of cell entry or cellular receptor for KSHV because of difficulties in transmitting the virus *in vitro.* However, KSHV has structural glycoproteins (e.g., gB, gD, and gH) used by other herpesviruses for cell entry and virion replication (243).

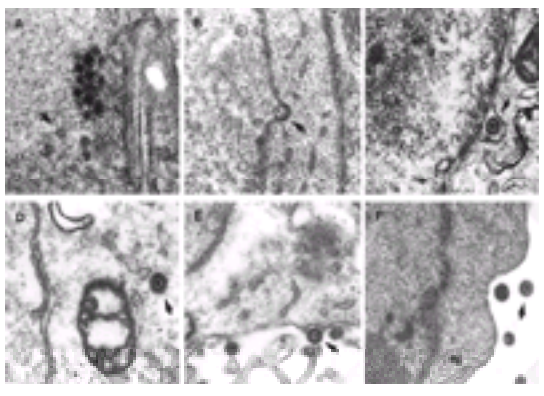

FIG. 3. KSHV virion maturation in the PEL-derived BCBL-1 cell line 48 hours after TPA stimulation. **A:** nucleocapsids forming in the cell nucleus, **B:** viral capsid budding through the nuclear membrane, **C:** virion in the perinuclear cistern after egress from the nucleus, **D:** complete encapsidated virion inside of a cytoplasmic vesicle, **E:** complete virions

budding at the cell membrane, **F:** free virus particles in the intercellular space (magnification 36,000x) Courtesy of Antonella Tosoni, L'Sacco Hospital, Milan.

Viral Replication and Infection of Cells

Like other herpesviruses, KSHV has two modes of replication: lytic, in which viral DNA is replicated by the virus-encoded polymerase and encapsidated into infectious virions; and latent, in which circular viral episomes replicate in tandem with host cell DNA using host cell replication machinery. Initiation of lytic replication results in cell killing (“lysis”) primarily through apoptosis (324), although the possibility of continued virion production without cell death has not been extensively studied.

Herpesviruses initiate lytic replication through highly choreographed transcriptional cascades in which a specific viral transactivator gene acts as a master control switch to turn on subsequent steps in lytic viral gene expression. Once the transactivator proteins are ex-pressed in response to cellular signaling, they in turn activate promoters of other viral genes in the lytic expression cascade. Viral genes needed for virus replication and encapsidation are sequentially activated either directly by the master transactivator or by secondary transcriptional regulators in a controlled series of early, delayed-early, and late waves of viral gene transcription. Individual KSHV genes, however, can also be regulated by tissue-specific cell-signaling pathways. Thus, some genes expressed during latency in some tissues may be up-regulated and expressed primarily during lytic replication in other cell types.

Two KSHV genes, *ORF K8* (129a,179) and *ORF50* (129,184,299), encoding the proteins K-bZIP and ART (activator of replication and transcription also called R4a or Lyta), respectively, are candidates for master lytic transactivators. These proteins are homologous to the well-studied EBV lytic transactivators Zta (ZEBRA) and Rta. The relationship between these KSHV lytic transactivators is not yet entirely clear, but Northern hybridization studies suggest that the ART may first transactivate the K-bZIP gene, *K8* (300), which subsequently acts at other viral promoters. In this case, expression of ART alone is sufficient to initiate all of the events leading to lytic replication (183). The DNA-binding elements in the promoters of KSHV genes responsive to ART have not yet been fully characterized.

Two putative lytic replication origins have been identified in the KSHV genome by sequence analysis that are the presumed initiation sites for genome replication (226). Like EBV, lytic replication can be triggered by chemical agents, including butyrate and the phorbol ester, 12- *O*-tetradecanoylphorbol-13-acetate (TPA or PMA) (202). TPA activates transcription through AP-1 signaling, whereas butyrate inhibits histone deacetylases involved in transcriptional repression. TPA also induces expression of histone acetyltransferases (195), suggesting that derepression of gene transcription is a critical factor in initiation of lytic replication. KSHV DNA is packaged into viral particle in the cell nucleus, and the virion is enveloped either in the Golgi apparatus or at the cell plasma membrane as it is exported out of the cell.

On entry into a new host cell, the linear KSHV genome recircularizes at its terminal repeat region to form the circular episome that will be replicated with the cell during latency. The membrane receptors allowing KSHV binding and entry into the cell are unknown. The episome is maintained in the replicating cell by the latency-associated nuclear antigen (LANA) encoded by *ORF73* (15). LANA is similar to the EBV EBNA1 protein (187,323). It binds the viral episome and tethers it to cellular chromosomes during mitosis, possibly through histone H1 interactions (66), resulting in segregation to daughter cells (15). The sites that LANA binds on the viral DNA, (the so-called origin-of-plasmid replication [ori-P]) have not yet been identified, although evidence suggests that the LANA-binding region lies within the terminal repeats (15a). This allows equal segregation of latent viral genomes to daughter cells during mitosis. During latency, KSHV gene expression is minimized, and the virus relies on the cellular nucleic acid replication machinery for survival. Silencing of viral genes during latency minimizes foreign antigen presentation to evade host immune responses.

The primary reservoir for persistent asymptomatic KSHV infection appears to be CD19+ B cells (3). Natural infection of endothelium, B cells, and possibly CD68+ monocyte-macrophage cells, prostate epithelia, and dorsal root sensory ganglion cells has been reported (3,29,63,74). Within KS tumors, virtually all spindle cells have detectable viral DNA and protein (33,86,255,292), but most cells are latently infected and do not generate encapsidated virus (232). Attempts to culture KSHV-infected tumor cells directly from KS lesions have been unsuccessful. Explanted KS spindle cells used in the past for studies on KS maintain PCR-detectable virus for only two to six passages, after which the virus is invariably lost from tissue culture (99,170). The spindle-like cells that grow out of explanted KS lesions may be uninfected endothelial progenitor cells or may be infected tumor cells that lose the virus with passage. In either case, the relationship of these tissue culture cell lines to KS tumors *in vivo* is unclear.

In contrast, KSHV can be readily cultured *in vitro* from PEL B-cell lymphomas and from peripheral blood cells from PEL patients (12,32,48,82,111,165,262). KSHV is maintained at high copy number in PEL cell lines (up to 50 to 150 genome copies per cell), and producer cell lines can be induced into lytic cycle replication by treatment with chemical agents to yield sufficient virus for purification by gradient centrifugation (218a,315,324).

In Vitro *Transmission*

Attempts to transmit the virus *in vitro* have until recently been limited to low-titered infection (104,201,212,260). Direct transmission from KS tumors and PEL cell lines to 293 cells demonstrates that tumor-derived virus is fully permissive for infection, although the achievable level of virus enrichment is low (104,260). Problems with virus transmission appear to be due to lack of appropriate recipient cell line rather than an inability to generate infectious virus. Nevertheless, extensive testing of dozens of established cell lines failed to identify appropriate recipient cells for transmission studies (260). Long-term infection and high-titered transmission have been recently described by Moses and colleagues (214). This was achieved using papillomavirus E6 and E7–immortalized dermal microvascular endothelial cells, which are

readily infected with supernatants from PEL cell lines. KSHV infection transforms E6 and E7–immortalized endothelial cells to grow on soft agar and lose contact inhibition. This occurs with only limited KSHV gene expression, suggesting that latently expressed genes are sufficient for complementing E6 and E7 in transformation. Confirmation of these results may allow more sophisticated analyses of KSHV genetics, assuming that deletion viruses can be generated and transmitted. Direct infection and transformation of primary bone marrow endothelial cells has also been reported, but like most other cell lines, high-titer infection is not sustained (101). These findings, however, are unique in that they suggest that paracrine effects from infected cells in maintaining endothelial cell proliferation may be important.

Genome Structure and Organization

The LUR of the KSHV genome is about 145 kb in length, contains at least 87 genes, and is flanked terminal repeat (TR) regions (Table 1). Varying numbers of TR units are present in each virus, and this variation may allow an assay of virus monoclonality within cells (150a,269). Each 801-bp repeat unit has high G+C content (85%) and contains putative packaging and cleavage signal sequences (269). The gene organization of the LUR (Fig. 4) is similar to that of H. saimiri, and KSHV genes are consecutively named after their H. saimiri counterparts, except for the first three H. saimiri genes, which are not represented in the KSHV genome (220,269). New genes, particularly spliced genes, continue to be described from transcription studies because the initial annotations of the genome were purposely conservative. KSHV genes that are not homologous to H. saimiri genes are given a K prefix (e.g., ORF K1 to K15) and include many of the cellular homolog genes. KSHV has no genes that encode proteins with direct sequence homology to known H. saimiri oncoproteins (STP and TIP) (152) or to EBV EBNAs and LMPs (see Chapter 74). As discussed in more detail later, some KSHV genes can functionally substitute for H. saimiri oncogenes (172) or are homologs of cellular genes induced by EBV oncogenes (210).

TABLE 1. *Features and functional properties of some KSHV regulatory genes*

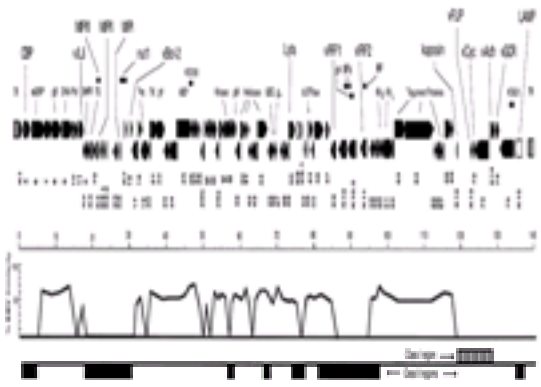

FIG. 4. Linear KSHV genome map showing open reading frames identified by sequence and transcript analysis. Genes are numbered after homologous H. saimiri genes or are given a K prefix if they have no apparent homology. Those loci in white have multiple alternative splice patterns. The middle panel shows the percentage amino acid similarity for each KSHV protein to the corresponding EBV protein, a measure of sequence conservation. Islands of highly conserved genes generally encode structural and replication proteins while intervening nonconserved regions frequently contain genes encoding proteins that alter host cell regulatory pathways. These blocks of conserved and nonconserved genes tend to have similar transcription patterns in PEL tissue culture cells (*bottom bar*). Class I genes are expressed constitutively (e.g. during PEL cell latency) and cluster at the right end of the genome (hatched box). Class II genes are expressed constitutively at low levels but are inducible during lytic replication (black boxes). These genes cluster in the nonconserved gene blocks. The remaining genes comprise class III genes (not shown for sake of clarity) corresponding to true lytic genes. These genes cluster in the conserved islands.

Viral Gene Transcription and Expression

Transcription Patterns in Cell Lines

Full-genome expression mapping has been performed using cDNA Southern hybridization of KS lesions (262), Northern blotting of the BC-1 cell line (271), and more recently by array hybridization (149). These techniques show that most viral genes are not expressed during latency, but are actively transcribed in lytic replication. Herpesvirus genes are usually classified into mutually exclusive categories of latent and lytic genes. Lytic genes can be further subdivided into immediate-early, early, and late expression patterns depending on the kinetics of transcription. This is a controversial

area of gene regulation because opinions differ on how individual genes should be classified and whether or not gene expression can be clearly divided between lytic and latent transcriptional programs.

KSHV has highly regulated transcriptional cascades like other herpesviruses. Initiation of lytic replication by ART results in specific genes being turned on to generate proteins ultimately involved in capsid formation and virus egress. Beyond this, several factors complicate classification of viral genes, such as differing expression programs occurring in different tissues, inability to use cycloheximide or polymerase inhibitors in tissue sections, and the unreliability of *in vitro* cell culture results when they are applied to *in situ* tumor sections (236). For example, some viral genes, such as *ORF K2* encoding vIL-6, are constitutively expressed in PEL cell cultures at low levels but are strongly induced by TPA treatment. This gene is constitutively expressed in hematopoeitic tumors but not KS lesions (209,237,291). Other genes, including *ORF K9* encoding vIRF, are expressed in cultured PEL cells but not the parental tumors from which these cells are derived (236). Finally, chemical inducing agents, such as TPA, may act on AP-1 sites in viral promoters that are not normally activated during natural lytic viral replication. In this case, viral genes would be activated by TPA treatment, which would not otherwise be activated in the course of lytic virus replication.

With these caveats in mind, PEL cells in culture provide an important model system for examining viral gene expression. Sarid and associates divided KSHV messenger RNA (mRNA) expression patterns into three broad classes using the BC-1 cell line that is under tight latent transcriptional control under unstimulated conditions (271). Class I transcripts are expressed constitutively and are not induced by phorbol esters, class II transcripts are expressed at low levels in unstimulated cells but are phorbol-inducible, and class III transcripts are detectable only after phorbol treatment. Class I genes correspond to latency genes, whereas class II genes appear to be expressed during both latency and lytic transcription. Interestingly, these classes of genes are least conserved with other herpesviruses and tend to be clustered together within the genome, forming “latency islands” of similarly regulated genes (see Fig. 4). Highly conserved genes involved in DNA replication and capsid formation also cluster and are expressed together during lytic reactivation (class III genes) (271). This implies that the evolutionarily successful architecture for the herpes virion has been conserved among herpesvirus species. In contrast, genes involved in herpesvirus latency are highly divergent and have evolved to match individual host cells for each herpesvirus.

The right hand cluster of latency genes (*ORF K13, ORF72,* and *ORF73*) is encoded on polycistronic messages expressed in most, if not all, infected cells and tissues (77,271,275,304). Latency transcript-1 (LT1) is a 6-kb message encompassing all three genes, whereas the 2-kb LT2 transcript initiates at the same start site as LT1 but has *ORF73* gene encoding the LANA spliced out. These transcripts (77,275,304) are constitutively expressed in PEL cells but are also cell cycle regulated, with highest transcription occurring in early G_1 phase (275). The KSHV cyclin protein, vCYC, encoded by ORF 72 has a long half-life relative to cellular D cyclins (P. J. Browning, personal communication); therefore, protein levels may not follow cell cycle kinetics. vCYC may also be encoded from a 1-kb transcript separate from LT1 and LT2 (77). LANA (*ORF73*) protein expression is constitutive and appears to be a reliable marker for the presence of the virus in all infected tissues and cells (86,236,255). An additional gene, *ORF K10.5,* lies outside of the right-hand latency cluster but is also constitutively expressed in PEL cells. Preliminary studies suggest that the *ORF K10.5*–encoded protein tentatively designated LANA2 is a B-cell–specific factor and is not expressed in KS tumors (265a).

Zhu and colleagues identified several immediate-early viral genes in PEL cells (belonging to Sarid's class II designation) that are resistant to the protein synthesis inhibitor cycloheximide after butyrate treatment, including *ORF50, ORF K4.2, ORF K8, ORF K8.2,* and *ORF4.5* (329) Based on the kinetics of their response to chemical agents and to the ART transactivator, Sun and colleagues (300) have designated a number of class II genes as early lytic and delayed-early lytic genes in PEL tissue culture cells. Examples of early genes include *ORF K2* (vIL-6), *ORF K3, ORF K4* (vMIP-II), *ORF K5, ORF K7* (polyadenylated nuclear transcript [PAN], formerly nut1, T1.1), and *ORF K8,* whereas delayed-early genes include *ORF2* (vDHFR), *ORF K6* (vMIP-I), *ORF16* (vBCL-2), *ORF K12* (kaposin), *ORF70* (vTS), and *ORF74* (vGCR). It is likely that proteins encoded by these genes play important early regulatory roles in preparing the cell for initiation of virus replication. Genes, such as K8 and K12, have alternatively spliced transcripts that belong to multiple classes of lytic gene expression (179,270).

The *K12* gene is an unusual and complicated locus that has both class I and class II transcription patterns in tissue culture (271). *K12* was originally named T0.7 based on the size of a 700-bp transcript isolated from a KS lesion (262). This transcript is now known to be an uncommon variant compared with most *K12* transcripts, which are 1.5 to 2.5 kb in length. *K12* encodes multiple proteins, termed kaposin A, kaposin B, kaposin C, and so forth, that use multiple alternative transcription and translation initiation start sites (270). In an intriguing study, Sadler and coworkers found that K12 protein translation occurs only infrequently at a predicted AUG start codon and more commonly begins at one of a series of upstream 5'-CUG codons. For most transcripts, translation then extends through the zppa repeat region but does so in different reading frames, depending on the initiation CUG codon used. Because the repeat unit (23 bp in length) is not divisible by three, each kaposin protein eventually encodes the same large internal acid repeat motif regardless of reading frame used* (Fig. 5). A small hydrophobic protein (kaposin A) encoded by the original T0.7 transcript but lacking the zppa repeat motif can transform Rat-3 cells (216), suggesting that some proteins encoded by this locus may be involved in cell proliferation. Because *K12* transcripts are abundantly expressed in all infected cells, they have been useful as *in situ* hybridization probes for KS lesions and related tumors (292).

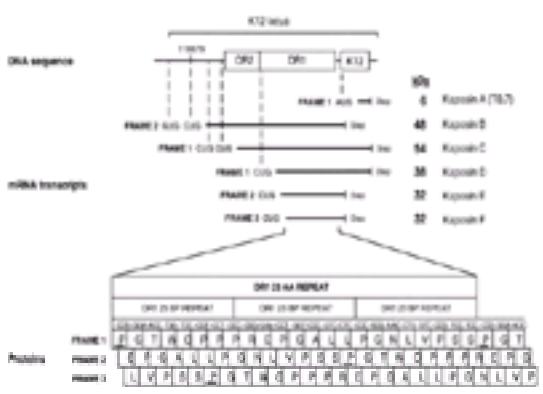

FIG. 5. Alternative transcription and translation start sites for proteins encoded by the K12 locus. Seven mRNA transcripts from this locus are shown encoding proteins with predicted sizes from 6 to 54 kDa. Except for the kaposin A (T0.7) transcript, these mRNAs initiate translation at noncanonical start codons (CUG or GUG instead of AUG) in different reading frames. Despite this, transcripts containing the zppa repeat region composed of two different direct repeat motifs (DR2 and DR1) produce peptides with the same repeating amino acid motif. An example is shown for the DR1 region which reoccurs in all three reading frames after 23 amino acids. The underlined proline is taken as an arbitrary start site for orientation purposes (modified from (270)).

ORF K7 encodes a second abundantly expressed 1.1-kb class II transcript called PAN that also has been used for *in situ* hybridization studies (261,298,327). PAN is primarily (but not exclusively) expressed after TPA treatment of PEL cells (271). Whereas *ORF K12* message is detectable in nearly all KS tumor cells as well as other KSHV-infected tissues, the *ORF K7* transcript has more limited expression compatible with lytic replication *in situ* (292).

Class III gene transcription in tissue culture cell lines is only detectable after lytic replication cycle activation. Typical class III genes include structural protein genes, such as the major capsid protein (*ORF25*) and the small capsomer-interacting protein SCIP (*ORF65*) genes. In general, transcription of these genes can be blocked by the viral DNA polymerase inhibitors phosphonoacetic acid or phosphonoformic acid that inhibit late gene transcription (100,300). Experimental results may differ between cell lines because different cells lines have differing proportions of cells spontaneously undergoing lytic replication. A small percentage of BCBL-1 cells (1% to 5%) continually enter lytic replication in culture, even in the absence of TPA or butyrate. BC-1 cells, in contrast, appear to be under tight latent control and do not express late lytic genes, such as the major capsid protein gene *ORF25* unless treated with phorbol esters (271).

KSHV Gene and Protein Expression in Human Tissues

Gene expression in infected tissues suggests different categories of KSHV latency that are reminiscent of those found for different types of EBV latency (see Chapter 74). In KS lesions, essentially all spindle cells are infected with KSHV, but the limited set of genes expressed includes LANA (*ORF73*), vCYC (*ORF72*), and kaposin (*T0.7, ORF K12*) (86,236). Rare tumor cells (about 1%) express the DNA processivity factor PF-8 protein (*ORF59*) and *T1.1,* indicative of lytic virus replication and virion production (50,155a,178,236). This expression profile is consistent with most KS cells being latently infected, whereas a minority cell population spontaneously undergoes lytic replication (232,292). vMIP-III protein (encoded by *ORF K4.1*), an angiogenic chemokine, has been found by immunoblotting KS tissues, but the cell types expressing this protein are not known (294). PEL tumors show a similar restricted pattern of gene and protein expression, with the exception that vIL-6 protein can be detected in 10% to 30% of cells (155a,236). In those PEL tumors co-infected with EBV and KSHV, EBV generally has a type I pattern of expression initiated from the Qp promoter, with EBNA1 being the only EBNA protein prominently expressed (142).

Infected cells in Castleman's disease lymph nodes have a strikingly different pattern of gene expression from PEL and KS tumors (155a,236). KSHV-positive cells comprise only a minority of cells in the Castleman's disease lymph nodes. These cells have features of immunoblastic B cells and are localized to 2% to 30% of cells in the mantle zone around reactive germinal centers (237). Although all these KSHV-infected cells express LANA, a substantial fraction also coexpress a variety of other proteins, including vFLIP, vIRF-1, vIL-6, PF-8 SCIP, K8-1, K10, and K11 proteins. It is unclear whether these cells are undergoing full lytic virus replication or have an expanded pattern of latent KSHV gene expression.

EPIDEMIOLOGY AND PATHOGENESIS

Diagnostic Assays

KSHV DNA can be detected in peripheral blood cells from only about half of infected persons using standard PCR protocols indicating that viremia is not prominent (88,213,317). Serologic assays for KSHV-specific antibodies are more sensitive than PCR for detecting either current or past infection with the virus. Improvements in assay formats are occurring rapidly, but current assays under optimal conditions can detect viral infection in more than 90% of infected patients. Antibody responses to LANA and other KSHV antigens appear to be lifelong in healthy adults; however, loss of detectable antibody occurs in 10% to 30% of end-stage AIDS patients with KS (116).

There are at least five serologic assays in current use, each with particular strengths and weaknesses. Indirect immunofluorescence assays (IFA) to detect LANA or lytic antigens are relatively inexpensive and simple but can be subjective. IFA is performed by incubating patient sera with infected PEL cells or isolated nuclei on microscopic slides, and specific antibody binding is measured using fluorescein-conjugated antihuman secondary antibodies. Patient sera with specific anti-KSHV antibodies fluoresce in characteristic patterns. LANA was the first serologic antigen identified by IFA (212), and this is still a standard assay because of its low rate of false positivity. A speckled nuclear pattern is seen in unstimulated whole cells (116) or isolated nuclei (157). The LANA IFA is very specific but generally less sensitive (about 80% sensitivity) than other assays. LANA can also be detected from Western blots of whole-cell lysates as a 226- to 232-kd doublet, although this procedure is more difficult owing to low protein electrotransfer (115).

Lytic antigen assays have higher sensitivity rates than LANA assays (more than 95%) but also carry the risk for cross-reactivity, especially at low serum dilutions. Cross-reactivity is the likely explanation of high human infection rates initially reported from lytic antigen assays compared with LANA testing (173). AIDS-KS sera are nonspecifically reactive to lymphocyte antigens at low dilutions (e.g., 1:10), which can be removed by adsorption with KSHV-negative lymphocyte lysates (116). Cross-reactivity in this assay can be markedly reduced by counterstaining with Evans blue and diluting

sera to 1:40 or more (287). Lytic IFA antigens have a diffuse cellular distribution after cells are treated with TPA or butyrate to induce virion protein synthesis (173,287). A purified whole virion enzyme-linked immunosorbent assay (ELISA) has been developed, which has similar characteristics as the lytic IFA (54).

Recombinant antigens from *ORF65* (180,284) and *ORF K8.1A* (51,177,249) used in an ELISA format show comparable sensitivity and specificity to the lytic IFA assays. SCIP (*ORF65)* is a small capsid antigen with some cross-reactivity to the homologous EBV antigen that can be eliminated by truncating the carboxyl portion of the protein (284) or through use of a peptide antigen (239). The *K8.1* gene encodes alternatively spliced glycoproteins termed *K8.1A* and *K8.1B* that may correspond to a 40-kd antigen identified by Western blotting BC-1 cells after butyrate treatment (203). *K8.1* can be examined in an ELISA or Western blot format. Other KSHV antigens have been examined but appear to be less appropriate for serologic screening owing to low sensitivity or cross-reactivity (4,168).

Evidence suggests that patient populations have heterogeneous serologic responses to defined KSHV antigens; therefore, combinations of antigen assays may achieve the best detection rates (168,284,329). Although early studies suggested wide differences in infection rates based on the type of antigen used (252), properly performed assays in standardized formats are actually highly concordant (284). These results are likely to be further improved with use of recombinant antigens (329), but conclusions based on early LANA IFA studies have been largely confirmed using the more refined antigen preparations (277).

Epidemiology

The existence of an infectious agent causing KS was predicted by the tumor's unusual epidemiology (21,123). Before the AIDS epidemic, “classic” KS was noted to have geographic clustering and occurred most frequently in elderly men of Mediterranean and Eastern European ethnicity. Evidence for an environmental (i.e., infectious) component to this disease comes from geographic differences in KS rates. More than 100-fold differences in classic KS rates occur in different regions of Europe. England and Wales have exceedingly low annual rates (0.14 cases per million), whereas rates in Sardinia and Sicily reach 24 to 30 cases per million per year. The United States has an intermediate rate estimated to be 3 cases and 0.7 cases per million men and women, respectively (144). Still higher rates of disease were present in Central African populations (“endemic KS”), where a particularly malignant form affects young children (13,61,330). The occurrence of KS is also increased among immunosuppressed transplant recipients and chemotherapy patients, particularly when the patient belongs to a high-risk ethnic group (241,247).

Regardless of the setting, country-wide incidence rates dramatically increased with the onset of the AIDS epidemic in the early 1980s. Evidence for an infectious etiology also became stronger with epidemiologic studies of AIDS patients because behavioral risk factors pointed toward a sexually transmissible agent (20). Homosexual male AIDS patients are 20 times more likely to have KS at AIDS diagnosis than comparably immunosuppressed male hemophilic AIDS patients (21). Studies also suggested that the agent spread internationally from the U.S. AIDS epicenters (New York, San Francisco, and Los Angeles) to Canadian and European homosexual communities (9,20,200). Having a bisexual male sex partner is also a risk factor for developing AIDS-KS among HIV-infected women. In contrast, people infected with HIV through blood transfusions rarely developed KS, indicating that HIV and the KS agent are not equally transmissible by blood-borne routes. Although HIV itself (particularly the tat protein) was considered a prime candidate for the KS agent before identification of KSHV, it could not be responsible for HIV-negative forms of KS, nor did it satisfactorily explain low rates of KS among HIV-infected hemophiliac patients and blood transfusion recipients.

Infection Rates in Various Populations

Isolation of KSHV DNA allowed use of PCR to test for the presence of the virus in both HIV-negative (classic, endemic, and immunosuppression-related) and HIV-positive forms of KS. Well more than 20 separate studies have demonstrated that viral DNA can be detected by PCR in at least 95% of KS tumors regardless of source. It is possible that a small percentage of KS lesions are free of KSHV infection, but it is now assumed that virus will always be detected in properly diagnosed and prepared specimens. The benefit of PCR in providing a simple and sensitive assay for infection is counterbalanced by its tremendous susceptibility to amplicon contamination. Some anomalous early studies resulted from undetected PCR contamination, which underscores the need for caution when using this technique in epidemiologic investigations. Confirmatory assays, such as direct Southern hybridization, should be used whenever possible. Serologic studies also support the near universal infection of KS patients with KSHV because specific antibodies are detectable in 70% to 90% of all KS patients and in almost 100% of KS patients who are not immunosuppressed (54,115,157,173,180,203,212,284,287,318,326).

Unlike most human herpesviruses, KSHV is not ubiquitous but is distributed according to a combination of geographic and behavioral risk factors (Table 2). KSHV infection rates generally parallel KS disease occurrence in different parts of the world. KS is uncommon in the United States, and KSHV seroprevalence among U.S. blood donors ranges from 0% to 10%, whereas seroprevalence rates above 50% occur in many African populations (10,19,35,116,121,136,173,196,230,284,285). Intermediate rates of infection are present for Italian blood donors (116,265,318) and other groups from Mediterranean countries where KS is more common (248,284). Two studies have demonstrated a gradient of KSHV seroprevalence along the Italian peninsula that matches local rates of KS disease occurrence. Highest rates of infection and disease are found in southern Italy—particularly Sardinia—and lowest rates are in northern Italy (43,318). Local population pockets having atypical KS rates such as in the Po valley also have high KSHV seroprevalence. In Japan, where KSHV seroprevalence among blood donors may be as low as 0.2% (109), KS is a rare tumor. Similar low rates of infection are present in HIV-seronegative people from Thailand (217), and KS is generally an uncommon opportunistic disease among AIDS patients in Asia. One unexplained finding is that KSHV infection rates are high in West Africa, although KS is reported to be uncommon (10,62). Data such as this suggests that other as-yet undescribed factors such as HLA (191), or other infectious cofactors may play a role in disease expression among people infected with KSHV.

KS incidence[a]	Regions	Population KSHV prevalence	Transmission	Risk groups
Low	North America, North Europe, Asia	<2%	Sexual	Homosexual men, STD attendees, transplant recipients
Intermediate	Mediterranean, Middle Eastern countries, Caribbean	5–20%	Sexual, nonsexual?	Homosexual men, STD attendees, transplant recipients, older adults
High	Africa, parts of Amazon basin	>80%	Nonsexual, sexual	Older adults, lower socioeconomic status

[a] AIDS-KS rates are highly dependent on local HIV infection rates and risk groups.

TABLE 2. *Patterns of KSHV infection and non–HIV KS*

KSHV seroprevalence in high-risk populations is dramatically higher than in the general population. Homosexual men can have asymptomatic infection rates approaching 40% (116,193,203), and KS is more frequent among HIV-negative homosexual men than the general male population (98,108). The only study from which population-based estimates of infection are available shows that 38% of homosexual men from San Francisco were LANA antibody positive in the early 1980s (193). Several studies record a dramatic decline in new KSHV infections since then, corresponding to declining KS rates that have been noted among AIDS patients (80,186,200,263). The reasons for this decline are not clear, but one explanation is that KSHV has low infectivity, and implementation of safe-sex practices effectively reduces its transmission in high-risk populations. Our current understanding of KSHV epidemiology helps to explain the long-standing mystery of why KS became a clinical hallmark for AIDS during the early manifestations of the epidemic. It is likely that the initial epidemic of AIDS-KS resulted from the collision between two simultaneous and independent viral epidemics of HIV and KSHV (200,227). It is therefore not surprising that homosexual men, who are independently at higher risk for HIV and for KSHV infection than heterosexual men, composed most AIDS-KS patients.

In many developed countries, women make up only 10% to 30% of the KS cases compared with men (26,130,140). Women are also less commonly infected than men in U.S. and Northern European KSHV serosurveys (126,157,319). Higher rates of infection generally occur among female sexually-transmitted disease clinic attendees, providing evidence that the virus might be heterosexually transmissible, but this mode is likely to be far less efficient than homosexual transmission (157,288). In Mediterranean and African populations, infection rates do not significantly differ between sexes (35,230,285,318), and a hormonal influence on disease expression seems likely because KS among people without HIV is still more common in men than women from these regions (117,145). Infection and disease are extremely rare among children in the United States and Europe, whereas it is far more common in parts of Africa (30,126,196,249).

Epidemiologic studies suggest that only a small percentage of healthy adults who are KSHV infected will develop symptomatic disease; therefore, KS is a rare tumor in countries with low infection rates. Symptomatic disease primarily occurs among people with acquired immunosuppression (for HIV-negative people, this may be due to advanced age or chemotherapy), resulting in loss of cell-mediated immune surveillance for virus-specific antigens (234). In contrast, KSHV prevalence was already high in some African countries before the introduction of HIV (230). Because of the African HIV epidemic, KS tumor rates have dramatically risen, and it is now the most frequently reported cancer in some African tumor registries, accounting for up to 50% of reported tumors among adult men (17,313). Public health efforts have not begun to address this problem, which is particularly important for African populations.

Transmission

Detailed knowledge of the epidemiology of KSHV transmission is currently limited; therefore, explanations of how KSHV infection occurs remain largely speculative. Seroepidemiologic studies suggest that KSHV has at least two major modes of transmission. Sexual transmission is the most common route of infection in low-prevalence, developed countries where infection is uncommon. In contrast, nonsexual routes of transmission predominate in endemic African regions where infection rates are high. The reasons for these differences and the precise mechanisms for human-to-human transmission are not known. Similarly, it is not yet clear whether sexual or nonsexual routes of transmission are the principal routes of infection in Mediterranean basin countries (Table 2).

KSHV can be shed into semen (reviewed in 28) and thus might be transmitted during vaginal or rectal intercourse. KSHV prevalence increases with the number of male homosexual contacts (193), and receptive anal intercourse has been found to be a risk factor for infection in some studies (200). However, virus can only be detected in semen samples from a minority of infected men, raising the possibility that shedding is episodic or that only a percentage of men are highly infectious. Shedding into the genital tract of infected women is uncommon; its significance for female-to-male transmission is unknown but probably low (42,319).

Infectious KSHV virions have also been detected in the saliva of KSHV-seropositive individuals, demonstrating replication of the virus in the oropharynx (164,239a,310). If KSHV is transmitted through oral secretions, the reasons that the virus has a sexual rather than an oral contact (e.g., kissing) transmission pattern are unclear (239a). Specific sexual practices such as oroanal and orogenital contact may help to explain this (131). Recent cohort studies point toward orogenital contact being a risk factor for transmission (84), although this does not fully explain why homosexual men have elevated infection rates.

Still less is known about how KSHV is nonsexually acquired in African countries, where infection can occur during childhood (154,230,285,331). Unlike developed countries, maternal–child transmission accounts for a portion of KSHV infections in highly endemic areas (196,286). This may not be due to vertical infection during the birth process because childhood infection appears common (188). KSHV infection continues to occur throughout childhood, and adolescence in

endemic regions (5,154,196) arguing for transmission through some form of casual contact. In this respect, KSHV epidemiology resembles that of hepatitis B virus (see Chapter 87), which has a low prevalence in developed countries, where it is principally sexually transmitted but is transmitted through unknown routes during childhood and adolescence in hyperendemic developing countries.

Transplantation-related KSHV transmission is an important preventable cause for KS. KS can be a complication of any form of transplantation, including bone marrow, cardiac, and renal transplantation. It develops in 0.1% to 1% of transplant recipients in low endemnicity settings (242) and in up to 5% of patients from Saudi Arabia, where KSHV infection is high (248). The risk for developing KS is significant for infected transplant recipients, with one study demonstrating 68% of KSHV-infected patients developing tumors during the course of follow-up (97). Recently, a new syndrome of bone marrow failure after transplantation due to KSHV infection has been described (186a). Disease can result from both reactivation of infection acquired before the transplantation (238) and *de novo* infection from the donated organ allograft (238,257,258). In contrast to organ transplantation, transmission from blood products is uncommon. KS rates are low among AIDS patients infected with HIV through blood transfusions, arguing for a low rate of transmission by this route. KHSV infection among intravenous drug users increases proportionally with frequency of drug use, suggesting that blood-borne KSHV transmission can occur but at lower rates than for other viruses, such as hepatitis C virus (45a).

Natural History of KSHV Infection

Characteristic clinical symptoms for primary KSHV infection have not been reported. Most primary KSHV infections are asymptomatic; however, in one well-documented case, an HIV-positive man developed a transient angioproliferative lymphadenopathy accompanied by a rise in LANA followed by a rise in *ORF65* antibody titers (229). Primary infection generates immunoglobulin M (IgM) and IgG antibody responses to LANA and lytic antigens. IgM titers are frequently transient, lasting for several months, although prolonged IgM responses can also be seen (258). Rising antibody titers may be predictive for KSHV-related disease (285), and the pattern of seroreactivity to specific antigens can evolve during the course of KS (128a,168).

The incubation period for disease caused by KSHV infection is primarily dependent on the immune status of the infected person rather than the length of time infected with KSHV. Examinations of transplant recipients receiving an infected organ give the most precise estimates for the minimum incubation period after infection. In one study, two patients developed KS within 26 months of receiving an organ from an infected donor (258); in another study, a patient developed MCD within 13 months and KS within 27 months after infection (238). Among HIV patients, risk for developing KS depends on patient CD4+ count rather than HIV viral load (263), and half of those destined to develop KS are infected at least 2 to 6 years before disease onset (115,263). Some patients, however, can develop clinical disease within months of primary KSHV infection. Longitudinal studies also suggest that those infected first with HIV and second with KSHV develop AIDS-KS more rapidly compared with KSHV-positive patients who later acquired HIV infection (263).

Immunologic control of KSHV infection in healthy adults is evident from clinical studies. HLA class I–restricted cytotoxic T-lymphocyte responses to proteins encoded by *K1, K8.1,* and *K12* are present in some infected patients but are lost with progression of AIDS-related immunodeficiency and onset of KS (234). Dramatic resolution of both KS and PEL tumors have been reported with highly active antiretroviral therapies in AIDS-KS patients (320). Similarly, posttransplantation KS can also resolve when patients are taken off immunosuppressive regimens. These findings are encouraging that preventative or therapeutic vaccines are achievable for controlling KSHV-related diseases.

Pathogenesis of KSHV-related Proliferative Disorders

The three neoplastic diseases associated with KSHV infection (KS, PEL, and some forms of MCD) represent a spectrum of proliferative disorders ranging from polyclonal hyperplasia to monoclonal neoplasia. Because of this, KSHV provides unique insights into the pathology of cell proliferation and its relationship to immune surveillance. All forms of cellular proliferation are considered tumors, but hyperplasia occurs through replication of normal, polyclonal cells operating from cytokine-driven regulatory mechanisms. Neoplastic proliferation implies dysregulated autonomous cellular expansion by abnormal cells that have undergone transformation. Prototypical cancers are aggressive, monoclonal conditions that rarely regress spontaneously. This does not imply that neoplastic disorders are cytokine independent, and in fact, neoplastic conditions characteristically show strong autocrine and paracrine cytokine dependence. Virus-induced cancers, however, can result from multiple independently infected cells undergoing clonal expansion, which resembles a polyclonal rather than a monoclonal disorder.

KS is a vascular tumor composed of proliferating spindle cells thought to be derived from an endothelial lineage, possibly from lymphatic endothelium (75,86). It frequently contains normal cells either as a result of infiltrative growth or inflammatory cell recruitment. Some studies suggest that KS tumor cells have a onoclonal cell origin as expected for a typical neoplasia (250,251,269), whereas others indicate that the tumor is a polyclonal hyperplasia (71,122). In part, these differences may be due to the admixture of normal and tumor cells, but it is also possible that KS lesions originate as polyclonal or oligoclonal diseases, which evolve into monoclonal disorders when single clones achieve growth advantage (113,150a,269). A more central issue to KS pathogenesis is whether or not KS spindle cell proliferation is caused by viral gene products or by external cellular inflammatory cytokines induced by infection. *In situ* localization studies show that most mature tumor cells contain virus (33,86,255,292). This implies that viral gene products directly impart a growth advantage within these cells, regardless of whether a tumor originated from a single clone or from a group of independently infected cells.

An alternate hypothesis states that an uninfected precursor lesion exists first, which secondarily attracts virus-infected cells through inflammatory cytokines (96,296). In this case, KSHV infection of KS lesions is a consequence rather than a cause of the disease. These conclusions are based on studies of early (patch) stage KS lesions in which only a minority of tumor cells have been found to be infected. The possibility of insensitive virus detection remains to be excluded, and it is difficult to reconcile this hypothesis with epidemiologic studies, indicating infection precedes onset of clinical disease

by up to 10 years (115,193,200,213,256,263,317).

Inflammatory cytokines, such as interferon-g, induce lytic KSHV replication as well as vasoproliferative cellular cytokines (208). Cellular cytokines, such as b-FGF, are likely to be central to maintaining tumor cells (93,95), but vigorous debate centers on whether b-FGF and other inflammatory cytokines induce KS lesions or are secondarily involved as an inflammatory response to viral infection of KS. HIV tat protein has also been implicated as a central mediator of KS pathogenesis (16,94,141,312), but clearly it is not involved in the development of HIV-negative KS, nor is HIV proviral DNA appreciably present in KS lesions (72). Although some angiosarcomas have been reported to be infected with the virus, pathologic discrimination between angiosarcoma and aggressive KS may be difficult, and these findings remain controversial (76,197,303,308).

In contrast to KS, PELs represent a more typical neoplastic process. PELs have unique clinical, immunophenotypic, and molecular characteristics. These B-cell lymphomas occur as malignant effusions in visceral cavities, although malignant cells can be found infiltrating adjacent visceral organs and in the peripheral circulation (32). Although most of these lymphomas are co-infected with EBV, dual viral infection is not invariable. In the setting of AIDS, the clinical course for most of these lymphomas is extremely aggressive, with a mean survival from diagnosis of 5 to 7 months (218). Implementation of highly effective antiretroviral therapy in cases has resulted in regression (320), which is atypical for a lymphoma. This “benign” course is reminiscent of that seen for PEL in HIV-seronegative individuals (295). Immunoglobulin rearrangements and surface expression of syndecan-1, coupled with lack of Bcl-6 expression, indicate that the KSHV-infected PEL cells are derived from preterminally differentiated post–germinal center B cells (46). PEL also express CD23, CD45, EMA, and other activation antigens. PEL cells, unlike cells from KS lesions, are fully transformed and tumorigenic in immunodeficient mice (32,245). A role for KSHV in other AIDS-related lymphomas, such as primary central nervous system lymphomas, has been proposed, but careful studies demonstrate that virus detection may be due to “spillover” of infected peripheral blood cells (47,110,112).

MCD is a reactive lymphadenopathy characterized by expanded germinal centers and proliferation of endothelial vessels within involved lymph nodes. Based on the microscopic features, MCD can be divided into two histologic subtypes: hyaline vascular and plasma cell variants. The former usually presents as a solitary, usually mediastinal, lesion that can be treated curatively by surgical excision; whereas the plasma cell variant tends to have a multicentric and malignant presentation. Although MCD is considered nonneoplastic, it often precedes development of non-Hodgkin's lymphomas (244). Dysregulated interleukin-6 (IL-6) production is the underlying abnormality that leads to the characteristic clinical and histologic manifestations of this disease (37). IL-6 signaling inhibits B-cell apoptosis by inducing BCL-XL (280), reducing B-cell turnover. IL-6 is also an autocrine growth factor for some B-cell neoplasias (1). KSHV-related forms of MCD appear to be caused by vIL-6 secretion (237), whereas KSHV-negative MCD probably results from local cellular IL-6 hypersecretion through other mechanisms. Most MCD tumors in AIDS patients have detectable KSHV infection, but only half of HIV-negative MCD patients are KSHV positive (289). KSHV-related MCD is more commonly the plasmacytic variant (64) and is clinically severe, with most patients dying within 2 years from a variety of disorders, including autoimmune hemolytic anemia associated with gammopathy (237).

Scattered KSHV-infected B cells are present in the mantle zone of germinal centers of KSHV-positive MCD tumors (237). These cells express a wide range of KSHV proteins, including early lytic gene products (155a,236). Whether the small percentage of KSHV-infected cells in an MCD germinal center are themselves neoplastic (analogous to EBV-positive Reed-Sternberg cells of Hodgkin's disease) remains to be determined, but monoclonal expansions of B cells have been found in some MCD cases (133,134,253). KSHV-infected cells in MCD germinal centers have been reported to have a lambda light-chain restriction pattern (85). vIL-6 secretion is most likely responsible for the B-cell hyperplasia surrounding uninfected B cells. Other viral and human cytokines may also contribute to MCD pathogenesis, particularly neoangiogenesis (7,14,31,306).

Attempts have been made to generate animal models of KSHV-induced proliferative disorders using whole virus or individually expressed viral genes. Primate infections with KSHV have not thus far led to clinically apparent disease. When KSHV is injected into human fetal liver and thymus implants in SCID mice, the virus replicates at low level before establishing a prolonged latency (78). No appreciable pathology was found in the infected tissues. Implantation of PEL cell lines into immunodeficient mice leads to lymphoma formation with local angiogenic hyperplasia (32,245). NIH3T3 cells overexpressing vIL-6 causes cell transformation and tumor formation, but also mouse B-cell hyperplasia and neoangiogenesis (7). One of the most convincing animal models for KS has recently been described using transgenic expression of the viral G-protein–coupled receptor (vGCR) under control of a B-cell promoter (322). These transgenic mice develop angioproliferative lesions that closely resemble KS lesions, suggesting an important role for vGCR in KS pathogenesis, presumably acting through paracrine mechanisms.

Molecular Piracy of Cellular Regulatory Genes

The most prominent feature distinguishing KSHV from other human tumor viruses is its extensive array of pirated regulatory genes (Table 1). Although the KSHV genome is unique because of these readily identifiable genes, other viruses frequently have functional equivalents (210). This is most clearly seen for EBV in which there is close correspondence between cellular genes induced by EBV infection and cellular homologs encoded by KSHV (Table 3). The recognizability of many KSHV genes make this virus a highly transparent model for understanding commonalities among tumor viruses. Other viruses modulate some of the same cell regulatory pathways, and it remains to be seen how far this correspondence principle can be extended to unrelated tumor viruses.

KSHV Genes	Effect	Cell protein induced by EBV	Responsible EBV proteins
ORF 4 (vCRP)	Complement inactivation?	C3bi/vCP2	EBNA2
ORF 42 (vIL6)	Growth factor	IL-6	gp350/220, LMP1
ORF 16 (vBCL2)	Anti-apoptosis	BCL2	LMP1
ORF K9 (vIRF)	IFN inhibition CBP/p300 binding		EBNA2
ORF 72 (vCYC)	Cell cycle control	Cyclin D2	EBNA2, EBNA-LP
ORF 74 (vGCR)	Cellular proliferation	EBI-1	EBV induced
ORF K1, K15 (LAMP)	Immunoreceptor signaling	–	LMP1, LMP2
ORF 50 (Lyta)	Lytic transactivator	–	BRLF (Rta)
ORF K8 (b-ZIP)	Lytic transactivator	–	BZLF (Zta, ZEBRA)
ORF 73 (LANA)	cis-P-binding protein	–	EBNA1

KSHV, Kaposi's sarcoma–associated herpesvirus; EBV, Epstein-Barr virus; IFN, interferon; CBP, CREB-binding protein.

TABLE 3. *Correspondence between KSHV and EBV regulatory genes*

Cytokines and Cytokine Receptors: vMIP-I, vMIP-II, vMIP-III, vIL-6, and vGCR

There are at least four secreted cytokines and a G-protein–coupled receptor encoded by KSHV. The cytokine genes (*ORF2, ORF4, ORF4.1,* and *ORF6*) cluster in a nonconserved 10-kb region on the left end of the genome between base pairs 17,000 and 27,000, whereas the G-protein–coupled receptor gene (*ORF74*) is at the far right-hand end of the genome (base pair 119,000).

*Viral Chemokines (*ORFK4, ORF4.1, *and* ORF6*)*

KSHV encodes three chemokines (chemoattractant cytokines) belonging to the macrophage inflammatory protein family. This group of related chemokines initiate signaling by binding to G-protein–coupled receptors and have a central dicysteine (CC) motif, which distinguishes them from CXC chemokines that include IL-8. The viral proteins encoded by *ORF K6, ORF4,* and *ORF4.1,* respectively, are termed vMIP-I (or vMIP1a), vMIP-II (or vMIP-1b), and vMIP-III (or BCK), based on their sequence similarity to cellular macrophage inflammatory protein (MIP) chemokines. Virus-encoded chemokines were initially considered to be unique to KSHV (209,225) and molluscum contagiosum virus (282), but functional chemokines have since been found in HHV-6 (334), human cytomegalovirus (CMV), and mouse CMV (189,240).

Although it was initially thought that the vMIPs might have a broad antagonist function to inhibit inflammatory responses (163), it has become clear that each vMIP binds to a specific receptor and initiates chemotactic signaling. Nonetheless, all three may share a similar functional activity in blunting the helper T-cell subtype 1 (T_H1) immune response (290). For example, vMIP-II specifically activates CCR3, the eotaxin receptor for eosinophils, while binding other receptors at low affinities (31). This broad receptor-binding range may be related to vMIP-II being a monomer in solution unlike most CC chemokines (182). vMIP-II also chemoattracts T_H2 lymphocytes that express CCR3, and this property may serve to inhibit recruitment of T_H1 lymphocytes during a nascent immune response. vMIP-I and vMIP-III bind CCR8 (67,92) and CCR4 (294), respectively, which are also T_H2 lymphocyte chemotaxis receptors. vMIP-II, like vMIP-I, binds CCR8 with high affinity, but results are contradictory as to whether this inhibits or activates (290) signaling. Only vMIP-III is actively synthesized and secreted in KS lesions (294). In contrast to KSHV, EBV encodes an IL-10–like cytokine, which may also serve to polarize immune responses toward a T_H2 profile.

Both vMIP-I and vMIP-II prevent HIV entry into cells by blocking the chemokine coreceptors used by both macrophage- and lymphocyte-trophic strains of HIV (31,209). Whether these proteins have significant activity at the concentrations achievable in patients is unknown, but they may serve as molecular starting points for generating synthetic broad-spectrum coreceptor inhibitors. All three viral chemokines are also highly angiogenic in chorioallantoic membrane assays, an unusual property for CC chemokines (31,294).

Viral G-Protein–coupled Receptor (vGCR, ORF74*)*

Like H. saimiri KSHV encodes a functional seven-transmembrane–spanning G-protein–coupled receptor with sequence similarity to the IL-8 receptor (49,132). vGCR is encoded on a bicistronic transcript, together with *ORF K13* (vAdh or vOX-2), as an early lytic gene in PEL cell lines (162). Stable vGCR expression induces cell transformation and secretion of the angiogenic vascular endothelial growth factor (VEGF) in NIH3T3 cells (11,14), but this gene has limited expression in KS lesions (162). KSHV vGCR is constitutively active in stably transfected cells in culture (11) and can be further activated by IL-8 and GRO-a binding (120,268). In contrast, the interferon-induced chemokine IP-10, which acts as a chemoattractant for T_H1 cells, inhibits vGCR signaling, as does vMIP-II (119). Activation of the receptor results in a Ca^{2+} influx and mitogen-activated protein kinase (MAPK) pathway activation, which may be responsible for cell proliferation induced by this viral gene (11). Transgenic vGCR expression in mice results in an angioproliferative disorder strongly resembling KS (322).

*vIL-6 (*ORF K2*)*

This secreted viral cytokine has 62% amino acid sequence similarity to its cellular counterpart (209,219,225). huIL-6 is an inflammatory cytokine that binds to a specific high-affinity receptor (IL-6Ra or gp80) and the low-affinity gp130 signal transducer molecule (reviewed in 139). IL-6 binding to gp130 leads to Jak-STAT pathway activation and activation of other pathways, such as MAPK, through the protein tyrosine phosphatase SHP-2 (161). Like the vGCR, constitutive overexpression of vIL-6 induces secondary VEGF secretion and promotes NIH3T3 cell transformation (7). vIL-6 is active in IL-6 bioassays, although it has a lower specific activity than the human cytokine (39,209,225). One important difference between the human and the viral cytokine is that vIL-6 can directly initiate signaling through the gp130 receptor without

use of the IL-6Ra co-receptor (206). Unlike haIL-6, vIL-6 makes contact with gp120 through hydrophobic interactions to induce reception dimerization-activation (57a). This may lead to activation of cells normally unresponsive to IL-6. Despite this, there is no clear indication that downstream signaling pathways differ between the two cytokines (233).

PEL cells are autocrine dependent on vIL-6 and huIL-10 (81,150). If vIL-6 inhibits an apoptotic B-cell responses initiated as a defense against KSHV infection, this results in the unusual situation whereby KSHV sets up a cross-species autocrine loop in which host cells become dependent on a parasite-derived cytokine. EBV does not encode an IL-6–like cytokine, but EBV LMP-1 induces huIL-6 secretion (90), and EBV-infected lymphoblastoid cell lines are autocrine dependent on huIL-6 (309). KSHV-infection and vIL-6 elaboration were proposed to play a role in multiple myeloma (23,264), but subsequent studies have failed to confirm this association (231).

Immune Regulatory Proteins: vIRF-1 and vIRF-2

KSHV vIRF-1 and vIRF-2 have low but significant homology to the interferon regulatory factor (IRF) family of transcriptional factors possessing a conserved amino-terminal DNA-binding domain (DBD) and a carboxyl-terminal transcriptional activation or repression region. The amino-terminals region of vIRF-1 corresponding to the DBD is poorly conserved and does not appear to bind specific DNA sequences (209). Two other genes and potential pseudogenes with even lower IRF homology are clustered with *ORF K9* in the midregion of the genome between base pairs 83,000 and 94,000.

The putative function of vIRF-1 is to inhibit interferon signaling in infected cells by blocking both class I and II interferon–regulated transcription (102,114,175,332). The mechanism by which this occurs is still under active investigation, but vIRF-1 does not compete directly with cellular IRFs for binding to interferon-stimulated response element (ISRE) sequences in interferon-responsive promoters. Interferon, which has an antitumor as well as an antiinfective role, induces cell cycle arrest, and up-regulates the cyclin-dependent kinase inhibitor, p21. vIRF-1 can reverse both of these interferon-induced effects, and expression of vIRF-1 in NIH3T3 cells results in a transformed phenotype (114,175).

One clue for the action of vIRF-1 comes from the adenovirus E1A protein, which inhibits interferon signaling by binding the CH3 domain of the transcriptional histone acetyltransferase coadaptors p300 and CBP (CREB-binding protein) involved in interferon-related transcription (25,325). vIRF-1 also binds p300 and CBP in the CH3 region (40). This provides a straightforward explanation of how vIRF-1 inhibits interferon-induced transcription. Transcriptional coadaptors, such as p300 and CBP, are recruited to promoters by transcription factors to acetylate core histones at the transcription start site. Histone acetylation relaxes condensed chromatin at the promoter, allowing RNA polymerase access to the start site and facilitating gene transcription. Therefore, the ability of vIRF-1 to interrupt this process could inhibit transcription of genes activated by interferon-pathway transcription factors.

The clinical significance of these effects is unclear because vIRF-1 is not expressed in KS or PEL tumors (236). These findings are nonetheless important in revealing the interactions between immune and cell proliferation signaling pathways.

A second IRF-like protein, designated vIRF-2 putatively encoded by *ORF K11.5,* has been described. vIRF-2, unlike vIRF-1, appears to be constitutively expressed at low levels in PEL cells and binds NF-kB enhancer DNA elements but not ISRE (41). vIRF-2 inhibits NF-kB–mediated transcription as well as activation of the interferon-a promoter by IRF1 and IRF3, which cooperatively activate this promoter together with NF-kB. Both vIRF-1 and vIRF-2, therefore, have potential to abrogate antiviral responses initiated by innate immune signaling pathways. If this strategy fails, evidence suggests that inflammatory cytokines, such as interferon-g, activate lytic virus replication, perhaps allowing the virus to escape to another uninfected cell or to a new host (208). A third protein with homology to cellular IRFs has recently been described. It is transcribed from the *ORF K10.5* gene as a spliced product and has low but detectable homology to human IRF4, primarily in a putative activation domain region (265a). This protein (LANA2) has generated interest because it is expressed constitutively in hematopoietic tissues and inhibits p53-induced apoptosis, thus serving as a primary candidate for contributing to B-cell transformation.

Antiapoptotic Proteins: vBCL-2 and vFLIP

Two KSHV genes, *ORF16* and *K13,* encode the vBCL-2 and vFLIP proteins, respectively, which have roles in preventing cellular apoptosis. BCL-2–like proteins are also encoded by EBV and H. saimiri, and primarily expressed during lytic replication, whereas EBV induces cellular BCL-2 during latent infection (137). KSHV vBCL-2 effectively inhibits apoptosis initiated either by Sinbis virus infection or by overexpression of the pro-apoptotic Bax protein (55,273), but study results are conflicting regarding whether it achieves this effect by heterodimerization with a cellular BCL-2 family partner protein. BCL-2 is minimally expressed in KS lesions and is markedly induced by TPA treatment of PEL cell lines.

vFLIP (FLICE-inhibitory protein) is encoded by *ORF K13* on latency-expressed polycistronic transcripts together with vCYC and LANA. Translation of vFLIP occurs through an internal ribosome entry site (IRES) present in the ORF72 gene, thus allowing protein to be made from polycistron C transcripts (25a,131a,182a). Other gammaherpesviruses, including *H. saimiri,* equine herpesvirus 2, and some poxviruses, encode similar proteins (24,307). Unlike KSHV vFLIP, the *H. saimiri* vFLIP is expressed as an immediate early gene during lytic replication (307). Cellular and viral FLIPs possess a single death-effector domain required for caspase-8 interaction but cannot interact with downstream signaling components and therefore act as dominant-negative inhibitors of caspase-mediated apoptotic signaling pathways (reviewed in 59). KSHV vFLIP inhibits Fas-mediated apoptosis (79), indicating that it may play an important role in preventing NK cells from killing KSHV-infected cells.

Cell Cycle–Regulatory Protein: vCYC

vCYC (*ORF72*) has well-conserved homology with cellular D-type cyclins, particularly in the region of the “cyclin box”

responsible for interactions with cyclin-dependent kinases (CDKs) (49,53). vCYC is constitutively coexpressed together with vFLIP on the LT2 transcript and with both LANA and vFLIP on the LT1 transcript. vCYC mRNA transcription is cell cycle dependent, with highest expression during early G_1 phase (275), but the protein lacks PEST sequences regulating cyclin protein turnover, resulting in a long protein half-life (P. Browning, unpublished observation).

Cellular D-type cyclins interact with CDK4 and CDK6 to direct phosphorylation of the tumor suppressor retinoblastoma (RB1) checkpoint protein involved in the G_1- to S-phase transition (44,185,301). RB1 is directly targeted by viral proteins from several small DNA tumor viruses and is probably indirectly inactivated by others (210). RB1 sequesters E2F transcriptional factors required for entry into S phase of the cell cycle and inhibits E2F-dependent promoters by recruiting histone deacetylases. D-type cyclins reverse this by phosphorylating RB1 and inhibiting its function, allowing transit across the G_1- to S-phase checkpoint. Overexpression of cyclin D1 (PRAD1, BCL-1) by chromosomal translocation overrides this tumor suppressor checkpoint and contributes to some B-cell and parathyroid tumors (215). Other cyclin–CDK complexes (e.g., cyclin B/CDK2) are involved in cell cycle regulation at other checkpoints.

Although KSHV vCYC complexes with CDK6 to phosphorylate RB1, leading to its inactivation and unscheduled S-phase cell cycle entry (53,125,176), the vCYC–CDK6 complex shows several important differences from the cellular CYC D–CDK6 holoenzyme. The vCYC–CDK6 complex is resistant to the cellular CDK inhibitors p16, p21, and p27 (302) and has a more promiscuous substrate specificity because it can direct phosphorylation of histone H1, which is not a substrate for cellular D cyclins (125,176). The vCYC–CDK6 complex also phosphorylates the CDK inhibitor (CDKI) p27 to induces its degradation (91,190) and induces the cyclin A promoter through E2F activation, but does not phosphorylate the RB-related protein, p107 (89). Thus, the KSHV-encoded cyclin can escape from CDKI regulation and may have substrate specificity that allows it to abrogate other cyclin- and CDK-regulated checkpoints beyond those targeted by cellular D-type cyclins. Overexpression of vCYC is toxic to cells and has not been directly assessed as a transforming viral protein.

Immunoreceptor Proteins: KSHV ITAM-Based Signal Transducer (KIST) and Latency-Associated Membrane Protein (LAMP)

KSHV encodes two membrane proteins, KIST and LAMP, which affect B-cell receptor (BCR) signaling (68). The K1 protein acts as a constitutively activated BCR, whereas LAMP inhibits some BCR signaling pathways. KIST is the first recognizable ORF in the KSHV genome and encodes a small transmembrane glycoprotein possessing a cytoplasmic immunoreceptor tyrosine activation motif (ITAM). The KIST ITAM is tyrosine-phosphorylated *in situ,* allowing recruitment of SH2 domain proteins, Syk pathway activation, and intracellular calcium mobilization (167,171). KIST overexpression causes transformation of Rat-1 fibroblasts and, when substituted for the *STP* oncogene in recombinant HVS virus, it can immortalize primary T lymphocytes and induce lymphomas in marmoset monkeys (172). KIST spontaneously forms multimeric complexes through cross-linking of the cysteine-rich external region. Aggregation of KIST, like BCR cross-linking, may be necessary for initiating its immunoreceptor signaling activity. Although the ectodomain of this transmembrane protein is highly variable between KSHV isolates, there is no current evidence that different KIST strain types have altered signaling activity or ability to induce proliferation.

ORF K15 encoding the latency-associated membrane protein (LAMP) is the last recognizable ORF on the opposite end of the genome from *ORF K1* (124,246). It lies in the highly divergent P and M variant regions, but *K15* genes from both P and M virus subtypes have a similar organization of eight exons, which generate a family of alternatively spliced transcripts. Surprisingly, the largest versions of the P and M subtype proteins are predicted to have only 29% amino acid identity to each other. LAMP proteins may consist of up to 12 transmembrane domains, depending on varying donor-acceptor splice site usage. Although numerous combinations of exons have been detected, all LAMP proteins have a hydrophilic C-terminal cytoplasmic tail encoded by exon 8, possessing protein motifs similar to those found in EBV LMP2A and LMP1 proteins. This region contains both a group III tyrosine motif and an src phosphotyrosine kinase SH2-binding motif present in the cytoplasmic domain of EBV LMP2A. It also possesses a PFQPADE motif similar to the TRAF-binding motif of LMP1, and protein–protein interaction assays demonstrate that it, like the corresponding region of LMP1, binds TRAF1, TRAF2, and TRAF3 (124). Further, epitope-tagged LAMP proteins also show membrane patching. In contrast to KIST, LAMP overexpression blocks calcium influx in response to BCR activation from cross-linking antibodies. This response is similar to EBV LMP2A (57). Taken together, the KIST and LAMP proteins appear to have many of the properties of EBV LMP1 and LMP2A. Future studies are needed to determine whether KIST and LAMP antagonize each other or whether they differentially affect different arms of BCR signaling pathways.

DNA Synthesis Enzymes

DNA synthetic enzyme genes, such as those found in the KSHV genome, are a central feature of herpesviruses (see Chapter 71). Rhadinoviruses have the most extensive sets of these genes among the herpesviruses (2,220,269). The corresponding cellular genes are under E2F transcriptional regulation controlled by the RB1 tumor suppressor protein (reviewed in 169,222, and 321). This leads to the possibility that the KSHV enzymes not only supplement enzymatic activities that may be rate limiting within the cell but also may overcome cellular mechanisms to regulate DNA synthesis. The KSHV DNA synthesis enzyme genes are generally expressed during initiation of lytic replication (271).

Most currently available antiherpesvirus drugs act by inhibiting the viral DNA polymerases, encoded by *ORF9* in KSHV. This may require phosphorylation of a prodrug by a viral thymidine kinase or phosphotransferase to generate the active drug. *In vitro* studies suggest that KSHV replication is most sensitive to cidofovir and foscarnet (phosphonoformic acid), with intermediate sensitivity being present for ganciclovir and relative resistance for acyclovir (45,156,199,223). Drugs such as penciclovir and BVDU show limited activity *in vitro.* Using recombinant viral proteins expressed in cell lines, Cannon and colleagues demonstrated that the KSHV phosphotransferase (*ORF36*) has higher activity than the viral thymidine kinase (*ORF21*) in phosphorylating ganciclovir, which converts it to an active form while the opposite is true for penciclovir (45).

Patient studies are largely consistent with these results in that patients at risk for AIDS-KS develop the disease at lower

rates after treatment with foscarnet for CMV retinitis (205,266). One study of high-dose oral ganciclovir for prevention of CMV retinitis demonstrated a 75% reduction in the rate of new KS lesions among high-risk AIDS patients (192). Little is known, however, about the activity of antiherpesvirus drugs on established KS lesions.

Several other KSHV enzymes are particularly worth noting because they may be unique targets for existing chemotherapeutic drugs (see Chapter 15). KSHV is the only human virus to encode a dihydrofolate reductase (*ORF2*), a potential therapeutic target for methotrexate and sulfonamide derivatives (58). The viral thymidylate synthase (*ORF70*) is another potential target for specific inhibitors.

Other KSHV Genes

The complement-binding protein or complement control protein homolog (vCBP or CCPH, *ORF4*) and the viral adhesion protein (vADH or vOX2, *ORF K14*) genes have not been experimentally examined. The protein encoded by *ORF4* is similar to the CR2 complement receptor (CD21). Other viruses, including poxviruses and HVS, possess similar proteins that inhibit C3 convertase activity and prevent activated complement deposition (103,194). vADH is predicted to be a transmembrane protein belonging to the immunoglobulin superfamily expressed together with vGCR on a bicistronic transcript (162,271,304). It possesses sequence similarity to adhesion molecules, such as N-CAM, and is highly homologous to the rat cell surface antigen OX-2 that inhibits T_H21 immune responses (128). HHV-6 and HHV-7 possess similar proteins that are not recognizably similar to vADH but are homologous to OX-2 and may have been independently acquired (127,224).

Some KSHV genes, such as *ORF K3* and *K5,* have counterparts only to uncharacterized human expression sequenced–tagged (EST) transcripts. An important clue to the functions of the K3 and K5 proteins was discovered by Coscoy and Ganem (65). Because other herpesviruses have specific mechanisms to inhibit class I major histocompatibility complex (MHC) peptide antigen presentation, these investigators surveyed the *K* genes in retrovirus vectors to find KSHV proteins that down-regulate class I MHC cell surface expression. They found that both K3 and K5 proteins sequester class I MHC to the endoplasmic reticulum from the plasma membrane through a unique mechanism. This has been confirmed by other groups (146,293) and may contribute to the reduced cytotoxic T-lymphocyte killing of PEL cell lines (36). K5 protein also downregulates the B7.1 and ICAM-1 immune accessory proteins. This may inhibit NK cell responses to cells lacking surface MHCI (145a.)

One gene that is not homologous to known cellular genes but deserves mention is the *ORF73* gene encoding LANA (158,160,255). *ORF73* is 4.5 kb and encodes a protein with a long internal acidic repeat region ending in a leucine zipper motif, flanked by basic regions at both the amino and carboxyl ends. The corresponding EBV EBNA1 repeat region inhibits peptide processing required for cytotoxic lymphocyte responses against EBNA1 antigen (174). In addition to its role in maintaining the viral episome (15), LANA may have a broader role in cell regulation during latency. LANA effectively inhibits p53-dependent transcription and apoptosis; thus, it is likely to be a major contributing factor to KSHV-induced cell transformation (107). LANA-1 also binds and inhibits RB1 (254a). In this regard, LANA has functional similarities to viral oncoproteins from other tumor viruses, such as SV40 T antigen. LANA directly binds histone H1 as well as the ubiquitin conjugating enzyme UBC9 and SUMO-1 (274).

Model for Viral Oncogenesis

Tumorigenesis alone is an unlikely reason for tumor viruses to encode oncogenes. Although tumors increase total infected cell burden, thereby increasing virus numbers, the virus is generally in a nontransmissible latent or defective state. This is particularly disadvantageous to the virus because tumors shorten the host's lifespan without increasing the opportunity of transmission to a new host. Tumor formation frequently occurs when a tumor virus infects a dead-end, nonnative or immunocompromised host and, therefore, is not likely to be an essential stage in the viral life cycle (210,221). This leads to the conclusion that virus-induced cancers, like nonviral cancers, are biologic accidents. What, then, are the benefits that some viruses derive from having conserved oncogenes?

The answer to this question may come from examining cellular targets of viral oncogenes. Most tumor viruses cause cell transformation by directly or indirectly inhibiting at least two major tumor suppressor proteins, RB1 and p53, which balance each other to prevent tumor cell formation (Fig. 6) These two pathways are functionally linked through feedback loops involving p14ARF (p19ARF in the mouse) and CDKIs. Inhibition of RB1 induces p14ARF, which in turn inhibits MDM2-mediated degradation of p53, resulting in increased p53 activity in cells in which RB1 has been inappropriately deleted or shut off (18,207,235,254,283). Activation of p53 leads to apoptosis through activation of pro-apoptotic signaling molecules, such as Bax. If this feedback loop is interrupted by a viral inhibitor, p53 drives the infected cell into apoptosis (27). However, in cells with intact RB1, p53 can also induce cell cycle arrest at the G_1- to S-phase and G_2- to M-phase checkpoints by inducing p21 and other CDKIs (38,56). Viral inhibition of RB1 therefore requires simultaneous inhibition of p53 for the virus-infected cell to survive.

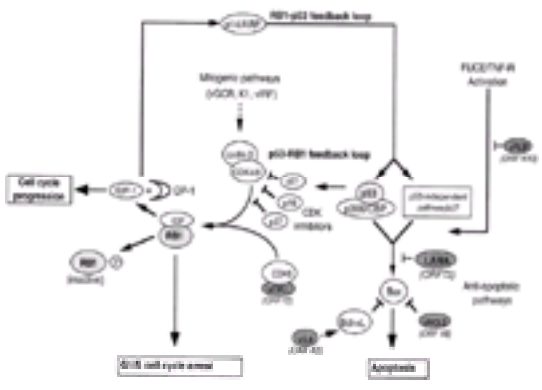

FIG. 6. Interrelationship between the RB1 and p53 tumor suppressor pathways commonly inhibited by tumor viruses.

Inhibition of the G1/S checkpoint protein RB1 leads to activation of p53, which controls apoptotic signaling, through a feedback loop mediated by p14ARF. p53 activation can in turn either arrest the cell by activating the cyclin dependent kinase inhibitor p21 or initiate apoptosis (cellular suicide). Sites of potential interaction with KSHV proteins demonstrated by experimental studies are indicated (potential but not proven interactions shown with dashed lines).

At least in broad outlines, KSHV possesses the machinery expected for a tumor virus (see Fig. 6 and Table 3). Despite the number and variety of viral genes capable of contributing to KSHV tumorigenesis, they are functionally similar to oncogenes found in well-established model tumor viruses. vCYC and LANA-1, for example, inactivate RB1 and possibly other cell cycle checkpoints. From a functional standpoint, this is similar to E1A and E7 oncoproteins. Other known *in vitro* transforming KSHV proteins (vGCR, K1, vIRF-1) may also provide mitogenic signals that complement or substitute for vCYC activity, depending on the tissue infected. vCYC activity could be expected to cause apoptosis, and this has been found to be the case when vCYC is expressed together with its kinase partner, CDK6 (228). Apoptosis through this vCYC mechanism is inhibited by vBCL-2 but not cellular CL-2. Because apoptosis also occurs in SAOS-2 cells, an osteosarcoma cell line homozygously deleted for both RB1 and p53, p53-independent apoptotic signaling pathways may be activated by vCYC as well.

The p53-inhibitory properties of LANA-1 make this protein the leading candidate to inhibit RB1-induced apoptosis (107), analogous to E1B and E6. Other KSHV proteins, including vBCL-2, vIRF-1, and vIL-6, also prevent apoptosis under specific circumstances, but LANA is ubiquitously expressed and regulated together with vCYC in all KSHV infected cells. KSHV gene analysis has proved valuable in uncovering new pathways that may be critical in cell transformation. Discovery of vFLIP led directly to identification of cellular FLIPs regulating Fas pathway apoptosis that may play a role in regulating tumor cell formation (143). Further, vIL-6 and vGCR experiments revealed that autocrine and paracrine cytokine processes play important roles in mitogenesis and inhibition of apoptosis.

What are the likely reasons that tumor viruses target these tumor suppressor checkpoints? There are at least two hypotheses, both of which have experimental support. One widely accepted explanation is that viral oncoproteins promote a proliferating state in the cell, allowing viral nucleic acids to be synthesized for virion replication (148). Under conditions in which nucleic acid synthesis is a limiting factor, RB1 inhibition drives infected cells into S phase, thereby priming cellular resources for virus replication. Viral proteins that inhibit apoptosis delay cell death so that optimal maturation and release of infectious virions can be achieved. Expression of viral oncoproteins would then result in two important preconditions of cancer cell transformation: dysregulated cell proliferation and blockade of cell death through apoptosis.

The alternative hypothesis, which does not exclude the possibility that viral oncoproteins also optimize virion production, is based on the idea that cells use tumor suppressor pathways to prevent and eliminate viral infections (210). Activation of innate immunity induces an antiviral state in the infected cell in part by activating key tumor suppressor checkpoints (Fig. 7). The p21 promoter, for example, is induced by interferon signaling as well as by p53 activation. By arresting cells from entering S phase through RB1 activation and *cMYC* repression, interferon signaling could thus effectively limit viral nucleic acid replication. Interferon signaling also sensitizes cells to p53-mediated apoptosis (305), which might kill virus infected cells. Other innate immune signaling pathways, such as the Fas-Fas ligand signaling pathway used by natural killer cells to kill virus-infected cells, initiates apoptosis using pathways shared with tumor suppressor control proteins. Thus, inhibition of innate immune pathways by a virus might inadvertantly initiate cell transformation (210). This is particularly appealing for KSHV because many of the proteins that have proliferative or antiapoptotic activities directly affect these immune pathways. Support for this idea also comes from the fact that proteins such as vCYC and LANA are expressed during virus latency and hence do not appear to be directly required for lytic virion production.

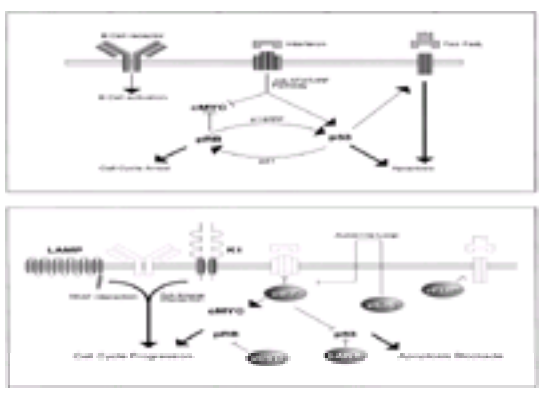

FIG. 7. Interactions of KSHV proteins, that may contribute to cell transformation and carcinogenesis, with innate immune signaling pathways. Innate immune signaling pathways act on tumor suppressor pathways to induce cell cycle arrest and apoptosis as a nonspecific response to viral infection. KSHV proteins involved in cell proliferation may therefore primarily serve to blunt these responses by blocking upstream innate immune signaling pathways, such as Fas and interferon pathways, or downstream effectors including RB1, p53, cMYC and Bax. B cell receptor (BCR) signaling can lead to either pro- or antiapoptotic responses. K1 mimics constitutive immunoreceptor signaling through Syk kinase interactions, whereas LAMP actually blocks some BCR signaling pathways through TRAF interactions possibly inhibiting pro-apoptotic BCR signaling.

Caution is required in applying this paradigm to human disease, particularly in the case of KSHV because of the atypical nature of the KSHV-related tumors. Detailed studies have not yet been performed to determine the effects of

combinations of KSHV genes expressed at levels that can be expected during natural infection. Many of KSHV genes involved in proliferative and antiapoptotic pathways are not expressed in KSHV-related tumors and do not directly contribute to disease pathogenesis. We now know that we have all the working parts for an automobile, but how they go together determines whether it will be a sports car or a city bus. Although PEL is an unambiguous lymphoma in which KSHV is likely to be contributing to the transformed tumor cell phenotype, the mechanisms for cell proliferation in KS lesion is more murky. Further, MCD is a hyperplasia rather than a neoplasia, and yet many of the suspected KSHV oncogenes are only naturally expressed in this disorder. Despite these reservations, the rapid advances that have been made already in KSHV research indicate that this virus promises to make a unique contribution to our understanding of tumor viruses and viral oncogenes.

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*A musical analogy to this is found in the composer Tom Johnson's piece, “Rational Melodies,” in which a 6-note melody is repeated in a 37-note rhythmic pattern. Each iteration of the pattern displaces the melody by one beat but the phasing of the melody returns after six iterations and the same piece is eventually played regardless of which note the piece is started on. According to the music commentator John Schaefer, similar rhythmic devices are common in medieval musical pieces.

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CHAPTER 83 Cercopithecine Herpesvirus (B Virus)

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B virus, endemic in macaque monkeys, has the unique distinction of being the only one of nearly 35 identified nonhuman primate herpesviruses that is highly pathogenic for humans. B virus has been positively linked with more than two dozen human deaths since its discovery. Five of those deaths occurred in the last 12 years, resulting from exposure to macaques with active viral infection. The unique biology of B virus includes its neurotrophism and neurovirulence. Untreated human B virus infections are associated with an extremely high mortality rate and, consequently, present unique and potentially lethal challenges for individuals handling macaques or macaque cells and tissues. Infection in humans is associated with breach of primary skin or mucosal defenses and subsequent contamination of the site with virus. Fomites and contaminated particulates or surfaces can serve as a source of virus infection as well. In one case, human-to-human transmission was noted and attributed to a shared tube of medication that resulted in virus infection at the broken skin site when a contaminated antiviral cream was applied to treat the patient's bite wound. Later, the same patient autoinoculated an eye. Of 25 human cases occurring during the 1980s and 1990s, out of a total 46 documented cases since 1933, 80% of individuals have survived infection because of the advent of antiviral therapies. In contrast, there is an 80% rate mortality in untreated patients. Timely antiviral intervention is an effective means of reducing B virus–associated morbidity and preventing a fatal outcome.

HISTORY

In 1932, a young physician (WB) was bitten by a monkey and later developed localized erythema at the site of the animal bite. This apparent localized infection was followed by lymphangitis, lymphadenitis, and, ultimately, a transverse myelitis. The demise of WB was ascribed to respiratory failure. At the time of WB's death, tissue specimens were obtained for laboratory investigation. Gay and Holden reported that an ultrafilterable agent recovered from neurologic tissues of WB was similar to herpes simplex virus (HSV) (48). The isolate was initially designated as “W” virus. Gay and Holden noted that it caused similar disease in rabbits infected by either intradermal or intracranial routes of infection. A rhesus macaque exposed to this virus showed no evidence of illness. These studies were expanded with the observation that B virus, deadly in this patient, caused disease strikingly different from HSV.

Within a year of this first report, Albert Sabin independently found an ultrafilterable agent that he identified as “ B” virus from tissues of this same index patient (101), naming the virus by the initial of the patient's last name. Thus, the virus subsequently has been called *herpes B virus, herpesvirus simiae, Cercopithecine herpesvirus 1,* or more simply, *B virus.* The lethality of B virus infection in rabbits was described by Sabin, who showed that infectivity was independent of route of inoculation. Experimentally infected dogs, mice, and guinea pigs showed no susceptibility, regardless of the route of inoculation (101). Both groups observed that B virus induced immunologic responses in an infected host similar to HSV-1 (97). The virus was also noted to share similarity with pseudorabies as well as with other viruses, including SA8 and two additional herpes nonhuman primate alphaherpesviruses recently described, HPV-2 and Langur herpesvirus (20,21,39,97,98 and 99).

By 1959, B virus had been identified as having caused 12 fatalities and 5 recognized survivors (18,68,91), suggesting for the first time that this infection may not always end in fatality for humans. Additionally, investigators reported antibody titers in a number of individuals with no clinical symptoms but a history of working with macaques (113). However, because serologic assays could not unequivocally distinguish HSV antibodies from those to B virus, the existence of seropositive humans without disease was difficult to substantiate. Once differentiation of humoral immune responses induced by these closely related viruses was possible, Freifeld and colleagues reported that, in a high-risk group of 325 subjects, antibodies were rarely, if ever, present in the absence of clinical symptoms (46). Since this report, a different cohort of individuals with no HSV antibodies has been found to have high B virus titers by enzyme-linked immunosorbent assay (ELISA) and Western blot (J.K. Hilliard, unpublished results). Thus, it remains controversial as to whether B virus infection in humans can occur in the absence of clinical symptoms.

During the decades since the initial reports of B virus infection, investigators have learned a great deal about the molecular biology of this virus and the illness it causes in humans and nonhuman primates, including its natural host, the

macaque monkey. Because of the neurovirulence this virus demonstrates in a foreign host, it is considered by some to be one of the most dangerous occupational hazards for those who work directly with macaques or their tissues or with monkeys who have been in contact with macaques.

DISTRIBUTION IN NATURE

B virus is endemic in macaques, one of the Old World monkeys widely distributed throughout Asia. All species of macaques tested thus far are able to serve as natural hosts. The virus can infect other nonhuman primate species; but in such cases, the infected animal is considered a foreign host, and the infection generally results in death within a relatively short time after onset. Transmission of virus can occur by direct contact between members of the *Macaca* genus, from infected animal to human, from virus-contaminated surfaces, or, in one case, from human to human, as extensively reviewed (27,35,41,42,43 and 44,64,69,70,73,74,90,117,122).

Prevalence estimations of B virus infection, using different serologic methodologies and sampling approaches in both wild and captive macaque colonies, have been noted to be variable (118). Spread of infection correlates with onset of sexual activity, which facilitates transmission of the virus among animals (106,120). Crowding of animals during transportation seems to accelerate the spread of infection as well (106).

B virus infection in the macaque species is mild and seldom associated with death of the animal, unless conditions prevail that facilitate generalized systemic infections (29). The frequency of active infections has been reported to be slightly more than 2%. These observations suggest that virus is only transiently present. This thought has prevailed because monkeys frequently had lesions only during a primary infection. Later, antibody-positive animals were found to shed virus periodically from mucosal membranes, including the eyes (65,106,118,120). Thus, isolation of virus from asymptomatic animals has been established (15,45,77,82,114,115 and 116).

THE VIRUS

Isolation and Growth Properties of B Virus

Because B virus causes fatal human disease in 80% of untreated cases, the Centers for Disease Control and Prevention (CDC) has published agent summary statements for laboratory studies using B virus. Growth of the virus is recommended in a BL-3 laboratory, whereas propagation should be strictly confined to a BL-4 facility (112). Initial isolation of B virus was achieved using rabbits for passage of the virus (47,48,101). Within a decade of discovery, the virus was grown on chorioallantoic membranes of embryonated eggs (20).

In 1954, B virus was reported to be present in rhesus kidney tissue that was used for preparation of poliomyelitis vaccines (124) and from rhesus central nervous system tissue (84). Subsequently, cultured cells, derived from monkey kidney and chick embryo, were found to support the replication of B virus (78,94). Infection of cells resulted in the development of large syncytial similar to those seen in Figure 1. The virus is relatively stable in cell culture media stored at 4°C and can be maintained for long-term storage at –80°C but not at –20°C. The virus has growth properties similar to HSV *in vivo* (20,97,98,99 and 100,102). Vero cells appear to be the best system for replication of virus.

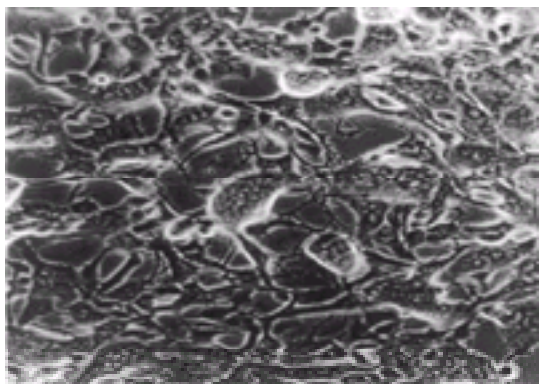

FIG. 1. Vero cell monolayer infected with B virus.

Replication kinetics of B virus follows a time course similar to HSV-1 and HSV-2 in cell cultures. Virus adsorbs to the surface of a susceptible cell within 30 to 60 minutes after infection. Host cell machinery is halted once the virus enters the cell. An eclipse of cell and viral activities is noted during the first 2 to 3 hours after infection, followed by synthesis of polypeptides early during infection. By 4 hours after infection, DNA synthesis increases dramatically and in parallel with the synthesis of viral polypeptides. Morphogenesis of the virus is also similar to HSV, as shown by electron microscopic studies (96). Infectious virus is detectable within 6 to 10 hours after infection, and both extracellular and intracellular virus levels plateau about 24 to 28 hours after infection, declining thereafter (56,94). B virus, like HSV, has been observed to express sequential classes of proteins, that is, immediate-early, early, and late proteins (56), and to possess glycoproteins and other structural proteins that were studied in detail with respect to their antigenic relatedness to HSV-1 and HSV-2 and later to other nonhuman primate alphaherpesviruses (36,55,81,88). B virus replicates to high titer in cell lines of Old World monkey origins, particularly in Vero, African green monkey, and vervet kidney cells, as well as rabbit kidney, BSC-1, and LLC-RK cells (62,65). Although B virus replication in cultured cells usually results in syncytial formation, as illustrated in Figure 1, some primary isolates produce cell rounding. Generally, cells balloon, fusing into polykaryocytes that expand outwardly as more cells become infected. In this manner, virus infection spreads through the entire cell monolayer, destroying cells. Eosinophilic intranuclear inclusions (Cowdry type A) can be visualized after fixation and staining infected cell monolayers; however, these inclusions are observed consistently neither in infected

animals nor in some humans with zoonotic infection (34,61). Thus, the presence of characteristic intranuclear inclusion bodies is not a reliable diagnostic marker of infection.

The B Virus Genome

B virus contains a double-stranded DNA genome of about 162 kb. One strain of virus originating from a cynomolgus monkey has been mapped and subcloned by Harrington and associates (52). The genome contains two unique regions (UL and US) flanked by a pair of inverted repeats, two of which are at the termini and two internally located, an arrangement that results in four sequence-orientated isomeric forms, as occurs with HSV. Figure 2 shows the comparative genome organization of B virus and HSV-1, as established by using HSV-1 genes to identify and locate specific B virus homologs (52). The overall size of the genome is slightly larger than HSV-1 (152 kb) and HSV-2 (155 kb). The guanosine and cytosine (G+C) content of the DNA has been calculated to be 75% based on the buoyant density of viral DNA. Eberle and Hilliard have published data that define the presence in B virus homology to HSV-1 and HSV-2, specifically for the genes of the glycoproteins gB, gC, gD, gE, and gG (38). Further examination of the sequences of the nine major glycoproteins showed that the 75% of the G+C content is conserved within most glycoproteins. Harrington and associates (52) have also shown that the location of genes within the UL regions of HSV and B virus was colinear. One gene rearrangement was described in an isolate that originated in a cynomolgus macaque. Homologs of HSV *US9* and *US10* genes are located upstream of the US glycoprotein gene cluster. This is in contrast to the downstream location of these genes in the HSV US region. This rearrangement was based on hybridization data and the proposed physical map of Harrington and associates (52). Sequence analysis (unpublished) of the prototype strain (E2490), which originated from a rhesus macaque, however, showed that B virus DNA is colinear in these same regions with the HSV-1 genomic arrangement (L.M. Perelygina and colleagues, 2000, unpublished data).

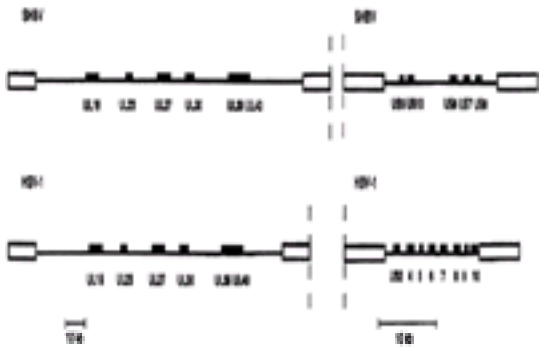

FIG. 2. Physical map of B virus as proposed by Harrington and colleagues (52).

To date, sequences for only a few B virus genes have been submitted to GeneBank, that is, homologs of gB, gD, gC, gG, gJ, and gI, largely covering the sequence of the US region (9,76,108). Because a number of laboratories are engaged in the sequencing of this virus, the entire genome should be sequenced within a short time. Each of the glycoproteins for which sequence information is available, except gG, has about 50% identity with HSV, slightly higher for HSV-2 than HSV-1. B virus gG is a homolog of HSV-2 gG and is closer in size to gG-2 (699 kb) than gG-1 (238 kb) (108). Glycoprotein sequences demonstrated that all cysteines are conserved, as are most glycosylation sites. This conservation suggests that B virus glycoproteins have similar secondary structure to that of HSV. Sequence analyses also suggest that B virus and HSV types 1 and 2 probably diverged from a common ancestor during the evolution of these pathogens.

Using restriction-length polymorphisms (RLPs) as a guide, several investigators have shown intrastrain variation among both human and nonhuman primate–derived isolates, the significance of which remains to be defined (57,59,109). Eberle and colleagues (40) postulated the possible existence of B virus isolates, which vary with respect to pathogenicity for nonmacaque species, based on the existence of three distinct B virus genotypes. These genotypes were found when collected data were analyzed phylogenetically; however, this postulate must be studied in a suitable animal model. In zoonotic infections, unfortunately, usually little is actually known about the species of the source animals. Where data are available, many rhesus macaques have been identified (90), targeting this species as the source of a unique, highly pathogenic strain that can cause zoonoses. Published case summaries that implicate other macaque species, and in one case a baboon, are difficult, if not impossible, to confirm (90).

Synthesis of Viral Proteins

About 23 major polypeptides have been identified by electrophoresis in denaturing polyacrylamide gels (Fig. 3), but more than 50 different polypeptides have been identified by immunoblot analysis. Each has been assigned an infected cell polypeptide number as an initial reference point (56). This number may be an underestimate of the total synthesized, but it serves as a basis for comparison in ongoing studies. The size of these infected-cell polypeptides ranges from about 10,000 to 250,000 daltons. More than 75% of the expected coding capacity of the viral DNA is accounted for by these infected cell polypeptides. At least nine bands from electrophoresed infected cell polypeptides containing viral glycoproteins have been thus far identified (Fig. 4). Many of these glycoproteins have been cloned and sequenced recently by two laboratory groups (9,10,40). The proteins encoded were mapped to genes by the US region, which was largely colinear to the HSV glycoproteins gD, gI, gJ, and gG, as previously described. Sequence analysis of selected genes showed that B virus is most closely related to herpesvirus papio 2 (HVP-2) (37). Although there are protein homologs in herpesviruses of New World monkeys, very little, if any, cross-reactivity exists between B virus and the New World monkey herpesviruses.

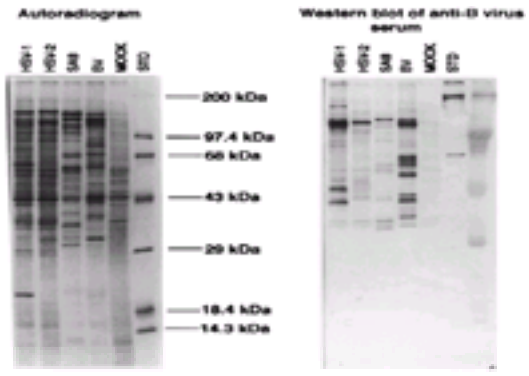

FIG. 3. Infected cell polypeptides of B virus–infected Vero cells electrophoresistically resolved on SDS-polyacrylamide gel electrophoresis and transferred nitrocellulose. **Left:** Result of autoradiography following electrophoresis of metabolically ^{35}S-methionine–labeled B virus–infected Vero cells 4 to 24 hours after infection. **Right:** Result of immunoblot of the exposed membrane using a rhesus sera pool of anti–V virus antibodies.

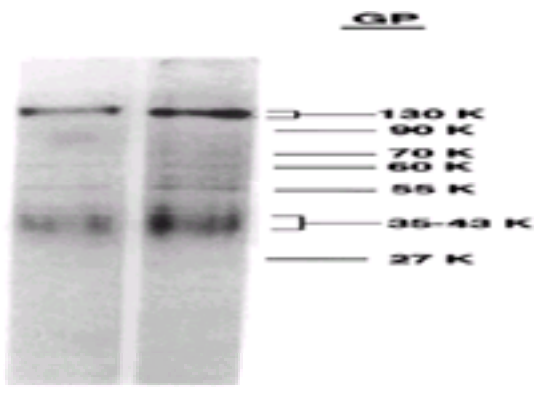

FIG. 4. ^{3}H-glucosamine, metabolically labeled B virus–infected cell glycoproteins. 20 µCi/mL of radiolabeled sugar was added to cells 4 hours after infection. Twenty-four hours after infection, monolayers were harvested and analyzed on SDS-polyacrylamide gel electrophoresis. Gels were subsequently exposed to Kodak RP X-Omat film for 24 to 72 hours for autoradiography.

The kinetics of protein and glycoprotein synthesis in infected cell culture are similar to that observed for HSV, although infectious virus was detectable slightly earlier—appearing 6 hours after infection. Both host cell DNA and protein synthetic activities appeared curtailed during the first 4 hours after infection, as shown in Figure 5. The polypeptides of B virus–infected cells, summarized in Table 1, may represent an overestimate of the primary gene products of the virus; however, this summary should serve as a fairly complete listing of B virus–induced polypeptides for future reference. As for glycoproteins, only glucosamine and some mannose are incorporated during the infection *in vitro.* B virus polypeptides and glycoproteins can be grouped into classes that differ in their relative rates of synthesis at different times throughout the virus replication cycle, as is characteristic of alphaherpesviruses (56).

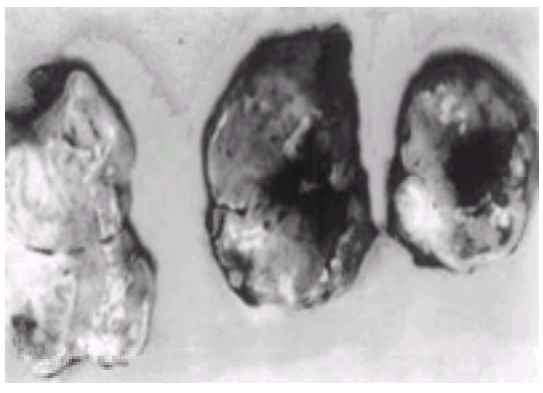

FIG. 5. Postmortem human tissues of upper cervical spinal cord showing adjacent sections of the cord with focal hemorrhage well defined and limited.

Probe DNA			SHBV fragments to which probe hybridized		
HSV-1 KpnI clone	Fragment	Gene function	Gene map site	BamHI	SstI
h	SstI	Glycoprotein D	(HSV .US6)	g	i
h	SstI	Glycoprotein E	(HSV .US8	g	p s'
h	SstI	Glycoprotein I	(HSV .US7)	g	q'
h	BamHI	Tegument phosphoprotein	(HSV .US9	r	c'
h	BamHI	Virion protein	(HSV .US10)	r	c'
n	XhoI/KpnI	Glycoprotein B	(HSV .UL27)	d	p2
d'	MluI	DNA polymerase	(HSV .UL30)	z	e'1
i	BstEI	Major capsid protein	(HSV .UL19)	j	s, y
s	KpnI/EgeII	Ribonucleotide reductase 1	(HSV .UL39)	m	f
s	KpnI/KpnI	Ribonucleotide reductase 2	(HSV .UL40)	p	f

HSV, herpes simplex virus; SHBV, simian herpes B virus.

TABLE 1. *Hybridization of plasmids containing HSV-1 DNA fragments to SHBV DNA blot membranes*

PATHOLOGY AND PATHOGENESIS

During the course of B virus infection, several observations are common to the natural host, the experimentally infected host, and infected humans. Those common factors are discussed initially, then specific details for each of these groups are provided. First, there are various pathogenetic outcomes in infected hosts. Evidence deduced from the literature suggests that route of infection has an important role in disease pathogenesis. Specific details must be studied further, but, in general, there are several common observations. The route of inoculation predicts differences in the time course of infection, development and spread through the central nervous system and visceral organs (e.g., spleen, adrenal, kidney, and, in some cases, even heart). There are routes of infection that are obviously unique to each of the categories of hosts: natural, experimentally infected, and human zoonotic infections. For example, venereal transmission of the virus is common in macaque hosts, whereas an intranasal route of virus delivery defines an experimental rabbit model or, in the case of human zoonotic exposure, accidental aerosolization of virus. However, these routes share one common feature: all require exposure of mucosal membranes to infectious virus.

The cells that come into contact with the virus initially are also important to the permissiveness of the infection. For example, nasal mucosa has been found to be a less ideal site for virus replication than lung (6,7 and 8,28,69). However, nasal mucosa is not entirely resistant because increasing titers of virus can be isolated from these sites. Another important consideration in B virus infection is the dose or quantity of the virus introduced initially into a host. The role of dose remains a challenging question for exploration. Both dose and route of inoculation are important factors with respect to the onset of pathogenesis. For example, a far greater quantity of virus is required to infect rabbits by aerosolization than by an intradermal route of inoculation, although practically, it remains unconfirmed whether these routes and doses are paralleled in human or nonhuman primates. Also, the dose may be an important factor in determining associated morbidity and mortality. The commonality of successful infection is the requirement of mucous membrane exposure early in infection. Another common feature of natural, experimental, or zoonotic infection is that B virus can be found in the central nervous system shortly after the onset of acute infection. But the distribution of virus and its consequences differs widely in natural infection as compared with infection failure of susceptible foreign hosts.

The Natural Host

The macaque monkey suffers little or no morbidity as a consequence of infection. Exceptions occur rarely and appear to involve specific accompanying factors, such as immunosuppression (29,107,125). Infection is self-limiting (1,11,73,74,120,121). Virus can replicate at the site of inoculation and induce a localized erythema. There is also evidence of a limited focal infection of liver and kidney in some macaques (73,74). Virus travels through the peripheral nerves from the site of inoculation to the representative dorsal route ganglia. Latent infection is established in the ganglia with intermittent reactivation of the virus throughout the life of the macaque (14,15,43,79,102,115,118). In rare cases, viremia has been observed (22,83,107). Virus has also been recovered from urine as well as multiple organs of animals. Reactivation from latency occurs in the natural host as judged by increasing antibody titers, recovery of virus by co-cultivation of sensory ganglia, and isolation of virus in the absence of detectable lesions (29,51,82).

During active B virus replication, isolation of virus can be readily accomplished from buccal, conjunctival, or genital mucosa sites (1,33). The frequency of active infection among seropositive macaques has been observed to be low, with relatively short time periods for successful virus recovery from mucosal sites (118,120).

Mucosal ulcers extend down to the papillary layer of the dermis. Two distinct zones have been described, namely a central area of necrosis and a surrounding zone of ballooning degeneration. Around the lesion, “normal” epithelium exists. An eosinophilic polymorphonuclear infiltration characterizes the histopathology of the lesion. Postmortem histopathologic examination of monkey tissues from animals euthanized at the time of active virus shedding reveals perivascular lymphocytic cuffing in sections of spinal cord. Examinations of latently infected but healthy animals failed to detect virus excretion from peripheral sites routinely, but virus can still be recovered from sensory ganglia (14).

Experimental Infections

Rabbits, mice, rats, guinea pigs, and chickens have been studied as B virus models, as previously mentioned (47,97,102). Disease is not a uniform consequence following B virus inoculation into mice and guinea pigs; however, several strains of B virus that have increased virulence for the mouse have been identified. One strain, identified as E2490, was avirulent for white rats and chickens; nonetheless, antibody developed after infection (64). Cotton rats infected by either intraperitoneal, subcutaneous, or intracerebral routes succumbed to infection with selected strains. Rats had typical hind leg paralysis secondary to transverse myelitis similar to symptoms in the rabbit. Reagan and colleagues selected by serial passage human isolates of B virus strains capable of infecting mice, hamsters, and white

chicks (92,93). With respect to experimental infections, review of the literature suggests that the rabbit is perhaps the most useful small animal model because virus replicates to high titers, making it a particularly good model for testing antiviral agents.

Using the rabbit or the mouse model, investigators have demonstrated that viral dose was important, depending on the route of inoculation, as would be predicted. Experimentally infected animals inoculated with low doses of virus intradermally developed only erythema that disappeared within a few days and was not associated with further apparent symptoms. In contrast, animals receiving a larger dose developed a necrotic lesion that was generally followed by central nervous system invasion (49,50). B virus subsequently appeared in the regional lymph nodes late after infection. These nodes drained the area of initial infection, and with time, necrosis of the infected nodes occurred, as seen upon postmortem examination. In the central nervous system, focal lesions are evident in pons, medulla, and spinal cord virus. Spread is most often facilitated by travel through the peripheral nerves, but, in rare cases, hematogenous route of spread in experimentally or inadvertently infected hosts can occur (29,49,50,98,111).

Cervical spinal cord and medulla oblongata are the primary sites of postmortem virus recovery. With time after infection, virus is also found in olfactory regions of the brain that may have been due to movement of the virus centripetally through the nerves innervating nasal mucosa. Perivascular cuffing and glial infiltration are characteristic histopathologic findings upon examination of brain tissue. Hepatic congestion is accompanied by infiltration of polymorphonuclear and mononuclear cells seen in the periportal areas of the liver. Scattered foci of necrosis can be found throughout the lobes of the liver. The presence of inclusions is visualized mainly in the regions of inflammation, around pyknotic or karyorrhetic hepatocytes. When lesions are present on skin, the depth of the involved tissue is significantly greater than that of mucous membranes, explaining perhaps the reason B virus can be recovered weeks or months later from these sites (34,60,61).

Development of animal models has been limited by the lack of antiviral drugs or a protective vaccine designed specifically to treat or prevent this infection. Because there is grave concern for individuals with this neurovirulent virus, should an accident occur, even when BL-4 laboratories are available, appropriate management protocols must be available.

Human Infection

Human B virus infection generally occurs through an occupational exposure to a macaque monkey shedding virus at a site that comes into contact with broken skin or mucosal membranes of the susceptible human. Several cases in which monkey contact had not occurred in years suggest that virus can be reactivated.

Review of all confirmed cases of B virus in humans can be summarized as follows. The most striking characteristic of human B virus infection is the involvement of the central nervous system, specifically the upper spinal cord and brainstem. These areas are the principal sites for virus replication, as observed from clinical, laboratory, and postmortem data. Initially, the infected individual experiences an influenza-like syndrome followed by numbness or parenthesis around the site of inoculation. An ascending transverse myelitis occurs during the final stages of the infection in humans, resulting ultimately in respiratory failure. Virus can be recovered at skin sites of inoculation for extended periods of time, and viral DNA can be detected generally in cerebrospinal fluid (CSF) by the time neurologic symptoms are experienced. CSF antibodies can also be detected. Generally, death is associated with complications of sustaining respiration with a ventilator. Cutaneous vesicular lesions are a source of virus isolation, even late in infection (85). Edema and degeneration of motor neurons are prominent. Even with advanced disease, Cowdry type A eosinophilic intranuclear inclusions can be found in only a few cases, and certainly not uniformly. Gliosis and astrocytosis are late histopathologic findings; thus, there can be evidence of myelitis, encephalomyelitis, or encephalitis, or combinations of each of these conditions (90).

Pathogenesis of B virus infection has been studied for each of the reported fatal cases and a few of the surviving cases (34,47,48,61,68,85,101). With fatal disease, generally, central nervous system lesions are localized within the upper cervical spinal cord, sometimes extending into the medulla and pons. In some cases, hemorrhagic infarcts are visualized in these areas, whereas in other cases, damage appears minimal despite the fact that the patient succumbs after prolonged ventilatory support. Discontinuing ventilatory support is nearly always followed by respiratory failure. In some cases, patients are alert but paralyzed, and in other cases, patients remain comatose. Surviving cases vary with respect to the level of morbidity, ranging from little or no neurologic impairment to more extensive central nervous system involvement (2,18,19,46). Some survivors experience slow neurologic decline, whereas others report few if any long-term effects. Several infected individuals have subsequently given birth to healthy children with no ill effects in either mother or infant. Monitoring of the vaginal canal for virus shedding in these individuals before delivery has produced negative results. In several reports, the ocular effects of B virus infection have been reported (86,95). Histopathologic examination of the eye revealed a multifocal necrotizing retinitis associated with a vitritis, optic neuritis, and prominent panuveitis. Herpes-like viral particles were identified in the involved retina by electron microscopy in one case. Postmortem vitreous cultures taken from both eyes and retinal cultures taken from the right eye in the later case were positive for B virus. From these observations, B virus can produce infection and destruction of retinal tissue similar to that of other herpesviruses. Ophthalmic zoster–like symptoms have been reported as well (45), and in one particular case, reactivation of latent infection was postulated.

To summarize human pathogenesis, the tissues and organs that become infected by B virus vary, perhaps according to the route of inoculation. If skin is the primary site of infection, the virus usually, but not always, replicates in the skin, resulting in localized erythema. Knowledge of the site of initial replication is useful for the development of guidelines for disease prevention and also for retrieval of a virus isolate that then allows unequivocal diagnosis. Subsequently, lymphangitis and lymph node involvement are observed. Although viremia has been documented in rabbits and monkeys, it has not been proved to occur in humans, although with the application of more sensitive assays (e.g., polymerase chain reaction [PCR]), more details of infection can be uncovered. Certainly, with lymphatic involvement, virus can spread, particularly to abdominal viscera, where it has been isolated previously. Nevertheless, spread through neuronal routes is the fundamental route of transmission of the virus, as it is with HSV, given the involvement of the spinal cord

and central nervous system. Visceral organs, including heart, liver, spleen, lungs, kidneys, and adrenals, demonstrate congestion and focal necrosis with variations in the extent of involvement from patient to patient. Recent human cases failed to demonstrate necrosis, but virus was isolated from adrenal, kidney, lung, and liver tissue collected at autopsy (34,61). In cases in which B virus infection is suspected, medical personnel should follow published guidelines at the time of injury or observation of symptoms of possible infection (23,24,25 and 26).

LATENCY

A characteristic of all herpesviruses is the ability to establish latency and to reactivate when provoked by the proper stimulus. B virus is no exception. Reactivated infection has been described in both wild macaques and established captive colonies (82,117,118,119 and 120). Unequivocal evidence of latent B virus infection in macaques came with studies on frequency of recovery of virus in monkey kidney cell culture systems. Wood and Shimada obtained six isolates from 650 pools of monkey kidneys, suggesting that at least 1% of macaque kidneys contain latent virus that can be reactivated in cell culture (124). Virus was also isolated from rhesus tissues by Boulter and Grant (14,15) as well as by co-cultivation from a variety of neuronal tissues, including gasserian ganglia, trigeminal ganglia, dorsal route ganglia, and spinal cord (116). Latent virus has also been isolated by co-cultivation of tissues from experimentally infected rabbits (116), further supporting the rabbit as a potentially acceptable animal model for B virus infections. Latency likely occurs after human infection.

As with human HSV infections, a prominent factor associated with reactivation of B virus in macaque monkeys appears to be stress, particularly that associated with the capture and shipment of animals from the wild to captivity. Shedding of virus after reactivation also occurs with illness and during the breeding season of the natural host. No information is yet available on the state of the viral DNA during latency or on the molecular or biochemical events associated with the establishment and reactivation of latent virus.

EPIDEMIOLOGY

Natural Hosts

Macaques

Most adult wild macaques are seropositive for B virus (106). However, colonies of animals exist in the wild that have been found to be predominantly seronegative. Each of these colonies was established apart from original natural habitats to meet escalating needs for seronegative animals by the scientific community. Thus, the epidemiologic pattern of virus infection was modified by human intervention. The seroprevalence in wild macaques, in addition to the noted high infectivity (118) and low morbidity within captive colonies (20), initially confirmed the macaque as the natural host. Recent studies among macaques belonging to captive breeding colonies demonstrated a high seroprevalence, a seroprevalence that increases after the onset of puberty (118,119). Increasing seroprevalence appears to be associated with sexual transmission within the colony (111,119). Infants and juveniles have a very low incidence of infection, judged by low prevalence of specific B virus antibodies (118). Despite antibody levels reflecting presence of maternal transplacental antibodies in young animals, it is of importance to note that all age groups have been found to be virus positive, indicating that routes of transmission other than sexual activity exist (1).

No particular species of macaque appears to be excluded as a natural host for B virus infection, although there are minimal or no data available from certain species. Although presence of antibodies has been confirmed in most of the different species of macaques, there has been speculation that virus isolated from certain species is less neurovirulent or less neurotropic than virus shed by rhesus macaques (12). Differences in the restriction endonuclease profiles of the different isolates from different macaque species have been reported, but the lack of available data on the types of macaques involved with each of the documented human cases does not permit rigorous evaluation of this hypothesis.

Virus shedding during either primary or recurrent infections has been noted to occur. Analysis of available data support that, in general, macaques shed virus for a longer duration during primary infection and, for short periods, even hours with reactivated infections. Levels of virus, as measured from mucosal swabs, range from 10^2 to 10^3 plaque-forming units (PFU).

Humans

B virus is an infection that humans rarely contract, but when they do, 80% of untreated cases result in death, as noted previously. Epidemiologic analysis indicates B virus is usually acquired through zoonotic transmission from either a macaque or infected cells or tissues from the animal. There is, however, one documented case of transmission from human to human, as previously noted, supporting the assumption that B virus can be transmitted similarly to HSV-1 or HSV-2. A recent fatal case resulted from exposure of ocular membranes to virus from a monkey in the process of being transported. This tragedy has refocused attention on an earlier report implicating this type of transmission in the epidemiologic analysis of zoonotic transmission of the virus (78). Interestingly, current case analyses suggest that categorization of risk levels with regard to the severity of injury is not useful. The low incidence of B virus infection in humans makes it difficult to reach statistical conclusions, but analyses of cases occurring during the 1990s supported the observation that immediate medical attention ameliorates, if not prevents, disease. Only minimal disruption of the protective skin layer or instillation directly to a mucosal membrane can result in initiation of infection. The level of virus required to initiate infection in humans remains unknown. Where data are available, rhesus macaques were most frequently implicated as the source of infectious virus in the known human cases, but alone, this is insufficient to conclude that rhesus-derived virus is uniquely important in the establishment of zoonotic infections. Other species of macaques, including baboons, have been linked to fatal zoonotic infections (90).

The incidence of zoonotic infections has been correlated retrospectively with periods of increased usage of macaques for biomedical research. Evaluation of past cases underscores that transmission of the virus is often associated with no

more than a superficial scratch or puncture, suggesting that once virus gains entry into a host, the ability to initiate disease is perhaps dose independent, at least in some cases. Again, however, this point remains unsubstantiated. Dose and route of entry are topics that require more study to clarify importance in zoonotic infection. Likewise, incidence and prevalence of zoonotic infections must be estimated cautiously because few data regarding the virus source are available from clinically recognized cases.

HOST IMMUNE RESPONSE

B virus antibodies have been studied in both the natural and the foreign hosts by a variety of methods, including serum neutralization with or without complement, competitive radioimmunoassay (RIA), multiple types of ELISA including competition ELISA, and Western blot. Limited serial observations have been available from both the natural and foreign host, but comparison of available data has been useful in that a relatively consistent response is induced in both hosts (3,4 and 5,63,66,67). The natural history of antibody development has been measured in wild macaques, captive colony populations, individually imported animals, experimentally infected macaques, zoonotically infected humans, and even vaccine trial recipients. The ELISA methodologies provide a rapid diagnostic tool with increased sensitivities of detection, and with enhanced specificity when competition protocols were used. The antibodies that develop in response to B virus infection in both humans and nonhuman primates are capable of neutralizing HSV-1 and HSV-2 as well as nonhuman primate alphaherpesviruses. Interestingly, the antibodies from HSV infections are not able to neutralize B virus, indicating the presence of virus-specific antigens unique to B virus (16,103). Sequence data have been useful in confirming the existence of B virus–specific epitopes (9,76,108).

The humoral immune response that develops after a B virus infection has a characteristic pattern (34,61,79). The glycoproteins induce antibodies early in the course of infection. Antibodies begin to appear within 7 to 10 days after the infection and consist of immunoglobulin M (IgM). Within 14 to 21 days after the onset of acute infection, IgG antibodies were present. In rare cases, the infected host remains persistently antibody negative despite virus isolation. The pattern of the immune response is somewhat altered in the cases of humans who have had a previous infection with HSV-1 or HSV-2 because viral antigens that are shared among the three viruses induce an anamnestic response toward shared protein or glycoprotein sequences. Neutralizing antibodies develop in both the natural and foreign host, but at significantly lower levels in the foreign host. The nature and specificity of the humoral responses make it possible to design enhanced serologic testing strategies to identify detectable antibodies rapidly and to provide the basis for future diagnostic strategies.

CLINICAL MANIFESTATIONS OF DISEASE

Observations of the clinical pattern of disease are important for rapid diagnosis of B virus infection in both macaques and humans. In the natural host, recognition of early infection allows removal of infected monkeys from captive colonies that are being established as B virus free. In B virus–free colonies, it is important to remove seropositive animals and isolate animals with equivocal results to prevent infection of other colony members, or in seropositive colonies to minimize risk to humans who have to handle them. Macaques are not treated with antivirals because the high prevalence of infection makes it cost prohibitive. In the case of human illness, early recognition of disease facilitates treatment with antivirals, principally such nucleoside analogs as acyclovir, valaciclovir, or famciclovir, a course that appears to lower the morbidity and mortality (34,61). Notably, immunosuppression (e.g., administration of corticosteroids) is often associated with reactivated disease, often mild, in the natural host (125), whereas other agents appear to be capable of facilitating systemic B virus disease culminating in death (27).

Humans exposed to B virus demonstrate clinically variable signs of disease. Most often, illness is apparent within days to weeks, but in some cases, there appears to be a delay in development of acute disease. The reasons for this delay are unknown and, although rare, delays may even range from months to years, making diagnosis difficult. Once symptoms appear, the clinical progression is associated with relatively consistent symptoms, including influenza-like illness, lymphadenitis, fever, headache, vomiting, myalgia, cramping, meningeal irritation, stiff neck, limb paresthesias, and urinary retention with an ascending paralysis culminating in respiratory failure, requiring ventilatory support. Cranial nerve signs, such as nystagmus and diplopia, are common in most published cases. Sinusitis and conjunctivitis have been observed in some (34,61). The array of symptoms may be related to the dose of virus with which the individual was infected or the route of inoculation. A summary of descriptions of human cases can be found in two comprehensive reviews (90,117).

A summary of reported cases indicates that the highest percentage of deaths occurs within a few weeks after onset of disease. In some cases, however, life was prolonged artificially for months or years. Incubation times from identifiable exposures to onset of clinical symptoms ranges from days to years, but most cases occur within days to months. Virus has been recovered from throat, buccal, and conjunctival sites as well as from lesions, vesicles, or injury sites as late as weeks to months after infection. Most clinical cases are associated with bites (50%), fomites, (8%) saliva (<5%), and aerosols (10%).

DIAGNOSIS

Nonhuman Primates

Macaques

B virus infection in macaque monkeys is identified by virus isolation, the presence of specific antibodies, or both (30,59,75). The neutralization antibody test was the predominant diagnostic tool in macaques and humans for many decades. The time required for the results of this test was often a drawback, and a more rapid test was required. Thus, the dot-blot, RIA, ELISA, and Western blots were developed (13,32,53,54,71,72,87). Three of these techniques (dot-blot, ELISA, and Western blot) rely on the use of monoclonal antibody. The results of these tests can be acquired in less than 1 day. These tests are available through commercial laboratories as well as through a national resource laboratory

subsidized through National Institutes of Health (NIH) National Center for Research Resources. All of these assays use B virus–infected cells for antibody detection, making such tests more effective than other types of assays that rely on HSV-1 (89). This is a particularly important point with respect to diagnostic tools used to identify signs of infection for the establishment of B virus–free colonies. Currently, there are no serologic tools that identify uninfected macaques; however, there are promising assays being developed for diagnosis of HSV-2 infections in humans that may be adapted for identification of B virus infection in macaques. When selecting an assay for detection of antibodies, the sensitivity and specificity of the test for a specific species of macaque should be known and considered in the final evaluation of the results. Tests dependent on monoclonal antibodies or recombinant reagents should have defined sensitivity and specificity for each macaque species to be tested. Finally, evaluation of a macaque is only optimal when analysis is performed on multiple samples at different dates, especially in cases in which antibody titer is low (less than 1:50). A constellation of different tests for deployment at varying time points after infection may be necessary in some cases to determine correctly the status of an animal with a very low antibody titer, particularly when such an animal is housed in a B virus–free colony (58).

Virus isolation is the gold standard for diagnosis of infected macaques. Unfortunately, virus isolation is not a particularly sensitive diagnostic tool, with the possibility of many false-negative results. Nonetheless, standard cell culture for virus isolation remains a valuable tool for the colony manager and for the veterinarian. Virus-positive cultures can be easily recognized with the unique cytopathic effect, but unequivocal confirmation requires either electrophoretic analysis of infected cell polypeptides or restriction endonuclease–digested DNA. More recently, several PCR tests have been described that can be used to verify the identity of the virus; however, this diagnostic tool for colony management is costly. Nonetheless, when a possible zoonotic infection must be confirmed, PCR may be beneficial for identification of B virus in macaques.

Nonmacaques

Other species of monkeys infrequently become infected with B virus, as previously discussed. These are usually animals that have been co-housed or housed in close proximity to B virus–infected macaques at some time. Because many, if not all, nonhuman primates harbor indigenous alphaherpesviruses, the important diagnostic point is to differentiate specific antibodies from cross-reactive ones. Euthanasia is generally advised in the case of a B virus infection in a nonmacaque monkey because it is likely that the animal will succumb and, in the meantime, would pose a great risk to anyone attempting to treat the infection. B virus has been identified in the patas monkey and colobus monkey (80,110,123). In each case, there was a major concern for the people responsible for care of the animal, particularly because these animals often have severe morbidity and are shedding virus. Currently, the most effective assay for diagnosis of B virus in a nonmacaque monkey would be a competition ELISA to facilitate discrimination between specific and cross-reactive antibodies similar to the challenge faced when diagnosing infection in humans.

Humans

The evaluation of clinical symptoms associated with an antibody- or virus-positive case is the gold standard for diagnosis of B virus infection in an exposed individual. Both serologic and virologic techniques are available for diagnosis of B virus–infected humans. The CDC has published specific guidelines for recognition and treatment of such infections (61). In the case of a suspected infection, several emergency resources are available. Contact with the CDC or laboratories recommended by the CDC (see guidelines) can expedite laboratory assistance for the clinician suspecting B virus infection. Generally, a rise in B virus–specific antibodies over several days during acute infection substantiates B virus as the etiologic agent. However, in other cases, data are equivocal, and decisions with regard to patient management must be based on a complex decision table collectively using all diagnostic tools, including clinical symptoms. Virus isolation remains the gold standard for diagnosis; however, virus isolation is frequently not possible even under the best of circumstances. Serologic diagnosis of B virus in humans is a complex task when an individual with a suspect infection has detectable antibodies as a result of a previous HSV-1 or HSV-2 infection. As discussed previously, significant cross-reactivity exists among these viruses. In the absence of these cross-reactive antibodies, diagnosis is rapid and straightforward, with confirmation using the neutralization assay, Western blot, or both. This was not the case before the development of rapid diagnostic competitive ELISAs and RIAs (71,72). The diagnostic tests for humans are performed currently by only a few facilities that have been licensed and have access to BL-4 containment laboratories for the preparation of B virus antigen.

Virus identification can be accomplished by isolation using conventional cell culture and, in clinical emergencies, by PCR (104). The identity of isolates should be confirmed by electrophoretic analysis of infected cell polypeptides or restriction endonuclease–digested DNA (56). The application of PCR is most helpful in the symptomatic patient if virus cannot be recovered. PCR is also a useful tool for monitoring the efficacy of antiviral interventions.

CONTROL OF B VIRUS INFECTION

Multiple levels of prevention can be used to prevent B virus infection in both humans and nonhuman primates, ranging from attempts to eliminate virus from colonies to designing methods to work safely in environments where there is increased risk for contracting this agent. The CDC has published detailed guidelines for maximizing protection of individuals working with macaque monkeys (23,25). Further, the NIH National Center for Research Resources has funded the development of B virus–free colonies for NIH-funded research involving these animals in an attempt to eliminate this virus from colonies used for biomedical research. Nonetheless, B virus–infected monkeys are plentiful and require handling that can be done safely if strict guidelines are followed, including barrier precautions.

When B virus is detected, it can be inactivated with either heat or formaldehyde (91). Other virus inactivators include detergents and bleach, but individuals who are working in a decontaminated area should still be alert for injury prevention. Minimizing fomites, however, decreases worker risk and reduces virus spread among animals. One B virus infection in a human was acquired from a cage after sustaining a scratch (61), underscoring that surface decontamination

can play an important role in infection control.

As early as the 1930s, attempts were made to identify an effective vaccine for protection of individuals who could be exposed to this virus while working with macaques or their cells or tissues. Limited vaccine trials have been performed in human volunteers (66,67); and although short-term antibody was induced, it was observed to wane quickly. Thus, the vaccine was not pursued further. Recently, a recombinant vaccine was tested and found to induce antibodies in macaques, but the duration of antibody persistence and efficacy remain to be assessed (10).

Antiviral therapy has been recognized as an effective prevention of infection prophylactic in human and animal trials, when administered early after exposure (17,31,34,61,126). Acyclovir and the related family of nucleoside analogs were noted to be effective when given in high doses (60), for example, acyclovir at 10 mg/kg intravenously three times daily for 14 to 21 days. Efficacy of therapy in cases of infection in humans has been monitored by inhibition of peripheral virus shedding in some cases and by reduction in cerebrospinal fluid antibodies or viral DNA load in others (34,61,105). Some physicians follow intravenous therapy with long-term oral suppression (acyclovir, valaciclovir, or famciclovir). Ganciclovir has a greater efficacy *in vitro* and thus was used in a few cases since 1989 with success. Interestingly, before 1987, in at least five retrospectively recognized cases, individuals fared well in the absence of antiviral therapy, but overall, the use of one of these nucleoside analogs is recommended by CDC. Generally, antiviral therapy is uniformly used in humans with clinical disease. Moreover, it is also used by an increasing number of facilities for postinjury prophylaxis or after laboratory results indicate an animal may have been actively infected around the time of the exposure. Postinjury prophylaxis has been performed with famciclovir or valaciclovir, and both have demonstrated efficacy *in vitro.* Recommendations and guidelines have been published by the CDC, as discussed previously, and can be readily accessed (60). Only a handful of physicians have had experience in the treatment of B virus zoonosis, and their participation and expertise has been important in the development of the CDC guidelines. There are other compounds that show a high degree of efficacy against B virus *in vitro,* but their clinical usefulness in the treatment of infections in humans must be further investigated owing to the toxicity of the compounds. Toxicity may be averted by lower doses of these compounds, but safety trials in animals are not complete.

Finally, with respect to prevention, the value of first aid after a potential exposure due to a bite, scratch, splash, or other suspicious injury is very important. Guidelines for wound cleaning are described in detail by the CDC. Every institution working with macaques should have an injury protocol with immediate availability of first aid, a secondary care plan, and last but not least, an infectious disease specialist who is a member of the institution's prevention and care response team.

CONCLUSIONS

B virus is usually a rapidly progressive, devastating disease that can be interrupted by effective use of antiviral therapies if deployed sufficiently soon after infection. The guidelines for treatment and prevention are widely published and can be rapidly accessed either through the CDC or the diagnostic resource using the World Wide Web address www.gsu.edu/bvirus. Other web sites are beginning to spring up as well and can be useful in learning more about B virus infections in both humans and nonhuman primates. Diagnostic techniques are rapidly improving to support clinical diagnosis and information regarding sample collection. Evaluation is available at any time to clinical care centers in case of emergencies.

With the newer diagnostic techniques, sensitivity of detection is improving, and the barriers posed by the high degree of cross-reactivity among this family of viruses are rapidly being diminished.

Because of the risk of human disease, precautionary methods must be followed in the workplace. Proper attention to the details of housing, management, and handling of macaque monkeys and organized exposure response measures using the CDC guidelines can minimize B virus zoonotic infections. Rapid identification of infection is essential for early initiation of antiviral drug therapy, which can prevent further mortality associated with this very interesting alphaherpesvirus.

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CHAPTER 84 *Poxviridae:* The Viruses and Their Replication

Bernard Moss

The *Poxviridae* comprise a family of complex DNA viruses that replicate in the cytoplasm of vertebrate or invertebrate cells. These viruses are of special interest because of their unique biologic properties and impact on human health. Two members of the family—variola virus and molluscum contagiosum virus—are obligate human pathogens, and many others can be transmitted to humans from other animal hosts (see Chapter 85). Variola virus can cause smallpox, a once common and devastating disease that altered human history. Smallpox was eradicated in 1977, nearly two centuries after the introduction of prophylactic inoculations with cowpox and vaccinia virus, through a dedicated effort spearheaded by the World Health Organization. Vaccination contributed to present concepts of infectious disease and immunity. Moreover, vaccinia virus was the first animal virus seen microscopically, grown in tissue culture, accurately titered, physically purified, and chemically analyzed. A once prevalent view of virus particles as packets of nucleic acid was revised after the discovery of RNA synthetic activity in purified vaccinia virions. This finding stimulated investigations that led to the discovery of transcriptase and reverse transcriptase activities in RNA viruses and to the elucidation of structural features of viral and eukaryotic messenger RNA (mRNA), including the 5' cap and 3' poly(A) tail. Recombinant DNA technology eliminated obstacles to working with these large viruses, and considerable progress has been made in elucidating the cycle of virus replication (Fig. 1). Discoveries of virus-encoded proteins that affect cell growth and modulate immune defense mechanisms provided new insights into virus–host relationships. In addition, the development of vaccinia virus as a live recombinant expression vector provided a powerful tool for immunologists and biochemists as well as an alternative approach to the development of vaccines against a variety of infectious agents.

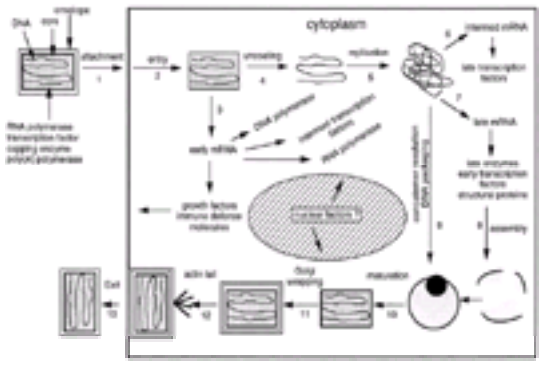

FIG. 1. The replication cycle of vaccinia virus. Virions, containing a double-stranded DNA genome, enzymes, and transcription factors, attach to cells (*1*) and penetrate the cell membrane, releasing cores into the cytoplasm (*2*). The cores synthesize early mRNAs that are translated into a variety of proteins, including growth factors, immune defense molecules, enzymes, and factors for DNA replication and intermediate transcription (*3*). Uncoating occurs (*4*), and the DNA is replicated to form concatemeric molecules (*5*). Intermediate genes in the progeny DNA are transcribed, and the mRNAs are translated to form late transcription factors (*6*). The late genes are transcribed, and the mRNAs are translated to form virion structural proteins, enzymes, and early transcription factors (*7*). Assembly begins with the formation of discrete membrane structures (*8*). The concatemeric DNA intermediates are resolved into unit genomes and packaged in immature virions (*9*). Maturation proceeds to the formation of infectious intracellular mature virions (*10*). The virions are wrapped by modified Golgi membranes, are transported to the periphery of the cell (*11*), and acquire actin tails (*12*). Fusion of the wrapped virions with the plasma membrane results in release of extracellular enveloped virus (*13*). Although replication occurs entirely in the cytoplasm, nuclear factors may be involved in transcription and assembly.

CLASSIFICATION

The general properties of *Poxviridae* include: a large complex virion containing enzymes that synthesize mRNA; a genome composed of a single linear double-stranded DNA molecule of 130 to 300 kilobase pairs (kbp) with a hairpin loop at each end; and a cytoplasmic site of replication. The *Poxviridae* are divided into two subfamilies, *Chordopoxvirinae* and *Entomopoxvirinae*, based on vertebrate and insect host range (Table 1). DNA sequencing has confirmed the genetic relationship between the two subfamilies. African swine fever virus shares some properties with poxviruses but is morphologically and genetically distinct and separately classified.

Subfamilies	Genera	Member viruses	Features
Chordopoxvirinae	Orthopoxvirus	Cowpox, vaccinia, ectromelia, monkeypox, raccoonpox, skunkpox, *Uasin Gishu, **vaccinia, *variola, volepox	Brick-shaped virion, DNA ~200 kbp, G + C ~35%, wide to narrow host range, variola (smallpox), vaccinia (smallpox vaccine)
	Parapoxvirus	*Bovine stomatitis, *Orf, pseudocowpox, paripox of deer, *sealpox	Ovoid virion, DNA ~140 kbp, G + C ~64%
	Avipoxvirus	Canarypox, *fowlpox, juncopox, mynahpox, pigeonpox, psittacinepox, quailpox, *peacockpox, *penguinpox, sparrowpox, starlingpox, turkeypox	Brick-shaped, DNA ~260 kbp, G + C ~30%, limited, arthropod transmission
	Capripoxvirus	Goatpox, lumpy skin disease, *sheeppox	Brick-shaped, DNA ~150 kbp, ungulates, arthropod transmission
	Leporipoxvirus	Hare fibroma, **myxoma, rabbit fibroma, squirrel fibroma	Brick-shaped, DNA ~160 kbp, G + C ~40%, leporids and squirrels
	Suipoxvirus	Swinepox	Brick-shaped, DNA ~170 kbp, narrow host range
	Molluscipoxvirus	*Molluscum contagiosum	Brick-shaped, DNA ~190 kbp, G + C ~60%, human host, localized tumors, contact spread
	Yatapoxvirus	Tanapox, *Yaba monkey tumor	Brick-shaped, DNA ~145 kbp, G + C ~33%, primates and T rodents
Entomopoxvirinae	Entomopoxvirus A	*Melanoplus melanoplus	Ovoid virion, DNA ~380–570 kbp, Coleoptera
	Entomopoxvirus B	**Amsacta moorei, *Melanoplus sanguinipes	Ovoid, DNA ~236 kbp, G + C ~18%, Lepidoptera and Orthoptera
	Entomopoxvirus C	*Chironomus solitor	Brick-shaped, DNA ~250–360 kbp, Diptera

*Probable member of genus.
**Biological member.
†Completely sequenced.

TABLE 1. *Family* Poxviridae

The *Chordopoxvirinae* consist of eight genera: *Orthopoxvirus, Parapoxvirus, Avipoxvirus, Capripoxvirus, Leporipoxvirus, Suipoxvirus, Molluscipoxvirus,* and *Yatapoxvirus* (see Table 1). Members of the same genus are genetically and antigenically related and have a similar morphology and host range. The orthopoxviruses have been studied most intensively. The prototypal member, vaccinia virus, has an unknown origin (41) and no known natural host, although it has been isolated from buffalo that probably contracted it from humans (149). DNA sequencing revealed that the genes common to vaccinia, variola, and cowpox viruses are greater than 90% identical (186,311,456,457), whereas limited data on orthopoxviruses indigenous to the Americas (e.g., raccoonpox virus and volepox virus) indicate that they are more genetically divergent (263). There are much less comparative data for individual members of the other poxvirus genera.

The *Entomopoxvirinae* have been divided into three genera based on the insect host of isolation (20). The prototypal members are listed in Table 1. The recent complete sequence analysis of the *Melanoplus sanguinipes* and *Amsacta moorei* viruses suggest that the Orthopteran and Lepidopteran members of genus B should be split into separate genera (1,40).

VIRION STRUCTURE

Morphology

Poxvirus virions are larger than those of other animal viruses and are discernible by light microscopy. The ultrastructural appearances of the particles varies according to the preparation methods. Vaccinia virions are visualized as smooth, rounded rectangles of about 350 × 270 nm by cryoelectron microscopy of unfixed and unstained vitrified samples (148) (Fig. 2). A 30-nm, membrane-delimited surface layer surrounds a homogenous core. Using the same technique, cores

produced by treating virions with a detergent, reducing agent, and deoxyribonuclease appear to be studded with 20-nm spikes. Two types of particles, C and M, are seen by negative staining of whole virions (359,546). The C form has a smooth exterior similar to the particles viewed by cryoelectron microscopy, whereas the M form has a beaded or tubular appearance also noted by freeze-etching (321). Incubation of virions with a nonionic detergent converts the M forms to C forms, but otherwise, the morphology appears unchanged (154). Further treatment with a reducing agent leads to removal of the outer coat, suggesting that disulfide-bonded proteins hold the latter together (154,422). Negatively stained images of cores, isolated by treatment with a detergent and reducing agent, have a rectangular shape; the wall of the core appears to be composed of an outer layer of cylindrical subunits 10 nm in length and 5 nm in diameter and an inner 5-nm thick smooth layer (154). Trypsin-sensitive structures called *lateral bodies* remain associated with cores prepared in this manner. In fixed and stained thin sections of virions, the core frequently appears dumbbell-shaped, with the lateral bodies in the concavities (126) (Fig. 3). It has been suggested that the surface tubules and the dumbbell-shaped cores result from nonisotropic drying because they are most clearly seen in dehydrated samples (148). Even so, an underlying structure must contribute to the acquisition of this highly characteristic appearance. Cylindrical elements that may take an S shape, or more complex flower-like structures, presumably representing nucleoprotein, have been visualized within poxvirus cores (227,390).

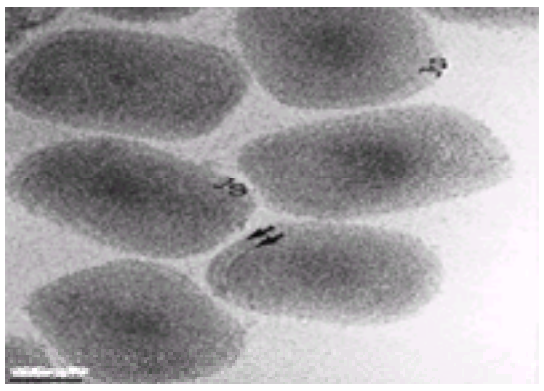

FIG. 2. Vitrified suspension of intracellular mature vaccinia virus particles observed by cryoelectron microscopy. The 30-nm thick surface domain (*S*) is delimited by arrows. (From ref. 148, with permission.)

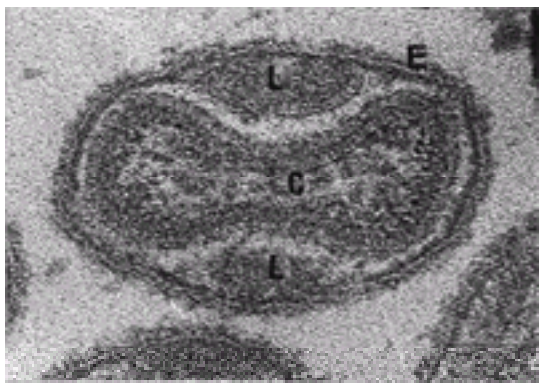

FIG. 3. Electron-microscopic image of a thin-sectioned intracellular mature vaccinia virus particle showing core (*C*), lateral bodies (*L*), and external membrane (*E*). (From ref. 393, with permission.)

The preceding descriptions apply to cytoplasmic infectious particles that are called *intracellular mature virions* (IMVs). *Extracellular enveloped virions* (EEVs), isolated from the tissue culture medium, contain an additional somewhat fragile lipoprotein envelope and have a lower buoyant density than IMVs (378,422). Thus far, EEVs from only vaccinia virus have been analyzed.

Chemical Composition

The large size of poxvirus virions facilitated their isolation by low-speed centrifugation and permitted accurate chemical determinations in the early 1940s (483). The principal components of vaccinia virions are protein, lipid, and DNA. These account for 90%, 5%, and 3.2%, respectively, of the dry weight (575), which has been estimated to be about 5×10^{-15} g (484). In contrast, about one-third of fowlpox virions is lipid (298). The lipid components of vaccinia virions are predominantly cholesterol and phospholipids (492,498), whereas fowlpox virions also contain squalene and cholesterol esters (298). Carbohydrate is present in the EEV as a constituent of glycoproteins. Spermine and spermidine (278) and trace amounts of RNA (420) have also been found in vaccinia virions, but their significance has not been established.

Genome

Poxviruses have linear double-stranded DNA genomes that vary from 130 kbp in parapoxviruses to about 230 kbp in avipoxviruses. All poxviruses examined have *inverted terminal repetitions* (ITRs), which are identical but oppositely oriented sequences at the two ends of the genome (176) (Fig. 4). The ITRs include: an A+T-rich, incompletely base-paired, hairpin loop that connects the two DNA strands (36); a highly conserved region of less than 100 bp that contains sequences required for the resolution of replicating concatemeric forms of DNA (136,324); variable-length sets of short, tandemly repeated sequences (557); and up to several open reading frames (ORFs). The ITRs are variable in

length owing to deletions, repetitions, and transpositions.

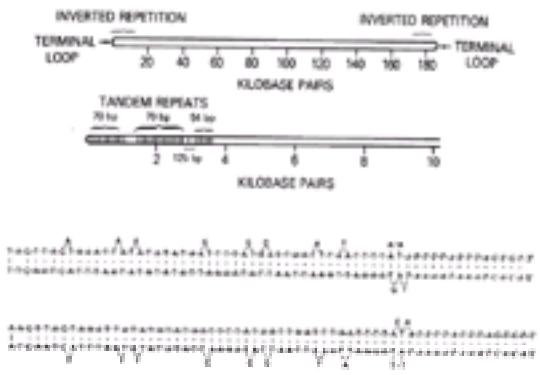

FIG. 4. Structural features of vaccinia viral DNA. A representation of the entire linear double-stranded DNA genome and an expansion of the 10,000-bp inverted terminal repetition are shown in the *upper part.* The nucleotide sequences of the inverted and complementary forms of the terminal loops are *below.* (From refs. 36 and 347, with permission.)

Complete genome sequences have been reported for six chordopoxviruses—vaccinia virus (19,186), variola virus (311,456), molluscum contagiosum virus (450), myxoma virus (89), Shope fibroma virus (552), and fowlpox virus (2)—and for two entomopoxviruses (1,40). There are about 100 ORFs that are represented in all chordopoxviruses and about 50 that are shared by chordopoxviruses and entomopoxviruses. Several generalizations can be made: ORFs are largely nonoverlapping, tend to occur in blocks pointing toward the nearer end of the genome, are usually located in the central region if highly conserved and concerned with essential replication functions, and are usually located in the end regions if variable and concerned with host interactions. The arrangement of the central genes is remarkably similar in all chordopoxviruses. The convention for naming orthopoxvirus ORFs was adopted before obtaining the complete genome sequence and consists of using the HindIII restriction endonuclease DNA fragment letter, followed by the ORF number (from left to right) within the fragment, and L or R, depending on the direction of the ORF. An exception to this rule was made for the HindIII C fragment; the ORFs were numbered from right to left to avoid starting at the left end of the genome, which is highly variable. For reference, the physical order of the 16 HindIII fragments of vaccinia virus is C, N, M, K, F, E, P, O, I, G, L, J, H, D, A, B. With the other poxviruses, the convention has been to number the ORFs continuously starting from one end.

Polypeptides

Consistent with their size and complex structures, poxvirus virions are composed of a large number of polypeptides. About 30 bands are readily resolved by one-dimensional polyacrylamide gel electrophoresis of vaccinia virus virions that have been purified from infected cells and disrupted with sodium dodecyl sulfate and a reducing agent, and considerably more can be detected by two-dimensional analysis (240). Twelve polypeptides have been localized near the exterior of the IMV by one or more of the following procedures: surface-specific labeling, sensitivity to proteases, extraction with nonionic detergents, and reactivity with virus-neutralizing antibodies (Table 2). Some of these proteins may form a disulfide-bonded matrix because both a reducing agent and a nonionic detergent are required for their release (233). There is evidence for physical association of the A17L, A27L, and A14L polypeptides in a complex (418). The L1R polypeptide is myristylated (405), and the A14L and A17L proteins are phosphorylated (55,143). Many of the IMV membrane proteins have unusual topologies: the A17L protein crosses the membrane twice with both the N- and C-terminal ends in the cytoplasm (54). The D8L (434) and the H3L (120) proteins are anchored by N- and C-terminal hydrophobic domains, respectively.

TABLE 2. *Nonenzymatic vaccinia virion components*

After IMVs are treated with a nonionic detergent and reducing agent, the cores can be recovered by low-speed centrifugation. Disruption of the core with deoxycholate releases a soluble enzyme fraction (described in the next section) from insoluble structural proteins. Four of the latter proteins, encoded by the F17R, L4R, A3L, and A10L ORFs, account for about 70% of the viral core by weight (see Table 2). The F17R 11-kd phosphoprotein and the L4R 25-kd polypeptide bind DNA and may correspond to nucleoprotein constituents (233). The mature G7L, L4R, A3L, A10L, and A12L proteins are processed from larger precursors at Ala-Gly-Ala/Ser motifs during morphogenesis (343,536). Vaccinia virus EEVs have a lower buoyant density than IMVs because of the additional lipid membrane and contain several unique

glycosylated proteins with type I or II topologies encoded by the A33R, A34R, A36R, A56R, and B5R ORFs (see Table 2) and by F13L, a nonglycosylated phospholipase (Table 3).

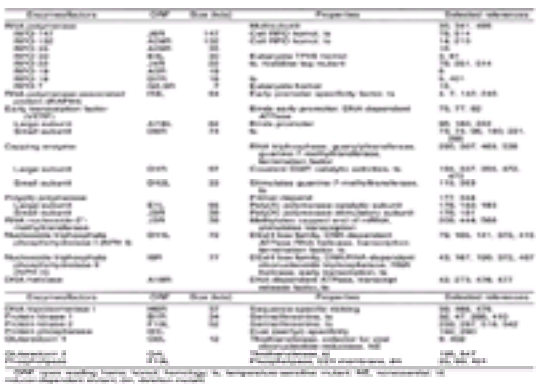

TABLE 3. *Vaccinia virion enzymes and factors*

Enzymes

Infectious poxvirus particles contain a transcription system (248,354) that can carry out *in vitro* synthesis of mRNAs that are polyadenylylated (247), capped, and methylated (544). A large number of virus-encoded enzymes and factors are packaged in the virus particle (see Table 3). Many of these, including the RNA polymerase, RNA polymerase-associated protein of 94 kd (RAP94), vaccinia early transcription factor (VETF), capping and methylating enzymes, poly(A) polymerase, and nucleoside triphosphate phosphohydrolase (NPH) I are directly involved in the synthesis and modification of mRNA. Additional enzymes may also be involved in synthesis of RNA or in virus assembly, protein processing, or DNA packaging.

VIRUS ENTRY

Studies of poxvirus entry into cells are complicated by the existence of multiple infectious forms that may use different and still unidentified cellular receptors and viral proteins (381,530). Although IMVs are commonly used by investigators to infect cells, they are only released by cell lysis; EEVs (68,380), and particularly the related *cell-associated enveloped virions* (CEVs) (61), are important for efficient cell-to-cell spread. IMVs are generally thought to enter cells by fusion with the plasma membrane or vesicles formed by surface invaginations (98,124,146,238). The inhibition of IMV entry by cytochalasin B and D suggests a role for actin filaments (381,530). In contrast to IMVs, some data suggest that EEVs enter cells by a mechanism that involves endocytosis followed by low pH disruption of the EEV outer membrane and fusion of the released IMV with endosomal membranes (228,530). The fusion of infected cells to each other, mediated by short exposure to a low pH, may mimic the latter process by disrupting the outer membrane of virus particles on the cell surface (146,187).

Poxvirus Receptors

A virus receptor is usually defined as a component on the cell surface to which a virus specifically binds and that mediates virus internalization. Because vaccinia virus can enter virtually all cell lines tested, there must be either many receptors or one that is ubiquitous. Thus far, however, no receptor has been unequivocally identified for any poxvirus. A virus-binding site, in contrast to a receptor, allows virus attachment but not necessarily entry. Evidence that IMV and EEV have different binding sites includes the efficiency with which the two virus types bind to different cell lines, differential effects of cell surface digestion with proteases, and the specific effect of a monoclonal antibody on binding of IMV (101,532). Heparin sulfate binds to vaccinia IMV and can inhibit infection by IMV of several orthopoxviruses and leporipoxviruses, suggesting that cell surface proteoglycans could serve as general mediators of poxvirus infection (106). It will be important, however, to rule out the possibility that heparin sulfate binding is an adaptation to passage in tissue culture. A candidate cell surface receptor was identified with a monoclonal antibody that blocked binding of IMV to cells (101). A suggestion that the epidermal growth factor (EGF) receptor serves as a portal of entry for vaccinia virus (305) has not been generally accepted (225). An exciting, albeit short-lived, proposal that poxviruses use chemokine receptors was based on studies with Shope fibroma virus (277). However, recent data (G. McFadden, personal communication) does not support this hypothesis.

Poxvirus Entry Proteins

Information about which viral proteins mediate cell entry and penetration is limited. At least four vaccinia IMV proteins—L1R, A27L, D8R, and H3L—have been implicated. Monoclonal antibodies to the L1R (231,561) and A27L (414) proteins neutralize the infectivity of IMV. In addition, the A27L protein plays a critical role in low pH–mediated cell-to-cell fusion, and an N-terminal domain binds heparin sulfate (187,217,537). The H3L protein also binds heparin sulfate, and a deletion mutant was reported to bind less efficiently to cells under some conditions (286), but not others (121). A role for the D8L protein was suggested by its ability to bind to cell surface chondroitin sulfate and by decreased binding of virions with a mutation in the *D8L* gene (218,274). Treatment of vaccinia virions with proteases increases infectivity, apparently by enhancing cell penetration (232).

A difficulty in studying entry of EEV is that purified preparations may contain damaged particles with exposed IMV membranes. In addition, deletion or repression of some genes encoding EEV membrane proteins may affect formation of the membrane, precluding an analysis of their role in entry. Repression of the *A34R* gene, which encodes a glycoprotein with a putative lectin domain, however, leads to increased production of EEV with reduced infectivity (319). Whether this

reduction is due to a defect in cell binding or later steps has not been determined. The B5R protein has also been implicated in cell entry because polyclonal antibodies to its extracellular domain neutralize EEV (173). Nevertheless, the extracellular domain does not appear to be essential for infectivity (203,312). Mutations of the hemagglutinin or the K2L glycoprotein result in the fusion of infected cells (448,524). However, neither the mechanism of fusion inhibition nor the roles of these proteins are understood.

Cell-to-cell spread is mediated most efficiently by CEVs, which resemble EEVs except that they are still attached to the plasma membrane of the parental cell (61). Vaccinia virus strains, such as WR, that produce mostly CEV form large, round plaques; whereas those that release large numbers of EEVs, such as IHD, form long comet-like plaques on cell monolayers under a liquid overlay. The different plaque phenotypes were mapped to a single amino acid substitution in the putative lectin domain of the A34R glycoprotein (62). Most orthopoxviruses have the WR-like sequence, suggesting that increased EEV may be an adaptation to spread in tissue culture. The extracellular domain of the B5R protein may also have a role in release of cell-associated virions (312). The efficient spread of CEVs is facilitated by their location at the tips of long microvilli that form by attachment of actin tails to the viral particles (119,499). Deletion of the *A34R, A33R,* or *A36R* gene prevents actin tail and microvillus formation and reduces the efficiency of virus spread (423,437,559,563). Presumably, the CEVs have the same outer membrane as EEVs, and details regarding their entry will be similar. A further discussion of these topics appears in a later section on EEV.

UNCOATING

After entry into the cytoplasm, virus cores are transported to juxtanuclear locations where they synthesize mRNA and undergo a second uncoating step. Electron microscopic images suggest that the nucleoprotein complex passes out through breaches in the core wall (123). Susceptibility of the virion DNA to treatment with deoxyribonuclease has been used as biochemical evidence of uncoating (241). Prevention of the uncoating process by inhibitors of transcription or translation (242) indicates a requirement for either a virus-induced or virus-encoded protein. A putative 23-kd uncoating protein with trypsin-like activity was partially purified from infected cell extracts (383). Viral particles in intermediate stages of disassembly have been isolated, and the polypeptide compositions have been analyzed (382).

GENE EXPRESSION

Programmed Expression of Poxvirus Genes

Studies primarily with vaccinia virus have led to a general understanding of how the DNA genomes of poxviruses are expressed within the cytoplasm of infected cells (see Fig. 1). A complete early transcription system is synthesized late in infection and packaged within the core of an infectious poxvirus particle, providing a mechanism for the initial synthesis of viral mRNAs. These early mRNAs encode enzymes and factors needed for transcription of the intermediate class of genes that in turn encode enzymes and factors for late gene expression (Table 4).

Stage	Factor	ORF	Promoter	Properties	Selected references
Early	VETF	A7L, D6R	Late	Heterodimer, DNA binding, ATPase	75, 77, 82, 98 73, 74, 1886, 2855
	RAP94	H4L	Late	Associated with RNA polymerase	4, 7, 142
Intermediate	VITF 1	E4L	Early	Homolog to TFIIS, also RNA polymerase subunit RPO30	428
	VITF 3	A8R, A23R	Early	Heterodimer	439, 440
	Capping enzyme	D1R, D12L	Early	Heterodimer, also functions to modify mRNA	2202, 540
Late	VLTF 1	D8R	Intermed	Interacts with self and VLTF 2	2553, 3144, 5885, 5965
	VLTF 2	A1L	Intermed	Interacts with VLTF 1, zinc binding	82, 2553, 2566, 3144, 5655
	VLTF 3	A2L	Intermed	Zinc binding	2203, 2553, 2554, 3775
	VLTF 4	H5R	Early	Stimulates transcription	2869

ORF, open reading frame.

TABLE 4. *Stage-specific transcription factors encoded by vaccinia virus*

Regulation of Early-Stage Transcription

Vaccinia virus early mRNA is detected within 20 minutes after infection and accumulates to maximal levels in 1 to 2 hours (28) (Fig. 5). About half of the vaccinia virus genome is transcribed before DNA replication (65,371), including genes encoding proteins involved in host interactions, viral DNA synthesis, and intermediate gene expression. When uncoating of the core is prevented by protein synthesis inhibitors, early mRNA synthesis is increased and prolonged (28,564), suggesting that under normal conditions, core disassembly leads to the disruption of the early transcription apparatus. By comparison, DNA replication inhibitors do not prevent uncoating and prolong early transcription to a limited extent. The rapid decline of steady-state early mRNA levels cannot be explained solely by cessation of transcription and is consistent with an enhanced rate of degradation of all classes of mRNA after virus infection (28,366). Rapid mRNA turnover may be a mechanism for eliminating cellular and viral mRNAs at the end of each temporal stage.

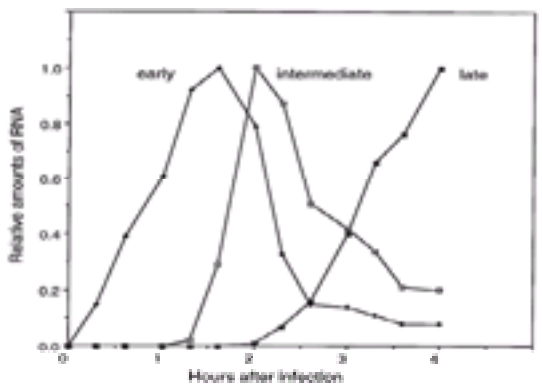

FIG. 5. Steady-state levels of representative early-, intermediate-, and late-stage messenger RNAs (mRNAs) in vaccinia virus–infected cells. Total RNA was isolated from infected HeLa cells at various times after infection and hybridized to antisense RNA probes specific for the 5' ends of mRNAs encoded by the C11R (early), G8R (intermediate), or F17R (late) open reading frames (ORFs) (28). After ribonuclease digestion, the protected probe fragments were analyzed by polyacrylamide gel electrophoresis and the radioactivity quantified. The numbers were normalized to the peak value in each case.

Early-Stage Promoters and Termination Signal

The transcription of an early gene is determined by an A+T-rich sequence located immediately upstream of the RNA start site. Saturation mutagenesis of a vaccinia virus early promoter defined a critical core region, from nucleotide –13 to –27, in which many single substitutions have a drastic effect on expression (131) (Fig. 6). A consensus core sequence AAAAAATGAAAAAA/TA is close to the optimal one defined by mutagenesis. Transcription initiation occurs with a purine, predominantly 12 to 17 nucleotides downstream of the core region. The intervening DNA, between the core and RNA start site, appears to have a spacer role, and there are no evident sequence requirements upstream of the core or downstream of the initiation site. Promoter sequences are conserved between poxvirus genera, partially explaining the phenomenon called *nongenetic reactivation,* which consists of rescue of a heat-inactivated poxvirus by co-infection with a second poxvirus (169,200). Thus, the heat-killed poxvirus provides the template and the second poxvirus provides the enzymes for transcription.

VACCINIA VIRUS PROMOTER SEQUENCES

	CORE			INITIATOR
EARLY	AAAA	T	GAAA TA	A/G
INTER	T	TT	AAA AA	TAAA
LATE	A/T-rich			TAAATG/A
	-30	-20	-10	+1

FIG. 6. Early-, intermediate-, and late-stage promoter sequences. Nucleotides that have a strong positive effect on transcription are shown. Positions of nucleotides in the nontemplate strand are relative to the RNA start site (+1). (From ref. 340, with permission.)

The 3' ends of vaccinia virus early mRNAs occur 20 to 50 bp downstream of the sequence TTTTTNT (574). Such termination sequences are present near the ends of most viral early genes but only rarely in their middle. When absent, the mRNA may extend through the next early gene downstream. *In vivo* studies suggested that the efficiency of termination is about 80% (152), although in some cases, it is much less, probably because of RNA secondary structure (282,296). TTTTTNT sequences have been noted near the ends of putative early genes in other poxvirus genera, suggesting a similar role in termination. As discussed later, the TTTTTNT sequence is actually recognized as UUUUUNU in RNA (474).

Enzymes and Factors for Early-Stage Transcription

A complete early transcription system is packaged within the core of an infectious poxvirus particle (248,354). Soluble extracts of vaccinia virus virions can transcribe an early promoter template *in vitro* to generate properly initiated and terminated mRNAs (421) and therefore provide a source of materials for characterization of the relevant enzymes and factors. The virion RNA polymerase is eukaryotic-like with regard to its size and subunit complexity (35). The subunits, ranging from 7- to 147-kd, are encoded by at least eight viral genes (see Table 3). There is a single copy of each large subunit, but the stoichiometry of the small subunits has not been accurately determined, partly because of polypeptide heterogeneity caused by alternative transcriptional and translational start sites. RPO147 and RPO132 are homologous to the corresponding large subunits of cellular RNA polymerase and resemble those of eukaryotes and archae (20% to 30% amino acid identities over the entire proteins) slightly more than those of bacteria. The vaccinia virus RPO30 subunit is about 23% identical in sequence to eukaryotic transcription elongation factor SII (TFIIS); RPO7, the smallest vaccinia virus RNA polymerase subunit, is about 23% identical in amino acid sequence with the smallest eukaryotic RNA polymerase subunit. Although the functions of the individual viral RNA polymerase subunits have not been investigated,

the large ones are likely to catalyze nucleotide addition based on homology with the cellular enzymes. Temperature-sensitive (ts) mutants with defects mapped to RNA polymerase subunits have been isolated (213,514). Mutant virions, propagated at the permissive temperature, generally contain active RNA polymerase, and early gene expression occurs even when cells are infected at the nonpermissive temperature. The phenotype of these mutants is a block in late gene expression, probably caused by aberrant assembly of the multisubunit enzyme.

An additional polypeptide is associated with RNA polymerase molecules in vaccinia virions (4,7,142). This RAP94 is specifically required for transcription of early promoter templates in conjunction with the VETF. RNA polymerase lacking RAP94 can transcribe single-stranded DNA nonspecifically or double-stranded intermediate or late promoter templates with the corresponding transcription factors. It seems likely that RAP94 interacts with VETF, although this has yet to be demonstrated. Synthesis of RAP94 occurs late, at the time of virion assembly, consistent with its exclusive role as a virion-associated early transcription factor. In this regard, RAP94 is distinct from the RNA polymerase subunits that are synthesized throughout the infectious cycle for intermediate and late transcription.

Transcriptional activity can be reconstituted *in vitro* with RNA polymerase and VETF, a heterodimer of 82 and 70 kd subunits (82,180). VETF, like RAP94, is synthesized only at late times after infection. The protein binds to the core region of early promoters and to DNA downstream of the RNA start site, thereby altering the conformation of the DNA (75,96). Single nucleotide substitutions in the core sequence of the promoter that decrease transcription also abrogate specific DNA binding (573). A DNA-dependent adenosine triphosphatase (ATPase) activity associated with the small subunit of VETF is not required for promoter binding but is essential for transcription, possibly through a promoter clearance mechanism (71,77,283). Complexes of VETF and RNA polymerase have been detected, suggesting that VETF may recruit RNA polymerase to the promoter (27,80,251,284). The elongation complex has a 3' RNase activity that permits resumption of transcription by stalled polymerase (197). A role for RPO30 has been suggested because the latter RNase activity is similar to one exhibited by eukaryotic RNA polymerase II in the presence of TFIIS, an RPO30 homolog.

A physical complex of RNA polymerase, VETF, capping enzyme, and NPH I (a DNA-dependent ATPase) can accurately terminate, as well as initiate, transcription on DNA templates containing an early promoter and TTTTTNT sequence (80,574). Although RNA polymerase and VETF can reconstitute the transcription initiation and elongation activities, capping enzyme and NPH I are needed to release nascent mRNA containing a UUUUUNU sequence from the transcription complex in an adenosine triphosphate (ATP)-dependent reaction (105,141,469,474). The physical interaction of NPH I with RAP94 may explain the specificity of this termination system for early transcripts (334). NPH I also serves as a polymerase elongation factor to facilitate read-through of intrinsic pause sites (141).

Because early viral transcripts made *in vivo* or *in vitro* are capped (64,544) and polyadenylylated (247), they structurally and functionally resemble eukaryotic mRNAs. RNAs synthesized by virus cores contain a cap I structure that consists of a terminal 7-methylguanosine connected by a triphosphate bridge to a 2'- *O*-methylribonucleoside. The N^7-methylguanosine component of the cap is required for mRNA stability and for binding of vaccinia virus mRNA to ribosomes, whereas the role of ribose methylation is undetermined (355). Capping occurs during transcription when the nascent RNA chains are about 30 nucleotides long (196). The steps in cap formation are: (a) removal of the terminal phosphate of the triphosphate end of the nascent RNA to form a pp(5')N-terminus, (b) transfer of a guanosine monophosphate (GMP) residue from guanosine triphosphate (GTP) to form G(5')ppp(5')N-, (c) transfer of a methyl group from *S*-adenosylmethionine to produce m^7G(5')ppp(5')N-, and (d) transfer of a second methyl group to form m^7G(5')ppp(5')Nm. The first three reactions are catalyzed by the virus-encoded 127-kd heterodimeric capping enzyme (307,308,538). The fourth step in cap formation, ribose methylation of the penultimate nucleoside, is mediated by a separate viral enzyme (34). The capping enzyme large subunit forms a covalent lysyl-GMP intermediate (114,362,431,472). The RNA triphosphatase and guanylyltransferase activities reside in an N-terminal segment of the large subunit (357,473), whereas a complex of the C-terminal part of the large subunit and the small subunit contains the N^7-methyltransferase activity (205,304). The nucleoside 2'-methyltransferase is a 39-kd protein that exists as a monomeric species and as a subunit of the poly(A) polymerase (444). High-resolution x-ray crystal structures of the nucleoside 2'-methyltransferase complexed to its methyl donor and mRNA cap have been determined (209,210). Viral mRNAs synthesized *in vivo* have additional base and ribose methylations that are catalyzed by cellular enzymes (64).

The enzyme that catalyzes poly (A) tail formation is a heterodimer of virus-encoded 55- and 39-kd subunits called VP55 and VP39, respectively (178,344). VP55 binds to uridylate sequences near the end of the RNA chain and catalyzes the processive addition of 30 to 35 adenylate residues before changing to a slow and nonprocessive mechanism (140,183). VP39 binds poly(A) and stimulates VP55 to add additional adenylate residues (181). Thus, VP39, which is present in an excess over VP55, serves as a processivity factor for the poly(A) polymerase as well as a methyltransferase. The two activities are independent because mutated forms of VP39 that lack methyltransferase but retain adenylyltransferase stimulatory activity *in vitro* have been produced (445,458). Genetic and biochemical studies indicate an additional role of VP39 as an RNA elongation factor for intermediate and late RNA synthesis (279,568), discussed later. The finding that the capping enzyme and termination factor and the ribose methyltransferase and poly(A) polymerase processivity factor function at the 5' and 3' ends of the mRNA is intriguing. Whether the association of such apparently disparate functions in the same enzymes provides a specific advantage or represents an economical use of proteins is unknown.

The minimal components for synthesis of correctly initiated, terminated, capped, and polyadenylylated mRNA were defined by *in vitro* reconstitution assays. However, additional enzymes may be needed within the virus core. One example is NPH II, an enzyme with RNA-dependent RNA nucleoside triphosphatase and RNA helicase (372,468) activities. Permeabilized mutant virions lacking NPH II have diminished transcription activity and produce longer than normal RNAs that remain virion associated, suggesting a role in transcription termination or extrusion (191). Studies with ts mutants suggest that the A18R DNA helicase has a role in early, as well as late, transcription (476,477). Biochemical studies indicate that the A18R protein, along with an unidentified cellular component, acts a transcript release factor (273). Virions deficient in the H1L serine-tyrosine phosphatase have low transcription activity, suggesting a regulatory role along with that of the virion-associated protein kinases (290).

Regulation of Intermediate-Stage Transcription

DNA replication precedes a shift in viral gene expression. Amino acid labeling (346,385) and transcription (28,541) studies indicated the existence of an intermediate class of genes that are expressed after DNA replication but before expression of the late genes (see Fig. 1). After synchronous infection, intermediate mRNAs were detected at 100 minutes, the time of peak early mRNA accumulation (see Fig. 5). Intermediate mRNAs reached their peak values soon after and then declined in quantity. Only seven vaccinia virus genes belonging to the intermediate class have been identified thus far (253,271,541), although additional ones exist (580). Three of these (*A1L, A2L,* and *G8R*) encode transactivators of late gene expression (253), and one (*I8R*) encodes the RNA helicase NPH II (467). Two have both early and intermediate promoters; of these, I3L is a single-stranded DNA binding protein that interacts with ribonucleotide reductase (130,521), and K2L (also called SPI-3) is a member of the serine protease inhibitor (SERPIN) superfamily with an unknown role (523,524).

The DNA replication requirement for intermediate gene expression may result from the inaccessibility of the genome within the infecting particle to the newly synthesized RNA polymerase and transcription factors. This hypothesis is consistent with transfection experiments showing that the DNA isolated from purified virus particles can serve as a template for intermediate and late transcription in the absence of DNA replication (253). The proposed inaccessibility of the parental DNA could be nonspecific owing to remaining virion proteins or to putative specific repressor proteins.

Intermediate-Stage Promoters

Mutagenesis of intermediate promoters indicated two important regions: a 14-bp core element separated by 10 or 11 bp from a 4-bp initiator element (29). The intermediate core element resembles that of early promoters in A+T richness but differs in specific sequence (see Fig. 6). The tetranucleotide TAAA serves as an initiator element of intermediate promoters. Intermediate-stage RNAs are initiated within the AAA triplet, but as discussed later, they contain additional A residues incorporated by a polymerase slippage mechanism. With a slight adjustment, early and intermediate promoter motifs can be accommodated within a single, dual-function, synthetic early-intermediate promoter (28), raising the possibility that such dual promoters occur naturally.

Analysis of some intermediate mRNAs by agarose gel electrophoresis indicated diffuse bands, equal to and longer than the coding regions, suggesting preferred sites of 3'-end formation that did not correlate with early gene transcriptional termination signals (28).

Enzymes and Factors for Intermediate-Stage Transcription

Intermediate promoter templates can be transcribed by extracts prepared from cells infected with vaccinia virus in the presence of an inhibitor of DNA replication (541). Further studies indicated a role for both viral and cellular proteins. The viral proteins (see Table 4) include capping enzyme, which acts by a cap-independent mechanism (202,539,540); VITF-1, which is encoded by the gene for RPO30, a viral RNA polymerase subunit with homology to eukaryotic transcription elongation factor TFIIS (426); and VITF-3, a heterodimer composed of polypeptides encoded by ORFs *A8R* and *A23R* (439). Unlike the early transcription factor, neither VITF-1 nor VITF-3 has ATPase activity or exhibits sequence-specific DNA binding. It is uncertain which component corresponds to the promoter melting factor (539). The cellular component, VITF-2, was extracted from the nucleus of uninfected HeLa cells but was distributed within the cytoplasmic and nuclear fractions of infected cells (427). Whether vaccinia virus infection induces transit of VITF-2 out of the nucleus, activates a cryptic cytoplasmic factor, or depends on newly synthesized VITF-2 has not yet been determined. A relationship between VITF-2 activity and certain examples of host range restriction has been suggested (503). One intriguing possibility is that VITF-2 provides a gate-keeper role between the prereplicative and postreplicative phases of the virus cycle by signaling whether a quiescent cell has been activated to allow optimal replication.

Genetic studies have provided evidence for positive and negative regulators of intermediate or late transcriptional elongation. Initial experiments pointed to similar effects on RNA metabolism of vaccinia virus mutants that mapped to the *A18R* gene, now known to encode a DNA helicase, and those produced by the antiviral drug isatin-b-thiosemicarbazone (IBT) (369). Subsequent experiments indicated that IBT-resistant mutants of vaccinia virus mapped to the RNA polymerase subunit RPO132 (111), whereas IBT-dependent mutants mapped to G2R, a protein of unknown function (322). The story became more intriguing when it was discovered that mutations in the *A18R* gene increased transcriptional readthrough of downstream gene sequences (571), whereas mutations of the *G2R* gene produced decreased-length mRNAs (57). Remarkably, *G2R* mutants rescued *A18R* mutants (113), and physical interactions between the proteins encoded by *G2R, A18R,* and *H5R* (a late transcription factor) were demonstrated (58,314). Recent biochemical studies suggest that the *A18R* helicase is a transcript release factor and acts in conjunction with an unidentified cellular protein (273). Similar genetic approaches led to the identification of the product of the *J3R* gene, which also serves as the cap 2'-*O*-methyltransferase and as a subunit of the poly(A) polymerase, as an mRNA elongation factor (279,568).

Regulation of Late-Stage Transcription

The transcription of late genes follows that of intermediate genes (see Fig. 1). In HeLa cells, late-stage RNA is detected at 140 minutes after synchronous infection with vaccinia virus and continues for about 48 hours (see Fig. 5). The persistent synthesis of late proteins reflects continued transcription because the half-life of late mRNAs has been estimated to be 30 minutes or less (366). Many late proteins, including the major virion components, accumulate in large amounts during this long period. Other late proteins include the factors that are specifically required for transcription of early genes, such as VETF and RAP94, as well as certain other virion enzymes (see Table 3). Although distributed throughout the genome, the late-stage genes cluster in the central region.

Late-Stage Promoters and RNA Processing Signal

Late-stage promoters may be considered in terms of three regions: a core sequence of about 20 bp with some consecutive T or A residues, separated by a region of about 6 bp from a highly conserved TAAAT element within which transcription initiates (132) (see Fig. 6). A synthetic promoter with exclusively T residues forming the core sequence was stronger than tested natural late promoters. Any mutations of TAAAT severely decreased transcription. G or A usually follows the late promoter TAAAT sequence; in the former case, the TAAATG transcription initiation sequence and the ATG translation initiation codon overlap. The seeming absence of an untranslated RNA leader in this situation was puzzling, until it was found that late mRNAs have a 5'-capped, heterogeneous-length, poly(A) sequence formed by RNA polymerase slippage (6,376,446). Poly(A) leaders are also present on mRNAs of a few early genes that have a TAAAT initiation site (5,235) as well as on intermediate mRNAs (28), suggesting that slippage on an AAA sequence is an intrinsic property of the viral RNA polymerase.

Most late transcripts are long and heterogeneous and lack defined 3'-ends (117,303). The early termination signal is not recognized by the late transcription system; consequently, TTTTTNT is frequently present within the coding region of late genes. Terminal heterogeneity, combined with transcription from both DNA strands, explains the ability of late transcripts to self-anneal or anneal with early transcripts to form ribonuclease-resistant hybrids (66,109). The vaccinia virus–encoded double-stranded RNA-binding protein (100) or RNA helicase (467) may prevent deleterious effects of the double-stranded RNA, although this idea is entirely speculative. Alternatively, the 5' poly(A) leader could compensate for the complementary RNA by providing a single-stranded binding site for initiation factors and the 40s ribosomal subunit, which would then move unimpeded by antisense RNA to the first AUG codon where ribosome assembly and translation occur.

An exception to the general 3' heterogeneity of late mRNAs was discovered (18). The cowpox virus late mRNA encoding the A-type inclusion protein has a 3' end corresponding to a precise site in the DNA template. The DNA sequence at this position encodes an RNA cis-acting signal for RNA 3'-end formation that can function independently of either the nature of the promoter or the RNA polymerase responsible for generating the primary RNA (214). There is evidence for induction or activation of a specific endoribonuclease that cleaves this RNA, which is then polyadenylylated. The number of late mRNAs that are processed in this manner remains to be determined. Therefore, poxviruses employ at least two mechanisms of RNA 3'-end formation. The first, operative at early times in viral replication, terminates transcription downstream of an RNA signal, whereas the second, operative at late times, involves RNA site-specific cleavage.

Enzymes and Factors for Late-Stage Transcription

Templates containing late promoters can be transcribed by extracts prepared from cells at the late stage of vaccinia virus infection (567). Late transcription factors were identified by the systematic screening of cloned DNA fragments: ORFs *A1L, A2L,* and *G8R* were necessary and sufficient for transactivation of a transfected late promoter reporter gene in vaccinia virus–infected cells that were blocked in DNA replication (253). An intermediate promoter regulates each of these late transcription factor genes (see Table 4), which is consistent with a cascade model of regulation. *In vitro* studies confirmed that the products of the *G8R, A1L,* and *A2L* genes are vaccinia virus late transcription factors (223,255,375,565,566), and they have been named VLTF-1, VLTF-2, and VLTF-3, respectively (see Table 4). In addition, ts and repressible mutations of *A1L* and *G8R,* respectively, block late gene expression under nonpermissive conditions (92,580). Both VLTF-2 and VLTF-3 can bind zinc (254), and yeast two-hybrid studies indicate interactions of VLTF-1 with itself and with VLTF-2 (314). *In vitro* studies indicated that the product of the early *H5R* gene, named VLTF-4, stimulated late transcription several-fold (269). Evidence has been obtained for a cell-derived late transcription factor that binds late promoters (193). Other studies demonstrated that the cellular transcription factor YY1 is found in the cytoplasm of infected cells and can bind to at least two late promoters (76), but it remains to be shown that this protein has a role in vaccinia virus transcription. Several studies have been cited as evidence for a role of host RNA polymerase II in late vaccinia virus gene expression (122), but this has not been demonstrated directly.

Posttranscriptional Regulation of Viral Gene Expression

Poxvirus mRNAs are made in the cytoplasm, and no evidence of splicing has been reported. As noted earlier, accelerated mRNA degradation induced by vaccinia virus facilitates rapid changes in viral mRNA populations (see Fig. 5), although the basis for this effect is unknown. The finding that intermediate and late mRNAs contain 5' poly(A) sequences, whereas such sequences are uncommon on early mRNAs, raises the possibility that this structural feature enhances translation. However, critical experiments to test such a hypothesis have not been reported.

DNA REPLICATION

General Features

Poxvirus DNA replication takes place in the cytoplasm, a characteristic shared only with African swine fever virus, and has been reported to occur in enucleated cells (386,398). Discrete cytoplasmic foci of replication, termed *factory areas,* were discerned by light and electron microscopic autoradiography and microscopic procedures (88,201,249).

Enzymes Involved in DNA Precursor Metabolism

Some poxviruses encode enzymes involved in the synthesis of deoxyribonucleotides, evidently to enhance DNA replication in cells with suboptimal precursor pools; whereas other poxviruses lack some or all of these enzymes and are dependent on the host. In orthopoxviruses, these enzymes include a thymidine kinase (26,216), thymidylate kinase (489), ribonucleotide reductase (478,510), and dUTPase (72). In addition, there is an incomplete guanylate kinase (488), raising the possibility that an intact form might be found in some other poxvirus. Leporipoxviruses are missing genes encoding the large subunit of ribonucleotide reductase, thymidylate kinase, and remnants of the guanylate kinase

(89,552); whereas molluscum contagiosum virus has no known enzymes involved in nucleotide precursor formation (450). Fowlpox virus encodes a protein related to human deoxycytidine kinase that is not present in the other poxviruses (266). The completely sequenced genome of the entomopoxvirus *Melanoplus sanguinipes* lacks all of the previously described poxvirus genes involved in nucleotide metabolism but has a thymidylate synthetase homolog (1). However, a homologous thymidine kinase gene is present in *Amsacta moorei* (40) and other entomopoxviruses.

The thymidine kinases encoded by orthopoxviruses, avipoxviruses, suipoxviruses, capripoxviruses, leporipoxviruses, and some entomopoxviruses are 20 to 25 kd and related in sequence to corresponding eukaryotic enzymes (35% to 70% amino acid identity), but not to the pyrimidine kinase of herpesviruses. The thymidine kinase gene is regulated by an early promoter, as befits its role in increasing the precursors for DNA replication. The vaccinia virus enzyme exists as a tetramer, has ATP- and Mg^{2+}-binding domains, and is susceptible to feedback inhibition by dTDP or dTTP (59). Although the thymidine kinase gene is not required for virus growth in tissue culture cells, deletion mutants are attenuated *in vivo* (86).

Thymidylate kinase catalyzes the next step in thymidine monophosphate (TMP) metabolism. The vaccinia virus gene encodes a 23.2-kd protein that can complement *Saccharomyces cerevisiae* mutants deficient in the homologous enzyme (224). The protein is expressed early in infection and is not required for virus replication in tissue culture.

The synthesis of ribonucleotide reductase, an enzyme that converts ribonucleoside diphosphates to deoxyribonucleoside diphosphates, occurs soon after vaccinia virus infection (480). Both the small catalytic subunit and the large regulatory subunit closely resemble their eukaryotic counterparts both structurally (70% to 80% identity) and functionally (215,479). Catalytic activity is inhibited by hydroxyurea, and drug-resistant mutants generate direct tandem repeats of the gene encoding the catalytic subunit (482). Mutation of the large subunit prevented induced ribonucleotide reductase activity in tissue culture cells without affecting replication (104). However, the mutant virus was mildly attenuated in a mouse model.

The hydrolysis of dUTP by the vaccinia virus dUTPase provides dUMP, an intermediate in the biosynthesis of thymidine triphosphate (TTP), and might also minimize dUTP incorporation into DNA (72). The protein is synthesized early in infection (481) and is nonessential for virus replication (388).

DNA Synthesis

The time of onset of DNA synthesis varies with different members of the poxvirus family and to some extent with the multiplicity of infection and cell type. In cells synchronously infected with vaccinia virus, DNA replication begins 1 to 2 hours after infection and results in the generation of about 10,000 genome copies per cell, of which half are ultimately packaged into virions (243,435). Studies depending on thymidine incorporation instead of hybridization for DNA quantification generally underestimate the length of the replication period, probably because of a decline in the activity of the viral thymidine kinase used for incorporation of the radioactive precursor through the salvage pathway.

A plausible model for poxvirus DNA replication begins with the introduction of a nick near one or both of the hairpin termini, followed by nucleotide addition to the free 3' end, strand displacement, and concatemer resolution. Nicking is supported by changes in the sedimentation of the parental viral DNA (394) (although the cleavage site remains to be defined), and labeling studies suggested that replication begins near the ends of the genome (396). The presence of single-stranded DNA is consistent with a strand-displacement mechanism, and the report of small DNA fragments covalently linked to RNA primers suggests lagging strand synthesis (158,159 and 160,395), but this needs further investigation with current methods. The presence of transient concatemeric DNA intermediates were demonstrated by the presence of junction fragments (37,352) and high-molecular-weight DNA (135,329).

Efforts to locate a specific poxvirus origin using a plasmid replication assay in transfected cells led to a surprising conclusion that any supercoiled plasmid replicated in cells infected with Shope fibroma (137) or vaccinia virus (330). The absence of any stimulatory effect of vaccinia virus DNA sequences resulted in speculation that poxviruses, unlike nuclear DNA viruses, do not require specific origin sequences. Recently, however, more specific results were obtained by transfecting a linear DNA molecule containing vaccinia virus hairpin ends (147). With this assay, an enhancing effect was found when the template contained the terminal 200 bp of the viral genome.

Viral Proteins Involved in DNA Replication

Several complementation groups of ts mutants that express vaccinia virus early proteins but are impaired in DNA synthesis have been found (112,317). One group contains mutations in the DNA polymerase, which has a mass of about 110 kd, an associated 3' exonuclease activity and sequence similarities with other eukaryotic and viral DNA polymerases (97,153,316). Certain codon substitutions confer resistance to inhibitors of DNA synthesis, providing information regarding the active site of the polymerase (134,153,505,506,519).

The second DNA-negative complementation group maps to the *D5R* gene, which encodes a 90-kd protein with a nucleic acid–independent nucleoside triphosphatase activity (163,165,429). An abrupt cessation of DNA synthesis occurred upon a shift to the nonpermissive temperature of cells infected with one of the *D5R* mutants, suggesting a direct but still unknown role in replication (164). The third DNA-negative complementation group was mapped to the *B1R* ORF, which encodes a serine-threonine protein kinase that is expressed early in infection, and is packaged in virions (32,288,410). An early protein encoded by the *H5R* gene has been shown to be a substrate for the B1R kinase (47), and interactions between these proteins was demonstrated by the yeast two-hybrid system (314). Although the H5R protein appears to be involved in late transcription (58,269), it has no known role in DNA replication. Two human homologs of B1R have been identified, but their functions are unknown (361).

Another vaccinia virus ts mutant impaired in DNA replication was mapped to the *D4R* ORF (331). A mutant with a deleted *D4R* gene was also impaired in DNA replication but could be propagated in a transfected cell line stably transfected with *D4R* (212). Both the *D4R* ORF and its Shope fibroma virus homolog encode functional uracil DNA glycosylases

(502,528). Because these enzymes remove uracil residues that have been introduced into DNA, either through misincorporation of dUTP or through the deamination of cytosine, a DNA-negative phenotype was surprising.

Additional ts mutants impaired in DNA replication were derived by directed mutagenesis of the *A20R* gene (264). The protein encoded by this gene interacts in the yeast two-hybrid system with the DNA glycosylase and the D5R protein, both of which are involved in DNA replication (314). Traktman (517) cited evidence that the A20R protein is the DNA polymerase processivity factor previously detected in infected cell extracts (315).

Vaccinia virus encodes a functional ATP-dependent DNA ligase that is not essential for viral replication in tissue culture, although it affects virulence as well as sensitivity to DNA-damaging agents (110,256). Whether DNA ligation is unnecessary for replication or this requirement is fulfilled by the cellular homolog is unknown.

Three of the putative replication proteins encoded by orthopoxviruses (the DNA polymerase, nucleoside triphosphatase, and DNA glycosylase) have homologs in all sequenced poxvirus genomes pointing to their central roles. B1R protein kinase homologs are present in all sequenced poxviruses except for molluscum contagiosum virus; putative processivity factor homologs are present in all except entomopoxviruses; an ATP-dependent DNA ligase is encoded by leporipoxviruses but not by either molluscum contagiosum virus or entomopoxviruses. *Melanoplus sanguinipes,* however, encodes a putative NAD^+-dependent DNA ligase (1).

Concatemer Resolution

The replication of the poxvirus genome involves the formation of concatemeric intermediates and their resolution into unit-length molecules. The concatemer junction consists of a precise duplex copy of the hairpin loop present at the ends of mature DNA genomes (see Fig. 4). Studies with ts mutants of vaccinia virus, as well as specific inhibitors, indicate that concatemeric forms of DNA accumulate when late gene expression is prevented (135,329). Resolution occurs upon allowing late proteins to be made, providing evidence that the concatemers are replicative intermediates.

Circular plasmids containing vaccinia virus (328) or Shope fibroma virus (138) concatemer junctions are converted into linear molecules with hairpin termini when transfected into poxvirus-infected cells. Using this assay, the structural and sequence requirements for resolution of concatemer junctions were determined by site-directed mutagenesis (136,318,324,327). The minimal requirement for resolution is two copies of the sequence T_6-$N_{7–9}$-T/C-A_3-T/A present in an inverted repeat orientation on either side of an extended double-stranded copy of the hairpin loop. Structurally and functionally similar resolution sequences are present in at least four poxvirus genera: *Orthopoxvirus, Leporipoxvirus, Capripoxvirus,* and *Avipoxvirus* (Fig. 7). The sequence of the intervening region, destined to form the hairpin loop, is not highly conserved but must be palindromic and less than 200 bp long. Further studies suggested that resolution is accomplished either by conservative site-specific recombination and oriented branch migration (325) or by nicking and sealing of an extruded cruciform structure (318).

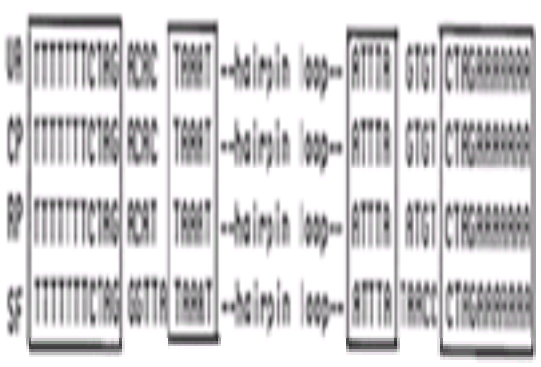

FIG. 7. Concatemer resolution sequence. The *boxed regions* represent conserved sequences necessary for concatemer resolution. Extended palindromic hairpin loop sequences are indicated. VA, vaccinia; CP, cowpox; RP, rabbitpox; SF, Shope fibroma.

Data showing that all conditional lethal mutants of vaccinia virus that are blocked in late gene expression are also defective in concatemer resolution at the nonpermissive temperature were interpreted as indicating a requirement for a specific viral late protein (135,329). However, the resolution sequence contains a functional late promoter (374,501) (compare Fig. 6 and Fig. 7). Therefore, it is unclear whether transcription of the resolution sequence, the translation product of a late transcript, or both are required.

There are at least three viral late proteins that might be components of a resolvase. One is a type 1 topoisomerase that structurally and functionally resembles its eukaryotic counterpart (39,261,470,475), except for a specificity for the sequence C/TCCTT (465). Similar sequence-specific topoisomerases are encoded by orf virus (260), entomopoxvirus (272,391), Shope fibroma virus (370), and molluscum contagiosum virus (226). Despite extensive biochemical analyses and evidence that the gene is essential for virus replication (466,471), its biologic role remains uncertain. The ability of poxvirus topoisomerases to resolve Holliday junctions, a key intermediate in both homologous and site-specific recombination, and generate hairpin ends suggest that this enzyme might be involved either in resolving concatemer junctions or in general recombination (370,449).

A 50-kd homodimeric DNase with nicking and joining activity (275,326,409) was recently identified as the product of the *K4L* ORF (M. Merchlisky, Food and Drug Administration, personal communication) and is present in vaccinia virus cores. The nicking and joining reaction requires no energy cofactor and generates 3'-P- and 5'-OH-termini. When the purified

enzyme was incubated with concatemeric junction fragments, cleavage occurred at the apex of the cruciform instead of at the base, which would be necessary for telomere formation (409).

Most recently, a bacterial-type Holliday junction resolvase was found to be encoded by the *A22R* gene of vaccinia virus and homologs are present in all poxviruses (174). *In vitro,* the recombinant A22R protein can resolve synthetic Holliday junctions or cruciform structures extruded by a supercoiled plasmid. Furthermore, a vaccinia virus mutant in which the *A22R* gene was repressed is defective in concatemer resolution (175).

Homologous Recombination

High rates of recombination occur within poxvirus-infected cells (168). Recombination has apparently occurred naturally between two leporipoxviruses—Shope fibroma virus, which produces benign fibromas in rabbits, and myxoma virus, the agent of myxomatosis—to form malignant rabbit fibroma virus (63), and between individual capripoxviruses (179). Recombination between the terminal sequences of poxvirus DNA may explain variations in the number of tandem repeats and translocations. Most extraordinarily, field and vaccine strains of fowlpox virus carry a nearly full-length and apparently infectious integrated avian retrovirus genome (204).

Recombination can also occur between virus-derived genomic DNA and transfected subgenomic DNA fragments, and this has been exploited to map and construct mutations and to insert genes for expression. Viral genomes rapidly eliminate direct repeats with the formation of intramolecular and intermolecular recombination products (31). Single- and double-crossover products, resulting from recombination between transfected plasmids and viral genomes (497), and intermolecular and intramolecular plasmid or bacteriophage DNA recombinants (162,373) have been detected in poxvirus-infected cells. Recombination does not require late gene products, and there appears to be a strong connection between recombination and replication (323,551). After phage lambda DNA was transfected into Shope fibroma virus–infected cells, heteroduplex formation coincided with the onset of both replication and recombination, suggesting that poxviruses make no clear biochemical distinction between these processes (170). Evidence for DNA strand exchange catalyzed by proteins from vaccinia virus–infected cells has been reported, and a derivative of the T4-replication–primed recombination model has been suggested (576). The roles in recombination of the poxvirus encoded topoisomerase and Holiday junction endonuclease, discussed in the previous section, remain to be determined.

DNA Replication Model

Although there are large gaps in our understanding of poxvirus DNA replication, the unique terminal structure of the poxvirus genome, evidence that initiation occurs near the ends of the molecule, and the presence of concatemer junctions in replicating DNA suggest a model similar to that proposed for replication of single-stranded parvovirus DNA. As depicted in Figure 8, a hypothetical nick occurring at one or both ends of the genome provides a free 3' end for priming. The replicated DNA strand then folds back on itself and copies the remainder of the genome. Concatemer junctions form by replication through the hairpin; very large branched concatemers can arise by initiating new rounds of replication before resolution occurs. Recombinational strand invasion may further contribute to the formation of complex multibranched molecules. After the onset of late-stage transcription, unit-length genomes are resolved, and the incompletely base-paired terminal loops, with inverted and complementary sequences, are regenerated.

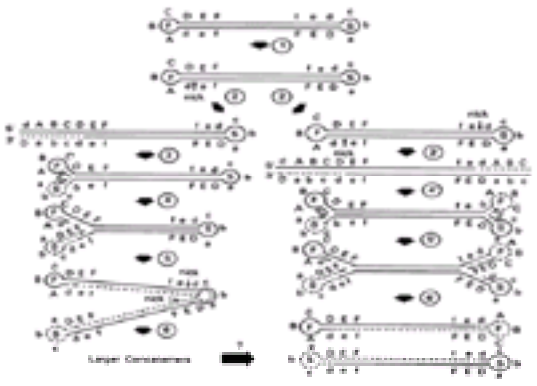

FIG. 8. Self-priming model for vaccinia virus DNA replication. *F* and *S* refer to the difference in electrophoretic mobilities of the two alternative inverted and complementary hairpin sequences present at the ends of the vaccinia virus genome. The scheme *on the left* assumes that the replication of a single DNA molecule is initiated at one end and continues to the other end to form a concatemer, whereas *on the right,* initiation occurs at both ends without concatemer formation. (From ref. 347, with permission.)

VIRION ASSEMBLY, MATURATION, AND RELEASE

Intracellular Mature Virions

Assembly begins in circumscribed, granular, electron-dense areas of the cytoplasm. The first morphologically distinct structure is a crescent (or cupulae in three dimensions) consisting of a membrane with a brushlike border of spicules on the convex surface and granular material adjacent to the concave side (Fig. 9, *panel 1*). Electron micrographs showing a single bilayer membrane with no apparent continuity with cellular organelles have been published by several groups (125,189,211). How such a membrane could form *de novo,* however, is unclear. One unprecedented and highly speculative possibility is the assembly of a protein lattice or scaffold with hydrophobic domains into which lipids could be deposited. An alternate view, more in line with cell biology, is that the viral membrane is composed of two tightly apposed membranes derived by a wrapping mechanism from the intermediate compartment between the endoplasmic reticulum

and Golgi network (434,492). A third scheme, in which vesicles bud from cellular organelles and then coalesce to form the viral membrane, is suggested by the accumulation of small vesicles in the viral factory area when morphogenesis is blocked by repression of the *A17L* (418,560) or *A14L* (419,520) genes. In subsequent stages of development, the crescent membranes are transformed into circular (or spherical in three dimensions) immature virions with a dense nucleoprotein mass embedded in a granular matrix (see Fig. 9, *panels 2 and 3*). Occasional electron-microscopic pictures suggest that the nucleoprotein enters the immature envelopes just before they are completely sealed (336). Further steps in maturation are inferred from selected images (see Fig. 9, *panels 4 to 6*).

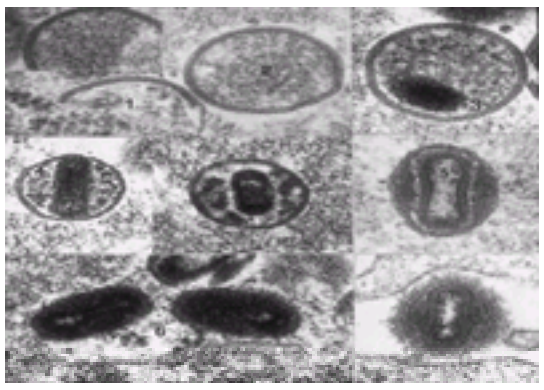

FIG. 9. Electron micrographs of developing and mature virus particles in thin section. (Courtesy of P. Grimley, Uniformed Services University of the Health Sciences.)

Studies of the early stages of morphogenesis have been facilitated by the combined use of drugs or conditional lethal mutants to arrest assembly and immunoelectron microscopy to locate viral proteins in the abortive structures. No viral membranes can be detected at their usual time of formation when viral DNA replication is stringently inhibited, consistent with the finding of late promoters on the membrane proteins. The earliest defect in morphogenesis occurs with a ts F10L kinase mutant (518,542). At the nonpermissive temperature, late viral proteins were made, but recognizable viral membranes were not seen. A similar phenotype was also described for a ts mutant in the *H5R* gene (139), which was surprising because other studies had indicated a role for this protein in RNA synthesis. When synthesis of the A17L or A14L proteins, which associate with each other, is repressed, small vesicles accumulate instead of a continuous membrane (418,419,520,560). There is, however, some difference in the locations of the vesicles that form with the two mutants and the block appears to be more stringent with the A17L mutant. Both the A17L and A14L proteins are phosphorylated apparently by the F10L kinase (55,143). In the presence of the antibiotic rifampicin, irregular viral membranes that lack spicules accumulate (345,358) (Fig. 10) Within minutes after removal of the drug, the membranes become spicule coated and assume a regular crescent shape (see Fig. 10), even in the presence of inhibitors of RNA or protein synthesis, suggesting that rifampicin directly interferes with assembly (189). The gene responsible for resistance of mutant viruses to rifampicin has been mapped to the *D13L* ORF, which encodes a 65-kd protein (30,509) that rapidly associates with the rifampicin body membranes after drug removal (332,493,534). When expression of the *D13L* ORF is repressed, morphogenesis of the viral envelope is blocked at the same stage as occurs with rifampicin (578). The 65-kd protein appears to concentrate on the inner side of the membrane, perhaps serving as a scaffold. A block at later stages, in which crescents and circular immature virus particles accumulated, occurred when expression of the A9L (572), L1R (406), or H3L (121,286) membrane protein was repressed.

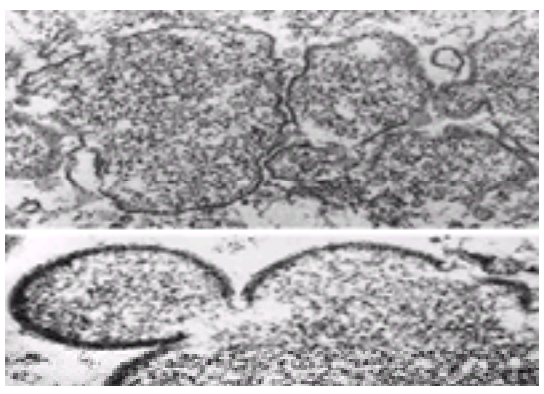

FIG. 10. Electron micrographs of thin sections of cell infected in the presence of rifampicin. **Upper panel:** HeLa cells were fixed and sectioned at 8 hours after infection in the presence of rifampicin. **Lower panel:** Cells were fixed and sectioned at 10 minutes after removal of rifampicin. (From ref. 189, with permission.)

Several vaccinia virus major core proteins have been detected within the immature particles visualized by immunoelectron microscopy (412,534) or isolated by sedimentation (442,535). Pulse-chase experiments indicated that several late polypeptides, including major structural proteins of the core, undergo proteolytic processing (343) at Ala-Gly-Ala/Ser motifs (280). Processing of the major structural proteins is coupled to virus assembly because their cleavage is prevented by rifampicin or mutants that interfere with early steps in morphogenesis (252). Repression of synthesis of certain core proteins, such as the F17R phosphoprotein or the I1L DNA-binding protein, halts assembly at an immature particle stage and prevents proteolytic processing (262,579). Under nonpermissive conditions, ts mutants with substitutions in the *I7L* ORF appear to be blocked at a slightly earlier stage, but protein processing was not examined (246). Repression of the *A32L* gene, a predicted ATPase, interfered with the packaging of DNA and led to the

formation of aberrant particles (95). The question of how the transcription apparatus becomes enclosed within the core of the assembling virus particle is intriguing. It seems unlikely that each enzyme has its own targeting signal. A clue to possible mechanisms was obtained by examining the noninfectious virus particles that formed when synthesis of RAP94, the RNA polymerase-associated protein that confers specificity for early promoters, was repressed. Such particles contained an apparently complete set of structural proteins as well as the early transcription factor VETF but lacked the viral RNA polymerase, poly(A) polymerase, capping enzyme, topoisomerase, NPH I, and NPH II (577). Such a specific effect could result if these proteins formed a complex that associates with promoter-bound VETF through RAP94. Evidence for an interaction between NPH I and RAP94 has been obtained (334). Reduced amounts of RNA polymerase in virions formed with a ts mutation in VETF (285) is also consistent with the latter model. Repression of synthesis of either subunit of VETF, however, leads to a more severe defect with accumulation of immature virus particles (221,222).

Because disulfide bonds usually form in the endoplasmic reticulum, the presence of intramolecular and intermolecular disulfides in cytoplasmic vaccinia virus core and membrane proteins (229,291,416,561) raise the possibility that poxviruses encode novel thiol oxidoreductases. Proteins with predicted C-X-X-C redox motifs include the vaccinia virus O2L and G4L glutaredoxins, which have been shown to have thiol transferase activities *in vitro* (9,195), and the E10R protein, a member of the eukaryotic ERV1/ALR family (450). Of these, the G4L and E10R proteins are conserved in all poxviruses and are essential for morphogenesis (452,547). Recent data suggest that the E10R and G4L proteins are components of a cytoplasmic disulfide bond pathway (453).

Occluded Virions

The IMV of some *Chordopoxvirinae* (e.g., cowpox, ectromelia, and fowlpox) become occluded in a dense protein matrix referred to as *A-type inclusions* to differentiate them from the sites of virus replication and assembly, which are sometimes called *B-type inclusions* (250). Presumably, the A-type inclusions are released into the environment after degeneration of infected cells. The major A-type inclusion protein of cowpox virus is a 160-kd species that may represent up to 4% of the total cell protein at late times after infection (172,377). Some cowpox virus mutants form inclusions without virions, implicating a role for the IMV-specific protein 4C (461,525). Although vaccinia virus does not form A-type inclusions, a smaller homologous protein is made because of a frameshift mutation (17,133). Both the cowpox and vaccinia virus inclusion proteins are myristylated (306).

The virions of *Entomopoxvirinae* are also occluded to protect them from the environment (51). After ingestion by a new larval host, infectious particles may be released in the alkaline pH of the gut. The sequences of homologous occlusion proteins, called *spheroidin* or *spherulin,* have been deduced from the ORFs of several entomopoxviruses (1,33,198,199,441). These proteins are cysteine-rich, about 100 kd, and lack homology to fusolin, the abundant 50-kd spindle-body protein of entomopoxviruses (127), the A-type inclusion protein of chordopoxviruses, or the polyhedrin protein of baculoviruses.

Extracellular Enveloped Virions

The movement of IMV out of the assembly areas to the cell periphery is microtubule dependent and requires the A27L membrane protein (438). The IMV become wrapped by additional membranes, derived from the trans-Golgi or early endosomal network, that contain viral proteins (206,230,336,443,516). Studies with mutant vaccinia viruses indicate that wrapping requires expression of at least one IMV membrane protein, the A27L 14-kd protein (417), and two EEV membrane proteins: the F13L 37-kd phospholipase (25,60,424) and the B5R 42-kd glycoprotein (156,558). Fusion of the wrapped particles with the plasma membrane results in the externalization of the virus, with loss of the outermost Golgi-derived membrane. Only some of the externalized virions are found in the medium as EEV, whereas the rest adheres to the cell surface (CEVs). The ratio of adherent to released virions varies with different cell hosts and different vaccinia virus strains (379), largely because of a single amino acid difference in the putative lectin-binding domain of the membrane protein encoded by the *A34R* ORF (62). The extracellular virions are important for virus dissemination (68,380). On cell monolayers, the CEVs can mediate efficient cell-to-cell spread and plaque formation, whereas the EEVs provide long-range dissemination (61).

The efficient cell-to-cell spread of CEVs appears to be due to their location at the tip of motile, actin-containing microvilli that have been visualized by fluorescence and electron microscopy (119,207,499). When the *A33R, A34R,* or *A36R* ORFs are not expressed, actin-containing microvilli are not formed, resulting in a small plaque phenotype (423,436,559,563). Nucleation of the actin tails depends on tyrosine phosphorylation of the A36R protein by Src-family kinases: the phosphorylated A36R protein interacts with the adaptor protein Nck, which results in the recruitment of the Ena/VASP family member N-WASP to the site of actin assembly (171).

VIRUS–HOST INTERACTIONS

Inhibitory Effects on Host Macromolecular Synthesis

Infection of tissue culture cells with vaccinia virus or other orthopoxviruses results in profound cytopathic effects (21), changes in membrane permeability (93), and inhibition of DNA, RNA, and protein synthesis. The effects on protein synthesis are dramatic (161,346). It seems likely that several factors may lead to the switch from host to viral protein synthesis and that the relative contribution of each factor may depend on the virus multiplicity, cell type, time of analysis, and use of metabolic inhibitors. Some experiments suggested that inhibition of host protein synthesis could occur in the absence of viral gene expression, thereby implicating a protein in the vaccinia virus particle. Candidate inhibitors are the surface tubules (313), the F17R phosphoprotein (389), and the B1R protein kinase (49). There is indirect evidence that some viral early proteins may allow translation of viral mRNA to continue under conditions that inhibit host protein synthesis (48,342). Other studies suggested that viral transcription is necessary for inhibition of host protein synthesis. For example, high concentration of actinomycin D and cordycepin prevented this inhibition (22). Also, in some cell lines, inhibition of host protein synthesis remains incomplete until after DNA replication. An inhibitory role for small poly(A)-containing RNA molecules was originally proposed to explain the effects on host protein synthesis that occur in

the presence of actinomycin D or by ultraviolet-irradiated virus (184,430). Subsequent reports, however, suggested that small poly(A)-containing RNAs may have an inhibitory role during normal virus infection (23,24,87). After several hours, most of the mRNA present in the cytoplasm of infected cells is viral, accounting for the predominance of viral protein synthesis regardless of translational effects (65,116). The shift in mRNA species results from the short half-life of all mRNAs following vaccinia virus infection (50,447), coupled with very active virus transcription and inhibition of host RNA synthesis and transport. Rapid degradation of actin and tubulin mRNAs has been demonstrated (411). Viral inhibitory effects on the synthesis or processing of cellular RNAs have been reported (50,239,447). This inhibition involves all classes of RNA and requires vaccinia virus protein synthesis; heated or ultraviolet irradiated virus was ineffective (384). The inhibition was correlated with the transit of RNA polymerase II activity from the nucleus to the cytoplasm (338,554). However, whole cell extracts of vaccinia virus–infected cells were no longer able to transcribe genes regulated by eukaryotic RNA polymerase II promoters, although they were still able to transcribe a polymerase III promoter (399). Nuclear DNA replication is also inhibited (144,244). Under certain nonpermissive conditions of vaccinia virus infection, the inhibition of host macromolecular synthesis may be accelerated. These situations include the restricted growth of vaccinia virus in Chinese hamster ovary cells, which can be overcome by the cowpox virus gene encoding a 77-kd protein (494); host range mutants of rabbitpox virus (351); and vaccinia virus K1L deletion mutants (185,387,503).

Stimulatory Effects on Cell Growth

Many poxviruses induce hyperplastic responses and even tumors in the skin of infected animals. These effects are pronounced with fowlpox virus (103), Shope fibroma virus (464), Yaba virus (364), and molluscum contagiosum virus (397). In the case of orthopoxviruses, the hyperplasia is due to secretion of a homolog of EGF (83,84,500). Leporipoxviruses have structural and functional homologs of EGF that are nonessential for replication in tissue culture but contribute to virulence (102,289,368). A gene encoding a mitogenic polypeptide with homology to mammalian vascular endothelial growth factor is present in the orf virus genome (555).

Viral Defense Molecules

Host immune responses can halt the spread of viruses and eliminate them. Immediately after virus invasion, nonspecific mechanisms involving interferons (IFNs), complement, cytokines, and natural killer cells predominate; subsequently, cytotoxic T cells and antibodies assume importance in defense (85). In response to these selective pressures, poxviruses encode multiple proteins that interfere with the induction or activity of complement and the principal cytokines (Table 5). The inhibitory proteins fall into three main classes: virokines, which are secreted from infected cells and resemble host cytokines or soluble immune regulators (268); viroceptors, which are altered cellular receptors that have lost their transmembrane anchor sequences and consequently are secreted from infected cells and sequester ligands (526); and intracellular proteins that interfere with signaling or effector pathways (407).

Virus protein	Role	Virus/Gene	Selected references
Secreted complement binding protein	Binds C4B, C3B; inhibits complement activation	Orthopox (VAC C3L)	236, 267, 268, 320, 433
Soluble IFN-γ receptor	Binds and antagonizes IFN-γ	Orthopox (VAC B8R); Leporipox (MYX M-T7)	12, 349, 350, 527
Soluble IFN-α/β receptor	Binds and antagonizes IFN-α/β	Orthopox (VAC B18R)	108, 504
Soluble TNF receptor	Binds and antagonizes TNF-α	Orthopox (CPV cmv5); Leporipox (MYX M-T2)	219, 293, 301, 485, 486, 526
Soluble IL-1β receptor	Binds and antagonizes IL-1β	Orthopox (VAC B15R)	10, 11, 496
Secreted IL-18–binding protein	Binds and antagonizes IL-18	Molluscipox (MC54L); Orthopox (ECTV v013/16)	67, 490, 569, 570
Secreted chemokine–binding protein	Binds and antagonizes CC chemokines	Orthopox (VAC 35 kD, Leporipox (M-T1)	13, 91, 186, 487
Secreted chemokine homolog	Binds and antagonizes CCR8	Molluscipox (MC148)	129, 279, 297
dsRNA-binding protein	Prevents activation of protein kinase PKR and 2,5A synthetase	Orthopox (VAC E3L)	45, 96, 190, 455
EIF-2α homolog	Inhibits protein kinase PKR	Orthopox (VAC K3L)	46, 54, 170
Caspase inhibitor	Inhibits caspase 1 to prevent processing of IL-1β and IL-18 precursors and procaspase 8 to block TNF and Fas-induced apoptosis	Orthopox (CPX crmA); Leporipox (MYX SERP2)	237, 265, 392, 407, 511, 581
Serine protease inhibitor	Inhibits serine proteases	Leporipox (MYX SERP1)	392, 393
FLIP (FLICE-like inhibitory protein)	Binds FADD and procaspase 8 to prevent TNF and Fas induced apoptosis	Molluscipox (MC159)	52, 220, 310
Glutathione peroxidase	Hydrolyzes peroxides from inflammatory cells or UV rays	Molluscipox (MC66)	469
Class I MHC homolog	Binds β2-microglobulin	Molluscipox (MC80R)	451

IFN, interferon; TNF, tumor necrosis factor; IL, interleukin; dsRNA, double-stranded RNA; EIF, eukaryotic translation initiation factor; FADD, Fas-associated death domain protein; FLICE, FADD-like IL-1β converting enzyme; MHC, major histocompatibility complex.

TABLE 5. *Poxvirus immune defense molecules*

Complement Regulatory Protein

One of the major secreted proteins of vaccinia virus consists largely of four, tandem, inexact copies of a 60–amino acid sequence known as the *short consensus repeat* (SCR) that is found in complement regulatory proteins (268). This vaccinia viral protein, called VCP, inhibits the classic and alternative pathways of complement activation through its ability to bind and inactivate both C4B and C3B (267,320,433) and contributes to virulence (236). B5R, an EEV membrane protein of vaccinia virus also has SCRs, but there is no evidence that it has complement inhibitory activity. Host proteins incorporated into the EEV membrane could also inhibit complement activation (531).

Inhibitors of Interferon and Interferon Transduction Pathways

Secreted proteins that bind to type I or II IFNs are encoded by poxviruses of several different genera. The IFN-g–binding proteins of myxoma virus (M-T7) and orthopoxviruses (vaccinia B8R) are similar in sequence to the extracellular domain of the IFN-g receptor and consequently bind IFN-g (12,350,527). Deletion of the gene encoding the IFN-g–binding protein of myxoma virus greatly attenuates disease in European rabbits (349). A protein from the supernatant of cells infected with tanapoxvirus has been reported to bind IFN-g and also interleukin-2 (IL-2) and IL-5 (157).

A type I IFN inhibitor is present in the supernatants and on the surfaces of cells infected with vaccinia and other orthopoxviruses (108,504). The protein has some sequence similarities with regions of cellular type I IFN receptor and binds IFN-a with high affinity but less species specificity than the receptors. Vaccinia viruses with a deletion of the type I IFN-binding protein gene are attenuated in mice, suggesting that the protein is a virulence factor.

Orthopoxviruses encode a double-stranded RNA-binding protein (e.g. vaccinia virus E3L) that prevents the activation of the IFN response protein kinase (PKR), which inhibits translation initiation (99,100,455). Vaccinia virus E3L deletion mutants show host range restriction and enhanced apoptosis, RNA degradation, and IFN sensitivity (45,258). Orthopoxviruses also encode a homolog of the translation initiation factor eIF-2a (e.g., vaccinia virus K3L) that inhibits eIF-2a phosphorylation by PKR (94,129). Deletion of K3L increases the IFN sensitivity of vaccinia virus (46). Therefore, the E3L and K3L proteins have overlapping roles in blocking IFN action.

Interleukin-18–Binding Proteins

IL-18 induces IFN-g production, acts in synergy with IL-12 to induces natural killer (NK) cell activation, T-cell activation and helper T-cell type 1 response polarization, and can protect mice from vaccinia virus infection (508). molluscum contagiosum virus (MCV) and orthopoxviruses encode secreted proteins that are homologous to the mammalian IL-18–binding protein and that bind IL-18 and block IFN-g production (67,490,569,570). An ectromelia deletion mutant induced elevated intraperitoneal NK cell activity and cytokine production and decreased virus replication compared with wild-type virus (67).

Tumor Necrosis Factor Receptor Homologs

Tumor necrosis factor (TNF) binds to its cognate receptors and induces proinflammatory responses or death of virus-infected cells. Leporipoxviruses (301,485,526) and orthopoxviruses (219,293,486) encode one or more soluble type II TNF receptor homologs that can block TNF-a–mediated cytolysis *in vitro* and attenuate virulence.

Interleukin-1 Receptor Homologs

IL-1b mediates a broad response to virus infection by binding to a high-affinity cellular receptor and triggering a signal transduction pathway. Orthopoxviruses encode homologs of the IL-1 receptor that bind IL-1b (11,496). Depending on the route of inoculation, deletion of the gene can reduce or enhance virulence (10,496). Frameshift mutations in the variola homolog preclude synthesis of an active protein.

SERPINS and Inhibitors of Cytokine Processing

IL-1b and IL-18 are produced by caspase-1 cleavage of inactive precursors. CrmA (also called SPI-2), a member of the SERPIN superfamily encoded by cowpox virus, was identified by its antiinflammatory properties and shown to inhibit caspase-1, a cysteine aspartic protease, and activation of IL-1b (265,407). An effect on activation of IL-18 is presumed, although it has not been shown directly. Further studies showed that CrmA protects cells against apoptosis by inactivation of additional caspases (581). Other orthopoxviruses and leporipoxviruses also encode CrmA homologs that inhibit caspase-1 (257,392). Attenuation of cowpox or rabbitpox viruses results from deletion of the *crmA* gene (515).

Orthopoxviruses and LeporIpoxviruses encode additional members of the SERPIN superfamily. SPI-1 and SPI-3 have been shown to complex with certain proteinases *in vitro,* but whether these proteinases are important targets *in vivo* is unknown (335,523). Deletion of SPI-1 causes a host range effect and nuclear changes but not other signs of apoptosis (335,462). Deletion of the *SPI-3* gene induces fusion of infected cells, but this activity does not require the SERPIN active site (523). Another SERPIN (SERP-1), encoded by leporipoxviruses, is secreted from infected cells, interferes with inflammation, and is required for virus-induced disease in rabbits (302). SERP-1 inhibits a number of serine proteases (plasmin, urokinase, tissue plasminogen activator, and a member of the complement cascade) *in vitro*, but whether any or all of these are significant biologic targets *in vivo* is unknown (292).

Chemokine Inhibitors

MCV encodes a CC chemokine homolog (MC148) that is predicted to be an antagonist based on the absence of the conserved N-terminal region (450). MC148 was found to bind specifically to the CCR8 chemokine receptor and to inhibit competitively the binding of I-309, the natural ligand (297). Other reports, based mainly on chemotaxis assays, suggest a broader activity (128,270).

Secreted proteins that bind to CC chemokines but lack sequence similarity to chemokine receptors are encoded by leporipoxviruses (M-T1) and orthopoxviruses (35-kd proteins) (13,91,188,487). These proteins prevent activation and chemotaxis of leukocytes. An influx of inflammatory cells into tissues infected with a rabbitpox virus 35-kd protein deletion mutant was described (188), although no difference in virulence was detected (309).

M-T7, the myxoma virus IFN-g receptor–binding protein, also interacts with chemokines but with low affinity through the heparin-binding site, suggesting that it could alter their tissue localization (276). The influx of leukocytes into tissues infected with a M-T7 deletion mutant could be due to loss of either the chemokine or IFN-g–binding activities.

Genes encoding homologs of seven transmembrane receptors have been identified in swinepox and capripox viruses (90,310), although their ligand-binding properties are unknown.

Apoptosis Inhibitors

CrmA was described previously as an inhibitor of caspase-1, the IL-1b–converting enzyme. However, CrmA and other orthopoxvirus homologs also block apoptosis activated through the Fas and TNF receptors (145,333,512) and potently inhibited caspase-8, the apical caspase in these pathways (581). CrmA also binds granzyme B, a serine protease associated with lytic granules released by cytotoxic T lymphocytes (CTLs) (400). It is difficult to evaluate the specific roles of CrmA during a natural infection. CrmA can protect target cells against Fas-mediated lysis by alloreactive CTLs

(300,512), but wild-type cowpox or ectromelia virus–infected target cells are not protected against lysis by class I major histocompatibility complex restricted CTLs (353). Although *crmA* deletion mutants replicate normally in most cell lines ithout inducing apoptosis, a cowpox *crmA* deletion mutant induced apoptosis in a pig kidney cell line but without affecting the virus yield (299,408). The effect on virulence caused by deletion of *crmA* or *SPI-2* has varied in different animal models (257,515).

MCV encodes two proteins with death effector domains (DEDs) (450). DEDs are also present in FADD and procaspase-8 and mediate their interaction after the binding of TNF or FasL to their receptors. One of these MCV proteins, MC159, binds FADD and protects transfected cells against death effector filament formation and apoptosis induced by Fas and other members of the TNF receptor superfamily (52,220,513,522). The role of the second DED-containing protein, MC160, is uncertain.

MCV encodes a selenocysteine-containing protein, MC66, that is homologous to cellular glutathione peroxidase and that can protect cells against the cytotoxic effects of hydrogen peroxide and ultraviolet (UV) irradiation (450,463). Peroxides may be formed by activated inflammatory cells and by UV light. A putative glutathione peroxidase is also encoded by fowlpox virus (2).

Ectromelia virus encodes a protein called p28 that contains a really interesting new gene (RING) finger motif that is required for virulence in mice (454) and that acts upstream of caspase-3 to block UV-induced apoptosis (69). Overexpression of the Shope fibroma virus RING finger protein N1R can also block apoptosis induced by UV light (70).

Several unrelated myxoma virus proteins prevent apoptosis. The protein encoded by the *M11L* gene localizes in the mitochondrial membrane and is required for virulence, to block myxoma-induced apoptosis of primary rabbit monocytes and staurosporine-induced apoptosis activation of caspase-3 (166,367). The M-T4 protein localizes to the endoplasmic reticulum of infected cells and is required for virulence and to prevent induction of apoptosis in a rabbit CD4+ T-cell line or primary rabbit lymphocytes (38,208). The M-T5 protein has a single ankyrin motif, is related to orthopoxvirus host range genes, and is required to prevent apoptosis in a rabbit CD4+ T-cell line (348).

CHOhr is a host range gene of cowpox virus that is disrupted or deleted from vaccinia virus strains and that is required for vaccinia virus replication in Chinese hamster ovary (CHO) cells (494). In the absence of the host range gene, there is a rapid cessation of host and viral protein synthesis and accelerated apoptosis in vaccinia virus–infected CHO cells (234,365,404). Apoptosis was induced at an unidentified viral postbinding step (403) and could be prevented by expression of the adenovirus *E1B-19K* gene or *Bcl2* gene in RK-13 (107,234).

The *E3L* gene of vaccinia virus was described previously as a double-stranded RNA-binding protein that protects against the action of protein kinase PKR and provides resistance to the action of IFN. *E3L* deletion mutants induced apoptosis in HeLa cells through failure to inhibit PKR (258,281).

EXPRESSION VECTORS

Several attributes of poxviruses have led to their extensive use as expression vectors (339). These include relative ease of formation and isolation of recombinant viruses, capacity for large amounts of DNA, relatively high expression, and wide host range. Expression has been achieved either by using poxvirus promoters or by employing bacteriophage RNA polymerases and cognate promoters. Recombinant viruses have been used for the synthesis of proteins *in vivo* or *in vitro* and as vaccine candidates.

CONCLUSIONS

The poxviruses are among the largest and most genetically complex of all animal viruses. Unlike most other DNA viruses, they replicate in the cytoplasm and encode many proteins that permit transcription and replication to occur outside of the nucleus. Gene expression is stringently regulated by a cascade mechanism, and virion assembly is a complex process that involves the formation of multiple membranes. Viral proteins are used for evasion of the host immune defense system.

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CHAPTER 85 Poxviruses

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The *Poxviridae* family comprises two subfamilies of double-stranded DNA cytoplasmic viruses that infect arthropods (*Entomopoxvirinae*) or vertebrates (*Chordopoxvirinae*). Vertebrate viruses are brick-shaped or ovoid (Fig. 1). Currently, there are eight recognized genera of vertebrate poxviruses (Table 1), which lack immunologic cross-protection (158). Species within each genus have distinctive biologic traits and genome sequences and may differ in geographic distribution and host range. Some vertebrate viruses may spread systemically, producing generalized dermal lesions after aerosol droplet infection of the respiratory epithelium or infection of the skin by contact; others produce single or multiple localized skin lesions after contact with infected animals or humans, mechanical transmission by insects, or exposure to contaminated fomites.

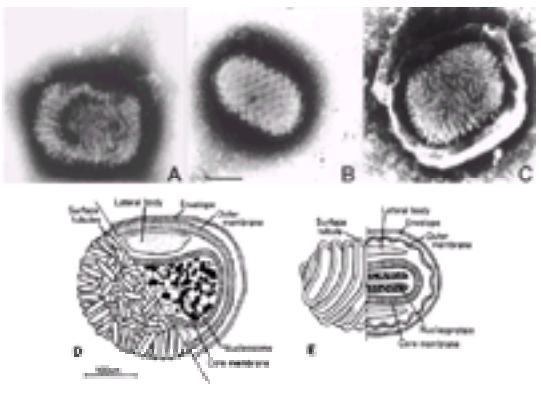

FIG. 1. Virion structure of orthopoxviruses (**A**), parapoxviruses (**B**), and yatapoxviruses (**C**) by negative staining and by diagram in **D** and **E.** The virion morphology of orthopoxviruses and yatapoxviruses is similar to the molluscipoxvirus (MCV; not shown)—intracellular mature virions (IMVs) are knobby and brick-shaped. Clinical material containing the yatapoxvirus tanapox virus often shows extracellular enveloped virions (EEV) (**C**); IMVs are more commonly seen in clinical material containing orthopoxviruses (**A**) or MCV. Parapoxviruses are ovoid and smaller, with a distinctive regular surface tubule that gives a criss-cross appearance (**B**). **D:** Drawing of brick-shaped virion, for example, vaccinia virus. Viral genome DNA and several proteins within the core are organized as a nucleosome. The core has a 9-nm thick membrane with a regular subunit structure. By negative staining, the virion core assumes a dumbbell shape with large lateral bodies occupying the concavities. The core and lateral bodies are enclosed within a lipoprotein shell, the outer membrane, which is about 12-nm thick. The outer membrane surface appears to consist of irregularly arranged surface

tubules, which produce the knobby or mulberry-like form. Most orthopoxvirus and MCV particles remain cell- associated and may be released by cytolysis; however, some virions may be released enclosed in an envelope (EEVs) composed largely of host cell Golgi membrane material associated with virus-specific proteins. **E:** Drawing of ovoid virion, for example, orf virus. The outer membrane consists of a single long tubule that appears to be wound around the particle. Negative-stained virions often show a criss-cross filament pattern as in **B**, because the opposing faces of the particle filament are visualized. The envelope is usually closely applied to the surface of the outer membrane (bars = 100 nm). (**A** courtesy of F.A. Murphy; **B** to **E** from ref. 73, with permission.)

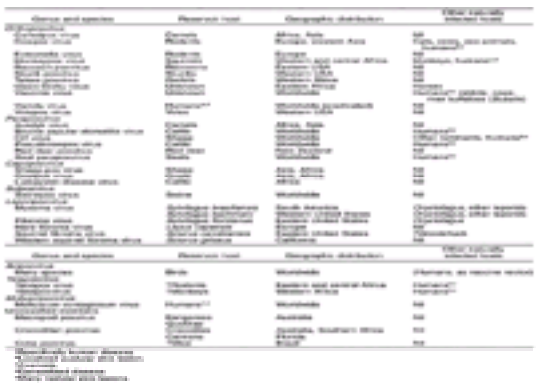

TABLE 1. *Host range and geographic distribution of genera and unclassified members of the subfamily Chordopaxvirinae*

Four genera contain species that may infect humans: *Orthopoxvirus, Parapoxvirus, Yatapoxvirus,* and *Molluscipoxvirus.* Four *Orthopoxvirus* species, three of which are zoonotic, may infect humans (162): (a) variola virus, the cause of smallpox, which is eradicated, is a strictly human pathogen producing a febrile pustular rash illness (72); (b) monkeypox virus, which is reported from nine West and Central African rainforest countries, mainly the Democratic Republic of Congo (DRC, formerly Zaire), is a zoonotic agent of a smallpox-like illness of low interhuman transmissibility (110); (c) cowpox virus is rodent-borne and indigenous to rodents in several European and a few western Asian countries, where humans appear to acquire a localized pustular skin infection by contact with infected rodents, or more likely, intermediate hosts, such as pet cats, cows, or other animals (9,40); and (d) the vaccinia virus subspecies buffalopox virus, which occurs mainly on the Indian subcontinent, causes localized oral or skin lesions after contact with infected dairy animals or drinking their milk (59,128). In addition, vaccinia virus strains used for smallpox vaccination form a dermal pustule and may cause postvaccinal side effects.

Members of the other two genera, *Parapoxvirus* and *Yatapoxvirus,* infect humans, usually as a zoonosis, and cause localized skin nodules. Three *Parapoxvirus* species may infect humans occupationally worldwide: (a) orf virus, which is transmitted by infected sheep, goats, or wild artiodactyls; (b) pseudocowpox virus (paravaccinia virus), which is transmitted by infected dairy cows, causing milker's nodule; and (c) bovine papular stomatitis virus, which is transmitted by infected beef cattle (192,193). Two species of *Yatapoxvirus,* tanapox virus and Yaba monkey tumor virus (YMTV), cause fever and localized single or multiple lesions after transmission, possibly by insects from infected African primates (57,109,198). Finally, molluscum contagiosum virus (MCV), the sole *Molluscipoxvirus,* is a strictly human virus that occurs worldwide, causing papular skin lesions by contact, sometimes sexually, and is an opportunistic infection in acquired immunodeficiency syndrome (AIDS) (13,183).

ORTHOPOXVIRUS INFECTIONS IN HUMANS

History

Smallpox

Fenner and colleagues (72) have provided a comprehensive description of smallpox and its eradication, and Hopkins (98) has described the historical, social, and political impact of the disease. Smallpox appears to have emerged some 5,000 years ago, presumably from wild animals, possibly by adaptation to an increasing human population of a rodent cowpox-like virus through an intermediate host such as cattle, which were domesticated some 12,000 years ago. The genome sequences of recent cowpox virus isolates appear to be the antecedents of those in other orthopoxviruses (204); including b-1 phylogeny inference of sequences for the hemagglutinin protein (Fig. 2). Moreover, a sign that the progenitor of variola virus was rodent-borne, like cowpox virus, appears in its DNA sequences (145), which encode three orthologs of the murine interstitial A-particle, long terminal repeat-promoted, placental-expressed (MIPP) protein.

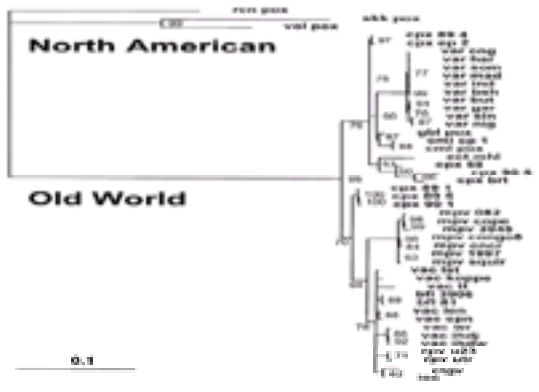

FIG. 2. Inferred phylogeny of orthopoxviruses. The open reading frames (~971-bp) coding for the hemagglutinin (HA) protein of different orthopoxviruses were compared by constructing an unrooted tree (S. Ropp, J. Esposito, et al., unpublished) using aligned (ClustalX) base sequences for input into the maximum likelihood software Puzzle 4.02 (218), opting for the Hasegawa model of substitution, neighbor-joining, a uniform rate heterogeneity model, raccoon poxvirus as the outgroup, and 100,000 tree-puzzling steps to obtain quartet maximum likelihood trees and a quartet puzzling tree with statistically significant taxon partition frequencies; the scale represents relative genetic distance. Virus sequences used for the analysis were the North American isolates raccoon poxvirus (*rcn-pox*); skunk poxvirus (*skk-pox*); and volepox virus (*vol-pox*), and the following strains or isolates of Old World orthopoxviruses: ectromelia mousepox Mill Hill (*ect-mhl*); cowpox from animals or humans (*cpx-89-1, cpx-89-4, cpx-89-5, cpx-90-1, cpx-90-5, cpx- ep-2, cpx-58*), and cowpox Brighton (*cpx-brt*); human monkeypox from West Africa (*mpv-082, mpv-3945, Congo-8, mpv-1997*), from an African squirrel (*mpv-squir*), strain Copenhagen (*mpv-cope*), and a red pock mutant of Congo-8 (*mpv-cncr*); vaccinia strains Lister (*vac-lst)*, Statens Serum Institute Copenhagen (*vac-koppe*), Lenny (*vac-len*), Copenhagen GenBank No. M35027 (*vac-cpn*), Western Reserve (*vac-wr*), International Health Department vaccinia variants (*vac-ihdw* and *vac-ihdj*), Chinese TianTan (*vac-tt*), rabbitpox Utrecht (*rpv-utr*), mutant rabbitpox (*rpv-u23*), buffalopox isolates (*bfl-3906* and *bfl-81*), the Brazilian smallpox vaccine strain (*ioc*), and a recent vaccinia-like isolate from Brazilian cattle and infected farmers (*ctgv*); variola virus isolates Congo (*var-cng*), Harvey (*var-har*), Madagascar (*var-mad*), Somalia (*var-som*), Bangladesh (*var-bsh*), India (*var-ind*), Butler (*var-but*), Brazil-Garcia (*var-gar*), Nigeria (*var-nig*), Sierra Leone (*var-sln*); Dahomey, Africa *Tatera kempii* gerbilpox (*gbl-pox*), and camelpox from Somalia (*cml-pox*) and Iran (*cml-cp-1*). Except for ioc and ctgv (sequences courtesy of N. Moussatché), the origins of the viruses are described elsewhere (62,125,154,181,196).

The earliest descriptions of smallpox date to western Asia and India about 3,000 years ago. By 700 A.D., smallpox had spread to China and Japan in the East and Europe and Northern Africa in the west. New World colonization provided for devastating transmission among Amerindians after introduction of the disease into the Caribbean by African slaves in 1518, with spread to Mexico and Peru by 1524, and its independent introduction from Africa into Brazil in 1555. From 1617, there were repeated introductions into North America from Europe and to a lesser extent from Africa, where slave trade had spread smallpox south of the Sahara in the 16th and 17th centuries. In 1713, infected travelers on a boat from India introduced smallpox into South Africa, and in 1789, Macassan visitors to northern Australia were likely the source of an epidemic among Aboriginals near the first European settlement in Sydney a year after the First Fleet arrived (32).

About 1000 A.D., smallpox spawned one of the first effective preventative measures for an infectious disease—immunization by intranasal insufflation of dried smallpox scab material in China, and in India the more popular practice of “inoculation” or “variolation,” which used pustule fluid or scab material applied variously into the skin. Such operations produced smallpox if the material contained active virus, but for unclear medical reasons, the consequent illness produced case fatalities of up to 2% rather than fatalities of up to 30% after natural infection usually acquired by aerosol droplet. The incubation period of variolation smallpox was much shorter than the 12 to 14 days normally associated with the appearance of the rash after a natural infection, and the rash often resolved more rapidly. Variolation did not change the virulence of the virus because contacts of mild cases of inoculation smallpox may have had severe illness and died. Despite the risks, the small-scale practice of variolation, which was used for several centuries in China, India, and western Asia, was introduced into Europe by the early 1700s and became common in some countries, notably England.

In 1798, following on folk medicine, which recognized that milkmaids who acquired cowpox were resistant to smallpox, Edward Jenner demonstrated protection from variolation by prior inoculation using cowpox lesion material, or as he called it, *variolae vaccinae* (smallpox of the cow) (108). The milder procedure, termed *cowpoxing,* was at first renounced by entrepreneur variolators, but it eventually became popular. By the late 19th century, vaccinia virus became the component virus in vaccine used virtually everywhere, but the historical details that changed the immunizing agent and the origin of vaccinia virus are obscure (8,92).

In time, health services improved and vaccine production became more scientific. By the 1950s, most industrialized countries had eliminated endemic smallpox by using vaccine prepared on the skin of cattle or sheep and suspended in bactericidal concentrations of glycerol. By the middle of the 20th century, freeze-dried vaccine became available. The availability of such a vaccine facilitated vaccination programs in remote smallpox-endemic countries, which was a key element of a Russian proposal by Viktor Zhdanov at the 1958 World Health Assembly (WHA) that the World Health Organization (WHO) should intensively pursue global eradication by mass vaccination (72).

Although smallpox was eliminated in several developing countries by 1965, it became clear that something more was needed, especially for the Indian subcontinent and sub-Saharan Africa. In 1966, WHO launched an intensified eradication program that paid special attention to vaccine quality control, surveillance and containment, a capacity for reliable diagnosis, and further research. Eradication was greatly enhanced by increased use of the surveillance and containment method, as eloquently demonstrated by William Foege and colleagues in West Africa. The method is sometimes referred to as *ring* vaccination, by which known and potential contacts of identified cases are immunized to contain an outbreak instead of using the more resource-demanding mass vaccination (54). By the mid-1960s, the use of the bifurcated needle for intradermal vaccination led to more efficacious vaccination by unskilled health workers and further augmentation.

By setting eradication priorities, the disease was gradually eliminated, country by country, continent by continent. The global collaborative efforts, especially those of Donald A. Henderson and colleagues, who facilitated the WHO program, saw the last natural case (a cook, Ali Maow Maalin) in Somalia in October 1977, and in 1979, the world was declared smallpox free (238). Routine smallpox vaccination of civilians was discontinued worldwide by 1982, although in the United States, routine vaccination of civilians had been stopped by 1971, except for travelers who could receive vaccine

until the late 1970s if going to endemic countries, and the U.S. military, which ended vaccination of recruits in 1990.

With smallpox eradicated, and after a tragic episode in 1978 involving an accidental lethal infection of Janet Parker, a medical photographer at the University of Birmingham, England, WHO initiated an effort to prevent reappearance of the disease by recommending that research on variola virus in laboratories, the only known source of the virus, should stop, and that all known variola virus samples should be sent to selected reference centers or destroyed (240). Beginning in 1982, a succession of recommendations were put forth by WHO to destroy the known variola virus samples after WHO had validated that the only known collections were at WHO Collaborating Centers in Atlanta at the Centers for Disease Control and Prevention (CDC) and in Moscow at the Research Institute for Viral Preparations (in 1994, the Russian government moved the samples from Moscow to the State Center for Virology and Biotechnology in Koltsovo). WHO itself delayed destruction several times, recommending that DNA cloning and sequencing of selected strains should be done to archive the genetic information of variola virus.

In the late 1980s, opposition to destruction surfaced, and WHO decided that the proposition should be vetted in the biomedical arena. As destruction issues were debated, they gained increasing public visibility, and intelligence agencies, mainly in the United States and the United Kingdom, voiced concerns that the collections would be needed for defensive research, because of a potential for bioterrorism that used possible clandestine sources of the virus. The probability of clandestinely held virus specimens was disclosed by a Russian official, Kanatjan Alibekov, who indicated after his defection that until the early 1990s, a civilian agency known as the Biopreparat in the former Soviet Union worked with the virus and the Russian military had weaponized the virus in a secret offensive biowarfare program (6). There are suggestions that Russia and some other countries may secretly retain the virus (6,93).

Essentially two scenarios developed over the WHO proposition for eliminating the known variola virus samples. Destruction proponents (141) raised the specter of undoing eradication by asserting that the risk of retaining the specimens in an unvaccinated world far outweighed any medical benefits from research using the virus, which produces its true pathologic effects only in humans; that the repository samples could be targeted by terrorists; and that the virus had a record of infecting laboratory workers, posing a biohazard. To address the possibility of bioterrorism, they argued that destroying the samples under aegis of the WHA would send a signal that such reprehensible acts are intolerable by all governments.

Destruction opponents (113,194) submitted that retention would offer otherwise unattainable advances, including more fully understanding the molecular basis of variola virus infection, which would allow development of antiviral drugs, particularly for immunocompromised people who could not be vaccinated if smallpox somehow reemerged. They contended that a broader knowledge would provide the best strategies to defend against malevolent use of the virus and that working with the virus in maximum containment facilities using vaccinated staff minimized the biohazard risk. In light of the smallpox bioterrorism potential, a pivotal argument for retention came to be antiviral drug research, which was fueled by an observation that some of the acyclic nucleoside phosphonates, including, 1-[(S)-3-hydroxy-2-(phosphonomethoxy)-propyl] cytosine (Cidofovir, Vistide), a DNA-polymerase inhibitor sponsored by the U.S. Food and Drug Administration to treat cytomegalovirus infection, suppressed variola virus growth in culture and inhibited certain orthopoxvirus diseases in experimentally infected animals (22,104). On the other hand, proponents advanced the position that rebuilding stockpiles of smallpox vaccine and human vaccinia immune globulin (VIG) and destroying the specimens would virtually eliminate the biothreat potential (93).

Although the United States had supported a WHO recommendation for destruction in 1996, on April 22, 1999, President William Clinton announced that the specimens at CDC would be retained for research that may help save lives should the disease somehow reemerge. The announcement followed an analysis by various U.S. government agencies and publication of a study by the U.S. National Academy of Sciences, Institute of Medicine, entitled “Assessment of the future scientific needs for live variola virus,” which recommended areas for research using live variola virus, considering the biothreat potential (104).

In May 1999, the 52nd WHA recommended postponement of destruction (240). Although destruction of all variola virus samples remains the goal of WHO and all WHA Member States, the destruction of the collections was delayed by the WHA to enable further internationally approved research on antiviral agents and improved vaccines and to permit high-priority investigations of variola virus genetic structure and its pathogenesis, in monkeys, a yet unproven surrogate system. In December 1999, poxvirus experts from several WHO regions assessed U.S. and Russian variola virus research agendas with the aim of reaching a global consensus on the timing of destruction, and early in 2000 and 2001, specific studies began using live variola virus in maximum containment laboratories of the WHO Collaborating Centers in Atlanta and Koltsovo, respectively.

Many experts on terrorism agree that the chance of smallpox reappearing is miniscule, but the consequences of a reappearance in humans would be enormous (93). In this light, the United States and several other countries set out to increase public awareness, revamp national policies, and improve the public health and emergency response infrastructure by strengthening national, state, local, and military defenses and promoting global condemnation of the malevolent use of variola virus and other agents of biologic and chemical terrorism.

Cowpox

Years before Edward Jenner's observations, cowpox was regarded as a sporadic disease of European cows, occurring as ulcers that could be transmitted from the teats onto the hands of milkers. Jenner's observation that milkmaids who had recovered from cowpox did not contract smallpox led to the practice of cowpoxing as a specific prophylactic against smallpox (108) until it was superseded by vaccination using vaccinia virus. Since the early 1970s, observations of cowpox in large cats in the Moscow Zoo in Russia and Whipsnade Park in England, and in domestic cats in England, led to conclusions that the cow is an ancillary host of cowpox virus and that cows, cats, foxes, and various zoo animals are sporadically infected from a rodent reservoir (40). Moreover, humans are probably infected by contact with intermediate infected animals, especially domestic cats, and not the rodent source (9). Support for indigenous rodents being the

reservoir derives from serosurveys of rodents, cattle, and humans, the fact that the virus remains indigenous to Eurasia, and that it has never appeared in exported cattle as has pseudocowpox, which is maintained by the bovine reservoir.

Vaccinia

The term *vaccination* (vacca, Latin for cow) was introduced in Plymouth in 1803 by Richard Dunning to describe the Jennerian method, and in 1881, Louis Pasteur honored Jenner by adopting the term to refer to *all* immunization methods. Jenner's writings indicate that although he had used cowpox lesion material initially, he also succeeded using lesion material from a now extinct equine disease “grease” because even then, cowpox rarely occurred. Over time, whichever virus it was that he and other vaccinators had used became passaged many times and in many ways, including in various animals and arm-to-arm in humans, probably also in smallpox patients; the passaging led to what is now regarded as a different species, vaccinia virus. Eventually, the selection of vaccinia strains that caused less fever, pain, and complications led to the strains now used (8,88,92,188,236). Sequencing of cowpox, variola, vaccinia, and monkeypox virus genome DNAs has substantiated that cowpox virus contains the most comprehensive set of genes, thus is the likely progenitor of contemporary orthopoxviruses, but it has not shed much light on the origin and derivation of vaccinia virus.

Lyophilized smallpox vaccine had several properties that made it one of the most successful vaccines in history, including its relative stability, ease of production and administration, and capacity to induce durable cellular and humoral immunity. Although recombination of DNA from one poxvirus into another had been reported beforehand, in 1982, marker rescue by recombination and expression of rescued sequences of other organisms by using vaccinia virus was described (139,173). The publications set the stage for development of vaccinia and other poxviruses into genetically engineered expression vectors, which have become widely used experimental tools, particularly in immunology and vaccinology (12,34,77,156,157,174,185).

Currently, in the United States, it is recommended that laboratory and health care workers at high risk for infection by human orthopoxviruses should be vaccinated within 10 years of their use (37; update available at http://www.cdc.gov/). In the United Kingdom, the decision to vaccinate is made on a case-by-case basis (2). Other countries have devised recommendations based on the U.S. and U.K. versions.

On teats of milking buffalo and dairy cows, a vaccinia subspecies, buffalopox virus, causes lesions that are similar to cowpox ulcers. Human skin lesions appear more like vaccinia pustules rather than the more severe lesions of human cowpox; oropharyngeal lesions also occur in humans, mainly by drinking unpasteurized milk from infected animals (128). DNA studies have suggested that buffalopox virus is related enough to vaccinia virus that it is a subspecies (59). Presumably, buffalopox emerged as a result of mass vaccination by transmission of human vaccinia to milking animals; outbreaks still occur in milking buffalo and cattle on the Indian subcontinent (161). Another vaccinia subspecies is rabbitpox virus, which has caused outbreaks of generalized disease in laboratory rabbits (171).

Monkeypox

Jezek and Fenner have extensively described monkeypox (110). In 1958, during days when subhuman primates from various parts of the world were mixed together in holding facilities to fill demands for polio vaccine, monkeypox was first reported as a disease of Asian cynomolgus monkeys. Monkeypox outbreaks with animal fatality rates of 3% to 48% continued to occur in several holding facilities during the 1960s, but no infections were reported among animal handlers, likely because they had preventative smallpox vaccination.

In 1970, the African origin of the disease became apparent when it was recognized that monkeypox virus caused a smallpox-like disease in humans in the DRC, and no such disease occurred in Asia. Since then, the illness has been observed as a rare zoonosis with low interhuman transmission among inhabitants of small villages in tropical rain forests in West and Central Africa. Because of the close resemblance between smallpox (Fig. 3) and human monkeypox (Fig. 4), following the declaration of smallpox eradication and recommendation to stop smallpox vaccination, WHO conducted a largely prospective intensified surveillance in the DRC from 1981 to 1986 that was aimed at determining whether human monkeypox could replace smallpox and pose a threat to eradication. The study identified 338 cases (86% under 10 years of age), including 33 deaths in five DRC provinces; 72% of cases were suspected to have acquired the disease from animals (Fig. 5). The number of first-generation cases among contacts not vaccinated against smallpox, which is protective, indicated a secondary attack rate of 7.5%, compared with the substantially higher 60%-on-average, secondary attack rate of smallpox. Ecological and serologic studies suggested that monkeypox virus has a broad host range, humans and primates being fortuitously infected, and that squirrels (*Funisciurus* species and *Heliosciurus* species), a usual food of the inhabitants, are involved significantly as wildlife hosts of the virus. It was concluded that monkeypox was a rare zoonosis that did not jeopardize eradication, primarily because of the relatively low secondary attack rate, the apparent low percentage of secondary cases (28%), and an infrequent finding of clusters of cases. Thus, monkeypox virus seems to be like cowpox virus, which is geographically confined to indigenous animals and not well adapted for interhuman spread.

FIG. 3. The rash of smallpox. The “smallpox recognition card” used during the Intensified Smallpox Eradication Programme of the World Health Organization, showing the nature and distribution of the rash of discrete, ordinary-type smallpox. (From ref. 72, with permission.)

FIG. 4. Human monkeypox. Front and rear views of a 7-year-old girl from the Democratic Republic of Congo with monkeypox, on the 8th day after the appearance of the rash. Note the enlargement of the cervical and inguinal lymph nodes, a feature not seen in smallpox. (From ref. 23, with permission.)

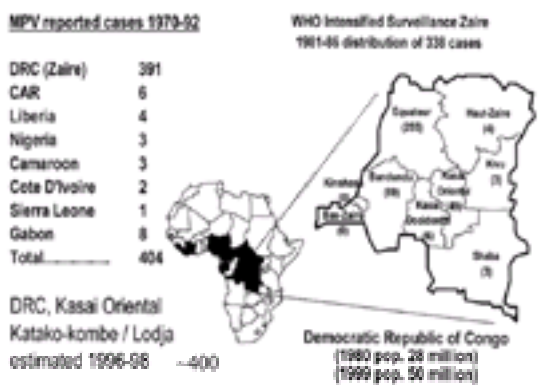

FIG. 5. Geographic distribution in West Africa rainforest countries of human monkeypox showing the location and number of cases verified mostly prospectively between 1970 and 1992. No cases were reported from 1993 to 1996 before limited retrospective studies in 1997 and 1998 in 78 villages of the Katako-Kombe and Lodja health zones, Kasai Oriental Province, DRC.

Few cases were reported after the intensified surveillance; however, in 1996, from February to August, 71 suspect cases, including 6 deaths, were reported from the DRC Kasai Oriental Province during heightened civil and military unrest in the country (159). Because of the unrest and consequent difficulty of obtaining prospective data, WHO conducted only three brief surveys using retrospective surveillance methods to obtain incidence estimates, two in 1997 and one that provided little data in 1998 (38,39,96). A review of the studies (241) indicated that retrospective case determination was obscured by a lack of surveillance continuity, use of disparate case definitions with attendant recall prejudices, and biases in case selection, ascertainment, and classification, particularly because of co-circulation of chickenpox, which is variably confused with human monkeypox. Given the limitations of such studies, laboratory test results of clinical samples from surveys in February and October, 1997 provided an estimate that just over half of 511 reported cases with febrile vesicular rash illness between February 1996 and October 1997, which were mostly children under 16 years of age, were most likely monkeypox. A secondary attack rate of 8% was estimated that was very much like the rate reported for 1981 to 1986. The review (241) concluded that human monkeypox is increasing in the region for various reasons, but the disease continues to be unsustainable by interhuman transmission and depends on repeated introductions from the animal reservoir; monkeypox still poses no threat to smallpox eradication. In addition, prospective intensified studies were encouraged, realizing that this would require a better public health system in the DRC and development of more specific and sensitive diagnostics. As in the 1980s, continued health education, and not reintroduction of smallpox vaccination, was recommended for disease prevention.

Infectious Agents

Several books and monographs about orthopoxviruses are available (12,49,74,143,227) (see Chapter 84). Descriptions of the biologic properties and the lineages of several vaccinia and variola virus strains are also available (68,88,188,236,237); most research is done using vaccinia virus.

Orthopoxviruses are large, brick-shaped particles (see Fig. 1) that replicate in the cytoplasm to produce intracellular mature virions (IMVs), which are released upon cytolysis. Some species (cowpox, ectromelia, raccoonpox, skunkpox, volepox) also occlude virions within cytoplasmic acidophilic-type inclusion (ATI) bodies, which may be protective. Poxviruses also have a method of viral dissemination from the cell in which a small proportion of IMVs become wrapped in an additional lipoprotein layer and released as extracellular enveloped virions (EEVs). Additionally, poxviruses may spread by enabling cell fusion, a feature that is influenced in orthopoxvirus-infected cells by several components,

including the viral hemagglutinin (HA) and a protein, termed SPI-3, with amino acid sequences resembling serine protease inhibitors.

To initiate infection, an IMV attaches to a cellular receptor, and the viral outer membrane fuses with the plasma membrane bilayer to enable release into the cytoplasm of the viral core complex containing the genome. During penetration of the virus into the cytoplasm, in the first stages of virion uncoating, a virion-associated RNA polymerase complex initiates transcription of the genome. Most cell functions shut down as the core elements become further dissociated and virus components become established in a cytoplasmic region of granular endoplasm termed the *viroplasm* or “viral factory,” which is devoid of ribosomes and organelles. In the viroplasm, the genome is replicated and progeny are assembled; the region comprises a basophilic inclusion. On the other hand, EEVs appear to attach to a distinct receptor and enter the cell by endocytosis or engulfment, forming a plasma membrane endosome in which the EEV tegument dissolves. In the endosome, the outer membrane of the tegument-less virion, like IMV, fuses with the enclosing plasma membrane, releasing the core into the cytoplasm (212).

As viral proteins become synthesized, morphogenesis of progeny virions initiates in the viroplasm by the formation of cup-shaped lipid bilayers (crescents in two dimensions) that contain, on the convex surface, spicules of viral protein. The membrane finally closes to form the outer membrane of a spheroidal immature particle (IV). For several years, the cup-shaped membrane was believed to originate by *de novo* synthesis because extensive electron microscopy studies indicated no connections between the lipid bilayer and cellular membranes (49). Recently, the outer membrane was suggested to originate from a tightly opposed pair of cell membranes in an intermediate compartment that is situated between the Golgi apparatus and the endoplasmic reticulum (213). The membrane pair was suggested to form the outer membrane by wrapping around the core and overlapping itself, like a macaroni shell. More recently, however, the seminal data (49) were substantiated by high-angle tilt electron microscopy observations of discontinuity between the viral and cellular membranes and detection of IV formation distal to the intermediate compartment, including in the nucleus (97).

The IVs mature into more complex structures containing a distinct nucleoprotein core that is not enclosed by a separate membrane (97) and appears dumbbell shaped in thin sections (49). Fully developed IMVs contain about 100 viral proteins, are infectious, and eventually are released by cytolysis. Before cytolysis, a minor portion of IMVs in the trans-Golgi network or tubular endosomes become further wrapped in a double lipoprotein layer. This production of intracellular enveloped virions (IEVs) appears to enable cellular actin polymerization behind the IEV, producing virus-tipped comet-shaped structures, which move erratically to the cell surface, where individual virions can be seen moving into separate microvilli (47). A microvillus may connect to an adjacent cell enabling virus transfer. The Golgi-derived lipid bilayer envelope of the IEV may also fuse with the plasma membrane in an exocytotic process that releases EEVs wrapped by the proximal layer. A portion of the EEVs may adhere to the plasma membrane as cell-associated enveloped virions (CEVs) (16). IEVs without actin tails may also reach the cell surface to become EEVs or CEVs, and some IMVs may become enveloped in some cells by budding (226). The envelope of the EEV contains about a dozen proteins, including viral and host complement control proteins that may inhibit virolysis. The envelope contains an HA glycoprotein, which is also intercalated into the plasma membrane of infected cells and antigenically distinguishes orthopoxviruses from other genera (206,212).

Neutralizing antibodies, as measured by standard pock- or plaque-reduction tests, block penetration of IMVs into cells, but these antibodies are ineffective in preventing EEV spread. Several studies have emphasized the importance of EEVs for spreading virus in culture and throughout the body, and the distinctive role of antibodies against envelope proteins, which are antibodies that aggregate rather than neutralize EEVs, preventing their release from the cell surface. Such antibodies may protect EEVs from IMV-neutralizing antibodies, but *in vivo,* the antibodies may augment cell-mediated clearance (212).

Orthopoxviruses, like other genera, contain a covalently closed, double-stranded DNA genome. Orthopoxvirus DNAs range in size from 170 to 240 kilobase pairs (kbp) and are composed of 34% G+C. DNA restriction mapping (62,138) has shown that 90 kbp composing the central region of the genome is highly conserved among species and strains and that the terminal regions are varied. DNA sequencing has shown that central region genes mostly specify virion structural components and enzymes associated with viral mRNA transcription and DNA replication. The terminal regions primarily specify genes associated with virus survival in the host, for example, genes for modulating immunopathogenesis and host range. To date, GenBank contains all or nearly all of the genome DNA sequences for several orthopoxviruses (in parentheses—accession number; reported sequences), including vaccinia strains Copenhagen (M35027; 191,737 bp), Tian Tan (AF095689; 189, 274bp), and Modified Virus Ankara (U94848; 177,923 bp), variola strains Bangladesh-1975 (L22579; 186,103 bp, fully sequenced), India-1967 (X69198; 185,575 bp), and alastrim Garcia/Brazil-1966 (Y16780; 186,986 bp). Additionally, large DNA segments of cowpox virus GRI-90 are reported (Y11842, Y15035, X94355), as are significant portions of other variola strains (U18337, U18338, U18339, U18340, U18341) and vaccinia strain Western Reserve (WR).

Among poxviruses, only members of the genus *Orthopoxvirus* produce an HA protein. Based on this trait, Esposito and colleagues have used HA sequences of several species and strains to estimate the phylogeny of orthopoxviruses (see Fig. 2). The estimate agrees well with phylogeny assessments based on dissimilarities of restriction maps (72,110) and coincides with the organization of orthopoxviruses by biologic criteria. The phylogram in Figure 2 shows a considerable divergence (75% sequence identity) between Old World orthopoxviruses and isolates from North America. The Old World species are 95% identical in sequence. The tree suggests that vaccinia and monkeypox viruses are more closely related to ectromelia virus, which itself appears related to a major clade of cowpox virus. Tatera gerbilpox, camelpox, and variola viruses appear more related to another major clade of cowpox viruses.

In the diagnostic laboratory, several of the biologic properties of human orthopoxviruses (Table 2) are useful for identifying and differentiating virus in lesion material (73), although polymerase chain reaction (PCR) amplification with or without restrictase cleavage of amplicons is proving to be more rapid and sensitive for routine diagnostic purposes (195). In experimental animal systems, infection by one orthopoxvirus species usually leads to protection against the others because of high antigenic similarity; the sera of animals infected with orthopoxviruses cross-react extensively.

Cross-absorption of immune sera against one species with antigen preparations of one or two other species has been used to resolve type-specific antigens (63,82,83,102,246); in one study (64), a predominant 73-kd viral protein was detected in variola-, vaccinia-, or monkeypox-specific immunoprecipitates.

Property	Variola	Monkeypox	Vaccinia	Cowpox
Host range	Narrow	Broad	Broad	Broad
Pocks on the chorioallantoic membrane (CAM)	Small, opaque, white	Small, opaque, hemorrhagic	Strains vary; large, opaque, white or hemorrhagic	Large, hemorrhagic
Ceiling growth temperature on CAM (°C)	37.5–38.5	39	41	40
Rabbit skin lesion	Small, transient, nontransmissible	Indurated, hemorrhagic	Strains vary; indurated nodule, sometimes hemorrhagic	Large, indurated hemorrhagic
Lethality for:				
Mice	Low	High	High to very high	Variable
Chick embryo	Low	Medium	Very high	High
A-type inclusion bodies	No	No	No	Yes
B-type inclusion bodies	Yes	Yes	Yes	Yes
Genome DNA size (kbp)	186	191	192	220

TABLE 2. *Biologic properties of orthopoxviruses that can infect humans*

Pathogenesis

In experimental monkey infections, variola and monkeypox viruses may induce systemic illness with fever and rash, generally resembling that in humans (see Fig. 3 and Fig. 4), although virulent human variola strains often produce an attenuated infection with reduced transmissibility (72,110). Cowpox and vaccinia viruses usually produce a localized dermal lesion with rare systemic complications after infection, the cowpox lesion being more severe in humans (74).

Generalized Infections with Rash

The developmental phases and physical appearance of smallpox is described in several publications (54,72,186,190). Our understanding of some details in the course of events can be derived from studies of monkeypox in monkeys with generalized disease (42), rabbitpox infection of rabbits (171), and ectromelia virus in mice (70). Careful autopsies of people who died of other causes during early-stage smallpox were rarely performed, and it was difficult to assess from postmortem examinations of acutely fatal cases the location(s) in the body that the virus multiplied before secondary viremia and concomitant prodrome symptoms began. The pathogenesis of monkeypox in humans probably is similar to that of smallpox, except that viral entry in primary infections from a wildlife source probably occurs through small lesions on the skin or oral mucous membrane, or by the respiratory route in cases of person-to-person infection (110).

Viral Entry and Incubation Period

During the smallpox rash stage, people with active disease may have transmitted virions by dissipation from the mouth simply by speaking or coughing. The inhalation of aerosol droplets from an erupting oropharyngeal enanthem passed the virus to the oropharyngeal and respiratory mucosa of a susceptible individual. Infection from fragmented dermal scab material, which consisted of inspissated virus, was probably responsible for the much lower transmission rates associated with crusted smallpox scabs. Initial infection of the mouth, trachea, and lungs produced no symptoms or local lesions that could be recognized clinically or by autopsy of people who died soon after infection. Whatever cells were initially infected passed the virus to respiratory tract macrophages, which by about day 3 would have entered the bloodstream and lymphatics, thereby reaching regional and draining lymph nodes by day 4 to 10. Infection was not contagious during the week or two of incubation before the development of the oropharyngeal enanthem and skin rash exanthem.

Spread Through the Body

As virus traveled into the most likely sites for early viral multiplication, the lymphoid organs (spleen, bone marrow, and lymph nodes), a prodrome of influenza-like symptoms with a fever of about 40°C developed quickly. As the fever descended from its peak, an oropharyngeal enanthem developed that involved the pharynx, uvula, larynx, and tongue, and less frequently the trachea and bronchi, from which virus was secreted for about a week. Cell-associated virus also localized to small dermal blood vessels, producing an exanthema. The rash developed over 3 days into pustules that felt velvety and “shotty,” as if filled with a birdshot, and eventually became umbilicated and may have fused with other pustules, causing skin sloughing.

Cell-associated virus was likely in the form of infected monocytes, which migrated from dermal vessels into the epidermis, where they transmitted the infection to cells of the basal layer. Sebaceous glands were particularly susceptible, and necrosis and edema followed, sometimes with splitting of the dermis. As part of the inflammatory response, polymorphonuclear cells migrated into the lumen of developing vesicles, rendering the contents pustular. Pustules then progressed to dried-up crusts, or scabs, within about 2 weeks. A phenomenon known as the *centrifugal spread* of the rash occurred by an unknown process that enabled distribution of more lesions on the extremities than on the trunk.

Patients were extremely toxemic and dehydrated at the peak of the rash, which was the critical time, resulting in life or death. At the peak of the rash stage, intestinal involvement may have led to vomiting, diarrhea, and severe dehydration, requiring fluid hyperalimentation. If skin lesions were confluent, with sloughing of the skin, it was difficult to administer intravenous fluids using a peripheral vein; central intravenous access was often attempted with variable success. Some physicians recount a fetid odor of variola major smallpox patients, but the reason or mechanism of its genesis is

unknown.

The Immune Response

After infection by most viral pathogens, infected macrophages and lymphocytes induce the production of interferon, which stimulates natural killer (NK) cell activity to permeabilize and kill virus- infected cells independent of conventional immunologic specificity (see viral immunology chapters in this book). Activation of the complement cascade also serves as a first-line host defense. Macrophages and dendritic cells process antigens into peptides that complex with products of the major histocompatibility complex (MHC) to present antigens to thymus-derived lymphocytes, of which there are several subclasses. CD8+ T lymphocytes (CTLs) respond promptly by specific clonal expansion to clear infected cells quickly. Type 1 CD4+ helper T lymphocytes play a role in inducing lasting CTL activity, and type 2 CD4+ helper T lymphocytes interact with B cells to produce the antiviral neutralizing and aggregating immunoglobulins that prevent subsequent infection. CTLs recognize antigen presented in conjunction with class I MHC, whereas the CD4+ helper cells recognize antigen presented in conjunction with class II MHC. CTLs recognize predominantly cytosol-derived proteins processed by antigen-presenting cells, such as dendritic cells, macrophages, and B cells, whereas helper T cells recognize exogenous antigens processed and presented by these same cells.

It is likely that variola virus infection of the oropharyngeal and respiratory mucosa led to infection of macrophages, and their lodgement in lymph nodes, bone marrow, and spleen provided for the network of T-cell interactions that stimulated the CTL-mediated killing of infected cells and the subsequent humoral immune response that produced neutralizing and other antibodies against a large assortment of viral antigens. Current evidence for other viruses suggests that NK cells and CTLs also play a subsequent role in antibody-dependent cellular cytotoxic killing of infected cells by mechanisms that may involve complement (ADCC response). The production of an array of cytokines and chemoattractant cytokines (chemokines) and concomitant receptor interactions are responsible for signal transduction processes that regulate the immune network. Classic experiments demonstrating cell-mediated immunity in the recovery of mousepox (44) and passive immunization protection from mousepox (67) and rabbitpox (18) substantiate the vital role of the cell-mediated response as crucial for viral clearance and the humoral response as important for resistance to subsequent infections with the same or related viruses.

In generally healthy individuals who became infected, a vigorous cellular immune response usually resulted in development of ordinary-type smallpox. However, if the cellular immune response was grossly deficient, the case may have presented as flat smallpox with skin lesions lacking a pustular infiltrate. Hemorrhagic smallpox was a rare form that occurred in patients with a defective immune response, which led to extensive virus multiplication in the bone marrow, where megakaryocyte destruction caused blood coagulation defects, and in the spleen.

Death or Recovery

The outcome of smallpox was either death or recovery with no persistence of the virus in the body. Pockmarks, the most common sequela, were the result of granulation tissue filling necrotic lesions. Lesions could occur all over the body, including on the soles and palms, which is a feature that is uncommon in chickenpox, a pustular rash illness sometimes confused with smallpox and human monkeypox. Smallpox lesions were usually most profuse on the face because of the large number of sebaceous glands there and the deeper pitting associated with their involvement (20). Blindness was an important but rare complication, usually occurring in cases in which there was malnutrition or secondary bacterial infection. Recovery was accompanied by prolonged immunity to reinfection with variola virus. In contrast, heterologous immunity, such as from vaccination, was less durable, especially in cases of variola minor alastrim virus infection.

Localized Infections

Infections with cowpox or vaccinia viruses ordinarily cause localized lesions at the site of their introduction into the skin, by inoculation or multiple puncture as in deliberate vaccination, or through small abrasions as with cowpox virus. Cowpox is generally more painful and produces somewhat larger lesions. The draining lymph node becomes swollen, and there is a brief viremia sometimes accompanied by a low-grade fever. Secondary lesions and complications occur sporadically, usually in individuals with immune deficiencies.

The Immune Response

Current knowledge indicates that macrophages, and very likely, Langerhans cells (11,94,163) in the skin, transport cowpox and vaccinia viruses to the draining lymph nodes, and there present viral antigens to immunocompetent lymphocytes. Specific antibodies, formed by B lymphocytes after interaction with type 2 CD4+ T lymphocytes, can prevent infection of susceptible cells by the virus. In contrast, CTLs provide the major mechanism for controlling and clearing any infection initiated by virus that escaped neutralization by preexisting antibody (e.g., after vaccination). Type 1 CD4+ T lymphocytes play a major role in inducing a lasting CTL response. In a naive host, the CTL response occurs first and is primarily responsible for clearing an infection (1,15), although antibodies formed soon thereafter may sometimes be important for preventing released virus particles from reaching sites not easily accessible to CTLs, such as the skin.

Experience with smallpox vaccination has indicated the importance of cell-mediated immunity in recovery from infection. For example, progressive vaccinia, the most dreaded complication, occurred in infants with defective cell-mediated immunity. In contrast, vaccinees with agammaglobulinemia but a normal cellular response recovered normally (78,79). Although shown for mousepox some time ago (15), production of vaccinia-specific CTLs in human vaccinees was recently demonstrated (61,197).

Pustulation following inoculation and migration of polymorphonuclear cells into the lumen of the vaccinal lesion have been shown to be reasonably good markers of smallpox vaccine capacity to induce cellular and humoral responses (148). The absence of virus replication of inactivated smallpox vaccines in the vaccinees, which led to absence of EEV

opsonizing antibodies and a less-than-comprehensive cell-mediated response, explains the failure of killed and component vaccines to fully protect and induce a durable long-lasting response.

Molecular Basis of Orthopoxvirus Pathogenicity

The collective viral and host molecular mechanisms in smallpox and human monkeypox that sometimes ended in death are largely unknown. Smallpox (and monkeypox) patients experienced a fever and toxemia that conceivably produced sufficient physiopathologic shock to induce fatal respiratory failure and heart attack, or encephalitides, which were usually fatal and affected smallpox cases somewhat more often than as a complication of smallpox vaccination. Such outcomes might have been influenced by the virus strain, secondary bacterial infection, immune complexes, the inflammatory response, malnutrition, or other factors likely operating concurrently.

For several decades, mousepox, caused by ectromelia virus in mice (70), has served as a paradigm of pathogenesis and immunology of an acute viral infection, including smallpox. However, in contrast to mousepox, in which death is from hepatic infection and decimation, autopsies of smallpox cases usually indicated that no vital organs were destroyed by variola virus infection (46) (no autopsies on human monkeypox patients are reported beyond characterization of skin lesions).

Poxviruses have adopted general and specific strategies to enable their growth in their hosts. General strategies include shutoff of cellular transcription, which is supervised by virus-controlled synthesis of its own components; the production of particles with different surfaces (e.g., IMVs, CEVs, and EEVs), which enhances viremia and augments attachment to and infection of different cell types; and the production by some species of virus-containing ATIs, which may ensure a concentrated inoculum and protect virus particles.

More specifically, sequencing and structure–function studies have indicated that vertebrate poxviruses have exploited their hosts by adopting cellular genetic information and adapting it to modulate aspects of infection, such as tissue and host range and the inflammatory reactions that lead to clearance mechanisms *in vivo.* How poxviruses acquire the cellular sequences is unknown, but the discovery of a reticuloendotheliosis retrovirus DNA integrated into fowlpox virus DNA (95) has suggested that both viruses may infect the same cell type, and there the retrovirus reverse transcriptase may produce provirus and copy cellular mRNAs for subsequent recombination into fowlpox virus DNA. Because no other poxviruses have been reported with retroviral sequences, other integrative mechanisms may be involved. Nevertheless, a viral strategy that usurps host inflammatory and other immune modulators suggests that the toxemia and fever are due to discordant reactions of cytokines, chemokines, cell adhesion molecules, growth factors, and other immunologic components.

Inflammation and Other Immune Modulators

The literature on poxvirus immune modulators and other elements involved in virus survival in the host is fast growing (5,30,48,66,149,166,182,209,211,215,232). Although many of the poxvirus modulators may react with their presumed substrates *in vitro,* providing functional information, the dynamic interactions of such components in the context of a naturally infected host are largely unknown.

Genome DNA analysis has indicated that orthopoxvirus species and strains differ in the extent of sequences specifying potential modulating proteins. Such sequences may be deleted without affecting virus growth in culture, but the encoded proteins are likely to be rather essential *in vivo* because they are directly related to surviving sufficiently in the host to produce progeny. Most hosts recover from poxvirus infections; hence, it is reasonable to assume that a prime purpose of viral expression of such modulators is to deal with antigen presentation in conjunction with class I and class II MHC and its consequences, the viral clearance process and immunologic memory.

There are no data on the capability of poxviruses to abrogate class II MHC presentation directly other than by shutoff of cellular transcription. However, myxoma virus has been shown to down-regulate the proportion of surface class I MHC epitopes, and there is evidence that some poxviruses can induce measurable, although less severe, down-regulation of surface class I MHC epitopes. Observations of early or late gene expression using recombinant vaccinia viruses have indicated a potential defect in presenting some foreign antigens that depends on the epitope and is antigen-specific, which has suggested that vaccinia virus can somehow compromise MHC-restricted antigen presentation, possibly by influencing proteosome function, or there may be class I MHC synthesis inhibitors or other competitive viral components.

Host processing of viral proteins is coordinated with up-regulation of class I and II MHCs in their respective antigen-presenting cells and association of infiltrating CD8+ and CD4+ T cells into the site of virus replication. The indication that poxviruses, including orthopoxviruses, are targeting antigen presentation stems from discoveries that some poxviruses encode secreted soluble receptors for interferon-g (IFN-g) and a variety of receptors for members of the tumor necrosis factor (TNF) superfamily, including TNF-a and lymphotoxin-a (LT-a) (5,166,211). IFN-a is a cytokine produced by CD8+ T cells and NK cells and potentiates IFN-a and -b, and TNF-a and LT-a are multifunctional cytokines usually specified by CD4+ T cells, NK cells, macrophages, monocytes, neutrophils, and B cells. The viral receptors mostly appear secreted from infected cells, except for a surface-associated TNF receptor of vaccinia strain Lister, which suggests that such proteins are extracellular decoys modifying host recognition of infected cells, rather than being intercalated into the plasma membrane, like cellular TNF receptors, to modulate signal transduction. At least one cowpox strain contains sequences for five different isologs of cellular TNF receptors (204), other orthopoxviruses may contain a lesser number or may have a truncated correlate, and some contain two copies by virtue of being in the DNA inverted terminal repeat. The time of expression of viral TNF receptors may be early or late after infection, and some may be produced much more abundantly by one virus than another (3,100,134,208).

Another viral homolog of a cellular receptor, or “viroceptor,” that appears rather significant for immune evasion is an interleukin-1b (IL-1b) receptor correlate known to be encoded by cowpox and some vaccinia strains, but not by variola and some vaccinia strains associated with high fever (4). The protein is soluble and secreted and thus is a probable

decoy *in vivo* for IL-1b, a cytokine produced by neutrophils, monocytes, and macrophages, which upon binding to its receptor, actuates signal transduction and production of other cytokines, including IL-2.

More than a dozen genes in orthopoxviruses specify proteins that contain ankyrin repeats. Because eukaryotic genes for ankyrin are located very close to genes encoding IL-1 and TNF receptors, which through their signal transduction processes can modulate nuclear factor-kB (NF-kB)-directed transcription, it is reasonable to consider that viral genes for proteins containing ankyrin might have been acquired to interface with cytosolic NF-kB (which itself derives from ankyrin-containing precursors) and its inhibitor, IkB (which also contains ankyrin). The interfacing may influence NF-kB translocation to the nucleus, where subtypes of NF-kB regulate transcription of various cellular genes for immune-modulating components (17,132).

Several orthopoxviruses express a soluble secreted homolog of the cellular receptor for IFN-a and -b (43,220), cytokines that can influence expression of class I MHC. IFN-a is produced by lymphocytes and occurs as approximately 15 different isologs, and IFN-b is a fibroblast glycoprotein. IFN-a can activate PLC-2A, which enables release of diacylglycerol and a precursor of proinflammatory prostaglandins and leukotrienes, arachidonic acid (31). The viroceptor for IFN-a and -b appears to block by interacting with moieties on the amino and carboxyl termini of IFN-a and -b (210,211), but the counterpart cellular receptor interacts only with the amino terminus and appears to function secreted and at the cell surface. The viroceptor may compete with the cellular IFN-a and -b receptor in its role as an inhibitor of signal transduction and prevent transcription of IFN-a–responsive genes.

At least two orthopoxvirus gene products have been implicated in modulating the antiviral effects of IFN-a and -b (81,106,107). One such protein encoded by most orthopoxviruses binds double-stranded RNA and inhibits the activation pathway associated with IFN-a induction of transcription of cellular 2',5'-oligo-A synthetase into isomers that can activate RNAseL, which may inhibit protein synthesis and protect the host from viral takeover by inducing apoptosis. Another comprises a homology of eukaryotic translation initiation factor eIF-2a that protects eIF2-a from P1/eIF2-akinase, which phosphorylates and inactivates eIF2 required by ribosomes for translation initiation, thereby inhibiting protein synthesis and reinforcing the 2',5'-oligo-A synthetase mechanism.

Associated with immune-modulating function of several poxviruses, including cowpox and vaccinia viruses, is an apparently multifunctional element, cytokine response modifier A (CrmA), which has been shown to inhibit caspase-1 (136,182,187). Caspase-1 is a cysteine protease involved in cleaving pre-IL-1b to IL-1b, which is produced mainly by monocytes and neutrophils, and possibly in blocking enzymatic cleavage of pre-IL-18 to IL-18 (211), an IFN-g–inducing inflammatory cytokine that is produced mainly by migrating macrophages, that activates NK cells, and that is needed for a type 1 helper CD4+ cell response. Regarding IL-18, some poxviruses, including orthopoxviruses, also produce at least one IL-18–binding protein (168,242).

CrmA may also interfere with proinflammatory leukotriene synthesis by interfering with an enzyme in the lipoxygenase pathway involved in producing 14,15-dihydroxyeicosate-traenoic acid from arachidonic acid, which is released upon membrane permeabilization (30,172). Further, CrmA and related proteins may also play a role in retarding CTL-mediated apoptosis by inhibiting the serine protease granzyme-B (184), which, along with perforin, is produced by CTLs in granules that help permeabilize infected cells and leads to antigen presentation and infected cell destruction.

In this regard, another apoptosis inhibitor encoded by orthopoxviruses appears to be a protein, designated CHO-hr, that influences viral infection of Chinese hamster ovary cells. The CHO-hr protein was first described as an effector of cowpox virus host range (214). CHO-hr contains ankyrin repeats and appears functionally similar to myxoma virus M11L protein (137), which may inhibit inflammatory signal generation resulting from apoptosis and has been shown to inhibit apoptosis in conjunction with the myxoma virus TNF-binding protein in primary rabbit peripheral mononuclear cells and RL5 rabbit CD4+ T cells.

A protein with amino acid sequences resembling the family of serine protease inhibitors termed serpin-1 or SPI-1, which is related in sequence to CrmA (also designated as the 38-kd protein or SPI-2) and is present in some orthopoxviruses, has also been implicated in apoptosis inhibition. More specifically, SPI-1 may interface with CrmA in Fas-mediated apoptosis (25); however, the *in vivo* effects of SPI-1 vary with the virus strain and animal model (121,222).

A 35-kd protein, which is similar in sequence to the superfamily of regulators of complement activation, is secreted by various orthopoxviruses and binds complement components C3b and C4b, thereby blocking antibody-dependent and alternate pathways in the complement cascade and inhibiting complement-augmented neutralization of virus particles (99,200). A protein in the envelope of EEVs is described that also resembles the superfamily members, but it may not bind complement (135); however, a few host complement control proteins have also been detected that are associated with the envelope of EEVs, which may provide resistance to virolysis (230).

Chemokines comprise a large family of low-molecular-weight secreted proteins that bind to specific seven-transmembrane G-protein–coupled receptors belonging to the rhodopsin superfamily. By a regulated electrostatic process of chemotaxis, chemokines recruit leukocytes into infected or injured sites in the host (10,117,178). Based on the number and arrangement of cysteines, the family consists of CXC chemokines, which attract neutrophils, lymphocytes, or fibroblasts or augment mitogenesis of fibroblasts; CC chemokines, which attract monocytes, B cells, T cells, eosinophils, and basophils; CX3C chemokines, which play a role in leukocyte adhesion and attract monocytes and T cells; and the C chemokine lymphotactin, which is chemotactic for lymphocytes but not monocytes. A wide variety of orthopoxviruses have been shown to secrete a soluble 35-kd protein that principally binds CC chemokines (210); other poxvirus genera may produce similar binding components, chemokine receptor-like molecules, or chemokine receptor antagonists (33,50,127,150). Myxoma virus, which is nonpermissive in mouse 3T3 cells, grows in 3T3 cells engineered to express certain chemokine receptors, particularly CCR5 (129), but this mechanism remains to be elucidated.

In addition to the immune modulators mentioned previously, some orthopoxviruses have a gene for a semaphorin-like protein that appears to bind to a B-cell 200-kd plexin-like transmembrane glycoprotein receptor containing a semaphorin

subdomain and may up-regulate intracellular adhesion molecule 1 (ICAM-1), induce CD-54, and reduce IL-6 production on human monocytes (45). Also, among the various TNF receptor–like proteins of cowpox virus (204) is a soluble secreted protein that binds CD-30 (D. Pickup, personal communication), a TNF-like ligand found on activated B and T cells, macrophages, and cultured NK cells that may modulate aspects of both the humoral and cellular immune responses.

Relative to controlling virus or host gene expression, various orthopoxviruses express zinc-finger proteins, phosphatases, and other kinases that probably play concerted roles in defining virus pathogenicity, including dUTPases, which may influence both neural and immunologic aspects of pathogenesis (116,229). Ectromelia virus expresses a zinc-finger–containing protein, p28, that may modulate viral replication and spread in macrophages (203). Several poxviruses, including orthopoxviruses, are predicted by sequencing or known to secrete a homolog of cellular epidermal growth factor, which may bind epidermal growth factor (EGF) receptor for roles, such as up-regulating macromolecular synthesis to facilitate cytoplasmic viral growth, inducing hyperplasia around foci of infection to provide virus susceptible cells, and affecting immune responses through affecting signal transduction (27,29,31). Vaccinia and other poxviruses express a 3b-hydroxysteroid dehydrogenase speculated to facilitate expression of immunosuppressive glucocorticoids (211). Finally, variola virus contains a correlate of the open reading frame C7L of vaccinia virus that influences the ability of vaccinia virus to grow in primate cells; however, amino acid 24 is changed in variola virus, creating an arg-gly-asp (RGD) motif (145), which is a feature of cellular adhesion proteins that bind integrins that modulate immune responses, including migration of lymphocytes across endothelial cells. Clearly, the plethora of determinants of poxvirus pathogenesis are not all discovered, and the complexity of their dynamic interplay in the host challenges our ability to molecularly define the pathogenicity of different poxviruses.

Pathology

Cytoplasmic inclusion bodies are a characteristic histopathologic feature of poxvirus infections. There are two types (Fig. 6): basophilic inclusions, or Guarnieri bodies (see Fig. 6A and Fig. 6B), which are made by all poxviruses and constitute the viroplasm or virus factory; and ATIs (see Fig. 6C and Fig. 6D), which may have an eponym and are produced by certain orthopoxviruses, cowpox (Downie body), ectromelia (Marchal body), raccoonpox, volepox, and skunkpox viruses (125), and some avipoxviruses, particularly fowlpox virus (Borrel body) (224). The five orthopoxviruses produce an ATI protein of 130- to 160-kd, which appears to provide a critical amino acid composition to form the inclusion; other orthopoxviruses produce a truncated polypeptide of 90 kd and do not form ATIs. The sequence variability of the orthopoxvirus ATI gene region has enabled development of a species-specific PCR assay (195).

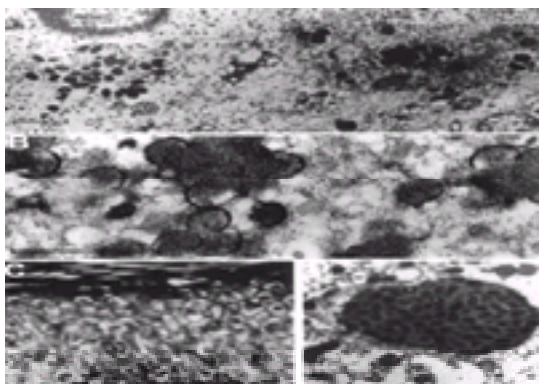

FIG. 6. Cytoplasmic inclusion bodies produced by poxviruses. **A** and **B:** Electron micrographs of viral cytoplasmic "factories"—B-type inclusion bodies that are produced by all poxviruses (B-type inclusions are basophilic by hematoxylin and eosin (H&E) staining.) **A:** Low-power electron micrograph of immature virus particles (IVs) on the *right* and nonenveloped intracellular mature virions (IMVs) on the *left.* **B:** Higher-power micrograph showing viroplasm containing the cupules of developing particles and immature particles. **C** and **D**: A-type inclusion bodies (ATIs), which appear late in the replication cycle of a few poxviruses, including the orthopoxviruses cowpox, ectromelia, raccoonpox, volepox, and skunkpox viruses and the avipoxvirus fowlpox virus; ATIs are eosinophilic by H&E staining. **C:** A-type inclusion body in cells in the skin of a mouse infected with ectromelia virus (H&E stain). **D**: Low-power electron micrograph of mature cowpox virions occluded within an ATI; some mutants show no occluded virions, and others show virions clustered around the outside of the inclusion body (103) (**A** from ref. 177, with permission; **B** from ref. 217, with permission; **C** from ref. 69, with permission; **D** courtesy of Y. Ichihashi.)

Smallpox

Hematoxylin and eosin stained sections of early-stage lesions in the skin and oropharyngeal mucous membranes of smallpox cases often showed B-type inclusions as round or oval, faintly basophilic bodies lying in the cytoplasm, usually close to the nucleus. In more advanced lesions, suitably stained sections showed faintly acidophilic granular inclusions occupying a large part of the cytoplasm of infected cells. These are seen particularly well in the epithelial cells at the base of vesicles and pustules.

The vesiculopustular skin rash, which appeared just after the viremic and toxemic prodrome, was the most outstanding pathologic feature of smallpox. Bras has described the evolution of the skin lesions (20; see also 54,72,186, and 190). The earliest change was dilation of the capillaries in the papillae of the corium, with swelling of the lining endothelium and infiltration of lymphocytes and histiocytes. Changes then appeared in the overlying epidermis. At first, a few cells in the middle layer of the epidermis became enlarged and vacuolated, and then great thickening of the epithelial layer occurred as cells in the malpighian layer proliferated and swelled. As the process of degeneration spread to neighboring cells in the middle layer, there was edema, the cell membranes ruptured, and a vesicle was formed, which was loculated,

with septa formed by the remains of incompletely destroyed cells. Polymorphonuclear leukocytes entered the vesicle from the dermis to produce a pustule, and the septa, which previously crossed the vesicle, disappeared. Fluid was absorbed from the pustule, drying the contents. Epithelial cells from the sides of the cavity grew under the residual mass of the exudate, so that finally a crusty scab was left, which consisted of degenerated epithelial cells, leukocytes, and cellular debris. Pockmarks, which were most severe on the face, were caused by destruction of sebaceous glands, followed by organization and subsequent shrinking of granulation tissue.

Because of the absence of an impervious keratin layer in oropharyngeal tissue, damaged epithelial cells, which were shed from oropharyngeal lesions that formed just as the fever began subsiding and immediately before the skin rash, produced highly infectious oropharyngeal excretions. There were few specific lesions in other organs, although small hemorrhages often occurred in the heart and liver, and endothelial cells throughout the body often showed hyperplasia and degenerative changes (21). In hemorrhagic, or “black,” smallpox, hemorrhages occurred under the serosa in the pericardium, pleura, and peritoneum; in the lungs, kidneys, liver, bladder, uterus; and occasionally in the bone marrow (170).

Human Monkeypox

Histologic examination of a papulonecrotic lesion from autopsy skin material from human monkeypox indicated that the skin lesions were like those in smallpox (216). In contrast to smallpox, generalized lymphadenopathy is a relatively common feature of human and simian monkeypox. No lymph nodes or other tissues from human monkeypox have been examined by histology to date, but in simian monkeypox, lymph nodes showed hyperplasia, medullary edema, dilation of sinuses, and occasionally degeneration and necrosis of cells in germinal centers; immunofluorescent staining showed large amounts of viral antigen in the necrotic foci (42,233).

Vaccinia and Cowpox

Vaccinia and cowpox skin lesions show some similarities to smallpox lesions. There is greater epithelial thickening and less rapid cell necrosis in cowpox than in vaccinia, and there is a greater involvement of mesodermal tissues. The most striking feature of cowpox is the presence of two types of cytoplasmic inclusions in virus-infected cells: rather faint, irregular, B-type inclusions; and several often large and somewhat homogenous ATIs (Fig. 6), which were first described by Downie (55). Some orthopoxviruses produce irregularly shaped ATIs (125) or ATIs with or without virions (103).

Clinical Features

Smallpox

The clinical features of smallpox are described with excellent photographs in several publications (54,72,186,190). Since the late 19th century, two varieties of smallpox have been distinguished on the basis of the severity of the disease and the mortality rate: variola major, the conventional disease of earlier times; and variola minor (called *alastrim* in South America), which was endemic along with variola major in the Americas and Africa. Although the rash was often quite extensive, alastrim produced a much less severe toxemia and a case-fatality rate among unvaccinated subjects of less than 1%, compared with 5% to 30% fatality rates for contemporary variola major outbreaks. DNA sequencing (144,146,205) has indicated distinct differences within the genome terminal regions of virus isolates from cases categorized clinically and epidemiologically as either variola major or minor, confirming earlier restriction mapping observations (60,62) that alastrim variola minor virus is a distinct subspecies. Alastrim spread through the Americas and Europe beginning early in the 20th century. On the other hand, some strains designated as variola minor viruses, such as that which occurred in Somalia in 1977, were not true alastrim virus by DNA analysis (62,146), but were much more like variola major virus, and probably represent attenuated variants.

The incubation period of ordinary smallpox was 10 to 14 days (Fig. 7). The onset of symptoms, or prodrome, was acute, with fever, malaise, headache, and backache. This phase, which was toxemic, lasted about 5 days; in pale-skinned subjects, onset of symptoms was sometimes accompanied by an erythematous rash or rarely a petechial rash, both of which were distinct from the vesiculopustular rash. On about the 3rd day after symptom onset, the rash began to form, first on the buccal and oropharyngeal mucosa, then on the face, the forearms, hands, and lower limbs, and then, within a day or so, on the torso. In contrast to chickenpox, in which periodic crops of rash arise, with the first crop appearing exactly at the peak of fever, smallpox lesions cropped up simultaneously just as the fever began to subside. On any part of the body, smallpox lesions were at the same stage of development, whereas in chickenpox, the lesions are at different stages. Smallpox lesions often arose on the soles and palms, which is uncommon in chickenpox.

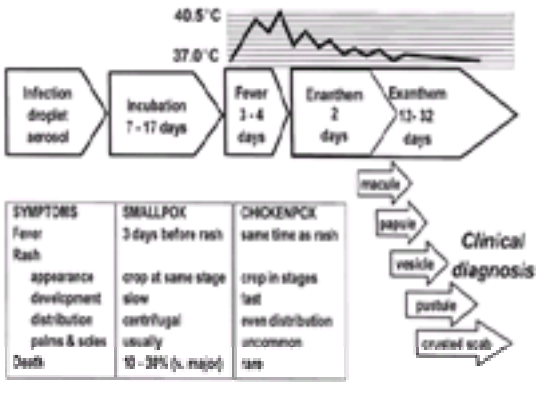

FIG. 7. The development of discrete ordinary smallpox. After aerosol droplet infection and incubation period, a prodrome, which includes a febrile response, is followed by development of an oropharyngeal enanthem and, soon thereafter, a dermal exanthem (the skin rash). The stages of rash development and a comparison of smallpox and chickenpox

symptoms are shown.

Smallpox lesions began as macules, which soon became firm papules, and then vesicles, which quickly became opaque pustules that were raised from the skin, tense, velvety, and firm to the touch as if filled with a small bead. About 8 or 9 days after rash onset, the pustules became umbilicated and then dried up, becoming crusty scabs by day 14 to 16. Within 4 days, most scabs had separated, except those on the palms and soles, which fell off last. The distribution of the rash, as well as its evolution, was a highly characteristic centrifugal spread, usually most profuse on the face and more abundant on the forearms than the upper arms and on the lower legs than the thighs, but relatively sparse on the trunk, especially the abdomen (see Fig. 3).

The previous description relates to what was called *ordinary* smallpox, which was by far the most common clinical form produced by both variola major and minor viruses. According to the severity of the rash on the face and upper limbs, ordinary smallpox was subdivided into confluent, semiconfluent, and discrete types (186). Alastrim was nearly always discrete, ordinary smallpox; however, the toxemia was much less, the individual lesions were generally less severe and smaller, and the rash healed quicker than in variola major.

Three other clinical types of smallpox were recognized: *flat*, *hemorrhagic*, and *modified*. Hemorrhagic smallpox was rare and associated with dermal petechia, bleeding from the conjunctiva and mucous membranes, very severe toxemia, and early death, often before skin lesions developed. Flat smallpox, which was also rare and mostly fatal, was characterized by intense toxemia and delayed appearance and slow evolution of skin lesions, which were usually flat and soft and nonsuppurative. The physiopathologic mechanisms and concurrent factors that influenced these rare forms are unknown, but they were not due to more virulent virus strains because infected case contacts usually developed ordinary smallpox. Hemorrhagic smallpox was more common in pregnant women than in other adults; flat smallpox was probably associated with particular defects in cell-mediated immunity. Modified smallpox, which was usually nonfatal, was seen in people who had been vaccinated, usually many years earlier; the rash evolved quickly, and the lesions were often more variable in size than in discrete, ordinary smallpox and were sometimes rather sparse.

Human Monkeypox

The clinical features of human monkeypox (see Fig. 4) are generally indistinguishable from those of ordinary smallpox, except there is often a much more pronounced enlargement of cervical and inguinal lymph nodes than in smallpox (110). Syndromes resembling flat-type smallpox have not been seen in human monkeypox, and what appeared to have been hemorrhagic-type monkeypox with liver and spleen involvement has been reported for two cases in Gabon (153).

Vaccinia

When vaccinia virus is deliberately introduced into the skin for vaccination, a papule appears at the site in 3 to 4 days, and by 2 to 3 days, this progresses to a typical umbilicated and multilocular *Jennerian* vesicle (Fig. 8). As with a smallpox lesion, the contents of a vaccination lesion rapidly become turbid upon infiltration of inflammatory cells, and the center becomes surrounded by erythema and induration, reaching maximum diameter by approximately day 11. At this time, the draining lymph nodes in the axilla may appear enlarged and tender; many patients develop mild fever. The pustule dries from the center outward, and the brownish crusted scab falls off in about 3 weeks, leaving a scar, which historically was used as the epidemiologic marker of vaccination, recognizable for many years.

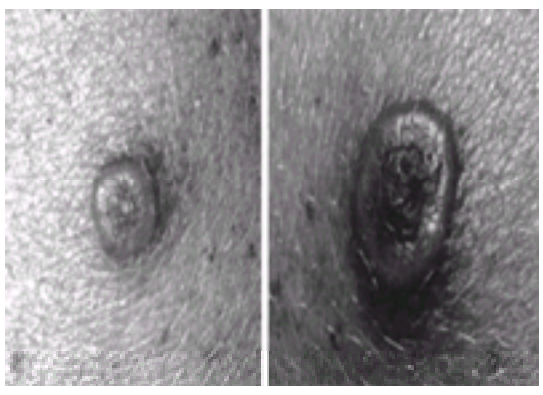

FIG. 8. Primary response to vaccination; typical vesiculopustular response, maximal at 8 to 11 days. **Left:** At 7 days. **Right:** At 11 days. (Courtesy of J. R. L. Forsyth.)

Complications of Vaccination

The occasional serious complications from smallpox vaccination (Fig. 9**A**, Fig. 9**B** and Fig. 9**C**) were more important than the slight morbidity that occurred from side effects, such as fever, lymphadenopathy, allergic skin reactions (Stevens-Johnson syndrome and urticaria), and auto-inoculation of some part of the body distant from the inoculation site, particularly ocular. From 1963 to 1968 in the United States, ocular vaccinia (see Fig. 9**D**) was observed in 348 people, including 259 vaccinees and 66 contacts (199); 22 cases showed corneal involvement, and 11 had residual visual defects.

FIG. 9. Severe complications of vaccination. **A:** Eczema vaccinatum in the unvaccinated contact of a vaccinated sibling. **B:** Progressive vaccinia (vaccinia necrosum), which was fatal, in a child with a congenital defect in cell-mediated immunity. **C:** Generalized vaccinia, 10 days after primary vaccination; benign course, no scarring. **D**: Ocular vaccinia after autoinoculation. (From ref. 72, with permission.)

Two large surveys of postvaccinal complications were reported (130,131) on the basis of vaccination as practiced in the United States in 1968 using vaccine manufactured by scarifying the skin of calves with the New York City Board of Health (NYBH) strain of vaccinia virus. Vaccination using this strain produced the following serious complications:

- Progressive vaccinia (vaccinia gangrenosa) (see Fig. 9**B**), which usually affected people with cell-mediated immunologic problems (78,79), occurred at a rate of about 1 per million vaccinations or revaccinations, and thus, congenital or acquired immunodeficiency diseases and pharmacologic immune suppression remain absolute contraindications to vaccination.
- Eczema vaccinatum (see Fig. 9**A**), which appeared only in people who had a history or suffered from eczema, a contraindication to vaccination, occurred at rates of 66 cases and no deaths among 14.5 million vaccinees, and 60 cases with one death among their several million contacts (vaccination under cover of VIG or treatment of established cases with VIG sometimes eased the severity of this complication).
- Generalized vaccinia, which may produce a rash covering the entire body (see Fig. 9**C**), occurred in 141 cases (no deaths) at 6 to 9 days after vaccination among 14.5 million vaccinees. The lesions resembled that at the inoculation site or sometimes varied in size; the prognosis was good because this side effect was not associated with immunodeficiency.
- Postvaccinial encephalitis, which was an unpredictable complication, occurred with widely differing frequencies in different countries (72,235).

In addition to these specific side effects, general encephalopathy was associated with a high viremia, particularly in infants younger than 1 year old; 4 cases with 3 deaths occurred among 614,000 vaccinees in this age group in the U.S. study. However, in older people, pathologic findings resembled other types of encephalitides, principally perivenular inflammation and demyelination (112). The incidence of this complication, which could not be predicted on the basis of preexisting disease in the vaccinee, was influenced by the virus strain used and was lowest with the calf skin Wyeth seedlot of the NYBH strain in the formulation; the U.S. survey showed 12 cases with one death among some 13 million vaccinees. Studies in the Soviet Union from 1968 to 1979 suggested generally higher side-effect rates in older primary vaccinees, including 313 cases of neurologic complications per million in people older than 5 years of age (87). Further information on complication rates outside the United States is available (88,188).

Attenuated or Recombinant Poxvirus Vaccines

During the latter days of eradication, attempts had been made to develop so-called priming vaccines, which were attenuated vaccinia strains that could be given before the standard vaccine to people who needed protection from smallpox but who were at risk for complications because of eczema or other contraindications. For example, safety tests were conducted in thousands of healthy people using variants of standard vaccinia strains that had been passaged hundreds of times on the chorioallantoic membrane (CAM) of chick embryos (e.g., the NYBH variant CV-I-78 in the United States and the modified virus ankara [MVA] strain in Germany) or adapted to grow at 30°C and plaque purified (e.g., the Lister variants LC16m0 and LC16m8 in Japan) (118,147,219). As eradication became a reality, further development of such strains as smallpox vaccines was virtually abandoned; however, as recombinant poxvirus vaccine technology developed, two of these strains emerged as potentially useful substrates for human vaccines: MVA, which replicates fully in avian cells and shows abortive infection in most mammalian cell lines and humans (34); and LC16m0, which is replication competent in most cell lines and shows high attenuation in experimental animals (219). In addition, a wide variety of other genetically engineered mutants with various attenuation traits and gene expression properties have been constructed for reasons of versatility. Several reviews on vaccinia recombinant vaccine candidates are available (77,133,156,157,174,180). Since the first recombinant vaccine candidates were produced in 1982 (139,173), no recombinant poxvirus vaccine has been sanctioned by regulatory agencies for routine use in humans, although a vaccinia rabies vaccine is being used to control wildlife rabies in several countries (24,175).

Cowpox

Downie (55) described lesions on people infected with cowpox virus from infected cows; lesions on humans appear similar to those evidently acquired from other cowpox-infected animals, commonly cats in Europe (9). One or more lesions usually appear on the hands; the thumbs, the first interdigital cleft, and the forefinger are quite vulnerable. Scratches or abrasions of the skin may determine localization of the lesions elsewhere on the hands, forearms, or face. Lesions resemble those of primary vaccination, passing through stages of vesicle and pustule before a scab forms. Local edema may be more pronounced than in vaccination, and lymphangitis, lymphadenitis, and often fever may persist for a

few days. There is virtually no encephalitis, but there may be multiple primary lesions, and generalized rash may occur. Children may have a more severe infection than adults.

Diagnosis

Differential Diagnosis

Smallpox

When smallpox was endemic, the diagnosis of most cases could be made easily from the distribution and evolution of the rash (54,72,186). Difficulties arose in outbreaks associated with importations into nonendemic countries, especially in cases of hemorrhagic smallpox, which were confused with meningococcal septicemia or acute leukemia. In situations in which their occurrence was not expected, modified-type variola major and ordinary-type variola minor were often confused with chickenpox, but the evolution and the distribution of the rash in the two diseases were usually distinguishable (see Fig. 7). More rarely, on first examination of a case, erythema multiforme or secondary syphilis might cause difficulties in diagnosis.

Human Monkeypox

Human monkeypox cannot be routinely differentiated from smallpox clinically, although enlargement of the cervical and inguinal lymph nodes is usual in human monkeypox and was virtually unknown in smallpox. With smallpox eradicated, the geographic localization of cases to African rainforest countries and other epidemiologic features help in making a clinical diagnosis; however, chickenpox, which is not uncommon in these countries, may easily be confused with monkeypox by local health care workers. Confirmation can be made by identifying the virus in skin lesions, such as by PCR (195), or by genus-specific antibody tests because no other orthopoxvirus is known in the endemic areas. Monkeypox-specific antibody tests, which use serum cross-absorption, have been employed to screen sera (102,165), but a gold standard species-specific test for orthopoxviruses has not been developed.

Vaccinia

Clinical diagnosis could be problematic for contact cases, but a history of contact with a recently vaccinated person or a laboratory source of vaccinia virus should arouse suspicion; the diagnosis can be confirmed by recovery and characterization of the virus. Buffalopox virus infection of humans has been reported from countries with domesticated river buffalo (*Bubalis* species). Since 1980, only India has reported infections in milking buffalo and dairy cattle, although horses and goats may sometimes become infected (59,128). Human infections are generally acquired occupationally or by drinking unpasteurized milk. The incubation period is about 7 days before vaccinia-like lesions appear on the hands or in the throat; there is virtually no interhuman transmission. Lesions usually heal by day 15 after infection. In some cases, differential diagnosis from infection by parapoxviruses must be made.

Cowpox

Cowpox in humans was traditionally associated with milking infected dairy cows in Europe, but many cases gave no history of contact with cattle; domestic cats now constitute a more important source of human infection (9). Occupationally acquired parapoxvirus infection sometimes must be differentiated from cowpox; parapoxviruses produce nonulcerating nodules, and cowpox virus causes pustular lesions (Fig. 10).

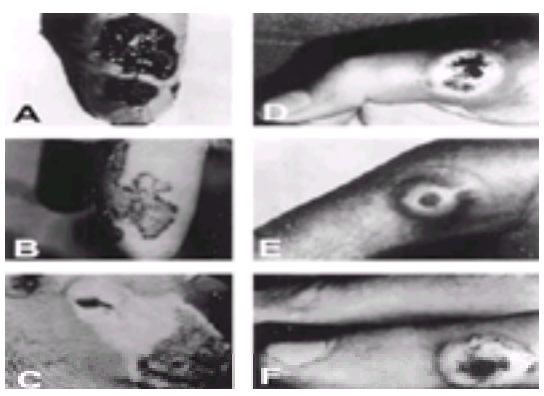

FIG. 10. Cowpox, pseudocowpox, and orf virus infections of animals (*left*) and lesions caused by these viruses on the hands (*right*). **A:** Cowpox ulcer on teat of cow, 7 days after onset of symptoms. **B:** Pseudocowpox (milker's nodule) virus infection of teat of cow. **C:** Orf virus scabby mouth in a lamb. **D:** Cowpox. **E:** Milker's nodule. **F**: Orf. (**A** and **B** courtesy of E. P. J. Gibbs; **C** courtesy of A. J. Robinson; **D** courtesy of A. D. McNae; **E** and **F** courtesy of J. Nagington.)

Laboratory Diagnosis

Laboratory methods for confirmation of orthopoxvirus infections are available (73,165,195). Laboratory diagnosis of an active infection is by discovery of characteristic virions in lesion material (see Fig. 1) and verification of genus and species by biologic or molecular tests, such as PCR assays. Histologic staining of viral inclusions in cells at the base of lesions may be used for diagnosis, but such methods are being displaced by techniques that use antibodies, PCR, or hybridization to detect virus *in situ.* Retrospective identification of infection uses convalescent-phase sera in various

assays, including enzyme-linked immunosorbent assay (ELISA), Western blot, and virus neutralization assays.

Clinical recognition of human monkeypox was made simpler by the eradication of smallpox from West and Central Africa; confirmatory diagnosis requires characterization of the virus in vesicle fluid or scabs and differentiation from chickenpox, usually by PCR. Cowpox is clinically distinctive, although laboratory confirmation by differential assays is important because of the rarity of the disease. Apart from buffalopox, vaccinia infections ordinarily do not need laboratory confirmation because the source of infection (vaccination) is usually known.

Treatment

Experimental orthopoxvirus infections of cell cultures and animals are variably susceptible to certain thiosemicarbazones; however, such drugs were found to be impractical for treating smallpox (72) and presently are not recommended to treat any other orthopoxvirus infection or postvaccinal complication. Presently, VIG is the usual treatment for eczema vaccinatum, accidental autoinoculation with vaccinia virus, and some cases of progressive vaccinia, although it has not been useful for treating postvaccinial encephalitis, and it may be contraindicated in auto-inoculation ocular vaccinia with keratitis (37,179). More recently, acyclic nucleoside phosphonates, like Cidofovir (Vistide) (51), and the antiviral ribavirin (120) have been used to inhibit growth in cell culture and animals of some orthopoxviruses, including variola virus in cell culture (104). Although no controlled clinical trials were performed, vaccination within 4 days after exposure to smallpox appeared to be an effective treatment (93).

Around the world, most institutions working with human orthopoxviruses (variola virus is restricted to WHO Collaborating Centers in the United States and Russia) usually vaccinate staff at risk for infection by administering their country's standard smallpox vaccine. If vaccination policies exist in different countries, they are generally patterned after recommendations used in the United States, which advise vaccination within 10 years for laboratory and health care workers who handle human orthopoxviruses (37; updates at http://www.cdc.gov/), and the United Kingdom, which advise obligatory vaccination for people handling monkeypox virus; otherwise, a case-by-case decision is made (2).

Epidemiology

Smallpox

Variola major case-fatality rates in outbreaks in the past millennium sometimes approached 30%, with considerable variations in different geographic regions that were probably influenced by concurrent factors such as nutrition status or quality of health care. During the WHO Intensified Smallpox Eradication Program (1967–1980), fatality rates were about 10% in African countries and about 20% in Asian countries, which suggested that the virus strain also played a role in the outcome of infection. In this regard, alastrim, which emerged in the late 19th century, was associated with a case-fatality rate of 1% or less in all affected countries (72).

Unvaccinated people of all ages were susceptible to smallpox, but mortality was highest in the very young and the elderly. Age incidence of infection depended on epidemiologic circumstances; where it was endemic in large population groups, smallpox was a disease of childhood. In rural populations, the disease might have been absent for many years, but then on its reappearance, people of varying ages would be susceptible, and an epidemic would ensue. When smallpox was introduced into virgin-soil communities in North, Central, and South America, in Central and South Africa, and in Australia, enormous epidemics ensued with very high mortality rates, in part owing to a breakdown of the social system because of simultaneous illness of so many adults in subsistence societies without food reserves.

One attack of smallpox provided solid immunity to reinfection. Vaccination with a potent vaccine gave complete protection against smallpox for about 5 years and varying degrees of protection after that. Before eradication, to provide an adequate margin of safety, the WHO Committee on International Quarantine specified that valid vaccination certificates for international travelers required successful vaccination to have been performed within the previous 3 years.

Human Monkeypox

The epidemiology of human monkeypox is quite different from that of smallpox; the disease is a rare zoonosis limited to tropical rainforest villages of West and Central Africa (110). Between 1970 and 1995, about 400 cases, most of which had been clinically diagnosed and verified by laboratory tests, were reported from eight African countries (see Fig. 5), mostly from the DRC (former Zaire). Of these, 338 cases, including 33 (9.8%) deaths, were discovered in five DRC provinces (1980 population, 5 million) during a largely prospective, intensified WHO surveillance from 1981 to 1986 that covered all of the DRC (1980 population, 28 million). In this study, 67% of the cases were substantiated by virus isolation, 30% by serology, and 3% by strong clinical data. Query data indicated that 72% of the 338 cases acquired the disease from wild animals, likely including infected squirrels caught to eat; and the secondary attack rate for people lacking cross-protective smallpox vaccination was estimated to be 7.5%. The study results indicated few clusters of cases and a dependence on an animal reservoir to sustain human infection, primarily because of the low (28%) interhuman transmissibility of monkeypox virus. Incidence modeling indicated that in an unvaccinated population, interhuman circulation of virus would not continue beyond a cascade of 14 human-to-human transmissions.

From February to September in 1996, 71 human monkeypox cases, including 6 deaths, were reported from the DRC (1999 population, 55 million). The reports prompted three WHO-sponsored, multiagency, short-term missions in 1997 to 1998 to identify cases, mostly retrospectively, because of heightened civil unrest. Although data from a 1998 mission was fragmentary, results from two epidemiologic surveys in 1997 suggested that a high proportion of cases (~75%) reported acquiring disease from another case, and there were several clusters of cases (38,39,96,159,239). In 1997, the survey teams visited 78 villages in the Katako-Kombe and Lodja areas (population, 0.6 million) in the Kasai Oriental Province in central DRC; 511 suspected active and retrospective cases were recognized (308 cases, including 6 active cases, were reported from the 1998 mission, but query data on only 50 cases were obtained). Of 42 cases in the rash stage that were discovered during the three missions, 26 were verified as monkeypox by virus isolation and PCR assays, in contrast to

the 1980s prospective study in which 227 of 338 cases were verified by virus isolation. The limitation to retrospective methods in the recent studies was compounded by a relative lack of expertise in clinically identifying monkeypox and differentiating it from chickenpox; 7 of the 42 active rash cases showed varicella virus by PCR assay. The missions were limited by a lack of surveillance continuity, use of disparate case definitions with attendant recall prejudices, and selection, ascertainment, and misclassification biases. Moreover, a critical weakness was dependence on invalidated serologic tests to confirm cases that met the epidemiologic case definition.

Despite the problems, some estimates were made. As found in the 1980s, the disease occurred mostly in children under 16 years of age, and the illness reported in 1996 to 1998 seemed no more severe than in the 1980s. A secondary attack rate was estimated to be about 8%, like that in the 1980s, which indicated a relative genetic stability of the virus over the years. DNA mapping and limited sequencing substantiated the close relationship of selected isolates from the DRC since 1970. In the 1997 to 1998 surveys, most reported cases occurred between June and September, a seasonality that was less clear in the 1980s study. The increased proportion of secondary cases, about 75% (28% in the 1980s), was attributed to reduced herd immunity caused by cessation of smallpox vaccination in the country by 1982. Misdiagnosed chickenpox was suspected to be responsible for some of the cases.

The limitations of the retrospective studies were likely insufficient to explain the increased number of monkeypox cases per capita compared with the 1980s. During a review at the WHO in 1999 (241), it was surmised that at least half of the 800 suspected cases in the Kasai Oriental that reported onset between February 1996 and July 1998 were monkeypox, and the rest were mostly chickenpox. WHO recommended that intensified prospective studies should be carried out as soon as possible to better define the estimated increased incidence since the 1980s and to understand the virus in the animal reservoir.

Vaccinia

Vaccinia virus ordinarily has no epidemiologic distinction because of little capacity to spread between humans; nowadays, it is mostly used for research. Vaccination was practiced during the eradication era on such a large scale that sometimes domestic animals, including cows, pigs, rabbits, and buffaloes, were accidentally infected with the virus, and humans were, in turn, infected from lesions on domestic animals (72). Unexpectedly, vaccinia virus was isolated in 1999 from farmers and dairy cattle in Northern Brazil (N. Moussatché, personal communication). During the latter days of the smallpox eradication era, certain strains of vaccinia virus (e.g., Lister, NYBH, and Tian Tan) became predominant for human vaccination as the rate of side effects for various vaccine strains in use became known (88,188).

Buffalopox

The most important veterinary infection with vaccinia virus is caused by buffalopox virus, which was thought to be a distinct species but is now regarded as a vaccinia virus subspecies as a result of DNA restriction endonuclease mapping of some isolates (59). During the WHO smallpox eradication program in the 1970s, buffalopox occurred in India, Egypt, and Indonesia, where water buffaloes, *Bubalis bubalis* (there are over 50 million in India), are used as domestic animals, and in certain countries had been used for vaccine manufacture (128). The disease has persisted in India since cessation of smallpox vaccination (161). Buffalopox is characterized by pustular lesions on the teats and udders of milking buffaloes and dairy cows; occasionally, especially in calves, a generalized disease is seen. Lesions may occur on milker's hands and face and in the mouth of people who drink unpasteurized milk from infected animals. Smallpox vaccination most likely had been protective. Several of the more recently isolated strains appear to have been maintained in nature by serial infection of buffaloes and dairy cows or possibly some wild animal host. Most isolates have somewhat different restriction maps compared with vaccinia strains used in India in the days of smallpox vaccination, although the differences observed were no greater than differences between a variety of vaccinia strains (59).

Cowpox

Cowpox virus is rarely contracted by humans directly from ulcers on the teats of infected dairy cows (see Fig. 10**A** and Fig. 10**D**); even in Jenner's day, “spurious cowpox,” which is caused by a parapoxvirus, was relatively much more common (36) and still is. Various studies have indicated that cowpox virus is transmitted inadvertently to humans, mainly by animals that acquire the disease from wild rodents that are indigenous to Europe and some adjoining Asian countries. Baxby and colleagues have studied cowpox epidemiology extensively over the years and have concluded that cowpox virus is restricted geographically to a region bounded by Norway, Northern Russia (includes Moscow), Turkmenia, Northern Italy, France, and Great Britain, probably because of limited ability of the virus to adapt beyond certain indigenous animals that constitute the reservoir (40). Moreover, based on their own studies and other reports, they concluded that the reservoir includes the following: bank voles (*Clethrionomys glareolus*), field voles (*Microtus agrestis*), and possibly wood mice (*Apodemus sylvaticus*) in Great Britain (except Ireland) and conceivably all of Europe; Turkmenian susliks (*Rhombomys opimus*) and gerbils (*Citellus fulvus*); Russian root voles (*Microtus oeconomus*) and possibly Russian (Georgian) gerbils (*Meriones libicus*); and Norwegian lemmings (*Lemmus lemmus*). Cowpox virus has also been isolated from Russian laboratory rats and humans who have handled infected rats and from zoo animals, but Baxby and colleagues have reasoned that the virus is not enzootic in rats, or else it would be found worldwide. It appears that domestic cats play a major role as intermediate hosts that transmit cowpox from rodents to humans, and possibly to horses, dogs, foxes, and various zoo animals, which may also be intermediate inadvertently infected hosts (9).

PARAPOXVIRUS INFECTIONS IN HUMANS

History

Humans acquire the following parapoxvirus infections, usually occurring as localized epithelial lesions contracted occupationally from infected animals and rarely from fomites: (a) orf, which is transmitted by sheep, goats, and several wild artiodactyls (e.g., bighorn sheep, mountain goats, reindeer), and is referred to in animals as orf, contagious pustular dermatitis, contagious ecthyma, or scabby mouth; (b) milker's nodule, which is transmitted by dairy cattle, and is referred

to as paravaccinia or pseudocowpox in these animals; and (c) bovine papular stomatitis, which is transmitted by beef cattle and possibly gazelles, and designated bovine papular stomatitis in these animals. Skin lesions on humans and some of the animals infected with these viruses are shown in Figure 10.

Reviews on parapoxvirus infections are available (90,152,179,192,193,244). Orf was first described as a disease of sheep in 1787, of goats in 1897, and of humans in 1934. Pseudocowpox lesions on the teats of cows constituted one of the forms of spurious cowpox about which Jenner cautioned should not to be used for smallpox vaccination (108). On rare occasions, humans have been infected with parapoxviruses of seals or possibly camels. In camels, parapoxvirus disease is termed *contagious ecthyma, amru,* or *ausdyk* (192,193). Camels may also acquire a rather severe generalized rash illness termed *camelpox,* which is caused by the orthopoxvirus camelpox virus, but the disease is not transmissible to humans (111).

Infectious Agents

Parapoxvirus virions are distinctive ovoid particles (see Fig. 1) that vary in size from 260 nm long by 160 nm wide (orf virus) to 300 nm by 190 nm (pseudocowpox virus). The outer membrane contains a tubular, threadlike structure, 10 to 20 nm wide and perhaps 1,000 nm long, that spirals around the particle, giving a characteristic cross-hatched appearance when opposing surfaces are viewed by negative-stain electron microscopy. Infectivity is resistant to ether and chloroform and is destroyed by heating at 58°C for 30 minutes. Orf virus is viable for long periods in dried scabs, which is an important feature for maintaining enzootic infection in sheep under field conditions.

The genome of parapoxviruses consists of a covalently closed, linear, double-stranded DNA of about 135 kbp and a 63% G+C (152,158,193). Within the genus, restriction patterns of DNAs of different parapoxviruses are more heterogeneous than DNAs of different orthopoxviruses (152), although Southern blot restriction mapping has indicated extensive sequence homology of different parapoxvirus DNAs within about 80 kbp of the central region and that the terminal region sequences are rather diverged and species specific (80). There is considerable synteny in the physical arrangement of parapoxvirus genes and the arrangement of orthopoxvirus genes; promoters and the function of several viral proteins are related, although the sequences are diverged. The demonstration (75) of nongenetic reactivation between parapoxviruses and orthopoxviruses—infection of cells with orthopoxviruses that are heat inactivated (which probably inactivates virion transcription enzymes) and reactivating their growth using parapoxviruses killed with ultraviolet light (which inactivates DNA)—has indicated that members of both genera have complementary components. Parapoxvirus promoter sequences can regulate transcription if they are inserted into orthopoxvirus DNA (152). Immune-modulating proteins specified by parapoxviruses include a vascular endothelial growth factor homolog that may effect angiogenesis in lesions, correlates of ovine IL-10 and an IFN-induced kinase inhibitor, and a secreted inhibitor of granulocyte-macrophage colony-stimulating factor (52,90).

Pathogenesis and Pathology

Parapoxvirus epidermal lesions are markedly proliferative. There are different incubation periods for orf virus in humans (3 to 14 days) and sheep (2 to 4 days), and the primary lesions differ clinically and histologically. Lesions in sheep appear as plugs penetrating the dermis (rete formation) and contain a massive accumulation of class II MHC-positive dendritic cells. Lesions in humans typically show a central umbilication with an erythematous margin and infiltration with monocytes and lymphoid cells more prominent around hyperemic capillaries and venules. Infection of endothelial cells may produce endothelial proliferation. The different lesions in humans and sheep suggest different viral clearance mechanisms (89,90,243,244).

The humoral immune response is generally similar in sheep and humans, although the durability of the response may be more in humans than sheep, and secondary infections producing milder lesions may occur in sheep. Early changes in proliferating keratinocytes include nucleolar enlargement and focal lysis of keratin fibrils (124). Extreme swelling of the cells results in ballooning degeneration, which, when accompanied by B-type cytoplasmic inclusion bodies and nuclear shrinkage, is pathognomonic of orf lesions. In humans, cellular and humoral immunity is significantly protective, although exceptions exist, such as among abattoir workers who may acquire repeated infections (193). In sheep, the cellular immune response appears more transient than in humans, and there appear to be different levels of susceptibility to reinfection of the udder, hind limb, and coronet of the hoof than other body parts—factors that might sometimes contribute to annual orf outbreaks in the same flock (193,244).

Clinical Features

Parapoxvirus lesions on humans, which occur through skin abrasions, are localized on the hands, sometimes on the face. Orf lesions on humans are 1 to 3 cm in diameter, and occasionally they are multiple. The lesion progresses from maculopapular to bullseye-like (red center, white ring, red halo) to a vascularized ulcerated nodule with central umbilication (see Fig. 10**F**), and finally to a crusted eschar with regenerating epidermis. The surrounding skin is inflamed, the lesion is painful, and there may be swelling of draining lymph nodes and low-grade fever.

Milker's nodule (see Fig. 10**E**) appears 5 to 7 days after exposure as a hemispherical, cherry-red papule that gradually enlarges to a firm, elastic, purple, and smooth hemispherical nodule that may reach 2 cm diameter and may be umbilicated when fully developed. Lesions are relatively painless but may itch. They are highly vascular, which explains the purple color, but they do not ulcerate. The granulation tissue makes up the mass of the nodule and gradually becomes absorbed; lesions disappear in about 5 weeks. The only evidence of a systemic effect is occasional slight swelling of draining lymph nodes.

Bovine pustular stomatitis lesions occur in and around the mouth of calves. Human infection, usually on the hands, is less common than orf or milker's nodule, probably because the physical contacts between animal handlers and beef cattle are less than between shearers and sheep and between milkers and dairy cattle (35).

Diagnosis

Diagnosis of human parapoxvirus infections is generally based on clinical and epidemiologic features, notably a history of contact exposure to infected animals. Laboratory verification is by observation of characteristic cross-hatched ovoid virions (see Fig. 1**B**) in lesion material; other laboratory diagnostic methods exist (73,165,195). Parapoxviruses do not produce pocks on the CAM, but they can be cultured from human lesion homogenates in primary bovine or ovine fetal kidney cells, primary ovine testis or muscle cells, or primary human amnion cells. Once in culture, the isolate usually can be passaged into established cell lines, such as human embryonic fibroblasts or monkey kidney cells. DNA restriction assay may be used for virus differentiation (80,152); efficacious, reproducible, type-specific PCR tests are not yet reported. Serologic cross-reactivity is demonstrable between parapoxviruses and certain orthopoxviruses and capripoxviruses, but cross-protection occurs only among members of the same genus. Western blot assays may be used to discern antibodies against parapoxvirus from other genera, and panels of monoclonal antibodies are described that may be useful for development of species-specific serologic assays.

Epidemiology

Orf is a common disease of sheep and goats worldwide, causing papules and vesicles sometimes around the nostrils and eyes and often on the skin of the lips of lambs and kids, which can lead to emaciation, infection of the udder of nursing ewes and dams, and consequent economic problems on farms. Human orf is a nonfatal occupational disease of animal handlers, including veterinarians, farmers, and shearers; thus, the disease is of industrial importance in countries where these animals are raised. Primary lesions on animals and humans cause more morbidity than lesions acquired by reinfection. Healing in humans and animals occurs in about a month, without scarring. Repeated infection of flocks and herds is largely due to the extended viability of virus in scabs that fall on pasture plants and the soil. In some countries, vaccination of lambs or kids with live, nonattenuated vaccine is practiced, which may lead to outbreaks on farms and human infections. Orf may be transmitted to humans from farmed or wild herds of musk-oxen and reindeer and by various other artiodactyls and wild ungulates, including deer, serow, chamoi, gazelles, bighorn sheep, and mountain goats (192). Humans have been infected by host animals kept as pets in petting zoos.

Pseudocowpox (paravaccinia) occurs in dairy herds in all parts of the world. The epizootiology of the disease in cows indicates that infection may be enzootic in cattle and may persist in small herds, in contrast to cowpox, which is sporadic (193). Milkers' nodules are contracted by contact with infected animals, and animals may be infected from the milkers' lesion through small abrasions on the teats or by mechanical transmission through teat cups of milking machines. Immunity sometimes appears to be short-lived. Cyclical waves of reinfection may occur in herds, especially in autumn and spring. Chronic lesions may occur, which are seen as mild scabbing. Beef cattle calves may transmit bovine papular stomatitis from lesions on their muzzles to their mothers' teats, which also occurs with pseudocowpox; lesions may also be chronic.

YATAPOXVIRUS INFECTIONS IN HUMANS

History

The genus *Yatapoxvirus* contains two species of African origin, tanapox virus and Yaba monkey tumor virus (YMTV); in monkeys, tanapox virus is designated Yaba-like disease virus (YLDV). Tanapox was first observed in epidemics of acute febrile illness associated with localized skin lesions in 1957 and 1962 among inhabitants of the flood plain of the Tana River in Kenya (58). The disease is endemic in this area and in the DRC (109). Epizootics of YLDV occurred in 1966 in macaques in three primate centers in the United States in which animal handlers were infected, apparently through skin abrasions (57).

YMTV was first recovered from subcutaneous tumors in captive rhesus (Asian) monkeys in Nigeria (167), but naturally occurring YMTV infections of humans have not been found in the field in Africa or Asia. Experimental subcutaneous or intradermal injection of YMTV into humans or monkeys, but not other tested animals, produced histiocytomas in the skin (85).

Infectious Agents

Tanapox virions (see Fig. 1**C**) in human lesions resemble orthopoxvirus particles, although most are enveloped (73). The yatapoxvirus genome DNA is ~145 kbp; the YLDV genome is sequenced (GonBank No. AJ293568; 144, 575 bp). DNAs of tanapox virus from Zaire and Kenya and YLDV from a monkey showed very similar restriction digest electropherograms. The patterns were rather different compared with YMTV DNA, although DNA of these viruses cross-hybridized extensively (122,126). YMTV has been used to reactivate orthopoxviruses nongenetically (225), indicating equivalent transcription components. A secreted 38-kd glycoprotein of tanapox virus binds to human IFN-g, IL-2, IL-5, and TNF-a (65,176).

Monkeys, but no other common laboratory animal, are susceptible to experimental infection with yatapoxviruses. Cross-protection tests and serologic assays have indicated that tanapox virus is related to YMTV (57). Neither species grows on the chick embryo CAM, but most strains grow in and form plaques in primate cell lines, such as CV-1 cells, producing distinctive focal lesions characterized by intense granularity, lipid vacuoles, and cell rounding; most isolates grow well at 33.5°C (58,73,126,165).

Pathogenesis and Pathology

Human tanapox lesions show marked epidermal thickening with extensive ballooning and degeneration of the prickle cell layer. The cytoplasm of swollen epidermal cells is filled with large, pleomorphic, granular, eosinophilic, B-type inclusions.

Nuclei are also swollen, with peripheral concentration of the chromatin and large nucleoli (57).

After subcutaneous or intradermal inoculation of humans or monkeys, YMTV produces histiocytic tumors that become suppurative. No true neoplastic proliferation occurs, and the lesions regress as the immune response develops (84).

Clinical Features

A detailed analysis of the clinical and epidemiologic features of tanapox as seen in 264 cases in the DRC has been reported (109) in which the diagnosis was confirmed by electron microscopy. The natural incubation period in humans is unknown, but in a person inoculated intradermally with 100 infectious particles, erythema and central thickening appeared by day 4 (57). Most patients have a mild fever before lesions erupt, sometimes accompanied by severe headache and backache, often with itching at the skin lesion site.

The appearance and evolution of a tanapox skin lesion is depicted in Figure 11. Initially, there is a small nodule without central abrasion, like an insect bite, which soon becomes papular and gradually enlarges to about 15 mm diameter by the end of week 2. An edematous zone and a large erythematous areola surround the nodule. The draining lymph nodes are enlarged and tender from about the 5th day after the appearance of the lesion, which may remain nodular, but usually ulcerates by the 3rd week and gradually heals within 5 to 6 weeks, leaving a scar. In Kenya, observed lesions were almost always solitary and on the upper arm, face, neck, and trunk (58); however, in the DRC, 22% of patients usually had two, and sometimes three or more, lesions, the maximum on one patient being 10 (109). Multiple lesions, although they differed in size, usually were close together and evolved together. In the DRC, the distribution of lesions was different from that seen in Kenya, with 72% being on the lower limbs, 17% on the upper limbs, 7% on the trunk, and 5% on the head.

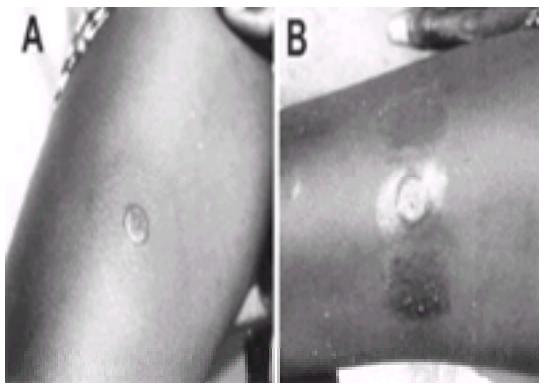

FIG. 11. Lesions produced by tanapox virus in a Congolese girl. **A:** 10 days after first appearance of lesion; **B:** 31 days after appearance of lesion. (Courtesy of Z. Jezek.)

Diagnosis

The clinical diagnosis of tanapox is based on the characteristic skin lesions occurring in parts of Africa where tanapox is known or in animal attendants who have had contact with monkeys infected with the virus (151). The slow evolution and lack of pustulation clearly differentiates tanapox from orthopoxvirus infection. Until a PCR test is developed, laboratory confirmation depends on demonstrating a high proportion of enveloped virions in lesion material, characteristic cytopathic effect in primate cell culture, failure to grow the virus on the CAM, and antigenic identification (165).

YMTV in monkeys produces large protuberant virus-containing histiocytic tumors (198). Humans are infected rarely, ordinarily by inadvertant inoculation or contact with infected monkeys. No natural infections have been reported since the original descriptions.

Epidemiology

Tanapox is a rare zoonosis, but neither the reservoir host nor the mode of transmission from wild animals to humans is known. The disease has been seen in Kenya and the DRC but may exist in other parts of tropical Africa. In Kenya, outbreaks in 1957 and 1963 were associated with periods of extensive flooding (58). In the DRC, cases occurred year-long, mostly from November to March, and the majority of cases occurred in the northwestern township of Lisala among people living within 300 meters of the Zaire River (109). Both sexes and all age groups were affected, and cases occurred much more frequently among people who worked or played close to the river than among men who engaged in hunting or worked as plantation farmers. Although clusters of cases occurred both in time and geographically, there was no indication of person-to-person transmission. Tanapox virus may be transferred to humans from monkeys or another reservoir host by biting arthropods, possibly acting as mechanical vectors (142). Mechanical transmission and human-to-human transmission may occur among animal attendants.

Tanapox virus inoculated intradermally produces skin lesions in both African and Asian monkeys (58). Analysis of monkey sera from various parts of the world for tanapox viral antigens revealed that New World monkeys were negative, but sera from several species of African primates contained antibodies (56). Because no studies of tanapox in other African wildlife have been made, it is not clear whether monkeys, like humans, are only incidental and not reservoir hosts. The disease profile in wild monkeys is unknown, but in colonies in which monkeys from various parts of the world are housed together, only Asian monkeys showed symptoms; the initial lesion was a pink, elevated papule that grew to a diameter of about 3 cm 1 week after appearance (91). Lesions regressed within 2 weeks, and usually only one lesion

appeared on an animal; occasionally, there were multiple lesions in some monkeys.

MOLLUSCUM CONTAGIOSUM

History

Molluscum contagiosum is a cosmopolitan human disease; several reviews describe the disease and the virus (13,26,179,183). In 1814, Bateman differentiated the infection from molluscum fibrosum (neurofibromatosis). In 1841, Henderson and Paterson independently described MCV intracytoplasmic inclusions (Henderson-Paterson bodies). In 1905, Juliusberg established the viral etiology, and in 1911, Lipschutz described “elementary bodies” in lesions. In 1931, Goodpasture and Woodruff described the inclusions and elementary bodies of molluscum contagiosum compared with those of fowlpox and concluded that the etiologic agent was a poxvirus. Since 1980, this relatively inconsequential illness at times has occurred in patients with AIDS as a tormenting opportunistic infection, which in Western countries amounts to a 5% to 20% incidence among homosexual men with AIDS (13).

Infectious Agent

Large numbers of MCV particles can be extruded from skin lesions, and large amounts of viral DNA can readily be extracted from virions. MCV has not been grown in established cell cultures, but virus from skin lesions has been propagated in human foreskin xenografts (28,76). The recognition of MCV transcriptional control elements in genetically engineered vaccinia virus indicates that MCV has a replication strategy similar to other poxviruses.

The MCV genome is a covalently closed, linear, double-stranded DNA of 190 kbp and 63% G+C. Restriction enzyme cleavage analysis of the DNAs of several hundred isolates from different parts of the world initially suggested two genome subtypes, subsequently a third, and recently a fourth (164), although the different DNAs cross-hybridize extensively by Southern blotting. The significance between restriction map differences and viral biologic differences is unknown; all DNA subtypes produce the same apparent disease.

Genomic DNA of MCV subtype I has been sequenced (GenBank No. U60315; 190,289 bp). Of 182 potential encoded proteins, 105 have homologs in variola virus DNA that are likely to be essential for virus replication based on correlation with known-function orthopoxvirus proteins; however, MCV generally lacks corresponding sequences for proteins involved in immune surveillance and modulation. Of the 77 predicted MCV proteins without correlates in orthopoxviruses, 36 show potential to be host-interactive homologs. *In vitro* studies indicate these are isologs of cellular glutathione peroxidase, class I MHC protein, two apoptosis inhibitors, two polypeptides that bind IL-18, and a chemokine antagonist (168,201,242).

Pathogenesis and Pathology

The typical molluscum contagiosum lesion consists of a localized mass of hypertrophied and hyperplastic epidermis extending down into the underlying dermis, without breaking the basement membrane and projecting above the adjacent skin as a visible tumor. There are increased mitotic figures in the germinal layer, above which are pathologic changes in the nuclei and cytoplasm. As the surface cells are approached, these lesions become more and more pronounced. Eventually, each cell is many times larger than normal, and the cytoplasm is filled with a large hyaline acidophilic granular mass known as the *molluscum body* (Fig. 12**A**), which pushes the nucleus to the edge of the cell and which may anatomically isolate the virus from the immune system. The core of the lesion consists of degenerating epidermal cells, with inclusion bodies, and keratin, which is produced by uninfected cells. Fully developed lesions are loculated, and there is little inflammatory reaction in the corium unless secondary bacterial infection has occurred. The tumor-like appearance of lesions is from hypertrophy of epidermal keratinocytes because of the presence of virus-packed inclusion bodies and hyperplasia of uninfected basal cells.

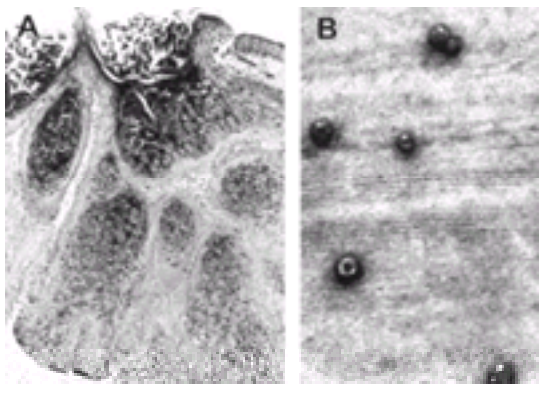

FIG. 12. Molluscum contagiosum. **A:** Section of a skin lesion of molluscum contagiosum. **B**: Lesions of molluscum contagiosum in humans. (Courtesy of D. Lowy.)

Lesions are relatively devoid of cells involved in the immune response, including NK and Langerhans cells, although T cells may be present in the perilesional dermis. Infected keratinocytes no longer display b $_2$-microglobulin (231), possibly because of complexing in the endoplasmic reticulum between an MCV homolog of MHC glycoprotein and cellular b$_2$-microglobulin (202). MCV does not appear to encode an epidermal growth factor homolog (183); however, intense staining of transferrin and EGF receptor has been observed on molluscum bodies, but not on surrounding uninfected keratinocytes (231). MCV appears to provoke little immunity; patients under immunosuppressive therapy, and some AIDS

patients, may have widespread and recurrent lesions. The lesions are noteworthy for the absence of reactive cells, and virus-specific antibodies are demonstrable in about 70% of patients (207); many patients develop antibodies after treatment for MCV (e.g., curettage). Antibodies against MCV do not react with different poxvirus genera. Lesions may persist in some patients for as little as 2 weeks or as long as 2 years, with no sign of inflammation, which may be an effect of an MCV chemokine antagonist (50). Repeated attacks are relatively common in situations in which opportunities exist for reinfection, such as in children's day care centers and among sexually promiscuous adults.

Clinical Features

Molluscum contagiosum is characterized by multiple, small 2- to 5-mm diameter noninflamed skin papules, scattered in a crop, which may arise on various parts of the body, including the torso in children and anogenital area in individuals who engage in anogenital sex. Occasionally, a single localized “giant molluscum” nodule about 10 mm in diameter may occur. Lesions rarely appear on the palms, soles, or mucosa but sometimes occur on the face, where there may be ocular involvement (179). The incubation period, as determined by inoculation of human volunteers, varies from 2 to 7 weeks. Lesions are pearly, flesh-colored, raised, and umbilicated in the epidermal layer of the skin (see Fig. 2**B**). Cropping occurs because of multiple simultaneous infections or localized mechanical spread. The lesions are painless, and at the top of each, there is often an opening through which a small white core can be seen. Infrequently, intrafollicular lesions occur as small, semitranslucent, pearly papules in the epidermis, which are difficult to identify because they lack the central umbilication. All DNA subtypes appear to produce similar lesions (183).

Solitary lesions may resemble pyogenic granuloma, keratocarcanthoma, or epithelioma. The disease is rather chronic, and lesions often persist for months or even years; ultimately, they resolve seemingly spontaneously and sometimes after trauma or bacterial infection of the lesion. Recurrences may be frequent, because of reinfection rather than latency. Lesions may be quite persistent and disfiguring in AIDS patients.

Diagnosis

Diagnosis can be made clinically from the characteristic appearance of the lesions and their chronic nature and can be verified by demonstrating “molluscum bodies” histologically or poxvirions by electron microscopy; diagnostic PCR methods are reported (169,221). Occasionally, solitary lesions on the face or neck may be misdiagnosed clinically as basal cell carcinoma; solitary tumors on the sole of the foot, although rare, also pose a clinical diagnostic problem.

Treatment

Commonly, the umbilicated core can be removed surgically, or the lesions may be treated chemically or by cryotherapy; however, lesions may recur and new ones form, particularly in AIDS patients in the anogenital area and on the head and neck. Lesions in children tend to disappear as they grow older. Topical treatment with DNA polymerase–blocking acyclic nucleoside phosphonates (Cidofovir) is promising (245).

Epidemiology

Infection occurs either by direct skin-to-skin contact or indirectly by means of fomites by virus entry through breaks in the epidermis or in the infundibular portion of the hair follicle. The incidence of disease is 2% to 8% in some populations and is more common among 10- to 12-year-old children in developed countries than in developing countries, such as Fiji and the DRC, where the peak incidence is reported between ages 2 and 3 years and 1 and 4 years, respectively. In New Guinea, a prevalence of 22% with an incidence of 6% in children younger than 10 years of age has been reported (13). The disease occurs sporadically, although it can become endemic, particularly in institutions such as children's day care or boarding schools, or in communities with poor hygiene and overcrowding. Contact transmission has occurred nosocomially, by tattooing, wrestling, or by swim team members sharing towels. Abundant lesions are common in atopic individuals who may acquire a patchy and irritable eczema around sites of infection known as *eczema molluscatum.* Genital molluscum sometimes coincides with other sexually acquired diseases, including AIDS, and may signal sexual abuse of a child; otherwise, children may acquire lesions on the arms, or most often, the upper torso.

The different DNA subtypes do not affect different body locations, as occurs in infections with human herpesviruses type 1 and 2. In one study of 261 patients, MCV subtypes I, II, and III were detected in a ratio of 226:32:3 (183). Usually, specimens from different parts of the body are the same DNA subtype as are isolates among contacts; however, on rare occasions, mixed infections have occurred. Genital MCV infection has recently become much more common based on studies in the United States and Great Britain (13).

POXVIRUS INFECTIONS OF VETERINARY IMPORTANCE

Except for smallpox and molluscum contagiosum, poxvirus infections of humans are zoonoses. Zoonotic poxviruses may cause significant livestock disease and consequent economic losses, and several poxviruses that do not affect humans are important pathogens of livestock. In addition, significant outbreaks in laboratory mice and rabbits, respectively, are caused by ectromelia and rabbitpox viruses. Recent literature is available detailing poxvirus infections of domestic and wild mammals, including ruminants, laboratory and captive animals, and rodents and lagomorphs (53,70,71,74,119,162,171,192).

Orthopoxvirus Infections

Camelpox

Camelpox affects *Camelus dromedarius* and *Camelus bactrianus* as a somewhat benign infection in adult animals but occurs more frequently in young camels as a severe generalized disease with extensive skin lesions (53,234).

Occasionally, there may be lesions in other organs, including the lungs, pharynx, trachea, heart, liver, and small intestine (123). Case-fatality rates may be as high as 25% in some epizootics. Sporadic outbreaks occur in virtually all camel-raising countries, particularly in Africa and southwestern Asia, where the camel is used as a beast of burden and milk animal (53,111). Camelpox virus has a narrow host range and can be differentiated from other orthopoxviruses by biologic characteristics (74), restriction mapping (62,189), and PCR methods (155,181). The camelpox virus genome DNA sequence closely resembles that of variola virus (G. L. Smith, personal communication). However, despite frequent exposure of unvaccinated humans to florid cases of camelpox, unequivocal human infection has not been seen. Camel parapoxvirus (ausdyk) may infect humans, and simultaneous orthopoxvirus and parapoxvirus infections of camels are reported (111,189,234).

Mousepox

Mousepox, also known as *infectious ectromelia* (impairment or loss of a limb), is caused by ectromelia virus and is known to occur only in laboratory mice, which acquire the disease through shipments of infectious laboratory mice and commercial products such as pooled mouse sera (53,70,171). Mousepox has been used as an animal model system for epidemiologic, pathogenesis, and immunologic studies. The infection has been enzootic in several mouse colonies in Europe, China, and Japan and has been imported into the United States, sometimes causing devastating outbreaks. About 40 episodes have been reported since 1950. A large outbreak at the National Institutes of Health in 1979 to 1980 led to U.S. government recommendations for stringent containment for studying mousepox. Ectromelia virus has been claimed to cause various human diseases, but never conclusively, more likely it was a contaminant that was not ruled out during diagnostic testing.

Depending on the mouse genotype, the disease may manifest either as a chronic form, characterized by ulcerating lesions of the feet, tail, and snout, or a quickly fatal illness, with extensive necrosis of the liver and spleen (Fig. 13**B** and Fig. 13**C**). Mice surviving the severe acute disease often develop a generalized pustular rash (see Fig. 13**A**). In this regard, lack of perforin, a component of NK cells, renders asymptomatic carrier C57BL/6 mice susceptible to acute illness (160), as does lack of a locus for active nitrous oxide synthetase II (115). Relevantly, ectromelia virus expresses an avidly binding TNF receptor homolog (134), which possibly modulates TNF-mediated cytolysis, releasing nitrous oxide. Sequencing of 32,318 bp of the right end of ectromelia-strain Moscow DNA showed at least six genes coding for immune modulator proteins, including an abundant chemokine-binding protein (41). Lesions in most organs, except the liver, are characterized by ATIs (Marchal bodies) in infected cells (see Fig. 6**C**).

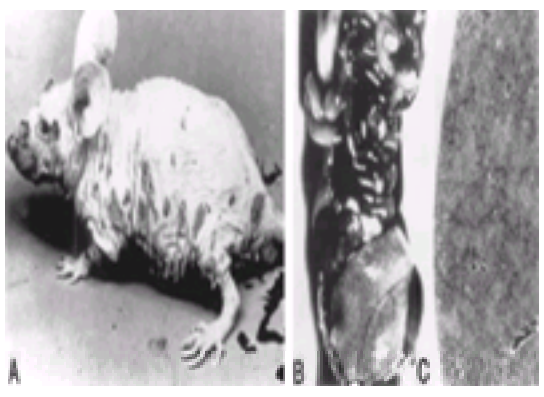

FIG. 13. Lesions of mousepox (ectromelia) in mice. **A:** The generalized rash as seen in a genetically susceptible hairless, nonathymic mouse. **B**: Swelling and necrosis of liver and spleen and hemorrhagic intestine in a susceptible mouse that died before rash appeared. **C:** Histologic appearance of necrotic liver. (**A** courtesy of Zentralinstitut für Versuchstiere, Hanover, Germany; **B** from ref. 7, with permission.)

Demonstration of poxvirus particles in lesion material by electron microscopy is diagnostic because no other poxvirus causes a naturally spreading disease in mice. If a positive result is found in an institution in a country normally free of mousepox, like the United States, other laboratory mice in the institution should be tested for infection, infected colonies should be destroyed, and the husbandry and laboratory areas should be disinfected.

Rabbitpox

At various times, laboratory rabbits in animal houses in Europe and the United States have been observed with a severe infection with rash, which turned out to be caused by the vaccinia virus subspecies rabbitpox virus (162,171). The virus involved was very similar to other strains of vaccinia virus in its restriction map and to so-called neurovaccinia virus in its biologic properties (68,74,236).

Other Species

A few other Old World orthopoxviruses are described (74,158,162,171). Uasin Gishu virus, a rare disease in horses in Kenya and Zambia, causes papular skin lesions, presumably contracted from a wildlife source. Three North American orthopoxviruses are known: volepox virus, isolated from lesions on *Microtus californicus* and *Peromiscus truii* in the Bay area of California; skunk poxvirus, isolated from lung tissue of a striped skunk (*Mephitis mephitis*) shot in Colfax, Washington; and raccoon poxvirus, isolated from lung tissue of North American raccoons (*Procyon lotor*) in Aberdeen, Maryland (125). A recombinant raccoon poxvirus multivalent vaccine candidate for pet cats is described (101); such vaccines appear sufficiently attenuated for vaccination by the intranasal or conjunctival routes (J. Orsorio, personal communication).

Capripoxvirus Infections

The genus *Capripoxvirus* includes lumpyskin disease virus (LSDV), which affects cattle in eastern, central, and mainly southern Africa, and sheeppox and goatpox viruses, which occur endemically in southwest Asia, the Indian subcontinent, and most of Africa, except southern Africa (162). Although the geographic distribution of the diseases may be different, the causative viruses are virtually indistinguishable by conventional serology and hardly distinguishable by DNA restriction assay. Such assays have indicated that African sheeppox virus is more like LSDV than goatpox virus. Although sheeppox and goatpox viruses are often considered host specific, in parts of Africa where sheep and goats are herded together, during an outbreak either virus may produce disease in both animals, indicating that some strains have a dual host range. Attenuated or inactivated vaccines are available in endemic countries; sheeppox vaccine can protect cattle from lumpyskin disease. Recombinant vaccine candidates that use various capripoxviruses to express genes for protective antigens of bluetongue and rinderpest viruses are described for geographic areas where outbreaks by these viruses overlap (14).

Clinical diagnosis presents few problems to veterinary clinicians familiar with capripoxvirus infections. Laboratory confirmation includes demonstration of brick-shaped particles in clinical material, and a PCR test is described for differentiating capripoxviruses from parapoxviruses (105). Capripoxviruses can be isolated in various cell cultures derived from sheep, cattle, or goats; B-type inclusions are formed in infected cells. A gene for a chemokine receptor homolog has been detected in some strains (33).

Sheeppox and Goatpox

Sheeppox and goatpox are clinically similar diseases exotic to the United States that can have mortality rates from 50% to 100%, mainly among young animals. They are the most important of all poxvirus diseases of domesticated animals because of the high transmissibility and significant economic losses (53). Sheeppox was apparently present in Europe as early as the second century A.D., and its infectious nature was recognized in the mid-18th century. Eradication was achieved in the Great Britain in 1866, but in contiguous European countries, it was more difficult to eliminate, probably because of the extensive live animal trade across borders.

After an incubation period of 4 to 8 days, there is a temperature rise, an increase in respiratory rate, edema of the eyelids, a mucous discharge from the nose, and a loss of appetite. One to 2 days later, cutaneous nodules 0.5 to 1.5 cm in diameter develop, which may be widely distributed over the body (Fig. 14**A** and Fig. 14**B**). The nodules usually scab and persist for 3 to 4 weeks, healing to leave a permanent depressed scar. Lesions within the mouth ulcerate and constitute an important source of virus for infection of other animals.

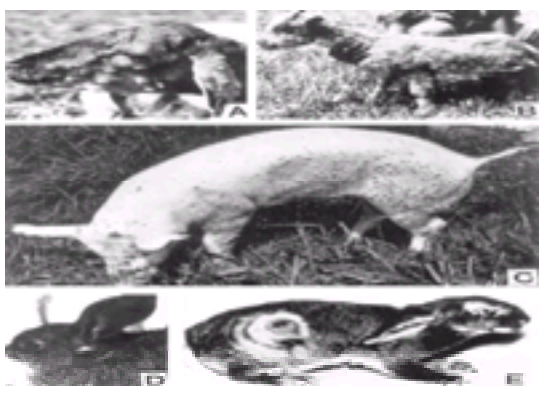

FIG. 14. Infections of domestic animals with poxviruses of other genera. Capripoxvirus infection in native breeds of sheep (sheeppox; **A**) and goats (goatpox; **B**) in Ghana. **C:** Suipoxvirus swinepox infection in pig showing generalized lesions. Leporipoxvirus myxoma virus infection in the native host the Californian rabbit, *Sylvilagus bachmani* (**D**) and the European rabbit, *Oryctolagus cuniculi* (**E**). (**A** to **D** from ref. 162, with permission; **A** and **B** courtesy of M. Bonniwell; **C** courtesy of R. Miller; **D** courtesy of D. C. Regnery.)

Scabs shed by infected animals remain infective for several months; environmental contamination leads to introduction of virus into small abrasions in the skin. The common practice of herding sheep and goats into enclosures at night in affected countries provides adequate exposure to maintain enzootic infection. During an outbreak, the virus is probably transmitted between sheep by aerosol; mechanical transmission of virus by biting arthropods, such as stable flies, may also occur.

Lumpyskin Disease

Lumpyskin disease affects cattle breeds derived from both *Bos taurus* and *Bos indicus* and was first recognized in an extensive epidemic in Zambia in 1929 (53). Since the 1950s, it has continued to spread progressively throughout Africa, first north to the Sudan and subsequently westward, appearing by the mid-1970s in most countries of western Africa. In 1988, the disease was confirmed in Egypt, and in 1989 a single outbreak occurred in Israel, the first report outside the African continent.

The disease is characterized by fever, lacrimation, nasal discharge, loss of appetite, and often generalized lymphadenitis and edema of the limbs followed by the development of nodular skin lesions. The skin nodules involve the dermis and epidermis; they are raised and later ulcerate and may become secondarily infected. Ulcerated lesions may be present in the mouth and nares; postmortem, circumscribed nodules may be found in lungs and alimentary tract. Healing is slow,

and affected cattle often remain debilitated for several months. Morbidity in susceptible herds can be as high as 100%, but mortality is rarely more than 1% to 2%; thus, the economic importance relates to a prolonged convalescence. The virus is probably mechanically transmitted between cattle by biting insects and is perpetuated in a wildlife reservoir host, possibly the African Cape buffalo. LSDV vaccines are used to control the disease.

Suipoxvirus Infections: *Swinepox*

Swinepox (see Fig. 14**C**), caused by the only member of the genus *Suipoxvirus,* is seen sporadically in swine-raising areas throughout the world (140). It is a mild disease with lesions restricted to the skin. When smallpox vaccination was common, some outbreaks of pox disease in swine were caused by vaccinia virus, but swinepox virus is now the only cause. Lesions may occur anywhere but are most obvious on the belly. A transient low-grade fever may precede the development of papules, which, within 1 to 2 days, become vesicles and then umbilicated pustules, 1 to 2 cm in diameter. The lesions crust over and scab by 7 days; healing is usually complete by 3 weeks. The clinical picture is characteristic, and it is seldom necessary to seek laboratory confirmation.

Swinepox is most commonly mechanically transmitted among pigs by the bite of the pig louse, *Hematopinus suis,* which is common in many herds; flies may also be mechanical vectors. Swinepox can be controlled by optimizing hygiene, including elimination of lice from the affected herd; no vaccines are available, or likely are necessary.

Restriction mapping of swinepox virus DNA suggested that it resembles an unclassified Brazilian poxvirus, Cotia virus (228). Sequencing has indicated several potential immune-mediating proteins encoded in the 175-kbp genome DNA of swinepox virus, including a chemokine receptor, but no such activity has been detected in swinepox-infected cell cultures (86). Swinepox virus has been used to develop recombinant vaccine candidates (223).

Leporipoxvirus Infections: *Myxomatosis*

The genus *Leporipoxvirus* contains five species, but only myxoma virus is of veterinary importance because it is a serious risk to breeders of European rabbits in California and Europe, and in the 1950s, myxoma virus was used very successfully to control a vertebrate pest, the feral European rabbit in Australia and Europe (71,119,192). Initially, in the Australian effort to control rabbits, more than 99% of rabbits died within 12 days of infection with a myxoma virus strain derived from a wild rabbit source (*Sylvilagus* species). However, milder virus variants emerged, and surviving rabbits produced resistant progeny, which has enabled the rabbit to continue as a troublesome pest. Myxoma virus recombinants expressing contraceptive proteins are being examined to control rabbits (191).

Myxomatosis was first observed in laboratory rabbits in Uruguay in 1896. The natural hosts of myxoma virus are the tapeti (*Sylvilagus brasiliensis*) in South and Central America and the brush rabbit (*Sylvilagus bachmani*) in California (brush rabbits are resistant the tapeti-derived myxoma virus). Myxoma virus causes a localized benign cutaneous fibroma (see Fig. 14**D**) in wild rabbits (*Sylvilagus* species) in the Americas and a generalized highly lethal disease (see Fig. 14**E**) in the European rabbit (*Oryctolagus cuniculus*).

Early signs of myxomatosis in the European rabbit are blepharoconjunctivitis and swelling of the muzzle and anogenital region, giving the rabbit a leonine appearance. Infected rabbits are listless, febrile, and sometimes, particularly with California myxoma virus, die within 48 hours of disease onset. European rabbits infected with Brazilian strains survive for 10 to 14 days, and subcutaneous gelatinous swellings (hence the name *myxomatosis*) appear all over the body. Virus transmission is usually by mechanical transfer by mosquitoes or rabbit fleas but can occur by droplet infection. Laboratory or hutch rabbits can be protected against myxomatosis by inoculation with the related rabbit fibroma virus or with attenuated myxoma virus vaccines.

Myxomatosis in European rabbits can be diagnosed by the clinical appearance, or by virus isolation in rabbits, on the chick embryo CAM, or in rabbit or chicken cell cultures. Electron microscopy of the exudate or smear preparations of the lesions reveal brick-shaped virions. Genome DNA restriction profiles can be used to differentiate species and strains (119,171). The complete genome sequences, encoding about 168 genes, are reported for the 161,744-bp DNA of Lausanne myxoma virus (GenBank No. AF170726) and the 159,857-bp DNA of Shope fibroma virus (GenBank No. AF170722). Leporipoxviruses express a variety of components for immune modulation in rabbits, many of which have counterparts in other poxviruses (66,166). Expression of certain chemokine receptors on cells markedly influences the growth of myxoma virus and possibly other poxviruses in some cells (129).

Avipoxvirus Infections

Avipoxviruses infect poultry and many species of wild birds, and virus species exhibit extensive DNA and serologic cross-reactivity (224). Viruses are generally recovered from skin lesions and given names related to their hosts (e.g., fowlpox, canarypox, turkeypox, pigeonpox). Mechanical transmission by arthropods, especially mosquitoes, provides a mechanism for transfer of the virus between a variety of different species of birds. There appear be a number of highly diverged avian poxvirus species based on their pathogenicity in various avian hosts and the efficacy of cross-protection afforded by different avipoxviruses in different birds, which varies considerably. Comprehensive restriction digest mapping to classify avipoxviruses has not been done; this may be complicated by the fact that complete or partial avian retrovirus DNAs have been detected within the 300-kbp genome of various fowlpox field strains (95), suggesting that sequencing selected genes might provide a more useful approach to estimate their phylogeny.

Fowlpox

Fowlpox is a serious disease of poultry that occurs worldwide (162,224). Two forms exist that are associated with different modes of infection. The *cutaneous form,* which is the most common, results from infection by biting arthropods and is characterized by small papules on the comb, wattles, and around the beak. Involvement of the skin around the nares may cause nasal discharge, and lesions on the eyelids can cause excessive lacrimation and predispose poultry to

bacterial infection. In uncomplicated cases, healing occurs within 3 weeks, and immunity is solid. The other form of fowlpox is probably due to droplet infection and involves infection of the mucous membranes of the mouth, pharynx, larynx, and sometimes the trachea. This is often referred to as the *diphtheritic form* because the lesions, as they coalesce, result in a necrotic pseudomembrane that can cause death by asphyxiation; the prognosis is poor.

Extensive infection in a flock may cause a slow decline in egg production. Cutaneous infections cause little mortality and seldom present a diagnostic problem. The diphtheritic form is more difficult to diagnose and may be confused with vitamin A deficiency. Electron microscopy, virus isolation in avian cells or on the CAM, and DNA analysis can be used to confirm a clinical diagnosis; the 288,539-bp DNA genome of fowlpox virus APHIS challenge strain has been completely sequenced (GenBank No. AF198100). A 15-second antigen capture assay for fowlpox has been described (114).

Several types of poultry vaccine are available, including attenuated fowlpox virus and a pigeonpox virus vaccine. Vaccines may be applied to the thigh by light scarification, or orally by poultry drinking water. Avipoxviruses engineered to express immunizing antigens of other poultry pathogens are described (19). Several recombinant avipoxvirus vaccine candidates for humans or domestic mammals are reported; such vaccines replicate abortively in mammalian cells, producing no infectious progeny, and thereby providing a margin of safety in being nontransmissible in mammals (133,174).

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CHAPTER 86 *Hepadnaviridae:* The Viruses and Their Replication

Donald Ganem and Robert J. Schneider

Of the many viral causes of human hepatitis, few are of greater global importance than hepatitis B virus (HBV). More than 300 million people worldwide are persistently infected with HBV, and of these, a significant fraction develop severe pathologic consequences, including chronic hepatitis, cirrhosis, and hepatocellular carcinoma (22,23,289). The epidemiology, natural history, and therapy of HBV infection are reviewed by Hollinger in Chapter 87. Here, we focus on the cellular and molecular mechanisms of the replication of HBV and its related viruses.

For many years, insight into the fundamentals of the HBV life cycle was impeded by severe experimental obstacles posed by (a) the narrow host range of the virus (resulting in the absence of convenient animal models of infection and disease), and (b) the lack of cell lines that support productive HBV replication. However, the molecular cloning of the viral genome (150,334,392,489,556,558), the development of cell culture–based assays for viral replication (95,151,211,477,516,593), and the discovery of natural animal models of viral infection (335,338,342,514) radically changed this picture. These advances ultimately resulted in the development of recombinant HBV vaccine (344), the first successful recombinant vaccine for a human infectious disease, and the seminal discovery that these viruses replicate their DNA through the reverse transcription of an RNA intermediate (510). The latter finding established HBVs as distant evolutionary relatives of the retroviruses and stimulated the identification of clinically effective drugs based on the inhibition of the viral reverse transcriptase. The more recent development of transgenic mouse models of virus-induced liver injury (86) has led to new ways of thinking about the pathophysiology of viral clearance and persistence, with profound implications for the design of strategies for intervening in the natural history of chronic infection. Virology affords few better examples of the close relationship between research into the molecular aspects of viral replication and advances in clinical diagnosis and therapy.

HEPATITIS B VIRUS BIOLOGY: AN OVERVIEW

Primary HBV infection of susceptible adults results from sexual contact with an infected host or from parenteral exposure to virus-containing blood or blood products (154,217,219,269,289). Primary infection may be asymptomatic or may result in varying degrees of acute liver injury (acute hepatitis). Although such hepatitis can be severe, in most adults, the primary infection resolves (219,233). Host immune responses to viral antigens result in the clearance of infected cells from the liver and the removal of virions from the bloodstream; lasting immunity to clinically evident reinfection typically results (217; but see later section, Viral Pathogenesis and Immunity, for an important caveat). However, in a small proportion of infected adults (generally less than 5%), the primary infection does not resolve (233). These individuals go on to a persistent infection characterized by active viral replication in hepatocytes and variable (but usually substantial) levels of viremia. As in primary infection, the clinical manifestations of the persistent infection vary greatly: many patients are relatively symptom free, whereas others have varying grades of chronic liver injury and inflammation (chronic hepatitis B) (289). Although it is the minority outcome of primary infection, persistence is important for several reasons. First, most of the morbidity and mortality of hepatitis B virus infection results from the persistent infection. Some subsets of symptomatic patients (e.g., those with severe chronic active hepatitis B) have 5-year survival rates of less than 50%, with most deaths resulting from liver failure and its complications (289). Chronic hepatitis B patients who survive 25 to 30 years of viral persistence also have a markedly increased risk for developing heptocellular carcinoma (23), a dreaded malignancy that is usually fatal. Finally, the asymptomatic carriers are the major epidemiologic reservoir of infection: it is principally from them that spread of HBV to susceptible hosts occurs.

The reasons that some individuals resolve HBV infection whereas others do not remain poorly understood. Much correlative clinical evidence suggests that variations in host immune responses are a critical variable. For example, individuals with overt deficits in cell-mediated immunity (e.g., transplant recipients, patients with acquired immunodeficiency syndrome [AIDS]) are more likely to become chronic carriers than are fully immunocompetent hosts. The most biologically important example of this phenomenon is seen in the vertical transmission of HBV from pregnant mother to newborn baby. HBV transmission to babies usually takes place at the time of delivery, when the newborn is exposed to large quantities of viremic maternal blood during passage through the birth canal. The cellular immune

system of the neonate is known to be incompletely developed at birth; in this context, 80% to 90% of HBV exposures result in persistent infections (24,294,381,502). Much has been learned in recent years about the immunologic mechanisms governing viral clearance; these are discussed in more detail later (see Viral Pathogenesis and Immunity).

VIRION STRUCTURE

Particle Types

HBV is unusual among animal viruses in that infected cells produce multiple types of virus-related particles (21,103,444). Electron microscopy of partially purified preparations of HBV shows three types of particles: (a) 42- to 47-nm double-shelled particles (known as *Dane particles,* after their discoverer); (b) 20-nm spheres, usually present in a 10,000- to 1,000,000-fold excess over Dane particles; and (c) smaller quantities of filaments of 20 nm diameter and variable length (Fig. 1). All three forms have a common antigen on their surface, termed *hepatitis B surface antigen* (HBsAg). HBsAg is present in enormous quantity in the serum of infected hosts, with concentrations ranging from 50 to 300 µg/mL; most of the circulating pool of HBsAg is composed of 20-nm spheres, which can reach titers as high as 10^{12}/mL. This unprecedented amount of viral antigen in the circulation allows physicians to use direct viral antigen detection as a sensitive diagnostic test for HBV infection.

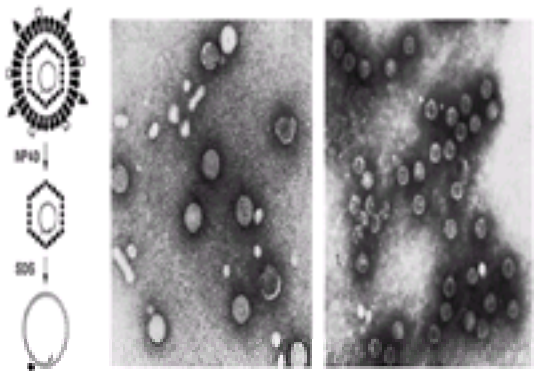

FIG. 1. The structure of hepadnaviral virions and subviral particles. **Left:** Schematic depiction of virion (*top*), core particle (*middle*), and virion DNA (*bottom*). The outer envelope of the virion contains three related surface glycoproteins (*L, M,* and *S*); the inner nucleocapsid contains a single capsid protein (*C*). The viral DNA contains a terminal protein (*oval*) attached to the negative strand and a short RNA (*wavy line*) attached to the positive strand. *Dashes* indicate single-stranded gap region on virion DNA. **Center:** Electron micrograph of hepatitis B virus particles, including virions, 20-nm spheres, and filaments. **Right:** Electron micrograph of virion cores produced by detergent (NP40) treatment of virions. (Experiment by June Almeida, United Medical School, Guy's Hospital, London, U.K.)

The Dane particle is the infectious virion of HBV; titers of Dane particles in the blood can range from less than 10^4/mL to greater than 10^9/mL. Its outer shell is a lipoprotein envelope containing the viral surface glycoproteins, originally detected serologically as HBsAg (35). These are the determinants against which neutralizing antibody (anti-HBs) is directed (217). The envelope can be removed by treatment with nonionic detergents, liberating an inner core particle or nucleocapsid of 25 to 27 nm. The major structural protein of the core is the C protein, a 21-kd basic phosphoprotein that was also originally detected serologically (218) and that is still frequently called *hepatitis B core antigen* (HBcAg) (442,511). Within the core is the viral DNA and a polymerase (P) activity (255,443) now known to be centrally involved in viral genomic replication. Purified cores also contain a protein kinase activity detected by its ability to phosphorylate C protein *in vitro* (2,135,165); because recombinant C and P proteins do not possess kinase activity, it is thought that this enzyme is of host origin. Whether it is truly an internal capsid component or merely tightly associated with cores during extract preparation is still uncertain, as is its biologic role (although there is no doubt that C protein is phosphorylated *in vivo*) (328,421,449,602,609).

The 20-nm spheres and filaments are composed exclusively of HBsAg and host-derived lipid (about 30% by weight); their principal lipids include phospholipids, cholesterol, cholesterol esters, and triglycerides (160,405,406). These particles lack nucleic acid altogether and hence are noninfectious. Nonetheless, in pure form, these particles are highly immunogenic and efficiently induce a neutralizing anti-HBs antibody response. Such 20-nm spheres, as purified from the serum of HBV carriers, in fact served as the initial form of HBV vaccine before the development of recombinant HBsAg preparations (269). Natural infection thus presents the seeming paradox of efficient progression despite the accompanying production of highly immunogenic particles that can elicit neutralizing host responses. How this comes about is not known; one school of thought is that the excess HBsAg particles function to adsorb neutralizing antibody and thus help shield virions from host defenses.

The Viral Genome

HBV virion DNA is a relaxed circular, partially duplex species of 3.2 kb whose circularity is maintained by 5'-cohesive ends (442,458,511) (Fig. 2). This molecule has an unusual structure in that its two DNA strands are not perfectly symmetric. The viral negative strand is unit length and has protein covalently linked to its 5' end (156,166,351). The existence of this terminal protein emerged from the observation that the viral DNA was extracted from the aqueous phase by phenol unless the genome was first treated with proteases. Restriction endonuclease digestion of viral DNA indicated that only terminal DNA fragments bearing the negative strand 5' end displayed this behavior, indicating linkage of the protein to the 5' end of negative-stranded DNA. By contrast, the positive strand is less than unit length and bears a capped oligoribonucleotide at its 5' end (309,472,581). The presence of these terminal structures explains the longstanding observation that neither 5' end of viral DNA can be phosphorylated by polynucleotide kinase. Importantly,

the positions of the 5' ends of both strands map to the regions of short (11 nucleotides) direct repeats (DRs) in viral DNA. The 5' end of negative-stranded DNA maps within the repeat termed DR1, whereas positive-stranded DNA begins within DR2 (309,352,472,581) (see Fig. 2). As detailed later, these repeats are importantly involved in priming the synthesis of their respective DNA strands.

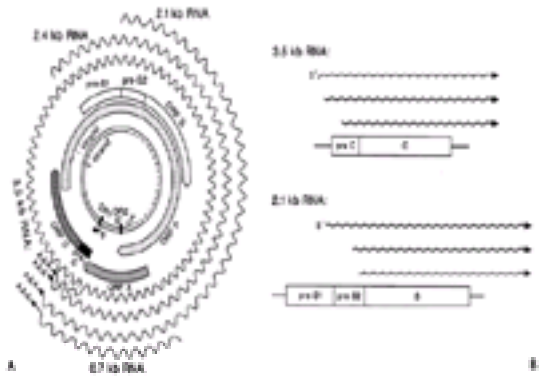

FIG. 2. A: Diagrammatic representation of the hepatitis B virus coding organization. *Inner circle* represents virion DNA, with dashes signifying the single-stranded genomic region; the locations of DR1 and DR2 sequence elements are as indicated. *Boxes* denote viral coding regions, with *arrows* indicating direction of translation. *Outermost wavy lines* depict the viral RNAs identified in infected cells, with arrows indicating direction of transcription. **B:** Fine structure of the 5' ends of the pre-C/C transcripts (*top*) and pre-S2/S transcripts (*bottom*) relative to their respective open reading frames.

Virion DNA thus contains a single-stranded region or gap of fixed polarity but variable length. Early studies showed that cores purified from extracellular virions contain a polymerase activity that can fill in this gap, generating a fully duplex genome (255,443,511). In this so-called endogenous polymerase reaction (EPR), the 3' hydroxyl of positive-stranded DNA serves as the primer, with synthesis extending along negative-stranded DNA templates. The newly synthesized product is therefore entirely of positive-stranded polarity, an asymmetry that is not simply reconciled with a standard semiconservative DNA replication scheme (see later).

Molecular cloning of HBV DNA extracted from Dane particles reveals a coding organization that is highly compact: every nucleotide in the genome is within a coding region, and more than half of the sequence is translated in more than one frame (150,558). As shown in Figure 2, four open reading frames (ORFs) are present in the DNA. The viral polymerase, the central enzymatic activity in genomic replication, is encoded by the *P* gene; ORF *P* also encodes the terminal protein found on negative-stranded DNA (37) (see later). The C (core) region encodes the structural protein of the nucleocapsid (392), whereas ORF *S*/pre-S encodes the viral surface glycoproteins (556).

The DNA sequence of HBV revealed a number of surprises not anticipated from earlier biologic studies. Chief among these was the existence of ORF *X,* a coding region whose product had not previously been detected. The product of ORF *X* is a complex regulatory protein that is required for viral infectivity *in vivo* and modulates the expression of heterologous and homologous genes in trans, at least in transient assays performed in cultured cells (451). How it does so is a matter of considerable debate and is considered in a later section.

Moreover, the coding organization of the genes for the known virion structural components (HBsAg and HBcAg) turned out to be more complex than expected. Early studies had suggested that the major component of HBsAg was a protein of 24 kd (30% to 50% of which is glycosylated, appearing as a second species of 27 kd), but small quantities of larger polypeptides often copurified with this material (164). The coding region for HBsAg was identified by aligning the translated HBV DNA sequence with the known N-terminal amino acid sequence of the 24-kd chain (556). This revealed that the reading frame for this antigen was open for about 400 nucleotides 5' to its AUG (initiation codon) and that this upstream (or pre-S) ORF could be divided into two subregions (pre-S1 and pre-S2) by the presence of two in-frame initiation codons (see Fig. 2). This coding organization provided an explanation for most of the larger polypeptides earlier observed in HBsAg preparations. The largest of these so-called pre-S proteins is the 39-kd L (pre-S1) protein, which is the product of initiation at the first AUG of the ORF (204,585). Initiation at the second AUG generates the 31-kd M (pre-S2) protein (326,327,401,503). Both proteins share the common C-terminal S domain and differ principally by the length and structure of their N-terminal (pre-S) extensions. Classic HBsAg, which contains only the S domain, is now more commonly referred to as the *S protein.*

Both pre-S–encoded polypeptides are quantitatively minor components of the circulating pool of S-related antigens, with the M protein accounting for about 5% to 15% of the total and the L protein typically representing only 1% to 2% or less of the total. Each protein exists in two isomeric forms, differing only by the presence or absence of S-domain glycosylation; in addition, the M protein contains an additional *N*-linked oligosaccharide on its pre-S2–specific domain (204,503). The L protein contains a further posttranslational modification, a myristic acid group in amide linkage to its amino-terminal glycine residue (403). Despite their low abundance, L chains are now thought to play a key role in viral assembly and infectivity (46,235,301,330).

Careful fractionation studies (204) have established that the three envelope glycoproteins are not distributed uniformly among the various HBV particle types. Subviral 20-nm particles are composed predominantly of the S protein, with variable quantities of M polypeptides and few or no L chains. By contrast, Dane particles are substantially enriched for L chains. Because L chains are thought to carry the receptor recognition domain (235,264,411), this enrichment may prevent the more numerous 20-nm particles from competing effectively with virions for cell surface receptors.

The coding organization of the core protein gene revealed a similar surprise: again, two in-frame AUGs were found in the

core ORF, with the classic HBcAg being the product of initiation from the more internal start codon (580). Initiation at the upstream AUG gives rise to a C-related protein that is not incorporated into virions but instead is independently secreted from cells, accumulating in serum as an immunologically distinct antigen known as *HBeAg* (159,345,388,448,498). The function of HBeAg remains enigmatic (see later).

CLASSIFICATION

The *Hepadnaviridae* Family

These unusual structural features clearly set HBV apart from the other families of animal DNA viruses. HBV is the prototype member of a family of related viruses known as hepadnaviruses (for hepatotropic DNA viruses). The first of the nonhuman hepadnaviruses to be discovered was the woodchuck hepatitis virus (WHV). In a colony of captive woodchucks (*Marmota monax*), veterinarians noted the frequent presence of chronic active hepatitis and hepatoma at necropsy (515); these features recalled the histopathology of advanced HBV infection. A search for HBV-like particles in serum from these animals ultimately culminated in the identification of WHV (514), a novel virus that is morphologically indistinguishable from HBV and whose genome shares about 60% nucleotide sequence identity with its human counterpart (149,268). A series of similar viruses have now been recovered from a variety of animal species, including the Beechey ground squirrel, *Spermophilis beecheyi* (ground squirrel hepatitis virus [GSHV]) (156,157,337,338), and the Pekin duck, *Anas domesticus* (duck hepatitis B virus [DHBV]) (336,337,342,497). Less well-characterized viruses have been recovered from arctic ground squirrels, wild herons, several varieties of wild and domestic geese, marsupials, and orangutans (496,527,530). All of these viruses share the following common properties: (a) enveloped virions bearing 3- to 3.3-kb relaxed circular, partially duplex DNA; (b) virion-associated polymerases that can repair the gap in the virion DNA template; (c) the production of excess subviral lipoprotein particles composed of envelope proteins; (d) narrow host range, growing only in species close to the natural host (14,285,336,422,475,542); and (e) production of persistent infections displaying pronounced (but not absolute) hepatotropism.

Table 1 summarizes the biologic properties of the most intensively studied hepadnaviruses. Although the similarities between these viruses outnumber the differences, there are important distinctions to be made among the individual members of this virus family. In general, the avian viruses are the most divergent: their viral genomes are smaller than those of the mammalian viruses and share little primary nucleotide sequence homology with them (334). Most avian viruses encode only two envelope proteins (L and S) rather than three (423,463) and lack the classic X coding region (although a small ORF beginning with a noncanonical initiator is present in this position and can be expressed in transfected cells (H. Will, personal communication). Although they too display a very narrow host range, their hepatotropism is less marked: viral antigens and replicative intermediates can be readily detected in several extrahepatic sites, including the pancreas, kidney, and spleen (193,194,195 and 196,220,247). In animals infected *in ovo,* the yolk sac is a major site of viral replication (520). Extrahepatic infection is also well-documented for HBV (412,447,605) and WHV (274,275 and 276,379), but the titers of their extrahepatic viral DNAs are generally much lower than those observed for DHBV. Nonetheless, even in the avian viruses, the liver remains the predominant site of virus production. Most strikingly, DHBV infection, although it can be associated with mild grades of hepatitis, is not strongly linked to the development of HCC. Although some DHBV-infected flocks in China and Japan have noted the occasional occurrence of hepatoma (234,383,606), infected birds in U.S. and European flocks (and laboratory-held animals) virtually never develop this lesion. It seems likely that the Asian flocks have been exposed to environmental cofactors that may accelerate HCC formation; dietary aflatoxin, a potent hepatic carcinogen, is a leading candidate for this cofactor role (100).

	HBV	WHV	GSHV	DHBV
Genome	3.2 kb	3.3 kb	3.3 kb	3.0 kb
ORFs	S, C, P, X, S, C, P, X	S, C, P, X	S, C, P	
Hosts	Humans Chimps	Woodchucks	Ground squirrels Woodchucks Chipmunks	Ducks Geese
Replication	Liver Kidney Pancreas WBC	Liver Kidney Pancreas WBC Other	Liver	Liver Kidney Pancreas Spleen Other
Diseases	ACS Hepatitis Cirrhosis HCC	ACS Hepatitis HCC	ACS Hepatitis HCC	ACS Hepatitis

ACS, asymptomatic carrier state; DHBV, duck hepatitis B virus; HBV, hepatitis B virus; GSHV, ground squirrel hepatitis virus; HCC, hepatocellular carcinoma; ORF, open reading frame; WBC, white blood cells; WHV, woodchuck hepatitis virus.

TABLE 1. *The hepadnaviridae family*

VIRAL REPLICATION

Overview of the Life Cycle

The peculiar asymmetries of virion DNA (and of the endogenous polymerase reaction) provided early clues that a mechanism other than semiconservative DNA synthesis is involved in hepadnaviral genomic replication. Examination of intrahepatic replicative intermediates revealed even more striking asymmetries. Infected liver cells harbor large quantities of negative strands of less than unit length, most of which are not associated with positive strands (340,578). In 1982, Summers and Mason (510) reported seminal experiments establishing that viral DNA replication proceeds not by conventional semiconservative DNA synthesis but by reverse transcription of an RNA intermediate. To do this, they first prepared subviral particles from DHBV-infected liver; these particles, unlike mature virions, incorporate labeled deoxyribonucleoside triphosphates (dNTPs) into both positive and negative strands of viral DNA, exactly as would be anticipated for authentic replication intermediates. Electron microscopy of the particles proved them to be immature (unenveloped) cores that were cytoplasmic in location; thus, most viral replication was occurring outside of the nucleus,

the principal site of host DNA synthesis. Importantly, the synthesis of negative-stranded DNA was resistant to actinomycin D, implying its template was not DNA, whereas positive-stranded synthesis was sensitive to this compound. In addition, a portion of newly made negative-stranded DNA was found in the form of RNA–DNA hybrids. These observations suggested that negative-stranded DNA was made from an RNA template, whereas the positive strand was copied from a DNA template—by inference the negative-stranded DNA whose RNA template had been removed.

These experiments predicted the existence of a full-length, unspliced RNA that would serve as the template for reverse transcription, and such an RNA was indeed identified soon thereafter (55,123,355). This RNA (sometimes called *pregenomic RNA* [pgRNA] to denote its role in genomic replication) has all the hallmarks of a transcript produced by host RNA polymerase II (pol II). But if pol II, a nuclear enzyme, is the enzyme responsible for genomic RNA synthesis, clearly a subpopulation of nuclear viral DNA molecules must exist to serve as its template. This function is provided by a nuclear pool of viral DNA consisting of 10 to 20 molecules/cell of unit-length, covalently closed circular DNA (cccDNA) (340,578). As the presumed viral transcriptional template, these molecules thus play in the hepadnaviral life cycle the role analogous to that of integrated proviral DNA in retroviral replication; however, in hepadnaviral infection, these molecules remain episomal. Presumably, incoming viral DNA is transported to the nucleus and converted to the cccDNA form. Consistent with this, cccDNA is the first novel virus-specific DNA species to appear after DHBV infection, preceding the accumulation of viral RNA (341).

These observations allow formulation of a model for the overall viral replicative cycle (Fig. 3). After receptor binding, virions deliver their nucleocapsids to the cytoplasm. These then translocate to the nucleus, where their genomic DNA is matured to the cccDNA form. This DNA is then transcribed by host RNA pol II, and the resulting RNAs translated to give rise to the P, C, pre-S/S and (in mammalian viruses) X gene products. Viral pregenomic RNAs are selectively encapsidated within core particles in the cytoplasm (124,510), together with the P gene product. Within this structure, viral DNA synthesis is initiated; after negative-stranded synthesis (and concomitant degradation of the RNA template), positive-stranded DNA synthesis occurs. Upon completion of genomic DNA synthesis, progeny cores bud into intracellular membranes, generally the endoplasmic reticulum (ER) or proximal Golgi (252,446), to acquire their glycoprotein envelope. Enveloped virions are then secreted through the constitutive pathway of vesicular transport.

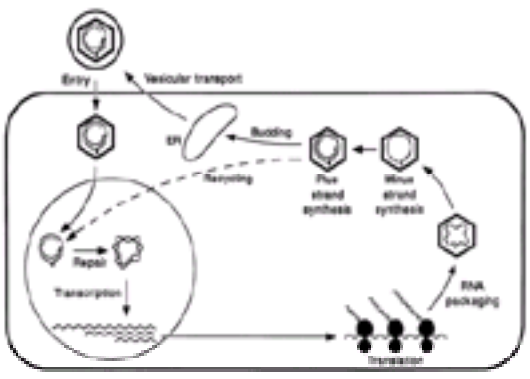

FIG. 3. Schematic depiction of the hepadnaviral life cycle. See text for details.

Many of the events schematized in Figure 3 can now be studied in cell culture. Although there are still no established cell lines that support infection with HBV virions (see later), primary duck hepatocytes freshly explanted directly from liver will support DHBV infection (152,424,546). These are terminally differentiated epithelial cell cultures that engage in little or no cell division and cannot be passaged long-term *in vitro.* Similar preparations of mammalian hepatocytes appear to support mammalian hepadnaviral infections, but much less efficiently (175,176,376). The reasons for this inefficiency are unclear, but HBV infection of primary human hepatocytes can be significantly enhanced by the addition of low concentrations of polyethylene glycol to the medium (175), suggesting that inefficient membrane fusion may be the cause.

Although they will not support virion infection, several well-differentiated and immortalized hepatoma cell lines (e.g., human HepG2 or HuH7, and chick LMH cells) support viral replication and release if cloned viral DNA is delivered by transfection (95,151,425,477,516,593). Presumably, the incoming transfected DNA functionally substitutes for cccDNA upon arrival in the nucleus, allowing the generation of the correct panel of viral transcripts; these can then encode the viral gene products necessary to complete all subsequent stages of the life cycle. Even fibroblasts can support hepadnaviral production after transfection by cloned viral DNA, provided that a heterologous promoter is used to drive expression of pgRNA (470), suggesting that transcription of pgRNA is a major determinant of liver specificity in wild-type hepadnavirus infection. Transfection of hepatocytes can also occur *in vivo* after the direct inoculation of cloned hepadnaviral DNA into the liver of susceptible hosts (471,497,579). In this case, progeny virions elaborated by the transfected cells establish a hepatic and systemic infection exactly as observed after virion inoculation. Similarly, HBV replication can proceed in transgenic mice bearing chromosomal copies of overlength HBV genomes (92,186). In these cases, the integrated transgenes serve the function of transcriptional templates for pgRNA production. Because mouse cells lack the HBV entry pathway, there is no horizontal spread of virus in these animals; however, such spread is not required to maintain viremia because every hepatocyte in the liver harbors a transcriptionally competent integrated viral genome from birth.

The availability of these systems has made possible mutational analysis of virtually all steps in replication. These systems have also revealed an important additional feature of the replication cycle: its noncytocidal nature. Primary cells supporting productive infection generally show no cytopathic effects and are morphologically normal (176,545,546); transfected hepatoma cells bearing viral replicative intermediates display growth rates identical to those of their untransfected parents (516). These facts agree well with clinical observations that many densely infected HBV carriers

have minimal or no hepatocellular injury and support the contention that such injury *in vivo* is largely the result of host immune or inflammatory responses (see later). The phenotype of HBV-positive transgenic mice bears further witness to this model: the livers of these mice (which are immunologically tolerant to the viral antigens) harbor high levels of viral replicative intermediates but remain histologically normal throughout life (185,186,354).

Attachment and Entry

Little is known about the earliest events in the viral life cycle. Enveloped virions must first bind to the cell surface, a reaction that has been best studied for DHBV. Binding occurs at 4°C, but at this temperature, no productive infection takes place. The binding reaction appears to have two components: a low-affinity, nonsaturable one and a high-affinity, saturable component (264). On warming to 37°C, entry and infection take place. After binding, a virus–host membrane fusion event must occur. For some viruses (e.g,. influenza) this occurs after endocytosis and is triggered by a pH-dependent fusion reaction governed by an envelope glycoprotein; in other viruses (e.g. paramyxoviruses, herpes simplex virus [HSV], and human immunodeficiency virus [HIV]), this is mediated by a pH-independent fusion reaction occurring at the cell surface. Experiments using inhibitors of endosomal acidification to examine the pH dependence of DHBV entry have come to divergent conclusions (377,441), but the weight of evidence favors a pH-independent mechanism (266,441).

The kinetics of DHBV uptake are odd: binding and entry appear to be slow, such that for maximal infection to occur, the cells must remain exposed to the inoculum for up to 16 hours (424); the basis of this is not understood. As for several other viruses, entry is blocked by suramin (404) and by some antienvelope monoclonal antibodies (MAbs) (80,81,284,286,612).

Much work has been done on the viral envelope components that may be involved in host cell interactions. For HBV and DHBV, several lines of evidence indicate that pre-S proteins participate in cellular receptor binding. Binding of HBV virions to human liver plasma membrane fractions is blocked by MAbs to the HBV pre-S1 domain; antipeptide antibodies to pre-S1 block adherence of HBV particles to HepG2 cells (370,411). Unfortunately, neither experiment is able to relate binding to productive infection. However, using recombinant DHBV envelope proteins, it has been shown that S proteins do not block DHBV infection of primary duck hepatocytes, whereas recombinant preparations containing both pre-S and S proteins do (264).

Another strong inference that pre-S determinants play a key role in receptor interactions comes from genetic studies of viral host range determinants. Hepadnaviruses typically are restricted to species close to that of their natural host, and DNA transfection experiments show that the block to efficient cross-species infection is at the level of virus entry (151,485). For example, the heron hepatitis B virus (HHBV) grows in herons but not in ducks or chicks, despite its substantial sequence homology with DHBV (496). If the normal entry pathway is bypassed by transfecting HHBV DNA into heterologous cells, replication proceeds normally. Consistent with this, pseudotyping experiments show that HHBV can be enveloped by DHBV envelope proteins and that these pseudotyped virions can efficiently infect duck hepatocytes (235). Examination of HHBV and DHBV chimeric envelope proteins in such pseudotyping assays indicates that pre-S determinants play the dominant role in host range determination (235).

Much less is known of the identities of the cellular receptors for hepadnaviruses. A large variety of proteins have been identified that bind to HBV envelope glycoproteins or to peptides derived from them. Some of these are serum derived, others are found in or on hepatocytes. A major problem has been that none of these molecules has been convincingly tied to infectivity. Mehdi and coworkers (346) have produced excellent evidence that HBV S determinants bind the serum protein apolipoprotein H, but this molecule is not an integral transmembrane protein of the hepatocyte and its role in infection is uncertain. Conceivably, it might play a role in delivery of virus from the periphery to the liver. Similarly, HBV S particles (HBsAg) have been shown to bind the phospholipid-binding protein endonexin 2 (207). The biologic significance of this remains unclear because the observed interaction may simply reflect the known ability of endonexin 2 to bind phospholipids, which are abundant in HBsAg lipoprotein. Because this binding does not involve pre-S determinants, it is unlikely to be the sole important component of attachment; it might, however, play some role in a postbinding membrane fusion event. Other studies have suggested possible roles for yet other molecules in viral entry (51,147,272,273,370,371 and 372,413), but no evidence that these proteins play a role in permissive infection has yet been forthcoming.

Progress in receptor identification has been greater in the DHBV system. In 1994, Kuroki and associates (278) identified a 180-kd surface glycoprotein (gp180) that binds to DHBV virions. Binding is specific to the pre-S region of the viral L protein (237,278,536,555) and is blocked by neutralizing (but not by nonneutralizing) antiviral MAbs. The species distribution of this protein mirrors the known host range of DHBV. All pre-S mutations that block this binding without affecting viral assembly destroy DHBV infectivity (237). Cloning of the cyclic DNA (cDNA) encoding this molecule revealed it to be a novel member of the carboxypeptidase family (279). The protein, also designated *carboxypeptidase D* (CPD), is enzymatically active, but this activity is separable from the virus-binding activity (125). Antibody to gp180 blocks DHBV infection of primary hepatocytes, as does soluble recombinant gp180 (S. Urban and H. Schaller, personal communication), demonstrating a key role for this molecule in viral entry. However, the protein is expressed on a wide variety of cell types in the animal, including many (e.g. fibroblasts) that are not thought to be susceptible to infection, and cDNA transfection into most nonpermissive cell lines (e.g., LMH cells) does not confer DHBV susceptibility (40,278). Thus, CPD is unlikely to be the sole component of the DHBV entry pathway; as in other virus systems, coreceptor proteins are likely to exist, and their identification constitutes one of the great remaining challenges to the field. Although gp180 is found in small quantities on the cell surface, most of it resides on intracellular (Golgi) membranes (40,279), suggesting that recycling of the protein between the cell surface and Golgi may occur. In accord with this, DHBV particles bound to CPD-expressing cells can be internalized (40). It is therefore possible that the accessory components of the viral entry pathway may act not at the cell surface but on intracellular vesicles bearing internalized DHBV.

The subsequent, postreceptor steps in hepadnaviral entry are even less well characterized (267). Cytoplasmic cores must be delivered to the nucleus, where their DNA is converted to the superhelical form. Nothing is known of this delivery process. Conceivably, microtubule-based motor systems might play a role in their transport across the cytoplasm to the nuclear envelope. Consistent with this, microtubule assembly inhibitors profoundly depress viral replication (D. Kedes

and DG, unpublished observations), but the exact mechanism of this inhibition remains uncertain (especially because these drugs display considerable nonspecific toxicity).

Once cores arrive at the nuclear envelope, they or their DNA contents must translocate across this structure. Some believe that core particles themselves are transported to the nucleus because (a) C protein is known to harbor nuclear localization signals in its C-terminus (120,390,601), and (b) the diameter of viral cores (about 25 to 27 nm) is just at the limit of the functional nuclear pore size as determined by experimental measurements in *Xenopus* species oocytes (136). However, the possibility that cores disassemble in the cytosol (perhaps at the nuclear pore) and deliver their DNA to the nucleus in nonparticulate form, as posited for adenovirus, has not been excluded (254). The exclusively cytosolic location of C protein in HBV transgenic mice (184,185) is sometimes cited as evidence in support of this notion. However, because such mice do not support cccDNA accumulation, it is difficult to know whether their phenotype is relevant to authentic viral infection—it may be that it is precisely their failure to transmit cores across their nuclear pore that underlies the absence of cccDNA in their nuclei. Recently, HBV core particle import into the nucleus has been examined in an *in vitro* system (631). Mature capsids purified from infected cells were presented to digitonin-permeablized cells in the presence of a cytoplasmic extract and adenosine triphosphate (ATP), a system in which soluble proteins competent for nuclear import are properly transported. HBV C protein accumulated efficiently in the target nuclei in particulate form. Interestingly, however, these particles appeared to lack viral DNA, suggesting that capsids might have released their genomes during or after import. Alternatively, cores might have disassembled during or after import, then reassembled within the nucleus once separated from their DNA.

Treatment of primary duck hepatocytes with glucagon has been shown to inhibit DHBV replication at a step occurring after receptor binding but before cccDNA formation—compatible with a block involving the cytoplasmic transport or nuclear translocation of cores (or their DNA) (209). Because glucagon modulates the activity of the cyclic adenosine monophosphate (cAMP)-dependent protein kinase A, it is tempting to speculate that at least one step in this process may be regulated by phosphorylation. However, the target of this phosphorylation remains unknown.

Once in the nucleus, the partially duplex viral DNA is repaired to the cccDNA form. This requires repair of the single-stranded gap, removal of the 5'-terminal structures (RNA and P protein), and covalent ligation of the strands. Several lines of evidence suggest that this process can be carried out largely by host machinery. The cloning of Dane particle DNA in *Escherichia coli* results in the recovery of fully duplex, superhelical plasmid clones in which the terminal structures have been correctly processed; thus, there would appear to be no need for viral machinery to effect these steps. Consistent with this notion, DHBV cccDNA formation in primary hepatocytes is not inhibited by phosphonoformate, a known inhibitor of P protein polymerase activity (267); however, the biochemical mechanisms by which the terminal structures are removed remain largely unknown.

Viral Transcription

Overview

Mammalian and avian hepadnaviruses use the circular 3-kb, cccDNA nuclear form of the genome as the template for viral transcription. Figure 2 shows the transcriptional organization of human HBV, which is similar to other mammalian hepadnaviruses. Avian hepadnaviruses differ from the related mammalian viruses in lacking an *X* gene ORF and an X promoter element. There are two classes of transcripts synthesized from cccDNA, known as the *genomic* and *subgenomic* classes, that have been identified in transfected cells in culture and in virus-infected liver. Both classes contain several transcripts, all are capped, and all are polyadenylated at a common polyadenylation sequence located within the core gene. The subgenomic transcripts function exclusively as messenger RNAs (mRNAs) for translation of the envelope pre-S1, pre-S2, S (also known as L, M, and S proteins) and X protein. The two genomic mRNAs encode the pre-C, C, and P ORFs. The C/P genomic mRNA is bifunctional, serving as the template for reverse transcription of the viral DNA negative strand (therefore known as *pregenomic RNA*), in addition to its role in translation of C and P protein products. The pre-C mRNA is not used for reverse transcription and functions only in translation of pre-C protein. Functional transcripts of the hepadnavirus genome are synthesized from the negative strand of the viral cccDNA template. RNAs transcribed from positive-stranded DNA, although detected in cells transfected in culture with viral genomic DNA, have not been found in infected cells or have not been found to encode viral polypeptides. None of the major hepadnavirus mRNAs contain classic introns. However, spliced viral transcripts have been detected for both avian and mammalian hepadnavirus mRNAs in cells transfected with genomic DNAs and in infected liver (74,508,517,529,586). Moreover, a spliced transcript encoding an alternately transcribed form of the S protein has been identified for the avian DHBV hepadnavirus in cultured hepatocytes and infected liver (375). Elimination of the spliced transcript by site-directed mutagenesis demonstrated that it is essential for viral replication in culture and in ducks. All of the hepadnavirus transcription elements, including promoters, enhancers, and the single polyadenylation signal, reside within viral genes that are actively transcribed and translated. This unusual arrangement reflects the compact organization of the hepadnavirus genome.

cis-Acting Transcription Elements

In HBV, the four overlapping ORFs are controlled by two enhancer elements and four (possibly five) promoters, corresponding to independent transcription from *pre-S1* (L protein), *pre-S2/S* (M and S proteins), *C* (core protein), *X* (X protein), and possibly *pre-C* (precore protein) genes (5,58,190,191,216,333,435,436,448,487,488,540,553,591,600,619). The fine structure and function of the individual HBV promoters have been very well characterized and shown in Figure 4. Most studies have investigated the activity of individual DNA fragments and used site-directed mutagenesis to then map transcriptional promoters that direct transcription of reporter genes. Several studies have also examined the transcriptional activity of DNA regions in the context of the virus genome. From these studies, it can be concluded that the *pre-C/C, pre-S1, pre-S2/S,* and *X* genes are controlled by independent promoters. Transcription from the pre-C/C promoter (5,216,448,592) is highly restricted to liver (216,431). Studies in transfected cultured cells suggest that transcription of pre-C and C RNAs is controlled by two physically overlapping, but functionally distinct, promoters that are differentially regulated (72,305,610,611). Studies have not yet verified the dual disposition of the pre-C and C promoters in infected cells. However, a frequently arising mutation in the presumptive pre-C promoter region has been documented

in HBV-infected individuals, which results in selective down-regulation of pre-C mRNA and protein (50,305,459). These results are fully consistent with the separate regulation of transcription by pre-C and C promoters. The liver-specific expression of *C* gene transcription is thought to result from the combined requirement for multiple liver-enriched transcription factors in the pre-C/C promoter. These include factors c/EBP, HBF1, HNF3, and HNF4 (64,73,112,158,189,249,321,322,430,437). The C promoter is thought to be controlled by both positive and negative regulation (431,611). Binding by the factor COUP-TF suppresses transcription from both promoters, HNF4 suppresses the presumptive pre-C promoter, and RXRa specifically enhances C promoter activity. Thus, part of the hepatotropism of HBV is likely attributable to liver-specific transcription of the C promoter, which is required for synthesis of pregenomic RNA.

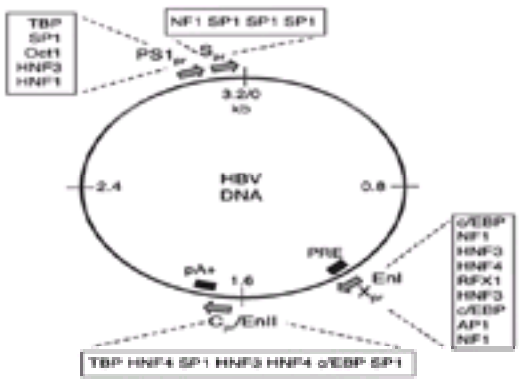

FIG. 4. Fine structure of hepatitis B virus promoter regions.

The HBV pre-S1 promoter directs transcription of the 2.4-kb large envelope (pre-S1 or L [large] HBsAg mRNA). It is located immediately upstream of the pre-S1 protein AUG initiation codon (64,65,434,436). The pre-S1 promoter functions weakly within the context of the virus genome, and when tested independently of other viral transcription elements, is consistent with the low level of pre-S1 protein observed during infection (5,434,436). The liver specificity of the pre-S1 promoter is derived from its dependence on liver-enriched transcription factor HNF1 (98). The S promoter directs transcription of several mRNAs of about 2.1 kb, which are translated to produce the pre-S2 (middle) and the S (small) HBsAg polypeptides (54,64,105,128,432,435,436,482,487,622). The S promoter is located within the coding region of the *pre-S1* gene and lacks a classic TATA sequence (5,64), likely accounting for the transcriptional stuttering that gives rise to mRNAs that either contain or lack the pre-S2 translation AUG initiation codon. The S promoter is constitutively active in a wide range of cell types, although it is more active in hepatocyte-related cell lines and in the liver of transgenic mice (7,11,64,91,105,128,132,133,499,501). The increased liver-specific transcription of the S promoter is probably related to the greater activity of enhancer II in hepatocytes (discussed later) (622).

The mammalian virus *X* gene mRNA is generally of low abundance *in vivo,* consistent with the low levels of X protein observed (101,102). When studied in transfected hepatoma cells independently of other viral elements, a region identified as the X promoter is considerably more active than in the context of the virus genome (5,190,434,488,540,543,592). In HBV, the X promoter is located adjacent to and downstream of enhancer I (EnI), which is within the ORF *P.* Based on transfection of cells with subgenomic HBV DNA fragments, the 5' end of the X mRNA appears to initiate at heterogeneous sites (488,540,543), consistent with the absence of canonical TATA and CAAT elements. X-promoter activity involves binding of both liver-enriched transcription factors (e.g., c/EBP) and ubiquitous factors (e.g., AP-1, ATF/CREB) and is strongly augmented by EnI (5,27,111,112,127,148,189,190,192,241,321,393,448,483,543).

Two genomic regions identified as EnI and EnII have been shown in HBV to fulfill the classic definition of transcriptional enhancer elements. These DNA fragments provide position- and orientation-independent stimulation of transcription when examined independently of viral genes in transient reporter assays (73,75,483,531,572,600,614). Regions corresponding to HBV EnI and EnII were also found to display DNase hypersensitivity patterns consistent with transcriptional activity when examined as integrants in transgenic mice (1), consistent with their identification as enhancer elements. Although both EnI and EnII can increase transcription from a variety of heterologous promoters, the activity of EnII is largely restricted to hepatic cells (600,615). HBV EnI functions in both hepatic and nonhepatic cells, although it more strongly activates transcription in hepatocytes (5,148,241,483,559,613,615). Accordingly, EnI has been shown to be activated by the binding of both ubiquitous and liver-enriched transcription factors, including c/EBP, HBF4, and HBLF (27,127,148,158,189,384,386,490,543). Although EnI is located adjacent to and upstream of the X promoter (and within the ORF *P*), it strongly up-regulates all of the major HBV promoters, both in heterologous reporter constructs and in the native context of the viral genome (5,225). WHV probably does not contain a region equivalent to HBV EnI (108). EnII is located within the ORF *X* and is adjacent to the pre-C/C promoter that directs transcription of pregenomic RNAs (600,615). EnII is a bipartite element that contains binding sites for, and is activated by, a constellation of liver-enriched transcription factors, including the c/EBP family of proteins, HBF1, HNF3, HNF4, and possibly a novel factor known as hB1F (189,249,283,307,308,321,322,431,433,625). One factor (E4BP4) has also been identified as a negative regulator of EnII, based on studies using artificial reporter constructs (283). In a similar manner, a negative regulatory element was identified within EnII that suppresses its activity (314). EnII strongly promotes liver-specific transcription of the pregenomic C promoter, and possibly X and S promoters, but not the pre-S1 promoter, for unknown reasons (108,216,308,322,506,572,614,624). It was also shown by mutagenesis of EnII that within the context of the viral genome, transcription of the viral genome is significantly (more than 10-fold) reduced with EnII impairment (576).

In addition to its role in activating viral transcription in a liver-specific manner, EnII plays an important role in oncogenesis by WHV. Unlike HBV, which integrates apparently at random, woodchuck hepatocellular carcinoma (HCC) tumors usually contain WHV sequences integrated from 2 to 200 kb away from the two *N-myc* gene loci (141,144,222). The genetic arrangement of WHV–cellular gene junctions of genomic integrants implicated WHV EnII in up-regulation of *N-myc*

genes (126,574). This is supported by studies using artificial constructs in which EnII was shown to be capable of directly activating *N-myc* genes (553,574).

Transcriptional Regulation of the Hepadnavirus Genome

The congested architecture of hepadnaviral transcriptional elements, with promoter and enhancer elements embedded within coding regions and physically adjacent or overlapping, necessitates complex regulatory controls. Studies have begun to examine transcriptional regulation of the hepadnavirus genome. The ratio of pre-S1 to pre-S2/S transcription appears to be one such form of regulation and is an important control point in the viral life cycle. The pre-S1 L protein product acts as an inhibitor of virus secretion (described later), and therefore its level must be tightly controlled. When assayed in reporter constructs independent of other viral genes, the pre-S1 and pre-S2/S promoters display similar activities, whereas in the context of the viral genome, the pre-S1 promoter is much less active (5). The S promoter down-regulates the pre-S1 promoter in the context of the HBV genome (54,323). Down-regulation is attributed to interference in pre-S1 transcriptional elongation, possibly by a CCAAT/NF-Y–binding element (323,324). Other complex regulatory circuits are emerging as well. In DHBV, an active C promoter appears to impair the usage of downstream pre-S and S promoters (228). The mechanism of promoter occlusion is thought to be involved, in which elongating transcription complexes originating from the C promoter impair pre-S1 and S promoters by passing through. Despite the fact that the pre-S1 promoter is located immediately upstream of the S promoter, there is no evidence for promoter occlusion in this complex arrangement.

Hepadnavirus Posttranscriptional Regulatory Element

RNA splicing in eukaryotes is tightly coupled to 3' end formation and export of the RNA from the nucleus to the cytoplasm (373). Consequently, intronless RNAs are poorly exported to the cytoplasm. A number of animal viruses encode intronless mRNAs that nevertheless efficiently accumulate in the cytoplasm. To overcome the export restriction to intronless RNAs, hepadnaviruses use a large RNA element known as the *posttranscriptional regulatory element* (PRE) (reviewed in 604). The PRE is located partially within the X promoter and ORF and downstream of the ORF *S,* indicating that it is contained in all viral transcripts (227,230,231,560). The PRE was shown to promote directly the export of unspliced transcripts. The HBV PRE and the HIV Rev response element (RRE), which performs a similar function, are largely but not completely interchangeable (113,231). However, the HBV PRE is not dependent on HBV proteins for export activity, whereas the HIV RRE requires binding by HIV Rev protein (227,230,231). The HBV PRE is conserved in mammalian hepadnaviruses, and a similar element has been described in WHV (114,630). The mechanism by which the PRE functions is not well understood. In contrast to the HIV RRE–Rev complex, the HBV PRE is not inhibited by the antibiotic leptomycin B, which poisons the nuclear export signal (NES) receptor CRM1/exportin-1 (387).

Processing of Viral RNA

The co-terminal nature of the viral transcripts (see Fig. 2) poses an interesting problem in the control of RNA processing. The subgenomic mRNAs are efficiently polyadenylated at a signal within ORF *C.* The genomic RNAs, being terminally redundant, contain two copies of this signal: one at the 5' end of the transcript and one at the 3' end. Thus, the transcriptional apparatus, which efficiently polyadenylates at this signal in subgenomic RNAs, must bypass this signal on its first pass in genomic RNA synthesis but use it efficiently on its second pass. (This problem is formally analogous to that encountered in retroviral genomic RNA synthesis, in which polyadenylation signals embedded in the 5'-LTR are ignored, but identical signals in the 3'-LTR are used).

Exactly how this problem is solved is still somewhat unclear, but the elements of the solution have at least been identified. Canonical eukaryotic poly(A) signals include the hexanucleotide AAUAAA, a cleavage site about 30 nucleotides downstream, and a U- or GU-rich element 3' to the cleavage site. Mammalian hepadnaviruses have polyadenylation signals that resemble these canonical signals, with one exception: the hexanucleotide is UAUAAA, which is known to function inefficiently. To bolster the efficiency of hepadnaviral RNA processing, the virus has evolved additional elements upstream of the hexanucleotide that enhance its use (455). Some of these elements are 5' to the genomic RNA cap site, such that full processing efficiency is possible only at the 3' end of the RNA. However, beyond this, it appears the the proximity of the 5' processing site to the 5' end of the RNA somehow drastically curtails its use (78,79). The mechanism by which cap-site proximity exerts this effect is unknown, but similar effects have been observed in cellular and retroviral transcripts (238).

X Protein and Its Role in the Virus Life Cycle

X Protein Is Required for Mammalian Hepadnavirus Infection in Animals

The small ORF located between EnI and the single polyadenylation signal of the HBV, WHV, and GSHV genome is referred to as the *X* gene, *HBx* or *pX,* originally reflecting a lack of evidence for gene expression. Subsequently, several groups showed that some infected individuals develop antibodies to X protein during natural infection (208,257,304), and X protein was detected by radioimmunoassay in some infected patients (407). The ORF *X* is contained within all of the mammalian hepadnavirus mRNAs, given its location just upstream of the single polyadenylation signal. However, a 0.7- to 1-kb RNA, corresponding solely to the ORF *X,* has been detected in hepatoma cells transfected with the entire HBV genome (191), and a specific *X* gene promoter was identified in transfected cells (190,509). It is assumed, but not proven, that the X protein is translated from the 0.7- to 1-kb transcript rather than from the core, pre-S1, or S antigen mRNAs through internal ribosome entry. An *X* gene–specific transcript was also detected in the livers of woodchucks chronically infected with WHV (102,253). In HBV-infected human liver tissue, a putative *X* gene mRNA was indirectly detected by reverse transcription–polymerase chain reaction amplification of X region–specific RNA (109,394). Although X protein has been detected in infected liver tissue (304,407,618), it is often difficult to do so because the protein is poorly immunogenic and has a short half-life (101,460). Consequently, it has been shown that immunohistochemical detection of X protein in infected liver tissue with certain antibodies can represent cross-reaction to cellular polypeptides (416,507). Nevertheless, it has been possible to unambiguously detect X protein in acute and chronically infected

woodchuck livers using high-affinity, multiple X protein–specific antibodies (102,240), where it is estimated to accumulate at 10,000 to 50,000 copies per cell (102). HBV X protein has been detected by a similar approach in infected human liver (416,507).

Seminal studies from Zoulim and colleagues (628) and Chen and associates (70) demonstrated that X protein performs an essential (but still unknown) function in WHV infection of woodchucks. Wild-type WHV genomic DNA, which expresses X protein, establishes an infection and an easily detectable viremia when directly injected into the liver of young woodchucks. Mutation of the *X* gene in a manner that does not disrupt the overlying Pol reading frame, either by elimination of the ORF *X* AUG codon or by severely truncating the COOH-terminus of the X protein, fully blocks establishment of infection. It was concluded that X protein performs an essential function in the infectious cycle of the virus, possibly to initiate or maintain viral replication, because there was no detectable level of WHV in serum or in liver tissue (628). This is consistent with a report that X protein expression strongly co-localized with active centers of WHV replication in the liver of chronically infected woodchucks (102). Consequently, HBV X protein is also thought to be essential for human infection. Although a replication function for X protein in viral infection still remains to be described in animal models, the first standard by which to consider various X protein activities is the requirement for viral infection in animals.

X Protein Is a Weak Transcriptional Activator

By comparing the location of the ORF *X* in the HBV genome to the genetic organization of complex retroviruses, it was proposed that the 154–amino acid (17-kd) X protein might possess transcriptional transactivating activity similar to the regulatory proteins Tat of HIV and Tax of human T-cell lymphotropic virus type I (HTLV-I) (350). HBV X protein was subsequently shown to possess a moderate ability (3- to 10-fold) to stimulate transcription of reporter genes (495,549). Subsequently, many reports demonstrated that HBV, WHV, and GSHV X proteins display a weak to moderate ability to stimulate transcription of a wide variety of enhancers and promoters (reviewed in 450 and 603). These include viral elements, such as the SV40 early promoter, the HIV LTR, the HSV tyrosine kinase promoter, and HBV EnI (303,480,486,488,495,549,584,616). Cellular promoter elements stimulated by X protein include those for interleukin-8, c-fos, c-myc, class I major histocompatibility complex, and tumor necrosis factor-a (TNF-a), among others (9,10,12,171,288,332,623; an extensive list can be found in 450). X protein also stimulates RNA pol I– and pol III–dependent promoters (adenovirus virion associated (VA) RNA, cellular transfer RNA [tRNA], and U6 elements) (6,8,282,563,564 and 565). RNA pol II transcriptional elements activated by the X protein usually contain a DNA-binding site for either NF-kB, AP-1, AP-2, c-EBP, ATF/CREB, or the calcium-activated factor NF-AT, among others (129,258,287,303,325,331,332,478,547,548,583). Biochemical evidence for activation of DNA-binding activity of NF-kB, AP-1, ATF/CREB, and NF-AT was also obtained (15,29,83,287,331,367,504,577,583).

The effect of X protein on HBV, WHV, and GSHV transcription elements is consistent with the weak transactivation observed for non-HBV transcriptional elements. When studied within the context of the whole viral genome in transfected hepatoma cells, most studies found that expression of X protein stimulates a modest increase (threefold to eightfold) in accumulation of viral mRNAs (94,263,295,362,594,628), although no effect on transcription was also observed in certain cell lines (34). These results imply that stimulation of transcription may not be a critical function of X protein during infection. Weak up-regulation of viral transcription is also not consistent with the absolute requirement for X protein in WHV infection (628). If the major function of X protein is to stimulate viral transcription slightly, then in its absence, there should be only a concomitant decrease in viremia. In contrast, serum and tissue levels of virus are actually undetectable in the absence of X protein expression (628).

Although it is possible during whole animal infection that X protein might stimulate viral transcription much more strongly than in cultured cells, there is presently no evidence to support this contention. Transgenic mice have been developed to express the HBV X protein specifically in the liver. When crossed with mice that express a reporter gene controlled by the HIV LTR, liver-specific expression of the reporter was increased by only three to six times (13). Therefore, X protein only modestly stimulates transcription of a wide variety of different elements, whether expressed in the context of the whole viral genome, independently in transfected cells, or in the liver of experimental animals. The importance of this weak transactivation activity in establishment of infection and virus replication remains to be determined in relevant animal models.

Mechanism of X Protein Action

Overview

A considerable amount of effort has been directed toward understanding the fundamental molecular activity of X protein. Although there has traditionally been little consensus regarding a common mechanism of action of X protein, several patterns have emerged, which are summarized here and reviewed later.

1. X protein is found largely in the cytoplasm and in small amounts in the nucleus of cells, whether expressed independently of other viral genes in transfected cells, in the context of HBV or WHV genomes, or in infected livers of humans and woodchucks (101,102,115,206,240,287,380,416,492,507,577). A few studies have described X protein as a predominantly nuclear protein in cell lines (202,214,488). In two of these instances (214,488), the nuclear location of X protein can be explained by the coexpression of SV40 large T antigen, which mediates nuclear accumulation of certain proteins, including X protein (115,479). Cytoplasmic X protein has not been found in association with organelles (115), although there is the possibility of an association with proteasomes (226,492).

2. X protein probably functions by several distinct mechanisms, which include direct interaction with components of the transcriptional machinery and stimulation of cytoplasmic signal transduction pathways.

3. The molecular actions of X protein are not restricted to transcriptional activation. Several studies concur that a fraction of X protein interacts with a probable DNA repair protein, known as UVDDB, which might affect viral replication,

pathogenesis, and carcinoma (26,30; for an extensive review, see 56,296, and 493). Whether X protein interaction with UVDDB alters nucleotide excision repair is presently unresolved. Using essentially similar assay systems to measure excision repair fidelity and frequency, several groups observed a slight to moderate decrease on expression of X protein (26,177,245,420), whereas another report did not (30). X protein has also been widely reported either to induce or sensitize cells to induction of apoptosis by pro-apoptotic cytokines and chemical agents in experimental cell systems and transgenic mice (30,84,261,505,528). However, in some systems, X protein is found to inhibit induction of cellular apoptosis (122,172,571), and the relationship of these observations to viral pathogenesis in animals and humans has not been investigated.

4. X protein is probably only weakly oncogenic at best, perhaps contributing indirectly to development of HCC (for an extensive review, see 4). Although X protein expressed in the liver of transgenic mice was originally reported to strongly promote tumorigenesis (259), this has not been found in subsequent studies, except perhaps at supraphysiologic levels of X protein expression (53,271,297). Moreover, studies implicating X protein interaction with, and impairment of, cellular p53 protein (134,541,570) have not been substantiated in more recent investigations (426, reviewed in 603). However, X protein was found to moderately promote development of HCC in the liver of transgenic mice coexpressing the *c-myc* gene (528) or when animals were exposed to hepatocarcinogens at levels that do not independently promote tumor development (102,494). Thus, it is probably correct to conclude that X protein expression is slightly pro-oncogenic.

X Protein Interacts with Several Components of the Transcriptional Apparatus

Most studies agree that X protein does not act by directly binding DNA (evidence reviewed in 603). Through interaction-cloning techniques using overexpressed proteins, it was shown that X protein associates with several components of the basal transcription machinery, including factors TFIIB and TFIIH, the RPB5 subunit of RNA polymerases, and TATA-binding protein (77,201,203,311,358,427,428). In these studies, coassociation of X protein and components of the basal transcription apparatus was observed in mammalian cells upon overexpression of both X and target proteins. Nevertheless, it is still possible that low levels of X-interacting complexes are sufficient to stimulate transcription but cannot be readily detected. X protein also directly interacts, *in vitro* and in cells upon overexpression, with the cyclic adenosine monophosphate (cAMP) response element–binding protein (CREB), a b-Zip family transcription factor (331,583). Evidence that X protein possesses a nuclear function in transcription, possibly by interaction with transcription components, is derived from three sets of observations:

1. X protein can stimulate transcription if tethered to a DNA-binding domain (202,478), which is consistent with, but does not prove, a direct mode of transcriptional activation.
2. X protein directly interacts with the basic leucine zipper region of CREB (583), which is involved in DNA-binding activity, and increases CREB affinity for its DNA-binding site by an order of magnitude (15,391,583). Occupation of the HBV EnI ATF/CREB-binding site is strongly enhanced *in vitro* by X protein interaction with CREB and correlates with CREB-dependent transcriptional activation of this element *in vivo* by X protein expression (4,15,399,583). A similar mechanism of action was described for X protein interaction with another b-Zip transcription factor, cEBPa (90).
3. X protein engineered to contain a nuclear localization signal, and shown to accumulate exclusively in the nucleus, strongly stimulates transcription from an EnI reporter construct in transfected cells, but no longer activates transcription dependent on factors NF-kB and AP-1 (115).

It should now be possible, using experimental approaches such as nuclear-targeted X protein, to determine whether nuclear activation of transcription occurs in the context of viral replication and whether it plays a role in the viral life cycle, pathogenesis, or transformation.

X Protein Stimulates Cytoplasmic Signal Transduction Pathways

Evidence from a number of groups indicates that X protein in the cytoplasm activates several related signal transduction pathways. It was first shown that dominant-inhibiting forms of the kinase Raf, an effector of the Ras-Raf-MAP kinase signal transduction pathway, could block X protein–induced activation of transcription factor AP-1–dependent transcription, as shown in transient transfection studies (99). It was then shown that a dominant-inhibiting form of Ras also blocks X protein activation of AP-1–dependent transcription (28,367,368). X protein increased Ras activity by increasing guanosine triphosphate (GTP) loading onto Ras, not by altering Ras-GAP activity (29). Importantly, the magnitude of Ras activation by X protein is not like that of growth factor stimulation, which strongly but transiently stimulates Ras. Rather, X protein only modestly stimulates Ras, but the activation is sustained or at least periodic (29,206). X protein stimulation of the Ras-Raf-MAP kinase pathway is critical for activation of transcription factor AP-1 in transiently transfected cells (28,29,97,99,115,262,367,368,504). More recently, X protein was found to stimulate Ras-Raf-MAP kinase signal transduction when expressed in the context of replicating HBV or WHV in hepatoma cells in culture (262,263). X protein stimulation of Ras was also found to be essential for its activation of RNA pol I– and pol III–dependent transcription in stably transfected insect cells (563,564 and 565). Thus, the ability of X protein to stimulate Ras is observed in a variety of cell types under different conditions. Retargeting X protein to the nucleus, by fusion to a nuclear localization signal, abolished activation of the Ras pathway (115). The activation of cytoplasmic signaling pathways by X protein is consistent with its predominant cytoplasmic location.

X protein was also shown to stimulate the stress-activated protein kinase (SAPK)-NH2-terminal-Jun kinase (JNK) pathway (29). This pathway is primarily activated by the Ras-related G protein Rac (107). Activation of the SAPK-JNK pathway by X protein was shown by several groups working with a variety of different cell lines, under different conditions (29,206,522; O. Andrisani, personal communication). Thus, there is good evidence that X protein stimulates Ras-Raf-MAP kinase and SAPK-JNK signal transduction pathways in the cytoplasm of a variety of different cells, whether expressed independently of other viral genes or from viral genomes during viral replication. It is not known whether these signal transduction pathways are activated by X protein in natural infection of animals and humans.

Although studies uniformly concur that X protein activates transcription factor NF-kB, whether activation of Ras or other

signaling pathways is involved is not resolved. One report found an essential requirement for X protein stimulation of protein kinase C in activation of NF-kB (258), whereas others did not (99,325,358,368,504). Two studies found that Ras signaling is necessary for NF-kB activation (115,504), whereas another did not (83). These discrepancies may result from the fact that NF-kB consists of multiple related proteins that may be activated to different extents in different ways (343,617). Related NF-kB proteins include p65/RelA, c-rel, and p105 (617). In most cells, NF-kB is sequestered in the cytoplasm, bound to inhibiting IkB proteins, which release NF-kB when phosphorylated by the IkB kinase (617). It has been reported that X protein directly binds to the cytoplasmic IkBa protein when both are overexpressed, mediating direct release of NF-kB and becoming transported to the nucleus (577). However, two previous studies did not detect direct interaction of X protein with endogenous NF-kB or IkB (83,504).

Activation of Ras by X protein is indirect because it has not been found in association with Ras, Ras-GAP, the GTP exchange factor Sos, or Ras adapter proteins Grb2 or Shc (29,262). More recent studies demonstrated that X protein activates Ras by activating the nonreceptor tyrosine kinases of the Src family, which are upstream activators of Ras GTPases (262). X protein constitutively activated Src kinases when expressed from WHV or HBV genomes during viral replication in cultured hepatoma cells and when expressed independently of the viral genome. Activation of Src kinases was modest (twofold to fourfold), as observed for X activation of Ras-signaling pathways. Additional evidence consistent with X protein activation of Src kinases includes X protein activation of the JAK-STAT signaling pathway (299), which is directly activated by Src kinases (544,607).

It is possible that some of the complications and contrasting results in the field of X protein research may be related to the finding that expression of the *X* gene in transfected cells can give rise to several related X proteins by alternate ribosome initiation at several in-frame AUG codons by leaky ribosome scanning (256,282,362,369,621). Although multiple X protein products have not been detected during replication of WHV in cultured cells (101,102), in transfected cells, they can apparently be synthesized from certain constructs and display somewhat different activities (282,362). Thus, expression of different related X proteins (whether biologically relevant or not) could influence different mechanisms of activation.

Possible Function of X Protein in Virus Infection and Replication

The molecular function of X protein in infection of animals and humans has not been investigated. However, studies have examined the function of X protein in WHV and HBV replication in cultured cells. Using cell culture systems, the activation of Src kinases by X protein, but not Ras, was found to stimulate HBV and WHV replication in hepatoma cell lines (263). In this system, ablation of X protein expression by mutation of the *X* gene impairs HBV and WHV replication 5- to 20-fold (94,263,295,347,362,594,628). Accordingly, inhibition of Src kinases impaired HBV and WHV replication 10- to 15-fold at the levels of reverse transcription of pregenomic core-associated RNA, and second-stranded DNA synthesis (263). Viral-specific transcription and translation were reduced only twofold to fourfold. However, it is not known whether X protein stimulates Src kinases during natural infection, nor whether the molecular function of Src kinases in viral replication is the same as in hepatoma cells. Importantly, activation of Src kinases cannot account for the entire function of X protein in viral replication in cultured cells because a constitutively activated form of Src only partially replaced X protein function (263). The mechanism by which X protein activates Src kinases has not been established, but it appears to involve calcium-dependent activation of the tyrosine kinase Pyk2 (M. Bouchard and R. Schneider, unpublished results), which is an upstream, calcium-dependent entry point into the Src pathway (110,302). Given X protein stimulation of calcium-dependent transcription factor NF-AT (287), it is possible that a fundamental activity of X protein is to release intracellular calcium, which could account for many of the reported activities of X protein.

Translation of Viral Gene Products

Hepadnaviruses have evolved multiple transcriptional and translational strategies to maximize the use of the limited coding capacity of their DNA. As previously noted, they make extensive use of overlapping reading frames (see Fig. 2), and within reading frames, they often employ multiple initiation codons to generate structurally related but functionally distinct proteins. Because of their 5'-scanning mechanism, host ribosomes tend not to initiate internally with high efficiency (277). Thus, to guarantee host ribosomes access to these internal AUG codons, the viral transcriptional program has deployed separate mRNA 5' ends just 5' to each AUG. In the case of the envelope proteins, it does this by (a) employing a dedicated promoter for the pre-S1 mRNAs, and (b) having the TATA-less S promoter display microheterogeneity of its start sites, such that some begin 5' to the pre-S2 AUG and others just 3' to it (58,499). In this way, both M and S proteins can be translated as the first ORF of a transcript (see Fig. 2).

Heterogeneous start sites also characterize the genomic promoter, whose RNA 5' ends bracket the pre-C AUG. Those initiating 3' to this AUG encode the C protein, the structural protein of the nucleocapsid (123,355,581). Those initiating 5' to this AUG encode the pre-C protein, which enjoys an altogether different biosynthetic fate. The pre-C region encodes a signal sequence that targets this protein to the secretory pathway (159,388,498). After cleavage of the signal in the ER lumen, the protein is transported through the vesicular transport system, undergoing cleavage of its basic C-terminal region in the process (242,243,462,498,569). Ultimately, it is secreted from the cell, accumulating in the extracellular medium (or serum) as the 17-kd protein known serologically as *e antigen* (HBeAg). In a minority of chains, ER translocation is aborted after signal peptide cleavage, and the resulting products are returned to the cytosol (159) or even the nucleus (390). The significance of these unusual events is uncertain. HBeAg immunoreactivity can also be found on the plasma membrane (462,466), although the mechanism of this cell surface localization is not well defined.

The function of HBeAg is still unknown. Clearly, it is dispensable for replication *in vitro* because nonsense or frameshift mutations within pre-C grow relatively well in cultured cells or in experimental animals (62,71,465,467). However, clinical studies of human HBV carriers show that during persistent infection, mutant viruses with pre-C lesions spontaneously and regularly arise, often coming to prevail over the wild-type virus after years of carriage (41,42,188,382,456,457,538). Transmission of these mutants confirms that they are infectious (198). This implies that there is some selection against HBeAg expression. Such a selection has also been seen in some experimental infections with mixtures of wild-type and pre-C–mutant DHBV isolates, although it is not a uniform occurrence in that setting (620). Because deliberate overexpression of pre-C can interfere with wild-type replication (186), it is conceivable that normal levels of pre-C

expression may confer a subtle growth disadvantage on the wild-type genome. Alternatively, host immunologic attack on HBeAg-bearing cells may select against cells infected by viruses expressing pre-C polypeptides. Whatever the mechanism of selection, all this raises a deeper paradox that is as yet unresolved: if there is a selection against pre-C products at the level of the individual host, how has the *pre-C* gene been maintained in the population? No entirely satisfactory answer to this question has yet been forthcoming. One proposal is that HBeAg may serve as a neonatal tolerogen (348); if so, transmission of pre-C–positive viruses could be selected for during vertical transmission because the induction of neonatal immune tolerance to HBV would promote the development of chronic infection in the infected baby. However, experimental studies do not show an important effect of pre-C proteins on immune function (440). Thus, the forces selecting for or against pre-C expression remain deeply enigmatic at present.

The genomic RNA species that initiates downstream of the pre-C AUG is the mRNA not only for C protein but also for P protein. Evidence that this is so comes from several sources: (a) it is the only identified viral transcript bearing the entire P coding region; (b) no viral transcripts have been identified with 5' ends just upstream of the P AUG, despite vigorous and sensitive searches; and (c) the fact that DHBV pregenomic RNAs are infectious (229) implies that P protein must have been translated from the input RNA. Examination of the coding organization of pgRNA shows that ORF *P* overlaps the 3' end of the upstream *C* gene; the P frame is +1 relative to the C frame. This organization recalls that of most retroviruses, whose upstream core gene (*gag*) overlaps the downstream polymerase gene (*pol*); there, however, the *pol* reading frame is –1 relative to that of *gag.* In those cases, the retroviral polymerase is expressed as a gag-pol polyprotein by ribosomal frameshifting in the overlap region (239). Early assumptions that this would prove true for hepadnaviruses seemed to be supported by the finding of proteins with both C and P immunoreactivity in HBV-infected liver samples (582). However, genetic studies with DHBV soon established that polymerase is made by *de novo* initiation at the P initiator rather than by C-P frameshifting. These studies showed that (a) mutation of the first P AUG to an ACU codon inactivates DNA synthesis; (b) stop codons in ORF *P* located 3' but not 5' to this AUG inactivate polymerase activity; and (c) *C* gene frameshift mutations do not impair the production of P protein (66,68,464). Subsequent experiments in HBV confirmed these findings and indicated that missense mutations in the P AUG could be reverted by construction of a new, in-frame AUG either upstream or downstream of the original mutation (244,454).

The mechanism by which ribosomes gain access to this internal AUG remains incompletely understood. Attempts to identify internal ribosome entry sequences of the type that function in picornavirus translation have failed, and *P* gene translation appears fully sensitive to poliovirus superinfection (66), suggesting that, like most translational events, it is cap dependent. Elaborate genetic studies have led to a model that invokes modified ribosomal scanning from the 5' end of genomic RNA. In this model, ribosomes scan from the cap site until they reach a small ORF overlapping the *C* gene; translation of this ORF allows bypass of a strong out-of-frame AUG that would otherwise occlude scanning further downstream, thereby allowing ribosomes that originated at the 5' end of the transcript access to the internal P AUG (140).

Genomic Replication

The Viral Polymerase

Early inferences that ORF *P* encoded the viral polymerase were strongly supported by the recognition of homology between this coding region and those of retroviral reverse transcriptases (532). Since then, many other reverse transcriptases have been identified from numerous sources, and all of them share sequence relatedness to the hepadnaviral enzyme. Sequence alignments between these coding regions reveal the existence of subregions with obvious homology to either other polymerases (including RNA-directed RNA and DNA polymerases) or to *E. coli* RNaseH (67,212,429,468). Mutational analysis of retroviral *pol* genes has confirmed that these regions indeed represent functional domains that control the polymerase and RNaseH activities of the retroviral enzyme (521). In hepadnaviruses, the polymerase homology domain is centrally located, whereas the RNaseH homology region is located near the C-terminus of the chain (Fig. 5).

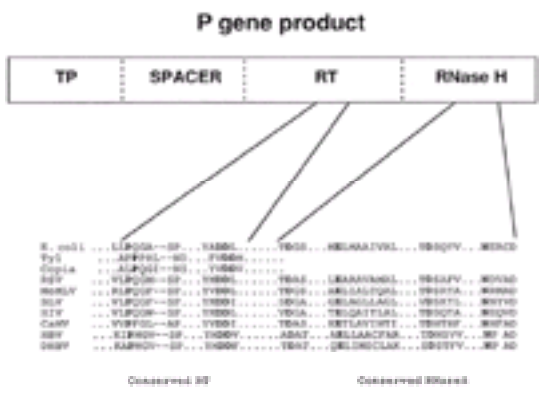

FIG. 5. Domain structure of the hepadnaviral P protein. **Top:** Schematic depiction of the functional domains of P protein. TP, terminal protein; RT, reverse transcriptase; RNaseH, ribonuclease H. **Bottom:** Amino acid sequence alignments with other RNA-dependent DNA polymerases; these homologies form the basis of the assignment of the RT and RNaseH domains.

In retroviruses and many retrotransposons, polymerase genes also encode protease and endonuclease activities, and recognizable homologies also characterize each of these activities. As might have been anticipated from the absence of a known role for integration in the growth of hepadnaviruses, no homology exists between ORF *P* and the integrases of other reverse-transcribing elements. Similarly, no functional protease homologies have been located within ORF *P* (or ORF *C*) (364), consistent with the fact that P protein is not processed from a C-P polyprotein.

The functional significance of these homology-based domain assignments has been validated *in vivo* through the study of mutant viral genomes bearing lesions in these regions. HBV and DHBV mutants bearing point mutations in highly conserved residues within the polymerase homology region are defective for viral DNA synthesis, whereas certain mutations within the RNaseH homology region allow negative-stranded but not positive-stranded DNA synthesis (67,130,429). Just upstream of the polymerase homology region, overlapping the pre-S coding domain, is a region of the *P* gene that is apparently unrelated to other polymerases and displays high sequence divergence between different hepadnaviral *P* genes. This region appears to define an unessential portion of the P protein because it tolerates a wide variety of substitution, deletion, and insertion mutations (18,67,306); 5' to it is the region implicated in the covalent linkage of the P protein to the viral DNA (see later). For this reason, the unessential region is often referred to as a "spacer" or "tether" region connecting the terminal protein (TP) domain to the polymerase and RNaseH domains (see Fig. 5).

Owing to the low abundance of P protein *in vivo,* it has been difficult to visualize P chains in virions. Attempts to do so by immunoblotting (329) or activity gel analysis (20,374) have often revealed species of less than full length, raising the question of posttranslational proteolytic processing. However, by engineering a protein kinase recognition site into the P chain (17), investigators were able to visualize directly encapsidated P chains after their phosphorylation *in vitro;* these studies strongly suggest that cores bear full-length P chains.

RNA Encapsidation

The classic experiments of Summers and Mason (510) indicated that virtually all reverse transcription takes place within subviral core particles, thereby implying that encapsidation of the genomic RNA template must represent the initial step in the genomic replication pathway. As noted previously, hepadnaviral genomic RNAs display microheterogeneities at their 5' ends (see Fig. 2), with the longer ones encoding pre-C proteins and the smallest one encoding the C and P gene products. Importantly, only the smallest genomic mRNA is encapsidated into the core particle (124), indicating that only this transcript (pgRNA) can serve as a template for reverse transcription; host RNAs, subgenomic RNAs, and even the closely similar longer genomic transcripts are excluded. (The basis of this fine discrimination among such closely related RNAs will be discussed later).

The viral proteins required for encapsidation have been identified by examining the impact of mutations in individual genes on pgRNA packaging. As expected, C protein is required to form the structure into which the RNA is packaged (212,293). Capsid formation is independent of pgRNA packaging: isolated *C* gene expression in heterologous hosts results in the accumulation of morphologically normal capsids devoid of pgRNA (33,93,388,448,580,626,627). However, such recombinant capsids do contain host and viral RNAs, which are encapsidated by virtue of the sequence-nonspecific nucleic acid–binding properties of the arginine-rich C-terminal domain of the molecule (33,153,199,363). (Deletion of this region still allows capsid formation, but not RNA encapsidation.) Assembly of capsids proceeds through C protein dimer intermediates (627); these dimers form very rapidly, perhaps even while the chains are still nascent (63). Once the dimers have reached a threshold concentration (estimated at 1 µmol), a sharply cooperative assembly reaction takes place to yield intact capsids (476). In general, no higher-order assembly intermediates can be detected *in vivo* between dimers and capsids. As a result, it is not yet known exactly where in this sequence the addition of RNA occurs.

The fine structure of recombinant HBV core particles produced by C protein expression in *E. coli* has been examined by cryoelectron microscopy (38) and x-ray crystallography (588). The individual C monomers have a unique folding pattern, unrelated to the b-barrel structure so often seen in capsid proteins in other viruses. Dimerization of these monomers occurs through association of two amphipathic a-helical hairpins, generating a four-helix bundle that protrudes from the capsid surface as a spike. Surprisingly, two different particle structures form in *E. coli*: T=3 capsids containing 180 C subunits, and T=4 structures containing 240 such subunits (38). Both particle types seem likely to exist *in vivo,* but it is unclear which can support formation of an infectious virion in the presence of pgRNA and the remaining viral proteins (or if both can do so). It is equally unclear what forces govern the assembly of C into these distinct structures.

In addition to the expected requirement for C protein, P gene products are also necessary for RNA packaging (16,212). Null mutations in ORF *P* result in cores with reduced or undetectable levels of pregenomic RNA. The P protein function required for packaging can be dissociated from those involved in DNA synthesis: many missense mutations that ablate polymerase or RNaseH activity are still competent for RNA encapsidation (212,429,453). This requirement for polymerase gene products in packaging of viral RNA is strikingly different from the retroviral case, in which only gag proteins are required.

How does the encapsidation machinery select the proper RNA? First, a cis-acting packaging signal is present on the encapsidated message. The location of this packaging signal was determined by assaying for the ability of hepadnaviral sequences to confer encapsidation upon heterologous transcripts to which they were fused. In HBV, a small (about 100 nucleotides) region from the 5' end of pgRNA (termed e) suffices to allow packaging (82,250,409). In DHBV, the corresponding e region is necessary but not sufficient; a second, noncontiguous region downstream of e is also required (57,213). Because of the terminal redundancy in pgRNA, the e element is present at both ends of the RNA. Interestingly, however, only the 5' copy is functional for RNA packaging; deletion of the 3' element has no impact on viral replication (213). How this position dependence comes about is unknown, but it helps to explain the exclusion of subgenomic RNAs from encapsidation (124). Although all viral transcripts harbor e sequences at their 3' ends, only the genomic RNAs harbor them in the 5' (functional) position (see Fig. 2); thus, subgenomic RNAs cannot be encapsidated.

Examination of the nucleotide sequence within e reveals a series of inverted repeats that can be predicted to fold into an RNA stem-loop structure (Fig. 6). This predicted stem-loop is phylogenetically conserved among all hepadnaviruses despite significant primary sequence variation in this region among the avian viruses (250). Consistent with this finding, direct biochemical analyses confirm that such a structure actually exists in HBV RNA (265,409). Mutational analyses have probed the regions of this structure that are functionally important for encapsidation (265,409,537). The lower stem (but only limited portions of the upper stem) must be base-paired, but primary sequence in this region is not critical; the

6-nucleotide bulge must be present, but again, primary sequence within the bulge can be radically altered without impairment of encapsidation. By contrast, the specific nucleotide sequence in the loop is critical, and base changes there are poorly tolerated by the packaging machinery.

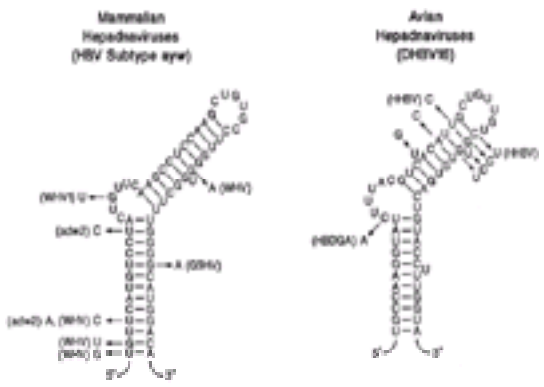

FIG. 6. Phylogenetic conservation of the e stem-loop structure in mammalian (*left*) and avian (*right*) hepadnaviruses. The positions of base changes in naturally occurring isolates are indicated.

What is the e recognition apparatus? The simplest model compatible with the genetic analysis of encapsidation is that P protein directly recognizes this element, and *in vitro* evidence for P protein–RNA interactions have been obtained by several groups (223,410). That this binding relates importantly to RNA encapsidation is supported by analysis of e mutations; all such mutations that block the P-e interaction *in vitro* block RNA encapsidation *in vivo.* However, there is one important discordance: loop mutations, known to strongly impair packaging *in vivo,* do not abolish P-e binding (410). This might mean that additional factors, possibly of host origin, may be binding to the loop region; alternatively (or additionally), lesions in the loop may subtly affect e structure. Searches for host proteins that bind to the apical loop have been unrevealing (400), but evidence for structural changes in e after P binding has recently been presented (25). Loop mutations that block encapsidation do not display these changes, suggesting that they are functionally significant (25).

The assembly of the P-e ribonucleoprotein (RNP) has recently been found to require host molecular chaperones and ATP (223,224). Addition of monoclonal antibodies to heat shock protein 90 (hsp90) blocks the ability of P synthesized *in vitro* to bind to e RNA, as does geldanomycin, a drug that blocks the association of hsp90 with its essential cofactor p23. (Interestingly, p23 also appears to bind directly to DHBV P protein, in a geldanomycin-resistant fashion.) The proteins p23 (and presumably hsp90) remain associated with the polymerase after encapsidation; this is unlike many other chaperone-assisted reactions, in which the association of chaperone and target is transient. However, clever temperature-shift experiments with a temperature-sensitive DHBV polymerase reveal that, after encapsidation and DNA priming, geldanomycin has no impact on subsequent reverse transcription (224). Thus, hsp90–p23 complexes act primarily to assist in the formation of an RNP competent for encapsidation and DNA priming (see later).

The next step in RNA encapsidation is presumably the association of the P-e complex with assembling core subunits. Detailed understanding of these events is lacking, but recent evidence indicates that HBV P protein produced in baculovirus-infected cells can interact noncovalently with C protein (R. Lanford, personal communication). Additional information has emerged from the study of structure–function relations within the DHBV P protein. By examining truncation mutants of DHBV P produced by *in vitro* translation, it has been shown that the C-terminus of P protein is dispensable for e binding; however, this same region is important for RNA encapsidation *in vivo* (212,410). This is compatible with a model in which the N-terminal two thirds of P protein binds pgRNA, leaving the C-terminus free to interact (directly or indirectly) with assembling C polypeptides. A full accounting of the mechanism of the encapsidation reaction will likely require development of an *in vitro* system in which the entire process is faithfully reproduced. The first step in this direction has been achieved: the assembly of “empty” HBV cores from C proteins produced in cell-free translation systems (312). However, no group has yet succeeded in observing P-dependent RNA encapsidation into such structures *in vitro.*

Thus, in hepadnaviruses, the encapsidation of polymerase occurs by a fundamentally different mechanism from that employed by retroviruses. The latter produce pol as a gag-pol polyprotein, which is assembled into capsids by interactions of its gag domain with assembling gag polypeptides. In hepadnaviruses, P protein is bound to the RNA that is destined for encapsidation, and noncovalent interactions, direct or indirect, between C and the P-containing RNP must exist to package the pregenome. Such interactions, however, must depend on a unique property of the P-e complex not shared by free P because DHBV genomes bearing e deletions produce cores that lack P protein as well as RNA (19). Perhaps one function of the stably complexed chaperones in the RNP is to maintain P protein in a conformation that is competent for interactions with assembling C subunits; evidence that P undergoes conformational changes after e binding has in fact been presented (525).

An interesting feature of the encapsidation reaction is the marked preference of the P gene product for its own mRNA during the packaging process. This is most easily demonstrated by cotransfecting a wild-type and P-negative mutant genome (which is differentially marked with a new restriction enzyme site). Examination of either the encapsidated RNA or the subsequently reverse-transcribed viral DNA reveals that the mRNA encoding the wild-type (functional) P gene product is preferentially encapsidated (90% to 95% of the encapsidated genomes are of wild-type origin) (212,293). The mechanistic basis of this cis-preference is unclear. One possibility is that the nascent P polypeptide co-translationally binds to its own mRNA. Alternatively, the levels of functional P gene product might be limiting, so that concentrations of P sufficient for encapsidation are found only in the vicinity of its own mRNA. In the case of HBV, the inefficiency in trans complementation can be at least partially overcome by deleting the encapsidation signal (16).

As noted earlier, the 5' ends of the genomic RNAs are heterogeneous, but only the shortest of these RNAs is encapsidated (124). Because all these transcripts contain an intact 5' copy of e, how is packaging of all but the shortest pregenomic RNA suppressed? Examination of the 5' termini of these RNAs indicates that all but the shortest transcript (i.e., pgRNA) include the upstream, in-frame pre-C AUG (see Fig. 2). Mutations that inactivate this AUG allow these longer messages to be encapsidated (365). These and other data suggest that translation through this region suppresses recognition of e. Because the shortest genomic RNA (pgRNA) lacks the pre-C AUG, such ribosome-mediated suppression cannot operate on this RNA.

Viral DNA Synthesis

The complex mechanism by which single-stranded pregenomic RNA is converted to partially duplex virion DNA is now understood in considerable detail. The current view of this reaction is summarized schematically in Figure 7. Pregenomic RNA is terminally redundant; its about 200-nucleotide redundancies (termed R) include the e stem-loop and a short sequence of 11 to 12 nucleotides termed *DR1.* Another copy of this same sequence is present near the 3' portion of the unique region of the RNA and is termed *DR2.* The potential importance of the DR sequences in viral DNA synthesis was recognized when the 5' termini of the viral DNA strands were precisely mapped. This showed that the 5' end of negative-stranded DNA mapped within DR1, whereas the 5' end of positive-stranded DNA mapped to DR2 (96,309,352,472,581). The exact roles of these sequences are discussed later.

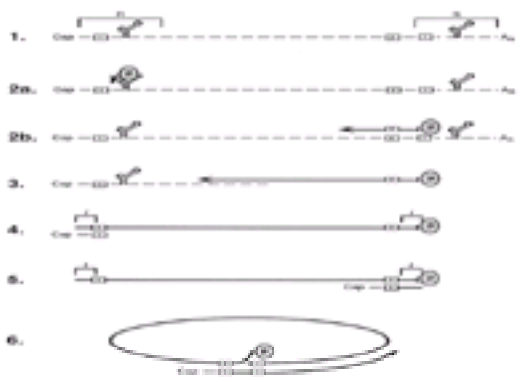

FIG. 7. The hepadnaviral reverse transcription pathway. Pregenomic RNA (*dashed line,* step 1) is capped and polyadenylated and has a large terminal redundancy (*R*). The locations of direct repeats 1 and 2 (DR1 and DR2) are shown as correspondingly numbered boxes, and the e stem-loops are indicated. Pregenomic RNA packaging into cores is initiated by the interaction of P protein with the 5' copy of e. P initiates reverse transcription at the 5' stem-loop and extends negative-stranded DNA (*solid line*) for 3 to 4 nucleotides (step 2a). P and the covalently attached nascent DNA are then transferred to the 3' copy of DR1 (step 2b), and the DNA is extended. During negative-stranded elongation, pgRNA is degraded by the RNaseH activity of P (step 3). When P reaches the 5' end of the template, its RNaseH activity leaves an RNA oligomer consisting of r plus DR1 sequences (step 4). This RNA oligomer is translocated and annealed to DR2, where it primes positive-stranded DNA synthesis (*lower solid line,* step 5). Positive-stranded elongation proceeds to the 5' end of the negative-stranded DNA template, including the sequences denoted as r. Because complementary r sequences are found at the 3' end of negative-strandeds DNA, a second homology-mediated template transfer can now circularize the genome. The positive strand is then extended for a variable length (step 6) to yield mature viral DNA.

Negative-Stranded Synthesis

Negative-stranded DNA has protein covalently linked to its 5' end (156,166,351). This so-called terminal protein was identified as the viral P protein by showing that viral DNA could be immunoprecipitated by anti–P protein antibodies (37). Limited proteolysis suggested that the N-terminal portion of the P polypeptide contained the site of covalent attachment to the DNA (18); this has now been directly confirmed for DHBV, in which the DNA protein joint has been precisely mapped to tyrosine 96 of the P protein (573,629).

Because of the fact that all nascent chains of viral DNA are found linked to P protein (351), virologists have for years suspected that the protein serves as the primer for DNA synthesis, in a fashion formally analogous to that established for the terminal protein of adenovirus DNA (61). This inference has now been directly sustained by a seminal experiment of Wang and Seeger (561). When DHBV P protein was translated *in vitro* (from a mRNA that also contains DR1 and e sequences) in the presence of ^{32}P- dNTPs, P protein chains become labeled by covalent attachment of nucleotides. Most product consists of P chains bearing bout 4 nucleotides; longer products (up to several hundred nucleotides) are present at a very low level. That this reaction reflects authentic viral DNA synthesis is indicated by the following: it is inhibited by mutations in the reverse transcription active site, and the sequence of bases at the DNA protein joint corresponds exactly to the authentic 5' end of viral DNA. In this reaction, the priming hydroxyl group is supplied not by a 3'OH of DNA or RNA but by the side chain of a tyrosine residue on P protein (573,629).

If P protein is the primer, what is the template for initiation? Because the 5' ends of viral DNA map to DR1, it was long assumed that the priming of negative-stranded DNA was templated by DR1 sequences. However, analyses of the DHBV priming reaction both *in vitro* (562,629) and in recombinant yeast expressing functional P protein (523,526) indicate that this is not the case. Rather, negative-stranded initiation is templated by sequences within the bulge of the e stem-loop. The involvement of e sequences in the reverse transcription reaction had previously gone unrecognized because *in vivo* e is required for the RNA packaging step that is a necessary precondition for DNA synthesis. Thus, defects in DNA synthesis due to e mutations could be accounted for entirely by their RNA packaging defect. However, both of the recombinant P protein expression systems mentioned previously were designed to be independent of DHBV packaging

functions. Under these conditions, the involvement of e in reverse transcription rapidly became apparent: deletions of e (or even of the e bulge alone) completely ablated all viral reverse transcription. Analysis of large numbers of more subtle e mutations reveals a close correlation between lesions affecting RNA packaging and those affecting DNA priming, suggesting that the same RNA protein recognition reaction underlies both processes (410). In accord with this, blockade of P-e RNP formation with anti-hsp90 or geldanomycin also blocks DNA priming (223,224). Taken together, these data all indicate that initiation of viral DNA synthesis occurs within e, not DR1 as initially supposed.

There is growing evidence that this critical step results in structural changes in both the RNA template and the enzyme that may be required to facilitate the subsequent events in the replication cycle. Beck and Nassal (25) have shown that the fine structure of e is subtly altered by P protein binding, and analysis of e mutants indicates that this change is correlated with competence for DNA synthesis. Similarly, the P protein may also undergo conformational changes following binding. This was initially revealed by an altered susceptibility of P to proteolytic digestion after contact with e RNA. All e mutations that allow DNA priming induce this change, whereas those that do not (including apical loop mutations that can still bind P but do not allow DNA synthesis) fail to induce the change (525). Thus, the e RNA might serve not only as the template for the polymerase but also as its allosteric effector.

The next events in replication are depicted schematically in Figure 7. After P-e binding, initiation occurs within the e bulge, generating an initial P-linked oligonucleotide of about 4 nucleotides. Because the sequences of the e bulge and DR1 share 4 nucleotides of identity, this DNA product can be transferred and annealed to DR1 sequences; from this position, the DNA can be extended by traditional elongation mechanisms (524,526,562). This strand transfer reaction has been studied in great detail but remains incompletely understood. It is clear that sequence homology between donor and acceptor sites is required for the reaction because disruption of the homology strongly impairs transfer (246,315,524). However, second-site mutations in DR1 that restore complementarity to a mutant e region do not always restore efficient transfer, indicating that homology, although necessary, is not sufficient for the reaction. Many other factors appear to be involved, including local sequence or structure surrounding the acceptor site, the position of the acceptor site relative to the donor, and the length of the transferred strand (317,524).

Both DR1 and e are represented twice in the pgRNA template. The fact that the 3' copy of e can be deleted from pgRNA without blocking viral replication (473,474) suggests that it is the 5' copy of e that is functional in initiation. This inference has been directly validated by genetically marking the 5' e with a sequence polymorphism and showing that this sequence marker appears at the 5' end of negative-stranded DNA after one round of reverse transcription (526,562). Analogous genetic tagging experiments indicate that it is the 3' copy of DR1 to which the nascent P-linked primer is transferred (473). Once this transfer reaction is complete, elongation can continue to the end of the RNA template. As negative-stranded elongation proceeds, degradation of the newly copied pregenomic RNA template occurs concomitantly (349,510); this is necessary to make negative-stranded DNA available to template positive-stranded synthesis. As in other reverse transcription reactions, this function is attributed to the RNaseH activity of the viral polymerase itself (429). Consistent with this notion, lesions in the RnaseH domain of P inhibit positive-stranded synthesis (67,429). The final product of this elongation reaction is a negative-stranded DNA that is actually terminally redundant by about 8 nucleotides (this shorter redundancy is known as r) (309,472,581).

Positive-Stranded Synthesis

After negative-stranded DNA synthesis is complete, positive-stranded DNA synthesis can begin. Positive strands are initiated at DR2 (309,472,581) (see Fig. 7), in a reaction primed by short (about 15 to 18 nucleotides), capped oligoribonucleotides derived from the 5' of pregenomic RNA; these include DR1 sequences and the 6-nucleotide 5' end of DR1. The viral RnaseH activity is also presumed to be responsible for the cleavages that generate these primers. The reaction does not proceed by sequence-specific endonucleolytic cleavage at the border of DR1 because nucleotide changes around the cleavage sites do not perturb either the specificity or efficiency of cleavage (500). However, when insertions or deletions are placed between the 5' end of pregenome and the 3' end of DR1 (316), the sites of cleavage remain a constant distance (15 to 18 nucleotides) from the 5' end of the pregenome. This indicates that the cleavages are somehow positioned by measurement from the end of the template and that the actual scission event is independent of the sequence at the cleavage site. A model for how this distance might be measured has been proposed (316).

The site of primer generation is many hundreds of bases away from the actual site of positive-stranded DNA synthesis at DR2. Thus, the newly cleaved RNA primer must be translocated to the 5' end of negative-stranded DNA to base-pair with DR2 for positive-stranded DNA synthesis to begin. Because the primer is being moved from a region (including r and DR1), where it is base-paired for 18 nt to a region (DR2) where it is base-paired for only 12 nt (see Fig. 7); this primer translocation is not thought to occur passively. Most likely, it is actively facilitated by proteins, but how this is accomplished remains a matter of conjecture. It is known that positive-stranded synthesis is very sensitive to mutations not only within the primer but also to lesions in several other regions of the template, including a region in the middle of the genome and others near DR2 and DR1 (200,315,318,319,357). The two terminal regions have the potential to base-pair with the central region (D. Loeb, personal communication), suggesting that these regions may be important in maintaining a structure in the template that facilitates the transfer, perhaps by juxtaposing the donor and acceptor sites.

Occasionally, primers that are cleaved fail to be translocated. This occurs at a low rate with wild-type virus genomes (about 1% to 5% of primers) but can be greatly increased by cis-acting mutations in and around DR1. Typically, primers that are cleaved but not translocated are instead extended from their original position, a process referred to as *in situ* priming. The result is fully duplex linear viral DNA (500). Such molecules are found in all stocks of hepadnaviral particles. Many of these duplex linear DNAs are dead-end products. However, recent work has shown that some of these species can participate in a variety of illegitimate recombination reactions, with interesting consequences. Although the viral life cycle is maintained by episomal DNA, integration of the genome into host DNA is known to occur at low frequency, an event that in some systems plays a role in hepatocellular carcinogenesis (see later). Analysis of these integrants suggests that a substantial fraction of them likely derive from double-stranded linear DNAs produced by *in situ* priming (168,169 and 170; W. Yang and J. Summers, personal communication). Illegitimate recombination can also circularize such linear DNAs, generating cccDNA forms with abnormal circle junctions. Some of these are competent to undergo additional rounds of transcription and reverse transcription, a process that has been termed *illegitimate replication*

(597,598 and 599).

Once the RNA primer is translocated, synthesis of positive-stranded DNA can begin. Elongation proceeds to the 5' end of negative-stranded DNA, at which point the template is exhausted and an intramolecular strand transfer is required to complete positive-stranded synthesis (see Fig. 7). This transfer is facilitated by the short redundancy (r) in the negative-stranded DNA template: annealing of the nascent positive strand DNA (by its r homology) to the 3' end of the negative strand circularizes the genome and allows continuation of positive-stranded synthesis. As for other hepadnaviral strand transfers, sequence complementarity is necessary but not sufficient for this process. Circularization can be affected by other variables, including the sequence and length of the transferred strand and the sequences adjacent to DR1 at the 3' end of negative-stranded DNA (200,315,318,319). Once accomplished, the strand transfer allows positive-stranded synthesis access to the rest of the negative-stranded DNA template. However, in most cases, synthesis does not proceed to completion (the exception is DHBV, in which 80% of the positive strands complete elongation) (310,352). The result is the characteristic single-stranded gap that is a hallmark of hepadnaviral DNA.

Why positive-stranded synthesis terminates prematurely is not entirely clear but might be related to the process of viral budding. The DNA of cytoplasmic core particles is substantially more immature than that of extracellular virions, suggesting that maturation of viral DNA synthesis is somehow coupled with envelopment and secretion of the Dane particle. In keeping with this, *P* gene mutations affecting viral DNA synthesis can strongly influence envelopment and release (162,576). Presumably, a structural change or posttranslational modification of the core particle occurs concomitant with reaching a certain stage in genome maturation (608), creating a signal for core envelopment. Once budded into the ER, the core particle would presumably no longer have access to cytoplasmic dNTP pools, and further DNA synthesis would be expected to arrest.

Viral Assembly and Release

20-nm Subviral Particle Assembly

As noted previously, 20-nm particles contain predominantly the S protein with variable amounts of M and only trace quantities of L subunits. With the advent of cloned HBV DNA, it became possible to express the surface protein coding regions in heterologous cells, leading to the discovery that cells expressing only the S polypeptide can assemble and secrete morphologically normal 20-nm particles. Thus, all of the viral information necessary for this assembly process resides in the S domain (290,313,401). Because the cellular machinery for particle assembly is present in virtually all vertebrate cells, expression of 20-nm particles is possible in a wide variety of expression systems (116,353,501). This has made possible the development of recombinant HBV vaccine, the first recombinant DNA-based vaccine licensed for any human infectious disease. Interestingly, yeast cells (the source of one widely used recombinant vaccine) do not secrete HBsAg and may not form intracellular particles at all. However, subparticulate lipoprotein aggregates of S subunits likely do form intracellularly and may rearrange during extraction to yield immunogenic particulate antigen (557,560).

How do 20-nm particles form in mammalian cells? Early electron microscopic studies of infected liver revealed that particles accumulated within cisternae of the ER (161), an observation also confirmed by later electron microscopic studies of transfected, HBsAg-positive cells in culture (395). Consistent with this is the fact that intracellular particles contain asparagine-linked high-mannose oligosaccharide chains, a modification specific to the ER (396). Subsequent biochemical studies have more precisely localized the major site of particle assembly to the so-called intermediate compartment between the ER and Golgi (232). Assembled complexes are then transported through the constitutive secretory pathway, traversing the Golgi complex, where their carbohydrates are processed to the complex, endoglycosidase H-resistant form (396). Transport of the complexes through the Golgi appears to be the rate-limiting step in export because all intracellular glycosylated S protein is found in the endoH-sensitive form, whereas all extracellular glycosylated HBsAg is endoH resistant.

S proteins are synthesized in the ER as integral membrane proteins. Their transmembrane topology has been determined in cell-free translation systems supplemented with ER-derived microsomal vesicles (117,119). In these experiments, nascent S protein chains were found to be oriented in the bilayer such that both N- and C-termini are in the vesicle lumen. To do so, the polypeptide must span the bilayer at least twice; many current models, based largely on theoretical considerations, envision up to four transmembrane passages (Fig. 8). (The S coding region contains three hydrophobic domains, encompassing residues 4 to 28, 80 to 100, and 164 to 221. Hydrophobicity and other considerations lead most to assume that all of these regions are located within the bilayer, generating the model of Figure 8. However, the disposition of the C-terminal hydrophobic domain has not been experimentally assessed.) This topology is achieved through the conjoint action of two topogenic signal sequences. Signal I corresponds to the first hydophobic domain and initiates chain translocation across the bilayer. It resembles conventional N-terminal signal sequences except for the fact that it remains uncleaved during translocation. Signal II (corresponding to the second hydrophobic domain) is a complex topogenic element that serves both as a stop-transfer sequence (anchoring the protein in the bilayer with residue 80 facing the cytoplasm and residue 100 facing the ER lumen) and a signal sequence directing the translocation of the distal region of the molecule. Thus, the region between signals I and II is a cytoplasmic loop; downstream of signal II is the major glycosylation site of S as well as its immunodominant surface epitope.

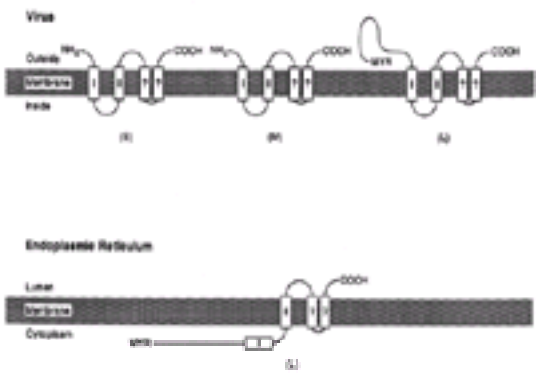

FIG. 8. Top: Proposed transmembrane topologies for the HBV L, M, and S glycoproteins in the virion envelope. *Rectangles* denote S-protein hydrophobic domains. [The transmembrane orientation of domains I and II have been addressed experimentally (87), whereas the disposition of the remaining hydrophobic regions remains speculative.] The *psi* symbol denotes *N*-linked carbohydrate. *Myr* is the myristate group in amide linkage to glycine at the N-terminus of L protein (292). **Bottom:** Transmembrane structure of L protein in endoplasmic reticulum membranes. Virtually all pre-S sequences reside on the cytoplasmic face of the membrane. (The disposition of S hydrophobic region I is here drawn as cytoplasmic, but this has not been directly validated.)

This transmembrane form of S is only a transient intermediate in particle formation; *in vivo,* this form is rapidly converted to a series of defined assembly intermediates (232,491), and *S* gene mutations affecting one or more of these steps have been examined (44,418). After membrane insertion, the transmembrane monomers aggregate and in the process exclude host proteins from the complex. (With one exception, in DHBV, subviral particles also harbor large quantities of the host chaperone hsp70, a known participant in many protein-folding reactions. Because hsp70 is a cytosolic protein, it must be interacting with cytoplasmically disposed regions of the viral envelope proteins. It is not clear, however, why so much hsp70 remains in the final particle because in most other folding reactions, the chaperone is released from its targets once folding is accomplished.) The aggregate then buds or extrudes into the lumen. It is likely that this extrusion event differs from more conventional viral budding reactions in that the “budded” product lacks a typical unit membrane structure. In addition, its lipid content is much lower than that of most cellular membranes, suggesting that that the host lipids may be substantially reorganized in the process.

This pathway shares many features with morphogenetic events postulated for the envelope proteins of other animal viruses: transmembrane insertion, aggregation, and budding. However, in other viruses, envelope glycoproteins remain anchored in the bilayer until interactions with other viral components (e.g., nucleocapsid or matrix proteins) trigger the budding step, thus ensuring that envelope proteins can exit the cell only by enveloping the nucleocapsid. The distinctive feature of the S protein is that it can carry out the entire assembly sequence without the involvement of other viral proteins. The result is the release of the subviral particles containing only envelope proteins—the distinctive signature of hepadnaviral infection.

In authentic viral infection, 20-nm particles frequently contain M as well as S subunits (204,503). In such particles, the pre-S2–encoded domain of the M protein is exposed on the particle surface, as demonstrated by (a) its reactivity with antisera specific for the pre-S2 domain of M, and (b) its ability to bind polymers of human albumin, an activity of uncertain biologic importance that is mediated uniquely by this domain (326,327,401). Given its surface location on the particle, in the transmembrane form of the M protein, this domain would be predicted to be translocated into the ER lumen (Fig. 9); this prediction has been experimentally confirmed (118). Because the pre-S2–encoded domain lacks a definable signal sequence, its transmembrane transport must be mediated by the downstream signals in the S domain.

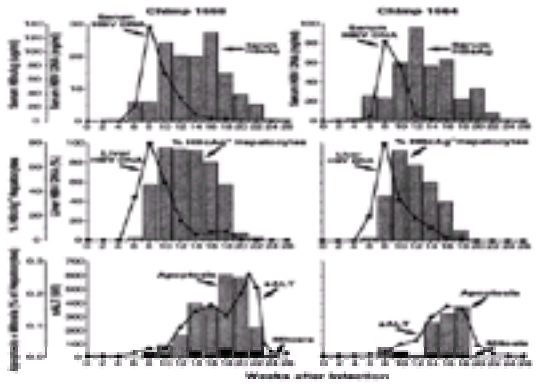

FIG. 9. Natural history of experimental HBV infection of two chimpanzees. **Left:** Chimpanzee, 1558. **Right:** Chimpanzee, 1564. **Top:** serum hepatitis B virus DNA levels and HBsAg levels as a function of time post inoculation. **Center:** Liver HBV DNA levels and the percentage of HBcAg-positive hepatocytes as a function of time after inoculation. **Bottom:** Serum transaminase levels and the percentage of hepatocytes undergoing mitosis (*black bars*) or apoptosis (*gray bars*) as a function of time after inoculation. (From ref. 187, with permission.)

As noted previously, natural 20-nm particles often lack detectable L protein (204). The reason for the low abundance of L chains on 20-nm particles became clear in experiments in which L chains were deliberately overexpressed in cultured cells. In cells infected with vaccinia vectors that express only the L protein, the L chains themselves are not secreted, despite the presence of an intact S domain that contains all the information required for the export process (76). This

suggests that the pre-S1–specific domain of L contains signals that promote intracellular retention of the chain and that can override the secretory elements in the S domain. When L chains are overexpressed in the presence of M and S chains, the ability of the M and S chains to be secreted is inhibited in a dose-dependent fashion (76,88,389,402); all three chains accumulate intracellularly (87). Presumably, this reflects the formation of mixed arrays of L, M, and S subunits, with the inhibitory influence of the pre-S1 domain now being conferred upon the entire aggregate. The inhibitory effect of L overexpression on S particle secretion explains the underrepresentation of L chains on 20-nm spherical particles.

The region of the pre-S1 domain that specifies the intracellular retention of HBsAg has been mapped by deletion mutagenesis to the extreme N-terminus of the molecule (281). The observation that this region of the L protein is myristoylated (403) raised the possibility that ER retention might be mediated by the affinity of this hydrophobic fatty acid for the ER membrane. However, point mutations that ablate myristylation have either no (281) or only incomplete (417) effects on secretion-inhibition, indicating that primary sequences in this region of the chain are important for this activity. The mechanism of the secretion-inhibition by L remains uncertain. Early models proposed that L chains might not be mobilized from their transmembrane state. However, subsequent studies (589) suggest that, when expressed in the absence of M and S proteins, L chains form intraluminal aggregates that remain trapped within the secretory system, where they can be found associated with the chaperone calnexin. This suggests that the block to release is distal to the budding step. Interestingly, in electron microscopic studies of the hepatocytes of transgenic mice overexpressing L chains (relative to M and S), 20-nm filamentous forms were frequently observed in the ER (87). This accords well with earlier observations that filaments are relatively enriched in L subunits (204) and suggests that filaments may arise in regions of the ER where local concentrations of L are high relative to S.

The overaccumulation of retained L-M-S aggregates can be injurious to the host cell. Hepatocytes of transgenic mice bearing such aggregates develop characteristic cytopathic effects (in the relative absence of inflammation) and are very sensitive to killing induced by interferon-a (IFN-a) and TNF (361). Morphologically similar cells, termed *ground-glass hepatocytes* by pathologists, are sometimes seen in biopsy specimens from acute hepatitis B, indicating that deregulation of envelope protein expression may make some contribution to the process of liver cell injury. The magnitude of this contribution, however, is uncertain, and is likely to be modest.

Dane Particle Assembly

Conceptual views of Dane particle assembly have been heavily influenced by the emerging understanding of the morphogenesis of subviral particles. Because all three HBV envelope proteins are expressed primarily on intracellular membranes rather than on the cell surface (389), virion formation almost certainly proceeds on intracellular membranes; EM studies consistent with this have been reported (252,446). However, an important distinguishing feature of virions is that they contain relatively large quantities of L proteins; in the best preparations, the ratio of L to M to S subunits was about 1:1:4 (204). Because these preparations are often contaminated to some extent with 20-nm particles, it is possible that the ratio of L to S chains in authentic virions is still higher. It is unlikely that subviral particles bearing this much L protein could be efficiently released; this suggests that distinctive mechanisms are involved in virion assembly and export.

To define the components required for Dane particle production, permissive HepG2 cells have been transfected with missense or nonsense mutants selectively affecting the HBV L, M, or S proteins and the ability of these mutants to support virion release assayed (45). Several conclusions have emerged from these studies: (a) unlike retroviruses, whose nucleocapsids can still bud out of cells in the absence of envelope proteins, no HBV cores are exported if the synthesis of all envelope proteins is aborted by nonsense mutations in S; and (b) both L and S proteins are required for virion formation and release. Although one report to the contrary has appeared (552), the weight of evidence is that M proteins are not required for assembly (45,137,457). Missense mutants affecting L myristylation also remain competent for virion assembly and secretion, although the unmyristylated virions are noninfectious (45,46,330,513). Myristylation may thus play its most important role in the adsorption, entry, or uncoating of virions on the next cycle of infection.

How the L protein contributes to viral assembly remains incompletely understood, but recent studies of its transmembrane topology have suggested new possibilities. Studies of HBV virion architecture reveal that the L protein–specific, pre-S1–encoded domain is exposed on the virion surface. For example, it is accessible to exogenous proteases as well as to pre-S1–specific MAbs (204,280,385). This would suggest that, like pre-S2, the pre-S1 domain in the transmembrane form of L should reside in the ER lumen. However, biochemical studies of HBV L chains synthesized *in vitro* in the presence of microsomal membranes indicated that this is not the case; rather, most pre-S–encoded L protein sequences are on the cytoplasmic surface (385). This result was subsequently confirmed by similar analysis of nascent HBV L chains *in vivo* (47,419) and is supported by other findings that indicate that pre-S1 regions are at least transiently cytosolic (see Fig. 8). For example, DHBV and HBV L chains can associate with cytosolic hsp70, through sites mapped to the pre-S region (320,518). Moreover, HBV and DHBV pre-S can undergo phosphorylation by cytosolic kinases (173,174,452). This phosphorylation has no impact on viral replication or assembly but is required for yet another known activity of L protein—its ability to up-regulate reporter genes in co-transfection assays (210).

This topology helps to explain how L might make a distinctive contribution to virion morphogenesis because it now can present unique determinants to nucleocapsids on the cytoplasmic face of the ER membrane. In one attractive model, capsid–L protein interactions trigger the budding event that incorporates the mature core into the viral envelope. This notion is supported by mutational studies examining the impact of pre-S topology on virion assembly. If the pre-S region of L is delivered to the ER lumen by an engineered N-terminal signal sequence, no virions can be formed, despite the fact that subviral particles can still be assembled and released (47). Examination of deletions and linker scanning mutations within pre-S confirms that only those mutations whose pre-S domain is cytosolically disposed are competent for virion assembly, and point to a region at the pre-S1–pre-S2 border as critical for virus production (43,48,49). The simplest model is that this region is directly involved in contacting the nucleocapsid.

We are left, however, with an important remaining problem: how does the pre-S1 domain wind up on the surface of the budded virion? One possibility is that nascent L chains exist in two orientations in the ER: one with luminal pre-S sequences and another with cytoplasmic pre-S domains. However, virtually all detected nascent chains have only

cytoplasmic pre-S determinants. A more likely scenario is that during or after budding, the components of the envelope undergo a dramatic rearrangement, resulting in delivery of the pre-S1 domain across the bilayer. How such a process would occur remains mysterious, but it happens with surprising efficiency; biochemical analysis of mature virions indicates that more than half of their constituent L chains bear pre-S1 regions that have been transposed to the virion surface (47).

Nuclear Delivery of Progeny Core DNA: cccDNA Amplification

Consideration of the overall replication cycle schematized in Figure 3 indicates that the central genomic species involved in viral persistence is nuclear cccDNA. Only cells that harbor this form of the genome can continue to produce virus. Because hepadnavirus-infected cells are not killed by viral replication, they may continue to grow and divide. (Admittedly, under normal circumstances, hepatocytes divide infrequently, but proliferation is often greatly enhanced during chronic infection as a result of inflammation and regeneration.) Thus, a mechanism must exist to ensure the inheritance of this molecule by both daughter cells after mitosis. In principle, this problem could be solved by equipping cccDNA with an origin for semiconservative DNA replication because all the machinery for this process exists in the nucleus; this would be analogous to the solution adopted by Epstein-Barr virus or papillomaviruses, for example. However, density labeling studies (545) indicate that this is not the case: all nuclear cccDNA is derived instead from the reverse transcription pathway. Because reverse transcription is a cytoplasmic process, this implies that mechanisms exist to deliver cytoplasmic reverse transcription products into the nucleus. For DHBV, such mechanisms operate efficiently early in the establishment of infection in primary duck hepatocytes, leading to the rapid accumulation of 10 to 20 or more copies of cccDNA per nucleus.

One possible way to account for the maintenance of a pool of cccDNA would be to imagine that it was replenished by reinfection of cells by extracellular virus. However, it was soon shown that cccDNA amplification could be observed in the presence of inhibitors of viral entry like suramin or neutralizing antibody; it also can occur after transfection of viral DNA into cells that lack the surface receptor, thereby eliminating the possibility of reinfection by released progeny virus (587). Thus, cytoplasmic cores bearing virion DNA can be shunted to the nucleus by an intracellular pathway. A key step forward was made with the recognition that DHBV mutants lacking the L envelope protein (or bearing certain point mutations in pre-S) (301) displayed greatly enhanced amplification, resulting in the nuclear accumulation of very high levels of cccDNA (512,513). This implies that the subcellular trafficking of cores is regulated: early in infection, when envelope proteins are at low levels, nuclear delivery is favored, allowing accumulation of cccDNA. This ensures that this infected cell will be stably colonized. Late in infection, with rising levels of envelope proteins, export of cores as virions is favored, allowing horizontal spread of infection to surrounding cells. Thus, in this view, nuclear transit is the default pathway; as envelope proteins accumulate, cores are shunted from this pathway to the export pathway.

Examination of DHBV pre-S mutants that up-regulate cccDNA accumulation reveals that these variants are often cytotoxic; presumably, cells do not tolerate high levels of cccDNA or the resulting elevated levels of viral gene products. The mechanisms of this toxicity are unknown but are not immune mediated because this phenomenon is also observed in cultured cells. Infection of animals with DHBV pre-S point mutants that display deregulated cccDNA gives rise to an early phase of liver injury, which subsequently resolves; this resolution is accompanied by the outgrowth of noncytopathic second-site revertants that restore normal cccDNA regulation (300).

VIRAL PATHOGENESIS AND IMMUNITY

Save for the rare pre-S mutants just described, hepadnaviruses are generally agreed to be noncytopathic. If the viral replication cycle is not itself injurious to hepatocytes, how does clinical hepatitis arise? And why do some individuals develop serious liver disease, whereas others escape with only minor pathologic changes? Correlative clinical data suggested early on that host immune attack on infected hepatocytes, a response normally designed for clearance of infection, might also be the prime source of the injury (and that variations in the magnitude or nature of this response might account for the variety of clinical outcomes in different hosts). Here we summarize the growing body of experimental evidence that supports this inference and outline the mechanisms now thought to be involved.

The study of immune responses to HBV has long been bedeviled by a number of experimental obstacles that have only recently been overcome. HBV grows only in humans and higher primates, a fact that has severely constrained immunologic investigation. Apart from the evident ethical constraints involved in human experimentation, all primate hosts are genetically outbred, making many experimental manipulations of immunity (e.g., adoptive transfer of sensitized lymphocytes) impossible. Similar difficulties apply to the study of the other animal hepadnaviruses (e.g., WHV and DHBV), all of which grow poorly or not at all outside of their outbred native hosts. Thus, efforts to understand immune responses to hepadnaviruses in their native hosts have been limited largely to descriptive studies. Nonetheless, such studies have provided a useful starting point for hepadnaviral immunology because any model for immune-mediated liver injury must account for their observations.

As noted at the outset of this chapter, most primary infections of immunocompetent adults result in variable degrees of acute hepatitis, following which viral infection is cleared. Figure 9 depicts the virologic, serologic, and pathologic events seen in a primary HBV infection, here studied in two experimentally inoculated chimpanzees (187). After an incubation period of 4 to 6 weeks, HBsAg and viral DNA become evident in the serum, peaking at about postinoculation week 8, at which time more than 75% of hepatocytes in the liver display HBcAg by immunohistochemical analysis. In DHBV and WHV, virtually 100% of hepatocytes are infected in a typical primary infection (247,248,251). Importantly, at the peak of virus production, there is little evidence of liver injury, either histologically or by measurement of serum transaminases—in keeping with the noncytocidal nature of the viral replicative cycle. However, beginning 1 to 3 weeks thereafter, serum markers of liver injury become dramatically abnormal, along with histologic evidence of inflammatory cell infiltrates and cell destruction—markers of cell-mediated immunity. A particularly important point is that a 10-fold decrease in intrahepatic (and serum) viral DNA is evident even before the onset of liver injury (see Fig. 9; more about this anon). Ultimately, viral DNA and antigens are cleared from liver and blood, inflammatory infiltrates resolve, and serum

antibody to HBsAg provides immunity to reinfection.

Given the enormous size of the liver and the huge burden of infection achieved, clearance of HBV and other hepadnaviruses during acute hepatitis is remarkably efficient. However, although this clearance process achieves eradication of clinically detectable infection, there is evidence that a few infected cells may persist in the hosts after successful resolution of acute disease. For example, very sensitive nested polymerase chain reaction assays can detect low quantities of viral DNA in liver and serum of some such hosts years after primary infection (438,439). Virus-specific cytotoxic T lymphocytes (CTLs) are also detectable at high frequency in most such cases many years later (438,439). In fact, such CTLs may display activation markers, suggesting that a few persistently infected cells remain in the body and serve as ongoing stimuli of such responses. Rare reports that organ donors with serologic profiles compatible with resolved infection can occasionally transmit HBV after liver transplantation are compatible with this notion (69).

What are the determinants of successful clearance? Efforts to answer this question have focused on comparison of HBV-specific immune responses in patients who clear primary infection with those who fail to do so. Particular emphasis has been placed on the role of CTLs in this process (86). In those in whom primary infection resolves, the CTL response to HBV is vigorous, polyclonal, and directed against most or all of the viral gene products (31,85,181,236,397,398). The breadth of these responses may be important in preventing the emergence of CTL escape mutants, although rare escape or antagonist mutations have been observed (32). By contrast, in chronic HBV carriers, CTL responses to viral antigens are weak or undetectable. Such carriers often display weak responses of helper (CD4+) T cells to viral antigens as well. The latter may not be due to deletion of such cells because successful reduction of viral loads with lamivudine can restore significant CD4+ cell proliferative responses to viral antigen (36), although this does not result in clearance of infection.

These correlative clinical data have focused attention on T-cell responses (especially CTL responses) to HBV as the key to understanding viral clearance and immunity. However, meaningful analysis of the biologic roles of antiviral CTLs had to await development of tractable experimental systems for the study of HBV immunity. This became possible with the development by Chisari and coworkers (86) and Moriyama and associates (354) of transgenic mouse models of HBV infection and disease. The models involve inbred mice bearing one of two classes of HBV transgenes: those bearing subgenomic fragments expressing one or more viral antigen, and those bearing intact, replication-competent viral DNA. The latter mice have the full complement of viral replicative intermediates in the liver and release infectious virus into the bloodstream. These mice display two important virologic differences from natural infection: (a) because they lack HBV receptors, there is no horizontal spread of virus between cells; and (b) for unknown reasons, they do not support cccDNA formation. However, their integrated HBV transgenes serve to replace the transcriptional template function of cccDNA. Most HBV transgenic mice do not experience liver disease because replication is noncytopathic and they are immunologically tolerant to the transgene products, which are viewed as self because their genes are present from conception. One exception to this is the case of transgenic mice that overexpress the viral L protein relative to S and M. These mice accumulate viral glycoproteins to pathologic levels in their ER and display cytotoxicity on this basis (87,88 and 89).

Although these mice experience no liver disease in the ground state, when sensitized T cells from an immunized syngeneic mouse are adoptively transferred into them, dramatic liver injury results (354). CTLs account for the bulk of this injury, which can be reproduced with individual CTL clones; CD4+ cells can also mediate liver injury, but do so with much lower efficiency and intensity (146,398). CTL-mediated injury is dependent on viral antigen expression in the recipient and is class I major histocompatibility complex restricted. These studies provided the first experimental proof that CTLs could mediate liver injury as well as participate in viral clearance and put notions of HBV immunopathogenesis on a firm experimental footing.

They also permitted a major insight into the role of these cells in viral clearance. In addition to producing liver cell injury, transfer of CTLs to mice bearing replicating HBV resulted in a dramatic decrease in viral replication (178,183). Careful quantitation of direct CTL-mediated killing in the livers of these animals revealed that the reduction in viral load achieved after CTL transfer was much greater than could be accounted for by the number of infected hepatocytes directly killed by CTL contact. In fact, large fields of viable hepatocytes surrounding isolated CTL infiltrates could be shown to have lost HBcAg and viral DNA. This suggested that paracrine, noncytopathic mechanisms might be operating to effect much of the observed viral clearance, an inference sustained by the discovery that this clearance could be blocked by administration of antibodies to TNF-a and IFN-g. It is now thought that these cytokines, which are released from CTLs after contact with their targets, are major effectors of the antiviral response seen to accompany CTL administration. The molecular basis of this response is now under active study; the cytokines appear able to reduce the accumulation of viral RNA (perhaps by engagement of the 2',5'oligo(A) synthase–RnaseL pathway) and also, in some settings, to destabilize viral capsid components (59,167,182,205).

If intrahepatic cytokine release is in fact an important determinant of HBV clearance, other stimuli to their release should also produce a similar result. This is supported by the finding that many other infectious agents that replicate in the liver of HBV transgenic mice can suppress HBV replication. For example, LCMV strains that replicate in hepatic macrophages (but not in hepatocytes) dramatically suppressed HBV replication in hepatocytes—a suppression that could be reversed with antibodies to TNF-a and IFN (in this case, IFN-a and -b) (179,180). Induction of the same cytokines was shown to mediate a similar suppression of HBV replication by hepatocyte superinfection with adenovirus or cytomegalovirus (60). Apart from their intrinsic interest, these reports may have direct clinical relevance because it has been observed that superinfection of human HBV carriers with hepatitis A virus or hepatitis C virus has occasionally resulted in the resolution of the underlying HBV infection (60).

Does this form of noncytocidal antiviral mechanism operate during normal viral clearance? The best evidence that it might comes from consideration of the experimental study of acute HBV infection of chimpanzees (see Fig. 9). Recall that a significant decrement in virus production was achieved in the period before histologic evidence of massive cellular infiltration or hepatocyte destruction. Local production of IFN-g and TNF is demonstrable during this interval, although the source of these cytokines is not yet clear. Recent experimental evidence indicates that cells from the innate immune system, possibly derived from the natural killer T-cell population normally resident in the liver, may be important sources

of these cytokines early in the infectious process, before the development of adaptive CTL responses (J. Baron and D. Ganem, unpublished results).

Could this understanding of clearance during acute hepatitis be parlayed into a strategy for the immunotherapy of chronic hepatitis B? The answer to this is presently unclear because the basis of the absence of HBV-specific CTLs from chronic carriers is not understood. However, if the burden of infected cells could be reduced by long-term administration of antiviral drugs, up-regulation of CTL responses by therapeutic vaccination—or modulation of cytokine production by other means (e.g., gene therapy)—might provide a way to eliminate the remaining reservoir of HBV-positive cells.

HEPADNAVIRUSES AND HEPATOCELLULAR CARCINOMA

Biology and Epidemiology

The first indication that chronic HBV infection might predispose to the development of hepatocellular carcinoma emerged from studies of the global epidemiology of HBV carriage (519). These studies revealed that HBV is not uniformly distributed among human populations. In Western Europe and North America, only about 0.1% to 0.5% of individuals are HBsAg carriers; however, in Southeast Asia and Subsaharan Africa, fully 5% to 15% of all humans are chronically infected with HBV. (The remaining areas of the globe have an intermediate prevalence of HBV carriage.) This pattern of HBV infection strikingly reproduces the known global epidemiology of HCC, a rare tumor in the West but long known as a leading malignancy in Africa and Asia. This remarkable finding prompted deeper investigation of this linkage.

In HBV-endemic countries, most HCCs arise in patients who are HBV carriers (22,154,514); even though carriers may represent 5% to 10% of the population, they represent 50% to 80% of HCC patients in those societies. However, the strongest evidence for an association comes from careful prospective studies done in Taiwan, in which large numbers of carriers and noncarriers were followed prospectively and deaths due to HCC recorded. These classic studies demonstrated that chronic HBV carriage is associated with a 100-fold increase in risk for HCC relative to noncarriers (22,23). This massive increment in cancer risk makes HBV one of the most important environmental risk factors in human cancer epidemiology.

In Taiwan (as in all HBV-endemic areas), most infections are acquired in the first decade of life (154). Thus, in these studies, most of the individuals being followed had been carrying HBV for many decades. From the age distribution of Taiwanese hepatoma cases, it can be inferred that most tumors arise after 30 or more years of persistent infection; relatively few cases occur in children and adolescents. Extrapolation of this estimate of relative cancer risk to Western societies should be done with caution. In the West, most infections are acquired in young adulthood, as a result of sexual transmission or parenteral drug abuse. Although few doubt that such infections present an increase in cancer risk, it is unclear if the magnitude of this risk is as large as in the HBV-endemic zones, if only because those infected as adults have fewer years at risk. Nonetheless, even in the West, the interval between acquisition of infection and HCC development remains at least 30 years (154).

Further support for the oncogenic potential of hepadnavirus infection came with the discovery of the animal hepadnaviruses. Studies of WHV infection showed that this virus is even more potent than HBV as a hepatic carcinogen: nearly 100% of woodchucks infected from birth with WHV develop HCC, starting at about 18 to 24 months of age (163,414,415,469). Uninfected woodchucks housed in similar circumstances do not develop HCC at all. The oncogenic drive accompanying this infection is so strong that many animals develop multiple independent hepatic tumors; even animals who experienced only a transient WHV infection (as judged by the presence of antisurface and anticore antibodies in the absence of surface antigenemia) display an increased prevalence of HCC (163). Ground squirrels infected with GSHV also display an increased prevalence of HCC, although the tumors are less frequent and arise much later in life (339,539). Because WHV and GSHV can both be grown in woodchucks (3,475), it has been possible to compare the oncogenic potential of both infections in a common host. These studies (469) showed that the biology just described is determined largely by the viral genome and not by the host: GSHV-infected woodchucks display HCC later in life and at a lower frequency than WHV-infected woodchucks. As noted earlier, studies of the oncogenic potential of DHBV have not clearly established it as a tumor virus. Although some DHBV-infected flocks in Asia have shown HCC, most laboratory-raised animals or commercial flocks in America and Europe have not exhibited HCC (100), suggesting that environmental factors (or cofactors) may account for a large portion of the oncogenesis in Asia. There is no evidence that Asian DHBV isolates (550) differ from Western isolates in their oncogenicity.

Viral DNA and Hepatocellular Carcinoma

When the tumors themselves are examined, some can be shown to produce one or more viral antigens, but many have extinguished all HBV gene expression. Nonetheless, most (85% or more) of such tumors harbor integrated viral DNA, often multiple copies per cell (39,481,484). The HBV integrants are usually highly rearranged, with deletions, inversions, and sequence reiterations all commonly observed (121,359); as expected, most of these rearrangements ablate viral gene expression. WHV insertions in woodchuck HCC in general display similar features (378). In addition, alterations of host DNA often accompany these integrations. Described alterations include deletions or repeats at the host–virus junctions (445,484,534,535,590) as well as (more rarely) large chromosomal translocations, some of which harbor HBV sequences at the interchomosomal junctions (533); the latter might have arisen by homologous recombination between viral integrants on two different chromosomes. Importantly, tumors are clonal with respect to these integrants; that is, every cell in the tumor harbors an identical complement of HBV insertions. This implies that the integration events accompanied or preceded the clonal expansion of the cells. Although much has been made of this fact, by itself, it puts HBV DNA at the scene of the crime but does not establish criminality.

How HBV integration is achieved is poorly understood. As opposed to its role in retroviral replication, integration is not an obligatory part of the hepadnaviral replication cycle; it is therefore not surprising that hepadnaviruses have no virus-encoded integration machinery. Presumably, HBV DNA is assimilated into the nucleus by host mechanisms; it is reasonable to suppose that this event proceeds by pathways akin to those operating in the integration of exogenous DNA

in transfected mammalian cells in culture. Such mechanisms likely involve topoisomerase I (461,566) and other host enzymes involved in DNA recombination and repair. A major problem that has confounded study of the integration process has been that the integrants characterized in HCC specimens are many years removed from the primary insertion event. During this (about 30-year) interval, there has presumably been strong immunologic selection against viral antigen expression; this is the likely force that has selected the genomic rearrangements described previously. Recently, it has become possible to examine the structure of integrants newly generated in cultured cells permissive for DHBV replication. In acute infection of primary duck hepatocytes, integrated DHBV DNA can be detected as early as six days after infection, although such integration is a rare event (estimated at 1 insertion per 1000 to 10,000 hepatocytes; W. Yang and J. Summers, personal communication). LMH cells stably transfected with overlength DHBV clones similarly accumulate new DHBV insertions during serial passage (168,169 and 170). In both systems, many of the resulting integrants have structures compatible with a derivation from the double-stranded liner genomes produced by *in situ* priming; in accord with this, mutant DHBV genomes that display enhanced *in situ* priming display an increased frequency of integration.

Models for Hepatitis B Virus Oncogenesis

The molecular mechanisms by which hepadnaviruses predispose to malignancy remain much debated. Models for how they might act fall into two broad categories, direct and indirect, according to the roles they envision for the viral genome and its products (155).

Direct Models

Direct models posit that viral DNA makes a direct genetic contribution to the lesion, either by providing cis-acting sequences that activate or suppress host growth-regulating genes or by elaborating trans-acting factors that alter the program of cellular growth control. The most straightforward version of such a model is that a viral protein might act as a dominant oncogene, much as src does in Rous sarcoma virus infection. However, hepadnaviruses carry no gene directly transduced from the host cell genome; in addition, hepadnaviral infection does not lead to growth transformation or immortalization of primary cells *in vitro,* although there is one report (215) of further growth deregulation induced by HBV X expression in a cell line already immortalized by SV40 T antigen. These facts, together with the prolonged incubation period between infection and tumor development, make it extremely unlikely that the direct action of a single viral gene product can itself transform a hepatocyte. However, they by no means exclude more subtle roles for viral proteins that might help initiate the loss of growth control, even if they cannot by themselves complete this process.

In this connection, two viral proteins are frequently mentioned. Primary attention has centered on the X protein, for two reasons: (a) it is highly divergent or absent in the avian viruses, the only hepadnaviruses not reliably associated with HCC; and (b) as a known regulator of signal transduction, it is easy to envision models for how such a molecule might affect cellular growth. The debate about X protein's role in oncogenesis has been powerfully influenced by reports that transgenic mice bearing X sequences under the control of the X promoter and EnI develop HCC (260,270). However, this effect has not been observed by other groups using X vectors driven by heterologous promoters (298), nor has comparable oncogenesis been seen in transgenic mice bearing other subgenomic or genomic HBV DNAs containing the X region (11,92,131,133). The reasons for these divergent results are obscure; possibilities include differing levels of X expression or the different genetic backgrounds of the mice used in different laboratories. One thing is certain: if X protein plays a role in oncogenesis, it cannot be necessary for the entire life of the transformed cell because most advanced tumors have extinguished all HBV gene expression. Thus, if X is required early in oncogenesis to promote cell proliferation, this requirement must be obviated at later stages of transformation.

The second viral gene product for which a role in oncogenesis has been postulated is the truncated M protein generated by occasional viral integration events (291,292,366). The roles ascribed to such factors are formally identical to those outlined previously for X protein, but experimental evidence supporting such roles is scant at present: many tumors cannot encode such M variants, and expression of these proteins in cultured cells does not lead to loss of growth control. Finally, rare viral integration events might be able to generate other novel proteins by fusion of viral coding sequences with adjacent cellular genes, analogous to the generation of novel oncogenic proteins by chromosomal translocations in other settings. One potential example of this surfaced in a human HCC, in which integration fused the HBV pre-S region to a retinoic acid receptor locus (104,106). However, such events have been documented extremely rarely and are unlikely to represent a major pathway of oncogenesis.

Most advocates of direct models favor the notion that viral sequences act primarily by contributing cis-acting sequences rather than coding regions. There is unequivocal evidence for this mode of oncogenesis in WHV infection. In this system, about 40% of all HCCs contain an integrated WHV genome within several kilobases of one of two *N-myc* loci; these *N-myc* rearrangements are readily detected by Southern blotting (145,197). *N-myc* genes are normally silent in adult liver but are transcriptionally activated by enhancer elements within WHV DNA (575); recent studies implicate EnII (and, less frequently, EnI) in this effect (138,139,143,551,554). Interestingly, *N-myc* transcription is elevated very early in the oncogenic process, even in premalignant nodules (595), and is observed in virtually 100% of cases. Moreover, among many tumors that lack *N-myc* rearrangements by conventional Southern blotting, integrated WHV DNA can be found in cis to *N-myc2* by pulse-field gel analysis, at distances up to 150 to 180 kb away from the activated locus (142). This raises the possibility that all WHV-associated HCC may be initiated by insertional activation of *N-myc,* with many cases resulting from previously unsuspected long-range activation events. Consistent with this, antisense inhibition of *N-myc* expression in a woodchuck hepatoma cell line reverts its growth phenotype (567), and overexpression of *myc* in the livers of transgenic mice gives rise to HCC (52,221,356). Tumors in such mice require many months to develop, consistent with a requirement for multiple additional genetic hits in the pathogenesis of full malignancy.

N-myc expression may also explain other known features of the biology of woodchuck HCC. For example, it has long been known that such tumors overexpress insulin-like growth factor-2 (IGF-2) (595). Recent studies show that *N-myc2* overexpression often triggers apoptosis, and this effect can be suppressed by exogenous IGF-2 (551,596). It is likely that the up-regulation of IGF-2 naturally observed in HCC is the result of selective pressure exerted by the insertional

activation of *N-myc* alleles by WHV.

Efforts to make a similar case for insertional activation by HBV have been largely unsuccessful. Dozens of human hepatomas have been screened for common sites of HBV integration (359,534). This is done by cloning HBV integrants and using the flanking host DNA as a probe to screen Southern blots of DNA from other HCCs for rearrangements of the corresponding host sequences. (This is the same strategy that was employed productively for WHV.) To date, no rearrangements common to many human HCCs have been detected; although a few isolated examples of insertions near interesting loci have been reported (104,106,568), these insertions appear to be unique to the tumors in which they were discovered. Similarly, deliberate screens of human HCCs for rearrangements of known host oncogenes have likewise been largely unrevealing. It should be emphasized, however, that all of these methods can only detect rearrangements within a few kilobases of the locus being probed; if long-range activation events of the type identified for WHV (142) operate in HBV infection, insertional activation might still contribute to the biology of HBV-induced HCC.

Indirect Models

The relative paucity of experimental support for direct models of HBV-induced oncogenesis has led to the growing popularity of indirect models. Here, the term *indirect* means that HBV genes and their products make no direct genetic contribution to the transforming event. Rather, HBV-induced liver injury (itself largely the result of host immune or inflammatory responses to infection) triggers a series of stereotypic host responses that lead to liver cell regeneration. Vertebrate hosts are known to respond to liver cell injury of many types with a brisk regenerative response (although the mechanism of this response is poorly understood). Under conditions of chronic regeneration, the increase in the number of cells undergoing DNA synthesis results in an increased probability of mutation; this in turn increases the probability that one or more of the resulting mutant cells will have a proliferative advantage. As this cell is preferentially expanded, the same logic predicts an increased opportunity for additional mutational lesions in its progeny (and so on, until full growth transformation is achieved). Thus, in indirect models, the role of HBV is to act as an agent of liver injury; everything else—in particular, the growth-deregulating mutations directly responsible for tumorigenesis—is contributed by the host. (The identification of host mutations in HCC is now an active area of inquiry that has turned up numerous candidate loci. Readers interested in this aspect of HCC research are referred to references 52 and 408 for recent review.)

Supporters of indirect models point to several compelling facts, chief among which is that most conditions in human medicine that lead to chronic liver injury are associated to one degree or another with a risk for HCC: alcoholic cirrhosis, a_1-antitrypsin deficiency, Wilson's disease, and so forth. Even hepatitis C virus, an RNA virus with no known DNA intermediate, can predispose to HCC during chronic infection. However, none of these precedents can fully explain why the magnitude of the HCC risk appears to be so much larger in chronic HBV infection. Strong experimental support for this hypothesis is derived from influential experiments in transgenic mice (89). These mice were engineered to overexpress the HBV L protein relative to the M and S proteins. As noted previously, when L is overexpressed, it leads to retention of all the viral envelope glycoproteins in the ER (87,88); this retention is toxic to cells, although the mechanism of this toxicity is unknown. Initially, the mice display only hepatocellular necrosis and accompanying regeneration. Over time, however, malignant HCC arises with great regularity.

Confusion has arisen over the proper interpretation of this experiment, in part because it uses HBV gene products to drive the liver injury. Strictly speaking, the experiment demonstrates that chronic liver injury promotes regeneration and HCC. Although it raises the possibility that viral envelope glycoproteins might contribute to this injury, it does not establish that this form of injury is relevant to clinical hepatitis B. Indeed, it appears unlikely that this would be so; most evidence favors the notion that the bulk of the injury is produced by immune attack on infected hepatocytes (86), and the relative levels of L protein in natural infection are nowhere near those achieved in the HCC-prone transgenic mice. Although hepatocytes with the histologic appearance of cells undergoing damage from deregulated envelope expression can be seen in hepatitis B, in most cases, they are not numerous enough to explain the regenerative drive that accompanies this infection.

A more recent transgenic mouse experiment has affirmed that immune-mediated injury mechanisms can drive hepatic oncogenesis. This study employed mice expressing HBV envelope components at nontoxic levels (akin to those encountered in natural infection) (360). The tolerized immune systems of such animals were ablated by lethal irradiation and replaced with bone marrow and splenocytes from syngeneic mice immunized with a vaccina virus expressing HBsAg. As expected, the recipients developed a chronic hepatitis due to immune attack of the sensitized donor cells on their antigen-expressing hepatocytes. By 17 months after transfer, nearly all such animals had developed HCC, whereas control recipients that received immunocytes from a tolerized (HBV-transgenic) donor developed only minimal liver injury and had a low incidence of HCC. Thus, immune-mediated injury is sufficient to drive hepatic oncogenesis; direct cytotoxicity from deregulated L protein expression is not required.

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CHAPTER 87 Hepatitis B Virus

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HISTORY

Viral hepatitis is a term that is reserved for infections of the liver caused by one of at least five distinct hepatitis viruses. The most notable sign of this disease is jaundice, an orange-yellow discoloration of the scleroproteins of the skin and conjunctivae seen with an increased plasma bilirubin resulting from faulty excretion of bile pigment by damaged hepatocytes.

The existence of a parenterally transmitted form of hepatitis was documented by Lurman (473) in 1885. He reported the development of jaundice in 15% of 1,289 shipyard workers in Bremen, 2 to 8 months after they had received smallpox vaccine prepared from human “lymph.” Subsequent outbreaks of hepatitis were eventually traced to the use of improperly sterilized syringes and needles among patients who required parenteral inoculations and among those receiving tattoos (224,283).

It was not until the late 1930s that the existence of hepatitis transmitted by parenteral inoculation of human serum was firmly established. In 1937, it was reported from England (522) that approximately 40% of 109 individuals receiving inoculations of a single batch of human measles convalescent serum developed jaundice after incubation periods of up to 114 days. This was followed by an outbreak in British troops given filtered human mumps convalescent plasma (62). These clinical entities were grouped together as “homologous serum jaundice” (504). In 1937, Findlay and MacCallum (222) described cases of jaundice occurring 2 to 7 months after inoculation of volunteers with an attenuated strain of yellow fever vaccine prepared with the addition of human serum to stabilize the product. Fox et al. (227) subsequently implicated human serum as the vehicle of transmission. They also observed that the incubation period of parenterally transmitted hepatitis was much longer than that described for infectious hepatitis. It is assumed that these outbreaks were caused by hepatitis B virus (HBV) infection, but a hepatitis C virus (HCV) etiology also cannot be excluded.

Before a serum-free yellow fever vaccine product was generally used, a widespread epidemic of hepatitis occurred in U.S. military troops early in 1942, resulting in 28,000 cases with 62 (0.2%) deaths (660). Studies conducted more than 40 years later clearly established that this epidemic was caused by HBV (677). As a result of these investigations, the risk of hepatitis associated with the use of pooled and dried human plasma or the administration of whole blood became well recognized (61,528,841).

Based on the epidemiologic differences observed between infectious hepatitis and homologous serum jaundice, the groundwork was laid for the definition of a second major form of viral hepatitis. The terms *hepatitis A* and *hepatitis B* were introduced by MacCallum (475) in 1947 to categorize infectious (epidemic) and serum hepatitis (homologous serum jaundice). These terms have been adopted by the World Health Organization (WHO) Committee on Viral Hepatitis to replace the myriad descriptions previously applied to these disorders (816).

Between the late 1950s and early 1970s, the studies of Murray (536) and Krugman et al. (411) were instrumental in further defining the seroepidemiologic relationships between hepatitis A and B, which eventually led to the evaluation of new methods for the diagnosis and immunoprophylaxis of these diseases (412). Two distinct types of hepatitis with different incubation periods and lack of cross-immunity were differentiated at the Willowbrook State School for the Mentally Handicapped (see also Chapter 26).

The chain of events that culminated in the discovery of HBV and an effective vaccine for the prevention of hepatitis B was a tortuous one. It began in 1963 when Baruch Blumberg, of the National Institutes of Health, began examining thousands of blood samples from diverse populations in a study designed to look for inherited polymorphic traits in different geographic areas of the world (91). To detect novel antigens, sera from multiply transfused hemophilia patients were used because it was postulated that such sera might contain antibodies against these unique proteins. In the course of the investigation, Blumberg discovered that a serum sample from an Australian aborigine contained an antigen that reacted specifically with an antibody in the serum of an American hemophilia patient. Subsequent studies showed that this Australia (Au) antigen was relatively rare in North American and western European populations but was prevalent in some African and Asian populations and among patients with leukemia, lepromatous leprosy, and Down syndrome (90). The additional association of the Au antigen (now designated the hepatitis B surface antigen, or HBsAg) with what was then known as serum hepatitis was not recognized fully until 1967. By 1968, other investigators, notably Prince (624) and Okochi and Murakami (578), had established that the Au antigen was found specifically in the serum of hepatitis B patients.

The discovery and characterization of HBsAg was a major breakthrough in hepatitis research because it permitted intensive study of the disease and of the nature of the infectious agent even though the virus itself had not yet been identified conclusively (17,103). In 1970, Dane et al. (168) detected the complete hepatitis B virion, a 42-nm double-shelled particle that consisted of an outer envelope composed of HBsAg and an inner core or nucleocapsid containing its own distinct antigen, the hepatitis B core antigen (HBcAg), in addition to a small, circular, partially double-stranded DNA molecule and an endogenous DNA polymerase enzyme (384,649). A third antigen, designated hepatitis B e antigen (HBeAg), was described subsequently by Magnius and Espmark (479). This antigen is a reliable marker for the presence of intact virions and thus for enhanced infectivity.

INFECTIOUS AGENT

Biologic Characteristics

HBV is the prototype virus for a family of DNA viruses called the *Hepadnaviridae.* Related viruses are found in woodchucks, ground squirrels, Pekin ducks, and tree squirrels. These viruses share certain features such as virion size and ultrastructure; a distinctive polypeptide and antigenic composition; a comparable DNA size, structure, and genetic organization; and a unique mechanism of viral DNA replication. Separate genera have been proposed for the mammalian and avian hepadnaviruses because of differences that exist in their molecular biology. A detailed review of the *Hepadnaviridae* and their replication that includes virion structure and the life cycle of HBV can be found in Chapter 86.

The stability of HBV does not always coincide with that of its envelope protein, HBsAg. Immunogenicity and antigenicity are retained after exposure to ether, acid (pH 2.4 for at least 6 hours), and heat (98°C for 1 minute, 60°C for 10 hours); however, inactivation may be incomplete under these conditions if the concentration of virus is excessively high. Exposure of HBsAg to 0.25% sodium hypochlorite for 3 minutes destroys antigenicity (and probably infectivity). Infectivity in serum is lost after direct boiling for 2 minutes, autoclaving at 121°C for 20 minutes, or dry heat at 160°C for 1 hour. More recent studies have shown that HBV is inactivated by exposure to sodium hypochlorite (500 mg of free chlorine per liter) for 10 minutes, 0.1% to 2% aqueous glutaraldehyde, Sporicidin (Ash Dentsply, York, PA) (pH 7.9), 70% isopropyl alcohol, 80% ethyl alcohol at 11°C for 2 minutes, Wescodyne (American Sterilizer, Medical Products Division, Erie, PA) diluted 1:213, or combined b-propiolactone and ultraviolet irradiation (95,394,625). HBV has been shown to retain infectivity when stored at 30° to 32°C for at least 6 months and when frozen at –20°C for 15 years.

Propagation and Assay of HBV in Cell Culture

Although HBV has been successfully grown in primary cultures of normal adult or fetal human hepatocytes (234,259,564), susceptibility wanes as the cells differentiate, and serial propagation over a prolonged period of time is limited by availability of human liver tissue. Nevertheless, these susceptible cell cultures remain important for the propagation of HBV *in vitro* and for the study of early events in the life cycle of the virus such as attachment, adsorption, penetration, and uncoating. In 1982, Will et al. (809) reported that cloned HBV DNA could infect chimpanzees after direct inoculation into the liver. Since then, a number of investigators have reported the successful transfection of established cell lines with cloned HBV DNA leading to the expression of HBV proteins and the sustained production of HBV DNA (683,731,780,824). The particles produced by the stably infected human hepatoma cell lines were infectious to chimpanzees (3,730). Although these established cell lines are not directly susceptible to HBV, they provide a model

whereby intracellular events of HBV replication and release of virions can be studied. Transfected cell lines also are useful for the evaluation of potential antiviral compounds (405).

Nonhuman HBV Infections

Chimpanzees and other high-order primates are highly susceptible to experimental induction of hepatitis B (42). The immunobiology of infection is similar to that observed in humans except that the disease is generally less severe. Although the chimpanzee model has played an important role in studies of viral inactivation, vaccine safety and efficacy, disinfection kinetics, infectivity determinants, immunopathology, and seroepidemiology, its role as a model for studying viral replication or for the evaluation of antiviral agents has been limited. In many instances, the chimpanzee is being replaced by other animal systems such as the woodchuck, Pekin duck, ground squirrel, and transgenic mouse (485) (see Chapter 86). By introducing HBV genomic and subgenomic fragments into the germline of transgenic mice, the viral expression of HBV proteins in tissue, viral replication, regulatory functions, evaluation of antiviral compounds, and the immunopathogenesis of disease, including carcinogenesis, can be studied (33,147,398,481,530,542).

PATHOGENESIS

HBV is a noncytopathic virus that causes a spectrum of liver diseases ranging from acute hepatitis (including fulminant hepatic failure) to chronic hepatitis, cirrhosis, and hepatocellular carcinoma. The majority of patients recover from their infection. Of those who become persistently infected, many have mild liver disease with little or no long-term morbidity or mortality.

The eventual outcome, extent, and severity of HBV infection depend on a variety of viral and host factors. Although host responses play a major role in disease pathogenesis and viral clearance, the natural history of HBV infection is often predicated on a variety of viral adaptive mechanisms. Frequently, viral mutations in critical regions of the viral genome are the result of these adaptive mechanisms.

Although the early events of primary HBV infection remain largely unknown, hepatocellular injury most likely begins with antigen recognition by HBV-specific cytotoxic T lymphocytes (CTLs) with the induction of apoptosis in HBV-positive hepatocytes. Recruitment by the CTLs of host-derived, antigen-nonspecific inflammatory cells—including macrophages, neutrophils, and other lymphocytes—leads to formation of necroinflammatory foci. Infiltrates of these inflammatory cells are primarily responsible for hepatocellular death. At this stage, clinical evidence of hepatitis becomes apparent with elevation of aminotransferases. During acute self-limited infection, the pathology is mild to moderate, and viral replication is effectively controlled by host immune responses with resolution of hepatitis. In some cases, these virus-nonspecific inflammatory responses are markedly augmented by yet-unknown host determinants or viral factors, resulting in massive hepatocellular injury and fulminant hepatitis. On the other hand, chronic hepatitis results from continuous hepatic injury as a consequence of ineffective viral clearance. In asymptomatic carriers, tolerance to HBV antigens confers little or no immune-mediated injury in the infected liver.

Primary Infection

Animal models have been used to elucidate many stages of infection in the pathogenesis of HBV. The virus gains entry into a susceptible host through parenteral or percutaneous routes. Once inside the host, HBV is transported through the bloodstream to the liver, which is the primary site of infection. Since hepadnaviruses can infect nonhepatocytes, the existence of an intermediate compartment of replication before infection of the liver has been proposed (404), but this remains controversial (71,366). Viral spread within the liver appears to be a rather efficient process, with potential infection of virtually all hepatocytes (71,367). The onset of viremia is inversely related to the dose of virus administered, and one virion may be sufficient to cause infection (365). Viremia can reach very high levels (>10^9 virions/mL) and may result in dissemination to other sites of potential viral tropism that include kidney, pancreas, testis/ovary, spleen, and lymph nodes (402). Whether immune activation occurs in the resident liver or other distant sites during primary HBV infection remains a subject of debate. Irrespective of the mechanism, a vigorous antiviral immune response is directed to the infected liver. In most situations, this activated immune response can rapidly and effectively control the viral infection despite extensive infection of hepatocytes (71,368,379). The molecular and cellular spectrums of these virus–host interactions are discussed later.

Host Responses

Although virus-specific immune responses are thought to be protective in many viral infections, these same responses also are the mediators of tissue pathology (838). They frequently trigger a variety of nonspecific inflammatory reactions in the host, augmenting the potential for cellular injury. Consequently, both virus-specific and virus-nonspecific mechanisms invariably lead to injury at the sites of viral replication, the extent of which depends on the vigor and character of the inflammatory response. From a simplistic point of view, successful clearance of virus requires a timely deployment of effective antiviral regimens, but at the same time the target and duration of attack has to be orchestrated with great precision and care to avoid excessive pathology. Detailed knowledge of these factors and their relationships is pivotal to our understanding of how HBV infection leads to hepatocellular injury. Figure 1 summarizes the multiple pathways of HBV immunopathogenesis, which are described in detail later.

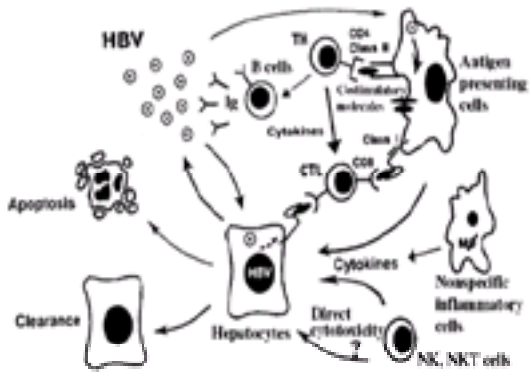

FIG. 1. Immunopathogenesis of HBV infection. See text for details. (Modified from Fig. 3 of ref. 148, with permission.)

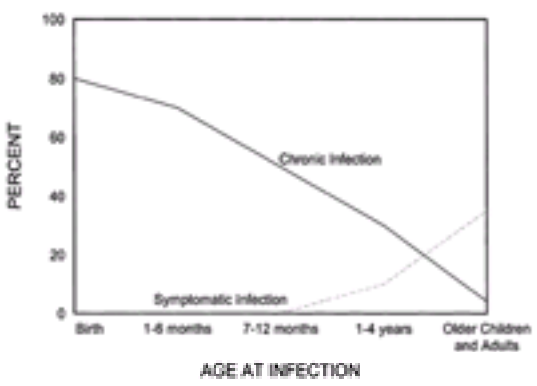

FIG. 3. Relationship between age and outcome after acute HBV infection. (Modified from M. Kane, personal communication, 1999.)

Innate Cellular Mechanisms

Following viral infection, higher eukaryotic cells have the innate ability to undergo programmed cell death (apoptosis) to terminate viral replication. Many viruses encode antiapoptotic gene products that function to oppose the cell death program triggered by viral infection and allow the virus to complete its replication within the infected cells (757). This mechanism is particularly relevant for viruses whose replication induces cytopathic changes. For noncytopathic viruses like HBV, the utility of such a mechanism is less obvious. In HBV DNA-transfected or HBV-infected cells, there is no evidence of apoptosis or cytopathic effect. However, in certain situations, as discussed later, direct hepatotoxicity can result from atypical patterns of viral replication and gene expression.

Certain HBV gene products, including HBX and pre-S1, have been implicated in activation of the NF-kB pathway (126), which is known to exert a potent antiapoptotic effect, particularly in hepatocytes (63). Further evidence suggests that HBX also may directly regulate cytokine-induced apoptosis (728). Induction of interferons is part of the innate antiviral responses generated by infected cells. Although HBV-infected cells may be more sensitive to interferon-induced cytotoxic effects (148), the predominant antiviral mechanism is most likely a noncytopathic one. Interferon-a has been shown to directly downregulate HBV transcription *in vitro* (652), whereas interferon-g may exert a potent inhibitory effect on various aspects of HBV replication (see later section, Cytokine Induction). Conversely, the HBV polymerase, possibly the terminal protein region, and the C-terminal region of the core protein have been shown to inhibit the cellular response to interferon-a/g and to suppress interferon-b gene transcription, respectively (225,807). Another cellular response to viral infection is the induction of heat-shock proteins including ubiquitin and other proteins involved in proteolysis; this response enhances the host cell's capacity to degrade proteins with abnormal conformations, such as viral proteins (657). Recent findings suggest that the proteasome complex, the predominant nonlysosomal pathway for intracellular protein degradation, may play an active antiviral role in degrading viral proteins following viral infection (673). Such a mechanism may be counteracted by HBV X protein during HBV infection (344).

Humoral Response

Activation of the humoral immune response with production of neutralizing antibodies prevents further viral spread and leads to subsequent elimination of circulating virus (591). This mechanism is particularly relevant for HBV infection because of the high level of HBV replication and viremia, which can reach more than 10^9 virions per milliliter in circulation. The antibody response to HBV envelope proteins is a T-cell-dependent process (514). In the majority of infected persons, antienvelope antibodies are readily induced and lead to viral clearance and protection from subsequent infection. This constitutes the basis for the successful formulation of an HBV vaccine based on the envelope proteins. However, a minority of infected persons fail to produce antienvelope antibodies, and this can contribute to the establishment of persistent infection. This deficiency resides at the level of T-cell nonresponsiveness, probably as a result of tolerance or anergy induction toward the HBV proteins (148,838). The molecular mechanisms underlying this deficiency, however, are largely undefined. On the other hand, the antibody response to HBV core protein is both T-cell-dependent and -independent (514). The anti-HBc response is the first serologic evidence of host immune response and often persists for years after viral clearance in infected individuals, even for life. Although chronically infected patients may be nonreactive to HBV core protein at the T-cell level (148), the alternative T-cell-independent mechanism appears to sustain the presence of an anticore response in these individuals. In contrast, antibody response to HBeAg, the other core gene product, is strictly T-cell dependent (514). Presence of antibodies to HBeAg is often associated with

recovery from active disease and is clinically used as the hallmark of response to treatment. However, this association does not imply a causal role for this antibody response in viral clearance. The seroconversion from HBeAg to anti-HBe merely indicates reduction of HBV replication and suggests a transition from an active to a quiescent disease state. In HBeAg-positive patients, antibodies to HBeAg frequently exist in immune complexes. Therefore, the status of HBeAg/anti-HBe is usually determined by a dynamic equilibrium between the titers of HBeAg (a marker of viral replication) and anti-HBe (a marker of host immune response). The role of anti-HBc/anti-HBe antibodies in HBV pathogenesis has not been defined. Cell surface forms of HBcAg/HBeAg on infected hepatocytes could be potential targets for antibody-directed cell cytotoxicity (510,669). However, this mechanism has not been shown to be operative in hepatocellular injury associated with HBV infection. In contrast, immune complex diseases with complement fixation, including glomerulonephritis, vasculitis, and cryoglobulinemia, which are major extrahepatic manifestations of HBV infection, can result from abnormally activated antibody responses to HBV antigens (e.g., HBsAg). Antibodies to viral polymerase and HBX proteins have been described in patients with HBV-associated chronic hepatitis and hepatocellular carcinoma, but their pathologic implications are unknown (793,800).

Cell-Mediated Immunity

The cellular immune responses directed against HBV have been studied in detail in both infected humans and transgenic mice expressing HBV gene products. Nonprimate animal models that are susceptible to hepadnavirus infection (woodchucks, ground squirrels, ducks) have provided limited utility in this venue of research, mostly because of a lack of immunologic reagents and inbred strains. Recent efforts to develop such reagents should lead to important insights into this complex process (505,508,544). In human infection, individuals acutely infected with HBV can mount a vigorous, multispecific, and polyclonal cellular immune response to HBV. In contrast, chronically infected patients have a weak or barely detectable anti-HBV response (148). This is true for both CD4 and CD8 responses. During acute hepatitis B, a vigorous human leukocyte antigen (HLA) class II–restricted, CD4+, helper-T-cell response to multiple epitopes of HBcAg/HBeAg predominates in virtually all patients. Such a response to HBV envelope antigens, however, is much less evident. It is not clear why this disparity exists, because individuals vaccinated with recombinant HBsAg are capable of mounting a strong class II–restricted, CD4+ response to the HBV envelope proteins (128,372). Possibly, an antienvelope T-cell response occurs early during the primary phase of infection but becomes exhausted or tolerant to the antigen because of exposure to high levels of virus or to inappropriate antigen presentation to the immune system (148,838). The relative absence of antienvelope T-cell response notwithstanding, the class II–restricted, CD4+ T cells specific for nucleocapsid probably play an important immunoregulatory role in the control of viral infection (148,218). By helping B cells produce neutralizing antienvelope antibodies and activating HBV-specific CTLs, this CD4+ T-helper population may direct the initial antiviral response. During chronic HBV infection, the once-strong nucleocapsid-specific CD4+ T-cell response becomes barely detectable, presumably because of a switch to T-cell exhaustion or nonresponsiveness. Again, the molecular mechanism of this switch is unknown. It is interesting to note that during reactivation or successful treatment of chronic hepatitis B, this nucleocapsid-specific T-cell response could reappear (97,484).

CTLs are believed to evolve teleologically in response to intracellular pathogens, particularly viruses. They possess the unique ability to detect and eliminate virus-infected cells through recognition of viral peptides in the context of HLA class I molecules. Using short synthetic peptides mimicking processed viral antigens and cells genetically engineered to express viral proteins, CTL responses to HBV in humans have been studied extensively (73,598,599,644,645 and 646). These studies indicate that patients acutely infected with HBV develop a strong, polyclonal, HLA class I–restricted CTL response that is directed against multiple epitopes in all viral proteins (547,599,645). Unlike the class II–restricted antienvelope response, the CTL responses to HBV envelope and pol are vigorous and multispecific. Similar to the class II–restricted CD4 responses, the class I–restricted CTL responses also are barely detectable during the chronic phase of HBV infection (218,643). Although these observations suggest that CTLs probably play a vital role in viral clearance, the critical factors determining the CTL responsiveness during HBV infection have not been defined. Since detailed studies on prospectively followed individuals acutely infected with HBV are lacking, the potential echanisms underlying the immunologic differences between patients who recover and those who progress to persistent infection cannot be delineated. Furthermore, the detection of HBV-specific CTLs in the peripheral blood and liver of chronically infected individuals, albeit with low frequency, suggests a pathogenic relationship between the indolent CTL activities and necroinflammatory liver disease associated with chronic hepatitis B (45,219,643). Therefore, the CTL response is a double-edged sword in HBV infection: A vigorous CTL response leads to viral clearance, whereas an ineffective CTL response results in chronic hepatocellular injury.

Although studies in humans have provided important correlative information, the molecular and cellular consequences of the immune response can be elucidated only in appropriate animal models. Transgenic mice have been used extensively to address these issues. Adoptive transfer of murine CD8+, class I–restricted HBsAg-specific CTLs into syngeneic transgenic mice expressing HBV envelope protein in the liver leads to acute hepatitis (27,148,530). Interactions between the HBV-specific T-cell receptors and the antigen-presenting HLA class I molecules on the hepatocytes cause scattered apoptosis of hepatocytes. By secreting cytokines, including interferons, the CTLs recruit a variety of antigen-nonspecific inflammatory cells into the liver, resulting in more extensive necroinflammatory injury of the liver that resembles acute hepatitis B in humans. The predominant infiltrating effector cells are the macrophages, which probably mediate the majority of hepatocellular injury in this model (28). A major consequence of these immunologic events is the marked inhibition of hepatocellular HBV gene expression and viral replication (148,262). Although the CTLs may contribute to this effect by direct cytolysis of HBV-expressing hepatocytes, a noncytolytic mechanism very likely plays a more substantial role. This concept also is supported by observations in the duck hepatitis B virus (DHBV) and woodchuck hepatitis virus (WHV) models, in which viral clearance is not accompanied by massive hepatocellular death (368,379). Interferon-g and tumor necrosis factor (TNF)-a produced by activated CTLs and other effector cells are the key cytokines involved in this mechanism (262). The effects of cytokines are discussed later.

These observations delineate a logical progression of immunologic events for HBV infection. However, transgenic models are not an infectious model. In the transgenic models, the complex virus–host interactions during the early stage of infection, which are critical in determining the manifestations of immune response and the outcome of infection, cannot be studied. Furthermore, the transgenic model does not offer the opportunity to study the events during a “curative response.” However, a recent study in chimpanzees (265) provided key insights into some of the unanswered questions

encountered in the transgenic models. This study, while supporting the noncytopathic mechanism as the major antiviral response responsible for viral clearance during acute hepatitis B, demonstrates that CTLs are probably not the primary mediator of this response. Instead, these results reveal that natural killer cells or other nonstandard T cells (g/d or NKT cells), which normally reside in the liver in relative abundance (65,167,270), may play an important role in this early phase of infection (127,265, and J. Baron and D. Ganem, personal communication, 1999). Furthermore, studies in the duck and woodchuck models suggest that accelerated hepatocyte turnover in infected liver may partially explain viral clearance during the recovery phase (379). However, these observations do not necessarily imply that CTLs are not important in controlling viral infection. The infiltration of CTLs, which occurred later in the chimpanzee study, probably mediates the control of the residual HBV infection. This is consistent with the observation that patients who have recovered from acute HBV infection harbor HBV-specific CTLs many years after viral clearance (644). It is believed that the CTLs persist as a result of continuous stimulation by low-level viral replication and in the meantime keep the infection in check. It is well established that low-level HBV virus can be detected in patients with serologic evidence of recovery (452,453,593,598,644,825,831,835). Furthermore, active HBV infection can be reactivated in these patients upon immunosuppressive therapy, and their sera or livers are capable of transmitting HBV infection (84,188,469,759).

Cytokine Induction

CTLs have been thought to be the principal effector in the clearance of viruses. However, studies in a variety of viral model systems point to the importance of cytokines in containing and eliminating the virus, in addition to their immunoregulatory functions (82). Upon antigen recognition, CTLs secrete a multitude of cytokines to activate other inflammatory cells, such as macrophages, that constitute a potent source of additional cytokines. The interferons are known to elicit a variety of direct antiviral factors in infected cells, such as protein kinase R, 2'-5'-oligoadenylate-activated RNase L, Mx protein, and inducible nitric oxide synthase (429). Recent studies in HBV animal models have provided compelling evidence that inflammatory cytokines, especially interferon-g and TNF-a, are the principal mediators of viral clearance during primary viral infection (262,263,266). These cytokines inhibit viral replication at several levels in infected hepatocytes. First, the cytoplasmic nucleocapsid particles that contain replicating viral genomes are rapidly eliminated (262). The kinetics of the elimination suggests that an activated degradative process may be the mode of action (266). Second, the cytokines induce a profound decrease in viral RNA level, most likely by a posttranscriptional mechanism (779). Third, the HBV covalently closed circular DNA (cccDNA) is susceptible to a similar noncytopathic control by the cytokines (265). Because the cccDNA appears to have a relatively long half-life in infected hepatocytes (152,379), an active mechanism of elimination may need to be evoked. Recent studies suggest that these cytokines may induce certain cellular nucleases and RNA binding proteins as a potential explanation for these noncytopathic actions (296,297). Finally, whether interferon-g and TNF-a mediate these effects directly or indirectly through a yet-unknown paracrine or autocrine mechanism on the infected hepatocytes is not known. Nitric oxide, a potent inflammatory mediator, has recently been implicated in this process (L. Guidotti and F. Chisari, personal communication, 1999).

Viral Pathogenic Determinants

Immune Escape

RNA viruses, retroviruses, and hepadnaviruses have high mutational rates because their polymerases do not have a proofreading capacity. This infidelity is beneficial to these viruses in terms of their ability to mutate their genomes in response to an active immune response. Many of them can mutate the binding sites of neutralizing antibodies on the viral surface proteins, as well as the viral sequences that are presented to the immune cells in the context of class I molecules, thus leading to the emergence of immune escape mutants. Immune escape mutants have been described to occur in both arms of the host immune response in HBV infection. Vaccine escape mutants have been detected in vaccinated infants born to HBV-infected mothers despite protective levels of anti-HBs (124), and in liver allograft recipients who were HBsAg carriers and who received hepatitis B immune globulin (HBIG) for prophylaxis (501). These strains contained mutations in the immunodominant region of HBV envelope protein, leading to poor reactivity with HBIG. Naturally occurring mutations in dominant CTL epitopes of HBV have been found to antagonize recognition of infected cells by HBV-specific CTLs (74). Many viruses, such as herpes simplex viruses, cytomegalovirus (CMV), and adenoviruses, encode gene products capable of preventing recognition of viral-infected cells by CTLs through inhibition of class I antigen presentation (582). Whether this mechanism is operative in HBV persistence is not clear.

Viral Strains

HBV has been classified into various serotypes/genotypes, which exhibit distinct geographic distributions (390,561,576). Whether they impart variable pathogenic potentials is not clear. The A genotype of HBV has been associated with better disease outcome and long-term survival, the C genotype with more severe disease, and the B genotype with development of hepatocellular carcinoma (383); however, the clinical significance of these relationships has yet to be firmly established. The precore stop codon mutation associated with fulminant hepatitis is much more prevalent in specific genotypes (such as D) than in others (such as A) because a genotype-specific nucleotide difference upstream of the stop codon mutation is involved in critical base-pairing with the mutation in the secondary structure of the HBV encapsidation signal (451). In addition, genotypes A and D have different replication efficiencies in tissue culture (47). Whether this difference can be equated to a pathogenic difference *in vivo* is not clear. This strain difference in pathogenicity also has been borne out by a study in which two naturally isolated WHV strains (WHV7 and 8) have different replication efficiency and pathogenicity in woodchucks (J. Gerin and B. Tennant, personal communication, 1999). Therefore, viral divergence may be linked directly to variant biologic behaviors.

Recent studies have identified and characterized viral mutations associated with fulminant hepatitis B. Two types of mutations—precore mutations leading to the HBeAg-negative phenotype (122,285,408,455,583) and core-promoter mutations resulting in enhanced replication (48,117,529,575,658)—have been described. Most reported precore mutations occur in codon 28 of the precore open reading frame, resulting in a stop codon that leads to absence of HBeAg synthesis (46). Other precore mutations, such as precore start codon mutations or deletions/insertions in the precore sequence resulting in HBeAg-negative phenotype, also have been reported. The described core promoter mutations cluster in a region of the promoter elements that contain binding sites to various transcriptional factors (117,636,832).

Some studies have suggested that two of the mutations confer diminished precore transcription leading to diminished HBeAg synthesis (117,661). Other studies have demonstrated that many of the core promoter mutations lead to a high replication phenotype (48,117,268). In addition, mutations in the core protein have been linked to severe or fulminant hepatitis B (198). However, these core mutations may reflect selection by a vigorous immune response during fulminant hepatitis, because they affect important T-cell epitopes (148). They also have been described during viral clearance and the active phase of liver disease in chronic hepatitis B (34,198).

Because all the mutations just described affect the core gene, it is worthwhile to briefly review the known and putative functions of the two core gene products (HBcAg/HBeAg). The HBcAg forms the nucleocapsid and is essential for viral replication and assembly. The HBeAg is synthesized as an alternative processing of the core gene and is a secreted circulating antigen. The gene product is not essential for replication nor for productive infection *in vivo* (143). HBeAg has been used clinically as an index of viral replication, infectivity, severity of disease, and response to treatment. Because HBeAg is evolutionarily conserved among all hepadnaviruses, it probably plays an important role in the life cycle of HBV.

The concept of HBeAg as an immunoregulatory protein has emerged from several lines of evidence (511). First, HBeAg may play a role in perpetuating viral infection during perinatal transmission (513). Perinatal infection from mother to infant is the most common form of HBV transmission in the world. Infants born to anti-HBe-positive, HBsAg carrier mothers are likely to develop acute hepatitis but less frequently to progress to chronicity (758). Studies in transgenic mice expressing HBeAg have demonstrated induction of immunologic tolerance to HBcAg/HBeAg *in utero* (513), although this phenomenon may be strain specific (647). Because HBeAg is synthesized as a soluble small protein, it may traverse the placenta, exposing the fetus to the antigen. Such an exposure may result in a partial tolerance of the infant's immune system to the virus. Following perinatal infection, the newborn infant, with the additional problem of a relatively immature immune system, may have limited ability to eliminate the virus. Although this is an attractive hypothesis, the role of HBeAg as a neonatal immunologic toleragen remains controversial (647).

Second, despite extensive sequence overlap with the core protein and sharing of the identified CD4 and CD8 epitopes (148), HBeAg has properties that are distinct from those of the core protein in terms of interactions with the host. HBeAg has been shown to induce a T_H2 immune response in mice, whereas HBcAg induces a T_H1 response (512,516). The T_H2 response to the HBeAg is dominant over the T_H1 response to the core antigen, resulting in depletion of the HBcAg-specific T_H1 cells *in vivo* (512). This event may have a profound effect on viral persistence. Experiments in mice have shown that circulating HBeAg is capable of abrogating the nucleocapsid-specific T_H1 response, which includes production of interferon-g and other cytokines essential for viral clearance. This concept is supported by studies in the woodchuck model in which WHV mutants negative for HBeAg are less likely to result in chronicity (143). In addition, HBeAg differs from HBcAg with respect to the antigen-processing pathway. Endogenously synthesized HBeAg can be presented through both the class I and II pathways (192), thereby inducing both CD8+ and CD4+ effector cells. This also may provide a plausible explanation for the emergence of HBeAg-negative mutants in chronic HBV infection. It is possible that CD4+ cytotoxic T cells exist that recognize only the HBeAg-expressing cells, not the HBcAg-expressing cells. Furthermore, despite extensive sequence overlap between HBeAg and HBcAg, HBeAg contains additional amino acids in the N-terminus, where a CD4 epitope has been mapped (192). Therefore, HBV mutants lacking HBeAg expression may have a selection advantage *in vivo*. Because overexpression of precore may inhibit HBV replication, HBeAg-negative mutants could be selected on the basis of a more efficient replication phenotype (264,425,662). However, studies of naturally occurring HBeAg-negative mutants *in vitro* and in animal models do not support this concept (47,143,769,832,836).

Although many questions remain unanswered, the HBeAg-negative mutants represent a true biologic variant of HBV with a distinct phenotype. If the HBeAg-negative mutants do have a biologic advantage over the wild-type virus, one would expect that these strains should be evolutionarily selected. However, wild-type strains still prevail over the mutants in the general population. For viral dominance, both intrapopulation and intrahost factors must be considered. Whereas the mutants may exhibit a selection advantage within an infected host during chronic infection, they may be disadvantaged in terms of transmission to the other hosts because they may be less efficient in inducing neonatal tolerance or causing chronicity. Therefore, the overall impact is probably more favorable to the wild-type strain.

Finally, the question remains as to whether the HBeAg-negative mutants are indeed responsible for more severe or fulminant hepatitis. The strongest evidence comes from studies of outbreaks of fulminant hepatitis B, in which transmission of a unique HBV strain to several unrelated individuals resulted in fulminant hepatitis (207,455,610,727,755,758). A compilation of all studies on fulminant hepatitis B revealed that the HBeAg-negative mutants were not universally present in all cases of fulminant hepatitis, nor were they completely absent in individuals with acute self-limited hepatitis, although the prevalence rate of these mutants was significantly higher in the former group (32,34,121,230,431,454,715). Therefore, these mutations may contribute to the development of fulminant hepatitis, but they are neither necessary nor sufficient for this outcome. On the other hand, a combination of viral mutations or host factors are probably involved in the pathogenesis of fulminant hepatitis. Recent studies also have demonstrated that core promoter mutations are commonly present in patients with severe or fulminant hepatitis B (268,432,575,658), and some of the mutations confer enhanced replication (48,117,529,630,716). During acute infection, enhanced replication may lead to a more widespread HBV infection in the liver. Because the core and pol are major targets of the host immune response, an increase in their expression resulting from enhanced replication also may render hepatocytes more vulnerable to immune-mediated injury. In addition, the absence of HBeAg production may be associated with a more T_H1-like, proinflammatory response. Taken together with the secondary nonspecific necroinflammatory infiltrate, this event may be more detrimental during the acute phase of HBV infection.

A vigorous and extensive immune response with enhanced viral replication may lead to massive liver injury and ultimately to fulminant hepatic failure. In addition, other unidentified viral pathogenetic factors also may contribute to the development of fulminant hepatitis. Therefore, viral factors, in concert with various host determinants directing the vigor and character of the antiviral inflammatory response, underpin the complicated interplay of virus–host interaction in the pathogenesis of fulminant hepatitis B.

Seronegative Infection

Other atypical infections include seronegative occult HBV infections. This heterogeneous group consists of HBsAg-negative patients who are either seronegative for all HBV markers or positive for anti-HBc and/or anti-HBs (452,593,598,644,825,831,835). Many of these patients also are positive for HBV DNA by polymerase chain reaction (PCR) in the liver or serum or both. Some of these patients have underlying liver disease, suggestive of ongoing hepatocellular injury from persistent HBV infection. Studies in the animal models have demonstrated long-term persistence of viral genomes in the serum and/or liver of animals who have biochemical and serologic evidence of viral clearance and recovery from infection (404,406,453). The important question is whether this represents ongoing viral replication, and therefore clinically significant infection, in terms of liver disease and transmission. Existing evidence supports the notion that this indeed indicates low-level viral replication, capable of transmission (453,759). A recent report on the WHV model demonstrated several well-documented examples of maternal–fetal transmission of occult WHV infection (157). Studies on liver transplantation revealed transmission of HBV infection to the recipients if the donors carried the anti-HBc marker (188). In addition, reactivation of HBV infection has been reported in patients with serologic evidence of recovery who were undergoing immunosuppression or chemotherapy (84,188,469,759). These observations, together with the immunologic studies previously described, provide compelling evidence that one may not be able to completely eliminate HBV infection. Patients with serologic evidence of recovery probably have low-level viral replication that is effectively controlled by an active immune response.

The possibility that these seronegative infections are caused by HBV mutants has been proposed. Although mutations have been reported in various regions of the viral genome (86,339,386,825), definitive evidence in support of a pathogenic role of these mutants is lacking. Furthermore, whether liver disease can result from these occult HBV infections is controversial. At present, there are no convincing studies in support of a causal relationship. Therefore, these occult HBV infections may not be clinically important except in the special situations described.

Abnormal Viral Gene Expression

There is accumulating evidence that abnormal expression of certain HBV gene products can result in cytotoxicity. First, “ground-glass” hepatocytes seen variably on histology of HBV-infected liver have been shown to contain high levels of HBsAg within the amorphous content of the ground glass (240,356). This intriguing histologic observation may provide a clue that abnormal expression or accumulation of HBV envelope gene products can lead to cellular injury. Second, overexpression of HBV large envelope protein (pre-S1-S2-S) in both tissue culture (93,821) and the transgenic mouse model (149) can result in a massive accumulation of the protein within the endoplasmic reticulum, accompanied by evidence of cytotoxicity with the appearance of ground-glass cells, and the eventual development of hepatocellular carcinoma (150). Third, liver pathology with minimal inflammation (fibrosing cholestatic hepatitis) can be seen in HBV-reinfected liver allograft recipients (436). Although the pathogenesis of this entity is unknown, it is possible that abnormal HBV replication in the transplantation setting may be partially responsible. Fourth, *in vitro*–generated pre-S1 mutants in the DHBV model have exhibited enhanced replication and cytopathicity in infected animals (446). Although there has been no direct proof that such a mutation or genotype exists in naturally occurring HBV strains, it is tempting to speculate that some HBV strains with this type of mutation may exist and cause more severe disease. Finally, high-level expression of HBX in tissue culture appears to sensitize cells to TNF-a-induced apoptosis, and possibly to induce other mechanisms of cellular injury (146,728).

Mechanisms of Viral Persistence

The outcome of a viral infection is determined by several opposing forces. On the one hand, the infected host commands a powerful armamentarium of antiviral defenses that target the virus for destruction and elimination. On the other hand, viruses have evolved sophisticated mechanisms to counteract, elude, and in some cases forestall these host defenses. If immune responses are more dominant, the infected host will clear the viral infection, whereas if viral mechanisms prevail, persistent and chronic infection will ensue. The balance of these competing forces underlies the molecular mechanisms of persistent viral infection. In HBV infection, establishment of persistent infection has been attributed to multiple viral and host factors. Although several *in vitro* studies have suggested that HBV gene products may potentially interfere with pathways of the immune response (225,344,807), there is no direct evidence that these mechanisms are operative *in vivo*. Many potential mechanisms of viral persistence have been discussed in the previous sections and are summarized in Table 1.

Host factors
Hepatotropism: tolerance or anergy induction
Infection of immunologically privileged sites: viral reservoir
Infection of lymphocytes: HBV-specific immunosuppression
Genetic modifiers: HLA or other genetic associations
Viral determinants
High-level replication: immune exhaustion
High rate of mutations: immune escape in antibody and cell-mediated immunity, antagonism
HBeAg: transplacental tolerance induction and dominance of T_h2 response
Miscellaneous: HBcAg and *pol* (interfere with interferon actions?), HBX (modify immune responses?)

TABLE 1. *Mechanisms of HBV persistence*

As the primary site of HBV tropism, the liver may be a key predisposing factor for viral persistence. Hepatocytes are poor

antigen-presenting cells because they do not express HLA class I and II and co-stimulatory molecules except during inflammation (467,524). Because of the lack of co-stimulation, presentation of viral antigens to T cells is more likely to induce anergy than activation (141). In addition, the liver is a principal site of death for circulating activated T lymphocytes (345). Once arriving in the liver, activated T cells may be short-lived and relatively ineffective as an anti-HBV effector mechanism.

As discussed previously, perinatal infection induces neonatal tolerance, probably because of the transplacental passage of soluble HBeAg. This tolerance is mostly toward the HBeAg–HBcAg complex, because infants born to HBV carrier mothers respond well to HBsAg immunization. During adult infection, several mechanisms may be operative. First, HBeAg may play a role in directing an immune response that is less favorable to viral clearance. Second, high viral load, typical of acute HBV infection, may induce immunologic anergy or tolerance by inappropriate antigen presentation to the immune system or by exhaustion of the antiviral CTLs. In support of this hypothesis, high doses of inoculum virus in the DHBV model are more likely to cause chronic infection (365).

Hepadnaviruses may infect lymphocytes, monocytes, and other nonhepatocytes (402,403,559,644). This has a twofold implication in viral persistence. First, as has been shown for other lymphotropic viruses, selected alterations of the immune response against HBV may result from infection of lymphocytes or antigen-presenting cells by HBV (838), although this has not been demonstrated experimentally. Second, infection in immunologically privileged sites that are protected from an anti-HBV response may function as a reservoir of persistent HBV infection (26,829). Finally, once a chronic infection is established, several mechanisms are potentially operative to perpetuate viral persistence. High mutational rates associated with hepadnaviral replication can lead to selection of immune escape mutants at both the antibody and cell-mediated levels. In addition, selection of antagonistic T-cell receptor epitopes could result in active suppression of CTL recognition of infected hepatocytes (74). However, this mechanism may be operative only when the CTL response is strong and focused predominantly on one epitope. Because the anti-HBV CTL response is typically polyclonal and multispecific, T-cell receptor antagonism may not play a substantial role in the overall mechanism of viral persistence.

Finally, certain HLA class II haplotypes have been associated with viral clearance and a favorable outcome of HBV infection (766). Other genetic modifiers of hepatitis B also have been reported (64). Because host response to infection is a complex process that is likely to be controlled by polygenic loci, these genetic associations may contribute to the overall outcome of infection and disease. However, whether these linkages imply a direct pathogenetic relationship or represent surrogate markers for other genetic loci of importance is unknown at present.

PATHOLOGY

HBV causes both acute and chronic liver disease. The pattern of liver injury is characterized by hepatocellular destruction, regeneration, and inflammatory infiltrates. Although these pathologic changes typically resolve completely after viral clearance, chronic infection is often accompanied by progressive fibrosis and architectural disarray that culminates in cirrhosis. The pattern of injury accompanying chronic viral hepatitis has distinct features and may be distinguished from other forms of liver disease, such as cholestatic liver disease, autoimmune hepatitis, metabolic liver disease, drug-induced hepatitis, and steatohepatitis. However, the pattern of injury seen with acute hepatitis B overlaps significantly with other causes of viral hepatitis. Because chronic hepatitis B takes decades to progress to end-stage liver disease, it is important to assess not only the pattern of injury but also the histologic severity and stage of hepatitis B.

Acute Hepatitis

The histologic features associated with acute hepatitis B are similar to those of hepatitis A (see Chapter 26), but hepatitis B is characterized by more extensive parenchymal abnormalities and less cholestasis and portal inflammation (356). Hepatocellular injury is marked by either ballooning degeneration or acidophilic bodies, which probably reflects two distinct mechanisms of hepatocyte damage. The former is characterized by swollen hepatocytes with fragmenting cell membranes, pale granular cytoplasm, and disintegrating nuclei. The latter alteration represents the histologic manifestation of apoptosis and is marked by a progressively shrinking cell, eosinophilic cytoplasm, and pyknotic nuclei. Focal hepatocellular necrosis, the histologic hallmark of acute viral hepatitis, is characterized by intralobular foci of necrotic or apoptotic hepatocytes surrounded by inflammatory cells including lymphocytes, macrophages, and, rarely, plasma cells. These necroinflammatory changes are distributed throughout the lobules with more extensive pericentral involvement (zone 3). Regenerative changes consisting of double-cell-thick hepatic cords, binucleated hepatocytes, and nuclear hyperchromasia often accompany necroinflammatory foci. Other histologic features include lobular inflammatory cell infiltrate, Kupffer cell hypertrophy, and sinusoidal reaction. Bile duct lesions such as ductular proliferation or cholestatic obstruction are rare events.

About 1% of patients with acute hepatitis B develop severe or fulminant hepatitis (441). Histologically, necrosis of a large area of liver cells (confluent necrosis) is evident. In more severe cases, the necrotic zones extend from zone 3 to adjacent vascular structures of the hepatic acini, forming bridging necrosis, which can be seen between central veins or between central veins and portal tracts. Submassive necrosis, in which bridging necrosis and parenchymal collapse are present extensively throughout the liver, often characterizes the fulminant form of acute hepatitis. Although most cases of acute hepatitis B resolve without histologic sequelae, the fulminant form often results in nodular regeneration, architectural distortion, and scar formation. This has been designated postnecrotic scarring, which is a nonprogressive lesion different from the cirrhosis of chronic hepatitis. It rarely leads to long-term morbidity or mortality.

Chronic Hepatitis

The histologic hallmark of chronic hepatitis B is inflammatory destruction of hepatocytes, accompanied by progressive fibrosis. The histologic classification of chronic hepatitis has undergone extensive modification to provide a more quantitative and clinically relevant description of the disease. The classical terms of chronic persistent, chronic lobular, and chronic active hepatitis are being replaced by revised grading and staging systems that consider the degree and

extent of necroinflammation and fibrosis (60,183,355,472). These histologic systems describe a continual spectrum of liver injury and disease activity and provide a more accurate assessment of prognosis and therapeutic effect.

The most widely used system is based on the modified Knodell scores (355). This system has two scores: one based on inflammatory activities (grade) and the other on fibrosis (stage). The grade is indicative of ongoing disease activity, whereas the stage represents the cumulative effect of the disease. The inflammatory component is divided into three compartments: the interface zone between the hepatocytes and the portal area (limiting plate), the portal area, and the hepatocellular parenchyma. Piecemeal necrosis or interface hepatitis describes the destruction of hepatocytes at the limiting plate, which forms a well-defined structure demarcating the hepatic parenchyma and the portal area. On microscopy, the smooth edge of the limiting plate becomes eroded and indistinct as the junctional surfaces of periportal hepatocytes are invaded by lymphocytes or macrophages (peripolesis) (Fig. 2A). Occasionally, lymphocytes are seen to actively penetrate the hepatocytes (emperipolesis) (see Fig. 2A). Piecemeal necrosis may be focal, involving only one or two hepatocytes with little destruction of the limiting plate, or it may be extensive, involving the entire circumference of the portal area with penetration of the inflammatory cells to a depth of several hepatocytes. In more severe cases, the necroinflammatory process extends into the parenchyma, reaching to the terminal hepatic veins or to other portal areas (bridging necrosis).

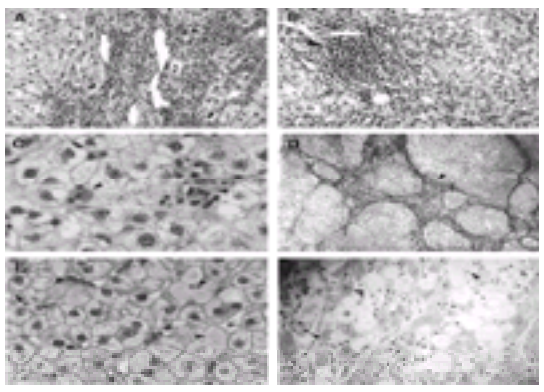

FIG. 2. Pathology of chronic hepatitis B. **A:** Piecemeal necrosis (interface hepatitis). *Arrowheads* indicate lymphocytic infiltrates disrupting the limiting plate of the portal area and invading the parenchyma (×200). **B:** Lymphoid aggregates. *Arrowhead* shows a dense lymphoid aggregate in the portal area surrounding the bile duct (×200). **C:** Lobular inflammation. Scattered apoptotic hepatocytes (*arrowheads*) with surrounding lymphocytes are shown in the hepatic lobule (×600). **D:** Cirrhosis. Thick bands of fibrotic tissues (*arrowhead*) encircling regenerative nodules (×100). **E:** Ground-glass hepatocytes (*arrowheads*) (×600). **F:** Fibrosing cholestatic hepatitis. Diffuse ballooning degeneration of hepatocytes and marked cholestasis with bile plugs (*arrowheads*) are shown (×200).

The portal area, while contiguous with the periportal compartment, is being considered separately because it delineates a distinct pathologic feature. When bound by the limiting plate, lymphoid infiltrates can fill and expand the portal areas, occasionally forming aggregates of variable density (see Fig. 2B). Like the primary follicles of lymph nodes, the lymphoid aggregates are composed of a mixture of B and T cells with small numbers of macrophages and dendritic cells. Rarely, the lymphoid aggregates resemble reactive germinal centers with a core of B and plasma cells and a mantle of B and T cells. Bile duct lesions (Poulsen lesions) also can be seen accompanying the lymphoid aggregates; they are characterized by reactive epithelial changes and lymphocytic infiltration without true cholestatic features. These histologic changes are more commonly observed in chronic hepatitis C infections, as is steatosis.

Parenchymal inflammation in chronic hepatitis B generally consists of small foci of lymphocytes and macrophages surrounding apoptotic bodies or necrotic (“drop-out”) hepatocytes (see Fig. 2C). Plasma cells and eosinophils can sometimes be seen in these foci. The relative abundance of plasma cells serves to distinguish chronic hepatitis from acute hepatitis. The parenchyma often shows areas of ballooning degeneration and focal collapse. There also may be features of regenerative activity such as hepatocellular mitotic figures, binucleated cells and widened liver cell plates. When these foci of lobular necrosis are the dominant features of the inflammatory pattern, it often is difficult to distinguish chronic from acute hepatitis. Regardless, this pathologic feature can be seen in many patients with chronic hepatitis B, particularly those with HBeAg-negative-mutant infection who experience periodic flares of symptoms and biochemical abnormalities (113). Occasionally, more extensive lobular necroinflammation with confluent, bridging, or panlobular necrosis can be seen. Often, these exacerbations resolve spontaneously with a return of the histologic features to minimal-to-mild inflammation. However, frequent and severe attacks can lead to more rapid progression to cirrhosis.

Although the pattern of inflammation characterizes the pathologic process of chronic hepatitis, it is the extent of fibrosis that determines the severity and prognosis of the disease. The earliest change is an expansion of the portal area by new collagen formation. Single hepatocytes or groups of hepatocytes are surrounded by newly formed connective tissue, forming active fibrous septa. When arranged in a tubular fashion around a central lumen, the liver cell groups form rosettes. Periportal fibrosis separates the hepatocytes from the limiting plate and disrupts the smooth edge of the limiting plate. These fibrous septations gradually extend from portal areas to other portal areas or to the terminal hepatic veins (bridging fibrosis). With continued inflammation, diffuse bridging fibrosis of varying width develops and, together with regenerative changes and acinar destruction, results in the distorted architectural pattern that we recognize as cirrhosis. Cirrhosis is defined as a diffuse change of the liver in which the normal architecture is replaced by regenerative nodules surrounded by bands of fibrosis (see Fig. 2D). Once patients reach this stage, end-stage (decompensated) liver disease ensues with high morbidity and mortality.

One unique feature of chronic hepatitis B is the presence of ground-glass hepatocytes (see Fig. 2E), which are rarely detected in acute hepatitis B patients. They are irregularly distributed and often appear in clusters. The hepatocytes are

slightly larger than normal and the cytoplasm contains a pale, eosinophilic, sharply circumscribed, finely granular, homogeneous inclusion that often displaces the nucleus to one side. Immunoperoxidase staining with HBsAg-specific antibodies demonstrates a high level of HBsAg immunoreactivity within the ground-glass material. By electron microscopy, large quantities of HBsAg-containing tubular and spherical structures are observed within the smooth endoplasmic reticulum. The pathogenesis of these cells is unknown but is thought to arise from impaired HBsAg secretion of the large envelope protein. An additional feature, often seen in the transplanted liver, is the entity of fibrosing cholestatic hepatitis (see Fig. 2F), which represents injured hepatocytes laden with HBV antigens, accompanied by excessive parenchymal fibrosis in the setting of little or no inflammation (436). Similar lesions also have been seen in the transplanted liver of HCV-infected individuals (843). Again the underlying mechanism is unknown. HBV mutants have been proposed to be associated with these two pathologic entities, but a direct causal relationship has not been established (93,98).

CLINICAL FEATURES

The course of hepatitis B can be extremely variable (Table 2). In addition to the inapparent or subclinical cases, patients may develop anicteric or icteric hepatitis. The terms *inapparent hepatitis* and *anicteric hepatitis* are often used interchangeably. However, the latter term should be reserved for those patients who develop clinical symptoms but who are not jaundiced. Patients with inapparent (subclinical) hepatitis have neither symptoms nor jaundice. These asymptomatic cases previously went unrecognized but can now be identified by detecting biochemical or virus-specific serologic alterations in their blood. Symptoms ranging from mild and transient to severe and prolonged may accompany clinical hepatitis. Patients may recover completely, progress to chronic hepatitis, or develop fulminant hepatitis and die. In general, the frequency of clinical disease increases with age (499), whereas the percentage of carriers decreases (Fig. 3).

Predicted parameter	Outcome (%)
Inapparent (subclinical) or anicteric disease	65–80
Icteric disease	20–35
Complete recovery	95–98
Chronic disease (% of total number infected)	2–10[a]
Mortality rate	
Based on infection	0.2–0.5
Based on icteric cases	0.5–1.5

[a]Data compiled for immunocompetent adults; rate in immunocompromised patients may be higher. Infection in the perinatal period leads to chronic disease in 70% to 90% of infants born to HBeAg-positive carrier mothers.

TABLE 2. *Predicted outcome after an infection with hepatitis B virus*

Clinical Phases of Acute Infection

Regardless of etiology, the course of acute viral hepatitis is similar and can be divided into four clinical phases: (a) the incubation period (time between exposure and the first day of symptoms or jaundice); (b) a prodromal or preicteric stage; (c) the icteric phase; and (d) the convalescent period (395). Thus, the clinician faces a formidable task in determining which type of hepatitis a patient has acquired, unless appropriate serologic assays are requested.

The incubation period for viral hepatitis type B ranges from 45 to 120 days (14,43,227,287,409,548,660). Incubation periods of less than 35 days or more than 150 days are unusual. Factors that might account for these extremes in HBV disease include (a) the size of the inoculum, (b) the route of infection, (c) co-infection with another hepatitis agent, (d) alteration of viral pathogenicity by chemical or physical means, (e) unusual virus–host interactions, and (f) the concurrent administration of specific antibody.

The influence of virus dose on the incubation period is suggested by several studies in which increasing the quantity of virus administered shortened the time of onset of hepatitis (13,43,410,625). The shorter incubation periods sometimes recorded for HBV in earlier studies may have reflected the presence of previously unrecognized non-A, non-B hepatitis agents in the inocula or, in rare circumstances, may have represented contamination of blood with hepatitis A virus (323). Occasionally, patients from high-risk groups enter studies already infected with HBV, and this can result in a spuriously shortened incubation interval.

Krugman and Giles (409) showed that the portal of entry may affect the incubation time. Volunteers who received an oral dose of HBV that was 50 times greater than that administered by the intramuscular route had an average incubation time that was approximately 1 month longer. Prolongation of the incubation period for HBV also has been observed after ultraviolet irradiation of infected plasma (43,549), after storage of pooled plasma at room temperature (537), and after heating (318), presumably because of a reduction of virus dose. It is noteworthy that the severity of clinical illness in these delayed cases is usually not affected, although progression to chronicity may be curtailed.

Variations in host response may affect the incubation period. Groups of children (409) or adults (334) who receive the same dose of HBV can have onsets of their disease that differ by as much as 1 to 2 months within each group. Among chimpanzees experimentally infected with HBV, the incubation period is found to be significantly shorter in the older animals (625). Finally, the late administration of high-titer HBIG or monoclonal antibody to HBV-exposed persons or chimpanzees during the incubation period may lead to a significant delay in the onset of hepatitis, sometimes exceeding 6 months (253,410,567).

A short prodromal or preicteric phase, varying from several days to more than a week, precedes the onset of jaundice. In over half of the patients, the prodromal state typically is characterized by mild fever, easy fatigability, malaise, anorexia, myalgia, nausea, and vomiting. It is felt that many of these symptoms may be mediated by interferon induction (see the section, Pathogenesis). In hepatitis A, the transition from well-being to acutely ill occurs abruptly (within a period of 24 hours) in over 60% of patients, whereas the onset is more insidious in hepatitis B. Weight loss of 2 to 10 pounds is common as a result of anorexia from disordered taste and smell (299,707). Older children and adults may complain of right upper quadrant pain or discomfort as a consequence of hepatomegaly, which usually precedes jaundice by 1 to 2 weeks. Severe abdominal pain and protracted vomiting are unusual manifestations of the disease and should lead the clinician to either question the diagnosis or suspect a more severe variant of the disease.

The icteric phase of acute viral hepatitis is ushered in by the appearance of dark, golden-brown urine (bilirubinuria) followed one to several days later by pale stools and yellowish discoloration of the mucous membranes, conjunctivae, sclerae, and skin. This icteric phase begins within 10 days of the initial symptoms in over 85% of the HBV cases (525,840). Fever, if present, usually subsides after the first few days of jaundice.

Physical examination of the patient with acute disease shows tenderness to palpation or percussion of the liver. Percussion often shows an enlarged liver, with a span of 16 cm or more. Measurement is essential because a reduction in size in a deteriorating patient often heralds massive necrosis. The edge is firm but not irregular. Approximately 5% to 15% of patients have splenomegaly. Generalized adenopathy is not a component of viral hepatitis A, B, or C. Palmar erythema and spider angiomata may be observed.

Occasionally, during acute viral hepatitis, more extensive necrosis of the liver occurs that leads to severe impairment of hepatic synthetic processes, excretory functions, and detoxifying mechanisms. This entity, designated fulminant hepatitis if it occurs during the first 8 weeks of illness, is characterized by the sudden onset of high fever, marked abdominal pain, vomiting, and jaundice, followed by the development of hepatic encephalopathy associated with deep coma and seizures (67,659,774). Although fulminant hepatitis B is uncommon when compared with infection, it is observed in up to 4% of the hospitalized cases (589). Ascites, a bleeding diathesis, renal dysfunction, and decerebrate rigidity lead to death in 70% to 90% of the patients. Overall survival rates for hepatitis B are lower than those observed for hepatitis A (33% versus 50%), but more than those of non-A, non-B hepatitis (19%) (68). However, within each of these populations careful evaluation of the patients can often differentiate those who will have a poor outcome from those who will survive. For example, mortality is highly correlated with age, survival being uncommon over the age of 40 years or under the age of 11 (66,532,563,774). Other clinical signs associated with a poor prognosis include a rapid decrease in liver size, prolongation of the prothrombin time to greater than 50 seconds, an elevated bilirubin level of greater than 17.5 mg/dL, often in the presence of a rapidly declining alanine aminotransferase (ALT) value, and encephalopathy developing within 7 days of the onset of jaundice (563). Factor V levels also may serve as a prognostic indicator (69).

Clinical Features of Chronic Viral Hepatitis

In the United States, chronic liver disease is among the 10 leading causes of death in men. Nearly 50% of these deaths are in individuals under 60 years of age. However, the vast majority of patients with chronic hepatitis B remain asymptomatic for many years even though there may be biochemical and histologic evidence of liver disease. Thus, clinicians are seeing only a part of the spectrum of pathology that is present in the community at any given time.

Some of the chronically infected patients may have no clinical or biochemical evidence of liver disease. To distinguish this group from patients with chronic hepatitis, they are often categorized as asymptomatic hepatitis B carriers or simply HBsAg carriers. De Franchis et al. (173) recently studied the natural history of chronic hepatitis B in a cohort of these carriers. At baseline, 96% of 92 patients were anti-HBe positive, and histologic abnormalities were normal or minimal in all but five who had only mild chronic hepatitis. During a mean follow-up of 130 months, liver enzymes remained normal in 85% of 68 patients who were extensively followed; 13% of these patients cleared their HBsAg. Among 21 HBsAg carriers who showed no biochemical changes during 10 years of follow-up, no histologic changes were detected. Spontaneous reactivation was a rare event (4% of 68 patients), and no hepatocellular carcinoma (HCC) was detected. Other investigators have reviewed the outcome of patients who have been histologically classified as having mild chronic hepatitis. Fewer than 10% of these patients progress to cirrhosis (637,679).

Patients with moderate to severe chronic hepatitis may have no symptoms, or they may be significantly incapacitated. Easy fatigability, anxiety, anorexia, and malaise are prominent complaints. At the time of the initial diagnosis, jaundice is uncommon. Ascites and pedal edema are seen in approximately 20% of patients, whereas fewer than 5% present with endogenous encephalopathy or variceal bleeding. Transaminases, bilirubin, and gammaglobulin levels are mild to markedly elevated. During follow-up, there may be a series of remissions and relapses. Remissions may last a few months to several years. During a relapse, aminotransferases may be markedly elevated and jaundice may be present. Predictors of progression to cirrhosis include hepatic decompensation, repeated episodes of severe acute exacerbation with bridging hepatic necrosis and/or high alpha-fetoprotein levels (greater than 100 ng/mL), acute exacerbations without HBeAg clearance, and HBV reactivation with the reappearance of HBeAg (163,459).

In one study (802), the estimated overall survival rates at 5 years were 97% for patients with mild chronic hepatitis (n = 121), 86% for those with moderate chronic hepatitis (n = 128), and 55% for severe chronic hepatitis patients with cirrhosis (n = 130). However, 54% of the original cohort were not contacted for this analysis. In another series (459), the overall annual incidence for developing cirrhosis among a group of 684 HBsAg-positive patients with chronic hepatitis was 2.4% among the HBeAg-positive group and 1.3% among anti-HBe–positive subjects. In a third study (174), the 5-year survival rate for compensated cirrhosis was 84% (97% for patients with HBeAg-negative cirrhosis and 72% for HBeAg-positive patients). No deaths occurred in any of the patients followed for an additional 5 years. On the other hand, a dismal 14% 5-year survival rate was observed in decompensated cirrhotic patients with ascites, jaundice, encephalopathy, and/or a history of variceal bleeding at entry into the study. Predictors of death included older age,

elevated bilirubin, ascites, and HBeAg positivity.

A number of hepatitis B patients will develop HCC (see the section, HBV and Hepatocellular Carcinoma). Clinical features include weight loss (77%), right upper quadrant pain (61%), fever (47%), and gastrointestinal bleeding (30% overall, and 65% among cirrhotics). On physical examination, ascites and hepatomegaly are common, whereas jaundice and a palpable mass are uncommon. The mean duration of symptoms before diagnosis is 6 months (range, 0.5 to 30 months). The tumor is relatively slow growing, with a median doubling time of 4 months (range, 1 to 14 months) (691,692). Metastatic spread is uncommon, the most frequent sites being the lung (36%), direct extension through the hepatic or portal venous systems (12%), adrenal glands (10%), skeletal tissue (10%), and brain (6%) (350). Persons at high risk of developing HCC include adult male, chronic hepatitis B patients with cirrhosis who contracted their disease in early childhood and who display serologic or histologic evidence of active HBV replication (HBV DNA, HBeAg, IgM anti-HBc, cytoplasmic HBcAg) (186,703). From 55% to 85% of patients with HCC have cirrhosis at the time of diagnosis (389,580). Conversely, only about 5% of patients with cirrhosis develop HCC. Other studies have shown incidence rates from 0.1% to 1.1% per year for male HBsAg carriers based on increasing age (51,498) unless cirrhosis is present, in which case the annual incidence of HCC ranges from 2% to 7% (460,579). Rates in female patients are almost six times lower.

Figure 4 illustrates the natural history and growth of HCC in an HBsAg-positive carrier (186). Acquisition of HBV in infancy often leads to a persistent infection, prolonged active viral replication, HBV DNA integration, and eventually cirrhosis. Malignant transformation occurs with clonal expansion of hepatocytes until HCC becomes detectable serologically, radiologically, and/or clinically. The mean 5-year survival rate of 25% to 60% varies depending on the size of the tumor, resectability, symptoms, and the presence or absence of alpha-fetoprotein (AFP). Patients with unresectable tumors have an overall median survival of 5 months for AFP-positive tumors and 10.5 months for AFP-negative tumors, depending on which treatment modality is selected (725).

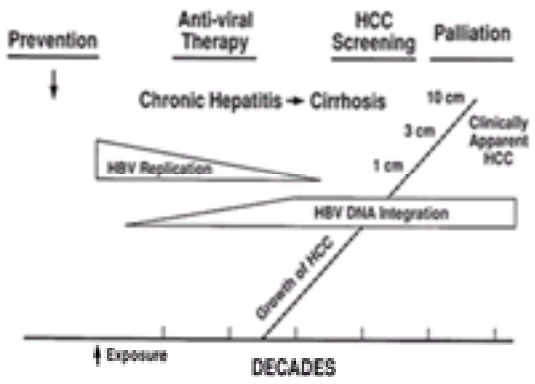

FIG. 4. Natural history and growth of HCC in an HBsAg-positive carrier. (From ref. 186, with permission.)

Extrahepatic Manifestations of Hepatitis B

Extrahepatic manifestations of viral hepatitis B are seen in 10% to 20% of patients. The nonhepatic disorders include (a) transient serum sickness–like syndrome, (b) acute necrotizing vasculitis (polyarteritis nodosa), (c) membranous glomerulonephritis, and (d) papular acrodermatitis of childhood (Gianotti-Crosti syndrome). Essential mixed cryoglobulinemia, although previously thought to be associated with HBV infection, is an uncommon clinical manifestation of this disease. Indeed, most patients with this disorder are found to be infected with HCV (235,498). Although the pathogenesis in several of these entities is unclear, most are believed to be mediated by virus-specific immune complex injury.

The major clinical features of the serum sickness–like syndrome are fever, skin rash, and polyarthritis (18,195,797). These disease traits usually precede the onset of jaundice by a few days to 4 weeks and may be the primary or only manifestation of anicteric HBV infection (195,724). Fever is generally more than 100°F and may exceed 102°F. Skin rashes typically consist of erythematous, macular, maculopapular, urticarial, nodular, or petechial lesions (672,801). The acute articular inflammation that develops is easily distinguished from the generalized arthralgia/myalgia that occurs in HBV infection. Joint involvement is usually symmetrical and is more commonly observed in female patients. It often involves multiple joints simultaneously but sometimes exhibits a migratory or additive pattern. Joint fluid examination is not diagnostic (195). Symptoms subside rapidly after the onset of jaundice but may persist throughout the course of illness. Neither recurrent nor chronic arthritis has occurred after recovery. This disorder resembles experimental serum sickness in which immune complexes activate the classic and alternative pathways of complement (797). Supporting this hypothesis are the following: the depression of complement levels in serum and synovial fluid of HBsAg-positive patients with arthritis (18); the detection of cryoprotein-immune complexes containing HBsAg, anti-HBs, and complement components; and the identification of complement, HBsAg, and IgM in cutaneous vascular walls of patients with urticaria.

One third to over one half of the patients with acute necrotizing vasculitis (polyarteritis nodosa) have HBsAg in their serum (250,507,773). The disease is uncommon in long-term carriers and thus appears to be a complication of a recent infection (267,498). Profound constitutional symptoms, high fever, anemia, and leukocytosis are prominent early manifestations of illness. Patients often have arthralgia, arthritis, renal disease (proteinuria and hematuria), hypertension, heart disease (pericarditis and congestive heart failure), gastrointestinal disease (acute abdominal pain), skin manifestations, and neurologic disorders [mononeuritis multiplex and central nervous system (CNS) abnormalities]. The disease is highly variable and has a mortality rate of approximately 40% within 3 years unless treated. Decreased levels of complement and circulating HBsAg–anti-HBs complexes have been demonstrated in addition to the presence of HBsAg and immunoglobulins in involved tissue (104,250,507,773). The diagnosis depends on finding the characteristic

arterial lesions on tissue biopsy. Angiography represents a simple and direct procedure for establishing the diagnosis (223).

HBV-associated nephropathy, especially membranous glomerulonephritis, has been described by several investigators in both adults and children (115,199,359,393,540,753,754). Liver disease may be minimal or absent in many of the patients, especially children. Although the incidence of HBV infection in cases of glomerulonephritis has not been established, it is frequently observed in countries with a high prevalence of HBV. Both HBeAg and HBsAg have been detected in glomeruli. Subepithelial electron-dense deposits are usually associated with HBeAg-immune complexes, presumably because of the low molecular weight of HBeAg, whereas the relatively large size of HBsAg (3.5 to 4.4 × 10^6 daltons) (see Chapter 86) results in the formation of larger immune complexes having a mesangial distribution (309,359,422,753). A good correlation has been found between nephropathy and serum HBeAg, but the same correlation is not seen with HBsAg. Remission of the nephropathy occurs in 85% to 90% over a 9-year interval and is associated with clearance of HBeAg from the serum (248,341,787).

A distinctive disease of childhood, papular acrodermatitis (Gianotti-Crosti syndrome) is irregularly, but not invariably, associated with hepatitis B infection and is believed to be mediated by circulating HBsAg–anti-HBs complexes (161,246,358). The skin lesion lasts 15 to 20 days and consists of lentil-sized, flat, erythematous, and papular eruptions localized to the face and extremities. It is accompanied by generalized lymphadenopathy, hepatomegaly, and acute anicteric hepatitis B with a predilection for the *ayw* subtype (246,358).

Laboratory Findings in Acute and Chronic Viral Hepatitis

The clinical diagnosis of acute hepatitis is made by biochemical assessment of liver function. The initial laboratory evaluation of a jaundiced patient should include the following tests: total and direct bilirubin, ALT, aspartate aminotransferase (AST), alkaline phosphatase, gamma-glutamyl transpeptidase, lactic dehydrogenase, albumin and total protein, complete blood count, and coagulation studies (prothrombin time, partial thromboplastin time). The aminotransferases are highly sensitive indicators of hepatocellular damage. They provide a quantitative assessment of the degree of damage sustained by the liver. Because the ALT is localized primarily to the liver, it is the most meaningful of the aminotransferases. The ALT level is significantly higher than the AST level in uncomplicated viral hepatitis. Bilirubin, albumin, prothrombin time, and partial thromboplastin time assess the synthetic function of the liver and are important predictors of survival. The prothrombin time accurately reflects early and potentially serious derangement in liver function, as does the factor V level (68,563). Ultrasonography can help exclude biliary tract disease.

The biochemical and hematologic tests that are useful for evaluating patients with chronic hepatitis are similar to those chosen for acute hepatitis. Evidence of portal hypertension associated with hypersplenism can be suspected in a patient with thrombocytopenia and/or leukopenia. Anemia may be present as a late manifestation of liver disease, but it also may signal evidence of a gastrointestinal bleed from esophageal varices. Hypoalbuminemia, hypergammaglobulinemia, elevation of the prothrombin time, hyperbilirubinemia, and creatinine are harbingers of more serious chronic liver disease and presumably cirrhosis, often confirmed by a percutaneous or laparoscopic liver biopsy. By determining the Child-Pugh classification of a chronic hepatitis B patient with cirrhosis (based on the presence or absence of clinical ascites, encephalopathy, prothrombin time, albumin level, and bilirubin), an excellent prognostic assessment of the patient can be made (353).

DIAGNOSIS

The terms used in identifying the various serologic markers of viral hepatitis B are shown in Table 3. An understanding of this nomenclature is essential for interpreting the results of the various serologic tests (Table 4).

Term	Abbreviation	Description
Hepatitis B virus	HBV	The 42-nm, double-shelled particle, originally called the Dane particle, that consists of a 7-nm outer shell and a 27-nm inner core. The core contains a small, circular, partially double-stranded DNA molecule and an endogenous DNA polymerase. This is the prototype agent for the family Hepadnaviridae.
Hepatitis B surface antigen	HBsAg	The complex of antigenic determinants found on the surface of HBV and on the 22-nm particles and tubular forms. It was formerly designated Australia (Au) antigen or hepatitis-associated antigen (HAA).
Hepatitis B core antigen	HBcAg	The antigenic specificity associated with the 27-nm core of HBV.
Hepatitis B e antigen	HBeAg	The antigenic determinant that is closely associated with the nucleocapsid of HBV. It also circulates as a soluble protein in serum.
Antibody to HBsAg, HBcAg, and HBeAg	Anti-HBs, anti-HBc, and anti-HBe	Specific antibodies that are produced in response to their respective antigenic determinants.

TABLE 3. *Nomenclature for viral hepatitis B*

Assay results			
HBsAg	Anti-HBs	Anti-HBc	Interpretation
Positive	Negative	Negative	Early acute HBV infection. Confirmation is required to exclude nonspecific or nonspecific reactivity (see text).
Positive	±	Positive	HBV infection, either acute or chronic. Differentiate with IgM anti-HBc (see text for exceptions). Determine level of replicative activity (infectivity) with HBeAg or HBV DNA.
Negative	Positive	Positive	Indicates previous HBV infection and immunity to hepatitis B.
Negative	Negative	Positive	Possibilities include HBV infection in remote past; "low-level" HBV carrier; "window" between disappearance of HBsAg and appearance of anti-HBs; or false-positive or nonspecific reaction. Investigate with IgM anti-HBc, and/or challenge with HBsAg vaccine (see text). Where present, anti-HBe helps validate the anti-HBc reactivity.
Negative	Negative	Negative	Another infectious agent, toxic injury to the liver, disorder of immunity, hereditary disease of the liver, or disease of the biliary tract
Negative	Positive	Negative	Vaccine-type response

TABLE 4. *Interpretation of HBV serologic markers in patients with hepatitis*

Course of HBV Infection

The clinical course and serologic changes that follow exposure to HBV represent a complex interaction between host, virus, and specific antigen. In a typical case of acute transfusion-associated HBV infection, HBsAg is first detected 7 to 9 weeks after transfusion (520), which is 1 to 3 weeks before the ALT level becomes abnormal and 3 to 5 weeks before the onset of symptoms or jaundice. It reaches a peak concentration during the acute stage of the illness, then slowly declines to undetectable levels within 4 to 6 months (Fig. 5). HBV DNA is detected 3 to 5 weeks after infection and up to 40 days before the appearance of HBsAg (average, 6 to 15 days), although newer, more sensitive HBsAg assays are closing this gap (118,419). The HBV DNA rises slowly and circulates at relatively low levels (10^2 to 10^4 genomic equivalents/mL) during the early HBsAg seronegative window period. In contrast, viral concentrations exceeding 10^9 HBV DNA genomic equivalents per milliliter are often present during the prodromal, acute, or chronic phase of the infection (118,168). As anticipated, communicability is highest when concentrations of infectious virus attain their highest levels in the blood.

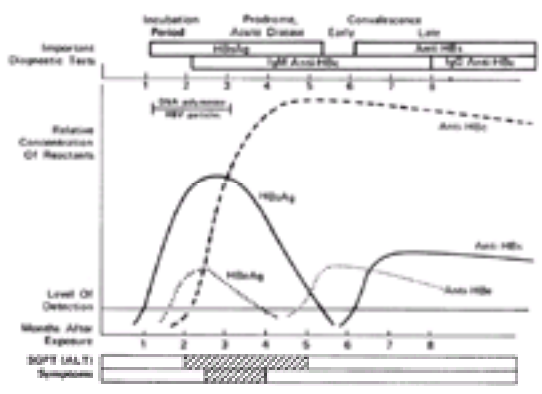

FIG. 5. Serologic and clinical patterns observed during acute HBV infection. (From ref. 317, with permission.)

HBeAg and IgM-specific anti-HBc are usually detected subsequent to the appearance of HBsAg (23,538) and concurrent with the onset of ALT abnormalities (116,158,243,417,650). Because the IgM-specific anti-HBc is produced in high titer (>1:1,000) in response to the synthesis of the nucleocapsid protein of the virion, its appearance in the serum is indicative of ongoing viral replication. These elevated titers decline to undetectable levels regardless of whether the disease resolves or becomes chronic, but the antibody may reappear during reactivation of HBV (see the section, Differentiating Acute Hepatitis from Chronic or Remote Infection). On the other hand, IgG-specific anti-HBc generally remains detectable for a lifetime.

During the early stages of convalescence and before HBsAg disappears, anti-HBe replaces HBeAg and signals (a) a reduction in viral replication and (b) the beginning of the resolution of the disease (329). Within 6 months, anti-HBe levels are no longer detectable in approximately one third of the patients. In 5% to 10% of the acute cases of hepatitis B, usually in life-threatening episodes such as fulminant hepatitis, HBsAg may not be detectable. Examination of the serum may show HBV DNA or IgM-specific anti-HBc (with or without anti-HBe) as the only indicators of active viral replication in this situation.

Termination of acute HBV infection occurs with the disappearance of HBsAg and the appearance of anti-HBs. It is believed that anti-HBs production may be initiated much earlier, but the antibody is not observed as a result of immune complex formation and removal in the presence of excess HBsAg. Anti-HBs generally persists for a lifetime in over 80% of the patients.

Those patients with acute hepatitis who maintain high levels of HBsAg concentration throughout the infection (317,319), or those whose serum HBeAg persists 8 to 10 weeks after symptoms begin to resolve, are most likely to become healthy carriers or to develop chronic liver disease. By convention, HBV carriers are identified by the persistence of HBsAg for at least 6 months. In approximately 5% to 10% of chronic carriers, anti-HBs also can be detected in low concentrations. Many of these patients have more serious liver disease.

In reviewing the natural history of chronic hepatitis B, loss of HBV DNA (as determined by relatively insensitive hybridization techniques) is followed by seroconversion from HBeAg to anti-HBe (38,458). This stage of disease is often accompanied by normalization of liver enzymes and remission of the disease (210,457). HBsAg may persist for years after the loss of HBeAg and its seroconversion to anti-HBe (100). Using the more sensitive PCR assay, HBV DNA usually remains detectable as long as HBsAg is present in the serum (407).

Diagnostic Assays

A number of assays are available to differentiate acute from chronic or remote HBV infection, to evaluate relative infectivity and prognosis, and to assess the immune status of the patient (319). Readers interested in details of the procedures listed later are referred to a recent review by Hollinger and Dienstag (317).

Among the many commercially licensed assays offered, the radioimmunoassay (RIA) and enzyme-linked immunosorbent assay (ELISA) are the most sensitive and specific. These assays can detect HBsAg in the 0.2 to 0.7 ng/mL range, and anti-HBs at a level of 1 mIU/mL. Within limits, the level of reactivity represents an approximation of the concentration of antigen or antibody present in a specimen. However, comparisons of results from one sample to another are usually meaningless unless paired samples are tested concurrently. Dilutions are often required because the capacity of commercial solid-phase systems is easily exceeded at relatively low concentrations (319). For example, most patients with acute HBV have HBsAg concentrations of 200 µg/mL or more, but the HBsAg assays reach saturation above 1 µg/mL. Similarly, anti-HBs assays reach a plateau at concentrations that approach values of 300 to 500 mIU/mL. Reactivities that are close to the cutoff values of the assay may signify a false-positive result.

Nucleic acid hybridization procedures (418,463,676,784,829) and the PCR assay (418,708) for the detection of HBV DNA in serum and tissue have been important in unraveling some of the intricate immunopathogenetic mechanisms of HBV disease and for evaluating the response to antiviral therapy. Filter or liquid-phase hybridization assays can detect quantities of HBV DNA sequences from 0.1 to 1.0 pg (33,000 to 330,000 genomic equivalents) within 24 hours, with a two- to tenfold increase after 5 days of autoradiography. A branched DNA signal amplification assay has a cutoff level of about 700,000 genomic equivalents per milliliter. In contrast, the PCR assay can detect less than 100 HBV DNA genomes per milliliter, a level that is over 300 to 7,000 times more sensitive than the hybridization techniques. Using this technique, virus has been detected 2 to 3 weeks before the appearance of HBsAg in experimentally infected chimpanzees and has continued to be detectable for 1 to 3 weeks after anti-HBs seroconversion (382). All HBsAg/HBeAg-positive patients have detectable HBV DNA in their serum, whereas only 70% to 90% of the HBsAg/anti-HBe-reactive patients are positive. All patients who recover from acute HBV are negative for HBV DNA. On the other hand, some chronically infected patients who have lost their HBsAg remain HBV DNA positive (see the section, Seronegative Infection).

Because the agents of viral hepatitis often cannot be distinguished clinically, and superinfection with HAV, HCV, or hepatitis delta virus (HDV) in an HBV carrier can occur—especially in groups at high risk of acquiring viral hepatitis—serologic means are required for their identification (313,317,319). To accomplish this, a variety of diagnostic profiles are available to the clinician to ensure that a misdiagnosis does not occur, because this may impinge on recommendations for prevention, prophylaxis, or therapy. Figure 6 is an algorithm that is designed to assist clinicians and diagnosticians in arriving at a correct diagnosis of (a) acute viral hepatitis A, B, C, or D; (b) chronic hepatitis B, C, or D; (c) past infection with HAV, HBV, or HCV; (d) the relative infectivity of the hepatitis B patient; and (e) the determination of hepatitis A or B vaccine status. Detailed interpretations of the serologic markers observed during infection with HAV, HCV, and HDV can be found in the appropriate chapters of this book.

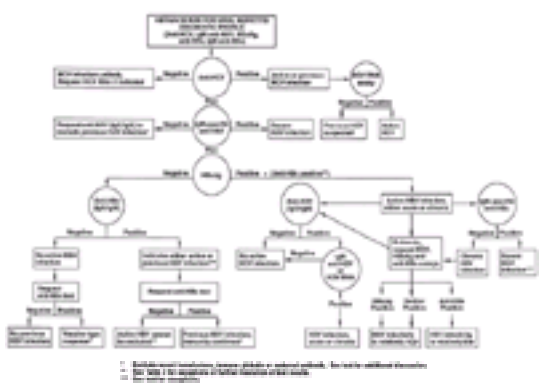

FIG. 6. Algorithm for the serologic diagnosis of HAV, HBV, HCV, and HDV.

Differentiating Acute Hepatitis from Chronic or Remote Infection

As summarized in Table 4 and illustrated in Figure 5 and Figure 6, detection of HBsAg in serum indicates that an active HBV infection, either acute or chronic, is present. Almost all HBsAg-positive sera from acute and chronic hepatitis B patients will be anti-HBc positive because it is only during the incubation period of acute hepatitis B, before liver abnormalities or clinical symptoms, that anti-HBc–positive reactions do not accompany the HBsAg response (see Fig. 5).

Patients with acute hepatitis B develop high titers of IgM-specific anti-HBc antibody commencing with the onset of aminotransferase abnormalities (158,415,417,443,602,650). These titers gradually decline over a 6- to 8-month period regardless of whether the disease is resolved or becomes chronic. By means of special research procedures, low concentrations of IgM-specific anti-HBc have been detected for up to 2 years in patients whose disease has resolved successfully, and in many chronic carriers (243,444,702,778). Among HBsAg carriers, the predominant component is a 7S IgM fraction, as opposed to a 19S component in acute disease (702,778). However, only a few chronic hepatitis B patients have an IgM-specific anti-HBc titer that is high enough to be detected in the conventional IgM anti-HBc dilutional assay. Even then, reactivity is relatively low when compared with that observed in acute disease. However, one event that can lead to a detectable IgM anti-HBc reaction in chronic disease is reactivation of HBV, especially after steroid withdrawal. Regardless, from a practical standpoint, the presence of IgM-specific anti-HBc at a dilution of 1:1,000 or higher (which is stipulated for that assay) is presumptive evidence of a recent acute infection (see Table 4).

Detection of anti-HBs and anti-HBc in an HBsAg-negative specimen normally indicates a previous infection with hepatitis B and confirms immunity against reinfection with HBV. However, in some hepatitis B–infected patients, especially in acutely ill neonates and in those with fulminant disease in which HBsAg expression by necrotic liver tissue is severely restricted, similar serologic results may be observed. In these situations, IgM-specific anti-HBc will distinguish between

acute disease or prior immunity.

The IgM-specific anti-HBc test also may provide assistance in evaluating HBsAg-negative sera from patients with hepatitis who are positive only for total anti-HBc (which measures both IgG and IgM anti-HBc) as shown in Table 4. Assuming that the solitary anti-HBc test result is not a false-positive reaction, the diagnostic alternatives include (a) chronic hepatitis B in which the HBsAg concentration is too low to be detected by conventional assays; (b) passively acquired anti-HBc [maternal–neonatal transmission, blood derivative therapy, immune globulin (IG) administration]; (c) previous hepatitis B infection with loss of detectable anti-HBs; and (d) acute HBV infection in which the sample was collected in the early convalescent stage of the illness when HBsAg has declined to undetectable levels (see Fig. 5). In this latter situation, the presence of a strongly positive IgM-specific anti-HBc result is definitive evidence for a recent (acute) hepatitis B infection. These sera also are likely to contain anti-HBe or, rarely, HBeAg. It is assumed that these patients are still infectious, although the degree of infectivity is muted by the low level of circulating virus present in the blood. Fortunately, fewer than 5% of acute clinical cases present with this serologic profile. Results can be verified and immunity documented by demonstrating anti-HBs seroconversion in 2 to 8 weeks.

In the absence of anti-HBs and high-titered IgM-specific anti-HBc, a serologic distinction between a chronic infection with undetectable HBsAg (an unusual event), or a past infection in which the anti-HBs level has declined to below the range of detectability, often is not feasible. Some of these patients could be co-infected or superinfected with HCV, which may interfere with the replication of HBV and result in a low-level HBV carrier (519). Testing these samples for HBV DNA by PCR may help resolve this issue, as would detection of HBV DNA in tissue from a liver biopsy. Another diagnostic approach is to challenge these patients with HBsAg vaccine and examine their blood for anti-HBs seroconversion 2 weeks later. Fewer than 3% of HBV-susceptible patients or chronic low-level carriers will generate anti-HBs levels of greater than 10 mIU/mL within this time interval. In contrast, the covert immune patient will generally develop an anamnestic response. Theoretically, completing the basic three-injection HBsAg immunization series in the initial nonresponders might separate the susceptibles from the low-grade carriers, because over 90% of the susceptibles should develop anti-HBs. The absence of anti-HBc in a specimen almost always excludes past or present hepatitis B, except in highly unusual situations (see the section, Seronegative Infection).

Relative Infectivity of Blood in HBV-Infected Patients

To determine whether the blood or body fluids of an HBsAg-positive patient are highly infectious or contain a relatively small concentration of infectious virions, the clinician can have the sample tested for HBeAg, anti-HBe, or HBV DNA. Because virtually all patients with acute hepatitis B are initially HBeAg positive (415,538), this diagnostic test should be reserved for chronically infected patients. HBeAg-positive specimens usually contain high concentrations of HBV DNA and infectious virions (>10^8 per milliliter of blood) (414,415,785), in contrast to anti-HBe–positive samples, in which the number of hepatitis B virions may be fewer than 100 per milliliter except in those individuals infected with precore mutants. As anticipated, HBeAg-positive patients are more likely to transmit hepatitis B sexually, percutaneously, or perinatally. In contrast, the HBV infectivity of persons who are HBsAg and anti-HBe positive is generally very limited. HBeAg is almost never detected in sera nonreactive for HBsAg.

As previously discussed (see the section, Pathogenesis), anti-HBe-positive carriers with precore mutations may have moderately high levels of HBV DNA. These patients can alternate between periods of apparent health and acute reactivation of their illness, or their clinical course may be very aggressive. When confronted with such a clinical picture, a specific evaluation for HBV DNA seems justified, especially if treatment with an antiviral agent is being contemplated.

Between 70% and 90% of infants born to HBV-infected mothers who are HBeAg positive are likely to become chronically infected, compared with fewer than 10% of infants whose mothers are HBsAg and anti-HBe positive. Fortunately, screening of mothers and immunization of at-risk neonates has reduced this concern (see the section, Prevention and Control). HBeAg, anti-HBe, and HBV DNA determinations also may be used to assess risk after an accidental needle-stick exposure or to judge the potential for sexual transmission.

Hepatitis B Immune Status

The conventional anti-HBs and anti-HBc assays are essential for determining the immune status of an individual to HBV, for evaluating risk factors associated with the transmission of HBV, and for assessing recovery or the progress of infected patients. Their presence confirms immunity to HBV, whereas their absence serves to identify those persons who are susceptible to HBV and who may benefit from being vaccinated. Both anti-HBs and anti-HBc results are necessary to render an appropriate interpretation concerning natural immunity to HBV. In a small percentage of patients, the concentration of anti-HBs may decline over time to undetectable levels, leaving the patient with an isolated anti-HBc result. As indicated previously, an anamnestic response to vaccination may serve to identify this group of individuals.

Anti-HBs is the principal neutralizing antibody of hepatitis B, and its appearance usually signifies recovery from infection (415). It has been detected in the IgG and IgM fractions of sera (427) and as an IgA antibody in secretions (569). Patients with preexisting anti-HBs are protected against acquiring HBV (44,314,327,427). Although this antibody is usually not detectable until the convalescent period (see Fig. 5), the occurrence of immune complexes leading to a serum sickness–like disorder during the prodromal phase of acute hepatitis B suggests that anti-HBs may be synthesized much earlier (16,195,320). When evaluating a response to the hepatitis B vaccine, only the anti-HBs test is required because the immunogen is HBsAg. Anti-HBc, a marker of viral replication (past or recent), remains nonreactive in this situation.

Depending on the circumstances, passive transfer of anti-HBs or anti-HBc may be observed after IG administration, or in neonates of mothers with recent or past hepatitis B infection. Because blood donations are pretested for total anti-HBc, passive transfer of anti-HBc and anti-HBs after transfusion of blood is unlikely. However, if a blood donor has been given the HBsAg vaccine, anti-HBs (but not anti-HBc) can be passively acquired by the recipient. Recognition of these possibilities can avoid an erroneous interpretation of serologic results.

Discordant or Unusual Hepatitis B Test Results

Profiles that yield discordant or unusual results require further evaluation (313). Factors leading to an isolated HBsAg, anti-HBs, or anti-HBc response already have been discussed. The most obvious isolated response encountered today is the presence of anti-HBs in HBsAg vaccine recipients, although solitary anti-HBc responses are becoming more evident, especially in patients infected with HCV. In general, an isolated antibody or antigen response should remain suspect until verified by repeat testing of the same sample or, preferably, by testing an additional sample.

Although a number of technical determinants may account for erroneous results, the testing of partially heparinized or severely hemolyzed blood, or the examination of sera from patients with coagulation disorders (258), still figures prominently in the occurrence of false-positive results, especially when testing for HBsAg. Most of the responses are low-positive reactions—near the cutoff value for the test—which is very uncommon among hepatitis B patients.

Anti-HBs reactivity in an HBsAg-positive patient is uncommon. It is most likely to occur (a) during resolution of acute hepatitis B, (b) in chronic carriers who have more serious liver disease, or (c) in carriers exposed to heterologous subtypes of HBsAg. Leading candidates for this type of response are parenteral drug abusers or individuals who receive multiple transfusions.

TREATMENT

Management of Acute Hepatitis B

There is no specific treatment for benign acute viral hepatitis. Thus, therapy should be supportive and aimed at maintaining comfort and adequate nutritional balance (for a complete discussion of this topic, see Management of Acute Viral Hepatitis in Chapter 26). It should be emphasized that there is no indication for the use of corticosteroids in the treatment of acute, uncomplicated hepatitis B, because they have no effect on the rate of resolution of the underlying disease process and may increase the rate of relapse (204,205). Furthermore, evidence has been presented that early treatment of acute hepatitis B with steroids may result in the development of persistent infection in a significant proportion of patients (208,667). Finally, the administration of HBIG to patients with fulminant hepatitis B is of no apparent benefit (4), and the therapeutic effectiveness of interferon or lamivudine on the course and prognosis of acute hepatitis B remains unknown.

Management of Chronic Hepatitis B

The goal of therapy for chronic hepatitis B is to suppress HBV replication to reduce symptoms, minimize chronic inflammation, and prevent progression to cirrhosis and HCC. Because the complete eradication of HBV infection often is not possible, the treatment is aimed at achieving a quiescent state of viral replication and remission of liver disease. Although the ultimate benefits on survival have not been demonstrated, several long-term follow-up studies on patients successfully treated with interferon have indicated a reduction in the complications that accompany end-stage liver disease (209,433,556).

Treatment is recommended for patients with persistently elevated serum aminotransferase values, active viral replication, and histologic evidence of moderate-to-severe inflammation or fibrosis. HBV carriers with normal aminotransferases should not be treated. Indications for other patient populations, including patients with severe extrahepatic manifestations, with human immunodeficiency virus (HIV) co-infection, with active alcohol or substance abuse, with renal disease, or with solid-organ transplantation, and patients on cancer chemotherapy or immunosuppressive agents, are less defined. Because serum biochemical tests and clinical evaluations often do not accurately reflect the extent of liver injury, a liver biopsy is recommended prior to initiation of treatment to confirm the diagnosis, to establish the grade and stage of liver disease, and to assess prognosis. Patients with contraindications for liver biopsy, such as coagulopathy, could be treated without a biopsy. The therapeutic end points employed in most clinical trials to define a complete response following the treatment of hepatitis B include HBeAg seroconversion to anti-HBe, clearance of HBV DNA from the serum (by a non-PCR assay), normalization of aminotransferases, and/or improvement observed in the liver biopsy. Because the first three parameters usually predict the last one, it is not necessary to perform a posttreatment liver biopsy unless in a protocol setting.

A number of agents have been used in the treatment of chronic hepatitis B. In general, corticosteroids, adenine arabinoside (and its water-soluble derivative), acyclovir, and dideoxyinosine have not been found to be beneficial and may even be deleterious (12,236). Several studies have shown that the administration of steroids to patients with chronic hepatitis B causes an increase in the serum concentration of HBsAg and HBV DNA and increases the expression of HBcAg in hepatocytes (424,634,655,783,819). Regulation of this response is via a glucocorticoid responsive element present in the HBV genome, which leads to the augmentation of an HBV enhancer element that presumably influences expression of specific HBV genes (782). In conjunction with this enhanced expression of virus and viral antigens during treatment with steroids, T-lymphocyte function is suppressed (280). The abrupt withdrawal of steroids elicits an intense, cell-mediated immune response that leads to the destruction of hepatocytes expressing these viral antigens. This event coincides with an increase in the serum ALT level, a decline in HBV DNA, and the development of symptoms of hepatitis. A similar response occurs after the discontinuation of cytotoxic chemotherapeutic agents. Either of these events has been associated with recurrence of disease and fulminant hepatic failure (81,233,331,437,765). In contrast, physicians must recognize that naturally occurring reactivation often culminates in substantial improvement of liver function, especially in HBeAg-positive carriers who are undergoing spontaneous remission of their disease (468). To administer steroids in this situation might abort this process and prevent clinical remission from occurring. Most of these individuals will eventually seroconvert to anti-HBe, a process that may take several months to develop. Currently, the only agents shown to have beneficial effects, and which are approved for the treatment of chronic hepatitis B, are alpha-interferon and lamivudine. Other agents under investigation are listed in Table 5.

Immunomodulatory agents
Nonspecific: interferons, thymosin, levamisole, IL-2, IL-12, etc.
Viral specific: therapeutic vaccines (pre-S/S vaccine, CTL epitope vaccine, and DNA immunization)
Cell based: adoptive immune transfer
Antivirals
Nucleoside analogs: lamivudine, famciclovir, adefovir, lobucavir, BMS-200475, L-FMAU, β-L-Fd4C, etc.
Glycosylation inhibitors: inhibit viral assembly
Gene therapy: antisense, ribozyme, targeted drug delivery, etc.

IL, interleukin; CTL, cytotoxic T lymphocyte.

TABLE 5. *Therapeutic approaches for hepatitis B infection*

Immunomodulatory Agents

Interferons are a class of molecules produced by higher eukaryotic organisms that have potent antiviral and cell regulatory (immunomodulatory) functions (605). Among the interferons, alpha interferon has been used successfully in the treatment of chronic hepatitis B (330). The recommended regimen is either 5 million units daily or 10 million units three times a week (or every other day) for 4 to 6 months. Side effects of interferon treatment are frequent and include constitutional symptoms, bone marrow suppression, neuropsychiatric effects, and the occurrence of autoimmune disease, especially of the thyroid.

During therapy, patients should be monitored frequently with hematologic and biochemical tests. Serologic assays for HBsAg, HBeAg, and HBV DNA level can be reserved for the end of treatment. Because seroconversion from HBeAg to anti-HBe can occur months after treatment (209,433,556), HBV markers should be followed at 3- to 6-month intervals. Transient increases in serum aminotransferase levels are common during therapy, particularly preceding HBeAg to anti-HBe seroconversion. This probably reflects the immune-mediated clearance of HBV-infected hepatocytes induced by alpha interferon. Typical patterns of treatment response to interferon are shown in Figure 7A and Figure 7B.

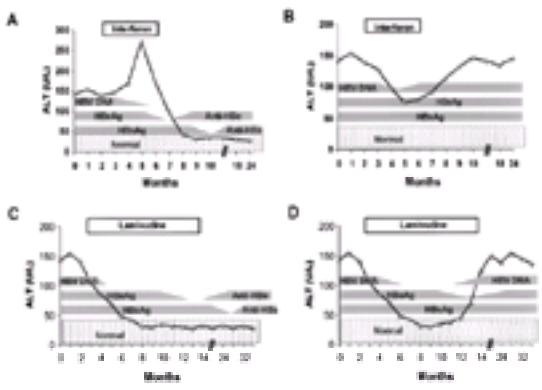

FIG. 7. Patterns of treatment response to interferon or lamivudine. **A:** Response to interferon. HBV viral levels gradually decline: Exacerbation of hepatitis with further elevation of ALT often precedes HBeAg seroconversion to anti-HBe. Many responding patients may experience late HBeAg seroconversion (after interferon treatment). Some patients also may experience HBsAg seroconversion to anti-HBs after treatment. **B:** Nonresponse to interferon. Transient ALT improvement and decline in HBV levels may occur, but no sustained response is noted. **C:** Response to lamivudine. HBV titers decline rapidly, usually to undetectable level (by a non-PCR assay) within 4 weeks. This is accompanied by improvement in ALT. HBeAg usually disappears gradually with or without anti-HBe development. HBsAg seroconversion occurs rarely. **D:** Development of lamivudine resistance. As in the responders, HBV titers decrease rapidly with ALT improvement but reappear late in the course of lamivudine treatment. The reemerging virus often carries lamivudine-resistant mutations, and relapse of hepatitis often accompanies viral resistance.

Several clinical, biochemical, and serologic factors are associated with a more favorable response to interferon treatment. They include low serum HBV DNA level, high serum aminotransferase concentration, active liver histology (inflammation and fibrosis on biopsy, but not cirrhosis), a short duration of infection, female sex, and absence of comorbid illnesses (330,814). The first two factors are the best predictors and probably reflect an underlying active anti-HBV immune response.

Based on a meta-analysis of 15 studies (Table 6) (814), a standard course of therapy resulted in the loss of HBsAg 6% more often in interferon-treated patients when compared to the natural loss of antigen seen in controls. Similarly, when compared to untreated controls, 21% of the treated patients more often lost HBeAg following treatment and 20% lost HBV DNA as determined by the relatively insensitive hybridization assay, which has a lower limit of detection of 1.5 to 3.0 pg/mL (500,000 to 1 million copies/mL). When tested by the more sensitive PCR methodology, most of these patients still have low levels of HBV DNA in their blood. Regardless, patients responding to therapy have a survival advantage over nonresponders.

Therapeutic intervention	Loss of HBsAG	Loss of HBeAg	Loss of HBV DNA
Alpha-interferon	7.8%	33%	37%
No treatment	1.8%	12%	17%
Difference	6%	21%	20%

Source: From ref. 814, with permission.

TABLE 6. *Meta-analysis of 15 studies on the efficacy of interferon in patients with HBeAg-positive chronic hepatitis B*

Children with active chronic hepatitis B (high serum aminotransferase concentrations) respond as well as adults to treatment with alpha interferon (654,712). Children with normal aminotransferase concentrations should not be treated, because they have a poor response rate (421). The standard regimen is 6 million units per square meter of body-surface area daily for 4 to 6 months.

Some patients with chronic hepatitis B have an atypical serologic pattern: elevated aminotransferase levels with a positive serum HBV DNA but no HBeAg. These patients are probably infected by a variant strain of HBV that has a mutation (or mutations) that inhibits the synthesis of HBeAg (see Viral Strains). Interferon therapy is effective in these patients, but the response rate is low and the relapse rate high (11).

Stimulators of HBV-Specific Immune Response

Patients with chronic hepatitis B have impaired anti-HBV cell-mediated immunity. Initial therapeutic approaches targeted at activating anti-HBV cellular responses have been attempted, but the antiviral effect has been weak (291). An alternative approach to overcome the tolerance and anergy of HBV antigens in chronically infected patients has been to use either the standard HBsAg vaccine or new pre-S–containing vaccines with or without novel potent adjuvants. Preliminary trials have shown some promise, but larger studies are required for confirmation and overall efficacy (164) (see also the later section, Immunization of HBsAg Carriers). Immunization with plasmid DNA-expressing HBV antigens has been shown to induce an immune response sufficiently robust to suppress viral replication in chronically infected animals (651). However, the duration and benefits of this response remain to be determined, and safety issues are a major concern. Finally, bone marrow transplantation from HBV-immune donors to HBV-infected recipients has been shown to lead to viral clearance in the recipients (435,697). Although this approach is not practical for therapy of chronic hepatitis B, a more viable option based on *in vitro* stimulation of autologous lymphocytes may be applicable. The anti-HBV immune response can be stimulated by *ex vivo* activation and reinfusion of immunocytes harvested from HBV-infected individuals. The *ex vivo* activation can be accomplished by either nonspecific [interleukin 2 (IL-2)] or specific (HBV antigens) stimulation. A preliminary trial using this approach showed some benefit (185).

Nucleoside Analogs

Most of the newer anti-HBV agents are nucleoside or nucleotide analogs that target the viral-encoded reverse transcriptase, inhibiting its activity and terminating DNA chain extension. Because the reverse transcriptases between the human immunodeficiency virus and HBV share structural and sequence homologies, many of the agents are effective for both viral infections.

Lamivudine is a cytosine nucleoside analog. It is phosphorylated by intracellular kinases to the active compound. The drug is rapidly absorbed, not altered by food, and has an *in vitro* intracellular half-life of 17 to 19 hours, making once-daily dosage possible. Hepatic impairment does not affect the pharmacokinetics of this product, but renal insufficiency may require dose adjustments. The dose used for HBV is 100 mg daily, which is lower than that for HIV (300 mg daily). Treatment of HBV patients with lamivudine is associated with a rapid and profound reduction of serum HBV DNA (median, five-log decrease) and an accompanying decrease in aminotransferase levels (189,239,420). Seroconversion from HBeAg to anti-HBe can be seen in 17% to 20% of patients (versus 6% to 7% of controls) after 1 year of lamivudine treatment. Biochemical and histologic improvements also are evident in those who respond to lamivudine. As with interferon, higher pretherapy levels of ALT influence HBeAg seroconversion (145,600). Reduction of HBV DNA to less than 10,000 genomes per milliliter by 20 weeks is also associated with HBeAg seroconversion (239). In those who seroconvert, remission is durable in most, histologic progression is slowed, and rates of HCC and liver-related deaths are reduced.

Lamivudine is well tolerated with few side effects. Therefore, long-term treatment has been proposed, as longer duration of therapy appears to result in increased rates of response. Because of an absence of bone marrow suppression, induction of autoimmune disease, and flares of aminotransferase values, lamivudine can be used in situations where interferon is not appropriate, such as decompensated cirrhosis, liver transplantation, and other immunosuppressed states (37,600). HBV carriers—even those with minimal liver damage—who are undergoing chemotherapy for malignancies should be prophylactically treated with lamivudine because of possible exacerbations of liver disease (19).

The major drawback of lamivudine is the emergence of drug-resistant mutants. In recent clinical trials, 14% to 39% of immunocompetent patients developed evidence of drug-resistant viruses (15,328,420,557). The emergence of the lamivudine-resistant mutants, usually occurring after 8 to 10 months of treatment, can be associated with increased serum HBV DNA and aminotransferase levels. Cessation of treatment is frequently associated with rapid emergence of the wild-type strain and can occasionally be accompanied by a marked flare of liver disease and even liver failure (456).

In addition, lamivudine-resistant mutants reemerge rapidly with retreatment, even at a higher dose of lamivudine (434). Lamivudine-resistant HBV strains contain mutations in the YMDD motif (Tyr, Met, Asp, Asp), which is part of the catalytic site (C domain) of the reverse transcriptase, and/or in the B domain (15). Mutation from methionine to valine or isoleucine at aa552 diminishes the binding affinity to lamivudine. Two other amino acid mutations in the B domain (V521L and L528M) occur primarily in combination with the YMDD mutation. However, despite these mutations, continual treatment is advocated to prevent relapse of the wild-type virus and to partially suppress HBV replication. Furthermore, this approach can lead to late anti-HBe seroconversion in some patients, which often is accompanied by an acute flare of hepatitis (456). Typical responses to treatment with lamivudine are shown in Figure 7C and Figure 7D.

Other nucleoside analogs, including famciclovir, ganciclovir, acyclovir, adenine arabinoside 5'-monophosphate (ARA-AMP), zidovudine, didanosine, and fialuridine have been tested clinically in patients with chronic hepatitis B (330). None of them have been shown to induce lasting beneficial effects on HBV DNA level and disease activity, and many of them are poorly tolerated or have significant toxicity.

Adefovir dipivoxil is an adenosine monophosphate analog that is a potent inhibitor of HBV reverse transcriptase *in vitro.* In recent clinical trials, adefovir has been shown to reduce serum HBV DNA by several logs with improvement of liver disease (290), and it appears to be clinically active against drug-resistant mutants of lamivudine (584,820). Therefore, lamivudine and adefovir may be promising as combination therapy to reduce the occurrence of drug resistance. Although adefovir is well tolerated, renal toxicity at higher doses may become a limiting factor. The effect is cumulative, with crystal formation injuring the proximal tubules and resulting in a Fanconi-like syndrome. However, this appears to be reversible with discontinuation of the drug. Other promising nucleoside analogs are 1-(2-fluoro-5-methyl-beta- L-arabinofuranosyl) uracil (L-FMAU), 2',3'-dideoxy-2',3'-didehydro-b- L-5-fluorocytidine (b-L-Fd4C), 5-fluorothiacytidine (FTC), and dioxolane guanosine (DXG), all of which have been shown to be potent inhibitors of HBV replication *in vitro* and in animal models (813,837). Unlike the fluorinated nucleoside analog fialuridine (496), the L forms appear to have no or little mitochondrial toxicity.

Novel Antiviral Approaches

Targeted delivery of antivirals to the liver has been developed to minimize side effects and reduce the dosages of the drugs. Preliminary studies have supported the utility of this approach (401,770). Development of antisense molecules, ribozymes, dominant negative mutants, or other gene therapy approaches to specifically inhibit HBV transcription or replication is theoretically possible and has shown some promises *in vitro* and in limited animal studies (80,565,805,818,839). However, these approaches are limited by a variety of obstacles including the foremost problem of efficiency and specificity of the delivery technology.

Combination Therapy

Interferon in combination with other immunomodulatory agents has been tested in various clinical trials but does not appear to be more effective than treatment with interferon alone. Results from a pilot study and a multicenter placebo-controlled trial showed that the combination of lamivudine and interferon may confer a marginally better response than lamivudine alone (539,665). Therefore, the more promising approach of combination therapy is probably in the use of direct antivirals such as nucleoside analogs.

EPIDEMIOLOGY

Prevalence and Risk Factors

HBV is a ubiquitous organism that is globally distributed. Although HBsAg has been found in the serum of certain species of nonhuman primates, humans remain the principal reservoir for HBV. In the United States in 1998, the number of cases of hepatitis B reported to the National Notifiable Diseases Surveillance System operated by the Centers for Disease Control (CDC) was 10,258, a rate of 3.8 cases per 100,000 population (140). This represented 28% of the total number of viral hepatitis cases reported. Approximately half of the hepatitis B cases occur in persons 25 to 39 years old. It is estimated that the true incidence of hepatitis B is 15 to 30 times the reported rate, of which about half are symptomatic. Since 1987, the number of reported cases of acute hepatitis B has declined by over 60%. This presumably has occurred as a direct result of universal immunization of neonates, vaccination of at-risk populations, life-style or behavioral changes in high-risk groups, refinements in the screening of blood donors, and the use of virally inactivated or genetically engineered products in patients with bleeding disorders.

To understand and appreciate the various epidemiologic patterns found in hepatitis B requires that the source of infectious virus be known. This is usually the blood, but the virus also has been detected in a variety of other bodily secretions and excretions, including semen (22,172,363,385,675), saliva or nasopharyngeal fluids (22,40,172,363,385,675,792), and menstrual fluid (169,352). Intravenous inoculation of chimpanzees with semen from HBV carriers (22), as well as the intravaginal instillation of semen in gibbons (675), has resulted in the transmission of hepatitis B. On the other hand, oral ingestion of semen failed to infect these animals. There is no convincing evidence that airborne infections occur (322). Feces have not been incriminated as a source of infection (287,474,503,548,549). Virus is sometimes present in urine (172,385,705), but this is probably of limited epidemiologic importance. Exceptions may occur when persons are exposed to chronically infected, diapered infants or to the urine of HBsAg-positive uremic patients undergoing hemodialysis (179). Gastric juice is not a source of HBV (684), but HBsAg has been detected in both bile and pancreatic juice (310,684). Pleural fluid, ascitic fluid, cerebrospinal fluid, tears, and breast milk also have been positive for HBsAg (120,176,668,750). From these reports, it should be assumed that all biologic fluids from HBV-infected patients may be infectious and capable of transmitting HBV. This is especially relevant when percutaneous or mucous membrane exposure has occurred, because this is the most efficient route for introducing the virus into the susceptible host. When you consider the high concentration of virus that may be present in the blood of an HBeAg-positive carrier and the stability of HBV (96), the possibilities for transmission are abundant.

Serologic surveys have documented the existence of hepatitis B throughout the world, including the most isolated and remote areas. The true prevalence of the disease can only be estimated because the collection of accurate data has been hampered by inadequate reporting, lack of laboratory confirmation, insensitive assays, and selection bias. Nevertheless, it is clear that the HBsAg carrier rate varies from country to country, with higher rates being reported from developing countries with primitive or limited medical facilities (Fig. 8) (35,83,89,260,711,733). Even within apparently homogeneous groups in the same country or area and among different ethnic groups, the HBsAg carrier rate may vary substantially (269,364,711,733,744,816).

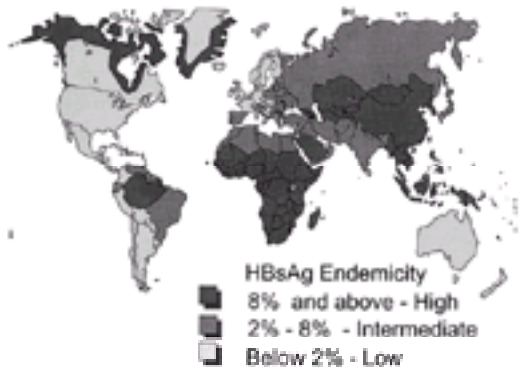

FIG. 8. Prevalence of HBsAg in the world. (From M. Kane, personal communication, 1999.)

There is no seasonal trend for HBV infection, and epidemics are unusual. Cumulative infection rates in the United States increase with age, with an overall age-adjusted prevalence of 4.9%, based on the presence of anti-HBc. Among the major racial groups, the rates were 2.6% in non-Hispanic whites, 4.4% in Mexican-Americans, and 11.9% in non-Hispanic black participants (502). The male-to-female ratio was 1.5:1. From these data, an estimated 1 to 1.25 million Americans are believed to have contracted chronic hepatitis B. Those with an enhanced likelihood of having acquired this infection include residents and staff of penal institutions and residential facilities for the developmentally disabled; multiply transfused patients (pre-1988); patients and staff of oncology or hemodialysis units and organ transplantation patients; male homosexuals and sexually promiscuous groups; users of illicit injectable drugs who share needles; close contacts of chronic carriers; sexual partners of patients with acute hepatitis; neonates born to women who have acute or chronic hepatitis B; and health care workers (particularly dentists, surgeons, and clinical laboratory staff). However, the availability of an effective vaccine, optimized blood donor screening, and better sterilization procedures for blood derivatives have dramatically altered the risk in many of these groups.

Based on data obtained from the Sentinel County Program sponsored by the Hepatitis Branch of the CDC (M. J. Alter, personal communication, 2000), acute hepatitis B cases resulting from illicit injecting drug use have decreased by 90% since 1988 (to 21%), whereas cases in heterosexuals who had sexual contact with a hepatitis B patient, with an HBV carrier, or with multiple partners declined by more than 40%. Homosexual activity is currently associated with 15% of the cases, whereas hepatitis B in health care workers declined from a range of 3% to 6% down to 1%, perhaps due, in part, to vaccination. Thus, heterosexual activity remains the single largest risk factor, accounting for 45% of the acute HBV cases seen between 1991 and 1997.

In endemic areas of Asia and Africa, the epidemiologic patterns of infection are different from those seen in North America and western Europe (735). Serologic surveys indicate that most infections in these geographic regions occur in infants and children as a result of maternal–neonatal transmission or early childhood contact, although percutaneous exposure with contaminated needles or following unsafe injections is always a possibility in these countries. An annual incidence of infection among children of 3% to 5% has been projected.

Modes of Spread

Percutaneous or Parenteral Transmission

Percutaneous or parenteral transmission is one of the principal modes of spread of hepatitis B in communities. The risk of acquiring infection following the administration of blood or blood components has undergone a dramatic reduction in recent years as a result of (a) the addition of sensitive and specific anti-HBc testing in 1986 to complement incremental improvements in HBsAg testing, (b) the use of an all-volunteer blood donor population, (c) improvements in assessing the qualification of donors and the implementation of donor self-deferral, and (d) the development of viral inactivation procedures in conjunction with the use of recombinant products to render blood components and derivatives safe. The current residual risk of receiving a unit of HBV-infected blood from a screened donor during the seronegative window period is estimated to be 1 in 63,000 [95% confidence interval (CI): 31,000 to 147,000] (118,671). New screening tests such as nucleic acid testing for HBV DNA and /or enhanced sensitivity of the HBsAg assay should reduce the risks even further.

Although the situation has changed dramatically, prevalence rates of hepatitis B are highest in hemodialysis patients and in adult hemophilic patients who, prior to 1988, received blood components and derivatives that had not been purified or virally inactivated. Up to 75% of HIV-positive and 33% of HIV-negative adult hemophilic patients have serologic evidence of hepatitis B infection as a result of exposure to contaminated blood derivatives (777), with active HBV disease being demonstrated in 12% and 4%, respectively.

In contrast to the current reduced risk of hepatitis B found among recipients of blood products, the hazards of

percutaneous exposure associated with repeated or one-time sharing of contaminated syringes and needles by users of illicit drugs continue to be responsible for many of the hepatitis B cases seen today in the United States (24,132). If followed long enough, most members of this group will develop clinical and/or serologic evidence of hepatitis B (238,252,679).

HBV infection is common in some, but not all, hemodialysis units and in unimmunized household contacts of hemodialysis patients (611,740,767). In 1997, a CDC survey of 3,077 hemodialysis centers in the United States, representing 253,001 patients and 63,054 staff members, revealed that the incidence of HBV infection had decreased from 3.0% in 1976 to 0.05% among patients, and from 2.6% to 0.05% among staff members, with the greatest decline occurring between 1976 and 1980 (767). Similarly, during the same period, the prevalence of HBsAg positivity among patients and staff declined from 7.8% to 0.9% and from 0.9% to 0.4%, respectively. The higher rate of chronic disease in patients can be attributed to a disease-related immunologic deficiency in many hemodialysis patients that prevents them from clearing HBsAg from the blood (or responding to vaccine). In 1997, 3.3% of the centers reported one or more newly acquired HBV infections, whereas 24.1% reported having one or more patients with chronic HBV infection. The virus generally spreads from patient to patient, or from patient to staff member, via contaminated equipment or the environment (169,214,709). Not only is the antigen stable on various surfaces for extended periods (211) but infectivity is retained (96). Reuse of dialyzers is not associated with any increased risk of HBV infection in either patients or staff. The use of hepatitis B vaccine was approximately 70% effective in preventing newly acquired HBV infection (518). By 1997, 47% of patients and 87% of staff members had received at least three doses of hepatitis B vaccine.

Health care personnel have a higher prevalence of HBsAg or anti-HBc than do those who have no occupational exposure to patients or blood products (450,595,666). In contrast, the recent incidence of clinical hepatitis is very low because of requirements for vaccination by the Occupational Safety and Health Administration (OSHA). The prevalence of hepatitis B markers increases with age and years of occupation (177,190,666) and is a function of the frequency of percutaneous and permucosal exposures to blood or body fluids containing blood (139,190,491). The unrecognized reservoir of HBV infection that is present in about 1% of the patients admitted to urban hospitals (216,464,480,492) represents the principal hazard to hospital personnel who handle blood or bodily fluids (438,449). In 1993, an estimated 1,450 health care workers became infected with HBV through exposure to blood and serum-derived body fluids, a 90% decrease from 1985 (139,685). An estimated 100 to 200 health care workers have died annually during the past 15 years because of the chronic sequelae of HBV infection.

Transmission of HBV by the percutaneous route also may occur as a result of inadvertent (or inapparent) inoculation after the use of shared razors, toothbrushes (531), and communal bath houses (105) or towels, and by tattooing (462,535,663,706), acupuncture (555), or ear piercing (374). In addition, transmission of hepatitis B has been documented after a human bite by an HBV-infected person (279,477). Engorged mosquitoes, bedbugs, and ticks, particularly if collected in homes or areas where HBsAg carriers reside, may be positive for virus or HBsAg (108,187,376,554,570). Under suitable circumstances, viral dissemination by mechanical transfer may occur, although the epidemiologic importance of this transmission pattern is still being debated (490). However, in one study, inoculation of HBsAg-containing mosquito tissue or the feeding of HBsAg-positive mosquitoes or ticks to susceptible chimpanzees failed to transmit HBV (70,377).

Contact-Associated Transmission

The hypothesis that contact-associated transmission of hepatitis B may exist comes from several disciplines. Between the late 1930s and mid 1970s, the traditional premise that hepatitis B is communicable only by a percutaneous route was altered when uninoculated contacts of icteric patients and familial clusters of hepatitis B cases were observed (252,277,303,354,741). Currently, one of the most important routes of transmission of HBV in the United States is by sexual contact with an infected person, either heterosexual or homosexual.

The successful experimental transmission of hepatitis B to children by the oral route using human serum containing high-titered virus, although with apparently minor clinical consequences, has been cited as supporting the possibility that oral transmission may occur under natural conditions (249,411). The subsequent detection of HBV in saliva (22,40,172,287,363,385,477,675,792), albeit at concentrations that were 100 to 10,000 times less than that found in serum (172,363), intensified interest in saliva as a mechanism for transmitting HBV. It has been postulated that saliva is contaminated by virus entering the mouth through the crevicular fluid at the gingival surface following rinsing of the mouth with water or after mastication (322). Alternatively, peripheral blood lymphocytes may be another source of HBV DNA in saliva (172,830). In three separate animal studies (22,40,675), five anthropoid apes were found to be susceptible to HBsAg-positive saliva, but only by parenteral routes (subcutaneous and intravenous). No hepatitis B occurred in the 13 primates that received HBV-contaminated saliva by the oral route. It is concluded that unless the inoculum is large or the concentration of virus is high, oral transmission by saliva or by objects contaminated with saliva is probably rare in nature, although percutaneous transmission via a human bite has been reported (119,279,477).

Hepatitis B has long been recognized as endemic in residential facilities for the mentally handicapped, no doubt reflecting problems of crowding and poor hygiene (409,411,416). A matched-pair susceptibility study evaluating equally exposed clients demonstrated that those with Down syndrome—like hemodialysis patients—frequently fail to develop specific antibody and are more likely to become chronic HBsAg carriers, whereas other mentally handicapped clients appear to resolve their infection successfully with the production of anti-HBs (321). The advent of immunization has rectified this situation so that clients are no longer acquiring HBV infection.

HBV transmission is a relatively common event among individuals residing in the same household with an HBsAg-positive carrier (277,289,603,604,741,791). In these situations, the occurrence of clinically overt infections is relatively uncommon. The actual mechanism of transmission is not always known, but sexual transmission or inapparent exposure through shared razors, towels, toothbrushes, oozing skin lesions, or fomite contamination of surfaces is frequently suspected.

Although all household contacts of a chronically infected individual are at high risk of infection, not all are equally likely to become carriers once infected (see also Fig. 3) (72,260,736). Siblings of carriers are found to be HBsAg positive more frequently than are parents (736). Transmission from HBsAg-positive carrier fathers to their offspring is relatively limited, in contradistinction to spread from carrier mothers to their children. Spouses of carriers also are infected frequently, but they are less likely to become persistently infected (72,260,736). Transmission of HBV from men to women appears to be more efficient than the corollary (590). Risk factors favoring transmission from HBsAg carriers to another household contact include the presence of high concentrations of HBV DNA or HBeAg in the serum of the carrier (603). Serologic evidence of intrafamilial spread is greatest in nonwhite families and increases with family size (736). The risk of infection also is increased in those families who adopt or care for HBsAg-positive children who come from high-risk areas of the world, such as India, Korea, Southeast Asia, Rumania, or Russia (560,790), because infants and children are more likely to be HBeAg-positive and thus are potentially more infectious.

In contrast to the rather pervasive transmission pattern seen in families exposed to chronically infected individuals, spread of hepatitis B among household contacts after acute disease appears to be a more limited process. At greatest risk is the spouse or sexual partner, with parents, siblings, or other domestic contacts having a much lower probability of acquiring hepatitis B (Table 7) (303,396,531,604).

		% Infected	
Investigators (ref.)	Number of susceptibles	Spouses or sexual partners	Nonsexual contacts
Mosley (531)	170	17.6	1.5
Peters et al. (604)	101	20.0	5.5
Koff et al. (396)	81	23.1	0.0

TABLE 7. *Secondary attack rates in household contacts of persons with acute hepatitis B*

Increasing promiscuity and the early age at which many teenagers now begin sexual activity have increased the incidence of venereal diseases, and thus probably hepatitis B as well, in these younger age groups. Rates of infection are especially significant among young homosexual men and prostitutes (232,292,362). Previously, about 7% of homosexual men were HBsAg positive, and nearly 60% of these also were HBeAg positive (159,201,734,737). The main reason for the high rate of infection among men having sex with other men is exposure to large numbers of sexual partners, coupled with the promiscuity of these partners. Men with monogamous relationships are at lower risk. Receptive rectal intercourse, especially when associated with rectal bleeding, increases the risk of transmission, implying that trauma to the rectal mucosa may facilitate entry of HBV (670). The frequency of disease in this group has decreased dramatically in recent years (132), as homosexuals have modified their high-risk behavior in an attempt to prevent HIV infection and have availed themselves of the hepatitis B vaccine.

Maternal–Neonatal Transmission

Perinatal and, uncommonly, transplacental transmission of viral hepatitis type B from an infected mother to her offspring have been documented often enough to suggest that this may represent the most important mechanism for maintenance of an HBV carrier population in certain areas of the world (493,506,571,674). It is estimated that at least 23% of the carriers in Asia and 8% of the carriers in Africa occur as a result of perinatal transmission (381). For the United States, 20% to 30% of the carrier pool is believed to result from perinatal and preschool transmission, even though only 1% to 3% of reported cases of HBV occur in children under 5 years of age (483).

Women with acute hepatitis B late in pregnancy or during the neonatal period readily infect their infants (506,674,768). On the other hand, neonatal transmission is infrequent when the acute infection occurs in the first or second trimester of pregnancy (674,704). From these data, it is concluded that the virus apparently does not cross the placenta easily. Women who are chronic HBsAg carriers also transmit the virus; however, the rate of infection varies among ethnic groups and in different parts of the world (180,588,718). For example, transmission from carrier mothers is unusual in most white populations (fewer than 10% transmit the virus) but is common among Asians (40% to 50%). In Senegal and other parts of Africa, infection of children more often occurs during the first few years of life, in contrast to Asia, where perinatal transmission is more common (59,430,718). This reflects differences in the prevalence of the HBeAg (e.g., virus burden) in these populations.

The most important feature of HBV infection in the newborn is its chronicity. From 70% to 90% of HBeAg-positive carrier women transmit the virus to their newborn infants (59,440,572,695,719), 80% to 85% of whom become chronic carriers. Among HBeAg- and anti-HBe–negative carrier mothers, most of whom are low-level HBV DNA positive, 10% to 12% of their infants will become chronically infected. On the other hand, babies born to mothers who are anti-HBe positive, especially if they are HBV DNA negative, seldom become infected, or the HBV infection is usually transient. The majority of infants who become carriers are asymptomatic and have variable elevations of their aminotransferase levels (718). The liver biopsy usually shows mild chronic hepatitis (674). However, some develop acute hepatitis (215,674), and severe icteric hepatitis has been reported in infants born to anti-HBe–positive carrier mothers (699). An estimated 25% of the carrier infants eventually succumb during adult life to the more serious complications of HBV infection, such as

cirrhosis or HCC (88,430,571).

Infants born to hepatitis B carriers and to women with acute hepatitis B late in pregnancy are usually HBsAg-negative at birth and develop antigenemia at 1 to 3 months of age. This coincides with the known incubation period for HBV if infection had occurred at the time of delivery and indicates that perinatal transmission from mother to infant probably occurs during labor or delivery in most cases. How the perinatal infection occurs is not known, but HBsAg has been detected in maternal blood, amniotic fluid, cord blood, breast milk, and vaginal secretions (101,180,440,588,674,718). The fetus swallows amniotic fluid *in utero* and frequently swallows maternal blood during passage through the birth canal. Gastric aspirates taken shortly after birth are often HBsAg positive (440). In contrast, several studies have failed to show a correlation between the presence or absence of HBsAg in cord blood and the eventual development of hepatitis B (440,588,674). Transmission also might occur through fetal monitoring sites, minor abrasions, or even across mucous membranes. Finally, it is suspected that 5% to 10% of infants born to HBsAg-positive mothers may be infected late *in utero* (52,375,720,751). Maternal red blood cells are known to cross the placenta during labor, and it is assumed that HBV also could cross the placental barrier at that time or even earlier.

Theoretically, the virus could be transmitted after birth through breast-feeding because HBsAg (and presumably HBV) can be detected in breast milk and could be present in maternal blood or serum that oozes from cracks or fissures around the nipple. However, in high-risk infants (i.e., those born to HBeAg-positive carrier women), breast-feeding has been shown to pose no added risk to the infant (58,180).

Nosocomial Transmission

In hospital settings, contamination of mucous membranes with infectious material may follow mouth pipetting accidents, incidental eye contamination, or direct contact with the eye or mouth by infected fingers. It also is not uncommon for members of the health care profession to acquire hepatitis B from patients following a needle stick or other sharp-instrument injury. Vaccination essentially eliminates this risk. On the other hand, transmission in the opposite direction—from hospital staff to patients—is infrequent. Nevertheless, health care providers who are acutely or chronically infected with HBV are potential sources of infection to their patients. In many situations, performance of exposure-prone procedures have been implicated. The largest published provider-related outbreak of hepatitis B (55 cases) was traced to an asymptomatic oral surgeon who was an HBsAg/HBeAg-positive carrier (648). Frequent hand cuts were suspected to be responsible for spread of the infection. At least six nosocomially related outbreaks (30 patients) were associated with obstetric–gynecologic surgeons (447,804). Clusters of cases in Britain from 1980 to 1983 involving 22 patients were traced to cardiac surgery units, a surgical registrar, and a perfusion technician (617). Other outbreaks (17 cases) involved an HBsAg-positive dentist who returned to practice after developing postnecrotic cirrhosis following acute hepatitis B (448) and an inhalation therapist with acute hepatitis B (710). The inhalation therapist was responsible for obtaining blood samples from indwelling arterial cannulas during open-heart surgery. She had a severe exudative dermatitis on both hands for which she was receiving topical and systemic steroids during the incubation phase of her illness, and she never wore gloves while collecting samples.

In contrast to these studies, prospective serologic surveys involving three chronic HBsAg-positive health care workers (an endocrinologist, a medical consultant, and a practical nurse) and a physician, food handler, and two dentists with acute hepatitis B have provided evidence that transmission from such personnel to their patients is highly unusual (20,810). Clearly, the real risk has not been established. However, in most instances, a number of interacting, fortuitous events seem to be necessary for transmission to occur. For example, the subjects are usually HBeAg positive and often unaware of their infection. In the limited number of studies in which transmission has occurred following exposure to an HBeAg-negative surgeon, a precore mutation was present (301,729). In these situations, measurable quantities of HBV DNA exceeding 250,000 copies per milliliter have been detected. In some studies, a medical condition that favors transmission was sometimes present. Finally, gloves often were not worn despite the fact that a traumatic-type procedure was being performed. For dentists and oral surgeons, the opportunities for blood droplet transmission after puncture wounds sustained in the oral cavity is well recognized (322). Potential control measures (e.g., use of gloves) should reduce the risk in most cases.

HBV and Hepatocellular Carcinoma

The incidence of HCC varies with geography, race, age, sex, and cirrhosis (581). HCC constitutes 90% of the primary malignant tumors of the liver observed in adults. Worldwide, liver cancer ranks eighth in frequency. It is the sixth most frequent cancer in men and eleventh most common in women. From 4% to 5% of the global cancer burden is attributed to this condition, and in developing countries it is the third most common cancer in males after the stomach and lungs (592,614). It is estimated that liver cancer may be responsible for more than 500,000 deaths annually throughout the world, with a male-to-female ratio of 3:1. In the industrialized nations of the world it is rare to relatively uncommon, yielding an age-adjusted incidence rate of less than 10 per 100,000 population per year in men from northern Europe, North America (except in migrants from high-risk countries such as China and Korea), Central and South America, and Australia. In the United States, HCC accounts for 2% to 3% of new cancer cases of the digestive organs, or approximately 4,000 to 6,000 cases per year (99,698,749). In contrast, in many areas of sub-Saharan Africa, Melanesia, Southeast Asia, China, and Japan, annual age-adjusted incidence rates from 23 to 48 per 100,000 among men have been recorded. The mortality-to-incidence ratio is close to or greater than 1, which indicates that most patients do not survive for more than 1 year.

A number of epidemiologic studies have shown a strong relationship between persistent or past infection with HBV and the eventual development of HCC. It is noteworthy that the frequency of HCC follows the same general geographic pattern of distribution as that of persistent HBV infection (see Fig. 8) (489). In cohort studies in developing countries, the incidence of HCC ranged from 240 to 281 per 100,000 HBsAg carrier-years (144,430).

Perhaps the most conclusive study designed to support the hypothesis that HBV plays a primary role in the pathogenesis of HCC was the prospective study of Beasley et al. (53; and R. P. Beasley, personal communication) in which 22,707

Chinese men in Taiwan were followed for a mean of 11.25 years. In this study, all but three of 194 men who subsequently died of HCC were in the original group of 3,454 men who were HBsAg positive. Nine of the 10 HCC patients who had seroconverted and had become HBsAg negative at the time of death were still anti-HBc positive (with or without anti-HBs). The annual incidence of HCC remained essentially constant over 10 years, implying that new liver cancers were continuously developing. There was an excess risk of dying from HCC among HBsAg carriers compared to noncarriers (relative risk of 217). A high carrier rate among the mothers of HCC infants supports the contention that HBV infection antedates the development of HCC and not the reverse. It also implies that offspring who are infected at birth are more susceptible to developing HCC in the future (50,430).

Although most HCCs occur in patients with cirrhosis, 15.7% to 45% appear to develop in the absence of cirrhosis (144,389,460,580,696), which is in contrast to HCV-associated HCC, in which over 90% occur in a background of cirrhosis. In patients without cirrhosis, the annual incidence of HCC is about 0.3%; this increases to 2% to 7% in male patients with cirrhosis (460,570) Rates in females are much lower. Factors accelerating this transition include hepatic decompensation, intermittent episodes of reactivation, duration of disease, alcohol intake, age, and, as indicated, male sex. Of the various types of cirrhosis, macronodular or mixed macro- and micronodular cirrhosis is more frequently associated with HCC than is micronodular cirrhosis. In this regard, it is noteworthy that HBV is more often associated with macronodular cirrhosis, whereas mixed macro- and micronodular cirrhosis is more often found in HCV-infected patients, with a predominance for the mixed type of cirrhosis (400).

HCC is common among Asian-Americans, in whom the prevalence of HBV markers is 10 to 40 times higher than in other ethnic groups living in the same area (744,749). Nearly all of the HBV infections are of the subtypes most prevalent in Asia (*adw* and *adr*). The age-standardized mortality rate from HCC of 131 per 100,000 among Chinese men in New York City was four times higher than that among black men (33.5 per 100,000), and 10 times higher than that among white men (12.5 per 100,000) (744). The excess mortality was observed at all age groups. These data indicate that Chinese-Americans are at high risk for both HBV infection and HCC, and they provide support for the hypothesis that the two conditions are causally related.

The incidence of HCC (and death from HCC) increases progressively with increasing age (53,388). However, the peak incidence of the disease may vary in different countries, especially in Mozambique, which has one of the highest incidence rates of HCC in the world, accounting for 65% of all malignant tumors in men and for 31% of the tumors in women (623). The shift toward a younger age group among Mozambicans compared with Chinese who are infected with HBV at a similar age (Fig. 9) suggests that exposure to environmental carcinogens, such as aflatoxin in Mozambique, may be an important determinant in these differences.

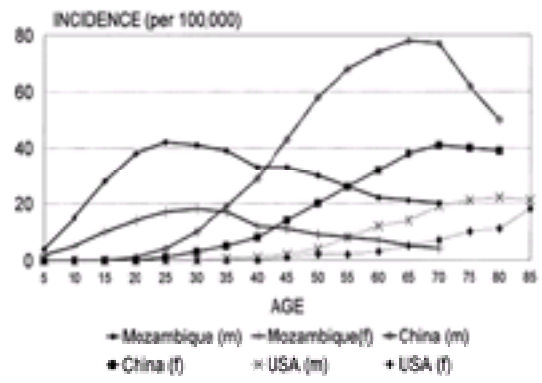

FIG. 9. Age-specific incidence rates of HCC for males (m) and females (f) in Mozambique, China, and the United States. (From F. D. Chen, personal communication, 1995.)

The emerging concept of the pathogenesis of HCC is that HBV infection in early life proceeds from chronic antigenemia to chronic hepatitis, then to cirrhosis, and finally to HCC, usually after a delay of 30 to 50 years (see Fig. 4) (92,690). Although both sexes may be equally infected at birth, men are more apt to develop chronic hepatitis (by a ratio of approximately 1.5:1). More men than women develop cirrhosis of the liver (by a ratio of 3:1), and an even greater proportion develops liver cancer (by a ratio of 6:1). Thus, at each progressive stage of liver disease, the ratio of men to women appears to double so that HCC becomes predominantly a disease of men (92,342,768). Additional factors accounting for the higher incidence rates in men might be their greater exposure to chemical carcinogens. Details concerning the cellular and molecular mechanisms of hepatocarcinogenesis of HBV and the role of environmental carcinogens can be found in Chapter 86.

PREVENTION AND CONTROL

HBV is a ubiquitous organism that is globally distributed. Over 75% of the world's population lives in areas where there are high levels of infection. It is estimated that more than 2 billion people have been, or are currently, infected with HBV, resulting in over 350 million carriers worldwide. Approximately 25% of these will eventually die from chronic liver disease or HCC, with as many as 1 million deaths per year being attributed to the ravages of this virus. Depending on the estimates, 2.5 to 4 times more people will perish from chronic HBV than from HIV. Since 85% to 95% of hepatitis B infections are vaccine preventable, emphasis should be placed on vaccination in resource-poor countries to limit the spread of this disease. Indeed, the greatest impact is being experienced in Asia, where over 75% of the world's chronic infections exist (estimated at 287 million, compared to 1.9 million infections in North America).

Prevention and control of hepatitis B depends on the proper selection and screening of blood donors, education of the populace regarding patterns of transmission, use of environmental procedures to limit the risk of infection, employment of

universal blood and body fluid precautions, and appropriate immunoprophylaxis (212,213). The latter includes a comprehensive vaccination strategy that focuses on prenatal testing of mothers, with vaccination of at-risk newborns and identified household contacts, universal immunization of all infants, and vaccination of adolescents and adults at high risk of infection.

The establishment of an all-volunteer blood donor population who donate periodically, combined with a stringent health screening process and mandatory testing of blood donors for HBsAg and anti-HBc, has led to a substantial reduction in transfusion-associated hepatitis B. Between 1981 and 1988, the number of posttransfusion hepatitis B cases reported to the American Red Cross declined by approximately 50% (194). This corresponded with the addition of anti-HBc testing of blood donors in 1986 that presumably identified low-level HBV infected individuals whose blood contained only anti-HBc (534). Currently, the residual risk of detecting an HBV infection in a donor sample is estimated to be 1 out of 63,000 units of blood processed (95% CI: 31,000 to 147,000) (118,671).

Acquiring hepatitis B is a well-recognized risk among health care personnel who work in blood-contaminated environments, such as surgical suites, hemodialysis units, obstetric services, gastrointestinal endoscopy suites, hematology and oncology wards, and blood-processing facilities (252,594,710,767). However, between 1985 and 1993, reported infections with HBV declined 90% among healthcare workers (133,137,139,685). The risk depends on the frequency of percutaneous and permucosal exposure and the concentration of the inoculum (190,247,274,686,762). The highest concentration of virus is found in blood and liver; thus, the most efficient route of transmission is percutaneous. Infection also can occur after direct mucous membrane contact, after oral ingestion of blood, or through contamination of skin lesions, open cuts, or abrasions. The risk of infection following a needle stick from an HBsAg-positive patient ranges from 19% in recipients of sera positive for HBeAg, to 2.5% among recipients of anti-HBe positive sera, a risk ratio of about 8:1 (806). Even risk following accidental inoculations with HBeAg-positive samples is variable and often associated with the concentration of virus in the sera (487). Larger doses of virus are apparently necessary to initiate infection via the oral route (see the section, Modes of Spread). Infections after fecal contamination have not been reported. Thus, enteric precautions are not necessary once the diagnosis of hepatitis B has been made. Other modes of transmission that have not been associated with HBV outbreaks include food-borne, waterborne, or airborne routes, or via casual contact.

Universal Blood and Body Fluid Precautions

Universal precautions represent an approach to infection control that should be used when handling human blood and body fluids (586). All body fluids are treated as if they are infectious, and methods should be devised to prevent contact with such material. Although the first line of defense is immunization, experience has taught us that not everyone will avail themselves of this proven method. Specific precautions include the use of gloves when handling potentially infectious materials contaminated with blood, when performing autopsies, or when dermatitis, cuts, or abrasions are present on the hand. Most inadvertent self-injections are the result of carelessness (e.g., recapping needles by a two-handed technique). Because many needle sticks occur while resheathing the needle, it is recommended that needles be discarded directly into puncture-resistant containers specifically constructed for this purpose, or that one-handed shielding and disposal equipment be used. Laboratory personnel should be prohibited from mouth-pipetting, and from smoking, eating, or drinking in their work area. Work surfaces can be decontaminated effectively with intermediate- to high-level disinfectants, such as 0.05% to 0.5% sodium hypochlorite or 2% aqueous alkalinized glutaraldehyde (95,682), or low-level disinfectants such as quaternary ammonium germicides and phenol-based disinfectants (629). Because of the known risk of transmission of hepatitis B by contaminated sharp instruments, only sterile disposable needles and stylets for finger puncture should be used. Plastic should be substituted for breakable glass whenever feasible. Protective garments should be worn when working with potentially infectious material and should be removed before leaving the work area. Masks in combination with eye protection should be worn whenever splashes, spray, splatter, or droplets of potentially infectious material may be generated and eye, nose, or mouth contamination can be reasonably anticipated. Autoclaving and the use of ethylene oxide gas are both acceptable methods for disinfecting metal objects, instruments, or heat-sensitive equipment (131,134,546).

Immune Globulin Immunoprophylaxis

Early Studies

After the remarkable success of human IG in the immunoprophylaxis of hepatitis A, it was only natural that similar studies would be instituted to determine if IG also could reduce the incidence of hepatitis B and the frequency of its complications. Data have clearly established that protection against reinfection with HBV resides with the anti-HBs fraction (21,314,327,426).

The development of sensitive and specific assays for the detection of the various hepatitis B antigens and antibodies has provided an opportunity to review the results of the early clinical trials on the protective efficacy of IG and to resolve some of the discrepancies (678). Before 1970, the preenrollment immune status of study participants could not be determined, nor could subclinical cases adequately be detected. Thus, attack rates could not be accurately evaluated. In addition, the important role of non-A, non-B hepatitis agents in causing posttransfusion hepatitis was not appreciated. However, perhaps the most significant unknown variable found in every study before 1970 was the concentration of anti-HBs present in the IG preparation. Retrospective analysis has shown that 50% to 80% of the commercial lots of IG manufactured before 1972 were devoid of detectable anti-HBs and that the titer of anti-HBs detected in the remaining lots was relatively low. In addition, as many as 0.8% of the IG preparations contained detectable amounts of HBsAg (332). These anomalies resulted from the pooling of plasma from HBsAg-positive carriers with plasma from immune individuals before the preparation of IG. It also has been argued that the efficacy demonstrated with IG in some of the earlier clinical trials may have been due to primary immunization with HBsAg that was present in some of the IG lots, rather than the result of neutralization of HBV by anti-HBs (680).

In 1972, routine screening of plasma donors for HBsAg was introduced, resulting in a sharp decline in the concentration

of HBsAg inadvertently added to donor pools destined for IG production. Concomitantly, the anti-HBs level increased from a mean titer of 1:4 in pre-1970 IG lots to a mean of 1:128 by 1978 (337). Since 1977, all tested lots of commercial IG contain anti-HBs at a titer of at least 1:100 by RIA (241). Human HBIG is similar to conventional IG except that it is prepared from plasma preselected for a high titer of anti-HBs. In the United States, HBIG has an anti-HBs titer of 1:100,000 or greater.

Side effects associated with the administration of IG are rare. However, the inadvertent administration of conventional IG by the intravenous route may lead to anaphylactic shock (41), and, rarely, single or repeated intramuscular injections can result in allergic reactions (49), especially in immunoglobulin-deficient children (794). Transmission of HBV, HAV, HCV, or HIV by intramuscular IG isolated from plasma by the cold ethanol fractionation technique has not been documented since screened plasma was introduced over 25 years ago. However, production lots prepared outside the United States by zinc or ammonium sulfate precipitation techniques have been implicated in outbreaks of hepatitis B (373,609).

Preexposure Immunoprophylaxis with Immune Globulin

In 1974, Szmuness et al. (739) reported that a conventional IG preparation with a low concentration of anti-HBs (1:16) and a special lot of high-titered HBIG, significantly reduced the frequency of hepatitis B infections among institutionalized children when compared with an untreated group. The HBIG appeared to protect by passive immunization, whereas passive–active immunization was more frequently observed in those who received the conventional IG. Regardless, persistent antigenemia was not observed in either the HBIG group or the conventional IG group, but it occurred in 14% of those for whom no treatment was provided.

Several preexposure immunoprophylaxis studies have been conducted in dialysis units among patients and staff (184,361,392,626). When HBIG was compared with no treatment (or a placebo), significant protection was inevitably observed. Concentrations of anti-HBs similar to that found in conventional IG were nearly as effective in preventing hepatitis B when compared concurrently with HBIG. Despite this success, the availability of a vaccine for hepatitis B and mandatory screening of blood donors for HBsAg and anti-HBc eliminates the need for HBIG in preexposure prophylaxis.

Postexposure Immunoprophylaxis with Immune Globulin

There are some situations in which postexposure prophylaxis with HBIG, usually in conjunction with vaccination, is essential or desirable. These include (a) exposure of an individual to hepatitis B–containing material by percutaneous inoculation, oral ingestion, or direct mucous membrane contact; (b) transient HBV contact through sexual exposure by the partner(s) of an acutely infected patient; and (c) fetal/neonatal exposure to mothers with hepatitis B. Several double-blind, randomized trials have addressed these issues, as shown in Table 8 (57,254,601,639,681). The conclusion reached from these studies is that HBIG administration (or conventional IG containing a lower dose of anti-HBs), used alone, is statistically and epidemiologically superior to a placebo or to no treatment in preventing hepatitis B following exposure. However, in some clinical trials, the conventional IG contained a small quantity of HBsAg, which may have resulted in active immunization. Timing of the initial dose of HBIG or IG after exposure and the concentration of HBV present in the source material are important factors in determining how successful prophylaxis will be after the administration of anti-HBs. For isolated episodes, effectiveness appears to diminish rapidly if administration is delayed for more than 3 days (254,533). Most of the regimens used today combine passive immunization with active immunization induced by vaccine, thereby providing immediate protection with more durable immunity.

Type of exposure	Investigators (ref.)	Summary of results
Needle stick	VA/NIAID, Seeff et al. (681)	HBIG > IG placebo[b]
	NHLBI, Grady et al. (254)	HBIG = IG[c]
Sexual contact	Redeker et al. (539)	HBIG > IG placebo[b]
	Perrillo et al. (601)	HBIG = IG[c]
Perinatal	Beasley et al. (57)	HBIG > placebo[b]

[a]Double-blind, randomized trials
[b]No detectable anti-HBs
[c]No placebo or untreated groups were evaluated. Instead, a comparison was made with IG containing low-dose anti-HBs. In these situations, it is difficult to determine protective efficacy, as results were comparable.
VA, Veterans Administration; NIAID, National Institute of Allergy and Infectious Diseases; NHLBI, National Heart, Lung, and Blood Institute.

TABLE 8. *Efficacy of IG and HBIG in the prevention of hepatitis B: postexposure prophylaxis studies*[a]

Maternal–neonatal transmission of HBV presents special problems in prophylaxis. Prevention of chronic liver disease and HCC during adult life are the ultimate goals of infant immunoprophylaxis, because most acutely infected infants are not overtly ill. A randomized, double-blind, placebo-controlled trial in Taiwan, in which 185 infants were followed for 15 months, clearly established the effectiveness of HBIG in preventing chronic infection in neonates of HBsAg-positive mothers (see Table 8) (57). This study indicated that repeated doses of HBIG (0.5 mL usually given within 1 hour of birth and repeated at 3 and 6 months) had a higher protective efficacy rate (PER) of 71% than a single, larger dose (1.0 mL) given at birth (PER of 42%). In two subsequent studies (642,827), large doses of HBIG given in multiple doses to neonates of HBeAg-positive mothers also were protective.

The urgent need to prevent HBV infection in the newborn has emphasized the importance of identifying mothers who are most likely to transmit HBV to their offspring. Women with symptomatic hepatitis B near the time of delivery are easily recognizable. Chronic carriers, on the other hand, are usually asymptomatic and can be detected only through testing of serum for HBsAg. Initially, an attempt was made in the United States to identify and test only those women who were likely to be carriers of HBV. This voluntary program was not successful and, in 1988, the CDC recommended that (a) all

pregnant women should be tested for HBsAg during pregnancy and (b) neonates born to HBsAg-positive mothers should receive HBIG and hepatitis B vaccine immediately after delivery (7). In 1991, this policy was formally revised by the Advisory Committee on Immunization Practices (ACIP) of the U.S. Public Health Service to include universal immunization of all infants, with the stipulation that infants born to HBsAg-positive mothers continue to receive HBIG as well.

Once treated, infants of HBV-infected mothers need not be isolated from their mothers. Whether to avoid breast-feeding is a complex issue. Where safe, alternative feeding methods are available, the potential extra risk of breast-feeding should be avoided. However, under conditions of poor sanitation in developing countries, bottle-feeding may greatly increase the risk of potentially fatal gastroenteritis. Here, breast-feeding should be strongly encouraged, even if there is a small additional risk of HBV infection. Conversely, no additional precautions are necessary once the infant receives prophylaxis.

Table 9 summarizes the salient features extracted from the various controlled and uncontrolled IG clinical trials described here.

Immune globulin containing anti-HBs (HBIG or conventional IG) is superior to placebo or no treatment in preventing hepatitis B.
Larger doses of HBIG or more frequent dosing intervals in HBV-exposed infants are associated with higher efficacy rates in preventing chronicity.
Persistent antigenemia after HBV infection is unusual after anti-HBs administration with HBIG or conventional IG.
A prolonged incubation period often occurs in persons who receive HBIG and who subsequently develop hepatitis B.

IG, immune globulin; HBIG, hepatitis B immune globulin.

TABLE 9. *Summary data from hepatitis B passive immunoprophylaxis trials*

Development and Safety of Established Hepatitis B Vaccines

Plasma-Derived Vaccines

Active immunization against hepatitis B in humans was initially investigated by Krugman and colleagues between 1971 and 1973 using a crude immunogenic preparation of HBV-containing serum (MS-2 strain; subtype *ayw*; titer of $10^{7.5}$ chimpanzee infectious doses per milliliter) (410,412,413). The serum was diluted 1:10 and heat-inactivated at 98°C for 1 minute. The heat-treated inoculum did not result in clinical or biochemical evidence of hepatitis in 29 vaccinated children, although anti-HBc seroconversion occurred in a few children. Between 4 and 8 months after receiving one to three inoculations, the group was challenged with the unheated HBV-containing serum. This was followed by complete protection in 17 (59%) and a transient, subclinical infection in three (10%).

These studies led to the development of more sophisticated hepatitis B vaccines harvested from the plasma of asymptomatic, apparently healthy human carriers of HBV by a series of steps that may include precipitation, ultracentrifugation, gel filtration, and/or affinity chromatography (5,110,200,308,627). These vaccines consisted of highly purified, formalin-inactivated (5,200,308,693) and/or heat-inactivated (641), alum-adsorbed, hepatitis B subvirion particles of HBsAg that were free of detectable nucleic acid. (The production processes and quality control measures used by various manufacturers for the plasma-derived vaccines can be found in ref. 200.)

The only plasma-derived hepatitis B vaccine manufactured in the United States was licensed in 1981 (Heptavax-B, Merck & Co., West Point, PA). It used a series of purification and inactivation steps designed to remove or inactivate not only infectious hepatitis B virions but also representatives of other groups of animal viruses known to cause human disease (130,307). It is noteworthy that each of the final purification steps (pepsin, urea, and formalin) will inactivate at least 10^{5} chimpanzee infectious doses of HBV per milliliter and all other classes of animal viruses, including the retroviruses (130,307,748).

In the late 1980s, Heptavax-B was replaced by recombinant yeast-derived vaccines and is no longer manufactured in the United States. However, plasma-derived vaccines are still being manufactured in North and South Korea, China, and Indonesia and are in development in Myanmar and Vietnam (701). Most of these vaccines are produced for local or regional consumption. Regardless, more than 30 million doses of plasma-derived vaccine have been distributed globally, and the safety and efficacy record for this product is impressive (see later). Immunogenicity is generally thought to be superior to the genetically engineered products and is increased in those plasma-derived products that use less stringent inactivation procedures such as formalin only (315,494), compared to heat alone (110,815), or heat and formalin (693,822).

Recombinant Yeast-Derived Vaccines

Yeast-derived recombinant vaccines were developed initially to counterbalance some of the perceived, but unfounded, concerns regarding the safety of plasma-derived vaccine. However, from the manufacturers' viewpoint, the fact that the production cycle could be decreased from 65 weeks to 12 weeks, batch-to-batch consistency could be more easily attained, and a continuous supply of material could be generated made the new technology very attractive. Thus, the licensure of a recombinant DNA–derived vaccine in 1986 eventually rendered the plasma-derived vaccine obsolete in the

United States (306).

Today several mammalian cell–derived [Chinese hamster ovary (CHO) cells] and yeast cell–derived recombinant vaccines are commercially available. Except for one of the mammalian expression systems (Genehevac-B, Pasteur-Merieux, Lyons, France), which consists of a pre-S2/S gene product, all other licensed vaccines consist of the major (S gene) product.

Current United States–licensed, yeast-derived vaccines of HBsAg are produced in large fermentation cultures of *Saccharomyces cerevisiae* cells, which carry a recombinant yeast expression vector (plasmid) consisting of specific promoter sequences from a yeast gene specifying a glycolytic enzyme, the S gene, and plasmid DNA from *Escherichia coli* (202,281,597,608). HBsAg is released from the cells by homogenization or disruption with glass beads, it is purified by clarification, ultrafiltration, chromatography, and ultracentrifugation, and it is then adsorbed onto aluminum hydroxide, after which thimerosal is added as a preservative (see discussion later on the thimerosal controversy). For a more complete description of the various production processes and quality control procedures for recombinant DNA hepatitis B vaccines, the reader is referred to the book by Ellis (200).

The two major yeast-derived hepatitis B vaccines that are licensed in most countries are Engerix-B [Smith Kline Beecham (SKB), Rixensart, Belgium, and Philadelphia, PA] and Recombivax HB [Merck (MSD), West Point, PA]. Other structurally and chemically similar commercial recombinant products are being produced in France, Japan, Cuba, South Korea, and India or are in various stages of development in several other countries. Before its adsorption with alum, Recombivax HB, but not Engerix-B, is treated with formaldehyde. Physicochemical characterization of the particles that are formed confirm the purity of these vaccines and their similarity with plasma-derived HBsAg based on molecular weight (24 kd), amino acid composition, peptide mapping by fast-atom bombardment mass spectrometry, electron microscopy, density in cesium chloride, and lysed composition. These vaccines contain less than 2% yeast protein and no detectable yeast DNA. Unlike the plasma product, the yeast-derived vaccine is not glycosylated. Nevertheless, immunologic analysis with monoclonal antibodies that have virus-neutralizing properties against two different serotypes of HBV indicates that the yeast- and plasma-derived vaccines contain epitopes known to be important in inducing protection after vaccination (286).

Safety Issues and Concerns

More than 500 million people have received the plasma-derived or genetically engineered hepatitis B vaccines. Local and systemic reactogenicities of the recombinant vaccines are comparable to those observed after an injection with plasma-derived vaccine (200,834). Andre (29) reviewed 122 clinical trials representing 40,140 vaccine doses of Engerix-B and found no clinically adverse effects. Soreness at the injection site was observed in 23% and induration in 8%. Fewer symptoms were seen in children than in adults. Clinical hypersensitivity to residual yeast antigens that might be present in the inocula has been cited rarely, even though most people harbor antibodies to the bakers' yeast, *S. cerevisiae* (107). In addition, new anti-yeast IgG specificities have not been elicited, nor has there been an increase in IgG-type antibodies (30,608). Thus, the risk of inducing an allergic reaction is negligible. However, anaphylaxis and life-threatening, immediate hypersensitivity reactions or an apparent hypersensitivity reaction of delayed onset that includes arthritis/arthralgia, fever, and dermatologic reactions (serum sickness–like) have been reported rarely (<1 event per 600,000 doses) (138; and package inserts of pharmaceutical companies). An association between hepatitis B vaccination and erythema nodosum or alopecia also may exist (812).

To date, there has been no confirmed scientific evidence that these vaccines cause chronic illnesses such as CNS demyelinating disorders (including multiple sclerosis), chronic fatigue syndrome, rheumatoid arthritis, or autoimmune disorders. Nor is there any clear causal association between administration of these vaccines and Guillain-Barré syndrome, sudden infant death syndrome (SIDS), transverse myelitis, optic neuritis, or seizures (6,278,558,687; J. E. Maynard et al., unpublished observations; H. S. Margolis, testimony before a subcommittee of the U.S. House of Representatives Committee on Government Reform, May 18, 1999). Two case-control reports from France (hospital based) and the United Kingdom (population based) failed to document a statistically significant association between hepatitis B vaccine and CNS demyelination disorders at the 5% level (226,726). However, both lacked sufficient power to definitively conclude that there are no risks, and vigilance to the possibility of a rare association should be maintained.

Recently, concerns have been raised about the administration of vaccines containing thimerosal to newborn babies. Thimerosal is an organic mercurial compound that has been added to many vaccines since the 1930s to prevent contamination with pathogens, which might occur during production or from imperfect aseptic handling. Adverse events, such as rashes, are rare, but exposure to much larger concentrations of mercury than are present in vaccines can cause neurotoxicity with learning disabilities. Unborn babies and newborn babies are at greatest risk, and high levels in pregnant mothers may result in brain damage to the unborn baby. However, there has been no evidence of harm from vaccines that contain 0.003% or 0.01% thimerosal. The risk, therefore, is theoretical, uncertain and extremely small. Regardless, because infant immunizations utilizing a number of vaccines containing thimerosal could theoretically raise mercury levels above some established regulatory agency limits, thimerosal has been eliminated from hepatitis B vaccines in the United States and Europe. In contrast, the WHO, because of cost and cold-chain considerations, is currently not recommending removing thimerosal from vaccines being used in developing countries.

Protective Efficacy Studies in Chimpanzees

Preexposure protective efficacy of plasma-derived and recombinant-derived hepatitis B vaccines has been established in susceptible chimpanzees, which have successfully withstood challenge with HBV after receiving the vaccine (200,300,494,627,631,632,664). Cross-protection by different serotype vaccines against opposing HBV subtypes has been observed; thus subtypes are not of major concern in formulating the vaccines because of the shared group *a* antigenic determinant. Postexposure immunization after an HBV challenge also has been found to be efficacious in chimpanzees.

Factors Influencing the Immune Response

Because the peak geometric mean antibody levels that are achieved after the basic immunization series determine how long antibody will persist at detectable levels, factors that influence immunogenicity must be considered. These can be separated into immunization factors and host factors (775). However, durability of immunity, based on the presence of circulating antibody, is only one aspect of protection. Retention of immunologic memory by partially differentiated antigen-specific B cells and specific helper T cells is necessary for rapid recall even when no antibody can be detected (307). How immunization or host factors influence this aspect of the immune system is less certain.

Immunization Factors

Dosage and Immunization Schedules

Several studies using age- and weight-matched subjects have shown unequivocally that the dose of vaccine administered to a subject is of considerable importance in determining the peak immune response achieved by that person (Fig. 10) (326,775,828,833). Not only is the immune response enhanced as the dose increases, but a more rapid response occurs, factors that could be important in postexposure prophylaxis.

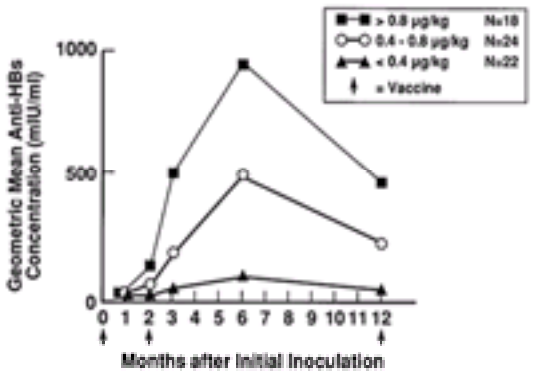

FIG. 10. Geometric mean anti-HBs concentration (mIU/mL) over time as a function of vaccine dose per kilogram of body weight. (Adapted from ref. 833, with permission.)

A number of vaccine schedules have been evaluated (316,369,833), but none has supplanted the current regimens. Using weight-matched subjects and a single lot of plasma-derived vaccine, Hollinger et al. (316) evaluated seven different schedules (Table 10). After three injections, 98% to 100% of the vaccinees developed anti-HBs, confirming the observation that seroconversion data have limited usefulness in comparing responses except during the first 3 months of a vaccine trial. The highest responses occurred when vaccine was given at 0, 1, and 6 months, and at 0, 2, and 4 months. In another study, injections at months 0, 1, and 12 achieved significantly higher anti-HBs responses when compared to a 0-, 1-, and 6-month schedule (370). It is important to recognize that although adherence to an optimal schedule is recommended, deviations will not lead to an adverse outcome if the primary series of three (or four) injections is completed within a reasonable period of time (273). A good response to the 0-, 2-, and 4-, or to the 0-, 2-, and 6-month schedule is encouraging because these regimens are similar to what has been proposed for the universal immunization of infants.

Vaccine schedule[a] (mo)	Anti-HBs response[b] Peak level, geometric mean (mIU/mL)	At final bleeding,[c] geometric mean (mIU/mL)
0-6	781	26
0-3-6	643	28
0-2-6	1,303	67
0-1-6	3,338	131
0-2-9	1,462	NT
0-2-4	2,848	155
0-1-4	771	61

[a]40 µg vaccine per injection
[b]Responders only
[c]28–30 months postinoculation
NT, not taken
Source: From ref. 316, with permission.

TABLE 10. *Maximum geometric mean anti-HBs levels attained during follow-up and at the conclusion of the study based on vaccine schedule*

Currently, two primary immunization regimens have been instituted. The usual schedule, consisting of three doses of vaccine given at 0, 1, and 6 months, is recommended for preexposure prophylaxis. An alternate four-dose schedule, with dosing at 0, 1, 2, and 12 months, was designed for immunocompromised persons, for infants born to infected mothers, and to hasten the immune response in travelers planning to go to high-risk areas. However, the addition of HBIG to the regimen for infants or travelers may neutralize this advantage. Although delaying the final injection for 12 to 18 months may eventually lead to high-peak anti-HBs responses, a potentially undesirable “window of vulnerability” occurring during the interval between months 1 and 12 (or 18) may restrict its use to groups who have a low immediate risk of acquiring

hepatitis B infection (370,833). Compliance and attrition also become issues when such delays are instituted.

Storage of Vaccine

Inadvertent freezing of alum-adsorbed, plasma-derived vaccine indicated that such a practice may be deleterious to immunogenicity (587). Although no additional clinical trials have been designed to verify this observation with recombinant vaccines, animal studies have confirmed that freezing reduces the immune response (193), presumably by altering the physical properties of the adjuvant and its relationship to the immunogen. Conversely, storage of a recombinant-derived vaccine at temperatures of 37°C for up to 4 weeks did not reduce the immunogenicity of the product in a significant manner (378). Potency is optimally preserved when vaccine is stored at 2° to 8°C.

Site and Route of Inoculation

Based on careful comparative studies, the site of inoculation has proven to be a critical factor. Persons administered vaccine into the adipose tissue of the gluteal region have lower seroconversion rates and geometric mean anti-HBs levels when compared with those who receive all of their injections intramuscularly in the deltoid region (72% and 85 mIU/mL versus 93% and 1,454 mIU/mL, respectively) (156,497,688). Such tissue is apparently a poor repository for vaccine (445).

Intradermal inoculation, which is safe and uses one-tenth the dose, has proponents throughout the world because of its potential for substantial reductions in cost. Whereas seroconversion rates are comparable to full-dose, intramuscular injections, the peak antibody levels are substandard (114,153,596,640). Horowitz et al. (338) concluded that the intradermal route was quite satisfactory as a mechanism for boostering individuals who had completed their basic intramuscular immunization series, but it does not improve response rates in non- or hyporesponders (220). Heijtink et al. (293) found that protective immunity and geometric mean concentrations were comparable when groups were given the same low-dose vaccine either intramuscularly or intradermally.

Methods to Enhance Immunogenicity

Adjuvanted vaccines can favorably influence the immune response. Alum (aluminum phosphate or hydroxide), the most common adjuvant, is clearly superior to aqueous or saline suspensions of vaccine (494,656). The use of other adjuvants and biologic response modifiers has been explored to enhance immunogenicity in nonresponders and hyporesponders and to reduce the number of injections from three to two, thereby improving compliance and providing earlier protection. The three new adjuvants that may fulfill these goals, at least partially, are (a) MF59, an oil-in-water emulsion consisting of the metabolizable oil, squaline, together with surfactants (295), (b) SBAS4, a combination of aluminum salt and monophosphoryl lipid A (761), and (c) DC-Chol, a cationic lipid (111). The MF59 adjuvant was combined with a new recombinant HBV subunit vaccine containing the pre-S2 and S antigens (295). The combination was up to 100-fold more immunogenic than Recombivax HB with a similar local and safety profile. It achieved levels after two doses (given 2 months apart) that were comparable to the 0-, 1-, and 6-month schedule used for the alum-adjuvanted licensed vaccine. Whether the enhanced response was secondary to the MF59 adjuvant alone or to the subunit vaccine containing Pre-S2 and S antigens (or a combination of the two) was not evaluated.

Several recombinant vaccines have been developed for human use that contain antigens from the pre-S and S regions of the S gene expressed in yeast or mammalian (CHO) cells (8,295,298,340,495,732,842). Immune responses to the S and pre-S regions are under major histocompatibility complex (MHC) control but are regulated independently. In animals, nonresponsiveness to the S region may be circumvented by the addition of pre-S antigens to the vaccine (515,517). Finally, antibodies to pre-S2 synthetic peptides neutralize HBV *in vitro* and *in vivo* in the absence of anti-HBs (360,550,764). Two genetically engineered vaccines containing the S antigen, pre-S1, and pre-S2 epitopes appear to have induced antibodies in S vaccine nonresponders, even in elderly patients or immunosuppressed patients (298,495,842). Whether these additions to HBsAg vaccines will be important for long-term protection from HBV infection remains to be determined in controlled clinical trials.

Host Factors

Age and Weight of the Subject

Increasing age, especially above 30 years, has been shown to correlate independently with a decreasing response to the vaccine (Table 11) (338,688,743,776). In one study, anti-HBs seroconversion rates declined from a relatively low frequency of 69% in persons 61 to 70 years of age to less than 39% in those more than 80 years of age (178).

	p value of F statistic		
Factor	Total (*N* = 62)	Age 10–30 yr (*N* = 32)	Age ≥31 yr (*N* = 30)
Age	<.05	NS	.0005
Weight	<.001	<.025	NS
Sex	NS	NS	NS
Dose	<.01	<.0005	NS

NS, not significant.
Source: From ref. 776, with permission.

TABLE 11. *Multiple linear regression of age, weight, sex, and dose on anti-HBs response to three doses of HEPTAVAX B at 8 months postinoculation*

Suboptimal responses occur in obese persons (156,775). Correspondingly, the immune response is inversely related to weight (Fig. 11) or, more precisely, the Quetelet index of body mass (weight/height ^{2}), especially in those subjects who are under 30 years of age (see Table 11). In this regard, it is noteworthy that one of the most important criteria for determining the intensity of the immune response in young adults and children is the dosage administered per kilogram of body weight (see Fig. 10). As indicated previously, a higher dose of vaccine per kilogram administered to any given individual will result in a more rapid immune response and lead to higher levels of anti-HBs (315,338,775,808,833).

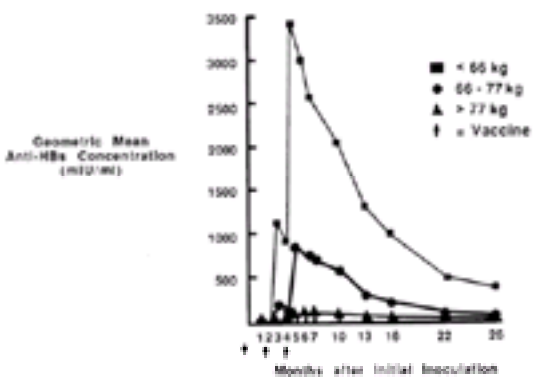

FIG. 11. Geometric mean anti-HBs concentration (mIU/mL) as a function of body weight observed in subjects receiving the same dose of HBsAg vaccine. (From ref. 316, with permission.)

Genetics, Immunosuppression, Smoking, and Gender

Genetics also may play a role in determining the strength of the immune response in individuals vaccinated under identical circumstances. Several studies have demonstrated an association between nonresponsiveness to the hepatitis B vaccine and certain HLA specificities, with accumulating evidence of an association between different HLA-DR alleles such as the HLA-DRB1 0701; DQB1 0202 genotype (495). This genotype not only was associated with a more rapid decline of anti-HBs following immunization with a vaccine containing S, Pre-S1, and Pre-S2 antigens, but also it was frequent among those individuals who remained nonresponders following booster inoculations. Other extended haplotypes (B44; DR7; FC31 and B8; DR3; SC01) also have been associated with nonresponders (166,616,786,798). The same histocompatibility antigens have been associated with the HBsAg carrier state and chronic active hepatitis, suggesting that these antigens may predispose individuals to detrimental cell-mediated immune reactions during an HBV infection in addition to modulating the immune response to HBsAg during vaccination.

Immunosuppressed groups, such as hemodialysis patients (109,261,397,488) and those on chemotherapy or infected with HIV (125,160,244,470), respond poorly to vaccination when compared with healthy individuals. About one third of hemodialysis patients who initially respond revert to being seronegative within 12 months of the initial vaccine dose. Smoking also appears to have an adverse affect on the immunologic response to HBsAg, presumably because of elevated levels of suppressor T cells that occur in smokers (94,338,688). Another study also found higher populations of suppressor T cells among nonresponders but failed to determine whether smoking was a factor (562).

Sex does not seem to play an important role in the immune response to hepatitis B vaccination. Although initial studies found that women often responded to the vaccine with higher peak antibody levels than did men (294,742), the key factor in this response may have been the weight of the vaccinee and not the gender, as discussed previously (i.e., no difference in immune response is observed when men and women are weight-matched) (see Table 11) (776).

Immunization of HBsAg Carriers

Vaccination of HBV carriers rarely generates an anti-HBs response. Indeed, nonresponders should be tested for HBsAg because anti-HBs is unlikely to develop in these individuals unless subtype differences exist between the immunogen and the HBV subtype infecting the host. In one of the earliest studies (191), 16 male carriers received two to six monthly injections of a 40-µg dose of plasma-derived vaccine (mean number of doses was 4.1). Some of the carriers received HBsAg having the same subtype as their chronic infection; others received a heterologous subtype vaccine. Circulating immune complexes were detected in six of the 16 vaccinees before immunization, and these levels did not increase subsequently. The 10 subjects who did not have prior evidence of immune complexes did not develop any complexes after inoculation. None of the individuals developed anti-HBs. Five carriers normalized their enzymes and three others showed a decline toward normal levels. However, evaluation of these changes was confounded by the fact that a downward trend was already occurring before vaccination in four of these subjects.

To determine whether other hepatitis B vaccines could ameliorate infection in HBV carriers, Pol et al. (615) vaccinated 46 chronic hepatitis B patients, who were HBsAg and HBV DNA positive, with three monthly injections of a pre-S2/S vaccine. Six months after the first injection, HBV DNA disappeared in 26% and an additional 17% showed a significant decrease (of more than 50%) in their HBV concentration. In another controlled trial by the same investigator (615), response to a pre-S2/S vaccine with HBV DNA disappearance was superior to that observed with an S vaccine (20% versus 4.8%) or with no vaccine (3.1%). No side effects or vaccine-induced escape mutants occurred.

Induction of Escape Mutants

HBV variants that have amino acid substitutions within the S gene involving the major antigenic “a” determinant have been detected in recipients of both plasma-derived and recombinant HBsAg vaccines (123,282,343,585). Vaccinated children contract these variants primarily through vertical transmission, although horizontal transmission can occur. These vaccination-associated escape mutants display a predominance of the G145R mutation and appear to circumvent neutralization by anti-HBs, raising a concern that they may be resistant to immunoprophylaxis. Although detected before the implementation of vaccination programs, universal immunization has hastened the accumulation of these mutants, which replicate independently of anti-HBs and remain stable over time. The G145R variant was recently found to be infectious and pathogenic in seronegative chimpanzees. The virus was used to challenge four chimpanzees that had been immunized with either the MSD or SKB recombinant vaccine (566). All chimpanzees were protected against infection, implying that the HBV variant was not a true neutralization-escape mutant. Thus, *a* domain–defective mutants presumably induced by vaccine may not pose a threat to vaccine responders as previously feared. Recently, vaccine escape mutants have been identified in patients with HCC. Mutations outside the “ *a*” determinant also have been detected.

Combined Vaccines

The availability of safe, efficacious HBsAg vaccines provides public health officials with an opportunity to reduce the incidence of HBV through mass immunization programs. To achieve this goal, more than 100 countries have integrated hepatitis B vaccine into their Expanded Program on Immunization (EPI), as recommended by the WHO (817). One approach was to physically combine the hepatitis B vaccine with other childhood vaccines, which would substantially reduce the number of injections that a child would require at each visit and lead to economic benefits (165). It was concluded that concurrent vaccination of neonates or infants with HBsAg and BCG or DTP–poliovirus vaccines yielded equivalent responses when compared with the results observed when the vaccines were given separately. However, lower measles and yellow fever antibody titers were detected when either of these vaccines was injected simultaneously with the hepatitis B vaccine. Recently, SKB has produced a tetravalent diphtheria–tetanus–pertussis and hepatitis B vaccine substituting acellular pertussis vaccine for whole-cell vaccine (245). The vaccine was safe, well tolerated, and highly immunogenic. In six pivotal vaccine trials, hepatitis A was combined with hepatitis B and administered to 843 healthy 17- to 60-year-old adults employing a 0-, 1-, and 6-month schedule (760). The combined vaccine appeared to be safe, well tolerated, and highly immunogenic, with responses that compared favorably with monovalent vaccines. Follow-up of 129 vaccinees at 48 months showed that all were still positive for anti-HAV and more than 95% were protected against hepatitis B.

Alternative Hepatitis B Vaccines

Although the current HBsAg vaccines have been shown to be safe and efficacious, a number of manufacturers continue to search for alternative vaccines that may be less expensive to produce and will provide greater immunogenicity, especially in immunocompromised hosts (see ref. 312 for a historical perspective on these vaccines). Except for the polypeptide preparations of HBsAg (325), and the DNA genetic vaccines or the pre-S gene products described previously, none of the other products appear likely to replace or supplement the current vaccines at this time. In this context, the rapid anti-HBs response that occurred in humans after an initial inoculation of an HBsAg polypeptide vaccine packaged in a micellar configuration and alum-adsorbed (75% seroconversion rate in 2 weeks), and the superior anti-HBs levels achieved (325), suggest that such an immunogen might be beneficial in postexposure prophylaxis when the early development of immunity is advantageous, or as a therapeutic vaccine against HBV in chronically infected carriers.

Early Clinical Trials

Homosexual Men

The first large-scale hepatitis B vaccine study in the United States in a high-risk group was initiated in 1978 by Szmuness et al. (746,747). Plasma-derived HBsAg (40 µg) was administered at 0, 1, and 6 months to 549 HBV-susceptible male homosexuals. An additional 534 homosexual men received a placebo containing alum, diluent, and formalin but not HBsAg. Fully 96% to 98% of the vaccinees developed an anti-HBs response at 9 months (3 months after the third injection). The protective efficacy rate (PER), computed by dividing the differences in attack rates between placebo and vaccine recipients by the rate in placebo recipients, was 92% with respect to clinical hepatitis B and 81% for all HBV events (including those with anti-HBc seroconversion alone). When recipients who had received their third dose of vaccine (and responded with adequate anti-HBs levels) were compared with the placebo group, the PER increased to 100%. Most important, no clinical or biochemical cases of hepatitis B occurred in anti-HBs responders who achieved anti-HBs levels above 10 mIU/mL. Of the 21 nonresponders, seven (33%) acquired hepatitis B, a rate similar to that of the placebo group. When hepatitis B occurred in these vaccine nonresponders, a typical course was observed. It is noteworthy that of the 14 vaccine recipients who became HBsAg-positive, one (7.1%) developed chronic antigenemia, which is comparable to the 10.4% rate observed in the 96 placebo recipients who became HBsAg positive.

During the first 60 days after randomization to the placebo or vaccine group (747), the number of HBV events was similar. It is presumed that many of these events resulted from exposure that occurred before or shortly after entry into the study. However, 77% of the placebo recipients developed clinical hepatitis B during this interval, whereas such events occurred in only 18% of the vaccine subjects. Thus, one might conclude that vaccination with HBsAg may abort hepatitis B or modify its course when given just before exposure or very early in the incubation period. These results of apparent benefit after postexposure prophylaxis are consistent with similar efficacy studies reported in chimpanzees (494,632).

A second study of similar design in homosexual men using 20 µg of a plasma-derived HBsAg vaccine yielded similar results regarding vaccine-induced immunity and efficacy (98%) (228). A similar PER of 77% has been recorded for

homosexual men followed for 3 years after being inoculated with 20 µg of a yeast-derived recombinant vaccine (251).

The wide spectrum of immunologic abnormalities that accompanies HIV infection, especially during the later stages of disease, has a profound effect on the anti-HBs seroconversion rate after vaccination with plasma- or yeast-derived HBsAg. Both the frequency of responders (33% to 56% for HIV-positive subjects versus 89% to 91% for HIV-negative controls) and the concentration of anti-HBs are decreased (160,244,470). It is recommended that active immunization should be performed at the earliest stages of the disease, before severe depression in cell-mediated immunity and defects in B-lymphocyte dysfunction occur.

Hemodialysis Patients and Staff

Clinical trials in hemodialysis patients and staff were initiated in France in 1975 by Maupas et al. (488). Table 12 summarizes the immunogenicity results from several of these hepatitis B vaccination studies (106,109,261,397,488). Seroconversion rates in uremic patients (and peak geometric mean anti-HBs concentrations) were substantially less (median of 60%) than those observed in staff members (median of 92% to 94%). Indeed, the anti-HBs response rate was inversely related to the degree of uremia present at the time of vaccination. Thus, vaccination should be considered before patients require dialysis. The PER ranged from 71% to 85% for the staff but was only 53% for the patients enrolled in large clinical trials (261,742). As was observed in the clinical trials that involved homosexual men, many of the HBV infections in the vaccine group occurred before the second month, indicating that viral infection probably existed before vaccination commenced (261). In one trial, the *ay* subtype of HBV accounted for 81% of the infections. Because an *ad* monovalent vaccine was used in this clinical trial, and the PER for the *ay* hepatitis B events was 85%, this study was the first to demonstrate subtype cross-protection in humans, thus confirming earlier results in chimpanzees. Results with a yeast-derived product suggest that doubling (or quadrupling) the dosage of vaccine that is routinely administered to adults and using a four-dose schedule may be optimal for hemodialysis patients (see the section, Guidelines for Preexposure Immunoprophylaxis and Booster Doses).

Investigators	Type of vaccine (manufacturer)	Dose (µg)	Schedule (mo)	Staff N	Staff %	Patients N	Patients %
Maupas et al. (488)	PD (Pasteur)	5	0-1-12	162	92	55	64
Guerry et al. (261)	PD (Pasteur)	5	0-1-2-12	170	94	43	93
Kohler et al. (397)	PD (MSD)[a]	40[b]	0-1-4	19	95	28	54
Brownell et al. (106)	PD (MSD)[a]	40[b]	0-1-4	9	89	24	92
Brugueras et al. (109)	YD (SKB)	40	0-1-2-6	–	–	43	86

[a] Injection in buttocks.
[b] Staff received half this dose.
PD, plasma-derived; YD, yeast-derived; MSD, Merck, Sharp and Dohme; SKB, SmithKline Beecham.

TABLE 12. *Seroconversion rates to hepatitis B vaccine in hemodialysis staff and patients*

Alaska Natives

Two vaccine studies were conducted in Alaska (305,500). In the first study, 1,581 susceptible Alaska natives were immunized with three doses of plasma-derived vaccine. Ten years after the first dose of vaccine, the annual incidence of HBV decreased from 50 to 0.93 HBV infections per 1,000 residents (795). None developed clinical illness or a sustained HBsAg response.

In a second study, 90% of an area's susceptible population (9,840 residents) were immunized, and all newborn infants were offered the vaccine at birth (500). The annual incidence declined from 215 cases per 100,000 population before immunization to 7 to 14 cases per 100,000 after vaccination (Fig. 12), primarily manifested as anti-HBc seroconversions. No carriers have been detected in recent years among infants immunized with vaccine only.

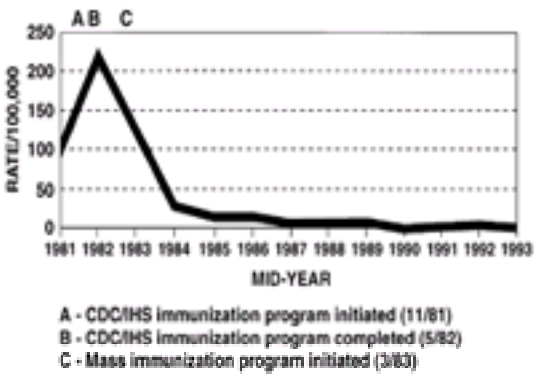

FIG. 12. Incidence of acute symptomatic hepatitis B observed in Alaska natives residing in southwest Alaska, 1981 to 1993. *Points A and B* represent the initiation and completion, respectively, of an immunization demonstration project in 17 villages. Mass immunization began in all villages at *point C.* (Adapted from refs. 500 and 795, with permission.)

Neonates

In contradistinction to the preexposure vaccination trials in homosexual men, hemodialysis patients, and Alaska Natives, early studies conducted in neonates were postexposure in principle. Current practice is to use a combination of passive and active immunization in these situations to provide prompt protection with HBIG, followed by long-term immunity with the vaccine, although this viewpoint is changing, especially in developing countries where cost is a factor. Concerns that HBIG might interfere with the active immune response of the vaccine have not materialized at the currently recommended doses. In one study in adults, doses of HBIG up to 3.0 mL failed to depress the active immune response to 20 µg of plasma-derived HBsAg given simultaneously at a different site (745). The usual dose of HBIG given to neonates is 0.5 mL. When concurrent administration of HBIG and vaccine are contemplated, different sites should be used.

A number of randomized, double-blind trials and open-label studies have been conducted in neonates of HBeAg-positive mothers to evaluate the effectiveness of HBIG alone, HBsAg vaccine alone (plasma-derived or genetically engineered), and HBIG plus HBsAg vaccine administered in close proximity to each other (Fig. 13) (31,57,312). The overall results of these studies indicate that the combination of HBIG and vaccine, or yeast-derived vaccine alone, is highly effective in reducing the incidence of chronic antigenemia in neonates born to mothers who are HBeAg positive. In 36 studies, PERs have ranged from 70% to 100%, with the median PER being 95% for the yeast-derived vaccine with HBIG compared to 91% for the plasma-derived vaccine with HBIG. Even better results should be obtained in infants of anti-HBe–positive mothers who are at a lower risk of acquiring an infection. In all of these studies, HBIG was administered after delivery, usually in the first 6 hours but certainly by 24 hours. Significantly delaying the time when vaccine is administered appears to lower the PER unless an additional dose of HBIG is given 1 to 3 months later. Except for this, the schedule chosen for vaccination is less critical. Interestingly, one of the better PERs was observed in a group of neonates receiving HBIG at 0 and 3 months, at which time vaccination was initiated with an “adult” HBsAg dose of 20 µg administered at 3, 4, and 9 months (52).

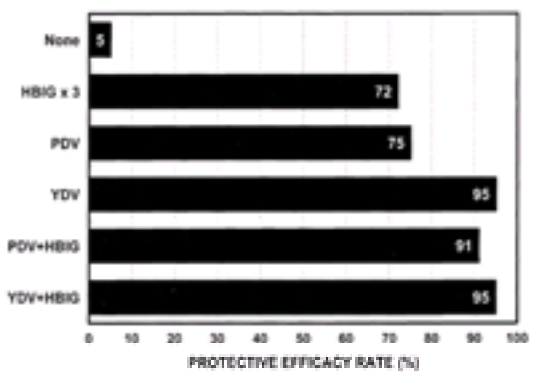

FIG. 13. Protective efficacy rates in neonates born to HBeAg-positive mothers after immunization with hepatitis B immune globulin alone, HBsAg vaccine alone, and a combination. HBIG, hepatitis B immune globulin; PDV, plasma-derived vaccine; YDV, yeast-derived vaccine. (Adapted from refs. 31,57, and 312, with permission.)

Because of the cost engendered in using combination therapy, a number of investigators in developing countries have opted to evaluate the PER of vaccine alone, including the use of the four-dose schedule. A wide range of responses has been generated with PERs that range from 90% to 95% for the yeast-derived products (three studies; median PER of 95%), to a range of 65% to 100% for the plasma-derived vaccine (11 studies; median PER of 75%) (see Fig. 13). The wide disparity that exists between the yeast-derived and the plasma-derived vaccines is perplexing, especially because the plasma-derived product is considered to be a superior immunogen in adults (311). Indeed, the suboptimal results obtained with plasma-derived vaccine are comparable only to that observed with HBIG alone, given as a series of three 0.5-mL doses at birth, 3 months, and 6 months of age (57). However, when interpreting these data, it must be recognized that the level of viremia present in a mother's serum can be approximated only with the HBeAg assay. Recent quantitative evaluation of sera for HBV DNA using molecular hybridization technology has shown that wide fluctuations in the concentration of virus exist in HBeAg-positive carriers (418). Because virus burden can significantly affect the outcome in an infected neonate, any comparisons between groups or vaccine regimens in the same or different communities must consider this important variable.

Guidelines for Preexposure Immunoprophylaxis and Booster Doses

Table 13 lists those individuals or groups for whom preexposure vaccination is recommended. If all members of these groups were immunized, the incidence of hepatitis B would rapidly decrease. However, except for infants and health care personnel, for whom governmental regulations mandate immunization of those exposed to blood-borne pathogens, attempts to recruit many of these individuals have been unrewarding. Therefore, control of this disease will require vaccination of all newborn infants regardless of the HBsAg status of their mothers (324). Universal screening of pregnant women for HBsAg will identify neonates at risk who would be given HBIG, in addition to vaccine, at delivery (see the section, Guidelines for Postexposure Immunoprophylaxis). The remainder of the infants could be vaccinated beginning at birth or in conjunction with the routine immunization program already established. Options for adding hepatitis B vaccine to the EPI schedule are in a state of flux because of the introduction of new formulations of childhood vaccines that are often combined with other vaccines. Regardless, protective levels of anti-HBs (³10 mIU/mL) are achieved when the hepatitis B vaccine is administered in a variety of regimens, including those begun soon after birth (620,720,822).

1. Infants (universal immunization)
2. Adolescents at high risk of infection
3. Persons with occupational risk (exposure to blood or blood-contaminated environments) and students of health-care professions before they have blood contact
4. Clients and staff of institutions for the developmentally disabled and susceptible contacts in day-care programs who are at increased risk from HBV carrier clients with aggressive behavior or special medical problems that increase the risk of exposure
5. Hemodialysis patients; vaccination before dialysis treatment is recommended
6. Recipients of frequent and/or large volumes of blood or blood components
7. Susceptible injecting drug abusers
8. Sexually active men and women (homosexual and bisexual men; persons with recently acquired sexually transmitted disease; prostitutes; promiscuous heterosexuals)
9. Susceptible inmates of long-term correctional facilities who have a history of high-risk behavior
10. Household contacts and sex partners of HBV carriers. Screen adoptees from countries with high HBV endemicity for HBsAg. If positive, vaccinate family members.
11. Populations with a high incidence of disease (refugees; Alaska natives; Asian-Americans)
12. International travelers to areas of high HBV endemicity if specific at-risk circumstances exist (includes military personnel identified as being at increased risk)
13. Transplant candidates preferably before transplantation

[a]Prevaccination screening is recommended to determine susceptibility where stipulated. Anti-HBc is the preferred serologic test for this purpose.
ACIP, Advisory Committee on Immunization Practices.
Source: Adapted from ref. 136, with permission.

TABLE 13. *ACIP recommendations for preexposure immunization with hepatitis B vaccine*[a]

Table 14 summarizes the recommended doses and schedules to be used for preexposure prophylaxis with the two hepatitis B vaccines currently licensed in the United States. Vaccine is to be administered by intramuscular injection in the anterolateral thigh of infants, or the deltoid muscle of children and adults, to achieve optimal protection. Longer intervals between the last two doses result in higher final titers of anti-HBs (273,370). If vaccination is interrupted after the first dose, the second dose should be given as soon as possible, with the third dose separated by an interval of at least 2 months. If the third dose is delayed, administration can proceed whenever convenient. For hemodialysis or immunocompromised patients, larger doses or an increased number of doses given either by the intramuscular or the intradermal route may result in a higher frequency of responders (206,371,717).

Group	Recombivax HB (0, 1, 6 months)	Engerix-B (0, 1, 6 months[a])
Infants and children (≤10 years)	5.0 μg	10 μg
Adolescents (11–19 years)	5.0 μg	10 or 20 μg
Adults (≥20 years)	10 μg	20 μg
Immunocompromised patients	40 μg[b]	40 μg[b,c]

[a]Alternate schedule with injections at 0, 1, and 2 months is designed for neonates born to HBsAg-positive mothers, for persons recently exposed to HBV, for immunocompromised patients, and for travelers to high-risk areas where a rapid response is desirable. A booster at 12 months is recommended.
[b]Dialysis formulation.
[c]Dosing schedule at 0, 1, 2, and 6 months is recommended.

TABLE 14. *Recommended dosages and schedules for preexposure prophylaxis with hepatitis B vaccines licensed in the United States*

To ensure that protective levels of antibody have been generated, blood should ideally be examined for anti-HBs concentration 2 to 6 months after the basic immunization series has been completed, although tests performed at a later time also can provide meaningful information (317). This is especially desirable for persons at high risk of contracting HBV, such as surgeons. A report from the laboratory that a test result is positive is not satisfactory because the level of protection may be inadequate. Based on quantitative anti-HBs results, vaccinees can be divided into four groups: (a) nonresponder, no detectable anti-HBs; (b) inadequate responder or hyporesponder, positive for anti-HBs, but with a concentration of less than 10 mIU/mL; (c) low responder, with a concentration of 10 to 100 mIU/mL; and (d) normal or adequate responder, with a concentration of greater than 100 mIU/mL. A fifth category, high responder, can be used for anti-HBs responses of greater than 1,000 mIU/mL. These designations have clinical relevance in that nonresponders or inadequate responders continue to be at risk of acquiring HBV, whereas risk of infection is low to absent in the other categories (Fig. 14) (275).

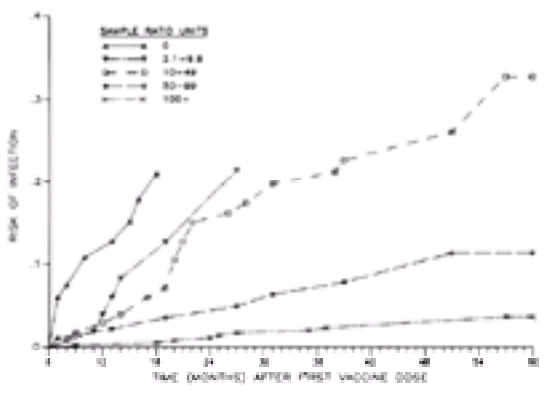

FIG. 14. Life-table analysis of risk of HBV infection according to the maximal anti-HBs level after three doses of vaccine. (From ref. 275, with permission.)

Although genetic factors may play a role in nonresponsiveness to hepatitis B vaccination, nonresponders or inadequate responders should be examined for evidence of HBV infection (HBsAg, anti-HBc, and/or HBV DNA) as another reason for

their inability to generate an adequate anti-HBs response (752). In the absence of this discovery, booster doses—consisting of one to three additional injections of vaccine in which the standard dose, or two to four times the standard dose (e.g., dialysis formulation; see Table 14), is administered—are recommended. In general, the anti-HBs concentrations remain low in these subjects. There is no evidence that nonresponders to plasma-derived vaccine will respond any differently to genetically engineered vaccines (803), but the use of hepatitis B vaccines that incorporate additional components of the envelope gene, and/or employ novel adjuvants, may be warranted (295,340,761,842).

Most experts will agree that low responders also should be encouraged to receive a booster inoculation 1 to 2 years after their last injection because breakthrough disease seems to occur earlier and more frequently in this group (see Fig. 14) (25,272). In the CDC study of homosexual men (272), 26% of 139 non- or hyporesponders had serologic and/or clinical evidence of HBV infection, with 44% of these subjects displaying HBsAg in the sampled specimen. Six of these subjects (4.3% of the total group) became persistently infected. In contrast to these data, long-term protection observed in persons who develop an adequate anti-HBs response after vaccination with the plasma-derived vaccine (greater than 100 mIU/mL) has been encouraging, as shown in Table 15 (482). In the CDC study cited earlier (272), only 11% of the 634 responders acquired HBV during follow-up, and none of these had clinical symptoms or became persistently infected. Only two (0.3%) had detectable HBsAg at the time they were sampled, and both of these individuals were positive for HIV. Most of the HBV events observed in vaccine responders were delayed for 4 to 9 years, and 72% occurred in those whose anti-HBs levels had declined to less than 10 mIU/mL. Similar results were found in another clinical trial in homosexual men (722), in the Yupik Eskimo trial in Alaska (795), in health care workers (482), in 104 infants of HBeAg-positive mothers (722), and in 654 infants of HBeAg-positive mothers in Taiwan (723).

Group[a]	N	Years of follow-up	Anti-HBs (%) <10 mIU/mL[b]	Anti-HBc positive (%)	HBsAg positive (%)
Homosexual men					
Hadler et al. (275)	634	9	54	11	0.3
Stevens et al. (722)	107	10	42	4.7	0
Alaska natives					
Wainwright et al. (795)	~1200	9-10	27	0.5	0
Health care workers	174	5	24	0	0
High-risk infants					
Hwang et al. (349)	654	5	7	7.0	0.5
Stevens et al. (722)	104	5	21	6.7	0

[a]Numbers in parentheses are references of specific studies.
[b]Anti-HBs level measured at follow-up.
Source: Modified from ref. 482, with permission, and from unpublished data (CDC).

TABLE 15. *Long-term protection from HBV infection among responders (anti–HBs>100 mIU/mL) to plasma-derived hepatitis B vaccine*

Whether to recommend a booster dose for vaccinees who have an adequate response to the vaccine remains controversial (25,275,300). One group of experts has gone on record as favoring at least one booster inoculation approximately 5 or more years after the basic series of immunizations (300). Another group of experts recommends that no booster dose is necessary for at least 10 years (135). However, for the moment it is comforting to know that few, if any, relevant clinical infections have been recorded among vaccine responders who have been followed for up to 10 years, even if they have lost detectable antibody (275,349,465,722,795). Unfortunately, in most of these studies, only limited numbers of vaccinees have been evaluated at these late time intervals, and most of these were administered the antigenically more potent plasma-derived product. In addition, the subjects being surveyed often come from high-risk groups that may be receiving sporadic natural boosters. Thus, whether the vaccine will confer lifelong immunity against chronic infection among all vaccine responders is not known. In the interim, at least one booster dose 5 to 10 years after completing the basic immunization series seems prudent, especially if the recently discontinued low-dose (2.5 µg) yeast-derived vaccine was previously administered.

It is the consensus of hepatitis experts that neither HBIG nor conventional IG should be used for preexposure prophylaxis, because active immunization with HBsAg is more effective and leads to long-term protection. In addition, there is no substitute for good personal hygiene, strict surveillance, and appropriate environmental control measures to limit transmission (129), not only of HBV but also of HCV and HIV.

Guidelines for Postexposure Immunoprophylaxis

Appropriate immunoprophylaxis can effectively prevent HBV infection following perinatal, sexual, household, or inadvertent exposure to the virus. HBIG is the mainstay of postexposure prophylaxis, although vaccine can provide an additional degree of protection in some settings, especially following perinatal exposure. Table 16 is a general guide to the recommended treatment that should be initiated following exposure to HBV, whereas Table 17 provides detailed current ACIP recommendations for postexposure immunoprophylaxis of infants born to HBsAg-positive mothers or of individuals having sexual contact with an HBsAg-positive person. When it becomes advisable to determine susceptibility prior to initiating postexposure prophylaxis, anti-HBc is the preferred serologic test for this purpose. In general, no postexposure prophylaxis is indicated for contamination of unbroken skin, for staff members who are providing routine care of patients with hepatitis B, for immune individuals, or for those who inadvertently share food or utensils with a person who subsequently develops hepatitis B, unless there are extenuating circumstances. It is hoped that all at-risk individuals should already have been immunized.

Type of exposure	Immunoprophylaxis
Perinatal	HBIG + vaccination
Sexual	
Acute infection	HBIG ± vaccination
Chronic infection	Vaccination ± HBIG
Household contact	
Acute case, no known exposure	None
Acute case, known exposure	HBIG ± vaccination
Chronic carrier	Vaccination
Inadvertent	
Percutaneous/permucosal	HBIG + vaccination

HBIG, hepatitis B immune globulin.
Source: Modified from ref. 136, with permission.

TABLE 16. *Guide to appropriate immunoprophylaxis following exposure to HBV*

	HBIG		Vaccine	
Exposure	Dose (i.m.)	Timing	Dose (i.m.)[a]	Timing
Perinatal	0.5 mL	≤12 hr of birth	0.5 mL	≤12 hr of birth
Sexual	0.06 mL/kg	≤14 days since last exposure	1.0 mL	Concurrent with HBIG (first dose)

[a]Each milliliter contains 10 μg Recombivax HB or 20 μg Engerix-B; subsequent doses at 1 and 6 months for either vaccine or the alternate four-dose schedule (0, 1, 2, and 12 months) for Engerix-B. HBIG and vaccine should be administered at different sites.
ACIP, Advisory Committee on Immunization Practices; HBV, hepatitis B virus; HBIG, hepatitis B immune globulin.
Source: From ref. 136, with permission.

TABLE 17. *ACIP recommendations for postexposure prophylaxis for perinatal or sexual exposure to HBV*

An algorithm has been devised for determining a particular course of action to take after exposure of health care workers or other individuals to a potential source of HBV (Fig. 15). If a puncture wound has been sustained, the person should be instructed to wash the affected area immediately with copious amounts of water and soap or 0.5% sodium hypochlorite, and to promote bleeding. Persons exposed to HBsAg-positive material from a known source should provide their own blood sample to the laboratory for evaluation and storage. Following this, they should immediately be given HBIG (0.06 mL/kg) and HBsAg vaccine at different sites unless records indicate prior immunity or active hepatitis B disease in the exposed individual, in which case no course of action is required. Under no circumstances should the injection of HBIG be delayed until the results of the HBV serologic tests become available. If a significant delay is anticipated in obtaining or dispensing the HBIG, conventional IG (0.06 mL/kg) containing anti-HBs could be substituted for the HBIG. This provides immediate (and perhaps sufficient) protection at a lower concentration of anti-HBs until the proper tests can be performed or HBIG can be located and dispensed. There is no precedent for recommending HBIG prophylaxis if HBV exposure has occurred more than 7 days earlier, but unusual circumstances occasionally warrant extending this time period up to 14 days.

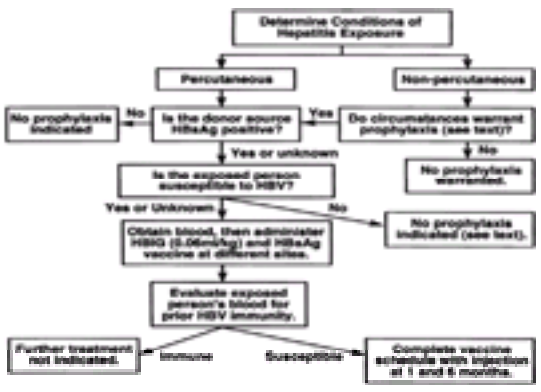

FIG. 15. Algorithm for postexposure prophylaxis of health care personnel exposed to a potentially infectious source of HBV. HBIG, high-titered specific hepatitis B immune globulin.

CHALLENGES FOR THE FUTURE

Both pre- and postexposure hepatitis B immunizations have been shown to provide immediate and long-term protection against HBV infection and to have significantly reduced the incidence of chronic infection and the onset of HCC. In comparing 1991 and 1997 birth cohorts, it is estimated that current vaccination programs have prevented more than 2.4 million people from becoming carriers and have reduced the number of hepatitis B–related deaths by 460,000. Combination vaccines are in production and experimental vaccines are continuing to be researched.

Although many vaccinees lose anti-HBs over 10 to 15 years of followup, no disease and no carriers have been detected in adults, and no clinical disease and only rare carriers have been observed in infant responders. Carrier prevalence has been reduced to below 1% in many countries where perinatal transmission is high and a national immunization program

is in place (Fig. 16). Because older carriers lose HBeAg, they are less likely to transmit disease, and when transmission does occur in an adult, the risk of chronicity is infrequent. As a result, the impact of vaccine programs has been greater than expected. Already, a reduction in liver cancer is being observed in Taiwan and The Gambia. This is significant as HCC is 100% fatal, represents a major cause of death in young men in Asia and sub-Saharan Africa, and is preventable by vaccination.

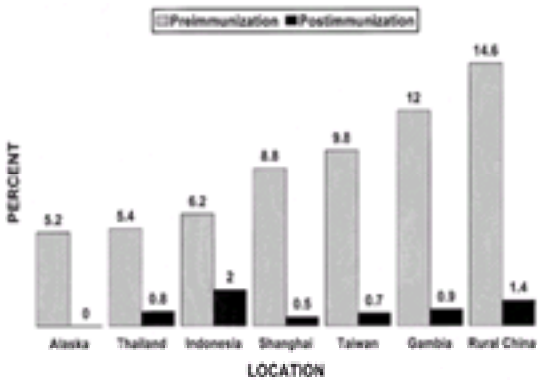

FIG. 16. HBsAg prevalence before and after the introduction of hepatitis B vaccination programs in selected high-risk areas in the world. (From M. Kane, personal communication, 2000.)

More than 60% of the countries of the world have a national program. Significantly, many Asian-Pacific countries have a national universal immunization policy that has achieved vaccination coverage rates of better than 70%. Countries with the lowest gross national product per capita are less likely to implement a program. The challenge for WHO and other international organizations is to (a) sustain existing programs, (b) assist those with economic problems to introduce the vaccine into their national immunization program by providing free vaccine, (c) increase coverage, (d) expand use to adolescent groups (catch-up immunization), (e) monitor and evaluate programs, and (f) encourage the development and deployment of combination vaccines. Unfortunately, many persons in sub-Saharan Africa, the Indian subcontinent, and in the Newly Independent States are not being vaccinated through national programs. The WHO has added hepatitis B vaccine to their recommended EPI schedule.

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CHAPTER 88 Hepatitis Delta Virus

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HISTORY

In the mid-1970s, Mario Rizzetto, a gastroenterologist in Turin, Italy, detected a previously unrecognized nuclear antigen in hepatocytes of patients with chronic type B hepatitis. The antigen resembled hepatitis B core antigen (HBcAg) in its obligatory association with hepatitis B virus (HBV) infection, but it rarely coexisted with HBcAg. The antigen was never found in biopsy samples from patients with non-B hepatitis, and it was not found in every patient with type B hepatitis. Similarly, only a proportion of such patients had antibody to the new antigen. The antigen was initially believed to be a previously unrecognized hepatitis B–specific antigen; it was called delta antigen and its antibody, anti-delta, and it was first reported in 1977 (79).

By 1980, transmission experiments in chimpanzees had confirmed that the delta antigen was a component of a transmissible pathogen that was defective and required co-infection with HBV for its replication (80). Simultaneously, a radioimmunoassay for anti-delta was developed (85), and it showed a high prevalence of anti-delta among hemophiliacs and drug users (83). In addition, it was established that hepatitis associated with expression of delta antigen was highly endemic in Italy, especially in the southern part of the country. Also, by the early 1980s, delta antigen was shown to be an internal component of a virus-like particle (Fig. 1 and Fig. 2) (81). The particle contained a small RNA, approximately 1.7 kb in size (Fig. 3), and both the delta antigen and RNA were enveloped with hepatitis B surface antigen (HBsAg), the envelope of HBV. In addition, the virus-like delta agent was shown to be associated with the most severe forms of acute and chronic hepatitis in many HBsAg-positive patients (96). Thus, in some cases, severe disease thought to be caused by HBV was actually the result of concurrent infection with the delta agent.

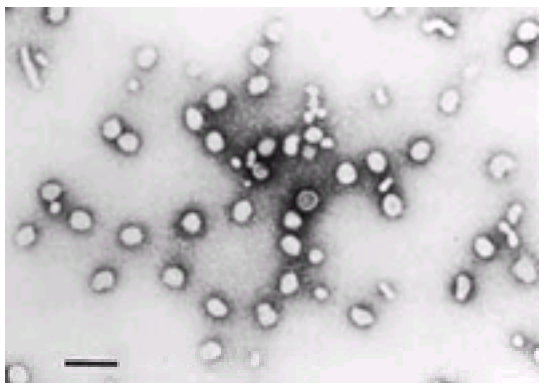

FIG. 1. Electron micrograph of the hepatitis delta virus. Peak fraction from a sucrose gradient. Stained with 1% phosphotungstic acid. Bar = 100 nm. (From ref. 35, with permission.)

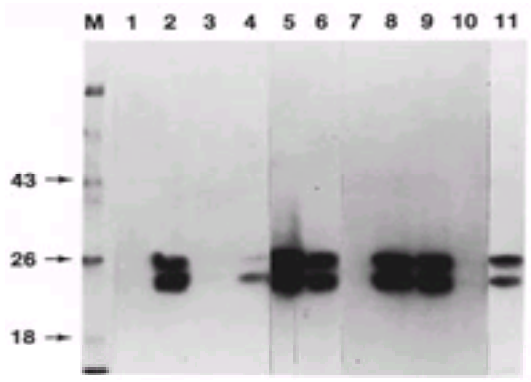

FIG. 2. Structural proteins of the hepatitis delta virus. Western blot of serum-derived HDAg with a human monoclonal antibody to HDAg. Extracts of HDAg from sera of five humans infected with HDV (*lanes 2 through 6*), two chimpanzees (*lanes 8 and 9*), and an HDV-infected woodchuck (*lane 11*) were analyzed. *Lane 1* contains an extract of an HDV-negative human serum; *lane 7*, an HDV-negative chimpanzee serum; and *lane 10*, an HDV-negative woodchuck serum. *Lane M* contains molecular-weight markers, and the relative molecular masses in kilodaltons are indicated on the left margin. (From ref. 4, with permission.)

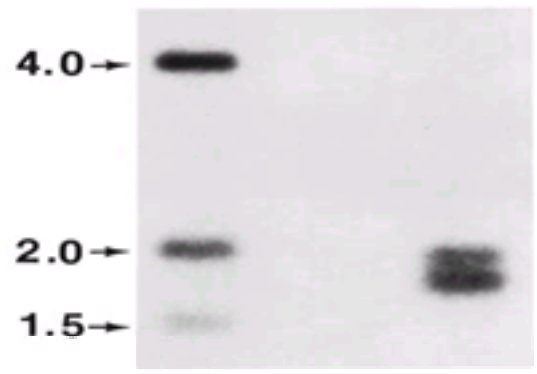

FIG. 3. Forms of hepatitis delta virion (genomic) RNA. Northern blot of RNA extracted from HDV particles and electrophoresed in 6% formaldehyde/1% sucrose gel. The two apparent sizes of HDV RNA represent linear and circular forms of the genome. Size markers (molecular weights in kilobases) are on the left margin. (Courtesy of F. Negro.)

By 1983, evidence for infection with the delta agent had been found on every populated continent (76). A year later, the virus was found to be the cause of severe hepatitis among indigenous populations of Venezuela (32), the first evidence outside of Italy, and the agent was making a major medical impact on populations not frequently exposed to blood. Subsequently the medical importance of the delta agent in South America was extended to include etiologic associations with traditionally severe hepatitis in Colombia (hepatitis de Sierra Nevada de Santa Marta) (48), and Brazil (Labrea black fever) (26), as well as more recently described severe hepatitis in Peru (94) and indigenous populations of Ecuador (53).

Also by 1983, the delta agent was considered sufficiently unique and medically important to be designated a distinct hepatitis virus, hepatitis delta virus (HDV), and the disease it caused was designated delta or type D hepatitis (78). By 1984, similarities between HDV and certain pathogens of plants, especially plant virus satellites, were noted. The cloning and sequencing of the HDV in 1986 confirmed the uniqueness of HDV among animal viruses and its similarity to a broad group of plant pathogens, including the viroids (110). Recent advances in our understanding of molecular features of HDV biology have revealed novel biologic pathways; these and the frequently devastating consequences of infection drive further research on this cause of acute and chronic liver disease. Thus, a chance observation by an inquisitive gastroenterologist resulted in the discovery of one of the most interesting and unusual of human pathogens.

INFECTIOUS AGENT

Classification and Structure

The approximately 36-nm hepatitis delta virus consists of an RNA genome and approximately 70 copies of a virus-specified nucleocapsid protein, hepatitis delta antigen (HDAg), which are enveloped by the surface antigens of the helper HBV. At 1.7 kb, the HDV genome is the smallest of the known human pathogens, and its limited coding capacity is reflected in the simple composition of the HDV particle. Because the sole viral protein is encoded by antigenomic RNA, HDV is a negative-stranded RNA virus. The envelope protein, which is required for HD virion formation and infection, is the only helper function provided by HBV. Its circular RNA genome and mode of RNA replication (see later) most closely resemble subviral plant pathogens, viroids, and virusoids. HDV is currently the only member of the *Deltavirus* genus (106).

Key Antigens

The nucleocapsid protein of HDV consists of two peptides, 24 and 27 kd in size (4,6) (see Fig. 2). The two species are related in that they share the same initiation codon and have common amino-terminal sequences, but they use different termination codons (110,111). Each is translated from a distinct mRNA species arising from a unique RNA editing event (64). The 24-kd and 27-kd species are referred to as HDAg-S and HDAg-L, respectively; both are serine-linked phosphoproteins with differing biologic functions in the virus life cycle (44,47). Dissection of the functional features of

HDAg has identified several essential elements: a nuclear localization signal (13,116) that targets HDAg to the nucleus where replication occurs; a coiled-coil domain (109) that mediates formation of HDAg dimers and higher-order structures; and arginine-rich motifs that are necessary for HDV RNA binding activity. The two forms of HDAg (S and L), which differ by the presence of an additional 19 to 20 amino acids at the C-terminus of HDAg-L, have distinct and opposed functions. HDAg-S is required for HDV replication, whereas HDAg-L inhibits HDV replication and is required for virion formation. An isoprenylation signal sequence at the C-terminus of HDAg-L is required for direct interaction between HDAg-L and HBsAg (28). The specific mechanism by which HDAg-S supports HDV RNA replication (and by which HDAg-L inhibits the process) is unknown. HDAg is not believed to act catalytically. It contains no known polymerase motifs, and no polymerase activity has been identified.

The HDV envelope consists of products of the HBV S and pre-S genes (see Chapter 86 and Chapter 87). The pre-S/S genes of HBV contain three start signals and a single termination codon. All three potential gene products are synthesized, and differential glycosylation results in six proteins or glycoproteins of varying molecular weights. The two smallest molecules are the most abundant, both in the HBV virion and in HBsAg-particles; they are designated *major* or *small* envelope proteins. The two intermediate-sized molecules are more abundant in virions than in HBsAg and are designated the *middle* proteins. The two largest proteins are also most abundant in virions and are called *large* proteins. The middle proteins contain a site encoded by the pre-S2 portion of the HBV genome that is a receptor for polymerized human albumin. The large proteins contain a separate site, encoded by the pre-S1 portion of the HBV genome, that is thought to bind directly to hepatocytes and mediate entry of the virus into cells. The large forms of HBsAg appear to be essential for initiation of infection because antibodies to pre-S1 and pre-S2 epitopes can neutralize the infectivity of HDV (103), and HDV particles containing only the small and large HBV surface proteins are infectious (100,101).

There is no evidence to date that the HBV-derived envelope proteins are further modified when they become the envelope for HDV, and surface epitopes unique to HDV have not been detected. As described later, HDV can use different hepadnaviruses as helpers under experimental conditions, and in each case the envelope of HDV has the physical and antigenic characteristics of the helper virus.

The HDV Genome and Its Replication

The 1.7-kb HDV RNA is structurally and functionally similar to plant viroid and virusoid RNAs (Fig. 4). It is single-stranded and circular, and it forms an unbranched rod structure with intramolecular base-pairing over 70% of its sequence (41,52,110). The only known gene product, HDAg, is coded for by the antigenomic strand. The RNA contains several functional and structural features (ribozyme sites, RNA editing, replication by host DNA-dependent RNA polymerase) that are essential for replication, and that distinguish HDV as a particularly interesting model system for RNA biology.

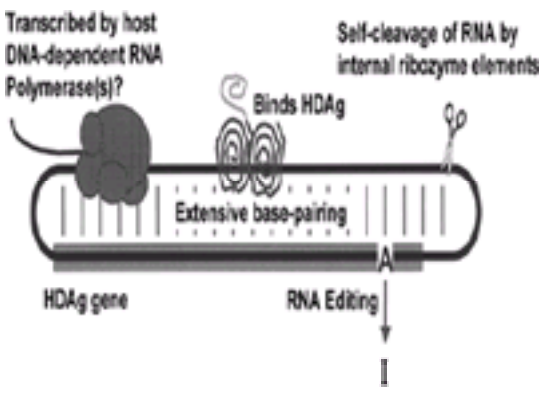

FIG. 4. Structure of the HDV RNA Genome. The single-stranded circular RNA genome is indicated by the *heavy black continuous line.* The genome has the ability to form an unbranched rod structure, in which approximately 70% of the bases are engaged in Watson-Crick pairs with counterparts from the opposite side of the circular RNA. In this unbranched rod structure, the region encoding HDAg (nt 1598-957) is on one side. The RNA editing site is at position 1012 in the antigenome. The region on the right-hand side contains the autocatalytic cleavage sites (ribozymes), one in the genome (nt 686) and the other in the antigenome (nt 900). The genome binds HDAg and is transcribed by a host DNA–dependent RNA polymerase. Numbering is according to Wang et al. (110).

Both the RNA genome and the antigenome contain closely related RNA structural elements of about 85 nucleotides (nt), termed ribozymes, that cleave the viral RNA at specific sites (43,91,112). Both ribozymes are present at one end of the unbranched rod structure (see Fig. 4), one in the genome and the other in the complementary antigenome. The ribozymes are central to the double-rolling-circle mechanism by which HDV RNA is replicated (7). According to this model, replication of genome and antigenome RNA from a circular template produces linear multimeric RNAs; these are processed by autocatalytic cleavage at the ribozyme sites to yield linear RNAs of unit length. In the final step of this process, unit-length RNAs are ligated to form circles; recent results suggest that a host RNA ligase is involved (75).

The HDV ribozymes are the only such elements known to be present in human viruses. They have been extensively studied biochemically, and a crystal structure was recently determined for the genome ribozyme (1,24). The HDV ribozymes are functionally similar to other ribozymes, including “hammerhead” ribozymes present in virusoid RNAs, in that they produce cleavage products with a 5'-hydroxyl and a 2',3'-cyclic phosphate. However, the HDV genome and antigenome ribozymes form similar complex, nested, double pseudoknot structures in which the active site is buried in a stable core reminiscent of protein catalytic sites (24,108). The catalytic sites formed by these unique structures enhance the cleavage rate, making the HDV ribozymes the fastest known self-cleaving RNAs—nearly 100-fold faster than hammerhead ribozymes.

HDV RNA replication begins with the circular RNA genome serving as the template for the synthesis of antigenome transcripts (Fig. 5). In step 1, two types of antigenomic RNAs are produced: a 0.8-kb mRNA that encodes HDAg-S, and greater-than-full-length transcripts. The mRNA is translated to yield HDAg-S, which promotes the transcription/replication process. The multimeric transcripts are processed to circular monomeric antigenome RNAs, as discussed previously. These circular RNAs then serve as templates for synthesis of circular genomic RNA by similar processing, with the exception that no genomic-sense mRNA is produced. Infected cells may produce over 100,000 copies of HDV genomic RNA.

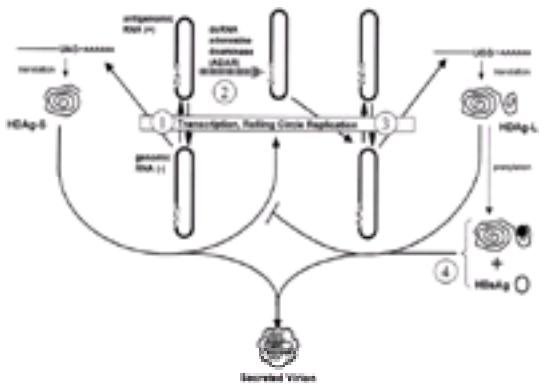

FIG. 5. Replication scheme of hepatitis delta virus. **1:** Replication begins with transcription of genomic RNA encoding the short form of HDAg (HDAg-S). Two types of antigenomic RNAs are produced: an mRNA that encodes HDAg, and a greater-than-unit-length antigenome. The latter undergoes autocatalytic cleavage via an internal ribozyme and is ligated by a host enzyme to yield circular antigenomes, which then serve as template for genome synthesis by a similar mechanism. HDAg-S is required for RNA replication. **2:** The adenosine within the stop codon for HDAg-S is deaminated to inosine in approximately 30% of antigenomic RNAs by a host enzyme. **3:** During subsequent RNA transcription, this change appears in the mRNA, which now encodes the long form of HDAg (HDAg-L), which inhibits RNA replication. **4:** The C-terminus of HDAg-L is prenylated by a host farnesyltransferase, facilitating interaction with the envelope protein (HBsAg) and subsequent virion formation.

The mechanism by which HDV RNA replicates its RNA is one of the more intriguing aspects of HDV biology. The virus does not encode a polymerase and there is no known RNA-dependent RNA polymerase in humans, the only known natural host of HDV. It is thus likely that HDV, by an undefined mechanism, corrupts the normal function of a host DNA–dependent RNA polymerase such that it functions on an RNA template. Sensitivity to alpha-amanitin and the presence of a 5' cap and poly(A) tail implicate RNA polymerase II for mRNA synthesis (29,37,49,50,56). Genome and antigenome synthesis are less sensitive to alpha-amanitin and appear to be differentially sensitive to inhibition by HDAg-L (55,56). The significance of these differences is not yet clear, but it could be explained by the involvement of different polymerase complexes for replication and transcription.

As noted previously, HDAg-S is required for HDV RNA replication, but HDAg-L, which inhibits RNA synthesis, is necessary for virion formation. HDV balances the conflicting but essential functions of these two gene products by using a cellular function known as RNA editing. Infectious RNA encodes HDAg-S. During RNA replication (see Fig. 5, step 2), a fraction (30% to 50%) of antigenome transcripts are “edited” by a cellular enzyme known as double-stranded RNA adenosine deaminase (9,64,65). This enzyme converts specific adenosines in RNAs to inosine. In HDV RNA, the edited position is within the stop codon for HDAg-S. Because of the different base-pairing tendencies for adenosine (which base-pairs with U) and inosine (which base-pairs with C), the edited antigenome eventually (after a round of replication and transcription; see Fig. 5, step 3) yields mRNA in which the HDAg-S stop codon has been changed to a tryptophan codon, allowing synthesis of HDAg-L. As increasing amounts of HDAg-L are produced, the mode of replication switches from RNA synthesis to packaging.

Propagation in Cell Culture and Biologic Characteristics

There is no practical *in vitro* cell culture system for the propagation of HDV. Primary hepatocyte cultures from woodchucks and chimpanzees have been infected with HDV, but replication was limited to a single cycle in a small proportion of cells (16,102,104). Although the HDV genome can replicate in a number of cells after transfection of HDV complementary DNA or RNA, infection appears to be limited to primary hepatocytes (27,42,102,104). Complete replication of the virus, including assembly and release, is limited to primary hepatocytes that are infected with a hepadnavirus or cultured cells cotransfected with HDV and hepadnavirus cDNAs (12,88,100,103,113). Such *in vitro* systems, which are capable of producing infectious HDV, have contributed greatly to our understanding of the molecular biology of this pathogen.

Infection in Experimental Animals

The host range for productive replication of HDV is limited to those species that support the replication of a hepadnavirus capable of supplying the essential helper function, the surface antigen envelope. These include the chimpanzee (hepatitis B virus) (80) and the eastern woodchuck (woodchuck hepatitis virus) (66).

Chimpanzees were shown to be susceptible to infection with HDV shortly after the discovery of delta antigen (80). Infection of the chimpanzee mimics that of humans, and much of our understanding of HDV infection is based on studies in this experimental model; both co-infection (simultaneous infection with HBV and HDV) and superinfection (infection of an HBsAg-positive subject) have been documented (74). Co-infection results in moderately severe hepatitis consisting of

a unimodal or bimodal elevation of alanine aminotransferase (ALT) activity and associated hepatitis. In unimodal disease, both HBcAg and HDAg are simultaneously expressed in the nuclei of infected hepatocytes; in bimodal hepatitis, one of the two antigens is usually predominant in the first episode and the other is expressed predominantly in the second episode (74). The order in which HBcAg and HDAg are expressed is probably determined by the relative titers of HBV and HDV in the infectious source. Regardless of the order, HBsAg is detected before HDAg in the blood. Co-infection of chimpanzees with HBV and HDV is followed by a transient immunoglobulin M (IgM) anti-HD response and a longer-lived immunoglobulin G (IgG) response. Superinfections of HBV carrier chimpanzees with HDV result in more severe disease. During acute HDV superinfection of HBV carrier animals, expression of markers of the HBV infection (HBsAg, HBV DNA) are suppressed. Co-infections of HDV and HBV usually result in acute, self-limiting hepatitis D. Superinfections of HBV carrier chimpanzees with HDV also have usually resulted in apparent acute, self-limiting hepatitis D, perhaps because most HBV carrier chimpanzees demonstrated relatively low-level replication of HBV, as measured by absence of HBeAg and presence of anti-HBe at the time of challenge. However, subsequent analysis with more sensitive assays of such anti-HBe–positive HBV carrier chimpanzees that had been superinfected with HDV showed that more than 50% of the animals actually had evidence of recurring low-level HDV replication (57). Furthermore, a chimpanzee that was HBeAg positive when challenged with HDV (25) demonstrated recurrent hepatitis D, with persistence of HDAg and other markers of HDV infection in the blood. The liver appears to be the only organ involved in HDV infection of chimpanzees. Hepatitis D in chimpanzees is generally more severe than other types of human viral hepatitis in this animal model (70).

Hepatitis D virus of human origin also infects woodchucks (*Marmota monax*) that are infected with the woodchuck hepatitis virus (WHV), causing acute or chronic hepatitis in a high proportion of animals (66,69). Hepatitis D in woodchucks is similar to that seen in chimpanzees; the liver is the only organ involved, despite the occurrence of WHV replication in extrahepatic tissues (58). Replication of HDV in woodchucks depends on the presence of replicating WHV, and the HBsAg envelope of HDV is replaced by the WHV envelope (WHsAg) (66). Hepatitis D virus that was passaged up to five times in woodchucks was readily transmitted back to chimpanzees with undiminished virulence (69,74). Serial passage of HDV in woodchucks resulted in a progressive shortening of the incubation period and the establishment of chronic HDV infection in most of the animals.

Transmission of HDV to mice has been reported (60). Infection, which could be achieved by intravenous or intraperitoneal inoculation, resulted in a single round of replication of the HDV genome in hepatocytes. The limitation to a single cycle of replication was probably the absence of a helper hepadnavirus to supply an envelope to the HDV nucleocapsid, thereby preventing release of complete virus and secondary infection of other cells. Efforts to develop transgenic mouse models of HDV RNA replication have been successful, and HDAg expression in a number of tissues has been demonstrated (62); also, DNA vaccination into skeletal muscles of mice leads to the development of anti-HD antibodies in nontransgenic animals, indicating that translation occurs in mouse cells (63).

Pathology and Pathogenesis

Direct pathologic changes in type D hepatitis are limited to the liver, the only organ in which HDV has been shown to replicate. The histologic changes, consisting of hepatocellular necrosis and inflammation, are those of acute or chronic viral hepatitis. There are no distinguishing features of the liver histology that differentiate type D hepatitis from other forms of viral hepatitis, except that the former tends to be more severe. The liver biopsy may contain only elements of acute hepatitis if co-infection with HBV and HDV has occurred, or elements of both acute and chronic hepatitis if acute HDV infection is superimposed on chronic HBV infection, or only the characteristics of chronic hepatitis if the patient is chronically infected with both HBV and HDV. In acute hepatitis, inflammatory cells, primarily lymphocytes and macrophages, are prominent in parenchyma and portal areas. Hepatocytes may be swollen and undergoing eosinophilic necrosis. Pathologic changes are often patchy and focal, except in more severe cases, when there may be confluent, submassive, or massive necrosis.

The histologic changes in chronic viral hepatitis are also those of inflammation and hepatocellular necrosis, but inflammation is largely portal and consists of lymphocytes, macrophages, and histiocytes. Necrosis of hepatocytes is usually evident but less severe than in acute viral hepatitis, and its distribution is typically periportal. A histologic lesion peculiar to HDV infection in northern South America, especially in the Amazon basin, is a microvesicular steatosis that gives rise to a characteristic alteration of the hepatocyte [a morula cell (71)] (Fig. 6). However, similar cells have been seen in severe hepatitis D elsewhere, including Africa and Italy. This severe form of HDV hepatitis in northern South America has been associated with a unique genotype of the virus (8) (see later).

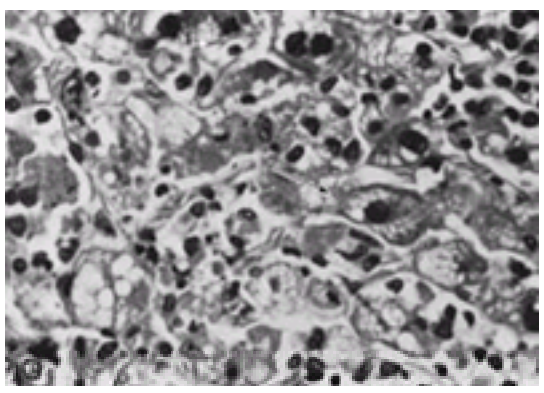

FIG. 6. Typical morula cells from a patient with hepatitis caused by HDV. (Courtesy of S. Thung.)

Only nonspecific electron microscopic changes have been observed in natural and experimental HDV infections, best

visualized in liver biopsy samples of experimentally infected chimpanzees (39). The most prominent of such changes are cytoplasmic tubular structures that represent alterations of the endoplasmic membrane and microtubular structures. Such structures are considered to be manifestations of exposure of hepatocytes to interferon, presumably produced in response to the viral infection (93).

The pathogenesis of type D hepatitis is poorly understood. A direct cytopathic effect of the small form of delta antigen, HDAg-S, in the absence of replication of the HDV genome has been reported (17). However, this has not been supported by data from liver biopsies (59); and the observation that some HDV-infected cell lines can survive for at least several cell divisions, indicating that HDV RNA replication is not acutely cytopathic (5); and from the failure to observe pathology of HDV expression in the transgenic mouse model (62). Nevertheless, the rate of loss of HDV-infected mice is most consistent with virus-mediated cell death (60). None of the hepatitis viruses have been shown to be directly cytopathic, and there is strong evidence supporting the notion that damage to hepatocytes is immunologically mediated, particularly for HBV (15). As with the other hepatitis viruses, peak replication of HDV precedes the peak histopathologic changes. In addition, replication of the virus has often been markedly diminished and the immune response begun by the peak of pathology; this adds credence to the belief that humoral or cellular immune mechanisms are involved. In the chimpanzee model of experimental acute HBV infection, viral clearance was associated with the noncytopathic antiviral effects of cytokines produced by non-T cells, whereas acute disease was associated temporally with T cells (30).

In addition to acute and chronic type D hepatitis, a third form of HDV infection, called latent infection, has been described in the orthotopic liver transplant setting in end-stage hepatitis D patients. Markers of HDV infection occurred in the absence of detectable markers of HBV infection, suggesting that HDV might replicate at low levels in the absence of HBV (97). Subsequent analysis with more sensitive assays showed that low-level HBV replication was occurring coincident with HDV replication, thus reaffirming the dependence of HDV on the HBV helper function and the absence of a true latent phase (95).

CLINICAL FEATURES

The clinical course of type D hepatitis is variable but is usually more severe than that of other viral hepatitides. After an incubation period of 3 to 7 weeks, the pre-icteric phase begins with the onset of nonspecific clinical symptoms of fatigue, lethargy, anorexia, and nausea, usually of 3 to 7 days duration. During the pre-icteric phase, biochemical markers of hepatitis, such as serum ALT and aspartate aminotransferase (AST) activities become abnormal. Viral replication is usually diminished during this phase.

The onset of the icteric phase, if it occurs, is characterized by the appearance of jaundice. Fatigue and nausea usually persist, and the serum bilirubin level becomes abnormal; this coincides with the appearance of clay-colored stools and dark urine, evidence of the liver's diminished ability to conjugate and excrete bilirubin. The most severe stage of clinical disease may coincide with a further decrease in the level of markers of viral replication. In patients with acute, self-limiting infection, convalescence begins with the disappearance of clinical symptoms. Anorexia and nausea disappear relatively early, but fatigue and lethargy may persist for weeks or months.

Fulminant viral hepatitis, the most severe form of acute disease, is a rare sequela of acute viral hepatitis but is approximately 10 times more common in type D hepatitis than in the other types of viral hepatitis. It is characterized by hepatic encephalopathy that is manifested by changes in personality, disturbances in sleep, confusion and difficulty concentrating, and, in severe cases, abnormal behavior, somnolence, and coma. Jaundice may be profound, but serum ALT and AST values may decrease as massive hepatic necrosis leaves few viable hepatocytes. The mortality rate of fulminant hepatitis is approximately 80%. Fulminant hepatitis is an indication for orthotopic liver transplantation.

Chronic viral hepatitis is a frequent sequela of type D hepatitis (see later). Unlike other forms of chronic viral hepatitis, chronic type D hepatitis is usually initiated by a clinically apparent acute infection. Symptoms are those of acute hepatitis but often less severe. Serum ALT and AST values are elevated, but serum bilirubin and albumin levels and prothrombin times may be normal, at least before cirrhosis develops. Approximately 60% to 79% of patients with chronic type D hepatitis develop cirrhosis (86), a figure almost three times higher than for type B hepatitis and type C hepatitis; a high proportion die of hepatic failure. Hepatocellular carcinoma (HCC) has been reported in some patients with chronic type D hepatitis. In a recent study of western European patients with compensated cirrhosis type B, HDV infection increased the risk for HCC threefold and for mortality twofold (23). It is unknown whether HCC is an effect of the underlying cirrhosis, a known risk factor for HCC, or a direct carcinogenic effect of the virus (2).

Because HDV infection has an absolute requirement for an associated HBV infection, acute disease occurs as one of two patterns, depending on the prior HBsAg status of the HDV-infected subject. Simultaneous infection of a susceptible individual with HBV and HDV (co-infection pattern) results in both acute type B hepatitis and acute type D hepatitis. The incubation period generally depends on the HBV titer of the infecting inoculum because the incubation of type B hepatitis is inversely related to the dose of virus. Depending on the relative titers of HBV and HDV, a single bout of hepatitis or two bouts of hepatitis, characterized by a biphasic pattern of serum ALT and AST values, may be seen. Usually, the first bout of hepatitis is HBV related; however, hepatitis B followed by hepatitis D and hepatitis D followed by hepatitis B have both been observed in experimental infections of chimpanzees (74). Co-infections of HBV and HDV are usually acute, self-limited infections.

In contrast, HDV infection of an individual chronically infected with HBV (superinfection pattern) causes a generally severe acute hepatitis with a relatively short incubation period that leads to chronic type D hepatitis in over 70% of cases. Superinfection with HDV is often associated with fulminant acute hepatitis and the most severe form of chronic hepatitis, chronic active hepatitis, often with cirrhosis. A subacute, rapidly progressive form of HDV superinfection has been seen in HBsAg carriers among the Yucpa Indians, an indigenous population of Venezuela (72). Such patients often die within 18 months of disease onset. This disease pattern may be related to a particular HDV genotype (see later).

DIAGNOSIS

Differential Diagnosis

Type D hepatitis should be considered in individuals who are HBsAg positive or who have evidence of recent HBV infection, especially if they have been exposed to blood or blood products or if they are members of a high-risk population or from a high-risk environment (see later). Other conditions to be differentiated from type D hepatitis include co-infection of hepatitis A virus or hepatitis C virus with HBV, superinfection of an HBsAg carrier with one of these two viruses, and exacerbation of a chronic HBV infection, with recurrence of clinical disease.

Laboratory Diagnosis

The typical serologic course of acute HDV co-infection is shown in Figure 7. Hepatitis B surface antigen, hepatitis B e antigen (HBeAg), and HBV DNA appear in the serum during the incubation period in a pattern characteristic of acute type B hepatitis. Antibody to HBcAg appears, coincident with onset of clinical disease; evidence of active viral replication has often disappeared by the peak of illness. Antibody to HBsAg appears later, during convalescence. The diagnosis of acute type D hepatitis is made on the basis of serologic tests for this virus. Antibody to HDAg (anti-HD) develops late in the acute phase of infection and may be present only transiently and in low titer. Assays for IgM anti-HD (21) and for virion-associated HDV RNA or HDAg in the serum (51,57,117) are more reliable markers of acute HDV infection. In the co-infection pattern, all markers of viral replication disappear in early convalescence, and IgM anti-HD and even IgG anti-HD disappear within months to years after recovery.

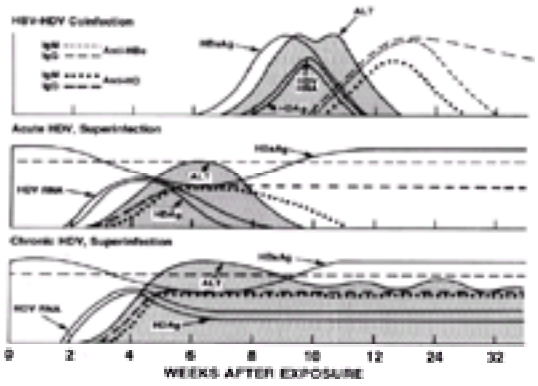

FIG. 7. The serologic patterns of type D hepatitis: Co-infection and superinfection. **Top:** Coexistent acute hepatitis B and hepatitis D. **Middle:** Acute hepatitis D superimposed on a chronic hepatitis B virus infection. **Bottom:** Acute hepatitis D progressing to chronic hepatitis, superimposed on a chronic hepatitis B virus infection. (From ref. 73, with permission.)

In contrast, superinfection with HDV usually results in persistent HDV infection. A typical serologic pattern for such hepatitis is shown in Figure 7. Evidence for HD viremia appears in the serum during the pre-acute phase and is followed by the appearance of IgM anti-HD and IgG anti-HD during the acute phase. Suppression of markers of HBV replication usually occurs during the acute phase but may be difficult to demonstrate without comparison with pre-HDV levels. Progression to chronicity is associated with high levels of IgM ant-HD and IgG anti-HD. These antibodies persist, as do positive tests for serum virion-associated RNA and HDAg (evidence for viremia) and intrahepatic HDV RNA and HDAg. In some experimental chronic infections of chimpanzees, only the antibodies persisted at constantly detectable levels. Viremia is indicative of virus replication and is associated with active liver disease (98). There is increasing use of molecular methods, such as polymerase chain reaction (PCR), to assess viremia in laboratory diagnosis of clinical disease (54).

TREATMENT

There is no specific treatment for type D hepatitis (77). Immunosuppressive therapy has been found to be of no clinical value (86) and is probably contraindicated, a situation that applies to the other forms of acute and chronic viral hepatitis as well. Because of the absolute dependence of HDV on HBV, therapies for HBV might be expected to control HDV also, but it is not certain that this is the case. For example, low to moderate doses of alpha-interferon have been shown to have an inhibitory effect on HDV replication (36,84), but, as with interferon treatment of HBV infection, beneficial effects were only transient. Massive doses of alpha-interferon [9 million units three times a week for 12 months, or 5 million units daily for up to 12 months (19,22)] have yielded a somewhat higher rate of remission, although most patients remained positive for HDV RNA whether or not their hepatitis improved, and despite the fact that relapse was common in the posttreatment period. In studies of the effectiveness of lamivudine in HDV-infected patients, neither HDV viremia nor liver disease was reduced (46). As mentioned previously, the only helper function that HBV provides for HDV is the envelope, and HBsAg circulates at very high levels in most HBV carriers. An effective therapy would probably require a marked suppression of HBsAg levels in order to impact on HDV, and current therapies for HBV do not achieve this.

Orthotopic liver transplantation has been relatively useful for treating fulminant acute and end-stage chronic hepatitis D. The results have been somewhat better than the disappointing results obtained with chronic HBV infection (97,107). Markers of continuing HDV replication are sometimes found in transplant patients shortly after transplantation, but liver disease does not recur unless significant HBV replication occurs. Recurrence of HBV in patients with both HBV and HDV infections is less frequent than in patients with HBV alone.

EPIDEMIOLOGY

Geographic Distribution

Seroprevalence studies of anti-HD in HBsAg-positive individuals have shown a worldwide, but nonuniform, distribution (32,68,82). Areas of high prevalence include the Mediterranean basin, the Middle East, central Asia, East Africa, the Amazon basin, and certain islands of the South Pacific (Fig. 8). Hepatitis D is less common in eastern Asia, although HDV has been implicated in up to 20% of cases of acute hepatitis in some populations of HBsAg-positive individuals in Taiwan, China, and India. High-risk populations in industrialized countries include hemophiliacs, users of illicit parenteral drugs, and others exposed to blood or blood products. HDV has been less frequent than infection with HBV or human immunodeficiency virus (HIV) infection among sexually promiscuous homosexual and heterosexual groups, suggesting that it is not typically a sexually transmitted disease, although such transmission may sometimes occur (18,99). Prevalence studies probably represent minimal values, because anti-HD can disappear after acute, self-limiting infection.

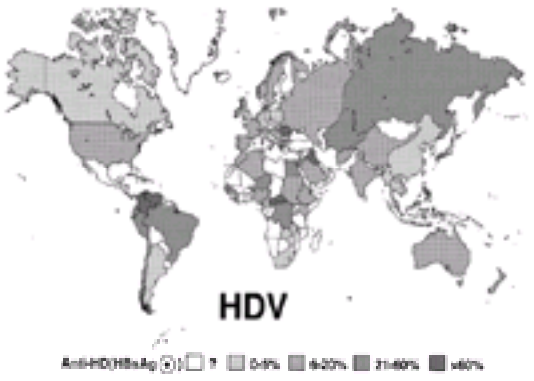

FIG. 8. The worldwide distribution of HDV infection, as measured by the prevalence of anti-HDAg in HBsAg-positive individuals with acute or chronic hepatitis. (Modified from refs. 31,68, and 82, with permission.)

Molecular studies of HDV isolates have revealed the existence of at least three major genotypes, with different geographic distributions (Fig. 9) (8,14,61,90,114). Whereas genotype I is found worldwide, genotypes II and III appear to be more geographically constricted to the Far East and northern South America, respectively. Genotype-specific support of HDV RNA replication by HDAg-S suggests that the genotype classifications have a functional as well as phylogenetic basis (11).

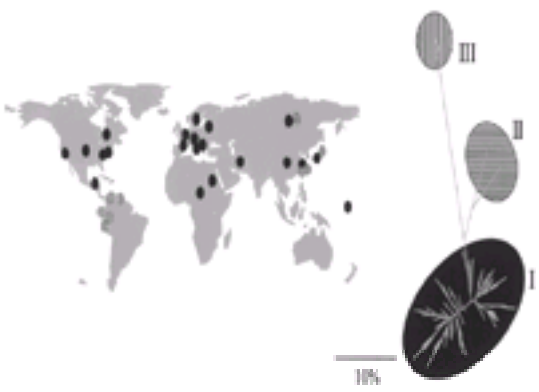

FIG. 9. Geographic distribution of HDV genotypes. Sequences of HDV isolates from different areas were compared by maximum likelihood phylogenetic analysis. *Horizontal bar* indicates approximately 10% sequence divergence. *Circles* on the map correspond approximately to geographic origin of isolates. *Filled circles*, genotype I; *horizontal stripes*, genotype II; *vertical stripes*, genotype III.

Morbidity and Mortality

The ratio of clinical to subclinical cases of HDV co-infection and superinfection is unknown, but the general severity of both forms of infection suggests that most cases are clinically important. The chronicity rate is 1% to 3% for co-infection and 70% to 80% for superinfection. The mortality rate of hepatitis D is 2% to 20%, 10 times higher than the rate for hepatitis B, the next most severe type of viral hepatitis. The patterns of disease associated with chronic HDV infection, however, vary in different epidemiologic settings, particularly with genotype I. In areas where HDV infection is endemic, such as southern Italy, a more slowly progressing, stable disease pattern appears to predominate, whereas in areas where HDV infection is not endemic, such as northern Europe and North America, the most severe forms of type D hepatitis frequently occur among intravenous drug users.

A very different pattern appears to be found in northern South America in association with genotype III. Whereas HDV is endemic in some communities, disease frequently follows a rapidly progressive course that is associated with significant morbidity and mortality. The varying patterns of disease could be related to a number of factors, including HDV genotype, characteristics of the HBV, variable responses in the host immune system, infection with other viruses, and substance abuse. The possible associations of genotypes II and III with mild and severe forms of type D hepatitis, respectively, is

particularly intriguing (8,10,115). Additional clinical, epidemiologic, and laboratory investigation of these genotypes will prove valuable in determining whether these associations are definitely related to HDV genotype, and in evaluation of disease mechanisms.

Age

Hepatitis D virus infects all age groups. It has been transmitted perinatally (with HBV) in Italy, but fortunately, HDV is not common in regions of the world, such as Asia, where perinatal transmission of HBV is common. Type D hepatitis affects children in the Amazon basin and has occurred among children with hemophilia in industrialized countries, especially before blood was screened for HBV. It also occurs among young adults over most of its geographic distribution and especially among users of illicit drugs in industrialized countries.

Epidemics

The epidemiology of HDV, which is principally transmitted by blood and blood products, is not conducive to epidemics in the classical sense. However, contamination of lots of clotting factor with HDV, as well as HBV, hepatitis C virus (HCV), and HIV, in past years (87) was responsible for the high incidence of chronic hepatitis among recipients of these products and the high prevalence of serologic evidence of infection (105). Small epidemics of severe type D hepatitis have occurred among drug addicts, and prolonged epidemics, leading to high cumulative attack rates, have occurred in some populations of drug addicts upon introduction of HDV into those populations (20,33,34,45,92). Ongoing outbreaks of HDV hepatitis are occurring in various regions of northern South America, especially the Amazon basin, and often involving fragile indigenous Amerind populations, such as the Waorani (53).

Immunity

Second cases of type D hepatitis have not been reported, suggesting that some form of immunity after acute, self-limiting infection exists; as mentioned previously, this may involve cellular immune mechanisms. Hepatitis B virus carrier chimpanzees superinfected with HDV were resistant to rechallenge with HDV 6 months later (74). Similar chimpanzees rechallenged 3 to 5 years after initial HDV infection, were protected or developed mild transient hepatitis. All of the chimpanzees were anti-HD positive at the time of challenge, but most lacked antibodies to the envelope of HDV (i.e., HBsAg). However, some of these chimpanzees still may have been replicating HDV at low levels at the time of rechallenge (57), and protection by interference rather than immunity could not be ruled out.

There is some experimental evidence in woodchucks that immunity to epitopes of the internal HDAg of HDV modifies the course of HDV infection (3,40). Indeed, vaccination against the internal HBcAg of HBV has also been shown to protect against type B hepatitis; again, cell-mediated immune mechanisms appear to be responsible. Clearly, antibodies to internal antigens such as HDAg and HBcAg do not protect, because both viruses are transmitted perinatally to infants who have high levels of these maternally derived antibodies.

Prevention and Control

Situations in which there is opportunity for repeated exposure to HBV constitute the major risks for co-infection or superinfection with HDV. The current policy of excluding blood donors with serologic markers of HBV infection has markedly diminished the chances of HDV transmission by blood transfusion. Similarly, screening of donors to large plasma pools used for the fractionation of blood products has diminished the risk of types B and D hepatitis from such preparations, but the inadvertent introduction of a single unit of plasma positive for HDV and HBV could contaminate the whole lot because of the high infectivity titers of these two viruses in blood (67). Practices designed to minimize the transmission of HBV, HCV, and HIV among drug addicts will also diminish the transmission of HDV to the extent that they are implemented. Implementation of better hygiene, especially control of weeping skin lesions, thought to be involved in the transmission of HDV in some primitive settings, may help control the spread of HDV and other blood-borne viruses.

Immunoprophylaxis against HDV is achieved by vaccination against HBV, because HDV uses the envelope proteins of HBV and has no detectable unique surface antigens. Hepatitis B vaccines currently available are highly effective in preventing HBV infection and the disease sequelae. Public health measures to interdict the transmission of blood-borne pathogens, combined with aggressive HBV vaccination programs at the national level, offer great promise for the control of type D hepatitis. Indeed, reports from Taiwan and Italy indicate decreasing rates of HDV transmission in these populations as a result of these wide-ranging control efforts (38,89). Unfortunately, some high-risk populations are among the most difficult to reach with such programs.

PERSPECTIVES

Over 300 million people in the world who are chronically infected with HBV are also at risk of contracting type D hepatitis. Such HBsAg carriers are refractory to vaccination with current HBsAg vaccines, and public health control measures depend on those strategies that limit reexposure to HDV through high-risk behavior. Future opportunities, based on laboratory animal studies, include the possibility of HDV-specific vaccines, but for the most part, limiting the burden of HDV disease will depend on the aggressive pursuit of HBV control, through both prophylactic and therapeutic intervention.

Fundamental studies of the HDV genome and its replication in recent years have permitted the application of modern molecular techniques to define the role of HDV in the evolution of liver disease. Additionally, our understanding of the similarity of HDV and other unusual subviral pathogens has stimulated a search for other unique transmissible agents with similar characteristics in humans and other animal species.

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CHAPTER 89 Hepatitis E Virus

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HISTORY

Hepatitis E was not recognized as a distinct clinical entity until 1980, when sensitive and specific tests for antibody to hepatitis A virus (anti-HAV) were first applied to the study of epidemic waterborne hepatitis in India (49,107). Such disease had long been thought to be caused by HAV, based on the epidemiologic and clinical similarities between these epidemics and well-characterized epidemics of hepatitis A occurring elsewhere. However, the patients in the Indian epidemics were all found to have serologic evidence of past, not recent, infection with HAV; the epidemics were therefore not epidemics of hepatitis A. Indeed, the studies of Indian hepatitis epidemics were initiated because previous seroepidemiologic studies had revealed a virtually universal exposure to HAV during infancy and early childhood in developing countries and it was therefore difficult to understand how large epidemics of waterborne hepatitis A could occur in young adults who would be expected to have immunity to infection with HAV. In fact, very few epidemics of waterborne hepatitis in developing countries of Asia and Africa have been linked to hepatitis A (3,48,79).

The first direct experimental evidence for the existence of an additional waterborne hepatitis agent was reported in 1983 by Balayan et al. (9), who described the successful fecal–oral transmission of hepatitis to a volunteer from patients with hepatitis A-like disease in Tashkent, Uzbekistan. The volunteer developed severe clinical hepatitis 36 days after ingestion of a fecal suspension from the patients. Virus-like particles, 27 to 30 nm in diameter, were detected by immunoelectron microscopy (IEM) in the feces collected from the volunteer between 28 and 45 days after exposure. After ingestion of the feces, the volunteer, who had been infected with HAV in the past, did not have a serologic response to HAV or HBV but did develop antibodies to the virus-like particles recovered from his feces. Balayan et al. also reported the transmission of hepatitis to cynomolgus monkeys inoculated with the fecal sample from Tashkent and the recovery of virus-like particles from their feces (9).

This form of non-A, non-B hepatitis came to be known as epidemic non-A, non-B or enterically transmitted non-A, non-B (ENANB) hepatitis, and the agent of this disease was subsequently found to be the major cause of sporadic hepatitis cases in regions where the epidemic form was known to exist (4,51,74). IEM and transmission to primates (see later) remained the only means of studying ENANB hepatitis until 1990, when Reyes et al. succeeded in cloning and sequencing a part of the genome of the virus (83). The complete sequence of the viral genome is now known, and the agent has been renamed hepatitis E virus (HEV) (79,83).

INFECTIOUS AGENT

Classification

The taxonomic classification of HEV is uncertain at present. Initial analysis of the HEV genome revealed three open reading frames (ORFs); putative, nonstructural proteins were localized at the 5' end, and structural protein(s) at the 3' end of the genome; and subgenomic messenger RNAs, detected in infected liver tissue, were superficially suggestive of a relationship with the caliciviruses (87), so the virus was classified in the *Caliciviridae* family. However, the order of the genes of HEV is not identical to that of typical caliciviruses: ORF 3 of the caliciviruses is located at the 3' end of the genome, whereas it overlaps the ends of ORF 1 and ORF 2 in HEV. Furthermore, although the sequence of HEV is not closely related to that of any other recognized virus, it most closely resembles the sequences of rubella virus, currently classified as a togavirus, and beet necrotic yellow vein virus, a plant furovirus (30). In addition, codon usage most closely resembles that of rubella virus (87). For these and other reasons, HEV was recently removed from the *Caliciviridae* family and is again unclassified (76). It has been proposed that, on the basis of genetic relatedness and genomic organization, the taxonomy of positive-sense RNA viruses should be reorganized. Under such a scheme, rubella virus, beet necrotic yellow vein virus, and HEV would be placed in separate but related families (55).

Virion Structure

Morphology

Hepatitis E virus is a nonenveloped, spherical particle, approximately 30 to 34 nm in diameter (9,11,90), with an indefinite surface substructure that is slightly less pronounced than that of the Norwalk agent (a calicivirus) but distinguishable from the smooth, featureless surface of hepatitis A virus (a picornavirus) (Fig. 1). However, on the basis of morphology, HEV cannot be reliably distinguished from other “small round viruses” found in feces. Analysis of electron micrographs of HEV particles by Markham rotational analysis provided images that suggested an icosahedral symmetry for HEV virions (90).

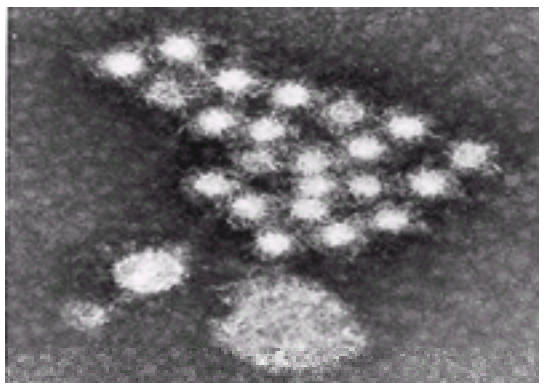

FIG. 1. Antibody-coated hepatitis E virus particles recovered from the stool of a patient with hepatitis E, in Mexico. Immunoelectron microscopy (×223,000). (Courtesy of D. Bradley.)

Chemistry

The buoyant density of hepatitis E viral antigen and/or virus particles has been reported to be 1.35 g/cm ^{3} (9) and 1.39 to 1.40 g/cm^3 (28) in CsCl. The buoyant density of HEV is 1.29 g/cm^3 in potassium tartrate/glycerol (11). The sedimentation coefficient of HEV is 183S (12). Hepatitis E virions appear to be unaltered by exposure to trifluorotrichloroethane (90). Survival of HEV in the intestinal tract suggests that the virus is relatively stable to acid and mild alkaline conditions. Although not specifically tested, HEV is likely to be inactivated by the same agents that inactivate hepatitis A virus. Hepatitis E virus contains an RNA genome enclosed within a capsid that is composed of one or possibly two proteins, but insufficient virus has been available for purification and direct chemical analysis to yield reliable information (87). It is not known whether subgenomic RNA transcripts are packaged in virions.

Genome Structure and Organization

The genome of HEV is a single-stranded, positive-sense, polyadenylated RNA molecule of approximately 7.5 kb in length (87) (Fig. 2). The genome of HEV consists of a 5' noncoding (NC) region of 27 to 35 nucleotides that is capped at its 5' end (42), followed by an ORF (ORF 1) of 5,073 to 5,124 bases. A second ORF (ORF 2) begins in coding frame two, 37 nucleotides 3' of the termination codon of ORF 1, and consists of 1,977 to 1,980 nucleotides. A 65- to 74-nucleotide 3' NC region is contiguous with the termination of ORF 2 and is terminated by a 3' stretch of 150 to 200 or more adenosine residues, at least in some strains. A third ORF (ORF 3), 366 to 369 nucleotides in length, overlaps ORF 1 by one nucleotide at its 5' end and extends 325 to 328 nucleotides into ORF 2 in coding frame 3. Thus, the HEV genome consists of three overlapping ORFs and utilizes all three coding frames.

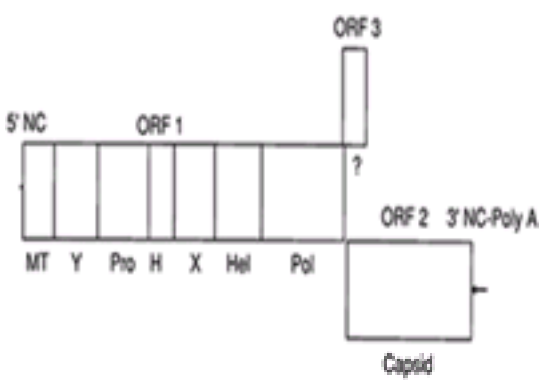

FIG. 2. Organization of the hepatitis E virus genome. The genome is a capped, single-stranded, positive-sense polyadenylated RNA molecule, approximately 7.5 kb in length. It has short 5' and 3' noncoding regions and three overlapping ORFs. All three coding frames are utilized. The nonstructural proteins are thought to be encoded within the large 5' ORF (*ORF 1*), and the capsid protein is encoded by the 3' ORF (*ORF 2*). The small, overlapping third ORF (*ORF 3*) encodes an immunogenic protein of unknown function. See refs. 30,34,35,42,54,55,82,87,94.

The functions of proteins encoded by the ORFs of HEV have not been directly demonstrated but, based on extensive sequence analysis of the HEV genome and identification of conserved motifs found in other viral proteins, ORF 1 is believed to encode the nonstructural proteins; ORF 2 is believed to encode the principal (or only) structural protein, the capsid polypeptide; and ORF 3 encodes a small immunogenic protein of unknown function (30,55,87). Starting from the 5' end of ORF 1 and extending to its 3' end, motifs characteristic of the following viral proteins have been identified (55):

1. A methyl transferase, consistent with the finding that the 5' end of the viral genome is capped (42)

2. A Y domain, a sequence of unknown function found in certain other viruses, including rubella virus
3. A papain-like cysteine protease, a type of protease found predominantly in alphaviruses and rubella virus (35)
4. A proline-rich “hinge region” that is thought to impart flexibility to the molecule and that contains a region of hypervariable sequence (82,94)
5. An X domain of unknown function that flanks papain-like protease domains in the polyproteins of other positive-strand RNA viruses (35)
6. A domain containing helicase-like motifs that are most closely related to the helicases of superfamily I (34)
7. The motifs of an RNA-dependent RNA polymerase, most closely related to supergroup III of viral RNA polymerases (54)

ORF 2 encodes a protein that contains a typical signal sequence near its 5' end, immediately followed by a region rich in codons for arginine and highly basic in charge. This region is believed to be involved in the encapsidation of the genomic transcript (87). The ORF 2–encoded protein contains potential glycosylation sites that are glycosylated *in vitro*, but it has not been established that these are functional *in vivo* (93,109). ORF 2 encodes a major immunogenic epitope at the extreme 3' end (108), as well as other important epitopes in the central region of the protein (61,97).

ORF 3 encodes a phosphoprotein that contains a signal sequence near its 5' end but lacks other identifiable motifs (110). It binds to the cellular cytoskeleton, but its function is unknown (82,110). This protein also contains an immunogenic epitope near its 3' end (87).

Genetic Heterogeneity of HEV

The genomes of several HEV strains from Asia (Burma, Pakistan, India, China, Nepal, Taiwan), Africa (Egypt, Algeria, Morocco, Chad, Nigeria), Europe (Italy, Greece, Spain, Austria), South America (Argentina), and North America (Mexico, United States) have been entirely or partially sequenced and are available through GenBank. Overall, the HEV strains appear to fall into at least four major and several minor genetic groups: Asian/African (Burmese, Indian, Pakistani, Central Asian, North African, and some Chinese strains), a second major Chinese genotype (China and Taiwan), Mexican, and U.S. strains. Other strains, recovered in Italy and Greece, are significantly different from all of these but are more closely related to the U.S. strains (Fig. 3) (85). Thus, genetic heterogeneity is regionally istributed, suggesting that HEV is an old virus that was geographically dispersed in the distant past. Although many strains of HEV from Africa are distinct members of the Asian/African genotype, there is evidence that other strains of HEV in the region have been introduced from Asia in recent times by religious pilgrims and laborers (32).

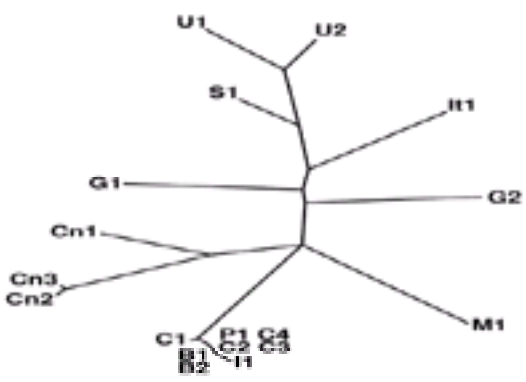

FIG. 3. Unrooted phylogenetic tree depicting the relationship of partial nucleotide sequences from ORF1 and/or ORF2 of HEV strains. Branch lengths are roughly proportional to the evolutionary relationship between sequences. Designations of these strains: B, Burmese; C, Chinese; P, Pakistani; I, Indian; M, Mexican; S, swine; U, United States; G, Greek; It, Italian; Cn, new Chinese. The isolates can be separated into Asian-African, Mexican, new Chinese, and United States-European groups. The last is very heterogeneous. (Modified from ref. 85, with pemission.)

Propagation and Assay in Cell Culture and Biologic Characteristics

Although the replication of HEV in cell culture has been reported (25,38,45), the utility of these systems has yet to be proved. The replication of HEV in primary cynomolgus monkey hepatocyte cultures has also been reported (88). Genomic and antigenomic HEV RNA was detected by reverse transcription–polymerase chain reaction (RT-PCR), and viral antigen was detected by immunofluorescence microscopy in cultures inoculated with stool suspension containing HEV. Infection of cultures has also been prevented by neutralization with sera containing anti-HEV, but not with normal sera (67,88).

Key Antigens

Hepatitis E virus encodes antigens reactive in IEM, enzyme-linked immunosorbent assay (ELISA), Western blot, and immunofluorescence microscopy. The first assay for detecting anti-HEV and the test with which the agent was first identified was IEM (9). From 1983 until 1990, IEM was the only assay for anti-HEV, and serodiagnosis was therefore extremely difficult. Antigens expressed from cloned cDNA derived from the genome of HEV were first used as the basis for serologic tests in 1991 (108). Major epitopes appear to exist in ORF 2 and ORF 3 (56,108). ORF 2 is relatively highly conserved (108). In contrast, ORF 3 is more heterogeneous (108). Thus, it is not surprising that serologic tests based on ORF 2 sequences are broadly reactive, whereas tests containing ORF 3 sequences may be more strain-specific (37,46), but exceptions have been reported (24). Mapping of linear B-cell epitopes in the three ORFs of HEV by the Geysen pin method revealed twelve such epitopes in ORF 1, three in ORF 2, and one in ORF 3 (44). Most of the epitopes identified in ORF 1 were clustered in the putative RNA polymerase gene region, near the carboxyl end of this ORF. It is not yet

clear how useful tests that incorporate expressed nonstructural proteins of HEV encoded by ORF 1 will be for diagnostic purposes. However, such a test may be important for differentiating between vaccine-induced and infection-induced immune responses when nonreplicating HEV vaccines are commercially available.

Although the B-cell epitope found in ORF 3 appeared to coincide with the epitope found in this region in expressed proteins, the major epitope found in expressed proteins of ORF 2 was not detected in the search for linear B-cell epitopes (44). Similarly, epitope mapping of ORFs 2 and 3 with longer synthesized peptides in standard ELISA tests confirmed some, but not all, of the results obtained with the Geysen pin method, suggesting that conformational epitopes are important in the immune response to HEV (47,60). In total, as many as 26 antigenic epitopes distributed among six antigenic domains have been reported to exist in the ORF2 gene product (47). However, only one neutralization epitope has been identified. It has been mapped approximately to amino acids 575 to 610 of this 660-amino-acid protein (86a).

Diagnostic tests based on synthetic peptides have been developed (66). Antigens derived from ORF 2 and expressed in *Escherichia coli* or insect cells have also been used for detection of anti-HEV by Western blot and ELISA (60,66). Western blot assays, although apparently specific, have been somewhat less sensitive than ELISA for detecting anti-HEV in some, but not all, studies. The entire ORF 2 has been expressed in insect cells (37,98), and at least a part of the expressed antigen spontaneously forms virus-like particles (61,98). In an extensive comparison of 12 different assays for IgG antibody to HEV, truncated proteins (approximately 56 kd) expressed from ORF 2 in insect cells were superior to proteins expressed in *E. coli* for detecting anti-HEV (66). Proteins expressed from ORF 3 were less sensitive for detecting anti-HEV. Peptides, whether expressed from ORF 2 or ORF 3, were the least useful in serologic tests (66). HEV antigens derived from ORFs 2 and 3 and expressed in *E. coli* have been incorporated into assays that are commercially available in Europe and Asia (24,62). These assays are useful for diagnosing hepatitis E, but they have failed to detect infections with some recently discovered genetic variants of HEV (85) and they are not sufficiently sensitive for seroepidemiologic studies (66).

Serologic Heterogeneity

Although moderate genetic heterogeneity has been identified among HEV strains (see earlier), evidence for serologic heterogeneity is limited and has been detected primarily with expressed antigens of ORF 3 (46,47,108). Cross-challenge experiments with the most diverse HEV strains in primates have demonstrated cross-protection following infection (14,90,98). Thus, all HEV strains studied to date appear to belong to a single serotype.

Infection in Experimental Animals, and Host Range

Various species of primates have been the animals of choice for transmission studies of HEV. Primates found to be susceptible to infection with HEV include Old World species such as chimpanzees, macaques (cynomolgus, rhesus, and pigtail monkeys), and African green monkeys; and New World species such as tamarins, owl monkeys, and squirrel monkeys (13,90,98). The most useful of these have been cynomolgus and rhesus macaques. The host range of HEV may be influenced by strain differences: chimpanzees and tamarins have yielded discrepant results in different laboratories (13,98). Hepatitis E has been transmitted to primates by the intravenous and oral routes, but the latter is less sensitive (36,100).

The course of infection in experimentally infected primates is similar to that in man (Fig. 4). The incubation period to peak serum levels of liver enzymes is generally 3 to 8 weeks, but shorter and longer incubation periods have been observed (13,92,98,102). Enzyme elevations are usually unimodal, but bimodal curves have also been observed (7,13,92,95,98,102). Peak viremia and peak shedding of HEV into the feces occurs during the incubation period and early acute phase of disease (9,14,63,95). Detection of HEV antigens in the liver generally precedes or parallels viremia and fecal shedding (57,63). Histopathologic changes in the liver parallel biochemical evidence of hepatitis, with resolution in all cases. As in hepatitis A, the immune response to hepatitis E in primates appears late in the incubation period or during the acute phase of illness and is characterized by a brisk IgM and IgG anti-HEV response. Also as seen in hepatitis A, viremia may persist for some time after the appearance of serum antibodies, suggesting the presence of immune complexes (96). Naturally acquired anti-HEV has been detected in wild-caught cynomolgus monkeys (7) and wild-caught (5) and captive rhesus monkeys (99); such animals are generally resistant to experimental infection with HEV (99).

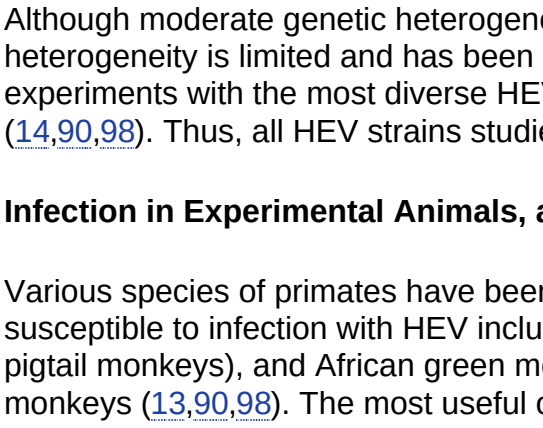

FIG. 4. Diagrammatic illustration of the clinical and serologic events in a typical case of acute hepatitis E. Antibody pattern is depicted as measured by ELISA. Viremia and fecal shedding patterns are based on PCR data. (Modified from ref. 78, with permission.)

Several other species of animals have been reported to be susceptible to infection with HEV or agents serologically

related to HEV. These include swine (10,70,75,104), sheep (103), rats (43,64), and mice (43). This has been established by the direct isolation of HEV from swine (70) and the transmission of HEV strains derived from humans to swine (69), lambs (103), and rats (64). In addition, anti-HEV has been detected in a high percentage of swine tested in many countries throughout the world, including the United States (21,68,70), in 70% to 80% of wild rats in the United States (41), and in varying percentages of other species of wild and domestic animals worldwide. An HEV-like agent has been recovered from cases of “big liver and spleen disease” of chickens, but its exact relationship to other HEV strains remains to be determined (74a). The role of animals as a reservoir of HEV for infection of humans is currently a subject of debate.

Stages of Replication

Because evidence for propagation of HEV in cell culture is quite limited (38,45,88), and because HEV is not closely related to any other well-characterized virus, little is known about its strategy for replication. The mechanism of attachment, entry, and uncoating of HEV is unknown, but it is assumed that the virus attaches to receptor sites on hepatocytes and possibly cells in the biliary tract and intestine. After uncoating, the 7.5-kb genome is probably translated via cellular mechanisms that recognize capped RNA. Cleavage of translated ORF 1 is probably achieved by cellular proteases. The motif of a papain-like protease has been detected in the sequence of ORF 1 (55), but it is not known whether it is functional. Replicative intermediate negative-strand RNA is probably synthesized by the viral polymerase, as are subsequent strands of positive-sense full-length (genomic and messenger) RNA, as well as at least two other messenger RNAs, a 3.7-kb subgenomic one and a 2.0-kb coterminal one (87).

Nothing is known about assembly and transport. The gene products of both ORF 2 and ORF 3 contain hydrophobic, signal-like sequences at their 5' ends (82), but it is not known whether they have a function. Nothing is known about release of the virus from infected cells.

PATHOGENESIS AND PATHOLOGY

The pathogenesis of hepatitis E is poorly understood. However, some speculative conclusions can be drawn from an analysis of the clinical and serologic events of a typical HEV infection (Fig. 4). Entry of the virus into the host is believed to be primarily by the oral route. The site of primary replication has not been identified, but it is presumed to be in the intestinal tract. It is not clear how the virus reaches the liver, but it is presumably via the portal vein. It replicates in the cytoplasm of hepatocytes (57) and is released into the bile and blood by mechanisms that are not understood. Based on limited studies of oral infection in a volunteer, viremia was first detected by RT-PCR 22 days after exposure and over a week before onset of disease on day 30 (20). Virus-like particles were found by IEM in the feces by 34 days after exposure. Liver enzyme values peaked on day 46. Anti-HEV was first detected on day 41 and was still detectable 2 years later. Similarly, in another, earlier volunteer study, HEV was detected in feces by IEM on day 28, and liver enzyme levels peaked on approximately day 42 (9). The individual volunteers in each of these studies recovered completely. Protracted viremia of 45 to 115 days has been reported in a small number of naturally infected patients (22,73).

As in hepatitis A, specific IgM and IgG immune responses occur early in the disease, usually by the time of onset of clinical illness. IgM anti-HEV disappears after several months; IgG anti-HEV persists, but at relatively rapidly decreasing levels shortly after infection (15). Anti-HEV can be detected for as long as 14 years after infection (52). It is not known whether such antibody eventually disappears or remains at low but detectable levels. Anti-HEV of the IgA class has also been detected in the serum of naturally infected individuals (19). The significance of such antibody is unknown.

Virus found in the feces of infected humans or animals is presumed to be the primary source of infectious virus in the environment. Because large quantities of HEV have been found in the bile of experimentally infected primates, it is assumed that the bulk of the virus in the intestinal tract originates in the liver (83). In this respect, hepatitis E is similar to hepatitis A.

The severity of HEV infections is, on average, somewhat greater than the severity of HAV infections. Mortality of hepatitis E has varied in different reports but has been as high as 1%, compared to 0.2% for hepatitis A (78). More important, however, is the severity of hepatitis E in pregnant women (1,8,16,48,79). The mortality of hepatitis E in pregnancy increases with each succeeding trimester and may reach 20%. In contrast, none of the other four recognized hepatitis viruses causes such severe hepatitis in pregnancy (71). The reason for the excessive mortality of hepatitis E in pregnancy is unknown. Fulminant NANB hepatitis occurring in nonendemic areas appears not to be caused by HEV (58).

Certain histologic changes in the liver of patients with hepatitis E are thought by some to be characteristic (81). The changes include focal necrosis with minimal infiltration and no localization to a particular zone of the lobule. Modest inflammation consisting predominantly of Kupffer cells and polymorphonuclear leukocytes is seen, and the focal lesions resemble drug-associated toxic hepatitis. Cholestatic hepatitis is often present, characterized by ballooning hepatocytes, cytoplasmic cholestasis, and focal cytolytic necrosis. An unusual “pseudoglandular” alteration of the hepatocyte plates has also been recognized in some epidemics and sporadic cases. The discrepancy between the time of appearance of viral replication in the liver and histopathologic and biochemical evidence of hepatitis suggests that HEV is not cytopathic and that the pathogenesis of hepatitis E is immunologically mediated, but there is no direct evidence for or against this hypothesis. It is not known whether HEV causes other sequelae or extrahepatic manifestations of infection. None has been recognized, except for excess fetal wastage, which has been reported in some (86,101), but not all (53), studies of pregnant females with fulminant hepatitis E. Premature deliveries with a high infant mortality (33%) have also been reported (86).

CLINICAL FEATURES AND COURSE

Individual cases of hepatitis E cannot be differentiated from other cases of hepatitis on the basis of clinical presentation (32,79). In fact, clinical features are of limited usefulness in the diagnosis of acute hepatitis caused by any of the recognized viruses (78). Sensitive serologic tests have revealed that not all HEV infections are clinically apparent (15).

However, in epidemics, the majority of patients will experience jaundice, anorexia, and hepatomegaly. Approximately half of the patients will experience abdominal pain and tenderness, nausea and vomiting, and fever. Like hepatitis A, hepatitis E never progresses to chronicity. However, persistence of IgM anti-HEV for at least 21 months was reported in one case (59). Recurrent (bimodal) hepatitis A is not uncommon, but comparable recurrence of hepatitis E has not been reported except in experimental infections of primates (7).

DIAGNOSIS

Hepatitis E should be suspected in outbreaks of waterborne hepatitis occurring among citizens of developing countries, especially if the disease is more severe in pregnant women. In the United States and Europe, hepatitis E should be suspected in a patient with hepatitis who has recently returned from an endemic region. Rarely, hepatitis E will occur in an individual who has not traveled to an endemic region. As with all types of viral hepatitis, serologic tests are necessary to establish a definite diagnosis. Specific tests for IgM and IgG antibodies to HEV have been developed and are commercially available in Europe, Asia, and Canada. As there are no plans at present to market them in the United States, the diagnosis of hepatitis E remains an experimental procedure here. Current tests are capable of detecting IgM anti-HEV in up to 90% of acute infections if a serum sample is obtained 1 to 4 weeks after the onset of disease (Fig. 5) (15,27,33,72). IgM anti-HEV reaches peak titers of 1:1,000 to 1:10,000 during the first 4 weeks after the onset of hepatitis (3,15). By 3 months after the onset of disease, IgM anti-HEV is no longer detectable in 50% or more of patients with hepatitis E (3,72).

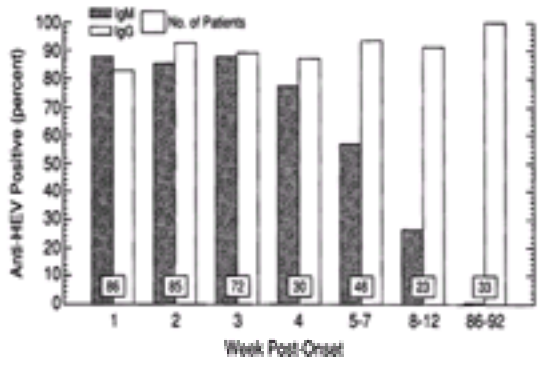

FIG. 5. Prevalence of IgM anti-HEV and IgG anti-HEV, as measured by ELISA, in patients with hepatitis E, by 1 week after onset of illness. (Data from refs. 3 and 15, with permission.)

A rising titer of IgG anti-HEV is also diagnostic. IgG anti-HEV peaks in titer (1:1,000 to 1:100,000) between 2 and 4 weeks after onset of hepatitis and diminishes relatively rapidly thereafter (3,15,24). It is not clear how long IgG anti-HEV persists at detectable levels: One study found that two thirds of pediatric patients became seronegative by 9 months after the onset of disease, whereas 100% of young adults were still positive 20 months after onset (15,33), and in another study, adults were still positive after 14 years (52). However, currently available serologic tests vary greatly in sensitivity and specificity (66).

IEM is an insensitive test that is positive in only approximately 10% of cases (91). Molecular approaches to diagnosis of hepatitis E have replaced IEM. RT-PCR is useful for detecting HEV in the blood and feces during the acute phase of infection. It has contributed to the identification of new genetic variants of HEV (69,85), but the sensitivity of the technique depends on a proper match between the HEV strain and the PCR primers (85). Because of this, serologic approaches to diagnosis remain the methods of choice.

TREATMENT

There is no specific treatment for hepatitis E. Aggressive management of fulminant hepatitis E may reduce the mortality of this complication in pregnant women (38a).

EPIDEMIOLOGY

Hepatitis with the clinical and epidemiologic characteristics of hepatitis E (peak clinical attack rate in young adults, high rate of fulminant disease in pregnancy, epidemics of waterborne disease) has been reported from Southeast and central Asia, the Middle East, northern and western Africa, and North America (Mexico). Epidemics in most of these regions have been confirmed serologically as hepatitis E, as has sporadic hepatitis occurring in many of the same regions (Fig. 6) (77). In fact, HEV is believed to be the most common cause of sporadic hepatitis in regions where the virus is endemic.

FIG. 6. Countries in which there is serologic confirmation or strong epidemiologic evidence of endemic and/or epidemic hepatitis E. (Modified from refs. 65 and 79, with permission.)

The age-specific clinical attack rate, with its peak among young adults, is striking (Fig. 7) (49,106,107). However, clinical disease has been found with similar frequency in all age groups in some epidemics (72), and clinical hepatitis E in children has also been reported (2,26,33,72). A male preponderance has been observed in some, but not all, epidemics. With the exception of several food-borne epidemics in China, all serologically confirmed epidemics of hepatitis E to date have been associated with fecally contaminated water. Cases of probable sporadic hepatitis E have been linked epidemiologically to consumption of raw or uncooked shellfish (17). The epidemiologic risk factors associated with most sporadic cases of hepatitis E have been difficult to identify. Person-to-person spread from cases to contacts has been reported, but this appears to be relatively uncommon (16,26). However, contact with a jaundiced person was reported in as many as 21% to 33% of sporadic HEV cases in three studies (32,50,51). Travel to an area where HEV is endemic has been a risk factor in a number of cases (18,29,32), and one hospital-related outbreak has been reported (84).

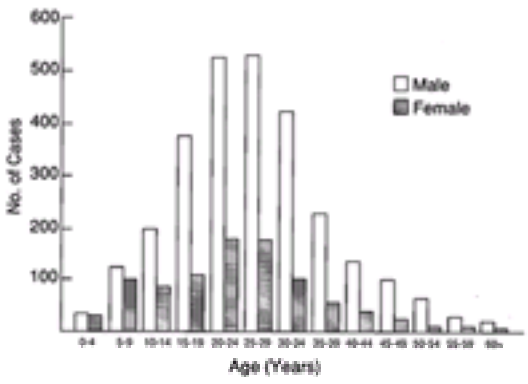

FIG. 7. The age-specific clinical attack rate of hepatitis in the large, waterborne epidemic of hepatitis that occurred in Delhi, India, in the winter of 1955 to 1956. [The epidemic was subsequently shown to be caused by hepatitis E virus (3).] (Data from ref. 106, with permission.)

The recent development of sensitive serologic tests for infection with HEV has permitted, for the first time, a more complete analysis of the worldwide distribution and seroprevalence of HEV infection. Surprisingly, with a few exceptions (28a), the prevalence of IgG antibody to HEV in suspected or documented endemic regions has been much lower than expected (3% to 26%), and the prevalence of such antibody in nonendemic regions has been much higher than anticipated (1% to >20%) (77). Except in epidemic settings (72) and a limited number of other instances (28a), the prevalence of anti-HEV in infants and children has also been quite low (6,59,62,72). The greatest increase in prevalence of anti-HEV appears to occur in young adults, the age group at highest risk of clinical disease (6,59,62). Above that age, the prevalence of anti-HEV has remained constant at 10% to 40%, with no difference between male and female prevalences (6,59,62). Although such a pattern of age-specific anti-HEV might suggest a cohort effect, signaling the disappearance of HEV from endemic regions similar to the disappearance of HAV from some regions previously (79), this appears not always to be the case: Virtually identical age-specific anti-HEV patterns were detected in the same population over a decade (6). Thus, in some settings, the epidemiologic characteristics of HEV appear to be quite different from those of most viruses, such as HAV, that are transmitted by the fecal–oral route (Fig. 8). The relatively rapid acquisition of antibody during adolescence and early adulthood is suggestive of a sexual component to the transmission of this virus, possibly through facilitation of the fecal–oral mechanism of transmission by intimate contact. However, there is no direct evidence for this at present. Interestingly, peak acquisition of anti-HEV in wild rat populations also occurs in juveniles and young adults (41).

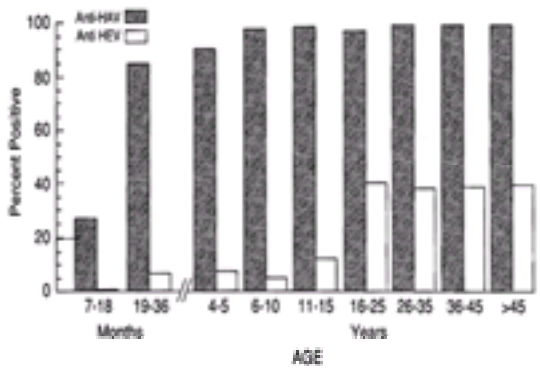

FIG. 8. The age-specific prevalences of antibody to hepatitis A virus and hepatitis E virus in a population of low socioeconomic status in Pune, India. Data were obtained by ELISA on sera collected in 1992. Virtually identical results were obtained with sera collected in 1982. Infection with hepatitis A virus occurred at an earlier age and in a higher proportion of the population than infection with hepatitis E virus. (Modified from ref. 6, with permission.)

The impact of HEV infections in various wild and domesticated animals on the epidemiology of hepatitis E in humans is the subject of much debate. The close genetic relationship between U.S. swine HEV strains and strains of HEV isolated from humans living in the same geographic regions suggests that some transmission from swine to humans may occur. This hypothesis is strengthened by the demonstration that swine and human HEV strains from the United States can infect both swine and chimpanzees (a surrogate of man) (69). In India, a similar association between waterborne hepatitis E epidemics and geographic proximity to wild rhesus monkeys (which appear to be naturally infected with HEV-like agents) has been suggested. An equally likely scenario is the zoonotic spread of HEV from wild rats to humans, as these animals can be found in close proximity to man worldwide. Until HEV strains recovered from rats can be fully characterized, this hypothesis cannot be tested.

The prevalence of anti-HEV in countries where hepatitis E is rarely, if ever, diagnosed is 10 times greater than the prevalence of antibody to hepatitis C virus in these same countries, where hepatitis C accounts for 10% to 20% or more of clinical hepatitides. This finding of anti-HEV in populations where clinical hepatitis E is rarely diagnosed suggests that subclinical infection, perhaps with attenuated or animal strains of HEV (zoonosis), occurs in these regions, or that currently available tests yield nonspecific results in some instances.

Molecular approaches to the epidemiology of HEV hold some promise. The relatively low titer of HEV in blood and feces limits the usefulness of hybridization without amplification. RT-PCR has been useful for confirming the results of serologic tests and for estimating the duration of infectivity of individual cases (20). RT-PCR has been used to detect HEV in contaminated water (40). However, it is much less sensitive than serologic tests for diagnosis of acute hepatitis E and of limited value for molecular-epidemiologic studies because of the relatively short duration of viremia and fecal shedding. Its main value in this regard has been the identification of HEV genotypes and their relationship to specific epidemics and geographic locations.

PREVENTION AND CONTROL

Interdiction

Improved personal and public hygiene has resulted in a marked reduction in the incidence of hepatitis A virus infections. It is assumed that such changes have had a similar, and probably earlier, effect on the incidence of hepatitis E (79). Indeed, hepatitis occurring in Europe prior to the 20th century, and believed then to have been hepatitis A, had the epidemiologic characteristics of hepatitis E (23). Thus, HEV, reportedly more labile and shed in lower titers than HAV, may have disappeared from more industrialized countries in the recent past, just as HAV is currently diminishing in importance in these countries. However, regions where HAV is still highly endemic may also harbor endemic HEV (6,59) (Fig. 8). Finally, the high prevalence of anti-HEV in domestic and wild animals, even in industrialized countries, suggests that possible zoonotic spread of HEV may continue in such regions, despite improved sanitation (5,41,68).

Immunoprophylaxis

Attempts to prevent or modify hepatitis E by the administration of normal immune globulin manufactured from source plasma obtained in the areas where HEV is endemic have been unsuccessful (16,39,50,105) or uncertain (89). Because most of these studies were performed before the development of sensitive and quantitative tests for anti-HEV, the lots of globulin employed were not tested for the presence or titer of anti-HEV. Because even populations in regions where HEV is endemic have a relatively low prevalence and titer of anti-HEV (see preceding), it is perhaps not surprising that unselected lots of normal immune globulin may not contain protective quantities of anti-HEV. Preliminary experimental studies in primates suggest that passively acquired anti-HEV does modify but does not prevent HEV infection (96). Hepatitis E immune globulin, prepared from plasma donors with high titers of anti-HEV or from neutralizing monoclonal antibodies to HEV (86a), may be useful for preventing hepatitis E during epidemics in the future.

There are at present no commercially available vaccines for the prevention of hepatitis E. However, candidate recombinant hepatitis E vaccines, expressed from ORF 2 in *E. coli* (80) or in insect cells (31,96,97), have protected against hepatitis E in nonhuman primates. One such vaccine is currently in clinical trials (97). It was safe and immunogenic in volunteers and is being tested for efficacy in Nepal, which has annual epidemics of waterborne hepatitis E.

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CHAPTER 90 Prions

Stanley B. Prusiner

Prions are infectious proteins that cause fatal neurodegeneration in humans and animals (156). The prion diseases, also referred to as the transmissible spongiform encephalopathies, are uniquely manifest as sporadic, genetic, and infectious illnesses. The only known component of the prion is a protein designated PrP ^{Sc}. The prion protein (PrP) gene found on the short arm of chromosome 20 of humans encodes a protein of 253 amino acids termed PrP ^{C}. Both PrP^{C} and PrP^{Sc} have the same covalent structure, but through a poorly understood process, PrP ^{C} is refolded into PrP ^{Sc}. The accumulation of PrP^{Sc} in the central nervous system (CNS) causes neurologic disease, and the refolding of PrP ^{C} is stimulated by the product of the reaction PrP ^{Sc}.

For many years, investigators classified scrapie, the prototypic prion disease of sheep, as a slow viral illness. Many aspects of scrapie in sheep and goats mimic viral diseases (Table 1). The term *slow viruses* was introduced by Bjorn Sigurdsson in 1954 to characterize the sheep diseases called visna and scrapie (184). He suggested that the long latency periods between inoculation and the onset of disease was a special feature of slow- or delayed-acting viruses. Eventually, the visna agent was shown to be a lentivirus, the same family to which the human immunodeficiency virus (HIV) belongs.

Scrapie is a transmissible disease.
Infectious pathogen is small and filterable.
Accumulation of the scrapie pathogen causes disease.
Distinct strains of the scrapie agent cause different patterns of disease, as measured by the length of the incubation time and the neuropathologic lesion profile.

TABLE 1. *Features of scrapie suggesting that it might be caused by a virus*

As early as 1967, several experimental findings by Tikvah Alper and her colleagues suggested that the infectious pathogen causing scrapie might be unusual (1,2). Most notable were the results of radiation inactivation studies suggesting that the scrapie might not contain a nucleic acid and that the infectious particle might be extremely small. On

one hand, those conclusions were vigorously challenged (165,166,167 and 168), and on the other, they prompted a myriad of hypotheses (Table 2). The most prescient of these speculations were those suggesting that the scrapie agent might be a protein. I. Pattison (143) proposed that the scrapie agent was a basic protein, and J. S. Griffith (72) published three hypotheses: that it was (a) a protein that induces its own gene transcription, (b) an antibody-like molecule stimulating more of itself, and (c) a protein that acquires a disease-inducing conformation. Although the first two hypotheses proved to be incorrect, the last resembles what was eventually discovered. Interestingly, the conformational proposal violated considerable data arguing that each protein has only one biologically active conformation (5,6).

Sarcosporida-like parasite
"Filterable" virus
Small DNA virus
Replicating protein
Replicating abnormal polysaccharide with membrane
DNA subvirus controlled by a transmissible linkage substance
Provirus consisting of recessive genes generating RNA particles
Naked nucleic acid similar to plant viroids
Unconventional virus
Aggregated conventional virus with unusual properties
Replicating polysaccharide
Nucleoprotein complex
Nucleic acid surrounded by a polysaccharide coat
Spiroplasma-like microorganism
Multicomponent system with one component quite small
Membrane-bound DNA
Virino (virus-like DNA complexed with host proteins)
Filamentous animal virus [scrapie-associated fibrils (SAF)]
Aluminum-silicate amyloid complex
Amyloid-inducing virus
Complex of apo- and co-prions (unified theory)
Nemavirus (SAF surrounded by DNA)
Retrovirus
Soil bacterium

Source: Adapted from Prusiner SB. Development of the prion concept. In: *Prion Biology and Diseases*. Cold Spring Harbor, N.Y.: Cold Spring Harbor Press, 1999, with permission.

TABLE 2. *Hypothetical structures proposed for the scrapie agent*

The tortuous path of the scientific investigation that led to an understanding of familial Creutzfeldt-Jakob disease (CJD) chronicles a remarkable scientific odyssey. By 1930, the high incidence of familial (f) CJD in some families was known (127,189). Almost 60 years were to pass before the significance of this finding could be appreciated (84,120,152). CJD remained a curious, rare neurodegenerative disease of unknown etiology throughout this period of threescore years (102). Only with the transmission of disease to apes after inoculation of brain extracts prepared from patients who died of CJD did the story begin to unfold (67).

Once CJD was shown to be an infectious disease, relatively little attention was paid to the familial form of the disease, as most cases were not found in families. It is interesting to speculate how the course of scientific investigation might have proceeded had transmission studies not been performed until after the molecular genetic lesion had been identified. Had that sequence of events transpired, then the prion concept, which readily explains how a single disease can have a genetic or infectious etiology, might have been greeted with much less skepticism (154).

Epidemiologic studies designed to identify the source of the CJD infection were unable to identify any predisposing risk factors, although some geographic clusters were found (19,45,74,117). Libyan Jews living in Israel developed CJD about 30 times more frequently than other Israelis (94). This finding prompted some investigators to propose that the Libyan Jews had contracted CJD by eating lightly cooked brain from scrapie-infected sheep when they lived in Tripoli prior to emigration. Subsequently, the Libyan Jewish patients were all found to carry a mutation at codon 200 in their PrP gene (61,70,85).

Although the brains of patients appear grossly normal upon postmortem examination, they usually show spongiform degeneration and astrocytic gliosis under the light microscope (Fig. 1). In all cases of Gerstmann-Sträussler-Scheinker disease (GSS) and variant (v) CJD, PrP amyloid plaques are found (213). Before PrP immunostaining was available, histochemical staining was used to examine brains from patients with kuru, and 70% of cases were thought to have amyloid plaques (103). The presence or absence of PrP amyloid plaques in sporadic and inherited CJD is quite variable (50).

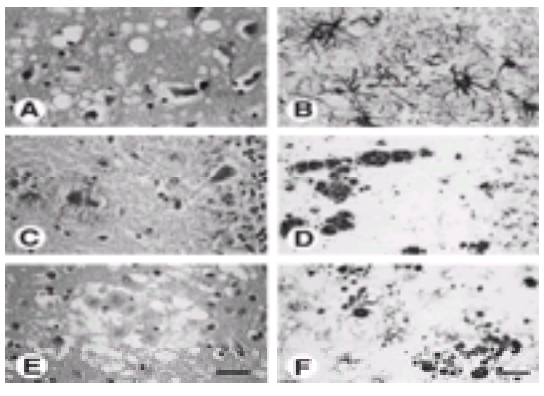

FIG. 1. Neuropathology of human prion diseases. Sporadic Creutzfeldt-Jakob disease (CJD) is characterized by vacuolation of the neuropil of the gray matter, by exuberant reactive astrocytic gliosis, the intensity of which is proportional to the degree of nerve cell loss, and rarely by PrP amyloid plaque formation (not shown). The neuropathology of familial CJD is similar. GSS(P102L), as well as other inherited forms of Gerstmann-Sträussler-Scheinker disease (GSS) (not shown), is characterized by numerous deposits of PrP amyloid throughout the CNS. New variant CJD (nvCJD) has clinical and epidemiologic features that suggest it was acquired by infection with prions. The neuropathologic features of nvCJD are unique among CJD cases because of the abundance of PrP amyloid plaques that are often surrounded by a halo of intense vacuolation. **A:** Sporadic CJD. Cerebral cortex demonstrates widespread spongiform degeneration (H&E). **B:** Sporadic CJD. Cerebral cortex immunostained with anti-GFAP (glial fibrillary acidic protein) antibodies demonstrates the widespread reactive gliosis. **C:** GSS. Cerebellum with most of the GSS plaques in the molecular layer (left 80% of micrograph); many but not all are periodic acid Schiff

(PAS)–reaction positive. Granule cells and a single Purkinje cell are seen in the right 20% of the panel. **D:** GSS. Cerebellum at the same location as (C). PrP immunohistochemistry after hydrolytic autoclaving reveals more PrP plaques than seen with the PAS reaction. **E:** Variant CJD. Cerebral cortex, showing the plaque deposits uniquely located within vacuoles (H&E). With this histology, these amyloid deposits have been referred to as florid plaques. **F:** Variant CJD. Cerebral cortex stained with PrP immunohistochemistry after hydrolytic autoclaving reveals numerous PrP plaques, often occurring in clusters as well as minute PrP deposits surrounding many cortical neurons and their proximal processes. *Bar* in (E) = 50 μm, and scale applies also to (A), (B), and (C). *Bar* in F = 100 μm, and scale applies also to (D). (Photomicrographs prepared by Stephen DeArmond.)

Human prion disease should be considered in any patient who develops a progressive subacute or chronic decline in cognitive or motor function. Typically, adults between 40 and 80 years of age are affected (169). The young age of more than 100 people who have died of vCJD in Britain and France has raised the possibility that these individuals were infected with bovine prions that contaminated beef products (34,46,213). Over 100 young adults have also been diagnosed with iatrogenic CJD from 4 to 30 years after receiving human growth hormone (HGH) or gonadotrophin derived from cadaveric pituitaries (105,148). The longest incubation periods (20 to 30 years) are similar to those associated with more recent cases of kuru (65,104).

In scrapie, kuru, CJD, and all the other disorders now referred to as prion diseases (Table 3), spongiform degeneration and astrocytic gliosis are found on microscopic examination of the CNS (215). The degree of spongiform degeneration is quite variable, whereas the extent of reactive gliosis correlates with the degree of neuron loss (121).

Disease	Host	Mechanism of pathogenesis
A. Kuru	Fore people	Infection through ritualistic cannibalism
iCJD	Humans	Infection from prion-contaminated HGH, dura mater grafts, etc.
vCJD	Humans	Infections from bovine prions?
fCJD	Humans	Germline mutations in PrP gene
GSS	Humans	Germline mutations in PrP gene
FFI	Humans	Germline mutation in PrP gene (D178N, M129)
sCJD	Humans	Somatic mutation or spontaneous conversion of PrP^{C} into PrP^{Sc}?
sFI	Humans	Somatic mutation or spontaneous conversion of PrP^{C} into PrP^{Sc}?
B. Scrapie	Sheep	Infection in genetically susceptible sheep
BSE	Cattle	Infection with prion-contaminated MBM
TME	Mink	Infection with prions from sheep or cattle
CWD	Mule deer, elk	Unknown
FSE	Cats	Infection with prion-contaminated beef
Exotic ungulate encephalopathy	Greater kudu, nyala, oryx	Infection with prion-contaminated MBM

BSE, bovine spongiform encephalopathy; CJD, Creutzfeldt-Jakob disease; sCJD, sporadic CJD; fCJD, familial CJD; iCJD, iatrogenic CJD; vCJD, (new) variant CJD; CWD, chronic wasting disease; FFI, fatal familial insomnia; FSE, feline spongiform encephalopathy; sFI, sporadic fatal insomnia; GSS, Gerstmann-Sträussler-Scheinker disease; HGH, human growth hormone; MBM, meat and bone meal; TME, transmissible mink encephalopathy

TABLE 3. *The prion diseases*

THE PRION PARTICLE

Perhaps the best current working definition of a prion is that it is a proteinaceous infectious particle that lacks nucleic acid (155). A wealth of data supports the contention that scrapie prions are devoid of nucleic acid and seem to be composed exclusively of a modified isoform of PrP, designated PrP^{Sc}. The normal cellular PrP, denoted PrP^{C}, is converted into PrP^{Sc} through a process whereby a portion of its a-helical and coil structure is refolded into b-sheet (139). This structural transition is accompanied by profound changes in the physicochemical properties of the PrP. Whereas PrP^{C} is soluble in nondenaturing detergents, PrP^{Sc} is not. PrP^{C} is readily digested by proteases, whereas PrP^{Sc} is partially resistant (137). Because prions appear to be composed entirely of a protein that adopts an abnormal conformation, it is not unreasonable to think of prions as infectious proteins (139,198). But we hasten to add that we still cannot eliminate the possibility of a small ligand bound to PrP^{Sc} as an essential component of the infectious prion particle.

In a broader view, prions are elements that impart and propagate variability through multiple conformers of a normal cellular protein. The species of a particular prion is encoded by the sequence of the chromosomal PrP gene of the mammal in which it last replicated. In contrast to pathogens with a nucleic acid genome that encode strain-specific properties in genes, prions seem to encipher these properties in the tertiary structure of PrP^{Sc} (12,155,198).

The discovery that mutations of the PrP gene caused dominantly inherited prion diseases in humans linked the genetic and infectious forms of prion diseases and presented another hurdle for investigators who continued to argue that prion diseases are caused by viruses. More than 20 mutations of the PrP gene are now known to cause the inherited human prion diseases, and significant genetic linkage has been established for five of these mutations (84, and reviewed in ref. 155). The prion concept readily explains how a disease can manifest as a heritable as well as an infectious illness. Moreover, the hallmark common to all of the prion diseases, whether sporadic, dominantly inherited, or acquired by infection, is that they involve the aberrant metabolism of the PrP (153).

Although PrP^{Sc} is the only known component of the infectious prion particles, these unique pathogens share several phenotypic traits with other infectious entities such as viruses. Because some features of the diseases caused by prions and viruses are similar, some scientists have difficulty accepting the existence of prions despite a wealth of scientific data supporting this concept (37,38,112,119).

MOLECULAR GENETICS OF PRION DISEASES

Once a PrP cDNA probe became available, molecular genetic studies were undertaken to determine whether the PrP gene controls scrapie incubation times in mice. Independent of the enriching of brain fractions for scrapie infectivity that led to the discovery of PrP^{Sc}, the PrP gene was shown to be genetically linked to a locus controlling the incubation time (28). Subsequently, mutation of the PrP gene was shown to be genetically linked to the development of familial prion disease (84). At the same time, expression of a Syrian hamster (SHa) PrP transgene in mice was shown to render the animals highly susceptible to SHa prions, which demonstrated that expression of a foreign PrP gene could abrogate the species barrier (176). Later, PrP-deficient (*Prnp*$^{0/0}$) mice were found to be resistant to prion infection and failed to replicate prions, as expected (23,157). The results of these studies indicated that PrP must play a central role in the transmission and pathogenesis of prion disease, but equally important, they established that the abnormal isoform is an essential component of the prion particle (153).

PrP Gene Dosage Controls Length of Incubation Time

Scrapie incubation times in mice were used to distinguish prion strains and to identify a gene controlling incubation length (53,177). This gene was initially called *Sinc*, based on genetic crosses between C57Bl and VM mice that exhibited short and long incubation times, respectively (53). Because the distribution of VM mice was restricted, we searched for another mouse with long incubation times. I/Ln mice proved to be a suitable substitute for VM mice; eventually, I/Ln and VM mice were found to be derived from a common ancestor. Subsequently, the PrP gene was shown to control the length of the scrapie incubation time in mice (27,131).

Overexpression of wt PrP Transgenes

Mice were constructed expressing different levels of the wild-type (wt) SHaPrP transgene (Tg) (176). Inoculation of these Tg(SHaPrP) mice with SHa prions demonstrated abrogation of the species barrier, resulting in abbreviated incubation times as a result of a nonstochastic process (159). The length of the incubation time after inoculation with SHa prions was inversely proportional to the level of $SHaPrP^{C}$ in the brains of Tg(SHaPrP) mice (159). Bioassays of brain extracts from clinically ill Tg(SHaPrP) mice inoculated with mouse (Mo) prions revealed that only Mo prions but no SHa prions were produced. Conversely, inoculation of Tg(SHaPrP) mice with SHa prions led only to the synthesis of SHa prions. Thus, the rate of PrP^{Sc} synthesis appears to be a function of the level of PrP^{C} expression in Tg mice; however, the level to which PrP^{Sc} accumulates appears to be independent of PrP^{C} concentration (159).

PrP-Deficient Mice

The development and life span of two lines of *Prnp*$^{0/0}$ mice denoted Zrch and Npu were indistinguishable from controls (24,118), whereas another line (Ngsk) exhibited ataxia and Purkinje cell degeneration at about 70 weeks of age (172). In the former two lines, with normal development, altered sleep–wake cycles (201) and synaptic behavior in brain slices have been reported (44), but the synaptic changes could not be confirmed by others (78,116). The lack of severe defects in these two lines of *Prnp*$^{0/0}$ mice were ascribed to adaptation, because PrP was absent throughout embryogenesis. However, bigenic mice expressing inducible PrP transgenes that were rendered PrP deficient as adults by the administration of doxycycline have remained healthy for more than 1.5 years (202). This argues against the adaptation hypothesis and raises the likelihood of surrogate proteins with functions overlapping that of PrP^{C}.

Prnp$^{0/0}$ mice are resistant to prions (23,157). *Prnp*$^{0/0}$ mice were sacrificed 5, 60, 120, and 315 days after inoculation with Rocky Mountain Laboratory (RML) prions, and brain extracts were bioassayed in CD-1 Swiss mice (Table 4). Except for residual infectivity from the inoculum detected at 5 days after inoculation, no infectivity was detected in the brains of the *Prnp*$^{0/0}$ mice (157). One group of investigators found that *Prnp*$^{0/0}$ mice inoculated with RML prions and sacrificed 20 weeks later had $10^{3.6}$ ID_{50} units/mL of homogenate by bioassay (23). Others have used this report to argue that prion infectivity replicates in the absence of PrP (38,112). Neither we nor the authors of the initial report could confirm the finding of prion replication in *Prnp*$^{0/0}$ mice (157,171).

Mouse	Time of sacrifice after inoculation with RML scrapie prions				
	5 days	60 days	120 days	315 days	522 days
	Log scrapie prion titers (ID_{50} units/mL ± SE)[a]				
Prnp$^{+/+}$	<1	3.9 ± 0.4	6.4 ± 0.3		
	<1	4.8 ± 0.3	7.1 ± 0.1		
	<1	4.6 ± 0.2	6.8 ± 0.2		
Prnp$^{+/0}$	<1	<1	5.1 ± 0.2		
	0.6 ± 0.7	<1	5.2 ± 0.6		
	1.2 ± 0.1[b]	3.4 ± 0.2	2.8 ± 0.1		
Prnp$^{0/0}$	<1[c]	<1	<1	<1	<1
	<1[d]	<1	<1	<1	<1
		<1			<1
					<1

[a]Titers are for 10% (wt/v) brain homogenates. Log titers of <1 reflect no signs of CNS dysfunction in CD-1 mice for >250 days after inoculation, except as noted.
[b]2/9 mice developed scrapie between 206 and 210 days after inoculation.
[c]2/9 mice developed scrapie between 206 and 225 days after inoculation.
[d]1/10 mice developed scrapie between 208 and 225 days after inoculation.
RML, Rocky Mountain Laboratory

TABLE 4. *Prion titers in brains of Prnp*$^{0/0}$ *and Prnp*$^{+/0}$ *mice*

Doppel Up-regulation in *Prnp*$^{0/0}$ Mice

Since a common approach to determining the function of a gene is the analysis of related genes, we searched for PrP-related genes by hybridization; however, these studies were uninformative (208). Vertebrate genomes contain a number of protein families sharing sequence homology and showing overlapping function (77), and because a number of them have been shown to be organized in clusters, we undertook sequencing of large cosmid clones containing the PrP

gene (113). Studies of regions flanking the human, sheep, and mouse PrP genes in cosmid clones failed to reveal additional open reading frames (ORFs) (113). Only when the sequencing of a cosmid clone isolated from a *Prnp*$^{b/b}$ mouse (I/LnJ-4) was extended downstream of PrP was a novel PrP-like gene found.

The locus *Prnd* is 16-kb downstream of the PrP gene, *Prnp*, and it encodes a 179-residue PrP-like protein designated doppel (Dpl) (132). *Prnd* generates major transcripts of 1.7 and 2.7 kb, as well as some unusual chimeric transcripts generated by intergenic splicing with *Prnp.* Like PrP, Dpl mRNA is expressed during embryogenesis, but in contrast to PrP, it is expressed minimally in the CNS. Unexpectedly, Dpl is up-regulated in the CNS of two *Prnp*$^{0/0}$ lines of mice (Ngsk and Rcm0), both of which develop a late-onset ataxia, raising the possibility that Dpl may provoke neurodegeneration (130,172). It has been suggested that PrP may have a role in the long-term survival of Purkinje neurons; however, it was puzzling that Ngsk and Rcm0 *Prnp*$^{0/0}$ mice should develop a fatal ataxia, whereas two other lines (Zrch and Npu) of *Prnp*$^{0/0}$ mice did not exhibit CNS dysfunction. In an effort to determine the role of PrP in this phenotype, Ngsk *Prnp*$^{0/0}$ mice were crossed with transgenic (Tg) mice overexpressing wild-type mouse PrP. This rescued the phenotype in Ngsk *Prnp*$^{0/0}$ offspring expressing PrP (135).

Our findings suggest that Dpl may provoke neurodegeneration in PrP-deficient mice—an observation that may explain why some lines of *Prnp*$^{0/0}$ mice develop cerebellar dysfunction and Purkinje cell death while others do not. The homology between Dpl and PrP suggests that these two proteins may share some biologic properties, and as such, Dpl may open new avenues of investigation in prion biology.

Our findings suggesting that Dpl may provoke degeneration in Purkinje cells force a reevaluation of Tg mice generated using the I/LnJ-4 cosmid. One explanation for spontaneous neurodegeneration reported in Tg(MoPrP-B) mice with high copy-number arrays of the I/LnJ-4 cosmid transgene is the overexpression of *Prnd* (206). In the case of the Tg(MoPrP,P101L) mice constructed with the I/LnJ-4 cosmid transgene (86,87), *Prnd* overexpression is an unlikely explanation, because similar results were obtained with Tg(MoPrP,P101L) mice constructed using the SHa Cos. Tet vector, which does not contain the *Prnd* gene (178,197,200).

Whether cerebellar degeneration in Zrch *Prnp*$^{0/0}$ mice expressing N-terminally truncated PrP transgenes is explained by Dpl overexpression remains to be established. The expression of N-terminally truncated PrPs carrying either codon 32–121 or 32–134 deletions caused degeneration of granule cells in the cerebellum of neonates, but the smaller 32–106 deletion did not (183). Interestingly, this granule cell dysfunction was mitigated by expression of wild-type MoPrP ^{C}. One interpretation of these studies is that PrP carrying either the 32–121 or 32–134 deletion assumes a Dpl-like conformation that is neurotoxic and results in the killing of granule cells because the transgene construct used in those Tg mice is expressed in granule cells but not Purkinje cells (57).

Dpl is the first PrP-like protein to be described in mammals, and because Dpl seems to be similar to PrP in causing neurodegeneration, the linked expression of the *Prnp* and *Prnd* genes may play a previously unrecognized role in the pathogenesis of prion diseases or other illnesses.

PRION PROTEIN STRUCTURE

Once cDNA probes for PrP became available, the PrP gene was found to be constitutively expressed in adult, uninfected brain (39,137). This finding eliminated the possibility that PrP ^{Sc} stimulated production of more of itself by initiating transcription of the PrP gene, as proposed nearly two decades earlier (72). Determination of the structure of the PrP gene eliminated a second possible mechanism that might explain the appearance of PrP ^{Sc} in brains already synthesizing PrP^{C}. As the entire protein coding region was contained within a single exon, there was no possibility that the two PrP isoforms were the products of alternatively spliced mRNAs (8). Next, a posttranslational chemical modification that distinguishes PrP ^{Sc} from PrP^{C} was considered but none was found in an exhaustive study (187), and we considered it likely that PrP^{C} and PrP^{Sc} differed only in their conformations, a hypothesis also proposed earlier (72).

When the secondary structures of the PrP isoforms were compared by optical spectroscopy, they were found to be markedly different (139). Fourier transform infrared (FTIR) and circular dichroism (CD) spectroscopy studies showed that PrP^{C} contains about 40% a-helix and little b-sheet, whereas PrP ^{Sc} is composed of about 30% a-helix and 45% b-sheet (139). That the two PrP isoforms have the same amino acid sequence runs counter to the widely accepted view that the amino acid sequence specifies only one biologically active conformation of a protein (5).

Prior to comparative studies on the structures of PrP ^{C} and PrP^{Sc}, metabolic labeling studies showed that the acquisition of PrP^{Sc} protease resistance is a posttranslational process (15). In a search for chemical differences that would distinguish PrP ^{Sc} from PrP^{C}, we identified ethanolamine in hydrolysates of PrP 27–30, which signalled the possibility that PrP might contain a glycosylphosphatidyl inositol (GPI) anchor (188). Both PrP isoforms were found to carry GPI anchors, and PrP^{C} was found on the surface of cells where it could be released by cleavage of the anchor. Subsequent studies showed that PrP^{Sc} formation occurs after PrP^{C} reaches the cell surface (31) and is localized to caveolae-like domains (71,195).

Computational Models and Optical Spectroscopy

Modeling studies and subsequent nuclear magnetic resonance (NMR) investigations of a synthetic PrP peptide containing residues 90 to 145 suggested that PrP ^{C} might contain an a-helix within this region (Fig. 2) (88). This peptide contains the residues 113 to 128, which are most highly conserved among all species studied (see Fig. 2A) and correspond to a transmembrane region of PrP that was delineated in cell-free translation studies. A transmembrane form of PrP was found in brains of patients with GSS caused by the A117V mutation and in Tg mice overexpressing either the mutant or wt PrP (76). That no evidence for an a-helix in this region has been found in NMR studies of recombinant PrP in an aqueous environment (54,93,162) suggests that these recombinant PrPs correspond to the secreted form of PrP

that was also identified in the cell-free translation studies. This contention is supported by studies with recombinant antibody fragments (Fabs) showing that GPI-anchored PrP^{C} on the surface of cells exhibits an immunoreactivity similar to that of recombinant PrP prepared with an a-helical conformation (146).

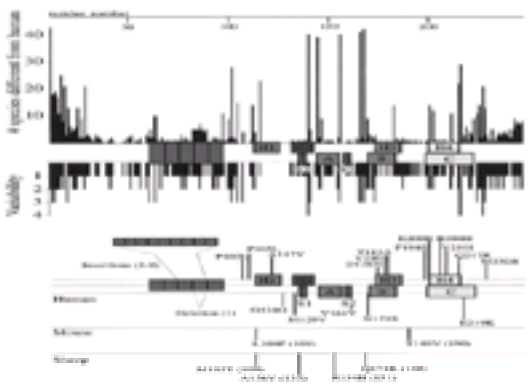

FIG. 2. Species variations and mutations of the prion protein gene. **A:** Species variations. The x-axis represents the human PrP sequence, with the five octarepeats and H1–H4 regions of putative secondary structure shown, as well as the three a-helices A, B, and C and the two b-strands S1 and S2. *Vertical bars* above the axis indicate the number of species that differ from the human sequence at each position. Below the axis, the length of the *bars* indicates the number of alternative amino acids at each position in the alignment. **B:** Mutations causing inherited human prion disease and polymorphisms in human, mouse, and sheep. Above the line of the human sequence are mutations that cause prion disease. Below the lines are polymorphisms, some but not all of which are known to influence the onset as well as the phenotype of disease. (Data compiled by Paul Bamborough and Fred E. Cohen.)

Models of PrP^{Sc} suggest that formation of the disease-causing isoform involves refolding of a region corresponding roughly to residues 108 to 144 into b-sheets (89); the single disulfide bond joining the COOH-terminal helices would remain intact, as the disulfide is required for PrP^{Sc} formation (Fig. 3D) (134). Deletion of each of several regions of putative secondary structure in PrP, except for the NH_2-terminal 66 amino acids (residues 23 to 88) and a 36-amino-acid stretch (Mo residues 141 to 176), prevented formation of PrP^{Sc} as measured in scrapie-infected cultured neuroblastoma cells (134). With a-PrP Fabs selected from phage display libraries and two monoclonal antibodies (MAb) derived from hybridomas, a major conformational change that occurs during conversion of PrP^{C} into PrP^{Sc} has been localized to residues 90 to 112 (146). Studies with an a-PrP IgM MAb, which was reported to immunoprecipitate PrP^{Sc} selectively (107), support this conclusion. Although these results indicate that PrP^{Sc} formation involves a conformational change at the NH_2-terminus, mutations causing inherited prion diseases have been found throughout the protein (see Fig. 2B). Interestingly, all of the known point mutations in PrP with biologic significance occur either within or adjacent to regions of secondary structure in PrP and as such could destabilize the structure of PrP^{C} or stabilize a transition state between PrP^{C} and PrP^{Sc} (42,88,162,163).

NMR Structure of Recombinant PrP

The NMR structure of recombinant (r) SHaPrP(90–231) was determined after the protein was purified and refolded (see Fig. 3A). Residues 90 to 112 are not shown in the figure, because marked conformational heterogeneity was found in this region, whereas residues 113 to 126 constitute the conserved hydrophobic region that also displays some structural plasticity (93,115). Although some features of the structure of rPrP(90–231) are similar to those reported earlier for the smaller recombinant MoPrP(121–231) fragment (162), substantial differences were found. For example, the loop at the NH_2-terminus of helix B is defined in rPrP(90—231) but is disordered in MoPrP(121–231); in addition, helix C is composed of residues 200 to 227 in rPrP(90–231) but extends only from 200 to 217 in MoPrP(121–231). The loop and the COOH-terminal portion of helix C are particularly important, as described later (see Fig. 3B). Whether the differences between the two recombinant PrP fragments are the result of (a) their different lengths, (b) species-specific differences in sequences, or (c) the conditions used for solving the structures remains to be determined.

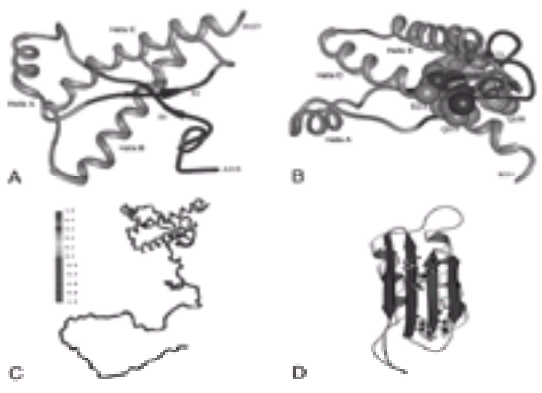

FIG. 3. Structures of prion proteins. **A:** NMR structure of Syrian hamster (SHa) recombinant (r) PrP(90–231). Presumably, the structure of the a-helical form of rPrP(90–231) resembles that of PrP^{C}. rPrP(90–231) is viewed from the interface where PrP^{Sc} is thought to bind to PrP^{C}. The color scheme is as follows: a-helices A (residues 144–157), B (172–193), and C (200–227) in *pink*; disulfide between Cys-179 and Cys-214 in *yellow*; conserved hydrophobic region composed of residues 113–126 in *red*; loops in *gray*; residues 129–134 in *green* encompassing strand S1, and residues 159–165 in *blue* encompassing strand S2. The *arrows* span residues 129–131 and 161–163, as these show a closer resemblance to the b-sheet (93). **B:** NMR structure of rPrP(90–231) is viewed from the interface where protein X is

thought to bind to PrP^C. Protein X appears to bind to the side chains of residues that form a discontinuous epitope. Some amino acids are in the loop composed of residues 165–171 and at the end of helix B (Gln-168 and Gln-172 with a low-density van der Waals rendering), and others are on the surface of helix C (Thr-215 and Gln-219 with a high-density van der Waals rendering) (96). **C:** Schematic diagram showing the flexibility of the polypeptide chain for PrP(29–231) (54). The structure of the portion of the protein representing residues 90–231 was taken from the coordinates of PrP(90–231) (93). The remainder of the sequence was hand-built for illustration purposes only. The color scale corresponds to the heteronuclear ^1H–^{15}N NOE data: *red* for the lowest (most negative) values, where the polypeptide is most flexible, to *blue* for the highest (most positive) values in the most structured and rigid regions of the protein. **D:** Plausible model for the tertiary structure of human PrP^{Sc} (89). Color scheme is as follows: S1 b-strands are 108–113 and 116–122 in *red*; S2 b-strands are 128–135 and 138–144 in *green*; a-helices H3 (residues 178–191) and H4 (residues 202–218) in *gray*; loop (residues 142–177) in *yellow.* Four residues implicated in the species barrier are shown in ball-and-stick form (Asn-108, Met-112, Met-129, Ala-133).

Recent NMR studies of full-length MoPrP(23–231) and SHaPrP(29–231) have shown that the NH_2-termini are highly flexible and lack identifiable secondary structure under the experimental conditions employed (see Fig. 3C) (54). Studies of SHaPrP(29–231) indicate transient interactions between the COOH-terminal end of helix B and the highly flexible, NH_2-terminal random-coil containing the octarepeats (residues 29 to 125) (54).

PrP Appears to Bind Copper

The highly flexible NH_2-terminus of recombinant PrP may be more structured in the presence of copper. Each SHaPrP(29–231) molecule was found to bind two Cu^{2+} ions at pH 6.5; peptide studies suggest that four Cu^{2+} ions will bind at pH 7.4 (17,203). Using tryptophan fluorescence assay, only Cu^{2+} was found to bind recombinant SHaPrP(29–231); other divalent cations including Ca^{2+}, Co^{2+}, Mg^{2+}, Mn^{2+}, Ni^{2+}, and Zn^{2+} did not bind to PrP (190). Earlier studies with synthetic peptides corresponding to the octarepeat sequence demonstrated the binding of Cu^{2+} ions (81,82), and optical spectroscopy showed that Cu^{2+} induced an a-helix formation in these peptides (128). More recently, PrP-deficient (*Prnp*$^{0/0}$) mice were found to have lower levels of Zn/Cu superoxide dismutase (SOD) activity than controls (18); SOD activity has been shown to mirror the state of copper metabolism (75). Measurements of membrane extracts from brains of *Prnp*$^{0/0}$ mice showed low levels of Cu, whereas Fe and Zn were unchanged, suggesting PrP^C might function as a Cu^{2+} binding protein (17). Interestingly, endocytosis of chicken PrP seems to be increased by Cu^{2+} (144), and renaturation of PrP^{Sc} appears to be enhanced by Cu^{2+} (125). Both Cu^{2+} and Zn^{2+} ions have been reported to modify the N-terminal truncation of PrP^{Sc} catalyzed by proteinase K, but the molecular basis underlying this phenomenon is not understood (205).

Disturbances in Cu^{2+} homeostasis leading to dysfunction of the CNS are well documented in humans and animals but are not known to be caused by abnormalities in PrP metabolism; Menkes disease is manifest at birth and is caused by a mutation of the MNK gene on the X chromosome, and Wilson's disease appears in childhood and is caused by a mutation of the WD gene on chromosome 13 (26,35,147,204). Both the MNK and WD genes encode copper transporting ATPases. Although both Menkes and Wilson's diseases are recessive disorders, only Menkes disease can be treated with copper chelating reagents. Interestingly, cuprizone, a Cu^{2+} chelating reagent, has been used in mice to induce neuropathologic changes similar to those found in the prion diseases (101,142).

PRION REPLICATION

In an uninfected cell, PrP^C with the wild-type sequence exists in equilibrium in its monomeric, a-helical, protease-sensitive state or bound to protein X (Fig. 4). We denote the conformation of PrP^C that is bound to protein X as PrP* (41); this conformation is likely to be different from that determined under aqueous conditions for monomeric recombinant PrP. The PrP*–protein X complex will bind PrP^{Sc}, thereby creating a replication-competent assembly. Additional experiments demonstrate that for PrP^C, protein X binding precedes productive PrP^{Sc} interactions (96). A conformational change takes place whereby PrP, in a shape competent for binding to protein X and PrP^{Sc}, represents the initial phase in the formation of infectious PrP^{Sc}.

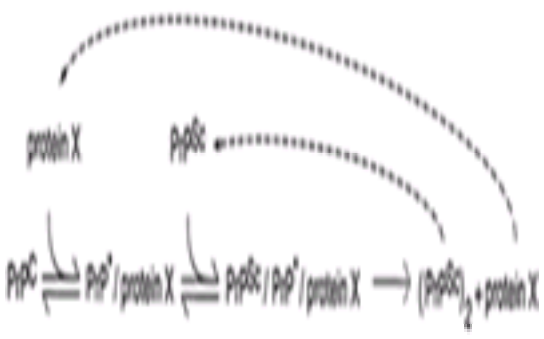

FIG. 4. Schematic diagram showing template-assisted PrP^{Sc} formation. In the initial step, PrP^C binds to protein X to form the PrP*–protein X complex. Next, PrP^{Sc} binds to PrP* that has already formed a complex with protein X. When PrP* is transformed into a nascent molecule of PrP^{Sc}, protein X is released and a dimer of PrP^{Sc} remains. The inactivation target size of an infectious prion suggests that it is composed of a dimer of PrP^{Sc} (10). In the model depicted here, a fraction of infectious PrP^{Sc} dimers dissociate into uninfectious monomers as the replication cycle proceeds, whereas most of the dimers accumulate in accordance with the increase in prion titer that occurs during the incubation period. The precise

stoichiometry of the replication process remains uncertain.

Several lines of evidence argue that the smallest infectious prion particle is an oligomer of PrP^{Sc}, perhaps as small as a dimer (10). On purification, PrP^{Sc} tends to aggregate into insoluble multimers that can be dispersed into liposomes (60). Some investigators argue that insolubility is a prerequisite for PrP^{Sc} formation and prion infectivity (30,64). Whether such arguments are correct remains to be established.

In attempts to form PrP^{Sc} *in vitro*, PrP^{C} has been exposed to 3 M guanidinium (Gdn) HCl and then diluted 10-fold prior to binding to PrP^{Sc} (95,106). Based on these results, we presume that exposure of PrP^{C} to GdnHCl converts it into a PrP*-like molecule. Whether this PrP*-like protein is converted into PrP^{Sc} is unclear. Although the PrP*-like protein bound to PrP^{Sc} is protease resistant and insoluble, this protease-resistant PrP has not been reisolated to assess whether or not it was converted into PrP^{Sc}. It is noteworthy that recombinant PrP can be refolded into either a-helical or b-sheet forms, but none have been found to possess prion infectivity as judged by bioassay.

INFECTIOUS PRION DISEASES

Transmission of infection is a rare cause of prion disease in humans. The infectious prion diseases include kuru of the Fore people in New Guinea, where prions were transmitted by ritualistic cannibalism (3,4,63). With the cessation of cannibalism at the urging of missionaries, kuru began to decline long before it was known to be transmissible. Sources of prions causing infectious CJD on several different continents include improperly sterilized depth electrodes, transplanted corneas, HGH, gonadotrophin derived from cadaveric pituitaries, and dura mater grafts (20). Over 90 young adults have developed CJD after treatment with cadaveric HGH; the incubation periods range from 3 to more than 20 years (13,148). Dura mater grafts implanted during neurosurgical procedures seem to have caused more than 60 cases of CJD; these incubation periods range from 1 to more than 14 years (33,56,110).

New Variant Creutzfeldt-Jakob Disease

In 1994, the first cases of CJD in teenagers and young adults that were eventually labeled new variant (nv) CJD occurred in Britain (212,213). The young age of these patients was unusual (9,16), and their brains showed numerous PrP amyloid plaques surrounded by a halo of intense spongiform degeneration (92). One French patient meeting these criteria later followed (34). These unusual neuropathologic changes have not been seen in CJD cases in the United States, Australia, or Japan (32). Both macaque monkeys and marmosets developed neurologic disease several years after inoculation with bovine prions (7), but only the macaques exhibited numerous PrP plaques similar to those found in nvCJD (111).

Have Bovine Prions Been Transmitted to Humans?

The restricted geographical occurrence and chronology of vCJD have raised the possibility that bovine spongiform encephalopathy (BSE) prions have been transmitted to humans. From 1994 until the last quarter of 1998, the incidence of vCJD seemed to be constant at about eight cases per year. This now seems to have changed with the report of 27 new cases in 2000 (212). This dramatic change in the incidence of vCJD raises the possibility that we are beginning to see a substantial increase in the number of patients dying of vCJD. Still, epidemiologic studies have not identified a set of dietary habits that distinguishes vCJD patients from apparently healthy people. Moreover, there is no explanation for the predilection of vCJD for teenagers and young adults. Why have older individuals not developed vCJD-based neuropathologic criteria? It is noteworthy that epidemiologic studies over the past three decades have failed to find evidence for transmission of sheep prions to humans (45). Attempts to predict the future number of cases of vCJD, assuming exposure to bovine prions prior to the offal ban, have been uninformative because so few cases of vCJD have occurred (46).

Strain of BSE Prions

Was a particular conformation of bovine (Bo) PrP^{Sc} selected for heat resistance during the rendering process and then reselected multiple times as cattle, infected by ingesting prion-contaminated meat and bone meal (MBM), were slaughtered and their offal rendered into more MBM? Recent studies of PrP^{Sc} from brains of patients who died of vCJD show a pattern of PrP glycoforms different from those found for sporadic (s) or iatrogenic (i) CJD (43,80). But the utility of measuring PrP glycoforms is questionable in trying to relate BSE to vCJD (185), because PrP^{Sc} is formed after the protein is glycosylated (15,31) and enzymatic deglycosylation of PrP^{Sc} requires denaturation. Alternatively, it may be possible to establish a relationship between the conformations of PrP^{Sc} from cattle with BSE and those from humans with vCJD by using Tg mice, as was done for strains generated in the brains of patients with the familial form of fatal insomnia (FFI) or fCJD (179,198). A relationship between vCJD and BSE has been suggested by finding similar incubation times in non-Tg RIII mice 310 days after inoculation with Hu or Bo prions (22).

Transgenetic Studies of BSE Prions

When Tg(BoPrP)$Prnp^{0/0}$ mice are inoculated with either nvCJD prions from humans or BSE prions previously passaged once through these same Tg(BoPrP)$Prnp^{0/0}$ mice (179), the resulting lesion profiles as measured by neuronal vacuolation, PrP^{Sc} distribution on histoblots, and PrP amyloid deposition as well as the incubation times were all indistinguishable (179a). Interestingly, Tg(BoPrP)$Prnp^{0/0}$ mice are also highly susceptible to natural and experimental sheep scrapie. Although the incubation times are similar, the neuronal vacuolation, PrP^{Sc} distribution on histoblots, and PrP amyloid deposition are extremely different.

We believe these data provide the first rational experimental studies that establish a clear link between BSE and nvCJD. Indeed, there is an eerie symmetry about these experimental results, where the one passage of BSE prions from humans is equivalent to the first passage from cattle through Tg(BoPrP) *Prnp*$^{0/0}$ mice.

Inherited and Sporadic Prion Diseases

For inherited and sporadic prion diseases, the major question is how the first PrP^{Sc} molecules are formed. Once these are formed, replication presumably follows the mechanism outlined for infectious disease. Several lines of evidence suggest that PrP^{Sc} is more stable than PrP^{C}, and a kinetic barrier precludes the formation of PrP^{Sc} under normal conditions. In the case of the initiation of inherited prion diseases, the barrier to PrP^{Sc} formation must be lower for the mutant ($DPrP^{C}$) than the wild-type, and thus DPrP* can spontaneously rearrange to form $DPrP^{Sc}$. Although the known mutations would appear to be destabilizing to the structure of PrP^{C}, we lack useful information about the structure of the transition state for either the mutant or wild-type sequences. Studies of PrP in the brains of patients who were heterozygous for the E200K mutation revealed $DPrP^{Sc}$(E200K) molecules that were both detergent insoluble and resistant to limited proteolysis, whereas most wild-type PrP was detergent insoluble but protease sensitive (62). These results suggest that in familial (f) CJD(E200K), insoluble wt PrP might represent a form of PrP* (62). In studies with Chinese hamster ovary (CHO) cells, expression of DPrP(E200K) was found to be accompanied by the posttranslational acquisition of resistance to limited proteolysis (114), but whether such cell lines expressing DPrP(E200K) produce infectious prions is unknown. It is noteworthy that levels of proteinase K used in the studies where D PrP(E200K) was expressed in CHO cells were lower by a factor of 10 to 100 compared to digestions of PrP^{Sc} derived from brain or scrapie-infected neuroblastoma (ScN2a) cells. Whether these alterations in the properties of DPrP(E200K) in CHO cells provide evidence for DPrP*, or such changes lie outside the pathway of $DPrP^{Sc}$(E200K) formation, remains to be determined.

Initiation of sporadic disease may follow from a somatic mutation and thus follows a path similar to that for germline mutations in inherited disease. In this situation, the mutant PrP^{Sc} must be capable of co-opting wt PrP^{C}, a process known to be possible for some mutations (e.g., E200K, D178N) but less likely for others (e.g., P102L) (198,200). Alternatively, the activation barrier separating wt PrP^{C} from PrP^{Sc} could be crossed on rare occasions when viewed in the context of a population. Most individuals would be spared, although presentations in the elderly with an incidence of about one per million would be seen.

Transgenic Mice Expressing Mutant PrP

With the identification of mutant genes causing familial forms of neurodegeneration, it became possible to model this process in Tg mice. The first mice expressing a mutant gene that developed CNS degeneration were those expressing the P102L mutation of GSS disease in a mutant PrP transgene (87). The age of onset of CNS dysfunction was found to depend on the level of expression of the mutant PrP transgene in Tg(MoPrP,P101L) mice. The higher the level of expression of the mutant PrP transgene, the earlier the age of onset (197). Additionally, Tg(MoPrP,P101L) *Prnp*$^{0/0}$ mice that were deficient for wild-type MoPrP exhibit a relatively uniform age onset of disease. Not only did the Tg(MoPrP,P101L) mice develop neurodegeneration spontaneously but they also transmitted disease to inoculated recipients that express the same transgene at a much lower level (86,197). These recipient mice, designated Tg(MoPrP,P101L)196/*Prnp*$^{0/0}$ (or Tg196), infrequently develop spontaneous neurodegeneration. Recently, a 55-residue synthetic peptide carrying the P102L mutation in a b-sheet conformation has been shown to induce disease in Tg196 mice (96a). This mutant 55-mer polypeptide can be refolded into at least two distinct conformations. When inoculated intracerebrally into Tg196 mice, 20 of 20 mice receiving the b-form of this peptide developed signs of CNS dysfunction at about 360 days, with neurohistologic changes that are pathognomonic of GSS. By contrast, 8 of 8 mice receiving a non-b-form of the peptide failed to develop any neuropathologic changes more than 600 days after peptide injections. We conclude that a chemically synthesized peptide refolded into the appropriate conformation can accelerate or possibly initiate prion disease. Also noteworthy is the production of neurodegeneration in Tg mice expressing mutant PrP with an expanded octarepeat region (40). This mutation is known to produce inherited prion disease in humans (138,150).

Mechanism of Prion Propagation?

From the foregoing formalism, we can ask, What is the rate-limiting step in prion formation? First, we must consider the impact of the concentration of PrP^{Sc} in the inoculum, which is inversely proportional to the length of the incubation time. Second, we must consider the sequence of PrP^{Sc} that forms an interface with PrP^{C}. When the sequences of the two isoforms are identical, the shortest incubation times are observed. Third, we must consider the strain-specific conformation of PrP^{Sc}. Some prion strains exhibit longer incubation times than others; interestingly, the levels of protease-sensitive PrP^{Sc} increase with prolongation of the incubation time, as described later (170). From these considerations, there exists a set of conditions under which initial PrP^{Sc} concentrations can be rate limiting. These effects presumably relate to the stability of the PrP^{Sc}, its targeting to the correct cells and subcellular compartments, and its ability to be cleared. Once infection in a cell is initiated and endogenous PrP^{Sc} production is operative, then the following discussion of PrP^{Sc} formation seems most applicable. If the assembly of PrP^{Sc} into a specific dimeric or multimeric arrangement was difficult, then a nucleation-polymerization (NP) formalism would be relevant. In NP processes, nucleation is the rate-limiting step and elongation or polymerization is facile. These conditions are frequently observed in peptide models of aggregation phenomena (30); however, studies with Tg mice expressing foreign PrP genes suggest that a different process is occurring. From investigations with mice expressing both the SHaPrP transgene and the endogenous MoPrP gene, it is clear that PrP^{Sc} provides a template for directing prion replication, where we define a template as a catalyst that leaves its imprint on the product of the reaction (159). Inoculation of these mice with $SHaPrP^{Sc}$ leads to the production of nascent $SHaPrP^{Sc}$ and not $MoPrP^{Sc}$. Conversely, inoculation of the Tg(SHaPrP) mice with $MoPrP^{Sc}$ results in $MoPrP^{Sc}$ formation and not $SHaPrP^{Sc}$. Even stronger evidence for templating has emerged from studies of prion strains passaged in Tg(MHu2M) *Prnp*$^{0/0}$ mice expressing a chimeric Hu/MoPrP gene as described in more detail later (155,198). Even though the conformational templates were initially generated with PrP^{Sc} molecules having

different sequences in patients with inherited prion diseases, these templates are sufficient to direct replication of distinct PrP^{Sc} molecules when the amino acid sequences of the substrate PrPs are identical. If the formation of this template were rate limiting, then an NP model could apply. However, studies of PrP^{Sc} formation in ScN2a cells point to a distinct rate-limiting step.

Cell biologic and transgenetic investigations argue for the existence of a chaperone-like molecule referred to as protein X that is required for PrP^{Sc} formation (200). As described later, mutagenesis experiments have created dominant negative forms of $DPrP^{C}$ that inhibit the formation of wt PrP^{Sc} by binding protein X (96). This implies that the rate-limiting step *in vivo* in prion replication, under conditions where PrP^{Sc} is sufficient, must be the conversion of PrP^{C} to PrP* because a dominant negative derived from a single point mutation could gate only a kinetically critical step in a cellular process. In the template-directed model, the conversion of PrP^{C} to PrP* is a first-order process. By contrast, NP processes follow higher-order kinetics [$(monomer)^{m}$, where m is the number of monomers in the nucleus]. The experimental implications of these rate relationships are apparent in transgenic studies; if first-order kinetics operate, halving the gene dose (hemizygotes) should double the incubation time, whereas doubling the dose of a transgene array should halve the time to disease. This quantitative behavior has been observed in several studies in mice with altered levels of PrP expression (25,27,157,159). The existence of prion strains that are conformational isoforms of PrP^{Sc} with distinct structures, incubation times, and neurohistopathology must also be considered in an analysis of the kinetics of PrP^{Sc} accumulation. Because the rate-limiting step in PrP^{Sc} formation cannot involve the unique template provided by a strain, differential rates of intercellular spread, cellular uptake, and clearance seem most likely to account for the variation in incubation times. This is consistent with results described later for eight prion strains in which the levels of protease-sensitive PrP^{Sc} correlate with the length of the incubation times (170). We have suggested that protease-sensitive PrP^{Sc} might serve as a surrogate marker for PrP^{Sc} clearance. In addition to these findings, different patterns of protease sensitivity and glycosylation for distinct prion strains support the proposal of clearance of PrP^{Sc} features in determining the length of the incubation time (12,43,185,198).

However, we hasten to add that NP models can provide a useful description of other biologic phenomena. Under conditions when the monomer is relatively rare and/or the conformational change is facile (e.g., short peptides), the NP model will dominate. However, when the monomer is sufficiently abundant and/or the conformational conversion is difficult to accomplish, the template assistance formalism provides a more likely description of the process.

Evidence for Protein X

Protein X was postulated to explain the results on the transmission of human (Hu) prions to Tg mice (Table 5) (199,200). Mice expressing both Mo and HuPrP were resistant to Hu prions, whereas those expressing only HuPrP were susceptible. These results argue that $MoPrP^{C}$ inhibited transmission of Hu prions—that is, the formation of nascent $HuPrP^{Sc}$. In contrast to the foregoing studies, mice expressing both MoPrP and chimeric MHu2M PrP were susceptible to Hu prions, and mice expressing MHu2M PrP alone were only slightly more susceptible. These findings contend that $MoPrP^{C}$ has only a minimal effect on the formation of chimeric $MHu2MPrP^{Sc}$.

Inoculum	Host	MoPrP gene	Incubation time (days ± SEM) (n/n_0)
sCJD	Tg(HuPrP)	*Prnp*$^{+/+}$	721 (1/10)
sCJD	Tg(HuPrP)/*Prnp*$^{0/0}$	*Prnp*$^{0/0}$	253 ± 2 (6/6)
sCJD	Tg(MHu2M)	*Prnp*$^{+/+}$	238 ± 3 (8/8)
sCJD	Tg(MHu2M)/*Prnp*$^{0/0}$	*Prnp*$^{0/0}$	191 ± 3 (10/10)

Source: Data with inoculum FG from ref. 200, with permission.
n, number of sick mice; n_0, number of inoculated mice.

TABLE 5. *Evidence for protein X from transmission studies of human prions*

When the data on Hu prion transmission to Tg mice were considered together, they suggested that $MoPrP^{C}$ prevented the conversion of $HuPrP^{C}$ into PrP^{Sc} by binding to another Mo protein but that it had little effect on the conversion of MHu2M into PrP^{Sc}. We interpreted these results in terms of $MoPrP^{C}$ binding to this Mo protein with a higher affinity than does $HuPrP^{C}$. We postulated that $MoPrP^{C}$ had little effect on the formation of PrP^{Sc} from MHu2M (see Table 5) because MoPrP and MHu2M share the same amino acid sequence at the COOH-terminus. This also suggested that $MoPrP^{C}$ only weakly inhibited transmission of SHa prions to Tg(SHaPrP) mice because SHaPrP is more closely related to MoPrP than is HuPrP.

Using scrapie-infected mouse neuroblastoma cells transfected with chimeric Hu/Mo PrP genes, we extended our studies of protein X. Substitution of a Hu residue at position 214 or 218 prevented PrP^{Sc} formation (see Fig. 3B) (96). The side chains of these residues protrude from the same surface of the COOH-terminal a-helix, forming a discontinuous epitope with residues 167 and 171 in an adjacent loop. Substitution of a basic residue at positions 167, 171, or 218 prevented PrP^{Sc} formation; these mutant PrPs appear to act as “dominant negatives” by binding protein X and rendering it unavailable for prion propagation. Our findings seem to explain the protective effects of basic polymorphic residues in PrP of humans and sheep (90,181,209).

Is Protein X a Molecular Chaperone?

Because PrP undergoes a profound structural transition during prion propagation, it seems likely that other proteins such as chaperones participate in this process. Whether protein X functions as a classical molecular chaperone or participates in PrP binding as part of its normal function, but can also facilitate pathogenic aspects of PrP biology, is unknown. Interestingly, scrapie-infected cells in culture display marked differences in the induction of heat-shock proteins (Hsp) (196), and Hsp70 mRNA has been reported to increase in scrapie of mice (99). Although attempts to isolate specific proteins that bind to PrP have been disappointing (136), PrP has been shown to interact with Bcl-2, Hsp60, and the laminin receptor protein by two-hybrid analysis in yeast (108,161). Although these studies are suggestive, no molecular chaperone involved in prion formation in mammalian cells has been identified.

MINIPRIONS

Using the four-helix-bundle model of PrP^{C} (88), each region of proposed secondary structure was systematically deleted and the mutant constructs expressed in ScN2a cells and Tg mice (133,134). Deletion of any of the four putative helical regions prevented PrP^{Sc} formation, whereas deletion of the NH_2-terminal region containing residues 23 to 89 did not affect the yield of PrP^{Sc}. In addition to the 67 residues at the NH_2-terminus, 36 residues from position 141 to 176 could be deleted without altering PrP^{Sc} formation. The resulting PrP molecule of 106 amino acids was designated PrP106. In this mutant PrP, helix A as well as the S2 b-strand were removed. Whether the structure of $PrP^{Sc}106$ can be more readily determined than that of full-length PrP^{Sc} remains uncertain.

Transgene-Specified Susceptibility

Tg(MHM2PrP106)*Prnp*$^{0/0}$ mice that expressed PrP106 developed neurologic dysfunction about 300 days after inoculation with RML prions previously passaged in CD-1 Swiss mice (191). The resulting prions containing $PrP^{Sc}106$ produced CNS disease in about 66 days on subsequent passage in Tg(MHM2PrP106) *Prnp*$^{0/0}$ mice. Besides widespread spongiform degeneration and PrP deposits, the pyramidal cells of the hippocampus comprising the CA-1, CA-2, and CA-3 fields disappeared in Tg(MHM2PrP106)*Prnp*$^{0/0}$ mice inoculated with prions containing $PrP^{Sc}106$. In no previous study of Tg mice have we seen similar neuropathologic lesions. The Tg(MoPrP-A) mice overexpressing MoPrP are resistant to RML106 miniprions but are highly susceptible to RML prions. These mice require more than 180 days to produce illness after inoculation with miniprions but develop disease in about 50 days when inoculated with RML prions containing full-length $MoPrP^{Sc}$.

Smaller Prions and Mythical Viruses

The unique incubation times and neuropathology in Tg mice caused by miniprions are difficult to reconcile with the notion that scrapie is caused by an as-yet-unidentified virus. When the mutant or wild-type PrP^{C} of the host matched PrP^{Sc} in the inoculum, the mice were highly susceptible. However, when there was a mismatch between PrP^{C} and PrP^{Sc}, the mice were resistant to the prions. This principle of homologous PrP interactions—which underlies the species barrier—is recapitulated in studies of PrP106 in which the amino acid sequence has been drastically changed by deleting nearly 50% of the residues. Indeed, the unique properties of the miniprions provide another persuasive argument supporting the contention that prions are infectious proteins.

Strains of Prions

The existence of prion strains raises the question of how heritable biologic information can be enciphered in any molecule other than nucleic acid (53). Strains or varieties of prions have been defined by incubation times and the distribution of neuronal vacuolation (53,59). Subsequently, the patterns of PrP^{Sc} deposition were found to correlate with vacuolation profiles, and these patterns were also used to characterize strains of prions (21,49,51).

The typing of prion strains in C57Bl, VM, and F1(C57Bl × VM) inbred mice began with isolates from sheep with scrapie. The prototypic strains called Me7 and 22A gave incubation times of about 150 and about 400 days in C57Bl mice, respectively (53). The PrPs of C57Bl and I/Ln (and later VM) mice differ at two residues and control incubation times (27,131).

Until recently, support for the hypothesis that the tertiary structure of PrP^{Sc} enciphers strain-specific information (153) was minimal except for the DY strain isolated from mink with transmissible encephalopathy (12). PrP^{Sc} in DY prions showed diminished resistance to proteinase K digestion as well as an anomalous site of cleavage. The DY strain presented a puzzling anomaly because other prion strains exhibiting similar incubation times did not show this altered susceptibility to proteinase K digestion of PrP^{Sc} (177). Also notable was the generation of new strains during passage of prions through animals with different PrP genes (177).

PrP^{Sc} Conformation Enciphers Variation in Prions

Persuasive evidence that strain-specific information is enciphered in the tertiary structure of PrP^{Sc} comes from transmission of two different inherited human prion diseases to mice expressing a chimeric MHu2M PrP transgene (198). In FFI, the protease-resistant fragment of PrP^{Sc} after deglycosylation has an M_r of 19 kd, whereas in fCJD(E200K) and most sporadic prion diseases, it is 21 kd (Table 6) (129). This difference in molecular size was shown to be the result of different sites of proteolytic cleavage at the NH_2-termini of the two human PrP^{Sc} molecules, reflecting different tertiary structures (129). These distinct conformations were not unexpected, because the amino acid sequences of the PrPs

differ.

Inoculum	Host species	Host PrP genotype	Incubation time (days ± SEM) (n/n)	PrP^{Sc} (kd)
Inherited prion diseases—mutant PrP^{Sc}				
None	Human	FFI(D178N,M129)		19
FFI	Mouse	Tg(MHu2M)	191 ± 4 (10/10)	19
FFI → Tg(MHu2M)	Mouse	Tg(MHu2M)	136 ± 1 (6/6)	19
None	Human	fCJD(E200K)		21
fCJD	Mouse	Tg(MHu2M)	170 ± 2 (10/10)	21
fCJD → Tg(MHu2M)	Mouse	Tg(MHu2M)	167 ± 4 (13/13)	21
Infectious and sporadic prion diseases—wild-type PrP^{Sc}				
BSE	Human	wt(M/M129)		19
nvCJD	Mouse	Tg(MHu2M)	483 ± 44 (6/6)	19
nvCJD → Tg(MHu2M)	Mouse	Tg(MHu2M)	169 ± 3 (6/6)	19
None	Human	wt(M/M129)		21
sCJD	Mouse	Tg(MHu2M)	191 ± 3 (9/9)	21
sCJD → Tg(MHu2M)	Mouse	Tg(MHu2M)	177 ± 3 (10/10)	21

Data from ref. 198.

TABLE 6. *Distinct prion strains generated in humans with inherited, infectious, and sporadic prion diseases and transmitted to transgenic mice*

Extracts from the brains of FFI patients transmitted disease into mice expressing a chimeric MHu2M PrP gene about 200 days after inoculation and induced formation of the 19-kd PrP^{Sc}, whereas fCJD(E200K) and sCJD produced the 21-kd PrP^{Sc} in mice expressing the same transgene (198). On second passage, Tg(MHu2M) mice inoculated with FFI prions showed an incubation time of about 136 days and a 19-kd PrP^{Sc}, whereas those inoculated with fCJD(E200K) prions exhibited an incubation time of about 170 days and a 21-kd PrP^{Sc} (155). The experimental data demonstrate that MHu2MPrP^{Sc} can exist in two different conformations based on the sizes of the protease-resistant fragments; yet, the amino acid sequence of MHu2MPrP^{Sc} is invariant.

The results of our studies argue that PrP^{Sc} acts as a template for the conversion of PrP^{C} into nascent PrP^{Sc}. Imparting the size of the protease-resistant fragment of PrP^{Sc} through conformational templating provides a mechanism for both the generation and the propagation of prion strains.

Interestingly, the protease-resistant fragment of PrP^{Sc} after deglycosylation, with an M_r of 19 kd, has been found in patients who died after developing a clinical disease similar to FFI (66,124,140). Since both PrP alleles encoded the wild-type sequence and a Met at position 129, these cases were designated sporadic fatal insomnia (sFI). At autopsy, the spongiform degeneration, reactive astrogliosis, and PrP^{Sc} deposition were confined to the thalamus as found in FFI (123,124,126). Extracts from the thalamus of an sFI patient transmitted disease to Tg(MHu2M)*Prnp*$^{0/0}$ mice. PrP^{Sc} accumulated primarily in the thalamus of these Tg mice and after deglycosylation, a 19-kd protease-resistant fragment was identified (124). These findings are similar to those noted earlier for FFI transmission to Tg mice (198). The results of FFI and sFI studies argue persuasively that the clinicopathologic phenotype is determined by the conformation of PrP^{Sc} and not the amino acid sequence (124).

Evidence for Different Conformations of PrP^{Sc} in Eight Prion Strains

Using a highly sensitive conformation-dependent immunoassay for measurement of PrP^{Sc} in tissue homogenates, eight different prion strains passaged in SHas were examined (170). Brains from SHas were collected when the animals displayed signs of neurologic dysfunction; the incubation times for the prion strains varied from 70 to 320 days. Most of the PrP in the brains of SHas with signs of neurologic disease was PrP^{Sc}, as defined by the b-sheet conformation. The level of PrP^{Sc} in the brains of these clinically ill animals exceeded that of PrP^{C} by 3- to 10-fold (Fig. 5A). The highest levels of PrP^{Sc} were found in the brains of SHas infected with the Me7-H strain; in contrast, the lowest levels were found in the brains of SHas inoculated with the SHa(Me7) strain (see Fig. 5A). Interestingly, the Me7-H and SHa(Me7) strains, both derived from Me7 passaged in mice (177), possessed similar denatured-to-native PrP ratios, but they accumulated PrP^{Sc} at quite different levels (see Fig. 5A and Fig. 5B). The highest denatured-to-native PrP ratio of all tested strains was SHa(RML).

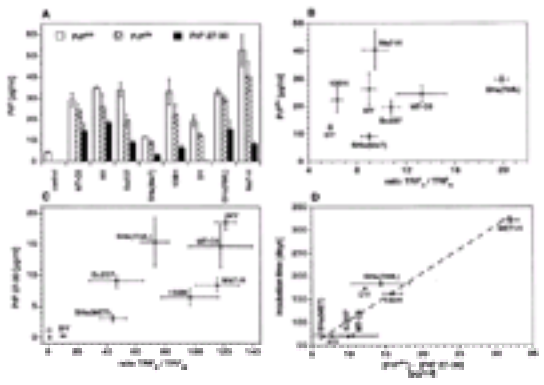

FIG. 5. Eight prion strains distinguished by the conformation-dependent immunoassay. **A:** Concentration of PrP^{Sc} and PrP 27–30. The *columns* and *bars* represent the average ±SEM obtained from three different brains of LVG/LAK Syrian hamsters infected with different prion strains and measured in three independent experiments. **B:** Ratio of antibody binding to denatured/native PrP and a function of concentration of PrP^{Sc} in the brains of Syrian hamsters (SHa) infected with different prion strains. Concentration of PrP^{Sc} and the ratio of antibody binding to denatured/native PrP were measured by the conformation-dependent immunoassay. **C:** Brain homogenates of SHa inoculated with different scrapie

strains and uninoculated controls, denoted *C*, were digested with 50 µg/mL of proteinase K for 2 hours at 37°C prior to the conformation-dependent immunoassay. **D:** Incubation time plotted as a function of the concentration of the proteinase K–sensitive fraction of PrP^{Sc} ([PrP^{Sc}]-[PrP 27–30]).

The apparent independence of the ratio of denatured-to-native PrP from the concentration of PrP^{Sc} became apparent after plotting both parameters in a single graph (see Fig. 5B). Each strain occupied a unique position, indicating differences in the conformation of accumulated PrP^{Sc}. Because the PrP^{C} concentration in each strain was less than or equal to 5 µg/mL and the PrP ratio for PrP^{C} was less than or equal to 1.8, the expected impact of the presence of PrP^{C} on the final PrP ratio was less than or equal to 15%.

Because only the most tightly folded conformers of PrP^{Sc} are likely to be protease resistant, we digested each of the brain homogenates with proteinase K prior to measuring the ratio of denatured-to-native PrP (see Fig. 5C). As shown, the positions of many strains changed when the protease-sensitive conformers of PrP^{Sc} were enzymatically hydrolyzed (see Fig. 5C). Most notable was the DY strain, which was readily detectable before limited proteolysis by immunoassay (see Fig. 5B) but became almost undetectable after digestion (see Fig. 5C), in accordance with earlier Western blot studies (11,12). Equally important, strains such as Sc237 and HY were marginally separated prior to proteinase K digestion (see Fig. 5B) but became quite distinct afterwards (see Fig. 5C).

These findings argue that Sc237 and HY are distinct strains although they exhibit similar incubation times of about 70 days when passaged in SHas (177). It is noteworthy that limited proteolysis of PrP^{Sc} from Sc237- and HY-infected brains produced PrP 27–30 proteins that were indistinguishable by migration in sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) as detected by Western immunoblotting (177).

When the incubation times of these eight strains were plotted as a function of the concentration of either PrP^{Sc} or PrP 27–30, no relationship could be discerned. Incubation times were also plotted as a function of the ratio of denatured-to-native PrP, and again no correlation could be found.

To assess the fraction of PrP^{Sc} that is sensitive to proteolysis during limited digestion with proteinase K, we subtracted the protease-resistant PrP 27–30 fraction (see Fig. 5C) from the total PrP^{Sc} (see Fig. 5B) for each of the eight prion strains. We asked whether the proteinase K-sensitive fraction of PrP^{Sc} ([PrP^{Sc}]–[PrP 27–30]) might reflect those PrP^{Sc} molecules that are most readily cleared by cellular proteases. The clearance of PrP^{Sc} is of considerable interest with respect to control of the length of the incubation time and other phenotypic features of prion strains (160). When the [PrP^{Sc}]–[PrP 27–30] fraction was plotted as a function of the incubation time, a linear relationship was found with an excellent correlation coefficient (r = 0.94) (see Fig. 5D).

The preceding results demonstrate that eight different strains possess at least eight different conformations (see Fig. 5B and Fig. 5C). Additional data argue that each strain is composed of a spectrum of conformations as revealed by limited protease digestion and GdnHCl denaturation studies (170). These findings contrast with the until-recently held notion that the primary structure of a protein determines a single tertiary structure (5).

How many distinct tertiary and quaternary structures can PrP^{Sc} adopt? The conformation-dependent immunoassay described here provides a rapid tool capable of discriminating the secondary, tertiary, and perhaps quaternary structures of a substantial number of PrP^{Sc} molecules.

As noted, for studies of strains passaged from humans with fCJD(E200K) and FFI, PrP^{Sc} must act as a template in the replication of nascent PrP^{Sc} molecules. Also as discussed, it appears likely that the binding of PrP^{C} or a metastable intermediate PrP* to protein X is the initial step in PrP^{Sc} formation, and that this is the rate-limiting step in prion replication (42,96,160). PrP^{Sc} interacts with PrP^{C} but not protein X in the PrP^{C}–protein X complex. When PrP^{C} or PrP* is converted into a nascent PrP^{Sc} molecule, protein X is released.

It also follows from these observations that the different incubation times of various prion strains should arise predominantly from distinct rates of PrP^{Sc} clearance rather than from the different rates of PrP^{Sc} formation (160). Thus, prion strains that are readily cleared should have prolonged incubation times, whereas those that are poorly cleared should display abbreviated incubation periods. We investigated this hypothesis by relying on the difference in brain PrP^{Sc} concentrations before and after proteinase K treatment as a surrogate for *in vivo* clearance of each prion strain. When clearance, as approximated by [PrP^{Sc}]–[PrP 27–30], was plotted as a function of the incubation time for eight strains, a linear relationship was found (see Fig. 5D). It is important to recognize that proteinase K sensitivity is an imperfect model for *in vivo* clearance and that only one strain with a long incubation time (exceeding 300 days) has been studied.

Although it has been suggested that asparagine (Asn)-linked carbohydrates (CHOs) specify prion strains (43), this proposal is difficult to reconcile with the addition of high-mannose oligosaccharides to Asn-linked consensus sites on PrP in the endoplasmic reticulum (ER) and with the subsequent remodeling of the sugar chains in the Golgi (55). Modification of the complex CHOs attached to PrP^{C} is clearly completed prior to PrP^{C} trafficking to the cell surface (15,31), which indicates that the Asn-linked CHOs of PrP^{Sc} do not instruct the addition of such complex-type sugars to PrP^{C}. It remains a formal possibility that specific prion strains preferentially recognize subsets of PrP^{C} glycoforms generated during protein maturation in the Golgi and then facilitate the conformational conversion of these glycoforms into nascent PrP^{Sc}.

Recently, evidence against the proposal that variations in the glycoform ratio encrypt prion strain properties has emerged. Patients with FFI have symptoms and neuropathologic profiles indistinguishable from patients with sFI (124,140). Although PrP^{Sc} from FFI patients shows relatively large amounts of the diglycosylated glycoform because of

the D178N mutation adjacent to one of the consensus Asn-linked glycosylation sites (129), wtPrP^{Sc} from sFI patients shows substantially less of the diglycosylated form (124,140). Following transmission to Tg(MHu2M) mice, FFI prions produced an identical pattern of neuropathology to that found in Tg mice infected with sFI prions, arguing that the same prion strain is responsible for the two forms of the disease (124,198). Furthermore, deglycosylated PrP 27–30 migrated at 19 kd whether it was obtained from patients with FFI or sFI, as well as from Tg mice inoculated with FFI or sFI prions (124). These findings with FFI and sFI also argue that the conformation of PrP ^{Sc}, not the amino acid sequence, determines the strain-specified disease phenotype. Further evidence arguing against any involvement of the pattern of glycosylation in specifying strain properties has emerged through the discovery that the PrP ^{Sc} glycoform ratios found in tonsil tissue differed markedly from those found in the brain of the same patient with nvCJD (79).

Mutagenesis of the complex-type sugar attachment sites (NXT ® NXA) seemed to increase the rate of PrP ^{Sc} formation in cultured cells (194) but resulted in prolonged incubation times in Tg mice and differences in the patterns of PrP ^{C} distribution and PrP ^{Sc} deposition in mice expressing mutant PrPs (51). These studies suggest that Asn-linked glycosylation might alter the stability of PrP, and, in the case of PrP ^{Sc}, that it results in various patterns of PrP ^{Sc} deposition. Thus, different clearance rates of PrP ^{Sc} may be important in determining not only strain-specific neuropathology but also the length of the incubation time (160).

Mechanism of Selective Neuronal Targeting?

In addition to incubation times, neuropathologic profiles of spongiform change have been used to characterize prion strains (59). However, recent studies with PrP transgenes argue that such profiles are not an intrinsic feature of strains (29,51). The mechanism by which prion strains modify the pattern of spongiform degeneration was perplexing because earlier investigations had shown that PrP ^{Sc} deposition precedes neuronal vacuolation and reactive gliosis (49). When FFI prions were inoculated into Tg(MHu2M) mice, PrP ^{Sc} was confined largely to the thalamus (Fig. 6A), as is the case for FFI in humans (198). In contrast, fCJD(E200K) prions inoculated into Tg(MHu2M) mice produced widespread deposition of PrP^{Sc} throughout the cortical mantel and many of the deep structures of the CNS (see Fig. 6B), as is seen in fCJD(E200K) of humans. To examine whether the diverse patterns of PrP ^{Sc} deposition are influenced by Asn-linked glycosylation of PrP ^{C}, we constructed Tg mice expressing PrPs mutated at one or both of the Asn-linked glycosylation consensus sites (51). These mutations resulted in aberrant neuroanatomic topologies of PrP ^{C} within the CNS, whereas pathologic point mutations adjacent to the consensus sites did not alter the distribution of PrP ^{C}. Tg mice with a mutation of the second PrP glycosylation site exhibited prion incubation times of more than 500 days and unusual patterns of PrP^{Sc} deposition. These findings raise the possibility that glycosylation can modify the conformation of PrP and affect either the turnover of PrP ^{C} or the clearance of PrP ^{Sc}. Regional differences in the rate of deposition or clearance would result in specific patterns of PrP ^{Sc} accumulation.

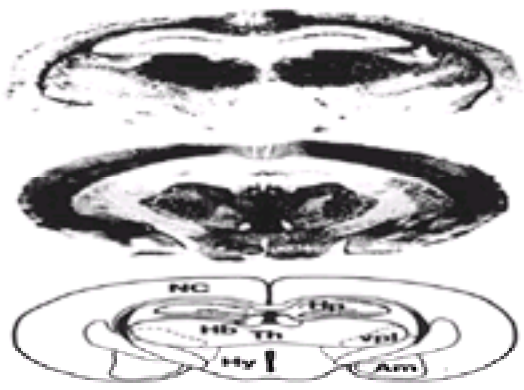

FIG. 6. Regional distribution of PrP ^{Sc} deposition in Tg(MHu2M)*Prnp*$^{0/0}$ mice inoculated with prions from humans who died of inherited prion diseases. Histoblot of PrP ^{Sc} deposition in a coronal section of a Tg(MHu2M) *Prnp*$^{0/0}$ mouse through the hippocampus and thalamus (198). **A:** The Tg mouse was inoculated with brain extract prepared from a patient who died of familial fatal insomnia (FFI). **B**: The Tg mouse was inoculated with extract from a patient with fCJD(E200K). Cryostat sections were mounted on nitrocellulose and treated with proteinase K to eliminate PrP ^{C} (193). To enhance the antigenicity of PrP ^{Sc}, the histoblots were exposed to 3-guanidinium isothiocyanate before immunostaining using a-PrP 3F4 monoclonal antibody (98). **C:** Labelled diagram of a coronal section of the hippocampus/thalamus region. NC, neocortex; Hp, hippocampus; Hb, habenula; Th, thalamus; vpl, ventral posterior lateral thalamic nucleus; Hy, hypothalamus; Am, amygdala.

FUNGAL PRIONS

Although prions were originally defined in the context of an infectious mammalian pathogen (151), it is now becoming widely accepted that prions are elements that impart and propagate variability through multiple conformers of a normal cellular protein. Such a mechanism must surely not be restricted to a single class of transmissible pathogens. Indeed, it is likely that the original definition will need to be extended to encompass other situations in which a similar mechanism of information transfer occurs.

Two notable prion-like determinants, [URE3] and [PSI], have already been described in yeast, and one in another fungus denoted [Het-s*] has been reported (36,47,210). Studies of candidate PrPs in yeast may prove particularly helpful in the dissection of some of the events that feature in PrP ^{Sc} formation. Interestingly, different strains of yeast prions have been identified (52). Conversion to the prion-like [PSI] state in yeast requires the molecular chaperone Hsp104; however, no homolog of Hsp104 has been found in mammals (36). The NH_2-terminal prion domains of Ure2p and Sup35 that are

responsible for the [URE3] and [PSI] phenotypes in yeast have been identified. In contrast to PrP, which is a GPI-anchored membrane protein, both Ure2p and Sup35 are cytosolic proteins (211). When the prion domains of these yeast proteins were expressed in *Escherichia coli*, the proteins were found to polymerize into fibrils with properties similar to those of proteolytically trimmed PrP and other amyloids (83,145).

Whether prions explain some other examples of acquired inheritance in lower organisms is unclear (109). For example, studies on the inheritance of positional order and cellular handedness on the surface of small organisms have demonstrated the epigenetic nature of these phenomena, but the mechanism remains unclear (58).

THERAPEUTIC APPROACHES TO PRION DISEASES

It seems likely that it will be possible to design effective therapeutics for prion diseases as our understanding of prion propagation increases. Because people at risk for inherited prion diseases can now be identified decades before neurologic dysfunction is evident, the development of an effective therapy for these fully penetrant disorders is imperative. Although we have no way of predicting the number of individuals who may develop neurologic dysfunction from bovine prions in the future, seeking an effective therapy now seems most prudent (155). Interfering with the conversion of PrP^{C} to PrP^{Sc} seems to be the most attractive therapeutic target. Both stabilizing the structure of PrP^{C} via the formation of a PrP^{C}-drug complex, and modifying the action of protein X, which might function as a molecular chaperone (see Fig. 3B), are reasonable strategies. Whether it is more efficacious to design a drug that binds to PrP^{C} at the protein X binding site or one that mimics the structure of PrP^{C} with basic polymorphic residues that seem to prevent scrapie and CJD remains to be determined (96,181). As PrP^{Sc} formation seems limited to caveolae-like domains (71,195), drugs designed to inhibit this process need not penetrate the cytosol of cells, but they do need to be able to enter the CNS. Alternatively, drugs that destabilize the structure of PrP^{Sc} might also prove useful.

The production of domestic animals that do not replicate prions may also be important with respect to preventing prion disease. Sheep encoding the R/R polymorphism at position 171 seem to be resistant to scrapie (90,209); presumably, this was the genetic basis of James Parry's scrapie eradication program in Great Britain 30 years ago (141). A more effective approach, using dominant negatives for producing prion-resistant domestic animals, including sheep and cattle, is probably the expression of PrP transgenes encoding R171 or basic residues at the protein X binding site (see Fig. 3B) (96). Such an approach can be readily evaluated in Tg mice and, once shown to be effective, could be instituted by artificial insemination of sperm from males homozygous for the transgene. Less practical is the production of PrP-deficient cattle and sheep. Although such animals would not be susceptible to prion disease (23,157), they might suffer some deleterious effects from ablation of the PrP gene (44,116,172,201).

Whether gene therapy for the human prion diseases will prove feasible using the dominant negative approach described earlier for prion-resistant animals depends on the availability of efficient vectors for delivery of the transgene to the CNS.

CONCLUDING REMARKS

Although the study of prions has taken several unexpected directions over the past three decades, a novel and fascinating story of prion biology is emerging. Investigations of prions have elucidated a previously unknown mechanism of disease in humans and animals. Although learning the details of the structures of PrPs and deciphering the mechanism of PrP^{C} transformation into PrP^{Sc} will be important, the fundamental principles of prion biology have become reasonably clear. Although some investigators prefer to view the composition of the infectious prion particle as unresolved (37,112,119), such a perspective denies an enlarging body of data, none of which refutes the prion concept. Moreover, the discovery of prion-like phenomena mediated by proteins unrelated to PrP in yeast and other fungi serves not only to strengthen the prion concept but also to widen it.

The discovery that prion diseases in humans are uniquely both genetic and infectious greatly extended the prion concept. To date, 20 different mutations in the human PrP gene, all resulting in nonconservative substitutions, have been found either to be genetically linked to or to segregate with the inherited prion diseases (see Fig. 2B). Yet, the transmissible prion particle is composed largely, if not exclusively, of an abnormal isoform of the PrP designated PrP^{Sc} (153).

Aberrant PrP Metabolism

The hallmark of all prion diseases—whether sporadic, dominantly inherited, or acquired by infection—is that they involve the aberrant metabolism and resulting accumulation of the PrP (see Table 3) (153). The conversion of PrP^{C} into PrP^{Sc} involves a conformation change whereby the a-helical content diminishes and the amount of b-sheet increases (139). These findings provide a reasonable mechanism to explain the conundrum presented by the three different manifestations of prion disease.

Understanding how PrP^{C} unfolds and refolds into PrP^{Sc} will be of paramount importance in transferring advances in the prion diseases to studies of other degenerative illnesses. The mechanism by which PrP^{Sc} is formed must involve a templating process whereby existing PrP^{Sc} directs the refolding of PrP^{C} into a nascent PrP^{Sc} with the same conformation. Not only will knowledge of PrP^{Sc} formation help in the rational design of drugs that interrupt the pathogenesis of prion diseases, but it may also open new approaches to deciphering the causes of and to developing effective therapies for the more common neurodegenerative diseases, including Alzheimer's disease, Parkinson's disease, and amyotrophic lateral sclerosis (ALS). Indeed, the expanding list of prion diseases and their novel modes of transmission and pathogenesis (Table 3), as well as the unprecedented mechanisms of prion propagation and information transfer, indicate that much more attention to these fatal disorders of protein conformation is urgently needed.

Over the past two decades, advances in our understanding of the prion diseases have helped refine our definition of neurodegenerative diseases. In addition to the more common neurodegenerative diseases just mentioned, there are less

prevalent maladies, including frontotemporal dementia, Huntington's disease, and the prion diseases (Table 7). With the exception of Huntington's disease, a minority of all of these neurodegenerative disorders exhibit an autosomal dominant pattern of inheritance, and the majority are sporadic. In all of these illnesses, including Huntington's disease, the onset of CNS dysfunction is usually seen in adults and the neurologic deterioration is progressive.

Neurodegenerative disease	CNS protein deposits	Mutant genes in familial disorders
Prion diseases	PrP^{Sc}; PrP amyloid plaques	PrP
Alzheimer's disease (AD)	Aβ; Aβ amyloid plaques Tau; neurofibrillary tangles	APP, PS1, PS2, $Apo\varepsilon^a$
Parkinson's disease	α-Synuclein; Lewy bodies	α-Synuclein, Parkin
Amyotrophic lateral sclerosis (ALS)		Superoxide dismutase
Frontotemporal dementia (FTD)	Tau; fibrils and pick bodies	Tau
Huntington's disease (HD)	HD; nuclear HD aggregates	HD

[a]ApoE genotype modulates the age of onset of both familial and sporadic AD.

TABLE 7. *Neurodegenerative diseases*

Abnormal protein deposits are found in all these diseases and the pathogenic process seems to evade immune surveillance. In Alzheimer's disease and the prion diseases, amyloid plaques are found, but the proteins in these plaques are different (68,122,158). In Alzheimer's disease, the plaques contain a peptide called Ab, which is derived from a large protein designated the amyloid precursor protein, or APP (97). In the prion diseases, the plaques are a nonobligatory feature of the illness (159); the plaques contain substantial amounts of PrP fragments (48,158,192). Mutations in the genes encoding these proteins are found in inherited forms of the disease (69,84). In familial Alzheimer's disease, mutations in the presenilin genes have also been implicated and are thought by some investigators to operate by modifying the processing of APP into Ab (14,164,180,214).

The time of onset of the prion diseases in experimental animals is determined by the level of PrP^{C} expression, the sequence of PrP, and the strain of prions (53,159,207). In Alzheimer's disease, the haplotype of apolipoprotein E (ApoE) greatly influences the age of onset of both the familial and sporadic forms of the illness (174). In Huntington's disease, the age of onset is governed by the size of the triplet repeat expansion encoding the polyglutamine segment (100).

Protein aggregates in the nuclei of some neurons can be observed in Huntington's disease (173,175), whereas in Parkinson's disease, intraneuronal inclusions called Lewy bodies are often found (186). Similarly, mutations in the genes encoding these proteins are found in inherited forms of the disease (73,149). In the inherited frontotemporal dementias and Pick's disease, mutations in the tau gene are the cause (91). In all of these diseases, astrocytic gliosis is seen. In some of these diseases, vacuolation in the neuropil is seen, but the most prominent vacuolation can be seen in the prion diseases where vacuoles may coalesce to create spongiform change. The degree of spongiform change is quite variable, whereas the extent of reactive gliosis correlates with the degree of neuron loss in CJD (121).

But prions may have even wider implications than those noted for the common neurodegenerative diseases. If we think of prion diseases as disorders of protein conformation and do not require the diseases to be transmissible, then what we have learned from the study of prions may reach far beyond these common illnesses.

Conformational Diversity

The discovery that proteins may have multiple biologically active conformations may prove no less important than the implications of prions for diseases. How many different tertiary structures can PrP^{Sc} adopt? This query not only addresses the issue of the limits of prion diversity but also applies to proteins as they normally function within the cell or act to affect homeostasis in multicellular organisms. The expanding list of chaperones that assist in the folding and unfolding of proteins promises much new knowledge about this process. For example, it is now clear that pro-proteases can carry their own chaperone activity where the *pro* portion of the protein functions as a chaperone in cis to guide the folding of the proteolytically active portion before it is cleaved (182). Such a mechanism might well feature in the maturation of polypeptide hormones. Interestingly, mutation of the chaperone portion of prosubtilisin resulted in the folding of a subtilisin protease with different properties from the one folded by the wild-type chaperone. Such chaperones have also been shown to work in trans (182). Besides transient metabolic regulation within the cell and hormonal regulation of multicellular organisms, it is not unreasonable to suggest that assembly of proteins into multimeric structures such as intermediate filaments might be controlled, at least in part, by alternative conformations of proteins. Such regulation of multimeric protein assemblies might occur either in the proteins that form the multimers or in the proteins that function to facilitate the assembly process. Additionally, apoptosis during development and throughout adult life might also be regulated, at least in part, by alternative tertiary structures of proteins.

Future Studies

The wealth of data establishing the essential role of PrP in the transmission of prions and the pathogenesis of prion diseases has provoked consideration of how many biologic processes are controlled by changes in protein conformation. The extreme radiation resistance of the scrapie infectivity suggested that the pathogen causing this disease and related illnesses would be different from viruses, viroids, and bacteria (1). Indeed, an unprecedented mechanism of disease has been revealed whereby an aberrant conformational change in a protein is propagated. The future of this emerging area of biology should prove even more interesting and productive as many new discoveries emerge.

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